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Nodulation signal specificity and perception in legumes

Jonathan Cohn

To the Graduate Council:

I am submitting herewith a dissertation written by Jonathan Cohn entitled "Nodulation signal specificity and perception in legumes." I have examined the final electronic 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.

Gary Stacey, Major Professor

We have read this dissertation and recommend its acceptance:

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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NODULATION SIGNAL SPECIFICITY AND PERCEPTION IN LEGUMES

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Jonathan Cohn

August 2000

This dissertation is dedicated to the loving memory of

my mother, Esther Cohn, who loved and supported

me throughout all of my endeavors in life.

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ABSTRACT

The protein products of the rhizobial nodulation genes synthesize lipo-chitin oligosaccharides (LCO) signal molecules, or Nod signals, that induce root hair deformation and induce nodule primordia on legume roots. These LCOs are generally tetra- and penta-lipo-oligosaccharides of *N*-acetyl-*D*-glucosamine with an acyl substitution on the non-reducing end and a variety of substitutions on the reducing end, depending upon the rhizobial strain from which they were produced. This study demonstrates that synthetic analogs have the same biological activity as natural product LCO. To determine structure-function relationships, a collection of synthetic and natural product LCOs was assayed on *Glycine soja* roots. All biologically active LCOs induced both root hair deformations and nodule primordia on *G. soja* roots. Structure-function studies indicate that there was interdependence on backbone length and the presence of substitutions on the LCO reducing end. Pentameric LCOs were active only if they were modified with a 2-*O*-methylfucosyl moiety on the reducing end. However, tetrameric LCOs were only active if they lacked this reducing end modification. Thus, *G. soja* recognized LCO without reducing end substitutions, despite the reported importance of these modifications for host range.

Structure function studies were also carried out on another tropical legume, rice bean (*Vigna umbellata*). LCOs containing a pentameric chitin backbone and a reducing-end 2-*O*-methyl fucosyl moiety were active on *V. umbellata*. In contrast to the studies with *G. soja*, a tetrameric LCO without any reducing end modification was not active on *V. umbellata*. A *Bradyrhizobium japonicum nodZ* mutant, which produces only LCO

without 2-*O*-methylfucose at the reducing end, was able to induce nodule structures on both *G. soja* and *V. umbellata*. Surprisingly, the individual, purified, LCO molecules produced by this mutant were incapable of inducing nodule formation on *V. umbellata* roots. However, when applied in combination, the LCOs produced by the *nodZ* mutant acted cooperatively to produce nodule-like structures on *V. umbellata* roots.

A recent study reported that a Nod signal binding protein from *Dolichos biflorus* is a candidate for a Nod signal receptor. This protein, LNP, was found to be an apyrase. To determine if apyrases might play a role in the early events in nodulation, putative orthologs of LNP were analyzed from the model legume *Medicago truncatula*. Four putative apyrase genes were identified from *M. truncatula*. Two of the genes identified from *M. truncatula, Mtapy1* and *Mtapy4* are expressed in roots, and are inducible within three hours after inoculation with *Sinorhizobium meliloti*. The level of mRNA expression of the other two putative apyrases, *Mtapy2* and *Mtapy3*, was unaffected by rhizobial inoculation. Screening of a bacterial artificial chromosome (BAC) library of *M. truncatula* genomic DNA showed that *Mtapy1*, *Mtapy3*, and *Mtapy4* are present on a single BAC clone, indicating that these apyrases are clustered on the genome. Screening of nodulation deficient mutant lines of *M. truncatula* revealed that two such mutant lines do not express apyrases to any detectable level. One of these mutant lines, *pdl*, is unable to produce cortical cell divisions in response to rhizobia. Conversely, apyrase mRNA is expressed at levels comparable to wild-type in another nodulation deficient mutant line, *lin*, which forms nodule-like structures in response to rhizobia. These data suggest a role for apyrases early in the nodulation response, before the involvement of root cortical cell division leading to nodule development.

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CHAPTER 1

INTRODUCTION

Bacteria of the family *Rhizobiaceae* and leguminous plants have the ability to establish a symbiosis in which the bacteria fix dinitrogen within a novel plant organ, the nodule. This symbiosis allows legumes to thrive in conditions of nitrogen starvation because the bacterial partners reduce nitrogen into ammonia, which is then transformed by the plant into a usable form of nitrogen. In return, the plant provides a readily available carbon source for the bacteria, necessary to process nitrogen inside the protective environment of the nodule. This relationship is believed to be unique to the members of the genera *Rhizobium, Bradyrhizobium, Azorhizobium, Mesorhizobium, Sinorhizobium, Allorhizobium,* and with very few exceptions, leguminous plants. The *Rhizobium*-legume symbiosis has been extensively studied for its possible agricultural benefits, such as attempts to improve crop yields and to transfer biological nitrogen fixation to non-leguminous plants such as rice (Stacey and Shibuya 1998).

The establishment of the symbiosis, and subsequent development of the nodule, involves a complex communication between the bacterial symbionts and their plant hosts. Signals produced by the plant, mostly flavonoids and isoflavonoids, act both as chemo-attractants to bring the bacteria into contact with the root surface and to trigger the induction of bacterial nodulation genes. These nodulation genes encode proteins involved

in the production and secretion of specific lipo-chitin molecules, Nod signals that initiate nodule organogenesis on plant roots.

Mechanism of Infection and Nodulation

Rhizobia, soil bacteria present in the rhizosphere, are initially drawn by chemoattraction to the roots of leguminous plants by the action of secreted signals, such as flavonoid and isoflavonoid compounds (Caetono-Anollé et al., 1998, Agilar et al., 1988, Barbour et al., 1991). Once in contact with plant roots, the infection process begins. Rhizobial attachment to legume roots is considered to be the first step of infection. Attachment to roots has been proposed to be controlled, in part, by host plant lectins (Bohlool and Schmidt, 1974, Dazzo, 1975). However, the results of these studies were difficult to reproduce, so the involvement of lectins in attachment is not clear. Rhizobial fimbriae (Vesper and Bhuvaneswari, 1988) have also been reported to be involved in rhizobial root hair attachment, as well as cellulose fibrils and a Ca^{2+}-dependant binding protein, rhicadhesin (Smit, et al., 1986, Smit et al., 1987). Efficiency of attachment has also been shown to be dependent on physiological conditions such as the growth phase of rhizobial cultures and nutrient availability (Roth and Stacey, 1991).

Prior to or upon root attachment, rhizobia respond to plant produced flavonoids leading to the induction of *nod* gene transcription. The *nod* gene products, in turn, synthesize the lipo-chitin Nod signals. It is these signals that cause curling, ("shepherd's crook"), deformation, and branching of plant root hairs (van Brussel et al., 1986). Due to the ability of Nod signals to cause root hair deformation, this phenomenon has been used

as a biological assay of their activity (Lerouge et al., 1990, Spaink, et al., 1991, Heidstra, 1994).

In the "shepherd's crook," formed by root hair curling, bacteria are entrapped in the host cell wall. The early events of bacterial invasion are poorly understood. However, it has been suggested that rhizobia, sequestered in the curled root hair, induce a lesion of the root hair cell wall by hydrolysis. It has been hypothesized that cell wall hydrolysis is due to the induction of bacterial hydrolytic enzymes, or that the bacteria may induce plant enzymes (Kijne, 1992). At this point, invagination of the plant plasma membrane occurs, and the plant deposits new cell wall material around the lesion formed by the bacteria. This deposit of new cell wall material presents itself as an inwardly growing tube, the infection thread. This infection thread is filled with dividing bacterial cells surrounded by an extracellular matrix. The infection thread takes advantage of the actively growing root hair cell tip by growing toward the inner wall.

In advance of the growing infection thread, cortical cell division takes place, which is initiated by bacterial Nod signals. As cortical cell divisions propagate, a nodule primordium forms on the surface of the root, and the infection thread grows toward these primordia and continues into the root cortex. Once the infection thread reaches a plant host cell, bacteria are released by endocytosis into the plant cell. Once inside the host cell, the bacteria go through poorly understood physiological changes and become bacteroids. Differentiation of rhizobia to the bacteroid state involves 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 a plant-derived membrane that surrounds them have collectively been called symbiosomes, the

functional units of nitrogen fixation (Roth et al, 1988). The symbiosome membrane acts as an interface between the symbiont and the host, as metabolites and signals are exchanged. Through this membrane, the product of nitrogen fixation, ammonium, is transported to the plant hosts. In return, carbon and assimilated ammonia are transported to the bacteria. The symbiosome membrane has also been postulated to prevent the bacteria from inducing a plant defense response (Nap and Bisseling, 1990, Werner, 1992).

There are two distinct types of nodules formed on legume roots. Generally, temperate legumes such as pea, alfalfa, and *M. truncatula* form indeterminate nodules. These nodule types are distinguished by the initial formation of nodule primordia in the inner cortex of host roots, a persistent, terminal meristem, and are generally cylindrical in shape (Dudley et al, 1987, Guan et al., 1995, Pawlowski and Bisseling, 1996). On the other hand, tropical legumes, such as soybean and *Vigna* spp. (e.g., *Vigna unguiculata* and *V. umbellata*) form determinate nodules that are characterized by their spherical structures and a non-persistent meristem. Primordia of determinate nodules initially form from divisions within the outer cortex of host roots.

Host Specificity in Nodulation

The nodulation process is host specific, in that, only certain rhizobial strains are able to infect specific legume hosts. The signals produced by both symbiotic partners control this specificity. Studies of compounds found in legume seeds and root exudates have revealed that there is a great deal of variety in the structure of the compounds and in their ability to activate *nod* gene expression in different strains of rhizobia (Rossen et al.,

1985; Mulligan and Long 1985; Firmin, et al., 1986 Peters et al, 1987; Kosslak, et al, 1987, Bergman et al., 1988, Sutherland, et al, 1990, Kape et al., 1991, Hungria 1991 a,b,; Kape, et al, 1991 Kape, 1992). It has been proposed that the differences in the chemical composition of the exudates produced by different legumes might have evolved so that specific bacteria are able to interact with their host plant. Furthermore, only certain compounds in these exudates, mostly flavonoids, are able to activate gene expression of specific rhizobial strains (Siqueira, et al., 1991). For example, flavonoid compounds produced by alfalfa, such as luteolin and narigenin, induce the expression of the nodulation genes of *Sinorhizobium meliloti* (Peters et al, 1987). In the case of soybean, the nodulation genes of the bacterial symbiont, *Bradyrhizobium japonicum*, are induced by a variety of isoflavonoid compounds including genistein and daidzein (Banfalvi, et al., 1988).

The protein products of the nodulation genes (*nod, nol,* and *noe*) of rhizobia are responsible for production and secretion of lipo-oligosaccharidic Nod signals. These genes can be divided into three classes. The first class, the regulatory genes, such as the *nodD* genes activate the expression of other nodulation genes in response to plant signal molecules. The second class, the common *nodABC* genes are found in all rhizobial species and are functionally conserved. The third class is the host specific nodulation genes (*hsn*), which play a major role in determination of host range. The presence or absence of various host specific *nod* genes has been shown to alter the structure of Nod signals. As will be discussed, the structure of the Nod signal plays a major role in determining host range. Due to their critical role in nodulation, it is not surprising that rhizobia have evolved complex regulatory pathways to control *nod* gene activation.

Transcriptional Regulation of *nod* Genes

The regulation of the nodulation genes of different rhizobial strains is controlled by a variety of parameters, but all require a functional NodD protein. NodD is required for the inducible expression of the nodulation genes (*nod*, *nol*, and *noe*), which are responsible for the biosynthesis and export of Nod signals (reviewed in Carlson, et al, 1993, Dénarié, et al, 1996). NodD belongs to the LysR family of transcriptional regulators (Schell, et al, 1993). NodD has been shown to bind to a conserved 47-bp DNA sequence upstream of the host inducible *nod* operon, which has been termed the *nod* box (Rostas, et al. 1986). The general model of NodD activation is that the NodD protein interacts with plant produced flavonoid molecules and that this interaction is essential for transcriptional activation of the *nod* genes (Day et al, 1999). In effect, NodD acts as a sensor of host plant flavonoids and an activator of *nod* gene transcription.

NodD genes control the first level of host specificity in the *Rhizobium*-legume symbiosis. NodD proteins from strains such as *S. meliloti* and *R. leguminosarum* bv. *trifolii*, which have narrow host ranges, respond to only a few flavonoids (Spaink, et al, 1987). Conversely, the NodD protein of the broad host range *Rhizobium* sp. NGR234 is able to respond to a wide spectrum of plant signal molecules (Horvath, et al. 1987, Spaink, et al, 1987). It is not surprising that mutations in *nodD* from one strain cannot always be complemented by the *nodD* gene from other rhizobial species (Horvath, et al. 1987, Spaink, et al, 1987). Interestingly, however, *nodD* point mutations have been found to extend the host range of certain rhizobia (McIver et al, 1989). It was found that transferring the $nodD_1$ gene from *Rhizobium* sp. strain NGR234 to *S. meliloti*, allowed *S. meliloti* to nodulate siratro (Horvath et al., 1987). However, the *nodD1* gene from *S.*

meliloti is incapable of complementing an NGR234 NodD1 mutant to restore the ability to nodulate siratro. In a similar set of experiments, it was found that the *nodD1* gene from *S. meliloti* was not able to complement a *nodD1* mutant of *R. trifolii* (Spaink, 1987). These results indicated that the NodD proteins of different rhizobia indeed function to activate *nod* gene expression, even though the various proteins respond to different plant signals. In support of this hypothesis, there are mutant and hybrid *nodD* alleles that can activate transcription of *nod* genes in the absence of flavonoids (Spaink et al, 1989).

Although, the NodD protein is present in all rhizobia studied, the number of copies of the gene varies from strain to strain. For example, *S. meliloti* wild-type strains contain three copies of the gene (Honma, et al., 1990), while wild-type strains of *B. japonicum* only contain two copies (Göttfert, et al., 1992). *R. tropici* has five *nodD* gene copies (van Rhijn, et al. 1994), while *A. caulinodans* and *R. leguminosarum bv. viciae* possess only one copy (Downie, 1985; Goethals, et al., 1990). It is not apparent that *nodD* copy number has any effect on host range. The broad host range *Rhizobia* sp. NRG234 is able to form a symbiosis with at least 110 genera of legumes and possesses two copies of *nodD* (Puepke and Broughton 1999).

The regulation of *nod* genes in some rhizobial strains (e.g., *S. meliloti* and *Rhizobium* sp. NGR234) is also controlled by another factor, SyrM (Mulligan and Long, 1989). When expressed from multi-copy plasmids, NodD3 and SyrM can induce *nod* gene expression in the absence of plant signals. It is believed that SyrM and NodD3 form a self-amplifying regulatory circuit (Swanson, et al., 1993).

A unique situation exists in the case of *B. japonicum*. Remarkably, it was found that mutations in either *nodD1* or *nodD2* do not result in a Nod$^-$ phenotype on any host,

which is the converse of the results found with similar mutations in other rhizobia (Nieuwkoop, et al, 1987). This discovery led to the identification of a new regulatory system for *nod* gene activation. Previously, it was discovered that the *B. japonicum nodV* and *nodW* genes were required for nodulation of the plant hosts *M. atropurpurem, Vigna radiata*, and *V. unguiculata* (Göttfert, et al., 1990). However, NodW was not required for soybean nodulation, the normal host of *B. japonicum*. The data suggested that NodV and NodW were acting in some fashion to extend the host range of *B. japonicum*. Subsequent to these findings, Sanjuan, et al. (1994) demonstrated that NodV and NodW were directly involved in the regulation of *nod* genes by isoflavone signals. These results indicated that *B. japonicum* possessed a mechanism for responding to plant flavonoid signal molecules that was distinct from the NodD regulatory circuit. It was later shown that NodVW comprise a classic two-component regulatory system (Loh et al., 1997). NodV is a sensor kinase that autophosphorylates in response to plant-produced isoflavones, and subsequently phosphorylates the response regulator NodW (Loh et al., 1997). It is possible that alternative hosts of *B. japonicum* exude compounds that are different from the signals produced by soybean, and that these signals interact directly with NodVW and not NodD1. This would explain the involvement of NodV and NodW in the host specific nodulation phenotype.

Negative Regulation of *nod* Genes

As is the case with many complex gene regulatory systems, *nod* genes are negatively regulated as well as positively regulated. For example *nod* genes seem to be necessary only during the early stages of nodulation, as they are repressed in bacteroids

(Schlaman, et al, 1991). In fact, it is becoming clear that the activation of *nod* genes, and subsequent production of Nod signals inside the nodule might be detrimental to efficient nodulation. One example of this comes from a study that showed that introduction of the *nodABC* genes of *R. leguminosarum bv. viciae* onto a multi-copy plasmid into the wild-type strain inhibited nodulation of pea (Knight, et al, 1986)).

It is possible that some components of the nodule environment, such as low oxygen concentrations, or nutrient limitation might affect *nod* gene regulation. It is generally accepted that the major carbon source for bacteroids is C_4 dicarboxylic acids, such as malate and succinate. Work by Yuen et al, (1996) showed that the addition of organic acids, such as malate and succinate to *B. japonicum* cultures significantly repressed *nod* gene expression. However, it is still not clear how *nod* gene expression is repressed *in planta.*

It is known that the transcription of *nodABC* and *nodSU* operons in *Rhizobium* sp. NGR 234 is undetectable 24 hours after the initial induction by purified flavonoids (Fellay et al., 1998), suggesting that bacteria might have some control over *nod* gene repression. The *nolA* gene product of *B. japonicum* has been suggested to function as a transcriptional repressor of the *nod* operon (Dockendorff, et al., 1994). The *nolA* gene was originally reported to be involved in genotype-specific nodulation of soybean (Sadowski et al, 1991). That is, certain genotypes of soybean are only capable of being nodulated by specific rhizobial strains. Transfer of the *nolA* gene into *B. japonicum* strain SD6-1C extended the host range of this strain to include a specific soybean genotype. More recent studies have shown that NolA activates *nodD2*, which then

represses *nod* gene expression (Garcia, et al., 1996). A summary of one of the recent models of nodulation gene regulation in *B. japonicum* is depicted in Figure 1.

The product of the *S. meliloti nolR* gene down regulates transcription of *nodD1, nodD2*, and the *nodABC* genes by binding to their promoters (Kondorosi, et al., 1989). It has been proposed that negative control by NolR might allow fine-tuning of *nod* gene induction by preventing over production of Nod signals (Kondorosi, et al., 1989, Kondorosi, et al., 1991). Mutations in either *nolR* or *nolA* resulted in significant reductions in nodulation capacity of host plants (Kondorosi, et al., 1989, Garcia et. al., 1996). In the case of *nolR*, this phenotype was believed to be due to over-expression of *nod* genes (Kondorosi, et al., 1991). Cowpea nodules infected by a *B. japonicum nolA* mutant were severely affected in their ability to fix nitrogen, possibly due to defective bacteroids development (Garcia et al., 1996). A similar phenotype was observed with a *Rhizobium* sp.NGR234 *nodD2* mutant, in that, this strain was unable to form nitrogen-fixing nodules on *V. unguiculata* or *Cajanus cajan,* possibly due to over expression of *nod* genes (Fellay, 1998).

Nod Signal Structure and Host Specificity

As indicated above, plant signal molecules such as flavonoids activate the nodulation (*nod*) genes of rhizobia. It is clear that different strains of rhizobia respond only to specific plant signals. This phenomenon controls one aspect of host specificity. The specificity of the symbiosis is also regulated to a great degree by specific chemical modifications to the bacterially produced lipo-chitin Nod signals (Figure 2, Table 1). Once the plant recognizes the specific Nod signal, the plant follows a predetermined

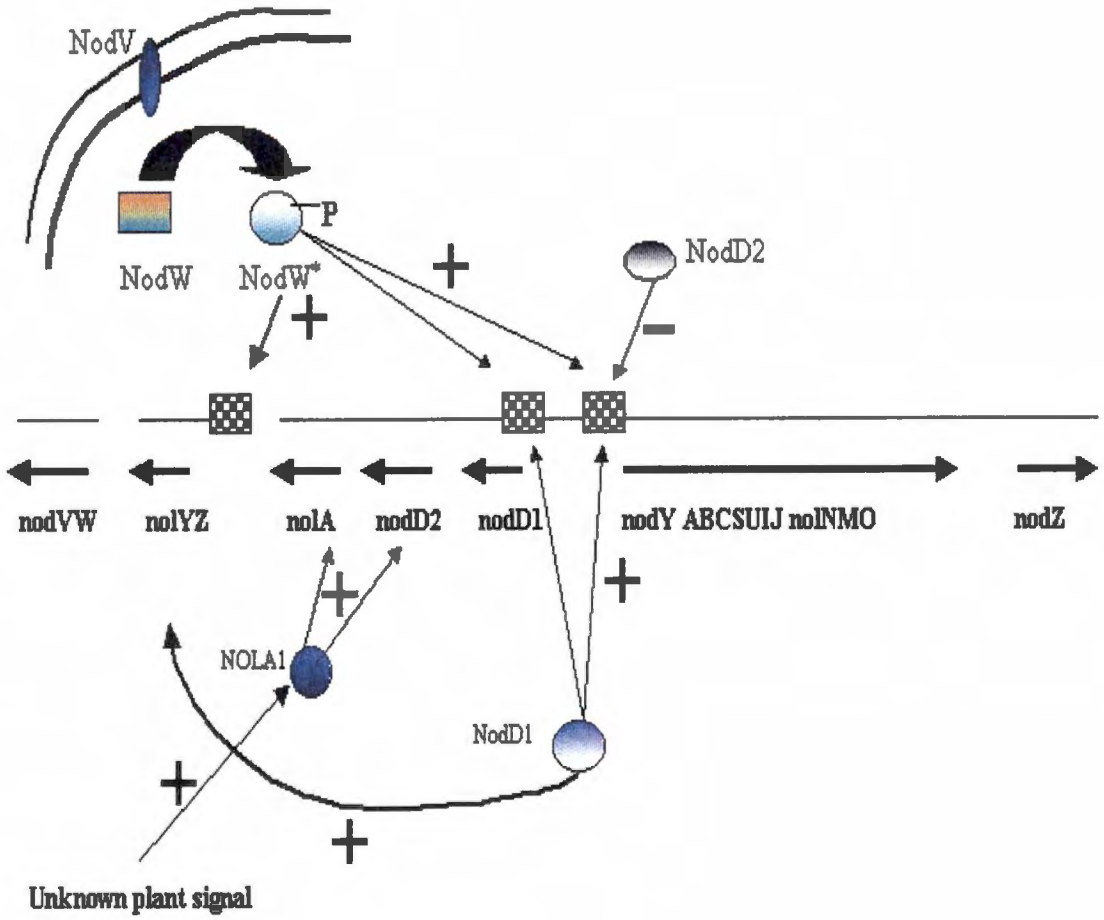


Figure 1. Diagram of a recent model of nodulation gene regulation in *Bradyrhizobium japonicum.* Two independent systems, NodD1 and NodVW, activate *nod* gene transcription in response to plant produced isoflavones as indicated in the text. The *nolA* gene encodes three distinct proteins. However, only NolA1 is shown because it is this peptide that is required to activate the transcription of *nolA* and *nodD2.* NolA also represses the expression of the *nod* gene operon in *B. japonicum,* including the genes *nodYABCSUIJ* and *nolMNO,* via the action of *nodD2.* Positive regulation is indicated by a 'plus' sign (+) next to the arrows indicating where regulation is taking place. In contrast, negative regulation in indicated by a 'minus' sign (-). This figure was adapted and slightly modified from a recent review by Day, et al. 1999.

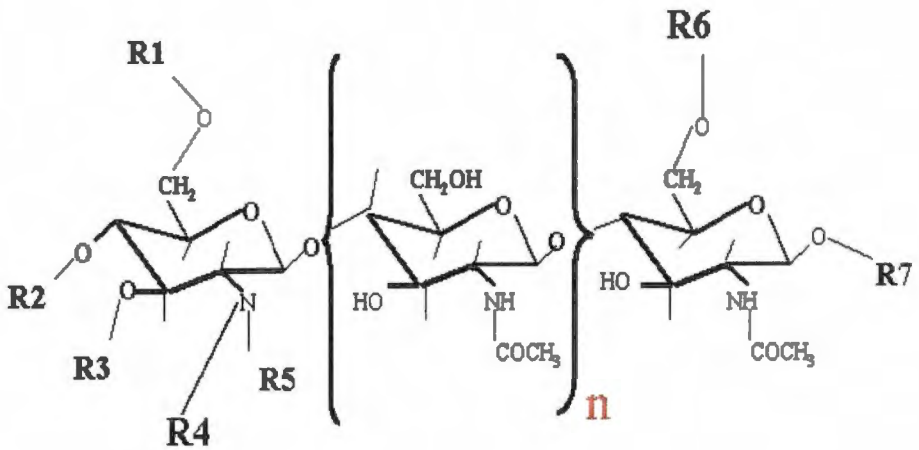


Figure 2. Structure of rhizobial Nod signal. Nod signals consist of a core structure of three to five β-1,4-inked N-acetyl glucosamine units that are modified at the non-reducing end at the R position by a fatty acid moiety of varying length. Substitutions are made at each of the indicated R-positions by different strains of rhizobia. Common differences in substitutions made are to the R2 and R4 positions indicated in the diagram. In this diagram, n represents the number of internal glucosamine units, which usually ranges from 1 to 3 in the Nod signal structures identified.

Table 1. Structures of selected Nod signals produced by various strains of rhizobia

Species	Host Plant	*n*	R1	R2	R3	R4	R5	R6	R7	Reference[1]
Bradyrhizobium japonicum	Soybean	2,3	Ac	H	H	C18:1 11 C18:1 9/C16:0 C18:1 9,Me C16:1 9	H	2-*O*-methyl fucose	H	1,2
B. elkanii	Soybean	2,3	Ac	Cb	H	C18:1/C16:0	Me,H	2-*O*-methylfucose	Glyceryl	2,3
Sinorhizobium meliloti	Alfalfa	1,2,3	Ac	H	H	C16:1/C16:2/ C16:3/(-1)OH C18 11	H	Sulfate	H	4,5,6
R. tropici	*Phaseolus*, *Leucaena*, *Medicago*, *Macroptilium*	3	H	H	H	C18: 11	Me	Sulfate, H	H	7
S. fredii	Soybean	1,2,3	H	H	H	C18:1 11	H	2-*O*-methyl fucose/ Fucose	H	8
Rhizobium. spp. NGR234	Very broad host range	3	Cb, H	Cb, H	Cb, H	C18:1 11/ Me C18:0/C16:0/ C16:1 11	Me	2-*O*-methyl fucose/ 3-S-methyl fucose/ 3 /4 Ac methyl fucose	H	9
R. leguminosarum bv. *viciae*	Pea, vetch	2,3	Ac	H	H	C18:1/C18:4	H	H or Ac	H	10,11
R. leguminosarum bv. *trifolii*	Clover	2,3	Ac	H	H	C18:1/C18:4	H	H or Ac	H	12,13
R. etli	*Phaseolus*	3	H	Cb, H	Cb, H	C18:1 1	Me	4-*O*-acetyl-*L*-fucose	H	14,15
Azorhizobium caulinodans	*Sesbania*	2,3	Cb	H	H	C18:1/C18:0	Me	Arabinose/ Fucose, or H	H	16
Mesorhizobium loti	*Lotus japonicus*	3	H	Cb	H	C18:1 11/C18:0	Me	4-*O*-acetyl-L-fucose	H	17

[1]References: 1= Sanjuan et al.,1 1992; 2 = Carlson et al., 1993; 3= Stokkermans, et al., 1996; 4= Lerouge, et al., 1990; 5= Schultze et al., 1992; 6= Roche et al., 1991; 7 = Poupot et al., 1993 8= Becferte et al., 1994; 9= Price et al., 1992; 10= Spaink et al., 1991; 11= Firmin et al., 1993; 12 = Spaink et al., 1995; 13= Orgambide, et al.., 1995; 14 = Cardenas et al., 1995; 15 = Poupot et al., 1995; 16= Mergaert et al., 1993; 17= Lopez-Lara, et al., 1995.

developmental pathway to form the nodule. The available data suggest that the appropriate Nod signal stimulates a defined signal transduction pathway leading to this programmed developmental event.

Identification of the Nod Signal

It became clear that the nodulation genes of rhizobia were responsible for the production of excreted molecules based on the observation that the sterile supernatants of bacterial cultures caused changes in root and root-hair morphology on the appropriate host plants (van Brussel, et al, 1986, Faucher, et al., 1989, Bengali, et al, 1989). These studies demonstrated that the excreted molecules were dependent on the activity of the common *nod* genes *nodABC*. Nod signals, also referred to as Nod factors, because they are the products of the *nod* genes, were originally purified from cultures of *S. meliloti* genetically engineered to overexpress *nod* genes (Lerouge, et al, 1990). The molecules were found to be hydrophobic, as they were present in butanol extracts of rhizobial cultures. The culture extracts were subjected to ion exchange chromatography and reverse phase HPLC. When analyzed by mass spectrometry, nuclear magnetic resonance, and chemical analysis, they were found to be lipo-chitooligosaccharides. Nod signals have now been identified from a variety of sources (see Table 1). Most of the Nod signals identified thus far consist of a chitin oligomer backbone of three to five β,1-4 linked *N*-acetyl glucosamine residues substituted with a fatty acid chain at the non-reducing end. (See Figure 2, Table 1).

Biosynthesis of the Nod Signal

It was discovered that the *nodA*, *nodB* and *nodC* genes are responsible for the biosynthesis of the core structure of the Nod signal. These genes have been referred to as the common *nod* genes, in that they are found in all rhizobial species studied. In a *R. leguminosarum* bv. *viciae* strain lacking the pSym plasmid, the presence of *nodD* and the *nodABC* genes was sufficient to produce chitooligosaccharides with four or five glucosamine residues, *N*-acylated with vaccenic acid (C18:1) (Spaink, et al., 1991).

NodC proteins from various rhizobial species share significant sequence similarity with chitin synthases, as well as cellulose synthases, hyalurone synthase, and the *Xenopus laevis* developmental protein DG42 (Atkinson, and Long, 1992; Bulawa, 1992; Débelle, et al., 1992; Semino and Robbins; 1995). Using radio-labeled UDP-Glc*N*Ac (*N*-acetyl glucosamine) and cellular extracts from a NodC overproducing strain, in vitro assays showed that NodC is able to polymerize Glc*N*Ac into chitin oligomers of up to five residues (Geremia, et al, 1994). For maximal activity the reaction requires free *N*-acetyl-*D*-glucosamine that cannot be substituted by glucosamine, galactosamine, or *N*-acetyl-*D*-glucosamine as acceptor molecules. Pulse-chase experiments indicated that chain elongation proceeds toward the non-reducing end of the oligosaccharide and that the reaction follows a single-chain mechanism (Inon, et al., 1995).

It is believed that NodC might control the degree of polymerization of the chitin backbone of Nod signals (Kamst, et al, 1996). In support of this hypothesis, the *nodC* gene of *S. meliloti* was found to specify the synthesis of chitin tetramers, indicating that it is a *hsn* gene (Roche, et al., 1996). In this study, the common *nodABC* genes and the *nodFL* genes of *S. meliloti* were transferred to *R. tropici*, allowing this strain to nodulate

alfalfa. The broad host range *R. tropici* is normally unable to nodulate alfalfa. The mutant *R. tropici* strain produced Nod signals that closely resembled *S. meliloti* Nod signals, with respect to chitin chain length and degree of unsaturation of the fatty acid moiety at the non-reducing end. Inactivation of the *S. meliloti nodABC* genes suppressed the ability of the hybrid *R. tropici* strain to nodulate alfalfa. The authors indicated that NodC is a determinant of host specificity, in that different alleles of *nodC* might be responsible for variations in chitin chain length of Nod signals produced by various strains of rhizobia (Roche, et al., 1996). However, the processes controlling chain length of the Nod signal are not well understood.

NodB is a chitin de-acetlyase that is specific for the non-reducing end of the molecule. Purified NodB of *S. meliloti* has been shown to de-acetylate oligo-chitin with two to six GlcNAcs but has no effect on monomeric glucosamine (John, et al, 1993, Röhrig, et al., 1994). NodB acts to deacetylate the non-reducing end of the Nod signal, preparing it for the addition of the acyl chain (John, et al., 1993, Kafetzopoulos, et al., 1993).

The final step in assembling the Nod signal's core structure is performed by the protein product of the *nodA* gene. NodA is an acyl transferase that catalyzes the addition of the acyl chain to the de-acetylated glucosamine residue at the non-reducing end of the chitin oligomer (Atkinson, et al, 1994, Rhörig, et al., 1994).

Nod signals have been purified from several species of rhizobia, and there are discrete differences in the structures of the molecules identified (reviewed in Cohn, et al., 1998) (see Table 1). Modifications to both the reducing and non-reducing ends of the core molecule have been shown to be important determinants of host specificity. That is,

variations in the structure of Nod signals controls the biological activity of these compounds on various legume roots. Differences in modifications to Nod signals purified from distinct strains of rhizobia are controlled by the presence or absence of host specific *nod* genes. Table 2 gives a list of some of the known nodulation genes and their proposed functions.

Modifications to the Reducing End

Modifications present on the reducing end of Nod signals from different strains of rhizobia play an important role in the host specificity of the symbiosis. For example, the products of the *S. meliloti nodH, nodP,* and *nodQ* genes act to sulfate the terminal, reducing *N*-acetyl glucosamine residue of a tetrameric core molecule (Lerouge, et al, 1990). NodH is a sulphotransferase and tetrameric Nod signals are the preferred substrate for sulfate transfer (Schultze, et al., 1995, Ehrardt, et al., 1995). NodPQ are required for production of the activated from of sulphate, 3'phosphoadenosine 5'phosphosullphate (PAPS) which is the substrate for NodH. The 6- *O*- sulphate modification has been reported to be absolutely required for nodulation of *Medicago sativa* (alfalfa), the normal host of *S. meliloti* (Lerouge et al, 1990). However, *S. meliloti* mutants unable to sulphate the core molecule retain the ability to nodulate an alternative host, *Vicia sativa* (vetch). Consistent with these observations, non-sulphated *S. meliloti* Nod signals are not biologically active on alfalfa roots, but are active on vetch roots. Introduction of the *S. meliloti nodHPQ* genes into *R. leguminosarum* bv. *trifolii*, or *R. leguminosarum* bv. viciae, neither of which possesses these genes, leads to the

Table 2. Function of the protein products of the known rhizobial nodulation genes. (modified from Dénarié et al., 1996)

Gene	Proposed Gene Product Function	Reference
Common *nod* genes		
NodA	Acetyltransferase	Röhrig, et al.1994, Atkinson, et al., 1994
nodB	Deacetylase	John, et al.., 1993
nodC	Chitin synthase	Geremia et al., 1994
nodM	D-glucosamine synthase	Baev et al., 1992
Host Specific *nod* genes		
nodE	β-Ketoacylsynthase	Demont et al., 1993
nodF	Acyl carrier protein	Shearman et al., 1996 Demont et al., 1993
nodH	Sulfotransferase	Roche et al.,1991 Ehrhardt et al., 1995
nodS	Methyltransferase	Mergaert et al. 1995
nodZ	Fucosyltransferase	Quinto et al., 1997
nodU	Carbamoyltransferase	Jabbouri et al., 1995
nodP	ATP-sulfurylase	Schwedock et al., 1994
nodQ	ATP-sulfurylase	Schwedock et al., 1994
noeJ	phosphate guanyltransferase	Fellay et al., 1995
noeK	phosphomannomutase	Fellay et al., 1995
noel	Dehydratase	Fellay et al., 1995
nolXWBTUV	cultivar–specific nodulation	Kovacs et al., 1995
nolK	NADS-dependent sugar epimerase	Mergaert et al., 1996
noeC	Arabinosylation	Mergaert et al., 1996
noeD	genotype-specific nodulation	Lohrke et al., 1998
noeE	Sulfotransferase	Hanin et al,, 1997,
noeI	2-O-methylation	Jabbouri et al., 1998
nolO	Carbamoyltransferase	Jabbouri et al., 1998
nodX	Acetyltransferase	Davis et al., 1998
nodL	Acetyltransferase	Berck et al., 1999
nolL	O-acetyltransferase	Berk et al., 1999
Regulation of nod gene Expression		
nodD	Transcriptional activation	Göttfert et al., 1992
nodV	Two component regulator	Göttfert, et al., 1989
nodW	Two component regulator	Göttfert, et al., 1989
nolA	Transcriptional regulator	Sadowsky et al, 1991
nolR	Transcriptional repressor	Kondorosi et al., 1989
syrM	Transcriptional regulator	Hanin et al., 1998
Secretion of Nod signals		
nodO	Secreted, pore-forming protein	DeMaagd et al., 1989
nodI	ATP-binding protein	Jabbouri et al., 1996
nodJ	Membrane protein	Cardenas et al. 1996
nodT	outer membrane protein	Rivilla et al., 1995
nolYZ	Type III secretion	Viprey et al., 1998

production of sulphated Nod signals and extends the host range of these strains to include alfalfa as a host (Lerouge, et al., 1990, Roche et al., 1991).

