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# Genetic and molecular analyses of host symbiotic genes and an in vitro regeneration system for Cicer arietinum L

Vijayashri Ganapathy Paruvangada
*University of New Hampshire, Durham*

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# Genetic and molecular analyses of host symbiotic genes and an *in vitro* regeneration system for *Cicer arietinum* L.

Paruvangada, Vijayashri Ganapathy, Ph.D.

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300 N. Zeeb Rd.
Ann Arbor, MI 48106

# GENETIC AND MOLECULAR ANALYSES OF HOST SYMBIOTIC GENES AND AN *in vitro* REGENERATION SYSTEM FOR *Cicer arietinum* L.

By

PARUVANGADA G. VIJAYASHRI
B.Sc.(Horticulture), University of Agricultural Sciences, 1986

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This dissertation has been examined and approved.

Dissertation director, Thomas M. Davis
Associate Professor of Plant Biology/Genetics

Subhash C. Minocha, Professor of Plant Biology/Genetics

Robert M. Zsigray, Professor of Microbiology/Genetics

Richard P. Blakemore, Professor of Microbiology

John J. Collins, Assistant Professor of Biochemistry & Molecular Biology.

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Date

To my husband Mr. Paruvangada Ganapathy

and

my mother Mrs. Vasanthalakshmi.

## ACKNOWLEDGMENT

My sincere thanks to all the sincere people at UNH.

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ABSTRACT

GENETIC AND MOLECULAR ANALYSES OF HOST SYMBIOTIC GENES
AND
AN *in vitro* REGENERATION SYSTEM FOR *Cicer arietinum* L.

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Ineffectively nodulating plant mutants PM405B, PM638A, and PM796B were used in molecular /genetic analyses of root nodule formation in chickpea. To establish the mode of inheritance of the mutant nodule phenotype in chickpea mutant PM638A, reciprocal crosses were made between PM638A and wild-type ICC640. The $F_2$ segregation data fit a 3 mutants: 1 wild-type monohybrid phenotypic ratio, indicating that ineffective nodulation is due to a monogenic dominant, nuclear mutation, tentatively designated as "$Rn_7$". Segregation analysis of $F_3$ progeny confirmed this model.

Early (ENod2) and late (Lb) nodulin cDNAs were used as heterologous probes to identify and study the expression of corresponding chickpea genes. ENod2- and Lb-homologous sequences were detected in the chickpea genome by Southern analysis. Northern analysis of root or nodule RNA extracted at different developmental stages indicated that chickpea ENod2 and Lb genes behave as early and late nodulin genes, respectively, and are expressed in a developmentally regulated nodule-specific manner. Comparison of nodulin gene expression in wild-type and ineffective nodules lead to the

following conclusions (1) the $rn_4$ (PM405B) and $Rn_7$ (PM638A) mutations do not prevent the expression of ENod2 gene (2) the $rn_4$ mutation eliminated detectible levels of Lb mRNA. $Rn_7$ mutation reduced levels of detectible Lb mRNA, and $rn_5$ (PM796B) mutation did not reduce Lb gene expression. (3) The symbiotic process in PM638A ($nod^+fix^-$) is blocked at a later developmental stage as compared to that in PM405B ($nod^+fix^-$).

To develop an *in vitro* regeneration system for chickpea, immature cotyledons were cultured on B5 basal medium with various growth regulators. Non-morphogenic callus formed in response to various auxins previously reported to induce somatic embryogenesis on immature soybean cotyledons. However, different concentrations of zeatin induced formation of white cotyledon-like structures (CLS) at the proximal end of cotyledons. No morphogenesis, or occasional formation of fused, deformed CLS, was observed in response to kinetin or 6-benzyladenine (BA), respectively. Maximum frequency (64%) of explants forming CLS, was induced by 13.7 $\mu$M zeatin plus 0.2 $\mu$M indoleacetic acid. Shoots formed at the base of CLS, proliferated in medium with 4.4 $\mu$M BA or 46 $\mu$M kinetin, and required 4.9 $\mu$M indolebutyric acid or 5.4 $\mu$M naphthaleneacetic acid to produce roots.

# INTRODUCTION

Biological N-fixation is a unique property exhibited by a small group of prokaryotic bacteria known as diazotrophs. During the course of evolution, legume plants have acquired the ability to obtain biologically fixed nitrogen because of their symbiotic relationship with diazotrophic bacteria of the family Rhizobiaceae. The legume-*Rhizobium* symbiosis is important both ecologically and agronomically. In addition, the legume-*Rhizobium* symbiotic system is a good model system for investigating some basic mechanisms of cell division, plant-microbe interactions, signal transduction and eukaryotic organ development. Accordingly, the legume-*Rhizobium* symbiosis is the subject of intensive research.