In the case of *Bradyrhizobium japonicum*, the major Nod signal produced is a pentameric signal molecule modified with a 2-*O*-methyl fucosyl moiety on the reducing end (Sanjuan, et al., 1992, Carlson, et al., 1993). As in the case of *S. meliloti*, the reducing end substitution found in *B. japonicum* Nod signals plays a critical role in host range determination (Stacey, et al., 1994). However the data suggest that the stringent requirement for a specific Nod signal structure may not exist in soybean. For example, a *B. japonicum nodZ* mutant is able to nodulate soybean with only a slight delay in nodulation (Stacey, et al., 1994). The modification to the reducing is critical, however, for nodulation of an alternative host, siratro (Stacey, et al., 1994). The *nodZ* gene encodes a fucosyl transferase that modifies the terminal, reducing *N*-acetylglucosamine of the normal pentameric Nod signal (Stacey, et al., 1994, Lopez-Lara, et al., 1997; Quinto, et al., 1997). Similar to the experiments above, transfer of the *nodZ* gene to *R. leguminosarum* bv. *viciae* results in the production of fucosylated Nod signals and an extension of host range to include soybean (Lopez-Lara, et al., 1997). These data argue for a strict requirement for a fucosylated Nod signal for soybean nodulation. However, *nodZ* mutants of *B. japonicum* that do not produce fucosylated Nod signals nodulate soybean normally (Stacey, et al., 1994). From these data, it would appear that the modification to the reducing end of the Nod signal is not important for nodulation of soybean, in direct contrast to the data obtained from the work with *S. meliloti* and alfalfa. However, there is one report that shows that the growth conditions of the plant might

have critical effects on the ability to nodulate. Ogawa, et al., (1991) showed that alfalfa indeed could be nodulated, albeit to a lesser extent, by a *nodH* mutant of *S. meliloti.* This would suggest that the sulfate moiety at the reducing end of Nod signals produced by *S. meliloti* might not be as critical for nodulation as presently believed. However, it could also be the case that this mutation is 'leaky'; that is, the mutant might still produce a low abundance of sulfated Nod signal.

Nod signals from *R. leguminosarum bv. viciae* strains (e.g. TOM) are decorated with a *O*-acetylation at the reducing terminus (Firmin, et al., 1993). This specific modification has only been detected in this particular species of *Rhizobium.* The strain TOM forms nitrogen-fixing nodules on primitive cultivars of *Pisum sativum* (pea) (e.g. cv. Afghanistan), but not on other cultivars (Davis, et al, 1988). Thus, this decoration has some effect on host specificity. *O*-acetylation of the Nod signals produced by *R. leguminosarum bv. viciae* is controlled by the protein product of the *nodX* gene (Firmin, et al., 1993). More accurately, NodX mediates *O*-acetylation of the reducing end of pentameric Nod signals, but not tetrameric Nod signals. (Firmin, et al., 1993; Hanin, et al., 1997). Remarkably, the *nodX* gene shares very little homology to *nodL,* another O-acetyl transferase present in *R. leguminosarum bv. viciae.* (Bloemberg, et al., 1994).

Modifications to the Non-Reducing End

Experiments similar to the ones described above have shown that it is the length and degree of unsaturation of the acyl chain of Nod signals that are critical determinants of their host specificity (Reviewed in Dénarié, et al., 1996). The *nodE* and *NodF* genes of *S. meliloti* and *R. leguminosarum* specify the synthesis of rare polyunsaturated fatty

acids found on the non-reducing end of their respective Nod signals (Table 2). *S. meliloti nodE* and *nodF* mutants are unable to produce wild-type Nod signals with C16:2 fatty acids at the reducing end, but replace the characteristic acyl chain with a vaccenic acid (C18:1). NodE of *R. leguminosarum bv. viciae* is required for the production of a C18:4 fatty acid chain at the non-reducing end of that strain's Nod signal. Recently, Demont-Caulet et al. (1999) have shown that the length of the fatty acyl chain of *S. meliloti* Nod signals is critical to their biological activity.

S. meliloti nodL mutants are significantly impaired in their ability to elicit infection thread formation of alfalfa, while *nodL/nodF* double mutants are unable to infect their hosts (Ardourel, et al., 1994). *nodL* shows significant sequence similarity to several bacterial acetyl transferases, and is responsible for acetylation of the C6 position of the non-reducing end glucosamine of *S. meliloti* Nod signals (Bloemberg, et al., 1994). The NodF sequence is similar to acyl-carrier proteins (Shearman, et al., 1986).

Some rhizobial strains produce *N*-methylated Nod signals via the action of the *nodS* protein product (Göttfert, et al., 1990; Lewin, et al., 1990). Mutations in the *nodS* gene of *A. caulinodans*, *Rhizobium* sp. NGR234, and *R. tropici* abolish nodulation of *Leucaena* and *Phaseolus* by these strains (Lewin, et al., 1990, Waelkens, et al., 1995). Thus, methylation of Nod signals is an important determinant of host range.

Biological Activity of Nod Signals

Purified Nod signals, at nanomolar to picomolar concentrations, elicit distinct morphological changes on the appropriate host plant. One of the earliest visible responses to Nod signals is the deformation of root hairs. The appropriate Nod signal stimulates

root hair deformations on all of the plants tested (Lerouge et al., 1990, Roche et al., 1991, Spaink, et al., 1991, Schultze et al., 1992, Price et al., 1992, Sanjuan et al., 1992, Mergaert et al., 1993, Carlson et al., 1993, Relic et al., 1993 Bec-Ferté et al., 1994,). The deformation process requires only 10 minutes of incubation with Nod signals (Heidstra et al., 1994). Deformation begins with swelling of the root hair tip, most likely due to cytoskeletal reorganization, and is visible within one hour post application of Nod signal. Clearly deformed root hairs, such as crooked hairs, have been reported to occur within three hours post inoculation (Heidstra et al., 1994).

Nod signals have been reported to have a variety of other responses related to nodule formation. However, the degree to which Nod signals stimulate these effects seems to be controlled by the plant host (See Table 3 for a list of the effects that Nod signals have). In the outer cortex of *Vicia*, Nod signals from its rhizobial partner, *R. leguminosarum bv. viciae,* induce formation of pre-infection threads and stimulate altered root hair growth (Van Brussel et al., 1992). At higher concentrations (>1nM), Nod signals are able to stimulate cell division and the formation of meristems that differentiate into nodule-like structures (van Brussel et al., 1992, Savouré et al, 1994, Stokkermans and Peters, 1994, Yang et al., 1994). The induced nodule developmental program includes stimulation of root cortical cell division and dedifferentiation of cell types. On some plants (e.g., *Medicago sativa*), application of the correct Nod signal induces nodule structures that contain bifurcating vascular bundles and a mitotic center in the appropriate region of the root cortex (Truchet et al. 1991). However, on other plants, incomplete or aberrant nodule development has been observed in response to Nod signal addition. For

Table 3. Time course of plant responses to *Rhizobium* and Nod signals. Slightly modified from a recent review by Day et al., 1999.

Time After Inoculation	Response	Reference
1-2 minutes	Bacterial attachment	Turgeon and Bauer, 1982
1-2 minutes	Ca^{2+} influx, Cl^- efflux in root hairs	Felle et al., 1995
2 minutes	Membrane Depolarization	Ehrhardt et al., 1992
2-10 minutes	Ca^{2+} spiking in root hairs	Ehrardt et al., 1996
5 minters	Intracellular alkalization of root hairs	Felle et al., 1995
5-10 minutes	Actin de-polymerization	Allen and Bennet, 1996
10 minutes	Root hair tip swelling	Heidstra et al., 1994
1 hour	Root hair deformation	Heidstra et al., 1994 Gehring et al, 1997
1 hour	Leghemoglobin induction	Heidstra et al, 1997
1-3 hours	*ENOD12* induction	Journet et al., Pichon et al, 1992
3 hours	*rip1* induction	Cook et al., 1995
8 hours	*ENOD5* induction	Vijn et al., 1995
5-17 hours	*CHS* induction	Mathesius et al., 1998
12 hours	Root hair curling	Turgeon et al., 1982
17 hours	*GH3* induction	Mathesius et al., 1998
24 hours	*PAL* induction	Krause et al., 1997
12-24 hours	*ENOD40* induction	Minami et al,1996a
12-24 hours	*ENOD20* induction	Vernoud et al., 1999
24 hours	Extensin mRNA	Arsenijevic et al., 1997
24 hours	*MtAnn1* induction	Niebel et al., 1998
24 hours	Visible infection thread	Turgeon and Bauer, 1982
24-96 hours	Cortical Cell Divisions	Turgeon and Bauer, 1982
24-96 hours	Bacteroid formation	Newcomb et al., 1979
5-6 days	*ENOD2* induction	Minami et al., 1996a
4 days	Development of nodule meristem	Turgeon and Bauer, 1982
4-10 days	Visible nodules on roots	

example, nodule primordia did not develop past the primordium stage and vascular bundles were not observed on *Vicia sativa* roots treated with the appropriate Nod signal (Spaink et al. 1991). Similarly, on *Sesbania rostrata* Brem. et Oberm., it was reported that Nod signals induced swellings at the base of secondary roots that, at a low frequency, developed into nodule structures (Mergaert et al. 1993). Nod signals produced by *Rhizobium* species NGR234 induced pseudonodules on siratro (*Macroptilium atropurpurium*) and cowpea (*Vigna unguiculata*) that resembled bacterially induced nodules (Relic, et al. 1993, Relic et al. 1994). On soybean roots, Nod signals from *B. elkanii* induced what appeared to be complete nodule structures (Stokkermans and Peters 1994), but the structures were not complete nodules. However, they appeared to reach a more advanced stage of development then seen on other plants such as *V. sativa* or *S. rostrata* (N.K. Peters, personal communication).

In addition to the morphological effects that have already been mentioned, Nod signals also stimulate other physiological changes that have been postulated to play a role in nodule formation. These effects include depolarization of root hair membranes and an increase in cellular calcium levels (Erhardt, et al., 1992, 1996, Heidstra, et al., 1997). In *Medicago sativa* root hairs, depolarization of the cytoplasmic membrane potential occurs within two minutes post application of Nod signals from *S. meliloti* (Erhardt et al., 1992). This effect required wild-type Nod signals sulfated at the reducing-end terminus and possessing an unsaturated acyl moiety. Ehrhardt et al. (1996) reported that specific Nod signals induced a quick rise in Ca^{2+} levels in alfalfa root hairs with subsequent periodic Ca^{2+} oscillations associated with the nucleus. The response was very specific; no response was observed when plants were inoculated with chitin oligomers or Nod signals

from *R. leguminosarum*, a bacterium that does not nodulate alfalfa. The rise in Ca^{2+} levels in root hair cells occurred very quickly, within ten minutes after inoculation with Nod signals. However, this delay indicates that it is probably not responsible for depolarization of the membranes (Erhardt, et al., 1996). These results are similar to what has been seen in mammalian cells in response to specific agonists (Clapman, 1995). If these events are indeed similar, the spiking noted in response to Nod signals might represent a 'calcium-induced, calcium release' in which Ca^{2+} could regulate its own release from internal stores through a positive feedback mechanism. However, Gehring et al. (1997) measured a rapid rise in cytoplasmic calcium levels in cowpea root hairs but failed to detect Ca^{2+} oscillations as reported by Ehrhardt et al. (1996). Differences in experimental material or methods may explain the differences in these two studies. Additionally, the measure of calcium in this study was taken from the root hair tip, which might just measure calcium that is involved with tip growth. More recent studies have attempted to examine the role of cytosolic Ca^{2+} concentration changes in regard to Nod signal transduction by using pharmacological agents, such as the Ca^{2+} ionophore A_{23187} and 2,5-di(t-butyl)-1,4-benzohyfroquinone (BHQ) (Felle, et al. 1998, Felle et al., 1999a). The results of these studies revealed that one of the earliest responses to Nod signals was a loss of Ca^{2+} from the root hair space and that this loss was necessary and sufficient to trigger down-stream responses to Nod signals (Felle et al, 1998). The Ca^{2+} ionophore A_{23187} mimicked the effects of Nod signals, in that it activated anion channels through which the cells lose Cl^- rapidly, causing depolarization of the membrane, followed by K^+ efflux (Felle, et al., 1998). BHQ, a known inhibitor of Ca^{2+}-ATPase in the ER of mammalian cells, was also able to mimic the effects of Nod signals. The results of these

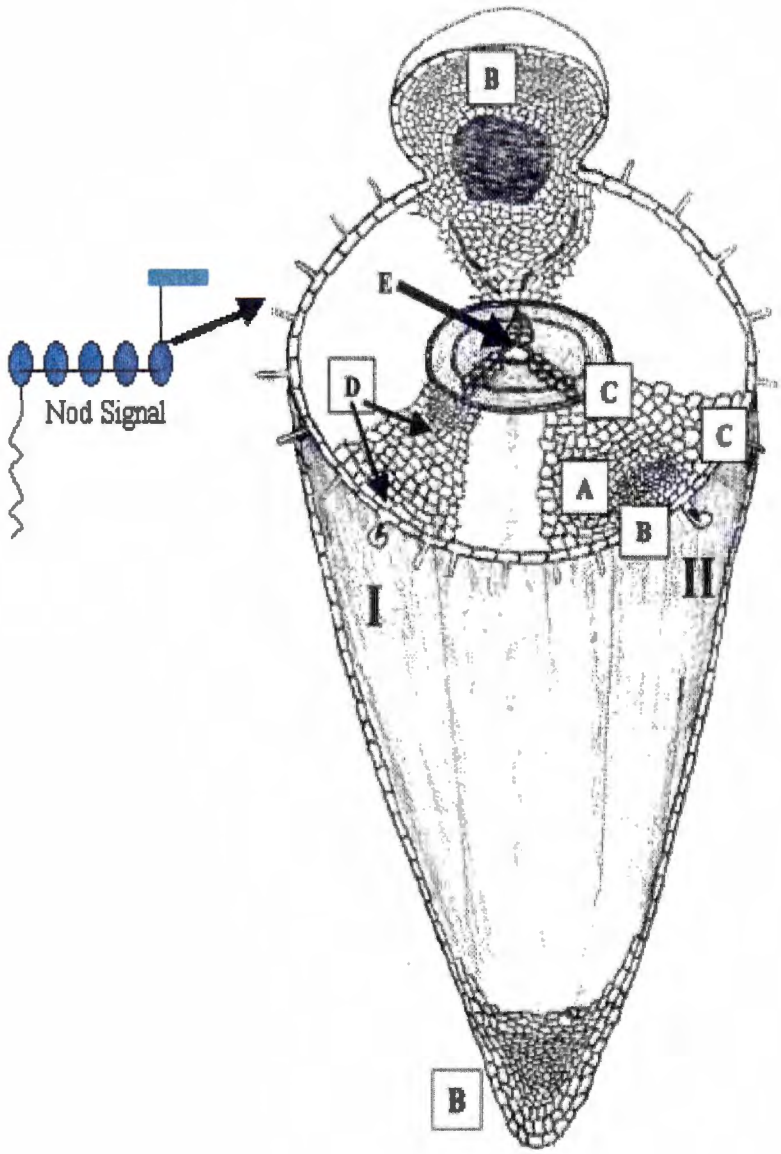
studies demonstrated that Nod signals cause an increase in cytosolic Ca^{2+} concentration of alfalfa root hair cells. In agreement with work by Pingret et al. (1998), the authors demonstrated that the Nod signal response was dependent on external Ca^{2+}. Another recent report indicated that the response to Nod signals depends on the stage of development of the root hair, and that changes in cytosolic Ca^{2+} concentrations may play different roles in Nod signal transduction (Felle, et al., 1999b). For example, changes in cytosolic Ca^{2+} concentrations in the apical part of the root hair may be related to root hair deformation. On the other hand, changes in cytosolic Ca^{2+} concentrations behind the root hair tip might be essential for amplification of the Nod signal and for transduction to trigger downstream events (Felle, et al, 1999b). Calcium is a well-established second messenger in plants and has been implicated in responses to pathogen elicitors and environmental stimuli (Roberts, 1992). Therefore, it is not surprising to see calcium implicated as a secondary messenger in the Nod signal response.

Plant Gene Induction in Response to Rhizobia and Nod Signals

ENOD40

Nod signal addition to plant roots, or inoculation with rhizobia, is known to induce the expression of proteins (i.e., nodulins) that are enriched in nodule tissue (See Figure 3). Nodulins induced rapidly upon rhizobial inoculation are termed early nodulins (i.e., ENOD) and have been postulated to play an important role in nodule development (Reviewed in Dénarié, et al., 1996, Long, 1996, and Cohn, et al., 1998). However, a specific biochemical function remains to be found for several *ENOD* genes. Some

Figure 3. Localization of early nodulin (ENOD) gene transcripts during nodule primordia formation. **I**, Representation of an indeterminate nodule primordium. **II**, Representation of a determinate nodule primordium. Note that the area of initial cell division in indeterminate nodules is closer to the root vasculature, while the first cell divisions in determinate nodules are closer to the epidermal cell layer. The information available on early nodulin expression has come from both determinate and indeterminate nodulating plants, so it is convenient to illustrate some of the pronounced differences in these two types of nodule structure. **A**, ENOD2 expression is most abundant in the cortical cells between primary and secondary meristems (not shown) and around the connecting vascular strand in determinate nodules. **B**, ENOD12 is expressed in both the actively dividing cells of nodules and in the meristematic region of the root cap. **C**, ENOD40 is first expressed in the pericycle of the root vascular bundle in advance of the induction of cortical cell division. After 72 hours, ENOD40 is strongly expressed in the pericycle and in the actively dividing subepidermal cortical cells. **D**, Increased levels of CHS1 and CHS3 have been detected in areas of nodule primordium formation, possibly altering endogenous hormone levels. **E**, root stele. A gradient may exist in which a factor from the stele, uridine, travels along the protoxylem poles, making cells in this area more sensitive to the action of Nod signals. (See text for more detail. Special thanks to my wife, Mrs. Susan Meadows for drawing the figure.)


B
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Nod Signal

ENOD genes are expressed in plant tissues other than the nodule, and orthologs have been found in non-leguminous plants, suggesting that they do not function exclusively in nodulation (Van de Sande, et al., 1996; Kouchi et al., 1999). However, the fact that many of these genes are expressed rapidly upon inoculation with rhizobia, or Nod signals, makes them very useful as molecular markers of Nod signal action on plants. *ENOD40* is first induced in response to both rhizobia and Nod signals in the root pericycle, and is subsequently expressed in dividing cortical cells (Kouchi and Hata, 1993, Crespi et al., 1994, Minami et al., 1996). Recent evidence indicates that ENOD40 might be an endogenous plant growth regulator. The sequence of *ENOD40* orthologs from several legumes, and two non-legumes, shows only a very short protein coding region (10-13 amino acids) at the 5'end and a significant 3' untranslated region (UTR) (Kouchi and Hata, 1993, Yang et al., 1993, Crespi et al., 1994, Vijn et al., 1995, Papadopoulou et al., 1996, Van de Sande, et al., 1996). The 3' UTR has been implicated as a possible riboregulator that might have a role in initiating plant cell divisions (Crespi, et al., 1994; Charon, et al., 1997). A recent study showed that over-expression of the *ENOD40* gene in transgenic plants induces dedifferentiation and division of root cortical cells (Charon, et al. 1997). These results suggest that Nod signal induction of *ENOD40* and subsequent modification of sensitivity to endogenous hormone levels may be directly involved in the de-differentiation of plant cells leading to nodule cell division. However, *ENOD40* induction in the absence of cell division has been reported (Minami et al., 1996a). Therefore, if this model is correct, other regulating factors must be involved.

Kouchi et al., (1999) reported the identification of an *ENOD40* ortholog from rice (*Oryza sativa*). In this study, *in situ* hybridization analysis indicated that *OsENOD40* is

expressed during the early stages of basipetal differentiation in the vascular bundles. *ENOD40* expression has also been detected in the vascular bundles of developing nodules (Yang et al., 1993, Minami et al., 1996a), thus the authors proposed that *ENOD40* orthologs from different plants might play a general role in cell differentiation.

ENOD2

ENOD2 has also been studied extensively, but less is known about its possible function in nodulation (Franssen et al., 1989, Van de wiel et al., 1990). The *ENOD2* gene encodes a (hydroxy)proline-rich protein that has been proposed to be a cell-wall protein, and the mRNA is exclusively localized in the parenchyma (inner cortex) of both determinate and indeterminate nodules (Van de Wiel et al., 1990). *ENOD2* expression has been demonstrated in alfalfa and *S. rostrata* roots in response to the addition of cytokinin (Cooper and Long 1994, Dehio and de Bruijn 1992). In addition, *ENOD2* expression is inducible by the application of auxin-transport inhibitors to alfalfa roots (Hirsch et al. 1989). *ENOD2* mRNA was shown to be inducible by inoculation of soybean roots (*Glycine soja*) with Nod signals. However induction required the cooperative action of at least two, structurally distinct molecules (Minami et al. 1996b).

ENOD12

Studies using both promoter-β-glucuronidase fusions and mRNA analysis of the *ENOD12* genes of alfalfa and *M. truncatula* have revealed that *ENOD12* is expressed within one to three hours post rhizobial inoculation (Scheres et al., 1990, Pichon et al., 1992, Horvath et al., 1993, Journet et al., 1994). Nod signals have been shown to be

necessary and sufficient to induce expression of this gene. Sequence analysis indicates that ENOD12 is a (hydroxy)proline-rich protein, similar to several other early nodulins (Scheres et al., 1990, Pichon et al., 1992). *ENOD12* has been shown to be expressed upon infection in the vicinity of the growing infection thread, and subsequently expressed in developing nodule tissues. Unlike some nodulins, for example *ENOD5, ENOD12* is expressed both in infected cells in the nodule as well as uninfected cells (Scheres et al., 1990). However, there have been reports that questioned the function of this gene during nodule development. For example, a *Medicago* subspecies lacking the *ENOD12* gene forms functional nodules (Csanadi et al., 1994). Thus, it appears that *ENOD12* expression is not required for nodule organogenesis or infection. However, it is possible that there are other, functionally redundant orthologs present in this subspecies, that may compensate for the lack of *ENOD12*. Regardless, the fact that ENOD12 is induced so rapidly in response to Nod signals makes it a useful tool for dissecting Nod signal transduction.

rip1

One gene that is rapidly induced upon inoculation with rhizobia, that does have a proposed function, is the *rip1* gene of *Medicago truncatula* (Cook et al. 1995). *rip1* mRNA is induced within three hours post rhizobial inoculation and is also inducible by Nod signals (Cook et al., 1995). The sequence of *rip1* indicates that it might encode a peroxidase, hence the name, Rhizobium induced peroxidase. The rapid but transient expression of this gene in response to inoculation with rhizobia suggests that an oxidative process may occur early in the symbiotic response of the plant. Oxidative processes are

known to be involved in the formation of cell walls and also play important roles in the defense response to pathogen attack (for reviews see Dangle et al., 1996, Hammond-Kossack and Jones 1996). Recent studies have indicated that an oxidative burst might be required for nodule formation. The data suggest that localized oxidative bursts, possibly due to *rip1*, in response to rhizobia might control the induction of downstream early nodulin genes (Senthil Ramu and Douglas Cook, personal communication). Addition of exogenous H_2O_2 was sufficient to induce the expression of early nodulin genes. This is supported by the fact that bacterial mutants that are defective in Nod signal formation do not induce the oxidative burst caused by wild-type rhizobia. Additionally, nodulation deficient plant mutants that do not display the very early responses to rhizobia, such as root hair deformation, are unable to produce localized oxidative bursts in response to rhizobia (Senthil Ramu and Douglas Cook, personal communication).

Annexins

Recently, a gene named *MtAnn1* was shown to be inducible by both rhizobia and Nod signals (Niebel, 1998). This gene has a high degree of sequence similarity to known annexin proteins. Annexins constitute a family of calcium-dependent, phospholipid-binding proteins identified in many eukaryotic organisms (reviewed in Moss 1997). These proteins generally are soluble proteins that can switch to a membrane- associated state in the presence of calcium. The role of annexins in either plants or animals is unclear. However, they have been implicated to play roles in transduction pathways of mitogenic signals, membrane trafficking processes, and the formation of voltage-dependent, ion-selective calcium channels (Moss, 1997, Raynal and

Pollard, 1994). Annexins from tomato and maize have been shown to have ATPase activity, and the tomato annexins are able to interact with the cytoskeletal element actin (McClung, et al., 1994, Calvert et al., 1996). An annexin from A*rabidopsis thaliana* has been proposed to play a role in the oxidative stress response (Gidrol et al., 1996). A function for the *MtAnn1* gene from *M. truncatula* in the nodulation process has been proposed based on reports of the possible roles of annexins in cytoskeletal rearrangement, and the fact that the gene is inducible by Nod signals.

ENOD20

Recently, MtENOD20 was shown to be a useful molecular marker of cortical cell activation in *M. varia* (Vernoud, 1999). ENOD20 was identified along with ENOD16 by low stringency screening of a *M. truncatula* genomic library with a pea ENOD5 cDNA probe. Both genes showed structural homology to copper-binding phytocyanins. *pMtENOD20-GUS* fusions showed that this gene is activated in inner cortical cells, corresponding to the sites of nodule initiation in indeterminate nodules. Additionally, it was shown that transcriptional activation of this gene is tightly linked to cortical cell activation using *Sinorhizobium meliloti nod* gene mutants that uncouple nodule organogenesis form infection. As is the case for many nodulins, the function of *ENOD20* is not known. However, it is still very useful as a molecular marker of Nod signal activity.

Structure Activity Relationship of Nod Signals

It is clear that the modifications made to the core structure of Nod signals (Figure 2) play an important role in host range determination. This was first demonstrated with purified Nod signals produced by *S. meliloti* (Truchet et al, 1991). As mentioned previously, Nod signals that lack a sulfate moiety at the reducing end are not active in biological assays on the normal host of *S. meliloti*, alfalfa (Truchet et al, 1991, Roche et al, 1992). Additionally, *S. meliloti nodH* mutants that do not produce Nod signals with the sulfate moiety are not able to nodulate alfalfa. However, *S. meliloti nodH* mutants retain the ability to nodulate an alternative host, vetch. In addition, only non-sulfated Nod signals are biologically active on vetch roots (Faucher, et al., 1989,Truchet et al, 1991).

As mentioned previously, Nod signals can cause calcium spiking in root hairs of alfalfa (Ehrhardt, et al, 1996). This spiking was shown only in response to Nod signals purified from a wild-type strain of rhizobia that are modified with 6-O-sulfate on the reducing end (Lerouge et al, 1990). Nod signals purified from *R. leguminosarum* bv. *viciae,* did not induce calcium oscillations on alfalfa root hairs. However, this cannot be solely attributed to the lack of a sulfate moiety. Nod signals produced by *R. leguminosarum* bv. *viciae,* although they are non-sulfated, are distinct from *S. meliloti* Nod signals, in that they are pentameric molecules that have quadruple unsaturated 18-carbon fatty acids on the non-reducing end (Spaink, et al, 1991).

In fact, changes in the acyl chain have been shown to be critical to the biological activity of Nod signals on alfalfa (Demont, et al, 1993, Ardourel et al., 1994, Demont-Caulet, et al. 1999). For example, *S. meliloti nodFE* mutants that produce Nod signals

that are *N*-acylated by a singly unsaturated, 18-carbon chain are severely affected in their ability to infect alfalfa root hairs (Ardourel, et al, 1994). In the study performed by Demont-Caulet, et al. (1999), the authors tested the ability of Nod signals with differing fatty acids at the non-reducing end to activate nodule-like structures (or cortical cell divisions) on alfalfa. The results of this work suggested that the length of the fatty acid chain is critical to biological activity. The wild-type reference Nod signal, modified with 16-carbon acyl chain that is doubly unsaturated, had the highest level of biological activity. Nod signals that were modified with acyl chains either greater or lesser than 16 carbons in length were much less biologically active compared to wild-type Nod signals. Additionally, Nod signals modified with 16-carbon acyl chains that contained only a single double bond were not as active as the wild-type reference molecule. The authors suggested that the fatty acid moiety might serve to insert the Nod signal into the membrane of plant roots cells.

In addition to studies of the acyl chain, the authors showed that the 6-*O*-sulfation at the reducing end, found in wild-type *S. meliloti* Nod signals, was essential to biological activity in their assay. These results support previous results mentioned earlier (Lerouge et al, 1990, Truchet et al, 1991, Erhardt et al, 1996), indicating the importance of the sulfate moiety for biological activity on alfalfa.

Although the 6-O-sulfate reducing end modification seems to be absolutely essential for biological activity of Nod signals on alfalfa, there have been reports that suggest that this requirement is not so stringent. For example, studies of the expression of the early nodulin gene ENOD12 indicated that a non-sulfated Nod signal was sufficient to significantly enhance *MsEnod12B* mRNA expression in the roots of alfalfa

(*M. sativa*) (Bauer, et al, 1994). These results are consistent with earlier results showing that *ENOD12* is inducible by heterologous Nod signals. Horvath et al, (1993) demonstrated that both *R. leguminosarum* bv. viciae Nod signals and Nod signals from *S. meliloti* were able to trigger the expression of the *ENOD12* gene in pea. *S. meliloti* Nod signals are not biologically active on pea in other biological assays. Pichon et al, (1993) reported induction of the *ENOD12* gene of *Medicago truncatula* by non-sulfated Nod signals. However, induction required four-fold higher concentrations of the non-sulfated Nod signal then the wild-type, sulfated Nod signal.

In addition to these studies, Gressent et al. (1999) demonstrated that the 6-*O*-sulfate moiety at the reducing end of *S. meliloti* Nod signals might not be required for binding to a putative receptor molecule identified in *Medicago* cell suspension cultures (Niebel, A. 1997). In this study, the authors examined the ability of different, substituted lipo-oligosaccharides to compete for a high affinity Nod signal binding site, NFBSII, present in the microsomal fraction of *Medicago varia* cell suspension cultures (Niebel, A. 1997). The results indicated that affinity of lipo-oligosaccharides for this binding site greatly depended on the structure of the acyl moiety. Theses results are similar to the results of Demont-Caulet et al. (1999) showing the importance of the acyl chain for biological activity of Nod signals on alfalfa. Additionally, O-acetylation of the non-reducing end sugar, but not sulfation of the reducing sugar influenced the affinity of lipo-oligosaccharides for NFBSII. Thus, this binding site might not be a bona fide Nod signal receptor, if sulfation of Nod signals is absolutely required for biological activity on alfalfa, as has been suggested.

All of the above studies have been performed on indeterminate nodulating plant hosts, and it is clear from these studies that these plants require a specifically modified Nod signal for biological activity (i.e., root hair deformation and cortical cell division). Studies with soybean, a determinate nodulating plant host, indicate that the requirements for biological activity of Nod signals on this plant are not so stringent. As mentioned previously, a *B. japonicum nodZ* mutant is able to form nitrogen fixing nodules on soybean (Stacey et al., 1994). The *nodZ* gene is responsible for the 2-*O*-methyl fucosylation of Nod signals produced by *B. japonicum* (Stacey et al. 1994, Lopez-Lara, et al., 1997, Quinto et al., 1997). However, fucosylation of the reducing end of *B. japonicum* Nod signals does play a critical role in the nodulation of an alternative host, siratro (Stacey et al., 1994). For example, a *B. japonicum nodZ* mutant is not able to nodulate this plant.

Minami et al. (1996a) showed that Nod signals of *B. japonicum* were able to activate mRNA expression of the soybean early nodulin gene ENOD40. In this study, it was also found that synthetic lipo-oligosaccharides and chitin pentamer, which were unable to induce cortical cell divisions, were able to induce the transient accumulation of ENOD40 mRNA. Chitin pentamer was found to induce ENOD40 mRNA within 24 hours after inoculation, but the level of mRNA transcript decreased to basal levels 6 days after inoculation. In contrast, wild-type *B. japonicum* Nod signal was able to induce a sustained increase in ENOD40 mRNA transcription. The results of this study suggested that, in the case of ENOD40, nodulation specificity is not determined at the level of initial gene expression.

Minami et al. (1996b) also showed that the *ENOD2* gene of soybean (*Glycine soja*) is inducible by Nod signals, but unlike *ENOD* genes previously studied, the induction of this gene required the cooperative action of at least two structurally distinct LCO (Lipo-chitooligosaccharide) molecules. These results indicated that differentiation of the parynchema, where *ENOD2* mRNA expression is localized (Van de Wiel et al., 1990), is not activated by single Nod signals. In contrast, it is well established that the differentiation of the cortex tissues is activated by single Nod signals (Truchet et al, 1991, Spaink et al., 1991, Mergaert et al. 1993, Stokkermans and Peters 1994). Due to the fact that *ENOD2* expression is inducible by the application of auxin-transport inhibitors and cytokinin (Hirsch et al., 1989, Dehio and de Bruijn, 1992, Cooper and Long, 1994), it is possible that the cooperative action of different Nod signals might result in an alteration of the phytohormonal balance in root cortical tissue. The study by Minami et al (1996b) showing that the action of multiple Nod signals is required for the induction of a gene thought to be involved in nodulation, gives a possible explanation for the fact that a given rhizobial species produces several structurally distinct Nod signals (Price et al., 1992, Schultze et al. 1992, Stokkermans et al. 1998).

Regulation of Nodule Induction

One of the more interesting questions relating to nodulation is how only certain root cells are triggered to divide by *Rhizobium* inoculation (Figure 3). In determinate nodulating plants, outer cortical cells divide first, while, in the formation of indeterminate nodules, the inner cortical cells are the sites of initial cell divisions (reviewed in Kijne, 1992, Roth and Stacey, 1991). Additional specificity is shown by the fact that, in both

types of plants, nodule primordia only form at what appear to be predetermined sites opposite the protoxylem poles. For several years it was hypothesized that specific cortical cells in this area of the root were cell cycle arrested (i.e., G2 arrested) and only these cells could respond to rhizobial infection (Savouré et al., 1994) . Savouré et al. 1994) measured the cell cycle state of alfalfa suspension cultures by examining *cdc*2, *cyc*2, and histone gene expression. This work showed that Nod signals stimulated cell cycle progression and mitosis. Yang, et al. (1994), using similar cell cycle markers, examined pea and alfalfa root cortical cells and showed that susceptibility to rhizobial inoculation did not correlate with a particular arrest in the cell cycle. Therefore, spatial control of cell division during nodulation must involve other regulatory mechanisms.

Libbenga et al. (1974) proposed that radial transport of chemical messengers from the root steele might control the spatial formation of nodule primordia in pea. In support of this hypothesis, extracts from the stele stimulated cortical cell divisions in sections of pea roots (Libbenga et al., 1974). Smit et al. (1995), recently identified uridine as the active component of the stele extract. It was proposed that a chemical gradient of uridine is established from the vasculature toward the protoxylem poles and this makes the cells more susceptible to Nod signal action. Previous results showing mitotic activation by Nod signal addition of narrow radial rows of cells opposite the protoxylem pole is consistent with this hypothesis (Yang et al., 1996). However, additional research is clearly needed to verify this hypothesis.

A consensus view is emerging that modification of the sensitivity of plant root cells to phytohormone and Nod signal action is likely involved in the initiation of nodule primordia formation. The previously discussed research on ENOD40 and uridine are

consistent with this notion. Some flavonoids that act to induce *nod* gene expression have been shown to be potent inhibitors of radial auxin transport (Savouré et al., 1994). As a possible positive feedback mechanism, *Rhizobium* inoculation induces the expression of the phenylpropanoid pathway leading to increased flavonoid synthesis (Savouré et al., 1994) . This increase in flavonoid synthesis may act, not only to produce more *nod* gene inducers, but also to modify the localized auxin levels within the root tissue (reviewed in Long, 1996, Cohn, et al., 1998). In support of this hypothesis, Mathesius et al. (1996) showed that GUS expression, arising from fusion with the promoters of the chalcone synthase genes CHS1 or CHS3 and β-glucuronidase, was significantly elevated in transgenic clover roots upon inoculation with either *R. leguminosarum* bv. trifolii or individual Nod signals. This increase in GUS expression correlated with sites of nodule primordia formation and a reduction in auxin transport as measured by the reduction in expression of the auxin inducible gene GH3 below the site of inoculation.

Recent work has also implicated ethylene in the spatial control of nodulation (Heidstra et al., 1997). Nodulation is inhibited by ethylene in several legumes (reviewed in Long et al, 1996). mRNA transcripts of ACC oxidase, which catalyzes the last step in ethylene biosynthesis, are expressed opposite the phloem poles of pea roots (Heidstra et al., 1997). As stated previously, nodule formation is generally initiated opposite the protoxylem poles (Savouré et al., 1994). Furthermore, when pea roots were grown in the presence of the ethylene inhibitors Ag^{+} or aminoethoxy-vinylglycine (AVG), a significant number of nodules formed opposite the phloem poles (Heidstra et al., 1997).

Negative Regulation of Nodulation

Nodule formation is also negatively controlled. For example, it is well established that the first nodules that form on the root suppress further nodulation via an unexplained feedback mechanism termed autoregulation (Gresshoff et al., 1992). Reciprocal grafts using wild-type soybean and the supernodulating soybean mutant NTS382 revealed that the autoregulatory mechanism was controlled by the genotype of the shoot (Delves et al., 1986). Recent work has implicated ethylene as a mediator of autoregulation, due to the fact that exogenous ethylene inhibits nodulation of several legume species (Heidstra et al., 1997). Support for this hypothesis comes from the study of a mutant of *M. truncatula*; *sickle.* The sickle mutation has the phenotype of insensitivity to exogenous ethylene (Penmetsa et al., 1997). This mutant line was found to be hyperinfected when inoculated with *S. meliloti,* a result consistent with a breakdown in the autoregulatory mechanism.

Although soybean nodulation exhibits autoregulation, exogenous ethylene does not inhibit nodule formation on most cultivars. However, some sensitive lines of soybean do respond to ethylene with a marked increase in chitinase production (Xie et al., 1996). It was suggested that this increase in chitinase might decrease Nod signal activity and, therefore, limit the number of effective rhizobial infections (Xie et al., 1996). This is an interesting result, due to the fact that one postulated model for Nod signal action relates to the ability of specific Nod signals to withstand chitinase degradation (Staehelin et al., 1994). It is intriguing to speculate that these enzymes might also be involved in the ethylene response.

Chito-Oligosaccharides as Endogenous Plant Hormones

The morphogenic and physiological effects of chitin lipo-oligosaccharides on plants have led to the suggestion that endogenous Nod signal-like compounds may exist in plants and act as signal molecules. At present, there is no firm evidence to support this notion. However, several lines of circumstantial evidence support the hypothesis. For example, some alfalfa lines form spontaneous nodules without the addition of exogenous Nod signals suggesting the presence of an endogenous signal (Truchet et al., 1989). Nod signals and chitin oligosaccharides have been shown to have developmental effects on non-leguminous plants, such as carrot and tobacco. De Jong et al. (1993) showed that Nod signal addition was sufficient to rescue somatic embryogenesis in a temperature sensitive line of carrot at the non-permissive temperature. Embryogenesis was also restored by the addition of an endochitinase, suggesting that chitinase action could be releasing a chitin molecule required for embryogenesis (de Jong et al., 1992). Schmidt et al. (1993) showed that expression of the *R. meliloti nodA* and *nodB* genes in transgenic tobacco significantly affected development. One explanation for these results is that these genes, encoding enzymes that modify the chitin Nod signal, are acting on endogenous chitin molecules within the plant. These data suggest that non-legumes can respond to Nod signal addition and that the response involves developmental pathways normally induced by classical phytohormones.

An exciting possibility is that chitin oligomers are an undiscovered class of developmental signals for both plants and animals. This speculation is supported by the discovery that DG42 (Differentially expressed in Gastrulation), a protein expressed in developing *Xenopus laevis* embryos, is a functionally interchangeable with the rhizobial

NodC protein, a chitin synthase (Semino et al., 1996). Homologues of DG42 and NodC have been found in zebrafish and mice (Semino et al., 1996). Conflicting reports indicate that DG42 is either directly involved in chitin-oligosaccharide synthesis or in the synthesis of hyaluronan (Varki, 1996). Hyaluronan is structurally similar to chitin, but is composed of interconnecting strands of β-1,4 linked N-acetylglucosamine and β-1,3 linked glucuronic acid. There has been at least one study indicating that chitin oligosaccharides may be playing a direct role in zebrafish development (Bakkers et al., 1997). Microinjection of the NodZ fucosyltransferase from *Bradyrhizobium japonicum* or of antibody raised against DG42 or into fertilized eggs of zebrafish led to severe defects in trunk and tail development.

Nod Signal Receptors: A Ligand In Search of a Mate

The diverse responses of plants to Nod signals and the precise nature of side groups needed for biological activity suggest that molecular recognition is accomplished by one, or possibly more plant receptors that interact with the bacterial signals. However, a bona fide Nod signal receptor has not been identified.

Chitin Binding Proteins

The fact that Nod signals act with high specificity at very low concentrations suggests that Nod signal perception is likely mediated by specific plant receptors. A Nod signal receptor should have the ability to respond to Nod signals at subnanomolar concentrations (i.e., at concentrations in which Nod signals have biological activity) with high specificity and possess the means to transduce this binding signal to other cellular

components. At present, there are no candidates that fulfill these requirements. High affinity, Nod signal binding activity has been measured in microsomal membrane preparations of cell suspensions of *Medicago truncatula* and *M. varia* (Bono et al., 1996). These experiments detected two binding activities; Nod Factor Binding Site I with a Kd=70-90nM and NFBS II with a much higher affinity (Kd=1.9nM). Additionally, chitin-binding sites have been reported from a number of different plant species (Shibuya et al., 1993, Shibuya et al., 1996, Baureithel et al., 1994, Day et al., unpublished). Recently, Day et al. (unpublished) identified an 85 kDa protein present in the plasma membrane of both suspension cultured soybean cells and soybean roots, that bound chitin octomers and pentamers with an approximate Kd of 35nM to 47nM, respectively. The discrepancy between the measures of binding affinities is likely due to differences in the preparation of the plasma membrane fractions from roots and suspension cultures, and not due to the presence of multiple binding sites (Day et al., submitted). As measured by competition studies, this site binds Nod signals, albeit at a much lower affinity than chitin octamers. Chitin tetramers showed approximately the same level of competition for the binding site as Nod signals (Day et al., submitted). The authors speculate that this protein is likely involved in a defense related response, and not involved in nodulation, as it is very similar in size and binding kinetics to the chitin binding protein identified in rice (Shibuya et al., 1996, Ito et al., 1996).

Multiple Receptor Models

Ardourel et al. (1994) proposed a model based on studies of *R. meliloti* nodulation mutants suggesting two Nod signal recognition events in alfalfa. This model postulated

the existence of a low stringency signaling receptor that mediates such events as root hair deformation, while a second, high affinity entry receptor was required to trigger rhizobial invasion. As mentioned previously, Minami et al.(1996a) undertook a related study utilizing the ability of purified and chemically synthesized lipo-chitin molecules to induce the expression of the early nodulin, *ENOD40*, in soybean roots. Early nodulins, such as ENOD40, are usually induced within 24 hours of inoculation. Minami et al. (1996a) found that *ENOD40* expression could be induced transiently by a simple non-acylated chitin pentamer. However, sustained expression of *ENOD40* required a soybean-specific Nod signal. In a second study, Minami et al.(1996b) examined the ability of specific chitin and lipo-chitin molecules to induce the expression of the early nodulin, *ENOD2*. In this case, no single chitin molecule induced *ENOD2* expression. As mentioned previously, *ENOD2* induction required the addition of a mixture of at least two chitin molecules, only one of which had to be a soybean-specific Nod signal. Taken together, the papers of Ardourel et al. (1994) and Minami et al. (1996a, 1996b), although differing in detail, argue for the existence of at least two Nod signal recognition events both in indeterminate and determinate nodulation. Whether such recognition is governed by two separate receptors or via some other mechanism remains to be determined.

A G-Protein Mechanism might mediate Nod Signal Transduction

A recent report proposed that Nod signal perception might be mediated by a classical G protein signal transduction mechanism (Pingret, et al., 1998). Using a series of pharmacological agents, in conjunction with transgenic plants expressing

p*MtENOD12*-GUS, the authors were able to show that Nod signals might indirectly interact with heterotrimeric G-proteins. In this study, it was shown that mastoparan, an activator of animal heterotrimeric GTP binding regulatory proteins, was able to stimulate transcription of the early nodulin gene *ENOD12*. In addition, pretreatment of roots with pertussis toxin, a well-characterized G protein antagonist, blocked the activity of Nod signals, as measured by *pMtENOD12-GUS* expression. Moreover, antagonists that interfere with phospholipase C activity and Ca^{2+} influx and release were shown to block the ability of Nod signals to stimulate *ENOD12* induction (Pingret et al., 1998). This study implied that Nod signals might interact with a member of the family of seven-transmembrane receptor proteins that have been extensively characterized in animal cells (Dohlman et al., 1991). However, this study used pharmacological agents that have not been extensively tested on plants. It is quite possible that the effects these chemicals have on nodulation are due to more general physiological effects on plant development. In addition, the function of the marker gene used in this study, *ENOD12*, is not known. As mentioned previously, *ENOD12* might not even be required for nodulation (Csanadi et al., 1994).

Chitinases May Play a Role in Nod signal Perception

Receptor-mediated Nod signal recognition is an attractive idea since it would explain the high specificity of Nod signal action. However, an alternative hypothesis has been proposed that would also explain Nod signal specificity. This model suggests that the ability to withstand plant chitinase activity may be an important determinant in Nod signal action and specificity. For example, Staehelin et. al. (1994) showed that various

purified *R. meliloti* Nod signals differed significantly in their ability to survive in the presence of chitinases produced by host plants. Specifically, sulfation at the reducing end of the chitin molecule prevented hydrolysis of the Nod signal. One could envision a system where Nod signal specificity is not determined by a specific signal-receptor recognition but by the ability of the chitin molecule to survive in the plant in the presence of potent chitinases. In the absence of conflicting data, this model is a viable alternative to explain how specific chemical modifications of the lipo-chitin Nod signal structure determines biological specificity.

Lectins

The carbohydrate structure of the Nod signal has led to the suggestion that a lectin could be a likely candidate for a Nod signal receptor. A postulated role for lectins in legume nodulation has had a long and controversial history. Interest in this idea was greatly stimulated by the work of Diaz et al. (1989). These workers constructed transgenic clover (*Trifolium repens* L.) roots expressing the pea lectin gene. Clover roots are normally nodulated very poorly by *Rhizobium leguminosarum* bv. *viciae*, the natural symbiont of pea. However, transgenic clover roots expressing the pea lectin gene were nodulated efficiently when inoculated by *R. leguminosarum* bv. *viciae*. Moreover, Diaz et al. (1995) showed, subsequently, that the pea seed lectin was oriented in the membrane of transgenic clover roots, in a fashion consistent with a role in Nod signal recognition. Further work showed that the sugar-binding site was critical for extending the host range of the transgenic clover roots, a finding consistent with the lectin binding to a carbohydrate molecule (Diaz, et al., 1995). These studies clearly showed that the pea

lectin can affect nodulation but did not provide unequivocal evidence that the lectin is a Nod signal receptor. For example, pea lectin has not been shown to specifically bind to Nod signals, nor is there an obvious method by which Nod signal binding could be converted to a cellular signal upon binding. However, a recent study repeated the results of Diaz et al (1989) by demonstrating that *Lotus corniculatus* nodulation host range can be extended by the presence of a soybean lectin gene (van Rhijn, et al., 1998). In this study, the authors introduced the soybean lectin gene Le1 into *L. corniculatus*, which is nodulated by *Mesorhizobium loti*. Transgenic plants possessed the ability to produce "nodule-like outgrowths" in response to the natural symbiont of soybean, *B. japonicum*. *B. japonicum* was not able to nodulate non-transformed wild-type lines of *L. corniculatus*. The authors also made transgenic lines with a mutated form of the Le1 protein. These plants did not respond to *B. japonicum* inoculation, indicating that the intact form of the protein was necessary to extend host range. An important feature of this study was the fact that Nod signals had the same effect on the transgenic plants carrying either the wild-type lectin gene or the mutated form. These results indicate that the lectin protein is not interacting with Nod signals. Moreover, results of this study indicated that the Le1 protein interacts with some component of the exopolysaccharide layer of the *B. japonicum* cells (van Rhijn, et al., 1998).

Another study recently published adds further support to the idea that lectins might play a role in nodulation. Diaz, et al. (2000) continued their study of the pea lectin, PSL, by transforming the *psl* gene into red clover hairy roots. Red clover roots normally respond only to the clover specific rhizobial symbiont *R. leguminosarum* bv. *trifolii*. Introduction of the *psl* gene into red clover hairy roots allowed the pea symbiont,

R. leguminosarum bv. *viciae* to induce nodules and nodule primordia on transgenic plants, similar to the results obtained previously with white clover hairy roots (Diaz, et al., 1989). *R. leguminosarum* bv. *viciae* is not able to nodulate red clover to any noticeable extent. This is unlike the previous study by Diaz et al. (1989) because *R. leguminosarum* bv. *viciae* does produce a weak response on wild-type white clover. The major finding in this study was that red clover roots transformed with the pea lectin gene responded positively to inoculation with Nod signals produced by a wide variety of rhizobia. All of the Nod signals tested on transgenic plants were active in cortical cell division assays, and in the formation of nodule-like structures. The authors of this study also reported that undecorated chitin oligosaccharides were mitogenic on transformed plants. Red clover hairy roots that had been transformed with a mutated form of the pea lectin gene did not respond to all of the Nod signal molecules. These plants only responded to Nod signals produced by their normal bacterial symbiont, *R. leguminosarum* bv. *trifolii.* These data indicate that the presence of the pea lectin gene somehow interferes with the normal barriers that prevent cortical cell division in response to improperly decorated Nod signals.

Recently, Etzler, et al. (1999) reported on a lectin, purified from the roots of *Dolichos biflorus,* that binds to Nod signals produced by *Bradyrhizobium japonicum. B. japonicum* can nodulate *D. biflorus.* This lectin possesses apyrase activity (nucleotide phosphatase) that is stimulated by Nod signal addition. Thus, the authors named the lectin gene LNP (Lectin Nucleotide Phosphohydrolase). This is an intriguing result since it suggests that this molecule possesses not only the ability to bind Nod signals but also enzymatic activity that may be involved in signal transduction. The *D. biflorus* lectin

LNP is unlike classical seed lectins but is apparently a member of a family of proteins found in both legumes and non-legumes (Roberts et al., 1999). In addition to the increase in enzymatic activity that the authors described, they also demonstrated that antibody raised against the protein was able to inhibit nodulation, indicating that this protein plays a critical role in the nodulation process. Day, et al. (2000) were able to repeat these results in soybean with antibody raised against an LNP ortholog from soybean. In this work, the authors also show that the soybean ortholog, GS52 is inducible within six hours after inoculation with the wild-type symbiont, *B. japonicum*, similar to early nodulin genes.

Apyrases

Apyrases, (ATP diphosphohydrolases, EC3.6.1.5) are enzymatic proteins that have been characterized in all prokaryotes and eukaryotes examined (Komoszyński and Wojtczak, 1996). The apyrases characterized to date have the common ability to hydrolyze ATP to ADP, AMP and orthophosphate. These enzymes are generally non-specific, in that they are able to use a variety of di- and tri-nucleotide phosphates as substrates, and are insensitive to inhibitors of P-type, F-type, and V-type ATPases (Lin, 1989, Komoszyński and Wojtczak, 1996). Apyrases have been grouped into two major classes of proteins; endo-apyrases, which have a catalytic domain inside the cell, and ecto-apyrases which have a catalytic domain outside of the cell (Komoszyński and Wojtczak, 1996). The biological function of these proteins is not very well understood, although there is a great deal of indirect evidence for functionality of most of the characterized apyrases.

Apyrases have been implicated in very diverse functions, such as blood platelet aggregation (Marcus and Safier, 1993), protein glycosylation in the Golgi lumen (Abeijon et al., 1993, Gao et al., 1999), neurotransmission (Sarkis and Salto, for review see Komoszyńsi and Wojtczak, 1996), phosphate metabolism (Thomas et al., 1999), and Nod signal recognition (Etzler et al., 1999). One function that has been proposed for apyrases in animals is to regulate blood platelet aggregation. Ectoapyrases present in the saliva of many insects that feed on blood can prevent blood platelet aggregation directly by decreasing the ADP concentration in damaged tissues (Marcus and Safier, 1993). Normally, the concentration of ADP in the extracellular space increases in response to damage. ADP is then able to bind to P_2 purinergic receptors on platelets, causing their concomitant aggregation (Marcus and Safier, 1993, Komoszyński, and Wojtczak, 1996). Apyrases found in animal cells have also been proposed to function in neurotransmission, also by interacting indirectly with P_2 purinoreceptors. These receptors have a high affinity for ATP and ADP, but a much lower affinity for AMP. Ectoapyrases present in synaptic membranes are thought to degrade ATP in the synaptic space. As the pool of ATP is hydrolyzed to AMP, the receptor is released, and its stimulation is broken (Marcus and Safier, 1993, Komoszyński and Wojtczak, 1996). In addition, apyrase effects on the ATP+ADP/AMP ratio modulate the action of 5'nucleotidase, an enzyme that is abundant in the synaptic space. This enzyme is inhibited by ATP and ADP, but is activated by AMP. Another interesting role for apyrases that has been reported is that they might be involved in the immune response. One apyrase found in humans, CD39, was reported to be a lymphoid cell activation antigen (Wang, et al., 1996, Wang, et al., 1998).

Two apyrase proteins identified from yeast (*Saccharomyces cerevisiae*), have been implicated in protein glycosylation (Abeijon, et al., 1993, Gao et al., 1999). These proteins are membrane-localized apyrases required for Golgi *N*- and *O*-glycosylation in *S. cerevisiae*. Mannosylation of *N*- and *O*-linked oligosaccharides was shown to be regulated by a GDPase that converts GDP to GMP (Abeijon et al., 1993). A deletion in the *gda1* gene of *S. cerevisiae* that encodes the GDPase protein resulted in several phenotypes related to an inability to glycosylate membrane proteins including; disruption of the elongation of *N*-linked carbohydrates of carboxypeptidase Y, blocking of the elongation of *O*-linked carbohydrate chains in mannoproteins and secreted chitinases, reduction in glycosylation of invertases, and defects in the biosynthesis of mannosylinositolphorylceramides. All of these proteins are believed to play a role in cell wall biosynthesis (Abeijon et al., 1993).

Recently, Gao, et al. (1999) reported that a homolog of GDA1, YND1 was an endo-apyrase that functioned in protein glycosylation. The authors of this work found that overexpressing YND1 in a ΔGDA1 background was sufficient to complement the mutation, implying that the function of these two genes is redundant. In addition, a double mutant, GDA1/YND1, was constructed that was severely affected in its growth rate compared to wild-type *S. cerevisiae*. Severe defects in cell wall integrity were also observed, further implicating the involvement of these proteins in cell wall biosynthesis

Apyrases that have been identified in plants have also been proposed to be involved in a variety of different functions. An apyrase protein identified in pea, pea NTPase, was originally purified from nuclei and was thought to play a role in mediating phytochrome responses (Chen and Roux, 1986, Chen et al., 1987). In one study, it was

reported that Ca^{2+}/Calmodulin stimulated apyrase activity of the pea NTPase (Chen et al., 1987). Hsieh et al. (1996) found that NTPase mRNA was expressed in the root tissues of light grown plants, and in etiolated plumules. However, the gene was not expressed in leaves of light grown plants, indicating that light was involved in the regulation of this gene. This is an interesting finding, due to the fact that the pea NTPase is a putative ortholog of LNP from *D. biflorus*. These data provide more circumstantial evidence that apyrases might play a role in nodulation, because it is well established that light inhibits nodulation.

The pea NTPase was recently reported to be involved in phosphate uptake and transport (Thomas et al., 1999). The authors demonstrated that transgenic *Arabidopsis* plants expressing the NTPase from pea showed enhanced growth compared to wild-type plants. The enhanced growth was reported to be due to the transgenic plants' increased ability to utilize inorganic phosphate, supplied as ATP. The authors also showed that the pea NTPase was able to complement a yeast Pi-transport mutant. Although the authors in this study claimed that the apyrase might be involved in phosphate transport, they did not provide a plausible explanation as to how the enzyme was involved in this system. This study did provide evidence, however, that the NTPase is an ectoapyrase and is localized in the plasma membrane of plant cells, as well as in nuclei, which was reported previously (Chen et al., 1987).

As mentioned above, LNP from *Dolichos biflorus* was recently shown to bind to rhizobial Nod signals (Etzler et al., 1999). The protein was found to bind Nod signals from a variety of rhizobia, but the highest apparent affinity for binding was to Nod signals from *B. japonicum* and *Rhizobium* sp. NGR234, both of which are able to

nodulate *D. biflorus*. Using fluorescent antibody labeling, Etzler et al., (1999) found that the protein was localized on the root hairs of *D. biflorus* plants. These results, taken together with the fact that the protein possesses apyrase activity that is stimulated by Nod signals, indicate that LNP might be involved in Nod signal recognition with the ability to transduce the signal via its enzymatic activity. It is important to note, however, that no receptor molecules thus far described possess nucleotide phosphohydrolase activity. Thus, it is difficult to imagine how LNP might function as a classical receptor protein.

Rational For Present Work

Although there is a great deal of information about the effects that Nod signals have on plants, relatively little is known about how plants perceive Nod signals. It has been known for some time that different rhizobial species produce a wide variety of structurally distinct LCO molecules. This structural diversity is believed to a play a major role in the host specificity observed in the symbiosis between legumes and rhizobia. Although a few studies have examined the structure-activity relationships of LCOs isolated from *Rhizobium* sp., these studies have been limited by their reliance on naturally produced material. Rhizobial strains such as *Rhizobium* sp NGR234, *Rhizobium fredii*, and *B. elkanii*, synthesize a wide variety of LCO molecules. However, it has proved very difficult to isolate all of the different molecular species. Therefore, we used synthetic LCO to perform a detailed structure-function analysis of LCO molecules on soybean. These studies were extended to include another determinate nodulating plant, rice bean (*Vigna umbellata*). The results of these studies revealed that the most important parameters for biological activity were fucosylation of the reducing

end of the LCO molecules and chitin chain length. Additionally, we found that fucosylation was required for biological activity on rice bean. However, this limitation can be overcome by the cooperative activity of multiple, structurally distinct Nod signals.

Studies were also performed on a family of apyrase genes found in the model legume *Medicago truncatula*. These genes are orthologous to a recently reported Nod signal binding protein isolated from the roots of *Dolichos biflorus* that was shown to be an apyrase protein. The results of this study revealed that at least two of the apyrase genes in *M. truncatula* are induced by the bacterial symbiont *Sinorhizobium meliloti*. The genes were also studied for their tissue specific expression, and the results indicate that the apyrase genes are differentially expressed in various plant tissues. Mapping studies indicate that at least three of the apyrase genes are tightly clustered in a short region in the *M. truncatula* genome. Additional studies indicate that the apyrase genes are not significantly expressed in nodulation deficient lines of *M. truncatula*. These data, along with the previous work showing that the *D. biflorus* apyrase protein binds Nod signals, suggests that these genes might play a role in early nodulation events.

CHAPTER 2

MATERIALS AND METHODS

Bacterial Strains and Plasmids

All bacterial strains and plasmids are listed in Table 4. Construction of some of the strains that were used in this work is described in the text.

Bacteria Culture Media and Growth Conditions

All strains of *Escherichia coli* were grown and maintained on LB medium (Sambrook et al., 1989) at 37°C. *Bradyrhizobium japonicum* and *B. elkanii* strains were grown and maintained on RDY medium (So et al., 1987) at 30°C. All *S. meliloti* strains and *Rhizobium* sp. NGR234 were grown and maintained on TY medium (Beringer, et al., 1974) at 30°C.

Antibiotics used in this study for selection and plasmid maintenance in all bacterial strains were: 100μg/ml ampicillin, 25μg/ml tetracycline, 50μg/ml rifampicin, 50μg/m kanamycin sulfate, and 100μg/ml streptomycin. Minimal medium (Bergersen, 1961) was used for growth of *B. japonicum* strains for isolation of Nod Signals. Minimal medium used in this study consisted of 0.3 g/L KH_2PO_4, 0,3 g/L K_2HPO_4, 0.1 g/L $MgSO_4 \cdot 7H_2O$, 1 mL of 1000X trace elements solution, 0.5 g/L NH_4NO_3, 0.4% glycerol, 0.0002 g/L biotin, 0.0001g/L calcium pantothenate, pH7.0.

Table 4. Bacterial strains and plasmids used in study.

Strain or Plasmid	Relavent Characteristic(s)	Source or reference
E. coli JM109	*endA1, recA1, gyrA96, thi, hsdR17, (r_k^- M_k^+), relA1, supE44, Δ(lac-proAB), F', traD36, proAB, lacI^{q}ZΔM15]*	Messing, 1983
E. coli INVαF'	F', *endA1, recA1, hsdR17, (r_k^-,m_k^+),* SupE44 thi-a, gyA96, relA1, F80lacZΔDM15D(lacZYA-argF)U169	Invitrogen, San Diego, CA
E. coli TOP10	F', *endA1, recA1, hsdR17, (r_k^-,m_k^+),* SupE44 thi-a, gyA96, relA1, F80lacZΔDM15D(lacZYA-argF)U169	Invitrogen, San Diego, CA
pPCR2.1	Ap^r, Km^r, T7 promoter	Invitrogen, San Diego, CA
pPCR-TOPO	Ap^R, Km^R	Invitrogen, San Diego, CA
pBLUEscript SK+/-	Ap^R	Stratagene, La Jolla, CA
pH3	Ap^R	D. Cook
pACTIN	Ap^R,Km^R	This work
B. japonicum USDA110	wild type, Cm^R	USDA, Beltsville, MD
B. elkanii USDA61	wild type	USDA, Beltsville, MD
NAD138	USDA110 *nodZ*::Tn*5*	Nieuwkoop, et al. 1987
S. meliloti ABS7M	wild-type	D. Cook
S. meliloti 1021	wild type, Sm^r	G. Walker, S. Long
S. meliloti SL44	1021 Δ*nodD$_1$ABC*	Sharon Long, unpublished
S. meliloti AK631	wild type	A. Kondorosi
S. meliloti AK1657	AK631 *nodC*::Tn*5*	A. Kondorosi
Rhizobium sp. Strain NGR234	very broad host range	A. Kondorosi
pJRC10	850bp TD-PCR product of *Mtapy1* cloned into PCR2.1	This Study
pJRC 30	340 bp 3' RACE product of *Mtapy1*, cloned into PCR2.1	This Study
pJRC42	650 bp 5' RACE product of *Mtapy1*, cloned into PCR2.1	This Study

Table 4 (Continued)

Strain or Plasmid	Relavent Characteristic(s)	Source or reference
pJRC63	1416 bp *Mtapy1* PCR product cloned into pBLUEScipt	This Study
pJRC201	450 bp RT-PCR product of *Mtapy2* cloned into PCR2.1	This Study
pKVO-1M6A	2.9 Kbp EST clone of *Mtapy2*	K. VandenBosch
pKVO-1M6B	2.9 Kbp EST clone of *Mtapy2*	K. VandenBosch
pJRC205	177 bp PCR product of *Mtapy2* cloned into pPCR-TOPO	This study
pJRC300	776 bp PCR product of *Mtapy3* cloned into pPCR-TOPO	This study
pJRC400	1017 bp 3' RACE product of *Mtapy4* cloned into pPCR-TOPO	This study
58M19	Bacterial Artifical Chromosome containing ~ 100 Kbp genomic DNA fragment from *M. truncatula*	D. Cook
52G10	Bacterial Artifical Chromosome containing ~ 90 Kbp genomic DNA fragment from *M. truncatula*	D. Cook

Plant Material and Growth Conditions

Glycine soja (PI 468397), *G. max*, and *Vigna umbellata* (Thunb.) Ohwi & Ohashi seeds were surface sterilized in 1% hypochlorite for 10 minutes, and washed with sterile, distilled water. The seeds were then treated with 0.01 N HCl for 10 minutes, and rinsed thoroughly with water. Prior to this step, *G. soja* seeds were scarified in concentrated H_2SO_4 for 5 to 10 minutes and washed thoroughly with sterile, distilled water. This step was necessary due to the very hard seed coat present on these seeds. The seeds were then transferred to moist, sterile Whatman paper in disposable plastic petri dishes and germinated in the dark for 2 days.

For germination of *Medicago truncatula*, seed were treated with concentrated H_2SO_4 for 7-10 minutes and rinsed thoroughly with distilled water. Seeds were then treated with 5% hypochlorite for 2 to 5 minutes and rinsed thoroughly with sterile, distilled water. *M. truncatula* seeds were allowed to imbibe in sterile, distilled water for 3 to 4 hours at room temperature with gentle shaking. The seeds were then transferred to moist, sterile Whatman paper in disposable plastic petri dishes and wrapped with parafilm. The seeds were vernalized at 4 °C in the dark for at least 48 hours. Seeds were then transferred to room temperature and germinated in the dark for an additional 24 hours.

For plants that were grown for bacterial inoculation studies, germinated seedlings were aseptically transferred either to sterile growth pouches (Mega Int., Minneapolis) or to aeroponic chambers. Growth of *M. truncatula* in aeroponic chambers was performed as described by Gallusci et al, 1991. Aeroponic chambers, or cassons, were constructed using plastic trashcans obtained from Walmart, Inc. Humidifiers were placed into the

bottom of the cassons and were held in place by a specially designed plexi-glass support that was donated by Douglas Cook at Texas A&M University. Defensor 5-S model humidifiers obtained from AxAir Ltd. (Switzerland) were used as a misting source for the inorganic nutrient media (Lullien et al., 1987). The lids of the cassons were constructed of black plexiglass to protect the plant roots from light. In order to support plant growth, the plexi-glass lids had approximately 1000 2mm diameter holes drilled through them. The lids of the cassons were fastened to, modified, trashcan lids so that they would fasten onto the tops of the casson tanks. In order to maintain humidity, a circular plexi-glass cover was constructed that would fit onto the lids of cassons, which were covered with fresh plastic film with every new use of the cassons. All parts of the cassons were thoroughly cleansed with soap and water and sterilized with 5% hypochlorite solution for at least 1 hour. The cassons were then carefully disassembled and every part of the casson was thoroughly rinsed with distilled water. Before introduction of plants into the cassons, the cassons were filled with 10 liters of sterile inorganic nutrient medium (Lullien et al., 1987). This method allowed for the growth of consistent plant roots, and consistent spraying of roots with bacterial inoculum.

For growth of all plants other than *M. truncatula*, ½ strength PNS was used (Wacek and Brill, 1976). *M. truncatula* were grown in inorganic nutrient medium (Lullien et al., 1987) when grown in pouches and when grown aeroponically. Plants were grown under a 16 hour day/ 8 hour night cycle. All plants excluding *M. truncatula* were grown at 28°C during the day cycle, and 22C° during the night. *M. truncatula* grown aeroponically received the same light conditions, but were grown at 22°C during the day and 18°C during the night.

Rhizobial Inoculation

Inoculation of Soybean and *Vigna* species

Bacteria were grown to log phase in Bergersen's minimal medium (Bergersen 1961) and diluted in sterile water to 10^7 cells per ml. Plants were inoculated 5 days post imbibition. Ten milliliters of rhizobial inoculum was applied per plant grown in sterile vermiculite pots and 1 ml per plant for plants grown in growth pouches. Plants were grown in a growth chamber (Percival Scientific, Boone, Iowa) with a light dark cycle of 16/8 h at 28/22° C with 70 to 80% relative humidity. Nodule formation was analyzed 12 to 18 days post inoculation.

Inoculation of *Medicago truncatula*

For inoculation of plants grown in aeroponic chambers, bacteria were grown to late log phase (OD_{600} 1.0-1.5) in TY medium (Beringer, et al., 1974) supplemented with the appropriate antibiotics. Bacteria were then centrifuged, washed and resuspended in sterile, distilled water. Bacteria were then added directly to nitrogen deficient plant nutrient solution in the aeroponic chambers (Lullien et al., 1987). Nodulation was rapid and uniform under these conditions. Plants were grown under a 16/8 hour light/dark cycle (22°Clight/18°C dark).

For inoculation of plants grown in growth pouches, bacteria were grown to log phase (OD_{600} = 0.5-1.0) in TY medium and an aliquot was transferred to fresh medium to monitor growth. Bacterial cultures were grown to an OD_{600} of 1.0 and washed by centrifugation. Bacterial cultures were resuspended in inorganic nutrient media to yield

approximately 10^7 cells/ml (OD_{600} = 0.1). 1ml of this cell suspension was added to each plant grown in pouches. Nodulation was also rapid and uniform under these conditions.

Nomenclature of Lipo-Chitin Oligosaccharides Used in Study

The accepted nomenclature for LCOs incorporates the structure giving nodulation-inducing activity and the bacterial species name from which the LCO was isolated. For example, the major LCO molecule produced by *Bradyrhizobium japonicum* is named Nod*Bj*-V(C18:1,MeFuc) and the major LCO produced by *Sinorhizobium meliloti* is named Nod*Sm*-IV(C16:2Δ2,9;Ac,S). In the case of *B. japonicum*, the molecule is a chitin pentamer with vaccenic acid (18:1) at the non-reducing end, and a 2-*O*-methyl fucosyl moiety at the reducing end (Sanjuan, et al., 1993). For the synthetic LCO molecules used in this study, we replaced the Nod and bacterial name designations with LCO to distinguish those molecules that are naturally produced and those that were chemically synthesized. By referring to the chemical nature of the synthetic molecule rather than its possible biological activity, any potential confusion is removed by calling a molecule a Nod signal when no biological activity has been demonstrated.

Synthesis of LCO

Synthetic LCOs were prepared in a steereocontrolled manner, as described previously (Ikeshita et al., 1994a, 1994b). Briefly, retrosynthetic bond disconnections were used to design a key intermediate, a modified chitin tetramer compound, so that any acyl group could be introduced efficiently at the final step of synthesis. Substitutioins to

the reducing end were made as previously described (Ikeshita et al, 1994a). Stock solutions were made from gavimetrically determined masses of synthetic LCO.

Root Hair Deformation Assays

Glycine soja seeds (PI468397) were surface sterilized as described above and used in root hair deformation (HAD) assays performed essentially as described by Bhuvaneswari and Sohleim (1985). In brief, seedlings were transferred to sterile tubes containing 1ml of plant nutrient solution (Wacek and Brill, 1976) and placed onto strips of sterile Whatman filter paper. LCO molecules were added at concentrations ranging from 10^{-8} to 10^{-15} M by dilution of a 1μM stock in 50% acetonitrile:water. A similar dilution of a 50% acetonitrile:water solution served as the negative control. Roots were kept in the dark by wrapping the test tubes with aluminum foil or dark paper. Roots were examined under a stereomicroscope for HAD 16 to 48 h post treatment. Deformation was scored in the zone of newly emerging root hairs and elongating root hairs from 2 to 10 mm from the root tip. Root hairs that were mature at the time of treatment were not included in the scoring. Types of deformation observed and scored were; swelling of the root hair tip, curving root hairs, and corkscrew-like hairs. All types of deformation were considered equally. For each LCO and control, 10 roots were scored for HAD on a scale of 0 to 3. The proportion of root hairs showing deformation was scored as follows: 0 for 0 to 10%; 1 for 10-25%; 2 for 30-60%; and 3 for greater than 60%.

Nodule Initiation Assays

LCO were diluted to 1mg/ml in 1% carboxymethylcellulose (CMC) (Sigma, St.

Louis, MO, USA) to increase the viscosity of the solution. Resuspension in CMC allowed LCO to remain on the roots for a longer period of time as compared to LCO resuspended in water alone. Spot inoculations were performed on *G. soja* (PI468397) plants grown in plastic growth pouches (Mega Int., Minneapolis, MN). The plastic pouches were aseptically cut open to reveal plant roots. Roots were spot inoculated in the region of emerging root hairs with a single spot of approximately 100nl of LCO dissolved in 1% CMC. The plastic was placed back over the roots, with sterile plastic straws in place to prevent the plastic touching the roots, or the LCO added, and taped into position. The plastic growth pouches were then returned to paper folders to protect the roots from light exposure. After 12 days of growth in the growth chamber, roots were examined to identify positive or negative responses to the LCO added. The plant response was scored as negative if no response detected, or positive if the following responses were detected; cortical cell divisions only, nodule primordia, or emerging nodule. For statistical analysis, all types of positive responses were grouped together such that the requency of a positive response with 100ng of LCO per spot was compared among the different LCO molecules tested. Differences in the frequency of positive responses were evaluated using Fisher's PLSD (Milliken and Johnson, 1984) as implemented in Statview for Macintosh (Abacus Concepts, Berkeley, CA). All statistical analysis was performed by Tom Stokkermans and Kent Peters while at the Ohio State Biotechnology Center (Ohio State University, Columbus, OH).

Spot Inoculation Assays Using Mixtures of LCOs

LCO from *B. elkanii* strain USDA61 and *B. japonicum* strain NAD138 were isolated as described in Carlson et al. (1993). The chemical structures of these LCO molecules were determined previously and are shown in Figure 4 and Table 6 (Carlson, et al. 1993; Stacey et al. 1994; Stokkermans et al. 1996). Concentrations were determined using an extinction coefficient of 1445 $cm^{-1}M^{-1}$ at 210 nm. To estimate the molar concentration of LCO in fractions that had been isolated as mixtures, the average molecular weight of the LCO molecules in the fraction was used. For example, the average molecular weight of LCO in peak 1 is 1366 g mol^{-1} (Table 11). For the mixtures used, the concentration of each peak in figure 7 was calculated and first resuspended in 50% v/v acetonitrile/ H_2O. Equal volumes of each solution which contained a single peak was mixed in a separate vial with a 1 mg/ml solution of the major LCO, Nod*Bj*-V(C18:1), produced by strain NAD138 and purified as described previously (Stacey et al. 1994). This solution was dried under nitrogen and resuspended to 1 mg/ml in 1% carboxymethyl cellulose. The LCO used in these experiments are the same LCO fractions used previously for structural analysis (i.e., Carlson, et al. 1993; Stacey et. al. 1994), excluding the LCO isolated in this work from NAD138. Synthetic LCO were kindly provided by Dr. T. Ogawa (The Institute of Physical and Chemical Research, Wako, Saitama 351-01, Japan).