Considerable progress has been made towards understanding the genetics, molecular biology, biochemistry, and physiology of symbiotic nitrogen fixation in different legume-*Rhizobium* symbiotic systems. Research has lead to identification, mapping and cloning of several plant and bacterial genes required for nodulation and N-fixation. Plant and bacterial mutants that block the symbiotic process at different stages have been very useful in gaining insight on the biological details of nodulation and N-fixation in legume plants. Existence of transformation and regeneration systems for some legume plants has facilitated studies on gene expression and regulation.

The legume-*Rhizobium* symbiosis begins with a series of complex biochemical and physical interactions between the soil bacteria and the host plant, and culminates in the

formation of highly specialized structures called root nodules. The bacteria within the nodules reduce, or "fix", atmospheric nitrogen to produce ammonia which is assimilated by the plant. The plant in return supplies the necessary reduced carbon source to the bacteria and an ecological niche in which the bacteria thrive and fix nitrogen.

During the different stages of the legume-*Rhizobium* symbiosis, gene expression is induced in the host plant and bacteria. Some of the bacterial genes involved in the symbiotic process are termed *nod* (nodulation), *hsn* (host-specificity), *eps* (exopolysaccharide), *lps* (lipopolysaccharide), *nif* and *fix* (related to N-fixation). Genes specifically expressed in the host plant in response to *Rhizobium* infection have been termed "nodulin genes", and their protein products are referred to as "nodulins" (van Kammen, 1984). Nodulin genes are classified as "early" and "late" based on the timing of their expression during nodule development. Early nodulin genes are expressed during the initial stages of infection and nodule formation, whereas late nodulin genes are expressed following infection and nodule formation, beginning just prior to or after the onset of nitrogen fixation (Govers et al., 1987b).

A number of legume nodulin cDNA and genomic clones have been isolated, and the tissue-specific expression of several nodulin genes has been described. Several early and/or late nodulin cDNA clones have been isolated from soybean (*Glycine max* L. Merr.)(Fuller et al., 1983; Franssen et al., 1987), alfalfa (*Medicago sativa* L.)(Dunn et al., 1988), pea (*Pisum sativum* L.)(Govers et al., 1987a; Scheres et al., 1990b), and cowpea (*Vigna unguiculata* L.) (Trese & Pueppke, 1991). Through *in situ* hybridization studies, the site of expression of many nodulin genes has been localized in pea and

soybean nodules (Scheres et al., 1990b; van de Wiel et al., 1990b). However, the exact function of most of the nodulin genes still remains unknown. The few well-characterized nodulins with known functions include leghemoglobin (Lb) (Verma et al., 1974; Bojsen et al., 1985; Davidowitz et al., 1991), glutamine synthetase (GS) (Tingey et al., 1987), nodule-specific uricase (N-35[1] or uricase II) (Legocki & Verma 1979; Nguyen 1985) and sucrose synthase (N-100) (Fuller et al., 1983; Thummler & Verma, 1987).

In contrast to the nodulin genes identified using molecular approaches, host genes identified by induced mutation (using mutagens such as $\gamma$ rays and ethyl methyl sulfonate) studies are referred to as "nodulation" or "symbiotic" genes. Many nodulation genes have been identified in legumes such as pea (Kneen & LaRue, 1984, 1988; Duc & Messager, 1989), alfalfa (Peterson & Barnes, 1981), soybean (Carroll et al., 1985a, 1985b; Gresshoff et al., 1988) and chickpea (*Cicer arietinum* L.) (Davis et al., 1985, 1986; Davis 1988). To date, none of the legume nodulation genes have been cloned or characterized at the molecular level and the functions of these genes are not yet known.

*In vitro* transformation and regeneration systems available for some legumes have led to new avenues for furthering research in the field of legume-*Rhizobium* symbioses. Transgenic *Lotus corniculatus* (birds-foot trefoil, a forage legume) plants have been used in studies on expression and regulation of the *Sesbania rostrata* leghemoglobin gene *glb3*, soybean Lb and N-23 genes, and a *Phaseolus vulgaris* glutamine synthetase gene (Forde et al., 1989; Jørgensen et al., 1988; Stougaard et al., 1986; 1990; Szabados et al.,

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[1] In the standard nomenclature used for nodulins, N-35 refers to nodulin-35, a protein with a molecular weight of 35kD.

1990). However, for other legumes including chickpea, regeneration and transformation systems have yet to be established.