Preparation of Root Tissue

Roots were cleared for analysis following the method of Truchet et *al.* (1989). A 3 cm piece of treated root sample, including the inoculation site, was cut from the root

and placed in 1% hypochlorite for 2 h. The roots were placed in 0.01% (w/v) methylene blue for 1 h, destained in 35% ethanol for 1 h and 70% ethanol for 10 to 16 h. The frequency of nodule initiation was monitored by observation under the stereomicroscope at 10 to 40 X magnifications. In most cases, roots were cleared and stained to identify true positives, but the nodule-like structures were often large enough that this was not necessary in most cases. This was the case for all inoculations performed on rice bean (*Vigna umbellata*). The structures formed in response to LCO on these plants were much larger than the structures formed on *G. soja*.

For sectioning, roots were fixed at room temperature for 12 to 24 hours with gentle agitation in 4% paraformaldehyde in 10mM sodium phophate buffer, pH 7.2 with 150 mM NaCl (PBS). The samples were rinsed in PBS and dehydrated with an ethanol series to 96% ethanol. Infiltration and embedding in JB-4 resin (Polysciences, Warrington, PA) were performed according to the manufacturer's specifications. Alternatively, nodule tissue was fixed and sectioned as previously described in Roth and Stacey (1989).

Microscopy and Photography

Microscopy and photography of *V. umbellata* nodule structures was performed by Tom Stokkermans (while at the Ohio State Biotechnology Center, Ohio State University, Columbus, OH). Longitudinal serial sections of 8mm thickness were cut and transferred onto drops of water on slides, heated to dryness, stained with 0.01% methylene blue for 30 min, and destained with 70% ethanol for 1 hour. Sectioins werere photographed with a

Nikon FX-35WA camera together with a Nikon AFX-IIA photomicrographic system on a Zeiss AxinKop Microscope at 100X and 200X magnification.

Nod Signal Purification and Analysis

LCOs were purified from *Bradyrhizobium* strains as previously described (Sanjuan et al, 1992). Briefly, bacterial cultures were grown in 3 mL of RDY medium (So et al, 1987), supplemented with the appropriate antibiotics, until late log phase (OD_{600} = 1.0 or greater). Aliquots were transferred to 100ml of fresh RDY medium, also supplemented with antibiotics until they reached an optical density between 0.5-0.8. Cultures were pelleted by centrifugation and washed once with minimal medium. The pellets were resuspended in 5 to 8 liters of minimal media (Bergerson, 1961) with no antibiotics and grown at 30°C until they reached an optical density of 0.1. Cells were then induced by the addition of soybean seed extract and allowed to grow for 24-40 hours. Soybean seed extract was prepared by incubating/shaking *Glycine max* cv. Essex seeds in a 50/50 (v/v) mix of water and ethanol (1ml/seed) for 12-24 hours at 37°C. The supernatant was filtered and concentrated to $1/10^{th}$ of the initial volume. 20 ml of this solution was added per liter of culture. Cultures were extracted using 1/3 volume of distilled 1-butanol. The butanol phase was allowed to separate by gravity over night and removed from the aqueous phase. The butanol extract was dried down by rotary evaporation and the residue was resuspended in acetonitrile/water (50:50, vol/vol). This mixture was then chromatographed on a silica gel 60 column (Pharmacia LKB, 1.6 X 100 cm). Fractions were eluted with a 60:40 acetonitrile/water gradient. The fractions were collected and those fractions with the most color, previously shown to be biologically

active, were further purified by reverse-phase HPLC (Waters 501 with a diode array detector), with a Pharmacia LKB SuperPAC Pep-S column (5-μm, 4 X 250mm) using a 20:80 acetonitrile/water gradient. Millennium software (Waters) was used to analyze the data. The purity of all samples was confirmed by mass spectrometry (MS).

Mass Spectrometry

Dr. Russell Carlson (Complex Carbohydrate Research Center, University of Georgia, Athens, Georgia) performed all mass spectrometry analysis of LCO. All mass spectra (MS) were obtained using a Jeol (Tokyo, Japan) SX/SX 102A tandem four-sector mass spectrometer, which was operated at 10kV accelerating potential. Ions were produced by fast atom bombardment (FAB) with xenon using a JEOL FAB gun operated at 6kV in a conventional FAB ion source. Spectra acquired for the first MS are averaged profile data of 3 scans as recorded by a Jeol complement data system. These spectra were acquired from 200 to 2000 m/x at a rate that would scan from m/z 0 to m/z 2500 in 1 min. A filtering rate of 100Hz and an approximate resolution of 1000 (a 10% valley) were used in acquiring these spectra. The samples were dissolved in dimethyl sulfoxide and 1ml aliquots were mixed with an equal volume of the FAB matrix, thioglycerol (TG), on the probe tip. The tandem MS analyses were performed on the monoisotopic mass ions. Collisional activation was performed in the third field free region, using helium as the collision gas. The helium pressure was sufficient to attenuate the primary ion beam by 75%. The collision cell was floated at 3kV, providing a collision energy of 7kV. Acquired tandem MS are averaged profiles of 4 scans as recorded by the JEOL complement data system.

Nucleic Acid Isolation and Manipulation

Plasmid DNA Isolation

Plasmid DNA was isolated from *E. coli* using either the alkaline lysis method (Sambrook, et al, 1989) or using the Wizard® Plus Miniprep DNA Purification kit (Promega, Madison, WI) according to the manufacturers instructions. BAC DNA was isolated using the MIDI Prep DNA isolation kit available from Qiagen (Qiagen, Valencia, CA) according to the manufacturer's instructions. DNA concentrations were determined using a DyNA Quant 200 fluorometer (Hoefer Pharmacia Biotech, San Francisco, CA).

Isolation of DNA Fragments

DNA restriction endonuclease fragments or PCR fragments used in cloning were separated by agarose gel electrophoresis and visualized by ethidium bromide staining Sambrook et al, 1989). The fragments were purified using a DNA Gel Extraction Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

Genomic DNA Isolation

Five day old seedlings of *M. truncatula* were ground under liquid Nitrogen in a mortar and pestle and genomic DNA was isolated using Promega's Wizard® Genomic DNA kit (Promega, Madison, WI) according to the manufacturers instructions.

RNA Isolation

Two methods were employed to isolate total RNA from *M. truncatula*. Initially, RNA was isolated using the Trizol® Reagent (Gibco BRL, Gaithersburg, MD) according to the manufacturer's protocol. The yields of RNA using this method were inconsistent, however, so a second protocol was also used. The method is essentially the Guanidine-HCl method (described in Sambrook et al, 1989) with some modifications (Cook et al., 1995). For RNA isolation, tissue samples were quick frozen in liquid nitrogen and stored at -80°C until use. The frozen tissue samples were ground to a powder by mortar and pestle in liquid nitrogen, resuspended in CSB buffer (7.5M guanidine HCl, 25mM sodium citrate, 0.5% sodium lauryl sarcosine, 0.1M β-mercaptoethanol), and homogenized in a polytron for 30 seconds. The homogenate was mixed with 2M sodium acetate, pH 4.0, extracted with an equal volume of phenol-chloroform (1:1), and centrifuged until the phases were well separated. The upper, aqueous phase was collected and precipitated with an equal volume of isopropanol for at least 1 hour at -20°C. RNA was recovered by centrifugation, resuspended in CSB, and again precipitated with isopropanol. The purified RNA pellet was resuspended in RNase-free (DEPC treated) sterile, distilled water and stored at -80°C after mixing with 3 volumes of absolute ethanol and $1/10^{th}$ volume of 3M sodium acetate, pH 5.2.

Isolation and cloning of *Mtapy1*

Reverse Transcription PCR

Total RNA was isolated from 3, 5, 7, and 9 day old *M. truncatula* roots using the Trizol® reagent (Gibco BRL, Gaithersburg, MD). The quality and quantity of RNA isolated was measured spectrophotometrically by optical density readings at 260/280 nm, and by agarose gel electrophoresis (i.e., by comparison to standards). RNA samples were treated with RNase free DNase I from Promega (Madison, WI) according to the manufacturer's protocol. DNase I was heat inactivated at 65°C for 15 minutes. RNA was then reverse transcribed using M-MLV reverse transcriptase from Promega (Madison, WI) using oligo-$(dT)_{12-18}$ as a primer, in the presence of the ribonuclease inhibitor RNasin (Promega, Madison, WI). All manipulations were carried out according to the manufacturer's protocol. Products were precipitated in 2.5 volumes of absolute ethanol with the addition of one-tenth volume of 3M sodium acetate, pH 5.5. The reverse transcribed products were resuspended in 50μL of sterile, distilled water. Five microliters of this solution was used in a polymerase chain reaction (PCR) including 10mM Tris-HCl (pH 8.0), 50 mM KCl, 2.5 mM $MgCl_2$, 2 units Taq DNA Polymerase (Promega, Madison, WI). The PCR reaction conditions varied according to the primer sets used. For cloning *Mtapy1,* degenerate primers were designed from conserved apyrase motifs present in pea (Hsieh, et al., 1996), potato (Handa and Guidotti, 1996), and LNP (Etzler et al., 1999). Primers were used in a final concentration of 2μM in all reactions. For this reaction, a 'touchdown' (TD) PCR method was employed, that involved steadily decreasing the annealing temperature of the reaction so that the primers

would bind the product initially at high stringency, then amplify this product under lower stringency, allowing for the degeneracy of the primers. The primers used for TD-PCR were TD-Apyrase forward, and TD-Apyrase reverse (Table 5). The conditions were as follows: 94°C, 1 min.; X°C 2 min.; 72°C 3 min., where X represents changing annealing temperatures. The annealing temperature was steadily decreased by 2°C every 3 cycles starting at 60°C and ending at 42°C. PCR products were visualized by agarose gel electrophoresis and ethidium bromide staining (Sambrook, et al., 1989) and compared to a known standard (λ DNA digested with StyI restriction endonuclease, both from Promega, Madison, WI).

Cloning of PCR Products

PCR products of the expected size, in this case 850bp, were cloned into the TA-cloning vector pCR2.1 using Invitrogen's TA Cloning ® Kit (Invitrogen, Carlsbad, CA). The ligation reaction from the kit was transformed in INVαF´ One Shot ™ competent cells (Invitrogen, Carlsbad, CA) according to the manufacturer's protocols). Transformed cells were plated onto LB medium supplemented with 100μg/mL ampicillin and 100μg/mL IPTG (Sigma, St. Louis, MO), and 25μg/mL X-Gal (Promega, Madison, WI). To confirm transformants, only white colonies were picked and grown in LB medium supplemented with ampicillin. Additional vectors used include Invitrogen's pCR2.1-TOPO (Invitrogen, Carlsbad, CA), and Promega's P-GEM-T-EASY (Madison, WI), using the respective manufacturer's protocols. Ligation products generated using pPCR2.1-TOPO were transformed into TOP10 chemically competent cells according to

Table 5. List of PCR primers used in this study.

Primer Name	Nucleotide Sequence
TD- Apyrase forward	5'-ATTGATGGAACCCAAGAAGG -3'
TD-Apyrase reverse	5'-GGY*AAAGM*TGATATGGCTTC-3'
3'RACEGSP1	5'-TGCTCGTTGATGGATTTGGC-3'
5'apy1GSP1	5'-ACCTTCATAATCTCAGCTCG-3'
5'apy1GSP2	5'-CTCCAAGATCCATTACTCCC-3'
5'Mt46fullORFE	5'-GGAATTCATGGTCTTACTTTGGCAAAACACC-3'
3'Mt46fullORFE	5'-GGCTTAAGTTAAACAAAATACATCATTCG-3'
Mtapy1 GS forward	5'-CTGGGGCTAATTTTAATGGATGC-3'
Mtapy1 GS reverse	5'-GTGGTACCCTCAATAGAAAAAACATGTCGG-3'
5'Mt46-2	5'- GGGGCAACTGCAGGTTTAAGGGC-3'
3'Mt46-2	5'-GAGCCAGATAAGATACACGGG-3'
Mtapy2 GS forward	5'-GATGCTGATGCGGTTACAGTGTTG-3'
Mapy2 GS reverse	5'-CCATGGCAGCCTCAGACTCA-3'
Mtapy3 GS forward	5'-GGTCACACATAATTCTCCCAACCC-3'
Mtapy3 GS reverse	5'-GCAGCGTTAAACTTGAGCG-3'
Mtapy4 GS forward	5'-GTTCAGTAACAGAAGTACCCTCAACG-3'
Mtapy4 GS reverse	5'-CACCTACTGTAATCTCTTTTCGTGGAC-3'
5'MtACTIN	5'-GCAGATGCTGAGGATATTAACCCC-3'
3'MtACTIN	5'- CGACCACTTGCATAGAGGGAGAGG-3'
pBeloBAC11left primer	5'AACGCCAGGGTTTTCCCAGTCACGACG-3'
pBeloBAC11 right primer	5'-ACACAGGAAAC AGCTATGACCATGATTACG-3'

*(Y = C/T; M = A/C)

the manufacturer's protocol (Invitrogen, Carlsbad, CA). Ligation products generated using P-GEM-T-EASY™ were transformed into *E. coli* strain JM109 as described in Sambrook et al. (1989). Plasmid DNA was isolated using the alkaline lysis method (Sambrook et al., 1989) and digested with EcoRI restriction endonuclease (Promega, Madison, WI). DNA digests were analyzed by electrophoresis on 0.8% agarose gels and strained for visualization of the DNA using ethidium bromide. Transformants with the expected size insert were again grown in LB medium and plasmid DNA was isolated using the Wizard® Plus Mini Prep Kit (Promega, Madison, WI) for automated DNA sequencing to confirm the clones.

DNA Sequencing

All DNA sequencing was performed by Dr. Neil Quigley (The University of Tennessee, Molecular Biology Resource Facility). DNA sequencing was performed using an ABI 373 DNA sequencer (Perkin-Elmer Inc., Foster City, CA) with the ABI Prism Dye Terminator Cycle Sequencing reaction kit. The initial sequence data files were edited following comparison with the same data displayed in four-color electrophoretograms before further analysis. In all cases, at least two independent clones were sequenced.

Extension of cDNA ends

Extension of the cDNA ends of the 850bp product was performed using the 5' and 3' Rapid Amplification of cDNA Ends (RACE) kit (Gibco BRL, Gaithersburg, MD), according to the manufacturer's protocol. For extension of the 3' end, the primer 3'apy1

GSP1 was employed; 3'RACEGSP1. For extension of the 5' end, the following primers were employed; 5'apy1GSP1, 5'apy1GSP2. Cloning and sequencing of the PCR generated products was performed as described above.

Isolation of the full length cDNA encoding *Mtapy1*

After sequence analysis of the 5' and 3' RACE products, primers were designed to amplify the coding region from the start (first in frame ATG) to the stop codon. Primers used for amplification of the full-length cDNA were 5'Mt46fullORFE and 3'Mt46fullORFE respectively. The sequence of these primers is available in Table 5. The primers were used in a RT-PCR reaction as described above using 5μM of each primer and Pfu DNA polymerase (Statagene, La Jolla, CA) in place of Taq DNA polymerase. The reaction was carried out as described above, except a single annealing temperature of 52°C was used.

Cloning of *Mtapy2*

To compare the full sequence of *Mtapy1* to known sequences in the database, a dbEST search was performed (Pearson et al., 1997). When compared to the EST (Expressed Sequence Tag) database dbEST, it was discovered that a very similar gene had been described from a root hair enriched cDNA library of *M. truncatula* (Accession number: AA660474; Covitz, et al, 1998). The available sequence was used to generate primers Mt46-2 forward and Mt46-2 reverse for use in an RT-PCR reaction (sequences of all primers is available in Table 5). The product of PCR was a 460 bp fragment that shared great sequence similarity to *Mtapy1* (85% similarity) and 100% sequence identity

to the EST. Cloning and sequencing was performed as described above. This cloning yielded plasmid pJRC201.

BAC Library Screening

A Bacterial Artificial Chromosome (BAC) library, available from the laboratory of Dr. Douglas Cook (Texas A&M University, College Station, TX) was screened using *Mtapy1* as a probe. For the probe, the 850 bp insert in pJRC10 was cleaved by digestion of the plasmid with EcoRI. The insert was the gel purified using a DNA gel purification kit (Qiagen, Valencia, CA). Southern analysis of colony blots was performed essentially as described in Sambrook, et al. 1989. Dr. Yung Wu Nam performed the initial BAC library screening in the laboratory of Dr. Douglas Cook. PCR analysis using primers specific to *Mtapy1* (TD-Apyrase forward, and Mtapy1GS reverse- see Table 5) confirmed that only BACs 52G10 and 58M19 contained this gene.

Identification of *Mtapy3* and *Mtapy4*

The 5' and 3' ends of the two BACs that were identified, 52G10 and 58M19, were sequenced using primers designed from sequence of the vector pBeloBAcII, used in construction of the BAC library (Nam, et al., 1999). Comparison of this sequence to the available databases led to the discovery that two additional putative apyrases were present in *M. truncatula* and appeared to be present in a cluster. Sequence comparison of the two additional apyrase-like genes indicated that they were both distinct from each other and distinct from *Mtapy1* and *Mtapy2*.

Primers were designed from the sequence of *Mtapy3* that were specific for this gene. The sequence of *Mtapy3* was obtained using a primer walking strategy on either total BAC DNA or on subcloned fragments of BAC DNA. Taesik Uhm performed most of the sequencing of *Mtapy3* (in Dr. Douglas Cook's laboratory at Texas A&M University, College Station, TX). The primers (Mtapy3 GS forward and Mtapy3 GS reverse- Table 5) were used to clone a partial fragment of the gene by RT-PCR. Total RNA was isolated from various tissues of *M. truncatula* wild-type line A-17, including roots, hypocotyls, stems, leaves, and flowers. RNA was reversed transcribed as previously described and used in a PCR reaction with primers Apy3 GS forward and Apy3 GS reverse. The resulting 776 bp PCR product was cloned into the TOPO cloning vector (Invitrogen, Carlsbad, CA) and the ligation product cloned into TOP 10 cells as described above. The resulting clone was referred to as JRC300.

For cloning *Mtapy4*, a single primer was designed to extend the 3'end of the gene from reverse transcribed RNA isolated from roots of *M. truncatula* wild-type line A17. The 3' RACE protocol used was essentially the same as the protocol described above for cloning of *Mtapy1*. The gene specific (GSP) primer used for extension of the 3'end of *Mtapy4* was Mtapy4 GS forward (Table 5). The PCR product obtained from the 3' RACE was cloned into the TOPO cloning vector (Invitrogen, Carlsbad, CA) and the ligation product was transformed into TOP 10 *E. coli* cells (Invitrogen, Carlsbad, CA). Cloning resulted in the production of plasmid pJRC400. The initial sequence of *Mtapy4* was a short region of approximately 600 base pairs that was obtained by BAC end sequencing. BAC end sequence data was provided by Taesik Uhm (Texas A&M University, College Station, TX).

Construction of a Contiguous Map of BAC Clones Around the Apyrase Cluster

Bacterial Artificial Chromosome (BAC) clones of *M. truncatula* genotype A17 were identified either by means of hybridization to high density filter arrays obtained from the Clemson University Genomics Institute (http://www.genome.clemson.edu), or by PCR-screening of a multiplexed DNA copy of the BAC library as described by Nam et al. (1999). BAC end sequencing was performed on whole BAC clones using primers complementary to the pBeloBAC11 vector ('left primer', and 'right primer'). Internal sequencing of selected regions of BAC clones was performed using a primer walking strategy, either on whole BAC clones or on DNA fragments subcloned into pBluescript (Stratagene, La Jolla, CA). The BAC contig shown in Figure 16 was extended by multiplex PCR, using primers designed to amplify sequences corresponding to the outermost BAC ends.

Genetic Mapping

Mapping of apyrase genes was performed by Taesik Uhm and Dong Jin Kim in Dr. Douglas Cook's laboratory at Texas A&M University (College, Station, TX). Genetic markers were developed based on BAC end sequence information obtained from BAC clones with homology to characterized *M. truncatula* apyrase cDNA products, or to the soybean RFLP clone B046. Briefly, oligonucleotide primers were designed based on the corresponding BAC end sequence information, and used to PCR amplify and sequence genomic DNA from *M. truncatula* genotypes A17 and A20. Comparing sequence differences between the parental genotypes against known restriction enzyme cleavage sites identified restriction enzyme polymorphisms. The resulting CAPS (cleaved

amplified polymorphic sequences) markers were mapped on a population of 93 F2 progeny from a genotype A17 X A20 cross (Penmetsa and Cook, 2000). Polymorphic DNAs were resolved on a 1.5% agarose gel and visualized by ethidium bromide staining. Primers, PCR conditions, and restriction enzyme information is given in Table 12. DNA was extracted from the mapping population by means of the Nucleon Phytopure kit (Amersham Life Sciences, Inc.), according to manufacturer's instructions.

Phylogenetic Analysis of *M. truncatula* Apyrase Genes

Phylogeny studies of apyrase proteins were performed by Todd Wood at the Clemson University Genomics Institute (Clemson, SC). Protein sequences were aligned using CLUSTALW (Thompson et al., 1994) under default parameters. End gaps were trimmed, leaving a 214-amino acid multiple sequence alignment for phylogenetic inference. Translations of EST sequences were generated using TFASTX3 (Pearson et al., 1997).

LNP protein sequences and rbcL sequences were aligned using CLUSTALW (Thompson *et al.*, 1994), running under default parameters. For both alignments, end gaps were trimmed. The final alignment for the LNP protein sequences contained 188 amino acid positions; the final alignment for the rbcL sequences contained 1310 nucleotide positions. DNA and protein distances were inferred using the DNADIST and PROTDIST programs of the PHYLIP package (Felsenstein, 1993), respectively. Bootstrap and neighbor-joining analysis were performed using the SEQBOOT and NEIGHBOR programs, also from the PHYLIP package. Trees were rooted using the out-group *Arabidopsis* sequences.

Southern Blot Analysis

Ten micrograms of genomic DNA isolated from five-day-old, *M. truncatula* plants (line A17) was used for Southern analysis. DNA was isolated using protocols described above. Genomic DNA was digested with *Bam*HI, *Hind*III, and *Eco*RI overnight at 37°C in reaction conditions specified by the manufacturer (Promega, Madison, WI). Digests were loaded onto a 0.8% agarose gel and electrophoresed for 1 hour at 100V. The DNA was then transferred to ZetaProbe® nylon membrane (BioRad Laboratories, Hercules, CA), according to the method described in Sambrook, et al, 1989. Hybridization and washing were performed at high stringency, according to the manufacturer's protocol. High stringency conditions included hybridization in 7%SDS, 0.25M sodium phosphate buffer (pH 7.2) at 65°C, and washing in 1% SDS, 20 mM sodium phosphate buffer, at 65°C. The 1416 bp fragment of pJRC63 (*Mtapy1*-see Table 4) was used as a probe and radiolabled with ^{32}P-αdATP using random primer labeling (Promega, Madison, WI).

Northern Blot Analysis

Total RNA was electrophoresed on denaturing 1% agarose, formaldehyde gels in 1X MOPS buffer (Sambrook, et al, 1989). Blotting of separated RNA was performed as described for Southern blotting procedures. In all cases, probes were radiolabeled with ^{32}P-αdATP using random primer labeling (Promega, Madison, WI). Hybridizations were conducted at 60°C in a solution of 7%SDS, 0,25M NaH_2PO_4, pH 7.0. After hybridization, membranes were washed successively in solutions of 0.1%SDS, 2X SSC (1X SSC is 0.15M NaCl, 0.015 M sodium citrate) (24 °C for 5 minutes); 0.25M

NaH_2PO_4, pH 7.0, 5%SDS (2X at 45°C for 30 min); 0.1%SDS, 0.1 X SSC (2X at 65°C for 30 min). Stripping the probes from the blots was performed by washing the membranes twice in 0.5% SDS, 0.1 X SSC at 90°C in a shaking water bath for 15 minutes.

Expression Analysis of Apyrase Genes from *M. truncatula*

RT-PCR Analysis of Gene Expression

Due to the high degree of sequence similarity between all of the putative apyrases from *M. truncatula*, a PCR based approach was used to distinguish the expression of the different genes. Gene specific primers were designed from the available sequence of all of the genes from regions of sequence divergence and from the 3' untranslated regions. All of the primers used are included in Table 5. These primers were first tested in PCR reactions against reverse transcribed total RNA isolated from roots of the wild-type line A17 of *M. truncatula* as previously described. In the case of *Mtapy3*, a combination of total RNA from various tissues was used, as previously described. The PCR products generated were sequenced to ensure that the amplified products were indeed from the expected genes. These products were cloned and used as probes in subsequent Southern analysis of the PCR products. In all of the reactions, primers designed to amplify the actin-2 gene were included as internal controls. For PCR reactions, 5 microliters of cDNA was used along with 5μM of each of the primers. The reaction conditions were the same as previously described, using the following parameters: 94°C for 45 sec., 52°C for 1 min., 72°C for 1 min., for a total of 25 cycles, followed by a 10 min. cycle at 72°C.

PCR products were separated by agarose gel electorphoresis and blotted onto nylon membranes as previously described. Southern analysis was performed as described previously. All blots were first probed with apyrase specific probes and then stripped and reprobed with an actin probe, the PCR product of the primers 5' MtACTIN and 3'MtACTIN (Table 5). All blots were counted for total radioactivity using an Instant Imager (Packard Instrument Co., Meriden, Connecticut). Total counts of each lane were measured for ^{32}P-labeled apyrase probe hybridization and ^{32}P-labeled actin probe hybridization respectively. The total counts of apyrase were divided by total counts of actin to yield a ratio of the relative intensity of hybridization of apyrase. The value obtained was multiplied by 100 to yield a value of relative expression, which was represented in graphical format.

Tissue Specific Expression

Total RNA was collected from five-day-old roots, hypocotyls, and cotyledons, from six-week-old stems, leaves, flowers, and 10-day-old nodules from roots inoculated with wild-type *S. meliloti* strain 1021 using the guanidine-HCl method described above. RNA was reverse transcribed and analyzed by PCR using the gene specific primers described above for each of the four genes (*Mtapy1* = Mtapy1 GS forward + Mtapy1 GS reverse; *Mtapy2* = Mtapy2 GS forward + Mtapy2 GS forward + Mtapy2 GS reverse; *Mtapy3* = Mtapy3 GS forward + Mtapy3 GS reverse; *Mtpay4* = Mtapy4 GS forward + Mtapy4 GS reverse- see Table 5). In each case, actin primers (Table 5) were added to the reactions as an internal control. PCR products were analyzed as previously described.

Expression of *MtApy1* in Response to Rhizobial Inoculation

RNA for some of these experiments was provided by Senthil Ramu, as indicated (Dr. Douglas Cook's laboratory, Texas A&M University, College Station, TX). Total root RNA was isolated from roots at various times between 0 and 6 hours after inoculation with *S. meliloti* ABS7. Each lane was loaded with RNA equivalent to 0.2g fresh weight of root tissue. The blot was probed with *Mtapy1* (the cDNA insert in pJRC10) stripped, and reprobed with histone H3 from *M. truncatula.* The histone H3 clone was a cDNA clone kindly provided by Dr. Douglas Cook. The membranes were subjected to autoradiography after hybridization and washing as described above.

Mtapy1 Expression in Response to Rhizobial Nodulation Mutants

Wild-type A17 plants were grown aeroponically as described above. Plants were inoculated with either wild-type *S. meliloti* strain AK631 or AK1657, an AK631 *nodC*:Tn5 mutant that does not produce any Nod Signals. Plants were also inoculated with either wild-type strain 1021 or with strain SL44, a 1021 $\Delta nodD_1ABC$ that does not produce Nod signals. 5cm sections were taken from the root tip at various times between 0 hours and 48 hours after rhizobial inoculation. Total RNA was isolated using the guanidine HCl method previously described. RT-PCR was used to analyze gene expression and was performed as previously described.

Mtapy1 Expression in Nodulation Deficient EMS Mutagenized Lines of *M. truncatula*

Several EMS mutagenized, nodulation deficient, lines of *M. truncatula* were kindly provided by Dr. Douglas Cook (Texas A&M University, College Station, TX;

Penmetsa and Cook 2000). The available lines were inoculated with wild-type *S. meliloti* strain ABS7. Roots of these plants were collected for analysis either 0 hours or 6 hours post rhizobial inoculation. RNA equivalent to 0.2 grams of fresh weight tissue was loaded onto each lane. The blot was probed with *Mtapy1* (the insert of pJRC10), stripped and rehybridized with Histone H3 as described previously.

Several of the mutant lines that were screened for *Mtapy1* expression were further analyzed. Wild-type line A17, and the mutants Poodle (*pdl*), Domi (*dmi*), and Lin (*lin*), (Penmetsa, 1998) were grown aeroponically and inoculated with wild-type *S. meliloti* strain ABS7. Root samples were taken 5cm from the root tip at various times from 0 hours to 24 hours. At various times from 48 hours up to 120 hours, a 5cm section was taken, excluding 0.5cm of the root tip for every 24 hours. This was done to try to accurately measure the level of gene induction in the same region of the root for the entire time course study. Total RNA was isolated using the guanidine HCl method described above. RNA equivalent to 0.2 g fresh weight tissue was used for loading each lane of a denaturing formaldehyde gel. Northern blotting and hybridizations were performed as described previously.

CHAPTER 3

RESULTS

Section I A: Structure Function Studies of Lipo-Chitooligosaccharides on *G. soja*

(Published in *Plant Physiol.* 1995. 108: 1587-1595.)

Biological Activity of A Synthetic LCO Analog is Similar to the Activity of LCO from *B. japonicum*

A collection of LCO molecules were synthesized based on the known structures of naturally occurring Nod signals produced by various strains of rhizobia (Sanjuan, et al., 1992, Carlson, et al., 1993, Spaink, et al., 1991, and Lerouge, et al., 1990) (Figure 4, Table 6). To determine whether synthetic LCO molecules have similar activities to those reported for naturally produced analogs, both synthetic and naturally occurring LCOs were evaluated using two biological assays. The first assay evaluated whether the LCOs were able to induce root hair deformation (HAD), which is one of the earliest visible plant responses to compatible rhizobia (Hiedstra, et al., 1994). The second assay evaluated the ability of the molecules to induce nodule primordia (i.e., activate cortical cell divisions), both of which are involved in nodule initiation (NOI). These two assays allowed us to evaluate both the ability of the molecules to initiate very early plant responses to Nod signals, and evaluate responses that require a sustained developmental program.

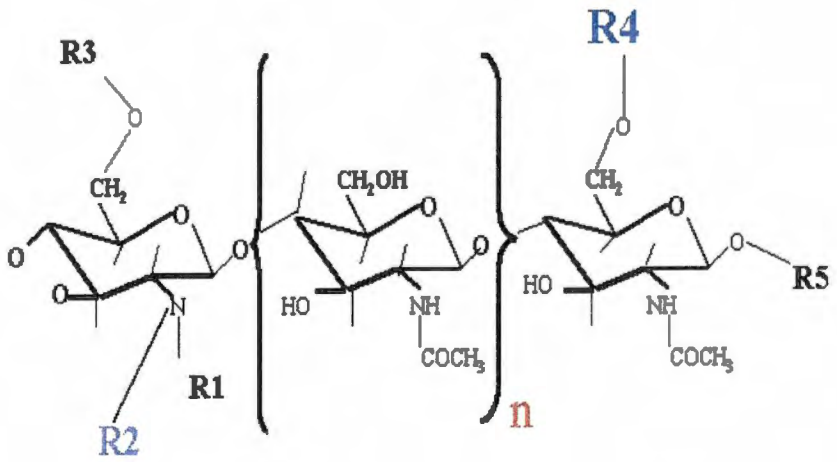

Figure 4. Chemical Structure of the LCO Molecules Used in this Study.
The Figure illustrates the structure of synthetic and natural product LCO used in the study. The LCO used in the study are listed in Table 6. Differences in chemical structure are indicated by the various modifications listed under the R-groups and under *n*, which indicates the degree of polymerization.

Table 6. Structures of synthetic and natural product lipo-oligosaccharides used in study. The R groups and *n* represent the groups labeled in Figure 4.

Lipo chitin-oligosaccharide	*n*	R_2	R_4	R_5
LCO-V(C18:1Δ11,MeFuc)	3	C18:1Δ11	2-*O*-MethylFucose	H
LCO-V(C18:1Δ9,MeFuc)	3	C18:1Δ9	2-*O*-MethylFucose	H
LCO-V(C16:0,MeFuc)	3	C16:0	2-*O*-MethylFucose	H
LCO-IV(C18:1Δ11,MeFuc)	2	C18:1Δ11	2-*O*-MethylFucose	H
LCO-IV(C16:0,MeFuc)	2	C16:0	2-*O*-MethylFucose	H
LCO-IV(C18:1Δ11,Fuc,R-Gro)	2	C18:1Δ11	Fucose	R-Glycerol
LCO-IV(C18:1Δ11,Fuc,S-Gro)	2	C18:1Δ11	Fucose	S-Glycerol
LCO-IV(C18:1Δ9,Fuc,R-Gro)	2	C18:1Δ11	Fucose	R-Glycerol
LCO-IV(C18:1Δ11,Fuc,S-Gro)	2	C18:1Δ11	Fucose	S-Glycerol
LCO-IV(C16:0)	2	C16:0	H	H
LCO-IV(C16:0,S)	2	C16:0	SO_3	H
LCO-IV(C16:1Δ2,S)	2	C16:1Δ2	SO_3	H
LCO-IV(C16:1Δ9,S)	2	C16:1Δ9	SO_3	H
LCO-IV(C16:2Δ2,9,S)	2	C16:2Δ2,9	SO_3	H
Nod*Bj*-V(C18:1Δ11,MeFuc)	3	C18:1Δ11	2-*O*-MethylFucose	H
Nod*Be*-V(C18:1Δ11,MeFuc)	3	C18:1Δ11	2-*O*-MethylFucose	H
Nod*Bj*-V(C18:1)	3	C18:1Δ11	H	H
Nod*Rl*-IV(C18:1Δ11)	2	C18:1Δ11	H	H
Nod*Bj*-IV(C18:1,Gro)	2	C18:1	H	Glycerol
Nod*Bj*-IV(C18:1)	2	C18:1	H	H
Nod*Bj*-V(Ac,C18:1)	3	C18:1	H	H

Several types of deformations were observed in HAD assays on *G. soja*, including swelled root hair tips, curvy root hairs, and corkscrew-like hairs (Figure 5). The natural product Nod*Bj*-V(C18:1Δ11, 2-*O*-MeFuc) and its synthetic analog, LCO- V(C18:1Δ11, 2-*O*-MeFuc), had similar activity in HAD assays at the concentrations tested (Table 7). The synthetic molecule showed HAD activity with as little as 10^{-15} M LCO. In spot inoculation assays, the synthetic LCO-V(C18:1Δ11, 2-*O*-MeFuc) also induced nodule-like structures that were morphologically similar to those induced by its chemically identical, naturally occurring Nod signal (Figure 6; Stokkermans and Peters 1994). In a previous examination of these structures, it had been shown that the natural product LCO from *B. elkanii*, Nod*Be*(C18:1Δ11, 2-*O*-MeFuc), was able to induce nodule-like structures that were very similar to nodules induced by bacteria (Calvert, et al., 1984, Stokkermans and Peters, 1994). The frequency of NOI response is statistically the same for both Nod*Bj*-V(C18:1Δ11, 2-*O*-MeFuc) and LCO- V(C18:1Δ11, 2-*O*-MeFuc) at 100ng per spot inoculation (Table 8). Thus, the synthetic analog, LCO- V(C18:1Δ11, 2-*O*-MeFuc), is able to initiate nodule structures on *G. soja* that resemble those that are initiated by the natural product, Nod*Bj*-V(C18:1Δ11, 2-*O*-MeFuc) at a frequency similar to the natural product. These data confirm that the synthetic analog has biological activity very similar to naturally occurring Nod signals.