Chickpea is the third most important pulse (edible, non-oilseed legume) crop in the world after bean (*Phaseolus vulgaris* L.) and pea (van Rheenan, 1991). Chickpea is a self-pollinating diploid with a chromosome number of 2n=16, and has a relatively (among legumes) small genome with a haploid DNA content of 1c=0.95pg (Patankar & Ranjekar, 1984). Taxonomically, the genus *Cicer* belongs to a monogeneric tribe Cicerae and is closely related to tribe Viciae which includes legumes of the genera *Pisum*, *Vicia*, and *Lens* (Smartt, 1990). Like its relatives in the tribe Viciae, chickpea forms indeterminate nodules that are characterized by the presence of a persistent nodule meristem. The chickpea endosymbiont is referred to as *Rhizobium ciceri* (Cadahia et al., 1986; Gaur & Sen, 1979; Kingsley & Bohlool, 1983). Neither organism in the chickpea symbiosis has received much research attention. The plant nodulation mutants that have been isolated in chickpea (Davis et al., 1985, 1986; Davis, 1988) are valuable tools for studying the host contribution to the nodulation process in chickpea-*Rhizobium* symbiosis. These plant mutants constitute useful research material for comparative studies since they are all isogenic lines and differ from wild-type only with respect to mutations in single genes.

Objectives

My research project had two overall goals: 1) to study plant genes involved in chickpea-*Rhizobium* symbiosis; 2) to contribute to the development of a genetic transformation system for chickpea. The specific objectives of my research project were to :

1) Determine the mode of inheritance of the mutant nodule phenotype in chickpea line PM638A.

2) Establish the existence of nodulin genes ENod2 and Leghemoglobin (Lb) in chickpea.

3) Trace the expression of chickpea nodulin genes during nodule development.

4) Compare ENod2 and Lb gene expression in chickpea wild-type and nodulation mutants.

5) Develop an *in vitro* regeneration system for chickpea of potential use for manipulating plant genes in chickpea-*Rhizobium* symbiosis using genetic engineering techniques.

The investigations carried out have been described in two self-contained chapters. **Chapter I** (covering **objectives 1 through 5** listed above) is concerned with the study of plant genes involved in the chickpea-*Rhizobium* symbiosis. **Chapter II** (covering **objective 6** listed above) is concerned with development of an *in vitro* regeneration system for chickpea, and has been published in a slightly modified form (Shri & Davis, 1992).

# CHAPTER I

Genetic and molecular analyses of host symbiotic genes in chickpea (*Cicer arietinum* L.)

# CHAPTER I

## SECTION A - LITERATURE REVIEW

A wealth of knowledge has accumulated on symbiotic nitrogen fixation in legume plants, particularly on genetics, molecular biology and biochemistry of N-fixation in *Rhizobium*. Extensive reviews on the biology of symbiotic interaction between legume plants and rhizobia have been published (Verma & Long, 1983; Rolfe & Gresshoff, 1988; Vance et al., 1988; Long, 1989a, 1989b; Nap & Bisseling, 1990; Sanchez et al., 1991; Franssen et al., 1992). This chapter presents a concise review of published work on legume-*Rhizobium* symbiosis, with major focus on research aimed at understanding the genetic contribution of the host plant in the symbiotic process.

### Taxonomy and genetics of *Rhizobium*

Bacteria that have symbiotic relationship with legume plants are grouped in the family Rhizobiaceae and are of the genera *Rhizobium*, *Bradyrhizobium*, or *Azorhizobium*. Different *Rhizobium* species infect specific host plants and based on host-specificity are classified into different compatibility groups (Table 1). Fast growing species of *Rhizobium* such as *R. meliloti*, and *R. leguminosarum* have symbiotic *nod*, *nif*, and *fix* genes located on large plasmids called "pSym". In contrast, in slow-growing species

**Table 1**: Cross-compatibility groups of *Rhizobia* and legume host plants.

| *Rhizobium* spp. | legume host |
|---|---|
| *R. leguminosarum* biovar (bv) viciae | *Pisum sativum*, *Vicia sativa* |
| bv. phaseolae | *Phaseolus vulgaris* |
| bv. trifolii | *Trifolium repens* |
| *R. meliloti* | *Medicago sativa* |
| *R. fredii* | *Glycine max* |
| *R. loti* | *Lotus corniculatus* |
| *R. ciceri* | *Cicer arietinum* |
| *Bradyrhizobium japonicum* | *Glycine max* |
| *Azorhizobium caulinodans* | *Sesbania rostrata* |

such as *Bradyrhizobium japonicum*, symbiotic genes are not plasmid-borne but are located on the bacterial chromosome (Long, 1989).

The bacterial *nod* genes fall into two general categories - a) the "common" *nod* genes and, (b) *nod* genes that are species-specific (also referred to as *hsn* genes). The common *nod* genes which include *nodDABCIJ* are functionally interchangeable between different species of *rhizobium* (Hooykass et al., 1981; Kondorosi et al., 1984; Djordjevic et al., 1985). Examples of *nod* genes in *R. meliloti* that are species-specific are the *nodH* and *nodPQ* genes, required for nodulation in alfalfa, and the *nodE* gene in *R. leguminosarum* which confers the ability to nodulate pea (Horvath et al., 1986; Djordjevic, 1987; Cervantes et al., 1989; Schwedock et al., 1989; Spaink et al., 1991).