Structural Requirements for Biological Activity of LCO on *G. soja*

All of the available synthetic LCO were used in both HAD and NOI assays to determine their biological activity on *G. soja* (Table 7 and Table 8). Because of the

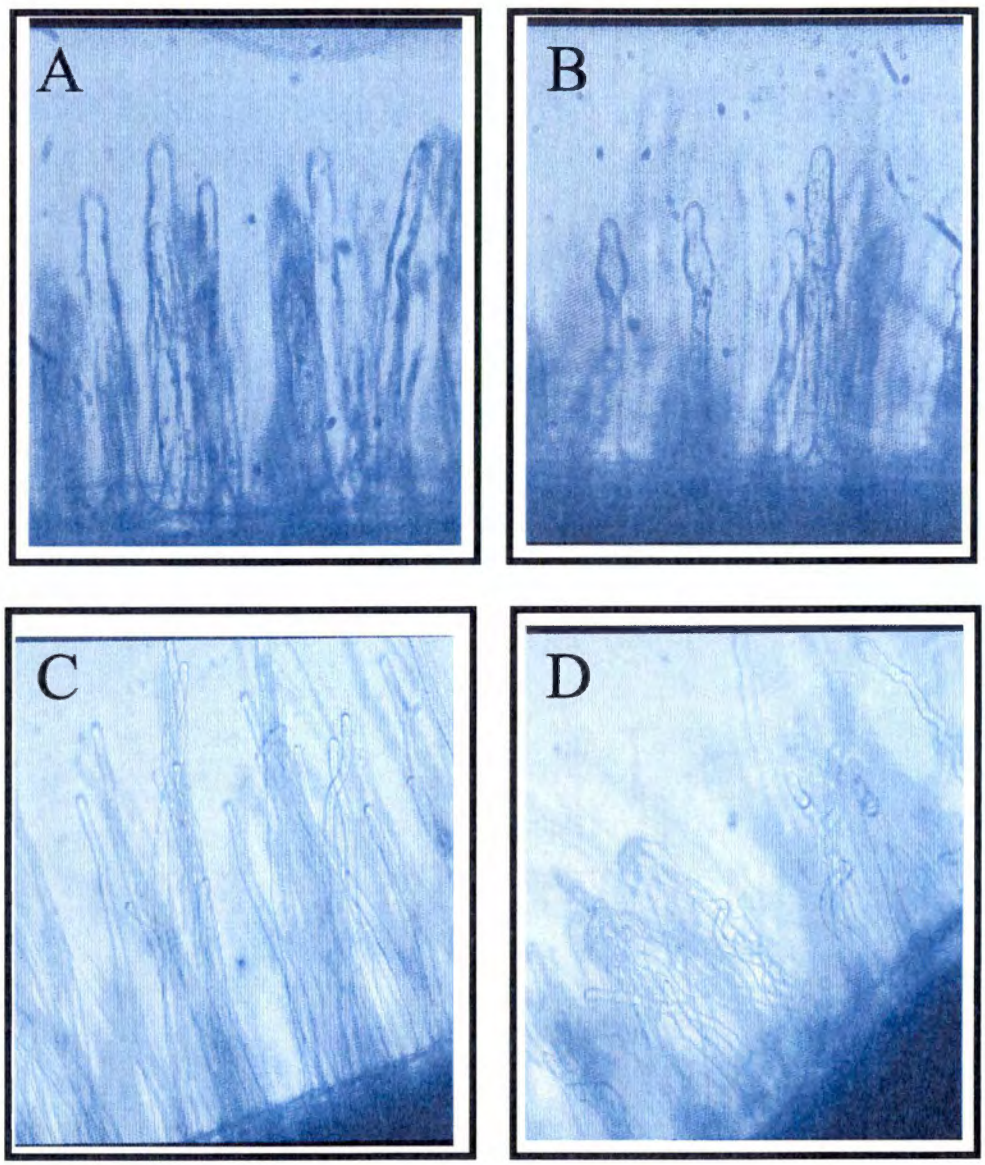

Figure 5. HAD (Root Hair Deformation) activity of synthetic LCOs on *G. soja* roots. Root hairs photographed 16 hours post treatment, 5 to 10 mm from the root tips. A) untreated control; B) treated with 10^{-8}M LCO-V($C_{18:1\Delta}{}^{11}$,MeFuc). Root hairs photographed 2 days post-treatment, 10 to 20 mm from the root tip. C) untreated control; D) treated with 10^{-8}M LCO-V($C_{18:1\Delta}{}^{11}$,MeFuc).

Table 7. HAD activity of synthetic and natural product LCO on *G. soja*

HAD Assay at Various Concentrations of LCO (M). - = Not Determined. Numbers are based on a numerical system of rating the degree to which different LCO were active on *G. soja* roots, as indicated in the text. The proportion of root hairs showing deformation was scored as follows: 0= 0 to 10%; 1 = 10 to 30%; 2 = 30 to 60%.

	Various concentrations of LCO used (M)						
LCO	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}	10^{-14}	10^{-15}
LCO-V(C18:1Δ11,MeFuc)	2	2	2	2	2	1	1
LCO-V(C18:1Δ9,MeFuc)	2	2	2	2	1	-	-
LCO-V(C16:0,MeFuc)	2	2	1	-	-	-	-
LCO-IV(C18:1Δ11,MeFuc)	0	-	-	-	-	-	-
LCO-IV(C16:0,MeFuc)	0	-	-	-	-	-	-
LCO-IV(C18:1Δ11,Fuc,R-Gro)	0	-	-	-	-	-	-
LCO-IV(C18:1Δ11,Fuc,S-Gro)	0	-	-	-	-	-	-
LCO-IV(C18:1Δ9,Fuc,R-Gro)	0	-	-	-	-	-	-
LCO-IV(C18:1Δ11,Fuc,S-Gro)	0	-	-	-	-	-	-
LCO-IV(C16:0)	2	2	1	1	1	1	-
LCO-IV(C16:0,S)	0	-	-	-	-	-	-
LCO-IV(C16:1Δ2,S)	0	-	-	-	-	-	-
LCO-IV(C16:1Δ9,S)	0	-	-	-	-	-	-
LCO-IV(C16:2Δ2,9,S)	0	-	-	-	-	-	-
Nod*Bj*-V(C18:1Δ11,MeFuc)	2	-	-	-	-	-	-
Nod*Bj*-V(C18:1)	0	-	-	-	-	-	-

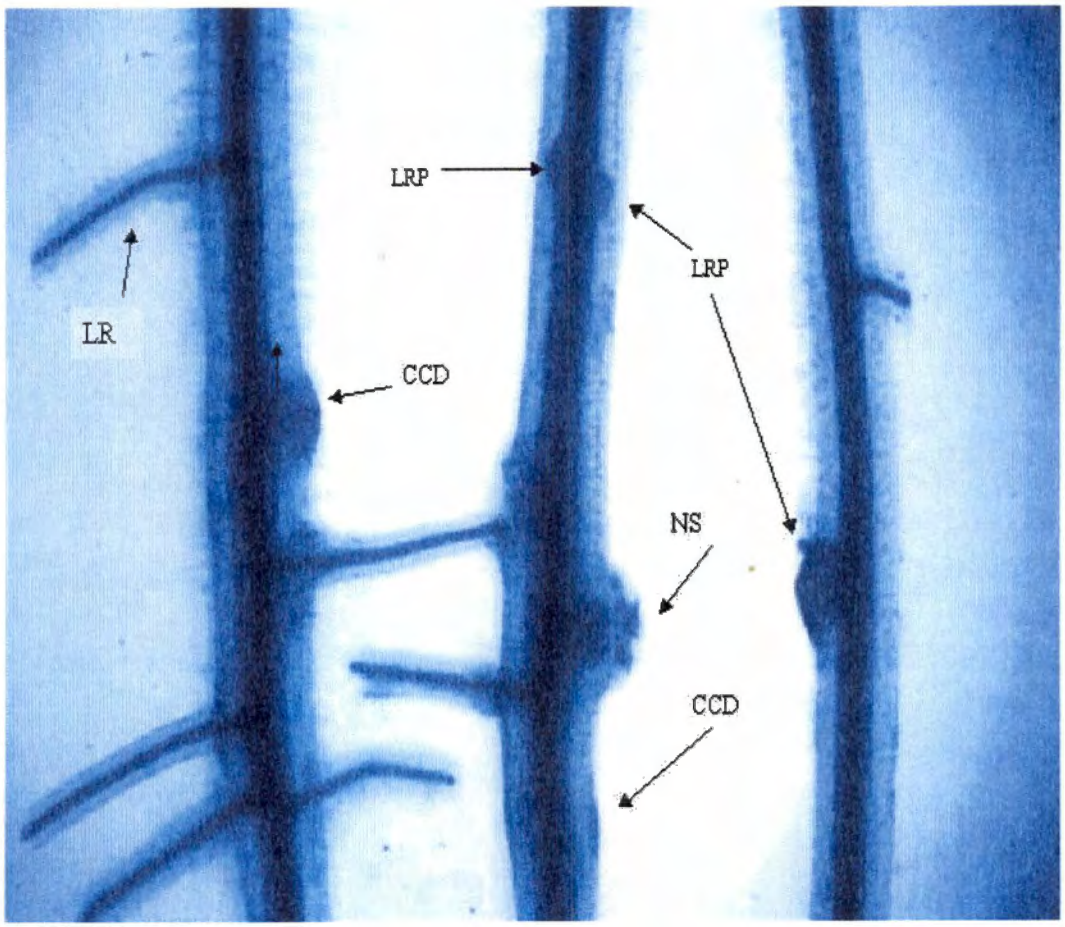

Figure 6. Nodule induction on *G. soja* roots in response to LCO-V(C18:1Δ11, 2-*O*-MeFuc). Roots of *G. soja* inoculated with 100ng LCO-V(C18:1Δ11, 2-*O*-MeFuc) and stained with methylene blue. Several types of structures are present, that can be easily distinguished by staining. Cortical cell divisions in response to LCO are labeled CCD and are indicated by arrows. Lateral root primordia are differentiated by the presence of dark staining of the vasculature that grows into the structure very early in its development. These lateral root primordia are labeled LRP. Mature lateral roots are indicated also by arrows and labeled LR. Nodule-like structures are labeled NS.

Table 8. NOI activity of synthetic and natural product LCO on *G. soja*. These data were repeated by Thomas Stokkermans in a double-blind experiment (while at the Ohio State Biotechnology Center, Ohio State University, Columbus, OH)

LCO Tested	NOI response	
	Mean percentage NOI response[a]	Total No. of plants (No. of assays)
LCO-V(C18:1 Δ11,MeFuc)	60(ab)	52(4)
LCO-V(C18:1 Δ9,MeFuc)	54(ab)	70(5)
LCO-V(C16:0,MeFuc)	41(b)	41(3)
LCO-IV(C18:1 Δ11,MeFuc)	0(c)	42(3)
LCO-IV(C16:0,MeFuc)	0(c)	28(2)
LCO-IV(C18:1Δ11,Fuc,R-Gro)	0(c)	78(4)
LCO-IV(C18:1Δ11,Fuc,S-Gro)	0(c)	85(4)
LCO-IV(C18:1Δ9,Fuc,R-Gro)	0[c]	15(1)
LCO-IV(C18:1Δ11,Fuc,S-Gro)	0[c]	15(1)
LCO-IV(C16:0)	41(b)	86(6)
LCO-IV(C16:0,S)	0(c)	28(2)
LCO-IV(C16:1Δ2,S)	-	-
LCO-IV(C16:1Δ9,S)	-	-
LCO-IV(C16:2Δ2,9,S)	0(c)	44(3)
Nod*Bj*-V(C18:1Δ11,MeFuc)	77(a)	98(6)
Nod*Bj*-V(C18:1)	0(c)	81(4)
Nod*Rl*-IV(C18:1Δ11)	45[c]	22(1)
1% CMC (Control)	0(c)	52(4)

Values are for 100ng per spot inoculation. Numbers followed by common letters in parentheses are not significantly different from one another at the 95%confidence interval as determined using Fisher's protected LSD (milliken and Johnson, 1984). [b]-, Not determined. [c]Not included in statistical analysis because the experiments were not repeated.

known response to LCO-V(C18:1Δ11, MeFuc), this molecule was used as a reference LCO, to which the biological activity of the other compounds was compared. Many of the compounds tested were available in very limited quantities, so they were not tested at the range of concentrations used for other compounds, such as LCO-V(C18:1Δ11, MeFuc). The limited supply of many of the LCO somewhat limited the conclusions that could be made about the relative biological activity of the various LCO. In general, however, the compounds that had HAD activity also had NOI activity. The results indicate interdependence of the length of the chitin backbone and the state of substitution of the reducing end of the molecule.

The importance of the fatty acid structure was tested by comparing the activities of LCO molecules that differed from the reference molecule only in the acyl chain at the non-reducing end of the molecules. LCO-V(C18:1Δ9,MeFuc) differs from the reference molecule only in the placement of the desaturation of the acyl chain, and has HAD activity similar to the reference molecule down to concentrations as low as 10^{-11}M (Table 7). In addition, the frequency of NOI activity in response to this molecule was similar to the reference molecule. These data indicated that the placement of the double bond in the acyl chain of Nod signals did not have a major impact on biological activity on *G. soja* at the concentrations tested. When the acyl chain length is reduced, as in the case of LCO-V(C16:0,MeFuc), HAD activity was slightly reduced. However, the frequency of NOI was not critically affected (Table 7 and Table 8). In this case, HAD activity was still similar to the reference molecule at concentrations as low as 10^{-9}M. The extent of nodule development differed significantly in response to this molecule when compared to the reference molecule, in that, none of the nodulation events developed into emerging

nodules. Because this molecule has two differences in the acyl chain relative to the reference molecule, both chain length and degree of unsaturation, it is difficult to determine if the change in activity relates to either of the respective alterations in structure.

The importance of the substitution at the reducing end of the molecule was tested by spot inoculation of Nod*Bj*-V(C18:1). This molecule had no biological activity in either the HAD or NOI assays. This result was similar to what had been previously published when the molecule was purified from a *B. japonicum nodZ* mutant (Stacey, et al., 1994). This molecule was tested several times and consistently had no biological activity.

The importance of the chain length of the chitin backbone of LCO was tested using LCO-IV(C18:1Δ11, 2-*O*-MeFuc). This LCO had no activity on *G. soja* in either the HAD assay or the NOI assay. This result indicated a strong dependence on the degree of polymerization of the chitin backbone for biological activity of LCO on *G. soja*.

There is a novel interdependence on length of the chitin chain and modification to the reducing end of LCO molecules in relation to their biological activity on *G. soja*. As indicated above, simply shortening the chitin backbone from a pentamer to a tetramer resulted in a total loss of biological activity, although the modification to the reducing end was identical to the reference LCO. A similar loss of activity was detected in all LCO with modifications to the reducing end. For example, LCO with a fucosyl or glyceryl substitution at the reducing end, as in the case of LCO-IV(C18:1Δ11,Fuc,Gro), had no NOI or HAD activity at any of the concentrations tested. Similarly, LCO with

sulfuryl modifications, such as LCO-IV(C16:2Δ2,9,S), had no activity in either assay even at concentrations as high as 10^{-8} M (Tables 7 and 8). This is very interesting due to the fact that this molecule is identical to the structure of the LCO produced by *S. meliloti* (Lerouge et al., 1990). It is well established that this bacterium is not able to infect soybean.

In distinct contrast to these results, we discovered that a tetrameric LCO with no reducing end modification, LCO-IV(C16:0), had activity in both the HAD and NOI assays (Tables 7 and 8). HAD activity was detected in concentrations as low as 10^{-12} M. However, none of the nodule initiations activated by this molecule resulted in emerging nodules (Stokkermans, et al., 1995). This LCO differs from the reference molecule in backbone length, reducing end substitution, and acyl modification. Therefore, the LCO molecule Nod*Rl*-IV(C18:1Δ11), that has an acyl substitution similar to the reference molecule, was used in a spot inoculation assay to determine if it was active on *G. soja*. This molecule was found to have NOI activity similar to the other active molecules (Table 7). These results indicated that tetrameric LCO without any reducing end modifications were active on *G. soja,* and that pentameric LCO were only active when they were decorated with the proper reducing end modification (i.e., a 2-*O*-methyl fucosyl moiety).

Section IB. Structure Function Studies of Lipo-Chitooligosaccharides on *Vigna umbellata*

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LCO Structural Requirements for Nodule Initiation on *Vigna umbellata*

Despite the large number of reports on the chemical structures and biological activity of LCO, there is limited data on structure activity relationships of LCO on different legumes (BecFerte et al., 1993; Carlson et al., 1993; Firmin et al., 1993; Lerouge et al., 1990; Mergaert, et al., 1993; Poupot et al., 1993; Price et al., 1992; Roche et al., 1992; Sanjuan et al., 1992; Spaink, et al., 1991; Stokkermans, et al., 1994, Relic, et al, 1993, Relic et al, 1994). In an attempt to learn more about the structural requirements necessary for LCO activity on different legumes, we tested several hosts of *B. elkanii* and *B. japonicum* using the spot inoculation assay. *V. umbellata* (Rice Bean) was found to respond well to the application of LCO.

To assay the LCO structural requirements necessary to induce NOI on *V. umbellata* roots, a collection of different natural product and synthetic LCOs, similar to those mentioned above, were spot inoculated onto *V. umbellata* roots. (Figure 4 and Table 9). In 10 separate experiments, application of 100ng of Nod*Bj*-V(C18:1Δ11,Mefuc) resulted in 60% or more of the plants showing a positive NOI response. To get more detailed information about the structural requirements of LCO for NOI activity on *V. umbellata*, several of the synthetic LCOs that were used in the NOI assays on *G. soja* were tested. For these studies, approximately 100ng (10^{-10}M) LCO was applied to each plant per spot (Table 9). No false positives were ever observed with the application of 1% CMC alone, so any synthetic LCO that induced a NOI response was

Table 9. NOI activity of synthetic and natural product LCO on *V. umbellata* roots

LCO Tested	NOI Activity[1]
LCOV(C18:1Δ11Mefuc)	+
Nod*Bj*-V(C18:1Δ11,Mefuc)	+
LCO-V(C18:1Δ9,Mefuc)	+
LCO-V(C18:1Δ11,Mefuc)	+
LCO-V(C16:0,Mefuc)	+
LCO-IV(C16:0)	-
LCO-IV(C18:1Δ,Mefuc)	-
LCO-IV(C18:1Δ11,Mefuc,RGro)	-
LCO-IV(C18:1Δ11,Mefuc,SGro)	-
LCO-IV(C16:0,S)	-
LCO-IV(C16:2,Δ2,9,S)	-

[1]100ng of LCO was spot inoculated onto 4 day old roots. A (+) score was given if LCO were able to induce macroscopically visible structures within 12 days post inoculation. A (-) score was given if no nodule structures formed.

considered to be a true positive.

The results of the NOI assays on *V. umbellata* were very similar to the results obtained above with *G. soja* (Table 9 and Table 10). This was very interesting, because we were never able to detect any nitrogen fixing nodules when we inoculated *V. umbellata* with *B. japonicum*, the bacterial symbiont of *G. soja*. However, *B. japonicum* was able to form pronounced, nodule-like structures on *V. umbellata* that were similar to the nodule structures formed in response to LCO. If the same Nod signals (i.e., fucosylated pentamers, which are produced by *B. japonicum*) are active on both plants, then one would expect that *B. japonicum* would be able to nodulate both plants equally well. However, this was not the case. It is possible that these results may have been due to our growth conditions.

Of the synthetic LCO tested on *V. umbellata*, only three were active; LCO-V(C18:1Δ11,Mefuc), LCO-V(C18:1Δ9, Mefuc) and LCO-V(C16:0,Mefuc) (See Table 9). Tables 9 and 10 show NOI activity as either positive or negative. A positive NOI response included macroscopically visible nodule structures within 12 days post inoculation with LCO.

To our surprise, the synthetic molecule, LCO-IV(C16:0), which was active on *G. soja* roots (Stokkermans et al. 1995; Minami et al. 1996a,b), was not active on *V. umbellata* (Table 10). This compound was tested several times at varying concentrations up to 10^{-8}M with consistently negative results. In all experiments, a positive control of 100ng (10^{-10} M) of NodBj-V(C18:1Δ11,Mefuc) was included. It was apparent from these data that only pentameric LCOs with a 2-*O*-methyl fucosyl moiety on the reducing end glucosamine were active on *V. umbellata.*

Table 10. NOI activity of LCO on *G. soja* and *V. umbellata* roots

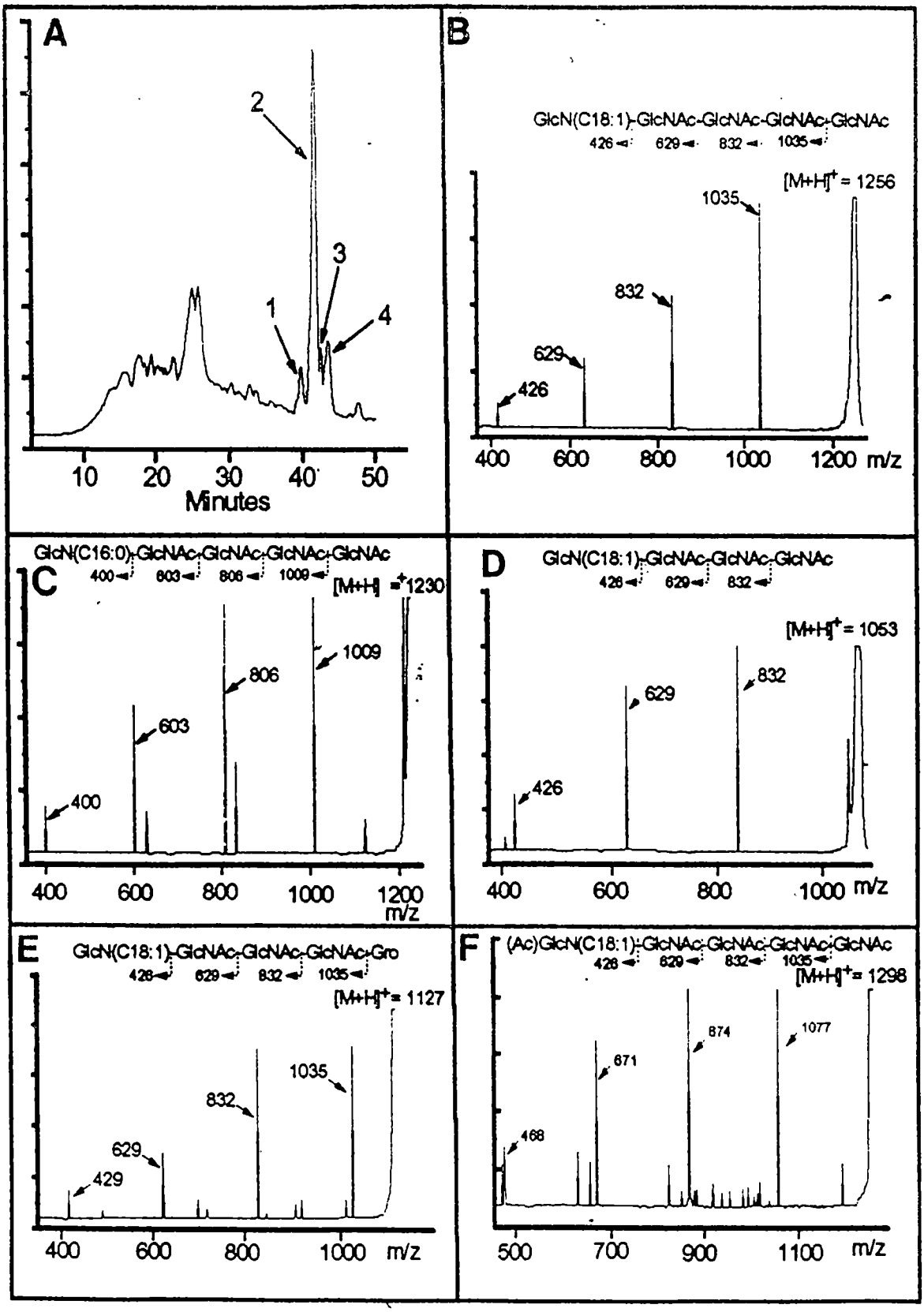
LCO Tested	*G. soja*	*V. umbellata*
LCO-V(C18:1Δ11,Mefuc)	+	+
Nod*Bj*-V(C18:1Δ11)	-	-
LCO-V(C18:1Δ9,Mefuc)	+	+
LCO-V(C16:0,Mefuc)	+	+
LCO-IV(C18:1Δ11,Mefuc)	-	-
LCO-IV(C16:0)	+	-

100 ng per spot of LCO added to 4 day old roots. A positive score was given if roots had macroscopic nodule structures within 12 days post inoculation. (-) indicates that no nodule structures formed.

LCO Produced By a *B. japonicum nodZ* Mutant

An HPLC elution profile of the LCO from the *B. japonicum nodZ* mutant, NAD138, is shown in panel A of Figure 7. NodZ is a fucosyl transferase that is responsible for the addition of 2-*O*-methylfucose at the reducing end of LCO produced by wild-type *B. japonicum* (Quesada-Vincens, et al. 1997, Quinto, et al. 1997). Previous studies had found that the major LCO produced by this mutant was identical to the major LCO produced by the wild-type strain USDA 110, however lacked any reducing end modification (Stacey, et al. 1994). The various Nod signals produced by NAD138 were distributed among four HPLC peaks. These four peaks contained five different structures that were identified by FAB-MS (spectra not shown), and by tandem MS-MS of the various $[M+H]^+$ ions. The tandem MS-MS spectra are shown in panels B-F of Figure 7. Peak 1 consisted exclusively of Nod*Bj*-V (C16: 0). Peak 2 contained approximately 85% of the total LCO and consisted of a mixture of Nod*Bj*-IV(C18: 1,Gro) and Nod*Bj*-V(C18:1). If one assumes that the various Nod signals ionize in the FAB source in an equivalent manner, then the relative intensities of their respective molecular ions would be indicative of their molar ratios. Based on that assumption the molecules in peak 2 were present in a 1:1 ratio. Peak 3 consisted of a mixture of Nod*Bj*-IV(C18:1) and Nod *Bj*-V(Ac, C18:1) in a 2.4:1.0 ratio. Peak 4 consisted of a mixture of Nod*Bj*-V(Ac, C18:1) and Nod*Bj*-V(C18:1) in a 1:1 ratio. It is likely that peak 4 consisted exclusively of Nod*Bj*-V(Ac,C18:1) and that the detection of Nod*Bj*-V(C18:1) is from the partial loss of the labile acetyl group subsequent to purification.

Figure 7. Chemical analysis of lipo-chitin oligosaccharides produced by the *Bradyrhizobium japonicum nodZ* mutant NAD138. In the upper left hand panel (A) is a HPLC chromatogram representing the elution profile of LCO produced by the mutant. The other panels B-F represent mass spectra-mass spectrometry data with molecular weight (M.W.) of each of the five compounds identified in the four peaks, and a description of the chemical structure related to each peak. See text for details.


A
2
3
1
4
10
20
30
40
50
Minutes
B
GlcN(C18:1)-GlcNAc-GlcNAc-GlcNAc-GlcNAc
426
629
832
1035
[M+H]+ = 1256
1035
832
629
426
400
600
800
1000
1200 m/z
C
GlcN(C16:0)-GlcNAc-GlcNAc-GlcNAc-GlcNAc
400
603
806
1009
[M+H]+ =1230
1009
806
603
400
400
600
800
1000
1200
m/z
D
GlcN(C18:1)-GlcNAc-GlcNAc-GlcNAc
426
629
832
[M+H]+= 1053
832
629
426
400
600
800
1000 m/z
E
GlcN(C18:1)-GlcNAc-GlcNAc-GlcNAc-Gro
426
629
832
1035
[M+H]+= 1127
1035
832
629
429
400
600
800
1000 m/z
F
(Ac)GlcN(C18:1)-GlcNAc-GlcNAc-GlcNAc-GlcNAc
426
629
832
1035
[M+H]+= 1298
874
1077
671
468
500
700
900
1100 m/z

Multiple LCO From NAD138 Are Required to Induce NOI on *V. umbellata* Roots

From the results of the NOI assay, it was assumed that the *B. japonicum nodZ* mutant, NAD138, would not be able to form nodule structures on *V. umbellata,* in contrast to wild-type *B. japonicum.* This assumption was made because this strain does not produce any LCO with the 2-*O*-methylfucose at the reducing end (Stacey, et. al, 1994). The product of the *nodZ* gene is a fucosyl transferase (Quesada-Vincens, et al. 1997, Quinto, et al. 1997). If the data above were correct, then this mutant would not be able to nodulate *V. umbellata*, because this plant requires pentameric LCO modified with 2-*O*-methyl fucose at the reducing end in order to form nodule structures (Table 9).

To our surprise, strain NAD138, was able to form nodule structures *V. umbellata* with only a slight delay in initial nodule formation (data not shown). However, like wild-type *B. japonicum*, the mutant strain was not able to form nitrogen-fixing nodules on *V. umbellata*. From a previous study, it was found that this same mutant was also capable of nodulating *G. max* and *G. soja* (Stacey, et al. 1994). One explanation for this apparent paradox was that the *nodZ* mutant likely produced a non-fucosylated tetrameric LCO. This idea arose from the above data with the synthetic LCO, LCO-IV (C16:0), which was shown to be capable of inducing a NOI response on *G. soja* roots. The detailed analysis of LCO production by the mutant strain NAD138 shown above, revealed that the *nodZ* mutant produced a compound, Nod*Bj*-IV(C18:1), which was postulated to be the factor necessary for *G. soja* nodulation. This natural product LCO was never applied to plants alone because it co-elutes from a reverse phase HPLC column with another LCO (see results above). However, as hypothesized, a peak that contains this tetramer was found to elicit a strong NOI response on *G. soja* roots, (data not shown). Since neither the fatty

acid chain length nor the degree of unsaturation strongly effects mitogenic activity on this plant (Stokkermans et al. 1995), we concluded that the low abundance LCO, Nod*Bj*-IV (C18:1) was responsible for the ability of the *nodZ* mutant to nodulate *G. soja*. However, these data were not sufficient to explain how this mutant can nodulate *V. umbellata*, since neither the mixture containing Nod*Bj*-IV(C18:1) nor the synthetic compound LCO-IV(C16:0), had activity on this plant (Table 10).

In an attempt to explain how strain NAD138 could nodulate *V. umbellata*, all of the individual peak fractions collected from HPLC eluates were tested using the spot inoculation assay. As seen in Table 11, none of the individual peaks showed any NOI activity. However, when pooled together, LCO produced by the *nodZ* mutant acted cooperatively to induce nodule-like structures similar to those induced by single, synthetic LCO.

From a previous study (Stacey, et al. 1994), we have in our collection purified Nod*Bj*-V(C18:1) that was analyzed by FAB-MS analysis and determined to be a single molecule. The HPLC peaks obtained in this study were purified on a different reverse phase column, possibly explaining the coelution of LCO. The previously purified compound Nod*Bj*-V(C18:1) was mixed with either peak two or three. Peaks two and three contained mixtures of Nod*Bj*-IV(C18:1,Gro) + Nod*Bj*-V(C18:1), and Nod*Bj*-IV(C18:1) + NodBj-V(Ac,C18:1), respectively. Neither of these peaks displayed any response when applied to *V. umbellata* roots alone. However, both peaks two and three were active when applied in combination with added Nod*Bj*-V(C18:1). Peak four likely contains only Nod*Bj*-V(C18:1). Addition of this peak with the previously purified compound to *V. umbellata* roots displayed no NOI activity (data not shown). These data

Table 11. NOI activity of LCO from *Bradyrhizobium japonicum* strain NAD138 on *V. umbellata* roots. Also included in the table, are the major product LCO produced by wild-type *B. japonicum* and the structurally identical synthetic LCO, that were used as positive controls.

LCO Tested	NOI Activity
LCOV(C18:1Δ11Mefuc)	+
Nod*Bj*-V(C18:1Δ11,Mefuc)	+
Peak 1[b] Nod*Bj*-V(C16:0) Nod*Bj*--IV(C18:1,Gro)	-
Peak 2[b] Nod*Bj*--V(C18:1) Nod*Bj*--IV(C18:1)	-
Peak 3[b] Nod*Bj*--V(Ac,C18:1) Nod*Bj*--V(Ac,C18:1)	-
Peak 4[b] Nod*Bj*--V(C18:1)	-
Mixture1: All peaks[b] Nod*Bj*--IV(C18:1,Gro) Nod*Bj*--IV(C18:1) Nod*Bj*--V(Ac,C18:1) Nod*Bj*--V(C18:1)	+
Mixture2: Nod*Bj*--V(C18:1) + Peak2: Nod*Bj*-IV(C18:1,Gro) Nod*Bj*--V(C18:1)	+
Mixture3: Nod*Bj*--V(C18:1) + Peak3: Nod*Bj*--IV(C18:1) Nod*Bj*--V(Ac,C18:1)	+

[a]100ng of LCO was spot inoculated onto 4 day old roots. A (+) score was given if LCO were able to induce macroscopically visible structures within 12 days post inoculation. A (-) score was given if no nodule structures formed See Methods and Results. [b]HPLC peak fractions (see text).

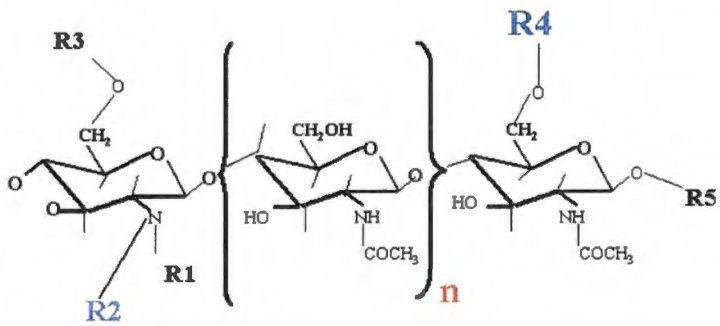
suggest that multiple LCO with distinct structures were required for NOI activity on *V. umbellata*. It is possible that a combination of pentameric and tetrameric LCOs is required for biological activity on *V. umbellata* roots. The fact that peaks two and three alone did not have any activity is difficult to explain. It is possible that the concentration of total LCOs in these peaks were not sufficient to produce a response. It is also possible that the ratio of the different LCOs is critical to their ability to signal the plant. This phenomenon was seen in our earlier work where we showed that multiple LCO, added in specific ratios, were required to initiate transcription of the soybean early nodulin gene ENOD2 (Minami et al. 1996b). In this study, we found that LCO-V(C18:1,Mefuc) had to be in a 1:1 ratio or higher with respect to LCO-V(C18:1,Mefuc) in order for the mixture to activate ENOD2 transcription in *G. soja* roots..

Peak 2, the most abundant peak, was inoculated onto *V. umbellata* roots in several different experiments and no activity was ever detected, even when the total concentration of LCO was approximately 10^{-8} M per spot. These data argue against the possibility that NOI induction by LCO produced by the *nodZ* mutant is simply due to a concentration effect. Therefore, we conclude that the combined effect of multiple LCO molecules produced by the *nodZ* mutant is the most likely explanation as to how this mutant was able to nodulate *V. umbellata*. Figure 8 depicts a summary of the structural requirements of LCO for biological activity on both *G. soja* and *V. umbellata*.

Aberrant Nodule Structures are formed on *V. umbellata* Roots in Response to LCO

To better determine the responsiveness of *V. umbellata* to LCO, roots were spot

Figure 8. Summary of LCO structural requirements for biological activity on *G. soja* and *V. umbellata.* The core structure of the LCO molecule is shown. The substitutions to the different R-groups are indicated in the table below. The biological activity is indicated by either a "+" sign or a "-" sign to indicate if the given LCO is biologically active or inactive respectively on the given plant species.



LCO Structure

				Biological Activity	
n	R4	R2	R3	*G. soja*	*V. umbellata*
3	2-O-Mefuc	C18:1Δ11	H	+	+
3	2-O-Mefuc	C18:1Δ9	H	+	+
3	2-O-Mefuc	C16:0	H	+	+
3	H	C18:1Δ11	H	-	-
2	2-O-Mefuc	C18:1Δ11	H	-	-
2	H	C16:0	H	+	-
2	H	C18:1Δ11	H	ND	+
3	H	C18:1Δ11	Ac		

inoculated with various concentrations of NodBj-V(C18:1,Mefuc), the most abundantly produced LCO from *B. japonicum*. The nodule structures that were induced by LCO were usually large, suggesting extensive nodule development. Additionally, several nodule structures were observed that contained emerging lateral roots (Figure 9). Similar lateral roots emerging from nodules were observed on *V. umbellata* roots that had been inoculated with the ineffective *B. elkanii* strain USDA61, or with *B. japonicum* strain USDA110, which also formed ineffective nodules in our assay. Many large nodule induced with LCO attained an irregular surface, as observed under the stereomicroscope (Figure 9, panel B). The normal appearing nodules, large nodules with an irregular surface, and nodules with emerging lateral roots were analyzed microscopically, as seen in Figure 3, panelC. *B. japonicum* USDA110 and *Rhizobium* sp. strain NGR234, which has a very broad host range, were unable to form nitrogen fixing nodules on *V. umbellata*, but were able to induce nodule structures similar to those formed upon addition of LCO (data not shown). These results might be explained by the fact that growth pouches were used in our assay system to facilitate the visualization of nodule structures. It should be noted that *Rhizobium* sp. NGR234 was recently reported to be able to infect *V. umbellata* when the plants were grown under different conditions (Puepke and Broughton, 1999).

From the section LCO-induced nodules, it is evident that the nodule structures of *V. umbellata* do not resemble normal determinate nodule development in several respects. First, none of the LCO-induced nodule structures contain a clear mitotic center (Figure 9C). Instead, the outer cortex of the nodule structure consists of smaller cells in which neither a clear mitotic center nor an epidermal layer can be distinguished. Second, a zone of large cells surrounds the vascular traces, and most of the peripheral cells in this

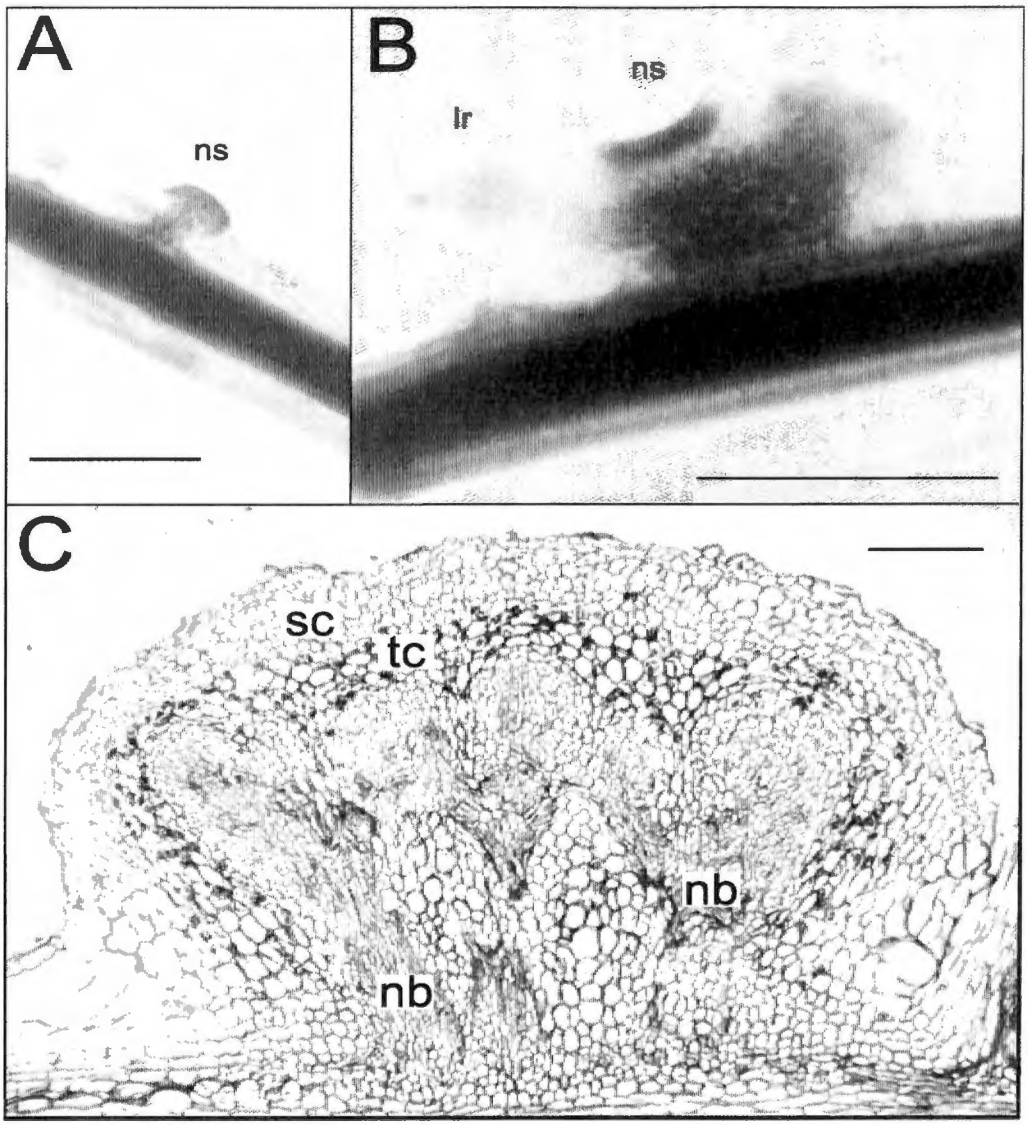


Figure 9. Micrographs of Nod signal-induced, nodule-like structures on *Vigna umbellata*. **A.** Whole mount nodule at emerged stage (ns) Bar = 1 mm. **B.** Whole mount nodule at emerging stage (ns) from which a lateral root (lr) has emerged. Bar= 1mm. **C.** Cross section of an emerged nodule demonstrating formation of multiple vascular traces. Bar = 100um. nb, nodular vascular bundle; tc, larger cells with thick cell walls; I, inner cortex of nodule structure; sc, small cells in outer cortex. Figure provided by Thomas Stokkermans and N. Kent Peters.

zone contain densely staining, thick cell walls. Third, some nodule structures contain multiple vascular bundles that often branch and that are positioned in a way that has not been observed in determinate nodules (Figure 9, Panel C; Sen, et al., 1986, Stokkermans, and Peters, 1994). Fourth, some of these vascular bundles give rise to lateral roots that emerge from the nodule structure (Figure 9, Panel B). These nodule-lateral root hybrid structures may contain only one central vascular bundle. However, some also contain a vascular bundle that bifurcates in the nodule structure portion and comes together again to form a central vascular bundle in the lateral root portion, as seen with a structure induced with ineffective rhizobia (Figure 9C).

Section II. Genetic Analysis of a Putative Lectin-Like, Nod Signal Receptor

Identification Of a Putative *Medicago truncatula* Ortholog of a Nod Signal Binding Protein from *Dolichos biflorus*

A promising candidate for a Nod signal receptor is an apyrase protein isolated from the roots of the legume *Dolichos biflorus*. This protein, LNP, was shown to be localized on the root hair surface and bind Nod signals from rhizobia capable of nodulating *D. biflorus* (Etzler, et al., 1999). Moreover, binding of the Nod signal to the apyrase stimulated ATPase activity, suggesting a possible mode for signal transduction. Unfortunately, *D. biflorus* is a little studied legume with limited information available on its nodulation properties, genetics, preferred symbiont, etc.

Recently, *Medicago truncatula* has been touted as a promising model plant for genetic studies of legumes (Barker, et al., 1990, Cook, 1999). A number of laboratories

worldwide are now working to develop this plant as a model system. The developing level of information on *M. truncatula* should be an aid to studies of nodulation. Therefore, a study was undertaken to examine the apyrase genes of this plant as an initial step in defining the role of these proteins in the nodulation response. Figure 10 depicts a picture of *M. truncatula*, comparing it to the model plant *Arabidopsis thaliana.* A *M. truncatula* cDNA was identified by touch-down-PCR (TD-PCR) using primers designed from conserved domains found in a variety of apyrases (Handa and Guidotti, 1996). The 850 bp fragment obtained by TD-PCR was extended using 3' and 5'-RACE (Frohman et al., 1988) to yield a full-length cDNA sequence. From the sequence generated from the RACE products, primers were designed to obtain the full-length gene using an RT-PCR based approach from RNA isolated from *M. truncatula* roots (see Table 5 for primer sequences). One of the clones generated was designated plasmid pJRC63, which contains the full-length cDNA that was designated as *Mtapy1*. See Figure 11 for the sequence of the full open reading frame.

Sequence of *Mtapy1*

Comparison of the sequence of *Mtapy1* to other genes in the non-redundant NCBI protein sequence database using a BLASTP (Altschul, et al., 1997) search identified several possible orthologs. We also identified possible orthologous genes using a dbEST search (Pearson, et al., 1997). Alignment of *Mtapy1* with the sequences of other known genes in the database revealed that *Mtapy1* contained all four of the putative conserved apyrase domains (Figure 12; Handa and Guidotti, 1996).

The sequence of *Mtapy1* predicts an open reading frame of 466 amino acids,

Figure 10. *Medicago truncatula* vs. *Arabidopsis thaliana.* The *M. truncatula* plant depicted on the left side of the figure was derived from vernalized seed. Therefore the plant depicted in the picture flowered at an earlier stage then plants that are not vernalized.

Figure 11. Sequence of the Open reading frame of *Mtapyl* showing DNA sequence and translated amino acid sequence. Genbank Accession number: AF288132.

```
   1  ATGGTCTTACTTTGGCAAAACACCAAGAATATGATGAACTTTATGACACTCATCACATTT   60
   1  M  V  L  L  W  Q  N  T  K  N  M  M  N  F  M  T  L  I  T  F    20

  61  CTTCTCTTCATTATGCCTTCAATTTCTTACTCCCAATATCTTGGAAACAACATATTACTC   120
  21  L  L  F  I  M  P  S  I  S  Y  S  Q  Y  L  G  N  N  I  L  L    40

 121  ACTAACCGTAAGATTTTCCCAAAACAAGAACCAATTTCCTCTTATGCTGTTGTGTTTGAT   180
  41  T  N  R  K  I  F  P  K  Q  E  P  I  S  S  Y  A  V  V  F  D    60

 181  GCTGGTAGCACTGGAAGCCGTGTCCATGTTTACCATTTTGATCAAAACTTAAATCTTCTT   240
  61  A  G  S  T  G  S  R  V  H  V  Y  H  F  D  Q  N  L  N  L  L    80

 241  CATGTTGGTAAAGATGTTGAGTTTTATAATAAGACAACGCCCGGTTTGAGTGCATACGCG   300
  81  H  V  G  K  D  V  E  F  Y  N  K  T  T  P  G  L  S  A  Y  A   100

 301  GATAATCCAGAAGAAGCTGCAAAATCTTTGATTCCACTTTTAGAGCAAGCAGAAAGTGTA   360
 101  D  N  P  E  E  A  A  K  S  L  I  P  L  L  E  Q  A  E  S  V   120

 361  GTTCCTGAGGATCAGCGCTCCAAGACACCCATTAGACTTGGGGCAACAGCAGGTTTAAGG   420
 121  V  P  E  D  Q  R  S  K  T  P  I  R  L  G  A  T  A  G  L  R   140

 421  CTTTTGAATGGGGATGCTTCTGAAAAAATACTGCAATCGGTAAGGGATTTGTTCAGCAAT   480
 141  L  L  N  G  D  A  S  E  K  I  L  Q  S  V  R  D  L  F  S  N   160

 481  AGAAGTACCTTCAATGTTCAACCTGATGCAGTTTCTATTATTGATGGAACCCAAGAAGGT   540
 161  R  S  T  F  N  V  Q  P  D  A  V  S  I  I  D  G  T  Q  E  G   180

 541  TGTTATCTGTGGGTGACAGTTAACTATGCATTGGGGAATTTAGGGAAAAAGTTCACAAAA   600
 181  C  Y  L  W  V  T  V  N  Y  A  L  G  N  L  G  K  K  F  T  K   200

 601  ACAGTGGGAGTAATGGATGTTGGAGGTGGATCAGTTCAAATGGCATATGCAGTGTCAAAG   660
 201  T  V  G  V  M  D  V  G  G  G  S  V  Q  M  A  Y  A  V  S  K   220

 661  TATACAGCTAAAAATGCTCCAAAAGTTGCTGATGGAGAAGATCCATACATTAAGAAGCTT   720
 221  Y  T  A  K  N  A  P  K  V  A  D  G  E  D  P  Y  I  K  K  L   240

 721  GTACTCAAGGGAAAACCATATGATCTCTATGTTCACAGTTACTTACACTTTGGTAGAGAA   780
 241  V  L  K  G  K  P  Y  D  L  Y  V  H  S  Y  L  H  F  G  R  E   260

 781  GCATCTCGAGCTGAGATTATGAAGGTCACTCGTAGTTCTCCTAACCCTTGCCTTTTAGCT   840
 261  A  S  R  A  E  I  M  K  V  T  R  S  S  P  N  P  C  L  L  A   280

 841  GGATTTGATGGGACCTACACATATGCTGGAGAGGAGTTTAAGGCCAAAGCCCCTGCTTCT   900
 281  G  F  D  G  T  Y  T  Y  A  G  E  E  F  K  A  K  A  P  A  S   300

 901  GGGGCTAATTTTAATGGATGCAAAAAGATAATTCGTAAGGCACTTAAATTGAACTATCCA   960
 301  G  A  N  F  N  G  C  K  K  I  I  R  K  A  L  K  L  N  Y  P   320

 961  TGTCCCTATCAAAATTGCACTTTTGGTGGAATTTGGAATGGCGGAGGGGGAAATGGACAG  1020
 321  C  P  Y  Q  N  C  T  F  G  G  I  W  N  G  G  G  G  N  G  Q   340

1021  AAACACCTTTTTGCTTCTTCATCTTTCTTTTACCTACCTGAAGATGTTGGTATGGTTGAT  1080
 341  K  H  L  F  A  S  S  S  F  F  Y  L  P  E  D  V  G  M  V  D   360

1081  CCAAAGACACCTAATTTCAAAATTCGTCCTGTGGATCTCGTGAGTGAAGCTAAGAAAGCT  1140
 361  P  K  T  P  N  F  K  I  R  P  V  D  L  V  S  E  A  K  K  A   380

1141  TGTGCATTAAACTTTGAGGATGCCAAATCCACTTATCCATTTCTTGCTAAGAAAAATATA  1200
 381  C  A  L  N  F  E  D  A  K  S  T  Y  P  F  L  A  K  K  N  I   400

1201  GCTTCATATGTATGCATGGATCTTATATATCAGTATGTGTTGCTCGTTGATGGATTTGGC  1260
 401  A  S  Y  V  C  M  D  L  I  Y  Q  Y  V  L  L  V  D  G  F  G   420
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1261  TTAGATCCATTGCAAGAAATTACATCGGGGAAGGAAATTGAATATCAAGATGCTGTTTTG   1320
 421  L  D  P  L  Q  E  I  T  S  G  K  E  I  E  Y  Q  D  A  V  L    440

1321  GAAGCTGCATGGCCTCTAGGCAATGCTGTAGAAGCCATATCATCGTTACCTAAATTTGAA   1380
 441  E  A  A  W  P  L  G  N  A  V  E  A  I  S  S  L  P  K  F  E    460

1381  CGAATGATGTATTTTGTTTAACTTAAGCC  1409
 461  R  M  M  Y  F  V  *  L  K     469
```

Mtapy1	53	SSYAVVF **DAGSTGSR** VHVYHFD	7
LNP	52	ESYAVIF **DAGSTGSR** VHVYRFN	73
pea NTPase	42	SSYAVVF **DAGSTGSR** IHVYHFN	63
potato apyrase	44	EHYAVIF **DAGSTGSR** VHVFRFD	65
GDA1	91	HKYVIMI **DAGSTGSR** VHIYKFD	112
Human CD39	47	VKYGIVL **DAGSSHTS** LYIYKWP	68

Mtapy1	128	KT **PI** RLG **ATAGLR** LLNGDAS	148
LNP	117	KT **PL** KLG **ATAGLR** LLDGDAA	136
pea NTPase	118	KT **PV** RHL **ATAGLR** LLNGDAS	138
potato apyrase	120	KT **PL** ELG **ATAGLR** MLKGDAS	140
GDA1	165	KT **PV** AVK **ATAGLR** LL.GDAS	184
Human CD39	124	KT **PV** YLG **ATAGMR** LLRMESE	144

Mtapy1	174	ID **G** TQ **EG** CYLWVTV **N** YALGKLGK	196
LNP	173	ID **G** TQ **EG** SYLWVTV **N** YLLGKLGK	195
pea NTPase	162	ID **G** TQ **EG** SYLWVTV **N** YALGNLGK	184
potato apyrase	164	LD **G** TQ **EG** SYMWAAI **N** YLLGNLGK	186
GDA1	210	MG **G** DE **EG** VFAWITT **N** YLLGNIGA	232
Human CD39	168	IT **G** QE **EG** AYGWITI **N** YLLGKFSQ	183

Mtapy1	201	TVGVM **D** V **GGGSVQ** MAYAVSKY	221
LNP	200	TVGVI **D** L **GGASVQ** MAYVSRNT	220
pea NTPase	190	TVGVE **D** L **GGGSVQ** MAYAVSKK	213
potato apyrase	192	TTATA **D** L **GGGSVQ** MAYAISNE	212
GDA1	240	TAAVF **D** L **GGGSTQ** IVFAVSEP	157
Human CD39	208	TFGAL **D** L **GGASTQ** VTFVPQNQ	228

Figure 12. Conserved apyrase regions from a variety of different apyrase genes. The sequences are compared to the deduced amino acid sequence of *Mtapy1*. Sequences that were aligned are LNP, pea NTPase, apyrase from potato, GDA1 from yeast, and human CD39. The conserved amino acids in this region, which are thought to be essential for enzymatic activity, are shown in bold.

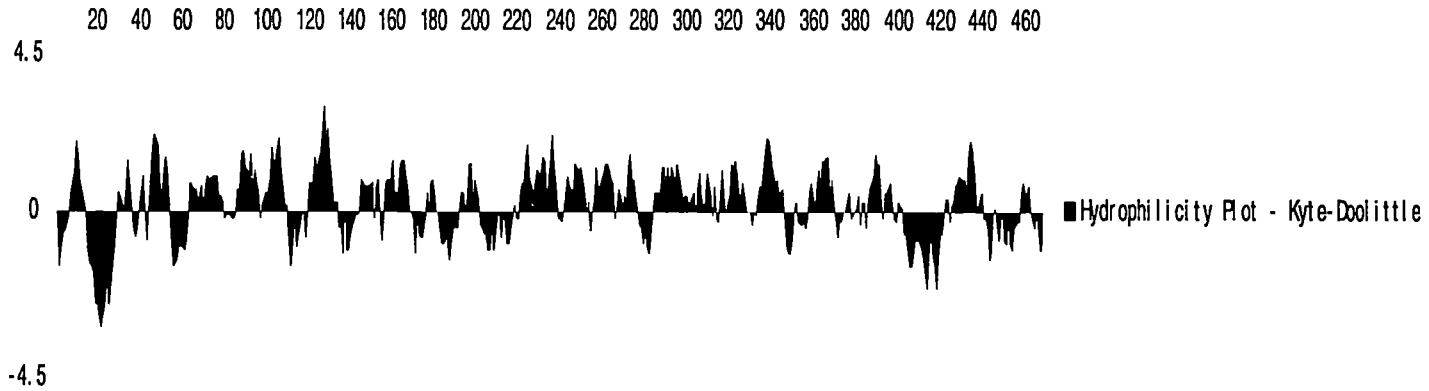


Figure 13. Hydropathy plot analysis of the predicted amino acid sequence encoded by *Mtapy1*. Analysis was performed using MegAlign™ sequence analysis software (DNASTAR Inc, Madison, WI). Amino acid positions are shown at the top of the plot, designated by number from the amino terminus.

encoding a protein with a calculated molecular weight of 51.6 kDa. Hydropathy analysis of the predicted protein sequence indicated that *Mtapyl* has a single strong transmembrane helix of twenty amino acids at the *N*-terminus (Figure 13). This transmembrane region was also predicted to be a cleavable *N*-terminal signal sequence according to the methods of von Heijne, et al. (1986).

The *Mtapyl* PCR Product Hybridizes to *M. truncatula* Genomic DNA

The *Mtapyl* PCR product generated from *M. truncatula* RNA was used as a probe against *M. truncatula* genomic DNA to ensure that the PCR product generated represented an actual *M. truncatula* DNA sequence. *M. truncatula* genomic DNA was subjected to restriction endonuclease digestion and blotted to nylon membranes as described in Materials and Methods. Blots were probes with ^{32}P-labeled *Mtapyl* DNA as described. As seen in Figure 14, the *Mtapyl* PCR-generated clone does indeed bind to *M. truncatula* genomic DNA under high stringency conditions. These data indicate that the *Mtapyl* clone isolated is indeed a gene from *M. truncatula*.

Mtapyl is Induced Rapidly in Response to *S. meliloti* Inoculation

Since the *D. biflorus* apyrase has been implicated in the nodulation process (Etzler et al., 1998), northern blot analysis was used to see if the level of apyrase mRNA was significantly regulated by rhizobial inoculation. We performed a time course of expression of *Mtapyl* using total RNA isolated from roots of wild-type *M. truncatula* line A17 inoculated with *S meliloti* strain ABS7M. As seen in Figure 15, *Mtapyl* mRNA levels increased significantly in response to *S. meliloti* within three hours after

Figure 14. Southern Blot analysis of the *Mtapy1* PCR generated product. Ten micrograms of *M. truncatula* wild-type line A17 Genomic DNA, digested with *Bam*HI, *Hind*III, or *Eco*RI, was loaded into each lane shown of a 0.8% agarsose gel. The blot was probed with *Mtapy1* (cDNA insert of pJRC63) and washed at high stringency (see Materials and Methods for details).

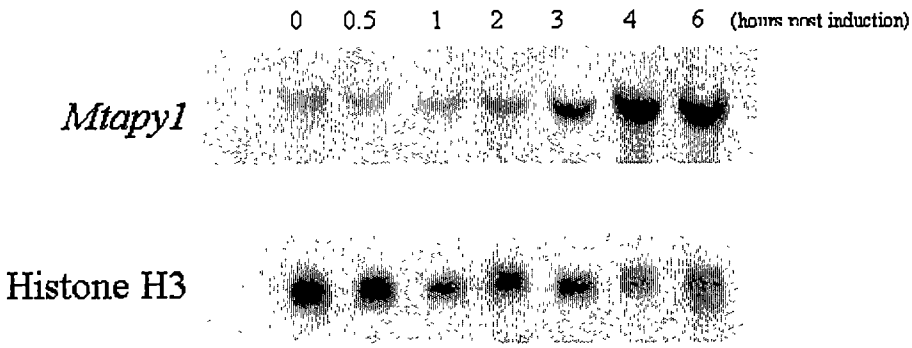


Figure 15. Northern blot showing the expression of *Mtapy1* in response to inoculation with *Sinorhizobium meliloti* wild-type strain ABS7M. Total RNA was isolated from roots inoculated 0, 0.5, 1, 2, 3, 4, 5, and 6 hours after inoculation with bacteria. Blots were also probed with Histone H3 cDNA as a RNA loading control. Each lane was loaded with RNA isolated from approximately 0.2 grams or root tissue. RNA for this blot was provided by Senthil Ramu (Texas A&M University, College Station, TX). See text for details.

inoculation.

Identification of a Second Putative Apyrase Gene From *M. truncatula*

A search of the dbEST sequence database (Pearson, et al., 1997) using the *Mtapy1* sequence for comparison identified a distinct apyrase that had been sequenced from a cDNA library made from *M. truncatula* root hair tissue (Accession number: AA660474; Covitz, et al., 1998). Using the sequence in the database, primers 5'Mt46-2 and 3'Mt46-2 (see Table 5) were designed for use in a RT-PCR reaction to clone the corresponding cDNA. As before, RNA was isolated from the roots of *M. truncatula* plants from 3 to 11 days post imbibition. RNA was reverse transcribed using MMLV-RT and the corresponding cDNA was used as a template in the PCR reaction. This approach yielded plasmid pJRC201, containing a partial cDNA clone of 430 bp. DNA sequence analysis clearly revealed that this PCR fragment was identical to the sequence found in the dbEST database but was distinct from *Mtapy1*. Therefore, this apyrase cDNA was designated *Mtapy2*. Sequence analysis was also confirmed by the identification of a second EST sequence from a different database (Accession number: AI974272). This EST, pKVO1M6B, was kindly given to us by Dr. Kate VandenBosch and the clone was sequenced. The overlapping regions in this sequence and the original EST sequence (Accession number: AA660474; Covitz, et al, 1998) indicated that they represent the same gene, namely, *Mtapy2*. The additional sequence information was critical for subsequent phylometric analyses (see below).

M. truncatula Apyrase Genes are Clustered on the Genome

Mtapy1 was used as a probe to screen a bacterial artificial chromosome (BAC) library containing large genomic DNA inserts from *M. truncatula* line A17 (Nam et al., 1999). Yung Wu Nam at Texas A&M University performed the initial BAC library screen. Two hybridizing BAC clones, 52G10 and 58M19, were isolated and confirmed by Southern blot hybridization (data not shown). As shown in Figure 16, a larger region of contiguous BAC DNA was identified by PCR amplification of the BAC library multiplex using oligonucleotide primers designed from BAC sequence information. DNA sequencing of BAC ends and internal sequencing with *Mtapy1* primers, identified five putative genes in this region, and two retrotransposon-like sequences. Additional sequence information for these five predicted genes was obtained using a primer walking strategy either on purified BAC DNA or subcloned BAC DNA fragments. Three of these predicted genes have their highest homology to characterized apyrase genes from legumes. One of these genes was verified as *Mtapy1* based on sequence identity, while the other two were tentatively designated as *Mtapy3* and *Mtapy4*.

The availability of numerous *Medicago truncatula* EST sequences in the NCBI GeneBank allowed the immediate verification of *Mtapy3,* and a thioredoxin-like sequence as transcribed genes. Gene specific primers were designed from the *Mtapy3* BAC sequence and used in a RT-PCR reaction from *M. truncatula* RNA to isolate a 460 bp PCR fragment that was cloned to produce plasmid pJRC300. In order to get enough sequence of *Mtapy4* to design gene specific primers, 3'RACE was used to extend the 3' end of this gene using the primer Mtapy4GS forward (see Table 5). This procedure yielded a 1017 bp PCR fragment corresponding to *Mtapy4*. This fragment was cloned

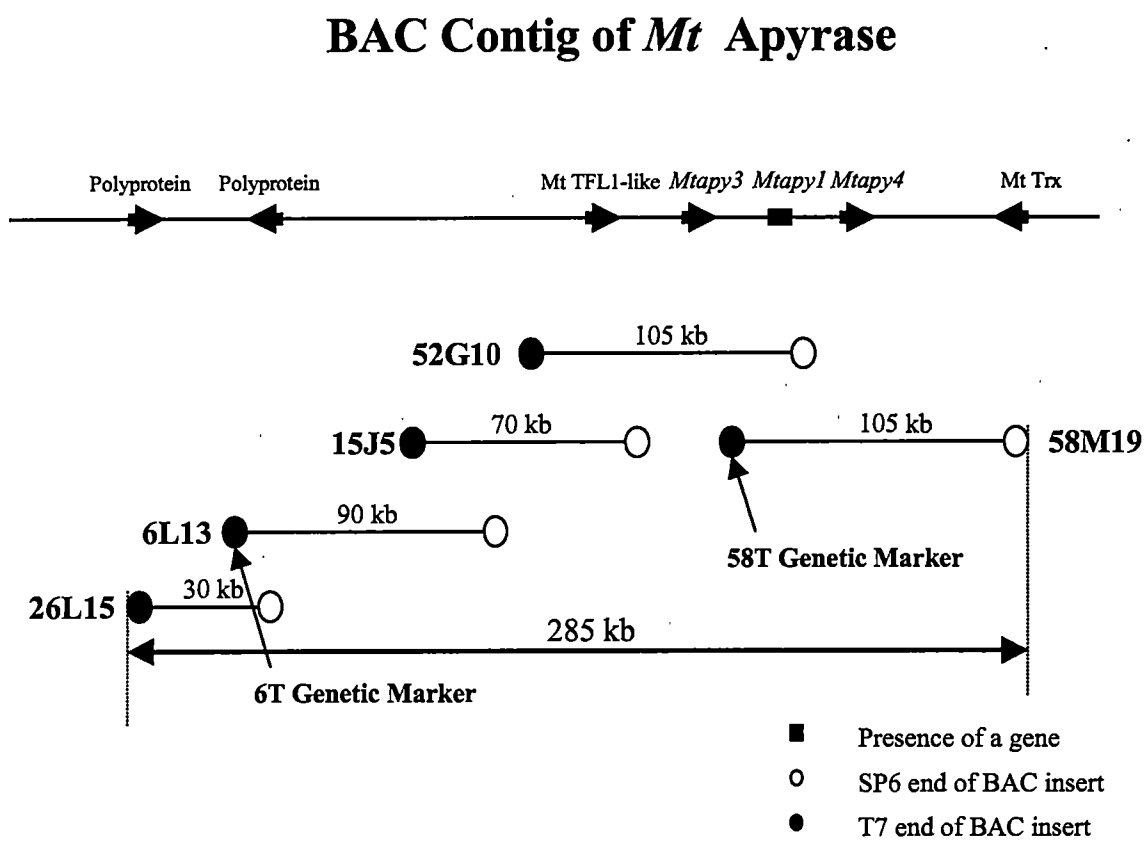


Figure 16. Contiguous map of Bacterial Artificial Chromosomes surrounding an apyrase cluster in *Medicago truncatula*. The map indicates the location of the *Mtapy1*(Genbank accession number AF288132), *Mtapy3, and Mtapy4* (Genbank accession number AF288133). Also note the position of the putative TFL1 ortholog. Sequence analysis of the BAC DNA also identified a thioredoxin-like gene (*MtTrx*) and two retro-transposon-like sequences as well as two polyproteins. One polyprotein is a Copia-like polyprotein (Genbank accession number AZ124289) and the other is a putative reverse transcriptase gene (Genbank accession number AZ124288).

into pCR2.1-TOPO (Invitrogen, Carlsbad, CA) to produce plasmid pJRC400. The cDNA inserts in plasmids pJRC300 and pJRC400 were sequenced. Comparison to the sequences of *Mtapy1* and *Mtapy2* indicated conclusively that *Mtapy3* and *Mtapy4* were distinct genes.

The BAC contig shown in Figure 16 contains a minimum of three apyrase genes with high homology to other legume apyrases. Similar, but less extensive analysis, was conducted with *Mtapy2*. Briefly, a single BAC clone, designated 20K21, was identified by means of hybridization to high-density filter arrays. BAC end sequencing of 20K21 revealed the presence of a putative gene with best protein homology to NADPH quinone reductase. Taesik Uhm and Dong Jin Kim at Texas A&M University performed the majority of BAC end sequencing and BAC contig analysis.

Genetic Mapping of *M. truncatula* Apyrases

Taesik Uhm and Dong Jin Kim at Texas A&M University performed the mapping studies presented here. To determine the genetic map position of the apyrase cluster (*Mtapy1*, *Mtapy3*, and *Mtapy4*), and of *Mtapy2*, co-dominant PCR-based CAPS markers were developed. Briefly, BAC end sequence information from BACs 6L13 and 58M19 (corresponding to the apyrase cluster) and from BAC 20K21 (corresponding to *Mtapy2*) was used to design oligonucleotide primers. PCR amplification and sequencing of genomic DNA from *Medicago truncatula* mapping genotypes A17 and A20 allowed the development of three CAPS markers, as detailed in Table 12. As shown in Figure 17, analysis of these CAPS markers on the basic mapping population of 93 F2 individuals

Table 12. Description of CAPS markers used for genetic mapping.

Marker Name	Marker Homology	Oligonucleotide sequence	PCR Conditions	Size of Product	Restriciton enzyme	Size of Cleaved Product
58T[a]	Mtapy4	AAGGGCTTTTAATTTCTGTCTG CCAAACCAATCTAAAATAATAAAC	90°C, 4 min 50°C, 30 s 72°C, 50 s	691bp	BsiEI	A17: 233bp + 458bp A20: 691bp
6T[b]	Mtapy contig	ATTTTAATTGAACGTATCTTTTT GAGGCTCCTAATTTTTGACTGTTG	90°C, 4 min 50°C, 30 s 72°C, 50 s	740bp	Bsp1286I	A17: 332bp + 408bp A20: 740bp
20S[c]	Mtapy2	GATGGTCTGGCAACTGT AGGGAGGACTTTTCTTAG	90°C, 4 min 50°C, 30 s 72°C, 50 s	409bp	RsaI	A17: 119bp +290bp A20: 691bp
DK427R	Soybean RFLP B046	CCAAACAAGGAAAAGTGTTGGTGTC AATGAGAAACTTTTGAAATTTAGGAT ACGATAG	90°C, 4 min 50°C, 30 s 72°C, 50 s	500bp	BsmAI	A17: 60 + 440 A20: 500

[a]58T corresponds to BAC 58M19.
[b]6T corresponds to BAC 6L13.
[c]20T corresponds to BAC 20K21.

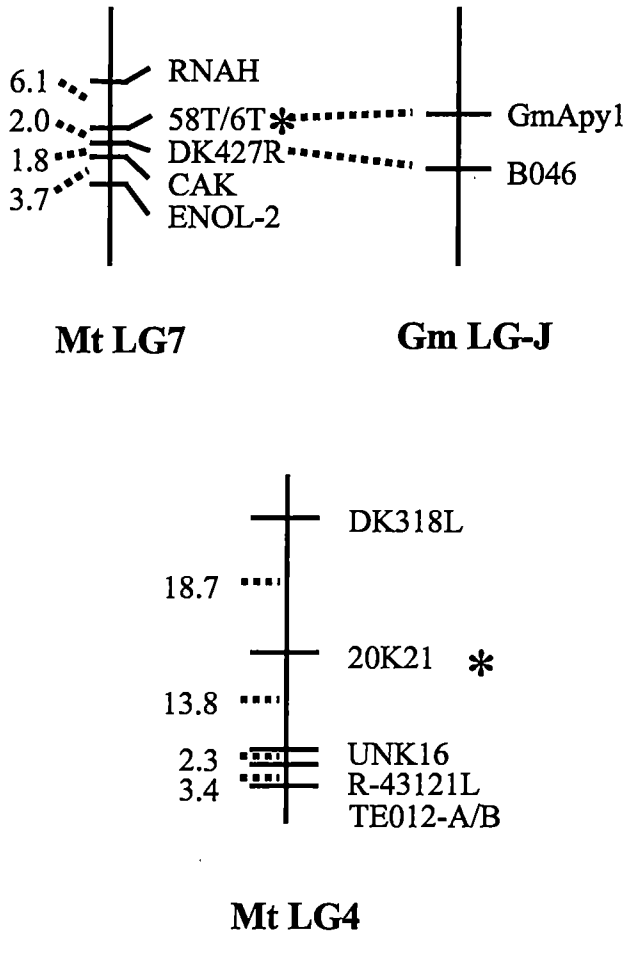


Figure 17. Genetic maps of apyrases from *M. truncatula.* The maps indicate that the apyrase cluster, including at least *Mtapy1*, *Mtapy3*, and *Mtapy4* is located on Linkage Group (MtLG) 7 based on analysis of the map positions of the 58T/6T genetic markers. *Mtapy2* was determined to be located on Linkage Group 4 based on CAPS markers developed from sequence analysis of the the BAC clone 20K21. An apyrase cluster was also found on *Glycine max* linkage group J (Gm LG-J), based on analysis of the map positions of two apyrases identified in soybean (i.e., GS50 and GS52; Day et al., 2000). The apyrase cluster in soybean (*G. max*) is closely linked to the B046 RFLP marker, which is orthologous to the 58T/6T marker in *M. truncatula.* Thus, the B046 homology is tightly linked to an apyrase cluster in both *Medicago truncatula* and soybean. These data are suggestive of conserved synteny between the two genomes, are consistent with close ancestry of these two apyrase gene clusters.

indicates that the apyrase cluster maps to *Medicago truncatula* linkage group 7, while *Mtapy2* maps to linkage group 4.

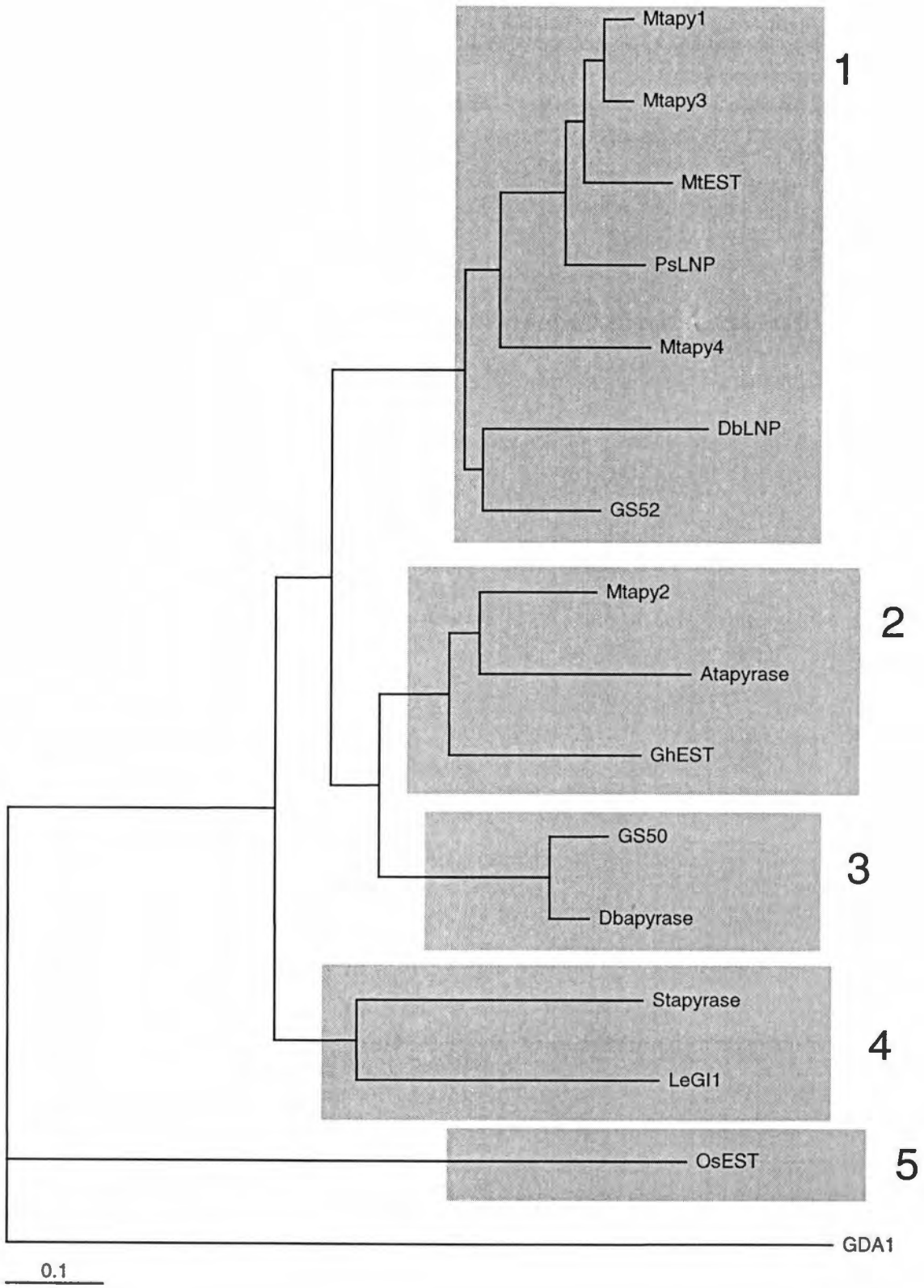
The position of the *Medicago truncatula* apyrase markers in Figure 17 is shown relative to selected core markers on these linkage groups (D.J. Kim and D. Cook, personal communication). Because several of the *Medicago truncatula* core markers were developed based on homology to mapped RFLP clones from the soybean genetic map, it is possible to identify putative syntenic relationships between the *M. truncatula* and soybean genomes. One such marker, designated DK427-R, maps in close proximity to the apyrase cluster marker 58T (Figure 17). MtDK427 has homology to soybean RFLP clone B046, the sequence of which (i.e. AQ841833) is similar to a putative cationic amino acid transporter from cotton (i.e. AI727823). In soybean, B046 maps to two loci, including marker B046b on soybean linkage group J. To ascertain if the *Medicago truncatula* apyrase cluster might share a conserved genome context with previously identified soybean apyrase genes (Day et al., 2000), we conducted a similar analysis with soybean apyrase genes GS50 and GS52. Briefly, three soybean BAC contigs were identified based on hybridization with GS50. One of these contigs also contained homology to GS52, thus identifying a probable apyrase cluster in soybean (data not shown). RFLP mapping of the GS52/GS50 contig allowed assignment of this putative apyrase cluster to soybean linkage group J, in close proximity to marker B046. Thus, the B046 homology is tightly linked to an apyrase cluster in both *Medicago truncatula* and soybean. These data are suggestive of conserved synteny between the two genomes, and are consistent with the close ancestry of these two apyrase gene clusters.