The *nif* and *fix* genes are required for N-fixation (Banfalvi, 1981). The *nif* genes include *nifHDK* genes which are also found in *Klebsiella pnuemoniae*, a free-living bacterium capable of N-fixation. *NifHDK* genes code for the structural components of the nitrogenase enzyme complex. All other *Rhizobium* genes that have a direct role in N-fixation but are not found in *K. pnuemoniae* are referred to as *fix* genes. Several reviews have been published in recent years and are excellent sources for obtaining detailed information on the different bacterial symbiotic genes (Long, 1989; Nap & Bisseling 1990; Sanchez et al., 1991; Franssen et al., 1992; Fisher & Long, 1992).

**A general overview of symbiotic N-fixation process**

The complex process of symbiotic N-fixation in legume plants can be sub-divided

into three main biologically distinct events - a) recognition of the host plant by bacteria, b) infection of the legume root, and c) nodule function.

Recognition of the host plant by bacteria

In legume plants, flavonoids exuded by the plant roots serve as signal molecules required for inducing the expression of bacterial nodulation genes. Flavonoids are three-ring aromatic compounds synthesized by the phenylpropanoid pathway. A unique spectrum of flavonoids is secreted by each legume species. Some of the flavonoid inducers are luteolin in alfalfa and diadzein in soybean (Peters et al., 1986; Kosslak et al., 1987; Peters & Verma, 1990). Flavonoids first interact with bacterial *nodD* gene product. This is the earliest interaction known in the sequence of events that take place in the symbiotic process. *NodD* gene product, a transcriptional activator, binds to the "nod box" which is a conserved upstream promoter element present in common *nod* and strain-specific *nod* operons (Mulligan & Long, 1985; Peters et al., 1986; Rostas et al., 1986; Long, 1989a).

Expression of the common nod*ABC* and host-range *nod* genes is required for the production of the bacterial signal molecules which induce root hair curling, cortical cell division, and nodule organogenesis in legume roots. One such bacterial signal molecule is an oligomer of *N*-acetyl glucosamine (Fisher & Long, 1992). The chemical structure of the bacterial signal was first elucidated in *R. meliloti*. The Nod factor secreted by *R. meliloti* is called NodRm-1 and is $\beta$-1,4-tetrasaccharide of D-glucosamine. The common

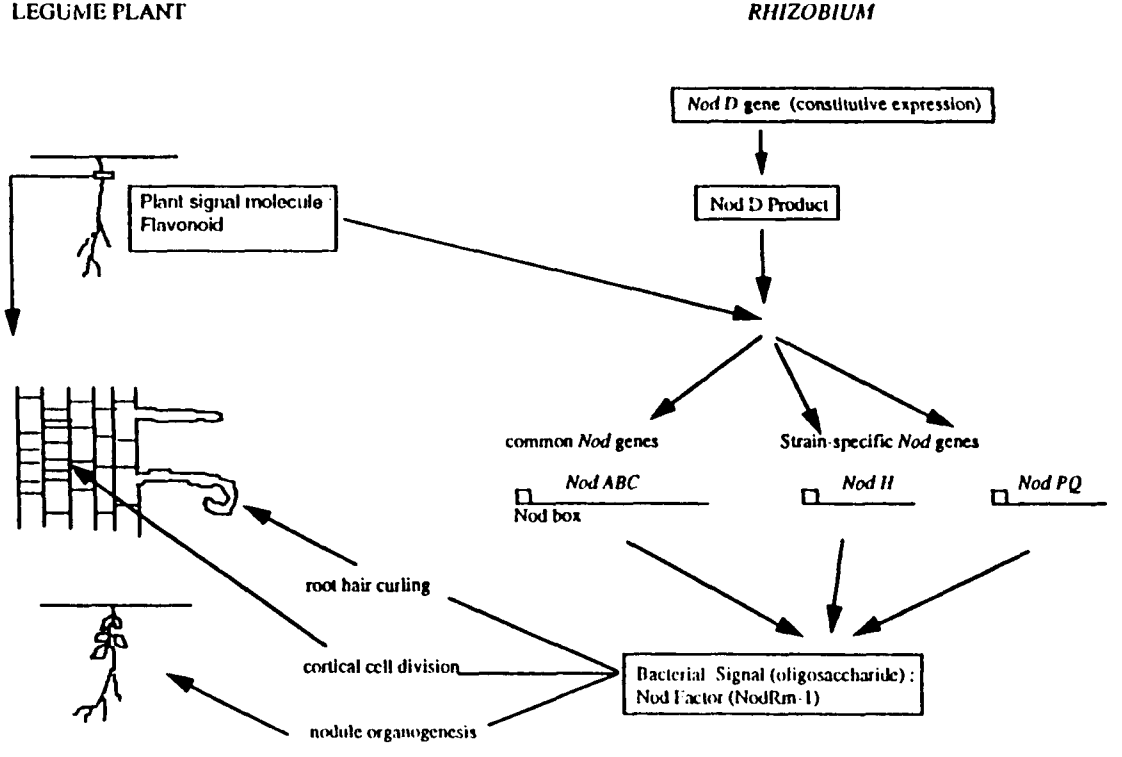
*nodABC* genes are required for the synthesis of the oligosaccharide component of NodRm-1, whereas the strain-specific *nodH* and *nodPQ* genes are required for converting the oligosaccharide factor into an active sulphated form (Lerouge et al., 1990; Schwedock & Long, 1990; Truchet et al, 1991). The early events that take place in plant-*Rhizobium* signal exchange are summarized in Fig. 1.

Infection of the legume root

The bacterial *exo*, *lps*, and *ndv* genes have a role in the infection process. Most of the *exo* genes are located on large plasmids and are involved in the synthesis of extracellular polysaccharides, including charged exopolysaccharides. Genes required for the synthesis of lipopolysaccharides and neutral glucans are referred to as *lps* and *ndv* genes respectively. Symbiotic defects can result from either the absence or the alteration of surface polysaccharides. Rhizobia with mutations in *exo*, *lps*, and *ndv* elicit so-called "empty" nodules with no infection threads, or with infection threads that abort prematurely, or are defective in releasing bacteria into plant cells (Finan et al., 1985; Leigh et al., 1985; Dylan et al., 1986; Halverson & Stacey, 1986; Carlson et al., 1987; Leigh et al., 1987; Klein et al., 1988; Long et al., 1988; Muller et al., 1988; van de Wiel et al., 1990).

The general process of infection and nodule organogenesis has been reviewed by several authors (Vance & Johnson, 1981; Downie & Johnston, 1986; Rolfe & Gresshoff, 1988; Vance et al., 1988). Bacteria enter the host root through root hairs in legume

**Fig. 1 :** Early events in host-symbiont signal exchange



hosts such as pea, soybean, and chickpea. First, there is entrapment of bacteria by the curled root hair. Next, the bacteria invade the root hair. A modified cell-wall is formed ahead of the invading bacteria. The bacteria, as they invade the root hair are enclosed within a newly synthesized structure called the infection thread, within which the bacteria multiply. Ahead of the growing infection thread, cell division takes place in the root cortex and a nodule meristem is formed. The infection thread penetrates cells in the root cortex. The release of bacteria into individual host cells takes place through the process of endocytosis. The bacteria released into the host cell cytoplasm are enclosed within a membrane called the peribacteroid membrane (PBM). The bacteria inside the infected cells in the cortex differentiate to form bacteroids.

The infection process has been studied in detail in some legume plants, examples of which are described below. In soybean, bacteria attached to epidermal cells and root hairs within minutes of inoculation. Marked root hair curling occurred within 12 hours after inoculation. Infection threads were observed in short, tightly curled root hairs (Turgeon & Bauer, 1982). Infection thread formation was observed within about 24 hr. Infection thread development was still limited to within the root hair 36-40 hr after inoculation. By 4 days post inoculation, a nodule meristem was formed in the cortex immediately contiguous to the infected root hair (Turgeon & Bauer, 1982). Cell division within the nodule meristem resulted in formation of the root nodule.

An extensive study on nascent root nodules in pea was done using flourescent microscopy and micro-autoradiography (Libbenga & Harkes, 1973). The authors found that root nodule initiation begins with cell division in the inner root cortex at a distance

from the advancing infection thread. After the invasion of bacteria, the infected inner cortical cells cease to divide. With the loss of initial meristematic center in the inner cortex, new meristematic activity is initiated in neighboring inner cortical cells. The peripheral layer and apical meristem of the nodule are not infected by the bacteria (Libbenga & Harkes, 1973).

The development of pre-emergent and early emergent stages of soybean and pea root nodules was studied microscopically (Newcomb et al., 1979). Soybean and pea nodules differ in their growth pattern. Soybean nodules are determinate in growth and pea nodules are indeterminate in growth due to the presence of a persistent nodule meristem. Newcomb et al. (1979) documented that in soybean, the central mass of infected tissue is derived mainly from the outer layer of root cortical cells, whereas the inner root cortical cells contribute extensively to the infected tissue of pea nodules. Detailed studies on early events in the infection process in a few legume hosts including alfalfa, cowpea, peanut (*Arachis hypoghea*), clover (*Trifolium* spp.) and soybean have revealed that - i) at any given point of time, infection events leading to the formation of nodules are restricted to only a narrow band of cells above the root tip ii) infectibility is developmentally transient and is lost rapidly in these epidermal cells as they mature (Bhuvaneswari et al., 1981).