Phylogenetic Analysis of Apyrases from *M. truncatula*

A recent report indicated that plant apyrases are separated into two distinct families based on phylogenetic studies (Roberts et al., 1999). It was suggested that LNP like genes from a number of different sources were members of a distinct class of apyrases that may be unique to legumes. A similar phylogenetic study was performed by Todd Wood at Clemson University with the apyrase sequences that we identified from *M. truncatula*. For phylogenetic analysis, 16 partial sequences of plant apyrases, and a yeast protein, GDA1 were aligned. One additional, recently submitted EST sequence of a putative apyrase from *M. truncatula* (MtEST, Accession number AJ388942) that is distinct from *Mtapy1*, *Mtapy2*, *Mtapy3*, and *Mtapy4* was included in the phylogenetic analysis. A neighbor-joining tree generated from this alignment shows that there appear to be at least five subfamilies of apyrases found in plants (Figure 18).

Subfamily 1 contains *Mtapy1*, *Mtapy3*, *Mtapy4*, and the newly identified *Mt*EST. This subfamily also includes the pea NTPase, LNP from *D. biflorus*, and GS52, a recently identified apyrase from soybean (Day et al, in press). Subfamily 2 consists of Mtapy2, an apyrase from *Arabidopsis thaliana*, and an EST identified in cotton (GhEST). Subfamily 3 consists of a second apyrase isolated from soybean, GS50 (Day et al., in press), and a second apyrase from *D. biflorus* that was identified subsequently to DbLNP. Subfamily 4 consists of the potato ayprase (Handa and Guidotti, 1996), and a tomato EST (LeGl1). Subfamily 5 consists of a single gene, represented by an EST identified in rice (OsEST) (GenBank Accession number AU065811). These results support the earlier results of Roberts et al. (1999), indicating that LNP from *D. biflorus* is a member of distinct family of apyrases that are present only in legumes. It is quite

Figure 18. Neighbor joining tree representing alignment of apyrases from selected sources. Sixteen partial sequences of plant apyrases and the yeast protein GDA1 were aligned for analysis. The apyrases fall into five distinct subfamilies. *Mtapy1, Mtapy3, Mtapy4*, a newly identified EST from *M. truncatula*, MtEST (Gen Bank Accession number AJ388942), LNP and GS52 (Day et al., in press) fall into the first class. *Mtapy2* was found to fall into subfamily 2, along with an *Arabidopsis* apyrase (AF156783), and an EST from cotton, GhEST (AI729322). Additional sequences include *G. soja* GS50 (Day et al, in press), *D. biflorus* Dbapyrase (AF156781), *Solanum tuberosum* apyrase (U58597), *Lycopersicon esculentum* EST cLED13N21 (AI488720), *O. sativa*, OsEST, (AU065811), and the *S. cerevisieae* GDA1 (6320793)


Mtapy1
Mtapy3
MtEST
PsLNP
Mtapy4
DbLNP
GS52
1
Mtapy2
Atapyrase
GhEST
2
GS50
Dbapyrase
3
Stapyrase
LeGl1
4
OsEST
5
GDA1
0.1

interesting that *Mtapy1*, *Mtapy3*, and *Mtapy4*, which are all clustered in the *M. truncatula* genome are all members of this apyrase family. It is possible that these genes arose from duplication events during the evolution of *M. truncatula.*

A further phylogenetic analysis was performed with the apyrases from *M. truncatula* to study their evolutionary relationships. To infer the phylogeny of the *Mtapy* sequences, amino acid sequences of known LNP proteins were aligned to the protein sequences of *Mtapy*1-4 using CLUSTALW (Thompson *et al.*, 1994). The apyrase of *Arabidopsis thaliana* was also included in the alignment as an out-group. The result of this analysis showed that *Mtapy1*, *Mtapy3*, and *Mtapy4* all group together with the LNP sequences, whereas *Mtapy2* is more closely related to the out-group apyrase from *Arabidopsis* (Figure 19). The appearance of multiple copies of *Medicago* apyrase sequences in the LNP clade strongly implies that gene duplication may be an ongoing phenomenon in this apyrase subfamily. To confirm this, a neighbor-joining phylogeny from *rbcL* sequences was constructed of closely-related species (Figure 19). The results showed that the topology of the two trees is not the same. Specifically, while the *rbcL* tree shows a substantial divergence between *Lotus/Glycine/Vigna* and *Pisum/Medicago* (bootstrap support 93%), the LNP clearly shows the *Glycine soja* sequence GS52 as the only outgroup (bootstrap support 100%). In the *rbcL* tree, *Lotus* is a sister taxon to the *Glycine/Vigna* clade, but in the LNP phylogeny, the *Lotus* sequence *Lj*LNP is a sister taxon to a clade formed of three *Medicago* sequences (*Mtapy1, Mtapy3, Mtapy4*) and the *Pisum* sequence *Ps*LNP. The conflicts between the *rbcL* and LNP trees can be explained as the result of gene transfer or multiple LNP paralogs that have not yet been sequenced. Because the LNP tree clearly shows three *Medicago* LNP paralogs (*Mtapy1, Mtapy3*, and

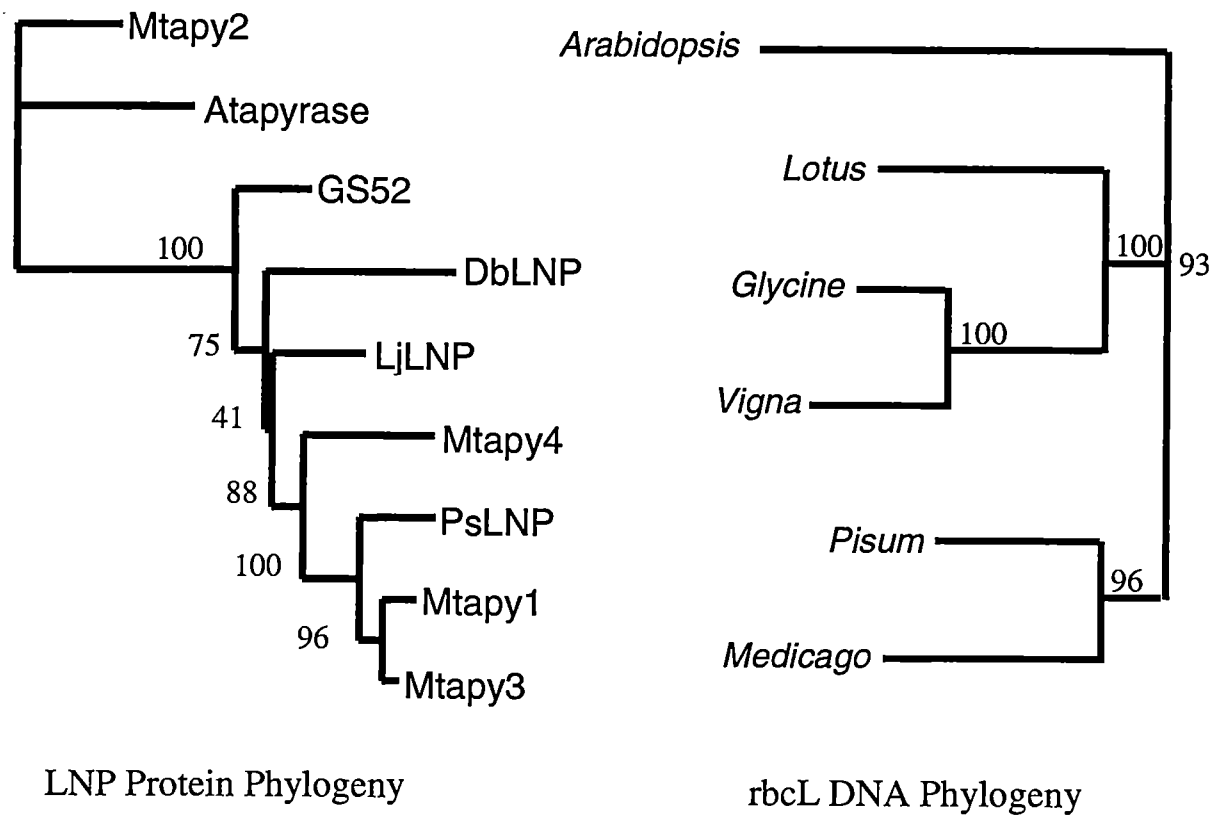


Figure 19. Phyologeny of LNP-like proteins compared to rbcL proteins from selected sources. The evolution of the LNP proteins has been markedly different from the evolution of the rbcL sequences from closely-related species. LNP protein phylogeny (left) was inferred by the neighbor-joining method using a 188 amino acid alignment of LNP proteins outgrouped using the apyrase from *Arabidopsis thaliana* (AF156783). Sequences shown are Mtapy2 (this study), GS52 (Day *et al.*, submitted), *Dolichos biflorus* LNP (AF139807), *Lotus japonicus* LNP (AF156780), Mtapy4 (this study), *Pisum sativum* LNP (Z32743), and Mtapy1 and Mtapy3 (this study). RbcL DNA phylogeny (right) was also inferred by the neighbor-joining method using a 1310 nucleotide alignment of rbcL DNA sequences outgrouped using the rbcL of *Arabidopsis thaliana* (U91966). Other sequences shown are from *Lotus corniculatus* (U74213), *Glycine max* (Z95552), *Vigna unguiculata* (Z95543), *Pisum sativum* (X03853), and *Medicago sativa* (X04975). Bootstrap values out of 100 bootstrap replicates are shown at nodes of both trees. Figure provided by Todd Wood (Clemson University Genomics Institute, Clemson, SC).

Mtapy4), undiscovered paralogs in other legume species would be the preferred explanation over gene transfer.

By closely comparing the *rbcL* phylogeny with the LNP phylogeny, we can propose a possible phylogeny of the *M. truncatula* apyrase sequences. *Mtapy1* and *Mtapy3* are both closely related to the *Ps*LNP, just as the *rbcL* phylogeny would imply for relationship of *Pisum* and *Medicago*. Thus, it seems likely that *Mtapy1* and *Mtapy3* resulted from a very recent gene duplication event, possibly unique to the *Medicago* lineage. Though clearly related to *Mtapy1* and *Mtapy3, Mtapy4* must have arisen from a duplication that took place before the divergence of *Pisum* and *Medicago*. Since it is not found in the LNP clade, *Mtapy2* is apparently more closely-related to the *Arabidopsis* apyrase, and likely arose from a duplication very early in flowering plant evolution.

Mtapy1 and *Mtapy4* are Transiently Induced After Inoculation with *S. meliloti*

Due to the high degree of sequence similarity shared between the apyrases identified in *M. truncatula,* we wanted to identify which of the genes were induced upon rhizobial inoculation. Using gene-specific primers, the level of *Mtapy1* transcript was significantly increased within three hours after inoculation. These data are consistent with our previous northern results using a radiolabelled *Mtapy1* probe (Figure 20). Quantification of the data shown in Figure 20 showed that the level of *Mtapy1* mRNA increased approximately three-fold within six hours post inoculation (Figure 21). Likewise, similar results were obtained using gene specific primers for *Mtapy4* (Figure 20). Quantification of these data indicated that *Mtapy4* induction by inoculation is slightly less than *Mtapy1* at three hours. However, three fold induction of the gene was

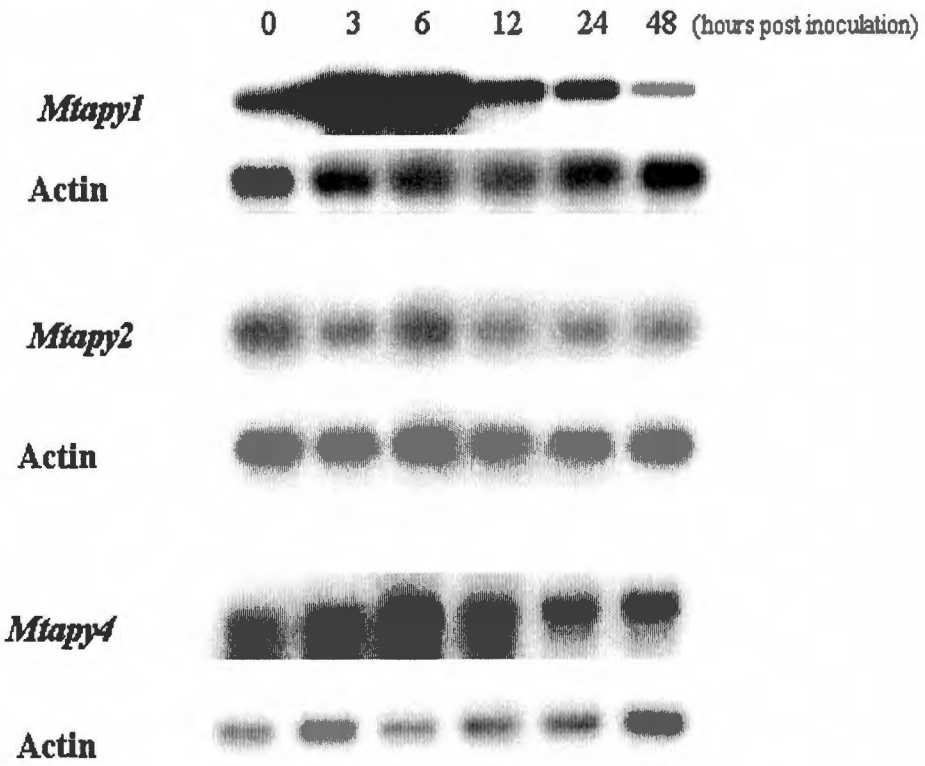


Figure 20. RT-PCR analysis of the level of mRNA expression of apyrase genes in response to wild-type *S. meliloti* strain ABS7M. RNA was collected from roots 0, 3, 6, 12, 24, and 48 hours post rhizobial inoculation. Actin primers were included in each reaction as a loading control. See text for details.

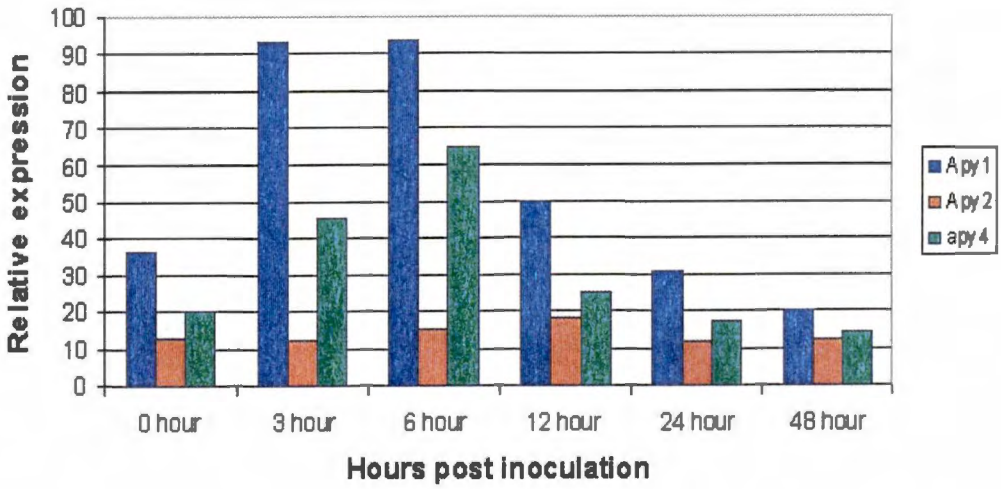


Figure 21. Quantification of the data shown in Figure 20. The relative level of expression was determined by the following calculation: (Total Counts Apyrase / Total Counts Actin) X 100 = relative expression. The standard deviation between relative expression values for two separate experiments was less than 10%.

apparent at six hours post inoculation (Figure 21). Both in the case of *Mtapy1* and *Mtapy4*, induction after inoculation with strain ABS7M is transient. The highest levels of expression are seen 3-6 hours post-inoculation with expression declining to basal levels by 12-24 hours after inoculation.

In contrast to these results, the level of *Mtapy2* mRNA was not significantly enhanced upon rhizobial inoculation (Figure 20 and Figure 21). In these experiments, *Mtapy3* mRNA was not detected, consistent with the lack of expression of this gene in roots (see below). For this reason, no data were available for induction of *Mtapy3*.

Apyrase Genes Are Differentially Expressed in *M. truncatula* Tissues

In order to analyze the tissue specific expression of apyrase genes in *M. truncatula*, we used gene-specific primers and RT-PCR to analyze mRNA levels in various tissues. We isolated total RNA from the roots, hypocotyls, and cotyledons of five-day-old seedlings, and from the stems, leaves, and flowers of five-week-old plants. These plants were vernalized at 4°C for 14 days to shorten the flowering time. In addition, we isolated total RNA from ten-day-old nodules that were harvested from plants inoculated with wild-type *S. meliloti* strain 1021. As shown in Figures 22 and 23, *Mtapy1* was expressed predominantly in roots, hypocotyls and nodule tissue. *Mtapy4* was also expressed in roots and nodules, but also showed significant expression in cotyledons, stem and flower. No expression was found in hypocotyls tissue. *Mtapy2* mRNA was detected in roughly equivalent amounts in all tissues. This is consistent with what has been found for the expression pattern of a putative apyrase identified in *Arabidopsis* (Serry Koh, personal communication). *Mtapy3* showed the highest level of tissue

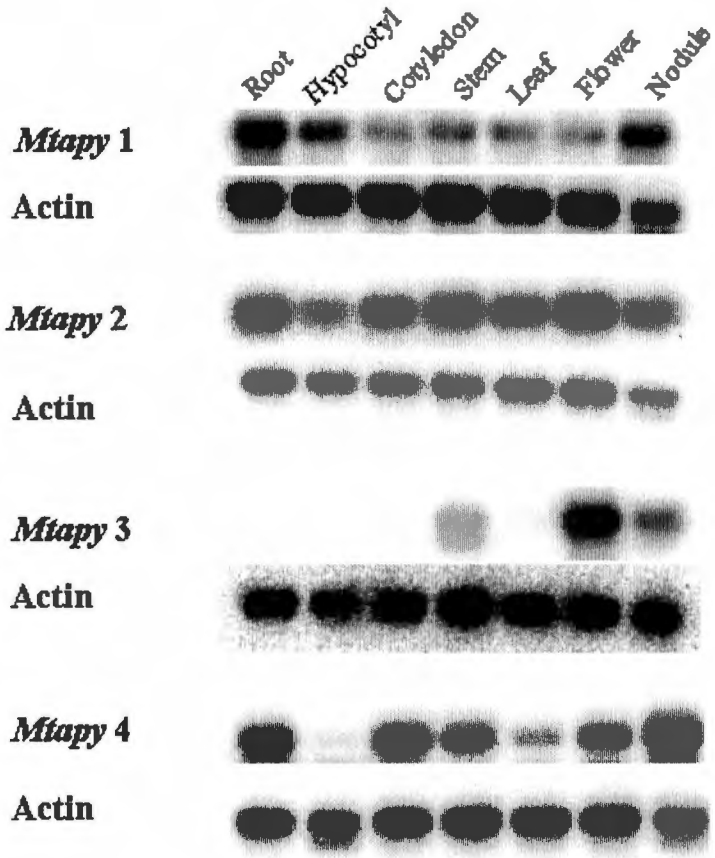


Figure 22. RT-PCR analysis of the expression of apyrase genes from *M. truncatula* in various tissues. Root=5 day old roots, hypocotyls=5 day old hypocotyls, cotyledon=5 day old cotyledon, stem=6 week old stems, flower=6 week old flowers, nodule= nodules isolated 10 days post inoculation with wild-type *S. meliloti* strain 1021. Actin primers were included in each reaction as a loading control. See text for details.

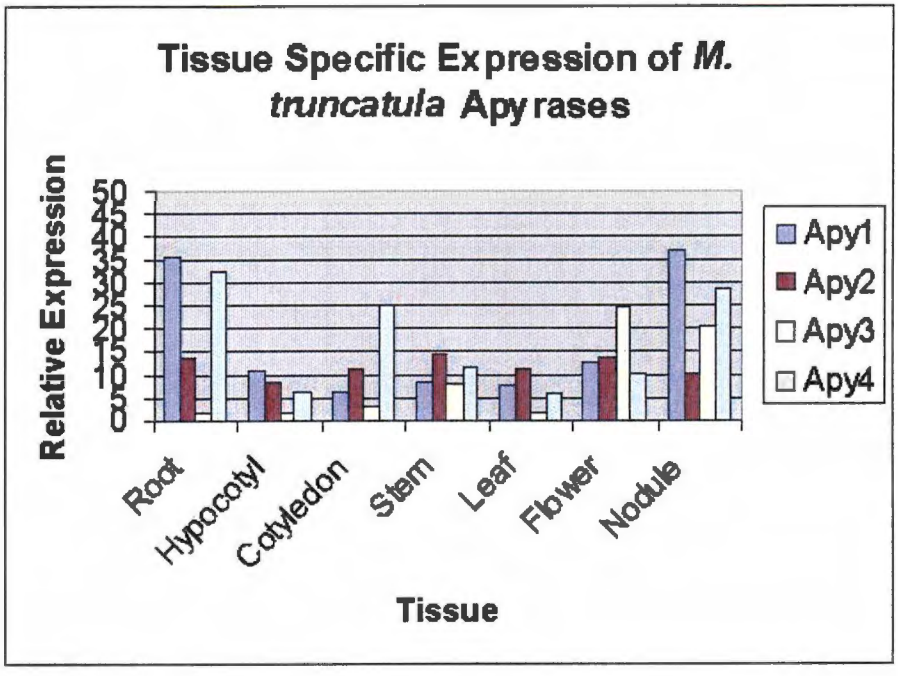


Figure 23. Quantification of the data shown in Figure 22. The relative level of expression was determined by the following calculation: (Total Counts Apyrase / Total Counts Actin) X 100 = relative expression. The standard deviation between relative expression values for two separate experiments was less than 10%.

Apyrase Expression in Response to a Nodulation Deficient Strain of *S. meliloti*

In order to determine if the increase in the level of apyrase mRNA expression in response to rhizobia was dependant upon Nod signals, we decided to test the response of roots to inoculation with a nodulation deficient line of *S. meliloti.* *S. meliloti* strain AK1657 is a mutant strain that is incapable of forming nodules on alfalfa (Kondorosi, et al., 1984). This strain carries a Tn*5* insertion in the *nodC* gene, therefore rendering it incapable of producing Nod signals (Kondorosi, et al., 1984). As seen in Figures 24 and 25 the level of *Mtapy1* mRNA was not significantly affected by inoculation with the *nodC*::Tn*5* mutant strain AK1657. However, the level of *Mtapy1* mRNA transcript increased approximately two to three-fold in response to the wild-type strain AK631. AK631 is the wild-type strain from which AK1657 was constructed. Similar results were obtained from RT-PCR analysis with *Mtapy4* (Figures 26 and 27). Again, the levels of *Mtapy4* expression were not significantly affected by inoculation with the mutant strain. However, when roots were inoculated with the wild-type strain, AK631, the level of *Mtapy4* expression increased over two-fold. These results suggest that Nod signal production by rhizobia is necessary for the induction of apyrase genes in *M. truncatula.* It is necessary to point out that the transient increase in *Mtapy4* mRNA levels detected when roots were inoculated with wild-type strain ABS7M was not apparent when roots were inoculated with strain AK631. Additionally, this experiment was repeated with another set of strains, SL44 (Δ*nodABC*) and the wild-type back ground strain 1021. Similar to the results above, no significant accumulation of either *Mtapy1* or *Mtapy4* mRNA was detected in response to SL44. However, no significant increase in the expression of either gene was detected when inoculated with the wild-type strain 1021, at

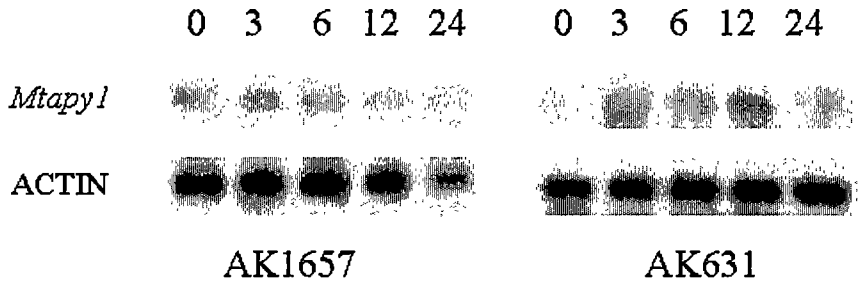


Figure 24. *Mtapy1* expression in response to a *nodC*::Tn*5* mutant of *S. meliloti.* Total RNA was isolated from roots inoculated with either the wild-type strain or the mutant strain at 0, 3, 6, 12, and 24 hours post inoculation. RNA was subjected to RT-PCR as described in the text, using *Mtapy1* gene specific primers (see Table 5). Actin primers were included in each reaction as a positive control.

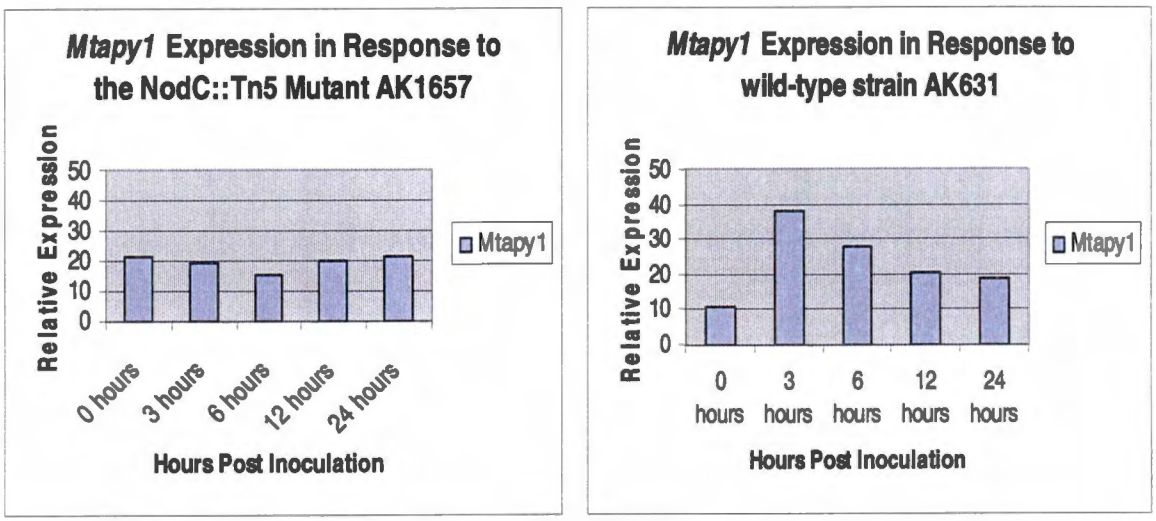


Figure 25. Quantification of the data shown in Figure 24. The relative level of expression was determined by the following calculation: (Total Counts Apyrase / Total Counts Actin) X 100 = relative expression. The standard deviation between relative expression values for two separate experiments was less than 10%.

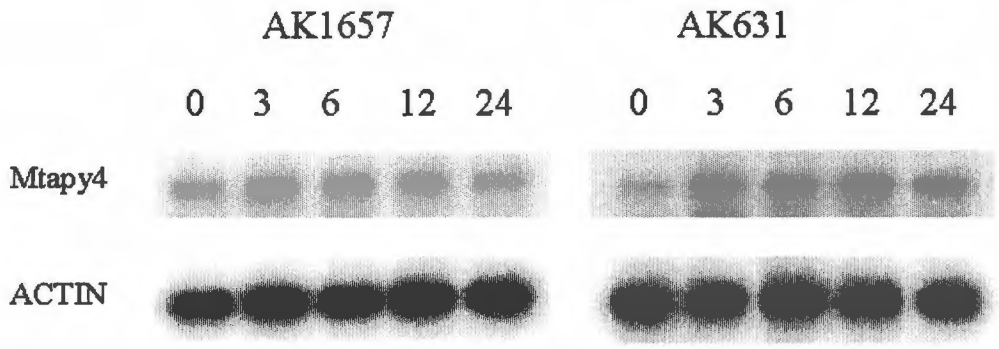


Figure 26. *Mtapy4* expression in response to a *nodC*::Tn*5* mutant of *S. meliloti.* Total RNA was isolated from roots inoculated with either the wild-type strain or the mutant strain at 0, 3, 6, 12, and 24 hours post inoculation. RNA was subjected to RT-PCR as described in the text, using *Mtapy4* genes specific primers (see Table 5). Actin primers were included in each reaction as a positive control.

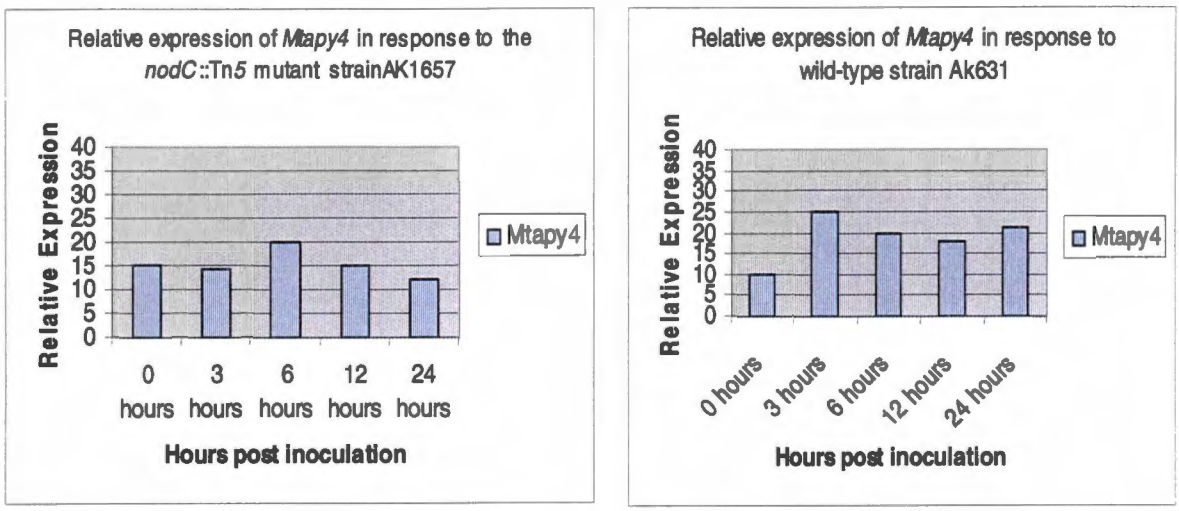


Figure 27. Quantification of the data shown in Figure 26. The relative level of expression was determined by the following calculation: (Total Counts Apyrase / Total Counts Actin) X 100 = relative expression. The standard deviation between relative expression values for two separate experiments was less than 10%.

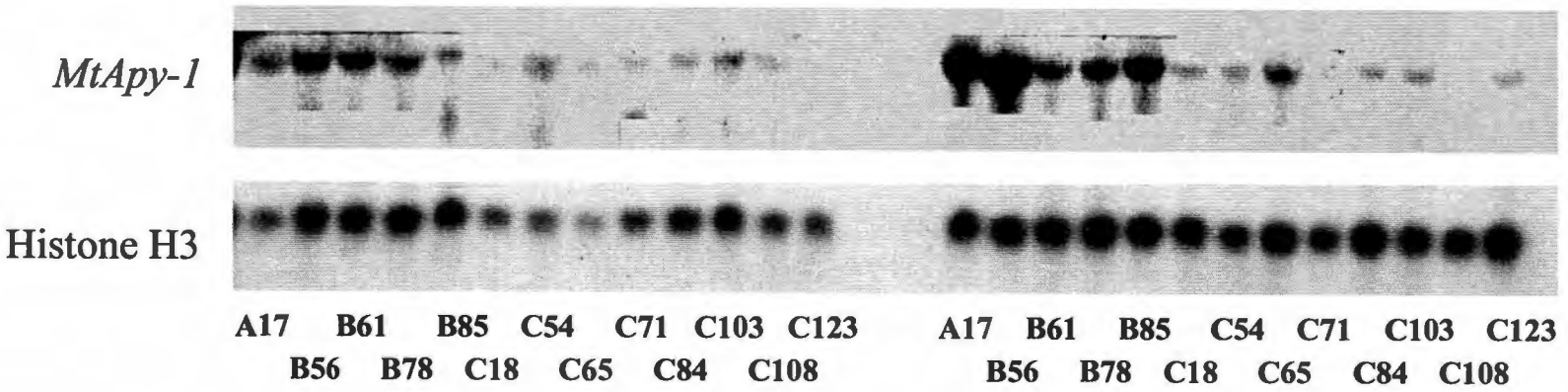
least not within the 24-hour time period studied. This might be due to growth conditions of the plants or bacteria. It might also be the case that this wild-type strain causes a delayed induction of apyrase genes. This would be consistent with findings that increases in *rip1* mRNA are significantly delayed in response to strain 1021 as compared to the wild-type strain ABS7M (Senthil Ramu, personal communication).

Apyrase Expression in Nodulation Deficient Mutant Lines of *M. truncatula*

If apyrases are important to the nodulation response, then plant mutants defective in nodulation may also show reduced or altered apyrase expression. To test this hypothesis, we initially screened several EMS-mutagenized, nodulation deficient lines of *M. truncatula* by hybridizing a ^{32} P-labeled *Mtapy1* probe to total RNA isolated from roots 0 and 6 hours after *S. meliloti* inoculation (Figure 28). Although some lines showed inducible expression similar to the wild type, others showed little or no *Mtapy1* expression even after inoculation. From these initial studies, we concluded that the mutant lines fell into three distinct classes based on the level of expression of *Mtapy1*.

The first of these classes includes mutant lines (e.g., B56,B61,B78) that showed no obvious differences in the level of *Mtapy1* mRNA expression when compared to the wild-type line A17. These plants displayed the same low basal level of *Mtapy1* expression as did wild-type plants, and the level of *Mtapy1* mRNA increased significantly after rhizobial inoculation. The second class of mutants had a very low basal level of *Mtapy1* expression. However, the level of *Mtapy1* mRNA rapidly increased after rhizobial inoculation. The lines that were included in this class of mutants include B85, C18, and C65. The third class of mutants displayed very low basal levels of

Figure 28. Northern analysis of *Mtapy1* expression in nodulation deficient lines of *M. truncatula.* Total RNA was isolated from several EMS mutagenized lines of *M. truncatula* at either 0 hours or 6 hours post inoculation with the *S. meliloti* wild-type strain ABS7M. The mutant lines are indicated below each lane in the figure. Line A17 is the wild-type positive control. Each lane represents total RNA isolated from 0.2 grams fresh weight tissue. The blots were probed with *Mtapy1*, stripped and rehybridized with Histone H3 cDNA. This blot was provided by Senthil Ramu at Texas A&M University.


MtApy-1
Histone H3
A17 B61 B85 C54 C71 C103 C123
B56 B78 C18 C65 C84 C108
A17 B61 B85 C54 C71 C103 C123
B56 B78 C18 C65 C84 C108

Mtapyl mRNA and the level of transcript did not significantly increase after rhizobial inoculation, as was the case for lines C54, C71 (Domi), C84, C103, C108, C123 and the mutant line Poodle (data not shown).

Three mutant lines, Poodle, Domi and Lin, were chosen for more detailed analysis, as these lines had been phenotypically characterized more rigorously than the other lines. As shown in Figure 29, little or no *Mtapyl* expression was detected in root tissue isolated from mutant lines Domi (*dmi*) or Poodle (*pdl*). As a positive control, *Mtapyl* mRNA levels increased upon inoculation in root tissue isolated from the wild-type *M. truncatula* line A17 (Figure 29). Consistent with previous results, the level of this expression peaked at approximately 6 hours and was undetectable by 12 hours post-inoculation. The level of *Mtapyl* expression in root tissue from the *lin* mutant showed a similar time course to that of the wild type, but expression was slightly delayed, peaking at 12 hours and reaching basal levels by 24 hours post-inoculation. Figure 30 illustrates the phenotypes of the mutant lines *dmi, pdl*, and *lin* after inoculation with wild-type rhizobia.