Dudley et al., (1987) reported that root cortical cell division is initiated prior to infection thread formation in alfalfa. Alfalfa roots infected with four nodulation defective (*nod*$^-$) mutants of *R. meliloti* were examined by light and electron microscopy (Hirsch et al., 1983). *Nod*$^-$ mutants failed to induce nodules on the host plant. One class of

*nod*$^-$ mutants did not induce root hair curling or penetrate host cells. The second class of *nod*$^-$ mutants induced some root hair curling and entered epidermal cells, but no infection threads were formed (Hirsch et al., 1983).

Nodule function

The root nodule serves as a source of fixed nitrogen for legume plants. The reduction of $N_2$ to ammonia is catalyzed by the bacterial nitrogenase enzyme complex, components of which are encoded by the *nifH*, *nifD*, and *nifK* genes (Long 1989b). The enzyme nitrogenase is very oxygen labile. The $O_2$ flux in the root nodule is maintained by the plant protein leghemoglobin (Lb). The apoprotein component of the Lb protein is encoded by the plant genome.

Ammonia produced by the bacteria is converted to amino acids in the plant cell cytoplasm by the action of two key plant enzymes, glutamine synthetase (GS) and glutamate synthase (GOGAT). Fixed nitrogen is mainly transported to the shoot in the form of amides asparagine and glutamine, or as ureides allantoin and allantoic acid. The ureides are the major nitrogen transport compounds in soybean, winged bean (*Psophocarpus tetragonolobus*), cowpea and french bean (*Phaseolus vulgaris*). Legumes such as chickpea, pea, clover and alfalfa are amide transporters (Sprent, 1980; Schubert, 1986).

In the functioning root nodule, it is important to maintain carbon flux to support bacterial and nodule respiration, and to provide carbon skeletons for the synthesis of

nitrogen transport compounds. Phosphoenolpyruvate carboxylase (PEPC) (Egli et al., 1989) and sucrose synthase (Thummler and Verma, 1987) are important regulators of carbon flux in the root nodule.

**Nodulin genes**

In response to *Rhizobium* infection, specific genes called nodulin genes are expressed in the host plant. Based on their timing of expression during the symbiotic process, these genes are referred to as early and late nodulin genes. The protein products of the nodulin genes are referred to as nodulins. Information available from published reports on legume nodulin genes is presented below.

Early nodulin genes

Early nodulin genes are first expressed during the initial stages of nodule formation and before the start of N-fixation. Many early nodulin genes have been identified and cloned in several legume plants but the exact functions of these genes, and their gene products are still not known.

Genes expressed in response to infection by *R. leguminosarum* in pea root hairs have been investigated (Gloudemans et al., 1989). Total root hair RNA from inoculated and uninoculated plants was translated *in vitro* and analyzed on 2-D gels. Two genes, RH-42 and RH-44, in pea root hairs were found to be expressed in response to

inoculation with *Rhizobium* (Gloudemans et al., 1989).

An early nodulin cDNA clone, pENod2, has been isolated from soybean (Franssen et al., 1987). The cDNA insert of this clone hybridized to nodule-specific RNA of 1.2 Kb. The RNA that was hybrid-selected by the cloned ENod2 DNA was *in vitro* translated and two nodulins with an apparent MW of 75 kD were produced. These nodulins are referred to as N-75 nodulins. The genes coding for N-75 nodulins appear to have a role in nodule morphogenesis (Franssen et al., 1987). Two genomic clones that code for ENod2 have been isolated from a soybean genomic library. The two clones have been designated pGmENod2A and pGmENod2B, and contain inserts of 16 kb and 25 Kb respectively (Franssen et al., 1989).

cDNA clones homologous to soybean ENod2 cDNA have been isolated in *Sesbania rostrata* (Strittmatter et al., 1989), pea (van de Wiel et al., 1990b), and cowpea (Trese & Pueppke, 1991). ENod2 gene expression is localized in the nodule parenchyma of pea and soybean nodules (van de Wiel., 1990b). The transcription of pea ENod2 gene takes place in nodule parenchyma cells as they develop from the nodule meristem (van de Wiel et al., 1990b). Genomic digests (*Hind*III and *Eco*RI) of *Medicago truncatula* cv "Jemalong" (a diploid relative of alfalfa) showed one band hybridization when probed with the soybean ENod2 cDNA clone, whereas at least four bands were present in tetraploid alfalfa DNA (Barker et al., 1990).

Several early nodulin cDNA clones other than ENod2 have been isolated in pea. A set of early nodulin cDNA clones ENod12, ENod3, ENod5 and ENod14 in pea was isolated by screening a nodule cDNA library with cDNA probes of RNA from nodules

of 10-day-old plants and uninfected roots (Scheres et al., 1990b). Through *in situ* hybridization, the ENod12 transcripts were detected in every cell of the invasion zone in the nodule, while ENod5, ENod3, and ENod14 transcripts were restricted to only the infected cells.

The predicted amino acid sequences derived from the nucleotide sequences of the cDNAs, in combination with the localization data, showed that ENod5 is an arabinogalactan-like protein involved in the infection process (Scheres et al., 1990b). Arabinogalactan proteins (AGPs) are widely distributed throughout the plant kingdom. AGPs are secretory proteins normally present in the extracellular milieu and are known to be involved in cell-cell communication or morphogenesis (Fincher et al., 1983). In contrast, both ENod3 and ENod14 had a cysteine cluster suggesting that these nodulins could be metal-binding proteins (Scheres et al., 1990b). Soluble compounds coded by common and host-specific nodulation genes of *R. leguminosarum* bv. viciae are known to be involved in eliciting ENod12 gene expression in pea (Scheres et al., 1990a).

Early nodulin cDNA clones VuA, VuB, VuC and VuD have been isolated in cowpea (Trese & Pueppke, 1991). VuA and VuB transcripts were expressed in nodulating cowpea roots. VuA clone had significant homology to soybean cDNA clone GmENod2. The expression of VuC and VuD transcripts was repressed in nodulating cowpea roots as compared to the nonnodulated control root (Trese & Pueppke, 1991).

Late nodulin genes with defined functions

Among all of the nodulin genes that have been identified to date, the functions are known for only a few of the late nodulin genes. These late nodulin genes are expressed after nodule formation and coincidentally with the onset of N-fixation. A brief description of the late nodulin genes whose functions are known is given below.

Leghemoglobin. Lb is a myoglobin-like protein with heme as the prosthetic group. Lb acts as an oxygen carrier, buffering the cell environment from wide fluctuations in oxygen concentration and ensuring adequate supplies of oxygen for both the host cell and bacteroid oxidative processes. Lb is very important for maintaining low oxygen concentration in the vicinity of the key enzyme nitrogenase (Appleby, 1984). Verma et al., (1974) were the first to report isolation and *in vitro* translation of Lb mRNA. Later, using immuno-gold staining on thin sections of pea nodules, Lb was found to be localized in plant cytoplasm of infected cells and not in the peribacteroid space (Robertson et al., 1984).

Three major forms of Lb called Lba, Lbc1, and Lbc2 have been detected in soybean nodules (Jensen et al., 1981). Lba differs from the two Lbc varieties in 6 amino acids, while Lbc1 and Lbc2 differ in only 1 amino acid. Detailed analysis of one of the isolated Lbc genes revealed the presence of three intervening sequences. Alignment of homology of all known globin amino acid sequences indicated that the splicing points of the first and third intervening sequences in the Lb coding region were located in the same

positions as the two interruptions found in all known globin-coding sequences including animal globins (Jensen et al., 1981). Soybean Lb genes constitute a complex family of genes including psuedo, truncated and functional genes (Brisson and Verma, 1982).

Southern blot analysis of *EcoR*I digests of soybean DNA revealed the presence of at least seven different Lb genes in soybean (Bojsen et al., 1985). In alfalfa, Lb is encoded by a small gene family; DNA sequences of seven cDNAs and two genomic clones have been determined (Davidowitz et al., 1991).

A chimeric soybean Lb gene (*Lbc3* 5′3′-cat) was introduced into the genome of a heterologous legume species, *Lotus corniculatus* (Jensen et al., 1986). Nodule-specific expression of the chimeric gene was found in root nodules formed on fully regenerated plants. Expression under control of the 5′ upstream region of the soybean gene was regulated at the level of transcription and followed correct developmental timing, indicating a conserved induction mechanism for Lb genes in legumes (Jensen et al., 1986).

Fragments of *S. rostrata* leghemoglobin *glb3* 5′ upstream region were fused to a β-glucuronidase reporter gene with its own CAAT and TATA box region (de Bruijn et al., 1989). The *glb3* CAAT and TATA box regions were found to be required for nodule-specific expression and several upstream enhancer-type regions were identified. Subfragments of *S. rostrata glb3* promoter region were examined for binding to *trans*-acting factors. The appearance of the *trans*-acting factor was shown to be developmentally regulated since the expected protein-DNA complexes were first observed about 12 days after infection, concomitant with the production of Lb proteins (de Bruijn

et al., 1989).

Soybean Lb cDNA has been used as a probe to detect the presence of leghemoglobin-like sequences in non-legume plants. Roberts et al. (1985) used a cloned cDNA partial copy of the soybean Lb mRNA to probe genomic DNA of actinorhizal plants including *Alnus glutinosa*, *Casuarina glauca*, *Ceanothus americanus* and *Elaeagnus pungens*. The hybridization patterns of the restriction fragments revealed some fragment size conservation between the DNA of soybean and the DNA of the actinorhizal plants which are taxonomically unrelated to soybean or to each other.