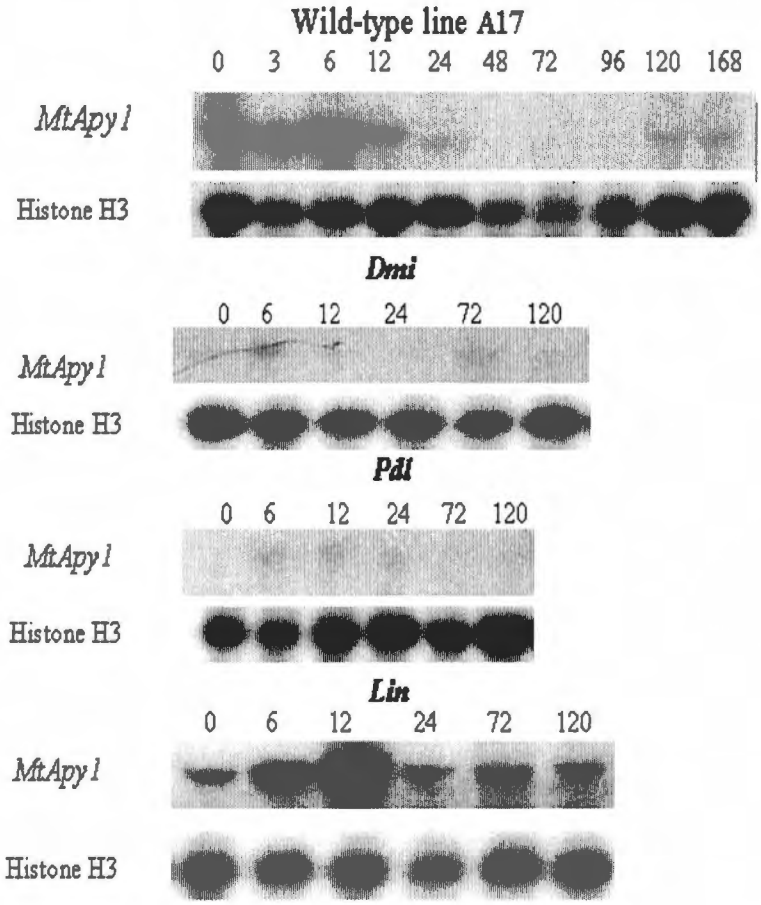
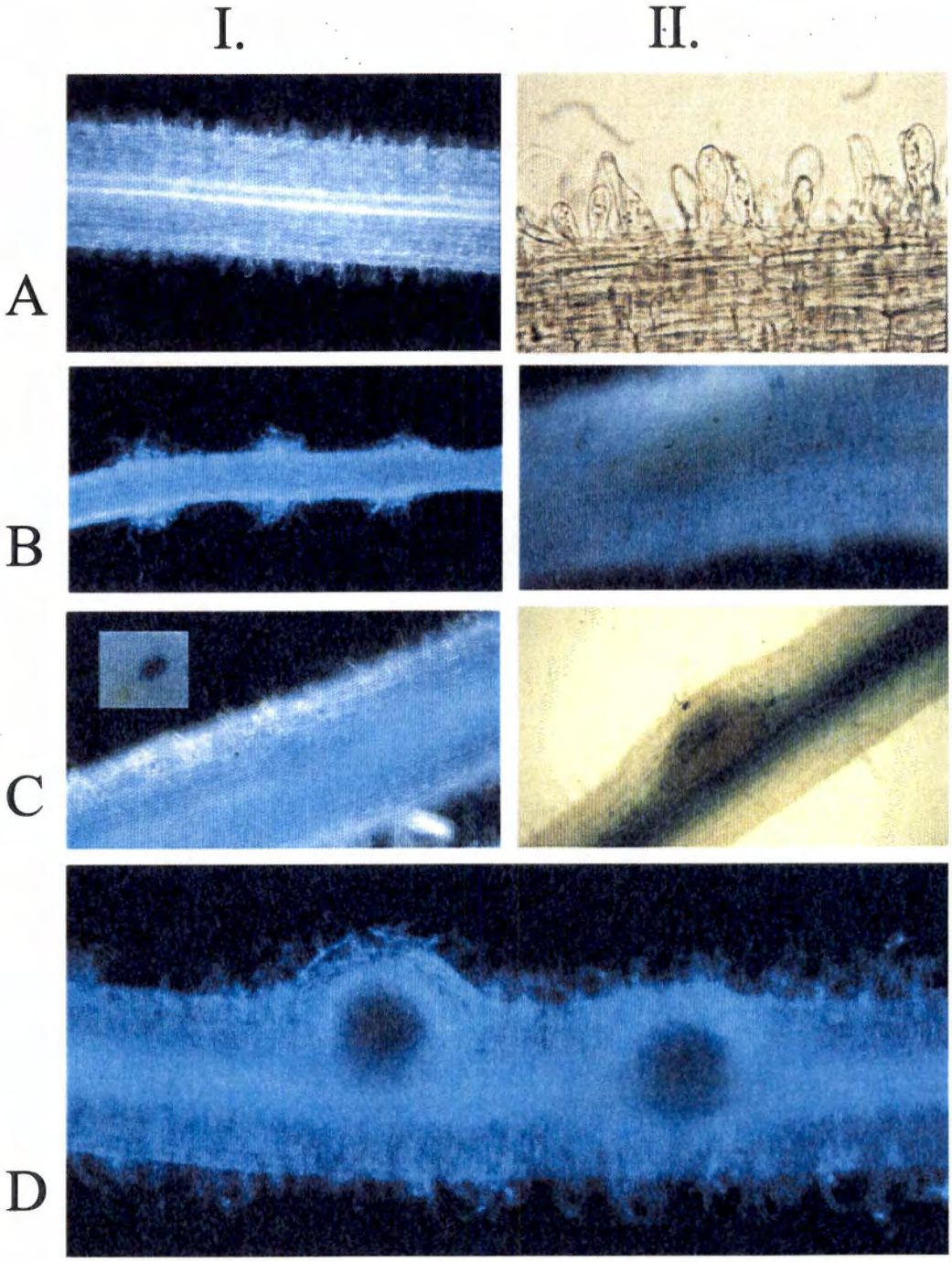


Figure 29. Northern analysis of *Mtapy1* expression in selected nodulation deficient lines of *M. truncatula.* The roots of *M. truncatula* lines A17, Domi, Poodle, or Lin were inoculated with the wild-type *S. meliloti* strain ABS7M. Total RNA was isolated from these plants at the times indicated in the figure, which represent hours after inoculation. Each lane corresponds to RNA isolated from 0.2 grams fresh weight tissue. Blots were probed with *Mtapy1* and stripped and rehybridized with Actin.

Figure 30. Figure showing phenotypes of Domi, Poodle, and, Lin compared to wild-type line A17 after inoculation with wild-type *S. meliloti* strain ABS7M. This particular strain harbors an IPTG inducible *lacZ* construct to enable visualization of bacteria infecting plant roots (Penmetsa and Cook, 1997). Panels AI, and AII show the phenotype of the *dmi* mutant. Panel AI shows that wild-type rhizobia have no affect on this mutant, panel AII shows that root hair deformations are not induced in response to this mutant. Panels BI and BII show the phenotype of *pdl*. Notice in panel BI the 'poodle-like' clusters of root hairs. Panel BII shows that rhizobia are able to infect the poodle mutant, but no cortical cell division is apparent after rhizobial inoculation. Panel CI an CII show the phenotype of the *lin* mutant. Panel CI shows that no infections proceed in this mutant. The inset in panel CI shows that infections do not proceed past the very early stages in this mutant. Panel CII shows the 'lumpy-infections' that are detected in the *lin* mutant. In panel CII , cortical cell divisions have taken place, however the nodule-like structures are not infected. Panel D shows the wild-type line A17, infected with wild-type *S. meliloti* strain ABS7M. Notice the full nodule structures that have formed and the dark blue staining indicating nodule occupancy by the bacteria.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CHAPTER 4

DISCUSSION

Section I. Structure Function Analysis of LCO Molecules on Legumes

Structure Function Studies of LCO on Soybean

The availability of a variety of synthetic LCO molecules allowed us to perform a detailed structure-function analysis of LCOs on soybean. In this study we were able to demonstrate that synthetic LCO molecules are biologically active to the same extent as naturally produced Nod Signals. Although there are now at least four published reports of the synthesis of various LCO molecules (Nicalaou et al., 1992, Wang et al., 1993, Ikeshita et al., 1994a, 1994b), there are few published reports of the biological activity of these molecules (Bono et al., 1995; Stokkerman's et al, 1995; Demont-Caulet, et al., 1999). This study demonstrated that synthetic LCO molecules were capable of inducing a sustained developmental program on soybean roots, including the deformation of root hairs and the formation of nodule-like structures. This showed conclusively that LCO molecules (or Nod signals) alone were sufficient to induce nodule development on soybean (*G. soja*).

The use of synthetic LCOs made detailed structure-function studies possible. Previously, such studies had been limited by the purification of naturally produced Nod signals, which are often isolated as mixtures of molecular species (Price et al., 1992; Carlson et al., 1993), as is evident in this study (see Figure 7). For this reason, the use of

naturally produced compounds limits the conclusions that can be made about the importance of specific substitutions to the LCO molecules. Additionally, most rhizobia produce a variety of LCO molecules. Genetically engineering rhizobial Nod signal production mutants can result in modifications to LCOs. However, the use of synthetically produced LCOs allows for a wider range of structurally distinct molecules to be studied. As has been the case in structure-function studies of oligoglucoside (Cheong et al., 1991, 1993) and xyloglucan-derived saccharide (Fry, 1989; Auger et al., 1992) signal molecules, the use of synthetic molecules has greatly enhanced our knowledge of structure-function relationships of LCOs.

In can be concluded from this study that the structural requirements for biological activity of LCO on soybean are quite complex. This complexity is similar to what has been reported previously for the activity of oligoglucosides (Hahn et al., 1989, Cheong et al., 1993), chitin oligomers (Hahn et al., 1989, Cheong et al., 1993), and xyloglucan oligomers (Hahn et al., 1989) on plants. It is apparent that the structural requirements for biological activity of LCO on soybean are not as stringent as the structural requirements for biological activity on indeterminate nodulating plants, such as alfalfa (Spaink et al., 1991; Truchet et al., 1991, Demont-Caulet et al. 1999). First, the acyl substitution at the non-reducing end of the LCO molecule is necessary for biological activity in our assays. However, the specificity of chain length and the degree of unsaturation was not critical for activity of LCOs at the concentrations tested. This is in contrast to what has been reported for LCO molecules that are active on indeterminate nodulating plants such as alfalfa and vetch (Spaink et al., 1991; Truchet et al., 1991, Demont-Caulet et al., 1999). For example, LCO molecules that differed from the

reference molecule, LCO-V(C18:1Δ11,MeFuc), only in the length of the acyl chain or the degree of unsaturation were active in both the HAD and NOI assays. In the case of alfalfa and vetch, active LCOs contain novel polyunsaturated fatty acids, with trans double bonds, such as C16:2Δ2,9 and C18:4Δ2,4,6,11, respectively. In contrast to this, LCO active on *G. soja* are decorated with common cellular fatty acids, such as *cis*-vaccenic acid (C18:1Δ11) and palmitic acid (C16:0). The novel desaturations found in the fatty acid moieties of LCO produced by *R. leguminosarum* biovars *viciae* and *trifolii* and by *S. meliloti* have been shown to be the result of the *nodEF* gene products (Spaink, et al., 1991, Spaink, et al., 1995, Demont, et al., 1993; see Table 2). *S. meliloti nodEF* mutants produce LCO that are *N*-acylated by vaccenic acid (C18:1Δ11) instead of the wild-type LCO that are substituted with polyunsaturated C16 fatty acids (Demont et al., 1993). These results suggest that the *nodE* and n*odF* gene products might also control fatty acid chain length. *S. meliloti nodEF* mutants demonstrate a greatly reduced ability to infect alfalfa, suggesting that the structure of the *N*-acyl chain is important for bacterial entry into root hairs (Ardourel, et al., 1994). It has also been demonstrated that the LCO produced by *S. meliloti nodEF* mutants are much less efficient at forming nodule-like structures than wild-type *S. meliloti* LCO (Ardourel, et al., 1994). A recent study examined the nodule inducing activity of synthetic LCO on alfalfa, concentrating on the structure of the acyl chain (Demont-Caulet, et al., 1999). LCO with polyunsaturated acyl moieties were found to be more efficient at developing nodule structures then LCO with only a single desaturation in the acyl chain (Demont-Caulet, et al., 1999). In contrast to the results obtained with soybean, the length of the acyl chain was critical for biological activity of LCO on alfalfa. For example, LCO with C16 fatty acids were more active in

their biological assay than LCO with C8, C12 or C18 fatty acids. Additionally, sulfated chitin tetramers with no fatty acid moiety were totally inactive. These results are consistent with recent results demonstrating that the acyl chain of LCOs is critical to their ability to bind to a plasma membrane localized Nod signal binding site, NFBSII (Gressent, et al., 1999).

The *nodEF* genes are not found in *Bradyrhizobium* (Nieuwkoop, et al., 1987), consistent with the finding that *Bradyrhizobium* species do not form LCO with multiple desaturations in the acyl chain (Sanjuan, et al., 1992, Carlson, et al., 1993). It is possible that the presence of the *nodEF* host range genes and the ability of legume hosts to recognize LCOs with multiple desaturated acyl moieties co-evolved, resulting in a more specific and efficient symbiosis. Indeed, *G. soja*, a determinate nodulating plant responds to a wider variety of LCO than alfalfa or vetch (indeterminate nodulating plants), suggesting that determinate nodulating plants are less discriminating symbionts than indeterminate nodulating plants.

Although a specific acyl moiety was not crucial for biological activity of LCO on *G. soja*, there is a novel interdependence of backbone length and reducing end modification that has not been reported for other legumes. In alfalfa, there is a dependence on the length of the chitin backbone of LCO molecules. Tetrameric LCO, Nod*Sm*-IV(Ac, C16:2,S), is 100-times more active in the NOI assay then a pentameric form of the molecule, Nod*Sm*-V(Ac, C16:2,S) (Schultz, et al, 1992). Additionally, a sulfate moiety at the reducing end of either LCO was reported to be absolutely required for biological activity on alfalfa (Schultz, et al, 1992). On vetch, both tetrameric and pentameric compounds are active in the HAD assay, but modification of the reducing end

residue resulted in the loss of activity (Spaink et al., 1991). In the case of *G. soja,* pentameric LCO with a 2-O-methyl fucose at the reducing end were active, while tetrameric compounds with similar reducing end substitutions were inactive. However, tetrameric compounds without any reducing end modification were active on *G. soja.* The interdependence of backbone length and reducing end modification on the activity of LCO on *G. soja* is similar to what has been reported for biologically active xyloglucan fragments (McDougall and Fry, 1989). Xyloglucan fragments with different side chains and backbone lengths have been reported to have an interdependent effect on activity (McDougall and Fry, 1989).

There have been a number of reported explanations for the various substitutions at the reducing end of LCO molecules produced by different strains of rhizobia (see table 3). The finding that the sulfate moiety present on LCO produced by *S. meliloti* was required for biological activity suggested that a very specific receptor molecule was responsible for recognition of this LCO in alfalfa. This hypothesis was seemingly supported by the identification of LCO molecules with a variety of substitutions at the terminal, reducing end sugar (Price, et al., 1992, Sanjuan, et al., 1992, Bec-Ferte, et al., 1993, Carlson, et al, 1993, Mergaert, et al., 1993, Pupot, et al., 1993; see also Table 1). The dogma was that receptor molecules of different legumes would recognize only LCO with the proper reducing end modification, i.e., LCO produced by the plant's natural symbiont. However, binding studies using radiolabeled Nod*Sm*-IV(Ac,$C_{16:2}$,S) to alfalfa root extracts demonstrated that a nonsulfated LCO could compete for a specific binding site, NFBSI, as effectively as a sulfated LCO (Bono et al., 1995). In addition, studies of the ligand specificity of a second high affinity binding site identified in *Medicago* cell

suspension cultures demonstrated that non-reducing end modifications were more important for ligand binding then reducing end modifications (Gressent et al., 1999). This second binding site, NFBSII, also did not discriminate between LCO with or without a reducing end sulfate moiety. These results indicate that the reducing end modification of LCO molecules might not be critical for the ability to bind to host plant receptors. Of course, these studies only described LCO binding proteins, not bona fide Nod signal receptors, so it is still possible that reducing end modifications are essential for Nod signal perception by a plant receptor.

An alternative hypothesis has been proposed that might also explain Nod-signal specificity. As mentioned in the introduction, this hypothesis suggests that the ability to withstand plant chitinase activity may be an important determinant in Nod signal action and specificity. For example, Staehelin et al, (1994) showed that various purified *S. meliloti* Nod signals differed significantly in their ability to survive in the presence of chitinases produced by host plants. Specifically, sulfation at the reducing end of the chitin molecule prevented hydrolysis of the Nod signal. It is possible that the specificity of LCO activity might rely on their ability to avoid chitinase degradation, and in their ability to bind a receptor. In the absence of conflicting data, this model is a viable alternative to explain how specific chemical modifications to the reducing end of the lipo-chitin Nod signal structure determines biological specificity. The data presented in this study do not address the question of the importance of the reducing end sugar substitutions for protection against endogenous hydrolytic activities of *G. soja.* However, the activity of LCOs without reducing terminal sugar substitutions demonstrates that a reducing end substitution is not required for signal transduction.

These data, taken together with the recent binding studies in *Medicago* (Bono et al., 1995, Gressent et al., 1999), suggest that all legumes might recognize similar features of the chitin backbone structure, with indifference toward any reducing end modification. If this is the case, then it might be possible that a Nod signal receptor is a modified chitin receptor. Indeed, chitin has been shown to induce several plant defense responses, and high affinity chitin binding proteins have been identified in legumes and non-legumes (Shibuya, et al., 1993, Baureithel et al. 1994, Day, et al., submitted). Chitin-derived fragments have been shown to induce phytoalexin production (Yamada et al., 1993) and membrane depolarization (Kuchitsu et al., 1993) in suspension-cultured rice cells. Similar biological activities have been found for LCOs. For example, LCOs have been reported to cause depolarization of alfalfa root hair membranes (Erhardt et al., 1992). LCO have also been shown to have biological activities on non-leguminous plant cells. LCOs have been demonstrated to induce somatic embryo development in a temperature sensitive, embryogenesis-defective line of carrot (De Jong et al., 1993). LCO molecules have also been reported to cause alkalization of the growth medium of tomato and soybean cells (Staehelin et al., 1994a, Day et al., 2000). Moreover, early nodulin genes have been identified in non-leguminous plants, suggesting similarities in the signal transduction pathways that are activated by either chitin or LCOs (Van de Sande, et al., 1996; Kouchi et al., 1999).

The identification of a chitin binding site in tomato supports the possibility that chitin perception in non-legumes and Nod signal recognition in legumes might share a common structural element, due to the fact that this binding site displayed a high affinity (Kd of 8nM) for the major Nod signal produced by *R. leguminosarum* bv. *viciae*

Nod*Rlv*-V(Ac,C18:1) (Baureithel et al. 1994). The recent identification of a high affinity chitin-binding site in both cell-suspension cultures and roots of soybean (Day et al., 1999) supports this hypothesis. In this study, it was found that Nod signal, Nod*Bj*-V(C18:1Mefuc), competed for binding as well as chitin tetramer or pentamer. However, none of these ligands had the same affinity for the binding site as the original ligand used in this study, chitin octamer (Day et al., 1999). It is possible that the binding site only recognized the chitin chain of the Nod signal. A further line of evidence that supports the idea that Nod signal receptors might be modified chitin receptors is data from Schlaman, et al. (1997), which showed that chitin oligosaccharides can induce cortical cell division in the roots of *Vica sativa* when delivered by biolistic microtargetting. These data suggested that the fatty acyl chain of LCOs was necessary for introduction of Nod signals into plant cells. It is important to note that the effect detected with O-acetylated chitin oligosaccharides was dependant upon the concomitant introduction of uridine into plant cells (Schlaman, et al., 1997). As stated previously in the introduction, uridine has been identified as the stele factor believed to be involved in nodule organogenesis.

The results of the present study support the hypothesis that the modifications made to the core structure of LCO might be involved in protection against hydrolytic enzymes, rather than that they are critical determinants for binding to a species-specific Nod signal receptor molecule.

Structure Function Studies of LCO on *V. umbellata*

It is clear that the structure of LCO molecules is critical for proper initiation of the morphogenic response, as specific chemical substitutions to the core structure have been proposed to play a role in controlling the host range of various *Rhizobium* species. Importantly, it has been shown that application of the correct LCO to certain legumes results in varying degrees of development that depends on the plant inoculated (Truchet et al. 1991, Spaink et al. 1991, Mergaert et al. 1993, Stokkermans, and Peters, 1994). To further investigate the role of LCO in nodule development, we attempted to determine what effect the major LCO produced by *Bradyrhizobium* species, namely Nod*Bj*-V(C18:1, Mefuc), would have on a variety of different legumes.

One of the tropical legumes studied, *V. umbellata,* displays a strong mitogenic response to LCO produced by *B. japonicum.* The results of a detailed study of the LCO structural requirements for nodule induction (NOI) on this plant closely resemble the results obtained using *G. soja* as a host plant. The only difference noted was the fact that *V. umbellata* displayed no response to the synthetic compound, LCO-IV(C16:0) previously shown to be active on *G. soja.* From our results with the spot inoculation assays, we concluded that only rhizobia that produced pentameric LCO with a reducing end 2-*O*-methyl fucose would be able to nodulate *V. umbellata.* We were able to test this theory using the *B. japonicum nodZ* mutant strain NAD138, which does not produce any LCO with a 2-*O*-methyl fucosyl moiety (Stacey, et al. 1994, Quesada-Vincens, et al. 1997). We predicted that this mutant would not be able to nodulate *V. umbellata.* Surprisingly, this mutant was able to induce nodule-like structures on this plant with a similar efficiency as the wild-type strain USDA110.

Results from earlier work in our laboratory revealed the importance of the cooperative action of LCO molecules in the induction of the early nodulin gene ENOD2 (Minami, et al. 1996b). These data stressed the requirement of rhizobia to produce multiple signals for the induction of plant genes involved in nodulation (Ardourel et al. 1994, Minami et al. 1996b). We have shown in this report that the action of multiple nod signals might also act to extend the host range of rhizobia. Pentameric LCOs with 2-*O*-methyl fucose reducing end modifications were sufficient to produce nodule structures on *V. umbellata*. However, the same developmental program can be induced via the action of multiple LCO that do not have this reducing end modification. This might explain how the *nodZ* mutant is able to form nodule structures on *V. umbellata*. These data suggest that mixtures of Nod signals might be important determinants of host range for rhizobia.

The results of this study also shed some light on earlier results showing that the *nodZ* mutant NAD138 was able to nodulate soybean (Stacey, et al. 1994). Results of a detailed analysis of LCO produced by the mutant indicated that it produced a tetrameric compound (Nod*Bj*-IV(C18:1)). This compound was similar to the synthetic LCO, LCO-IV(C16:0), that was shown to be active on *G. soja* in both the NOI and HAD assays. The only difference between the two molecules was the length of the acyl chain. The production of Nod*Bj*-IV(C18:1) by the *nodZ* mutant NAD138 likely explains the ability of this mutant to nodulate soybean.

The results of this study indicate that the production of multiple LCOs by rhizobia, plays a major role in host range determination. Even though it was apparent from the initial studies of synthetic LCOs on *V. umbellata* that a reducing end

modification was necessary for biological activity, it is now apparent that this was not the case. It is still possible that hydrolytic enzymes might be responsible for the species specificity of the *Rhizobium*-legume symbiosis. If this hypothesis were true, then it is possible that different plant species produce different chitinase enzymes that are responsible for the cleavage of Nod signals, if they are not properly decorated. In the case of *G. soja*, it is obvious that such chitinases would not be active on tetrameric molecules, as LCO-IV(C16:0) was found to be biologically active. In the case of alfalfa, it is apparent that chitinases are not active on LCOs with a sulfate moiety on the reducing end. However, endogenous alfalfa chitinases are active on non-sulfated LCO. The picture is more complicated in the case of *V. umbellata*. It is possible that chitinases produced by this plant are not able to degrade properly modified pentameric LCO. However, they are active on single pentameric and tetrameric LCO that are not properly modified, i.e., without any 2-O-methyl fucose. Thus, these molecules are not biologically active on *V. umbellata*. The fact that combinations of pentameric and tetrameric LCOs that do not have any reducing end modifications are biologically active brings into question the specificity of such a species-specific plant chitinase. It is quite possible that a plant chitinase in *V. umbellata* is able to bind unfucosylated pentameric LCOs with a higher affinity than unfucosylated tetrameric LCOs. If this was the case, then it is also possible that when combinations of unfucosylated LCOs, with differing chitin chain lengths, are added to *V. umbellata* roots, the chitinase binds preferentially to one LCO (e.g., the pentamer) and allows the other LCO (e.g., the tetramer) to bind to a receptor molecule, non-degraded. This hypothesis, although not entirely based upon facts, provides a possible mechanism that would explain the requirement of at least two,

structurally distinct, non-fucosylated LCOs for biological activity on *V. umbellata.* One critical argument that questions the theory that chitinases might control host specificity is the fact that Nod signals induce a biological response on legumes in the first few minutes after inoculation. For example, Nod signals produced by *S. meliloti* induce both depolarizations of root hair membranes, and calcium flux within ten minutes of their addition to alfalfa root hairs (Erhardt, et al., 1992, Erhardt, et al., 1996). It has been argued that if chitinases were involved in modifying Nod signals prior to their perception by host plant receptors, then they must be able to recognize, and modify Nod signals extremely quickly (i.e., in less than two to five minutes). Although this is still possible, it brings into question the validity of the chitinase theory of host specificity.

Section II. Genetic Analysis of a Nod Signal Binding Protein Using Orthologs from the Model Legume *Medicago truncatula*

Although there have been a great number of reports about the biological activity of Nod signals on plants, there is relatively little known about how Nod signals are perceived by legumes. As indicated in this study and others, Nod signals act with high specificity at very low concentrations. Therefore, Nod signal action is likely mediated through interaction with a specific, protein receptor. The search for such a receptor is an area of intensive research. To date, there have been very few reports of Nod signal receptor candidates (Diaz, et al, 1989, Bono et al., 1995, Niebel, et al, 1997, Etzler, et al. 1999).

A promising candidate for a Nod signal receptor is the apyrase protein, LNP, isolated from the roots of the legume *Dolichos biflorus.* This protein was shown to be

localized on the root hair surface and to bind Nod signals from rhizobia capable of nodulating *D. biflorus* (Etzler, et al., 1999). Moreover, binding of the Nod signal to the apyrase stimulated ATPase activity, suggesting a possible mode for signal transduction. Unfortunately, *D. biflorus* is a little studied legume with limited information available on its nodulation properties, genetics, preferred symbiont, etc.

In order to examine the possible role that apyrases might play in the nodulation response, we undertook a study to examine the apyrase genes of the model legume, *Medicago truncatula.* Recently, *M. truncatula* has been touted as a promising model plant for genetic studies of legumes (Barker et al., 1990, Cook et al., 1999). Therefore, it was decided that this plant would be an excellent choice for these studies.

Four apyrase genes were isolated. Due to the close sequence similarity among these genes, we utilized gene-specific primers and RT-PCR to examine mRNA expression levels. Consistent with a possible role in nodulation, mRNA levels of both *Mtapy1* and *Mtapy4* were rapidly elevated upon inoculation of roots with *S. meliloti.* The expression pattern of these two genes classifies them as early nodulins. There are only two other examples of legume genes that respond this quickly to rhizobia (Pichon, et al., 1992, Journet, et al., 1994, Cook, et al., 1995). The gene encoding ENOD12 has been reported to be inducible by rhizobia within one hour after rhizobial inoculation (Pichon, 1992, Horvath, et al., 1993, Journet, et al., 1994). At present, there is no known biochemical function for the proline-rich ENOD12 protein. In fact, a *Medicago* subspecies that does not express *ENOD12* is not affected in its ability to nodulate (Csanadi, et al., 1994). One early nodulin that does have a proposed function is *rip1* (*Rhizobium* induced peroxidase). *Rip1* mRNA was transiently induced in *M. truncatula*

roots within three hours after *S meliloti* inoculation (Cook, et al., 1995). This expression pattern is similar to what was seen for both *Mtapy1* and *Mtapy4*. *Rip1* has been postulated to play a role in oxidative processes that occur very early in the nodulation response (Cook, et al., 1995)

RT-PCR analysis of gene expression shows that induction of *Mtapy1* and *Mtapy4* after *S. meliloti* inoculation is transient, reaching a peak of expression 3-6 hours post-inoculation but returning to basal levels by 12-24 hours. These results are similar to what has been recently found for the expression pattern of an apyrase, GS52, identified from soybean (Day et al., 2000). The mRNA levels of this gene showed a transient increase in response to inoculation with the soybean symbiont, *Bradyrhizobium japonicum* within six hours after inoculation. As shown in this study, the data clearly demonstrated that the ability to produce Nod signals by rhizobia was necessary to induce *Mtapy1* and *Mtapy4* mRNA transcription. If these apyrases are indeed Nod signal receptors, it seems, at first, counterintuitive to see the level of expression of these genes increase in response to their agonist (i.e., rhizobia). However, these results are consistent with several reports of the activation of receptor genes in response to agonist addition in other systems (Eriksson et al., 1991, Ng et al., 1997, Kisselgof and Oettgen, 1998, Helmrath et al., 1998, Perera et al., 1999).

The data indicated that *Mtapy2* and *Mtapy3* were not induced in response to rhizobial inoculation. However, the *Mtapy2* gene was identified as an EST sequence from a cDNA library generated from mRNA isolated from *M. truncatula* root hair tissue. The root hairs are the site of rhizobial infection and, therefore, this location would be consistent with a role in nodulation. Although *Mtapy3* mRNA expression was not

detected in roots, there was appreciable expression in nodule tissue. In fact, the tissue expression pattern of *Mtapy3*, found in nodules and flowers, is similar to the expression pattern found for the early nodulin gene ENOD40 (Crespi, et al., 1994). Thus,the possibility that this gene plays a role in nodulation cannot be ruled out.

Hybridization and sequence analysis placed *Mtapy1*, *Mtapy3*, and *Mtapy4* close together on the *M. truncatula* genome. Indeed, these three genes are found on the same BAC clone (i.e., 52G10). PCR analysis indicated that *Mtapy2* was not present on BAC 52G10, 58M19 or other BACs found in this region of the genome (Figure 4). However, at this time, due to limited sequence information, we cannot rule out the possibility that the apyrase gene cluster identified in *M. truncatula* contains more than three members.

The mapping data showed that Mtapy3 on BAC clone 52G10 is closely linked to a putative gene that shares significant sequence similarity to the terminal flowering (*TFL1)* gene from *Arabidopsis thaliana* (Ohshima, et al., 1997). The *TFL1* gene controls inflorescence meristem identity in *Arabidopsis* (Shannon, et al., 1991). The elevated expression of *Mtapy3* in flowers and the close proximity to the TFL1 ortholog on the genome suggests the possibility that an enhancer element may coordinately control both TFL1 and *Mtapy3* flower expression. Similarly, rhizobial induction of the closely linked *Mtapy1* and *Mtapy4* genes could be coordinately controlled.

If apyrases play an important role in the nodulation response, then plant mutants defective in nodulation may show an altered profile of apyrase expression. Indeed, Northern analysis of EMS-generated, nodulation-defective lines of *M. truncatula* identified mutants defective in apyrase expression. For example, little or no *Mtapy1* mRNA expression was seen in root tissue from the POODLE (*pdl)* or DOMI (*dmi)*

mutants. Mapping data indicated that the apyrase cluster shown in Figure 16 is located within 2 centimorgans of the *pdl* locus (data not shown). Therefore, it is possible that the apyrase genes are clustered in an area of the *M. truncatula* genome that contains other symbiotically relevant loci.

Dmi (Does not make infections) is a mutant line that does not display any of the physiological responses normally elicited by inoculation with rhizobia (Penmetsa, 1998, Penmetsa and Cook, 2000; see Table 3 for a list of these responses). For example, the *dmi* mutant is deficient in root hair curling and root hair deformation in response to rhizobial inoculation. This suggests that the *dmi* mutation blocks nodulation very early in the signal transduction pathway leading to nodulation. In contrast, p*dl* is a mutant line that does display some of the early responses to rhizobial inoculation (Prabhu, 1998). For example, the *pdl* mutant does display curled root hairs and some infection thread formation in response to rhizobial inoculation. However, in contrast to the wild type, infection events in *pdl* were never observed to progress beyond the root epidermis and inoculated plants do not develop nodule primordia. Backcrossing the *pdl* mutant to the wild-type A17 line indicated that the mutation is a single recessive allele (Penmetsa, 1998). In addition to the nodulation phenotype, *pdl* displays a root phenotype as well. The roots of these plants are shorter and thicker than wild-type plants, and have clusters of root hairs resembling a poodle; hence the name (Penmetsa, 1998).

In contrast to the results with the *dmi* and *pdl* mutant lines, northern analysis of mRNA isolated from roots of the *Lin* (lumpy infections) mutant revealed that the apyrase transcript is transiently expressed with a time course slightly delayed from that seen in the wild type. This delay may not be significant and simply a result of slight delayed

growth or development in this mutant. The *lin* mutant shows many of the initial plant responses to rhizobial inoculation. In contrast to *pdl* and *dmi*, *lin* roots inoculated with wild-type *S. meliloti* exhibit cortical cell divisions leading to the development of a nodule primordium (Penmetsa, 1998). However, infection thread formation does not persist past the root hair. The numbers of infections that are initiated are also greatly reduced in the *lin* mutant as compared to the wild-type line A17. Taken together, the rapid induction of *Mtapy1* expression upon inoculation and the differential expression of this gene in *dmi/pdl* in relation to *lin* suggest a role for apyrases in the early events of nodulation that precede the induction of root cortical cell division leading to development of a nodule primordium.

The fact that apyrase expression is absent in the *pdl* mutant suggests that apyrases might not be involved in the earliest steps of Nod signal reception as has been hypothesized (Etzler, et al., 1999). Although the *pdl* mutant line is nodulation deficient, this mutant does display some of the early responses to Nod signals, such as root hair deformation and curling (Prabhu, 1998). These data suggest that *pdl* is able to respond to Nod signals, i.e., it likely still has a Nod signal receptor. However, this mutant obviously is deficient in the early events leading to nodule formation. It is possible then that apyrases in *M. truncatula* are not directly involved in Nod signal perception, however they might be necessary for down stream events in Nod signal transduction. Alternatively, the inductionof apyrase mRNA synthesis may be a later, positive feedback response initiated by an initial apyrase-Nod signal interaction.

This study did not directly address the question of whether or not the apyrase genes are Nod signal receptors. In order to address this question, these genes must be

expressed and their products analyzed biochemically. Several attempts were made to express *Mtapy1* as a recombinant protein in *E. coli*. However, the full-length protein was never purified in sufficient amounts to perform any biochemical analysis. It was found that the protein product of *Mtapy1* was toxic to the *E. coli* cells used for amplification of the protein product. For these reasons, we are still only able to assume that the apyrase genes identified in *M. truncatula* are indeed enzymes.

Evidence That Apyrases Play a Role in Nodulation

The data from Etzler et al. (1999), showing that Nod signals are able to competitively inhibit binding of LNP to solid phase chitin oligomers is circumstantial evidence that this protein plays a role in nodulation, possibly as a receptor. The structure of the predicted protein of both *Mtapy1* and LNP indicate that it is possible that these proteins might be present in the plasma membrane, similar to other well-characterized receptors. However, this region is likely a cleaved signal sequence, therefore it is possible that these proteins are not membrane bound. Further studies are necessary to provide information on the subcellular localization of these proteins. The available information indicates that these proteins are distinctly different from all receptor proteins identified. Although this does not rule out the possibility that apyrases are Nod signal recepetors, it does bring into question how they might function as receptor molecules.

There is further circumstantial evidence provided from the Etzler, et al. study and others that indicates that apyrases do play a role in nodulation. First, the *D. biflorus* apyrase, LNP, as well as being a Nod signal binding protein, is also predominantly expressed in roots. Moreover, immunofluorescence microscopy indicated that the protein

was localized to root hairs. This localization is consistent with the proposed location of suggested Nod signal receptor molecules, due to the fact that the first known responses to Nod signals occur in root hairs. Second, the enzymatic activity of LNP is increased in response to Nod signals. Although it is difficult to imagine how apyrase activity would be involved in signal transduction, the possibility that LNP does play a role in transducing the Nod signal cannot be rule out. Third, roots pretreated with antibodies to LNP are inhibited in their ability to respond to rhizobia. These data are further supported by the recent report that roots of soybean pretreated with antiserum made against a putative soybean ortholog of LNP, GS52, were inhibited in their ability to respond to rhizobia (Day et al., in press). Fourth, the expression in roots of an ortholog of LNP purified from pea is inhibited by exposure to light (Hseih, et al, 1996). This is consistent with a role in nodulation, because light is known to inhibit nodulation. The data obtained with the *M. truncatula* apyrases also indicate that apyrases might play a role in nodulation, due to the fact that the level of apyrase mRNA increased rapidly in response to inoculation with rhizobia. Furthermore, nodulation deficient lines of *M. truncatula* that are unable to produce many of the very early responses to rhizobia (e.g., Domi) do not produce any detectable level of apyrase mRNA. Although these lines of evidence are circumstantial, the accumulating information argues convincingly that apyrases likely play an important role in early nodulation events.

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VITA

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