Nodule-specific glutamine synthetase. Ammonia produced as a result of symbiotic N-fixation in legume plants is converted to glutamine by the nodule-specific enzyme glutamine synthetase.

GS is an octameric enzyme encoded by a small family of genes in common bean (Cullimore & Bennett, 1988). Several different forms of the enzyme that are specific to roots, leaves or nodules have been identified (Cullimore & Bennett, 1988).

The two nodule-specific forms of GS, GSn1 and GSn2 have been studied in detail in french bean (Cullimore et al., 1983). French bean roots contain only a single form of the enzyme, which appears to be identical to nodule GSn2. GSn1 and GSn2 have similar kinetic and physical properties, identical native and subunit molecular weights. The two forms present in nodules can be separated due to slight differences in charge by ion-exchange chromatography and native PAGE (polyacrylamide gel electrophoresis) (Cullimore et al., 1983). cDNA clones (pGS341, pGS134 and pGS197) encoding three

different GS mRNAs have been characterized in french bean (Tingey et al., 1987). Northern blot analysis showed that both pGS134 and pGS341 cDNA inserts detected a 1.4 Kb GS mRNA, for which steady state levels increased 10-20 fold upon nodule formation (as compared to leaf and root tissue). In contrast, a cDNA insert of pGS197 detected a larger GS mRNA (1.5 Kb) which was specific to leaf poly (A)$^+$ RNA (Tingey et al., 1987).

Nodule-specific asparagine synthetase. The enzyme asparagine synthetase catalyzes the formation of asparagine from aspartate and glutamine. Two classes of pea AS cDNAs AS1 and AS2 which encode homologous but distinct polypeptides have been isolated and characterized. Both AS1 and AS2 mRNAs accumulate to high levels in N-fixing root nodules compared to roots. Analysis of nuclear DNA revealed that AS1 and AS2 are encoded by single genes in pea (Tsai and Coruzzi, 1990).

Nodule-specific aspartate aminotransferase. AAT catalyzes the reversible reaction in which aspartate is formed from glutamate and oxaloacetate. In N-fixing lupine and alfalfa nodules, there is enhanced expression of nodule-specific isoform of AAT, namely AAT-$P_2$ and AAT-2, respectively (Griffith & Vance, 1989; Reynolds & Farnden, 1979). cDNA clones coding for the nodule-specific form of AAT, have been cloned from lupine and alfalfa root nodules (Gantt et al., 1992; Reynolds et al., 1992). Alfalfa AAT-2 is encoded by a small gene family and the AAT-2 mRNA expression in nodules is several-fold higher compared to that in leaves and roots (Gantt et al., 1992).

Nodule-specific uricase II (Nodulin-35). Tropical legumes such as soybean primarily transport fixed nitrogen as ureides allantoin and allantoic acid. The enzyme uricase catalyzes the formation of allantoin from uric acid.

N-35, a 35,000 molecular weight protein present in soybean root nodules, is a subunit of uricase II. This nodulin is present in root nodules irrespective of the effectiveness of N-fixation in the nodules (Legocki and Verma, 1979). By using immunofluorescent techniques, N-35 has been localized to the uninfected cells of the nodule (Legocki and Verma, 1979). The protein is not detected in uninfected plants, bacteroids and in free living *Rhizobium* (Legocki and Verma, 1979). A cDNA clone corresponding to N-35 has been isolated by using mRNA from immuno-precipitated polysomes (Nguyen et al., 1985). Southern blot analyses revealed that there are several *EcoR*I fragments containing N-35 sequences in soybean DNA. Three of these sequences were isolated from a soybean genomic library. Nucleotide sequence analysis showed that the complete gene extends to almost 5000 bp on two *EcoR*I fragments and the coding region (309 codons) is interrupted by 7 introns, ranging in size from 154 to 1341 bp (Nguyen et al., 1985). Dot blot analysis of RNA from roots, leaves and nodules revealed that uricase II is transcribed in nodules sampled 9 and 21 days after infection, but is not expressed in roots and leaves (Nguyen et al., 1985).

Nodule-specific sucrose synthase (Nodulin-100). Sucrose synthase in nodules works primarily in the direction of cleavage of sucrose to form UDP-glucose and D-fructose.

The soybean N-100 gene encodes the subunit of nodule sucrose synthase. Sucrose

synthase is a homo-tetrameric enzyme. This tetramer dissociates rapidly into monomers in the presence of heme (Thummler & Verma, 1987). Sucrose synthase activity increases rapidly during nodule development and decreases during senescence. As the degradation of Lb occurs during senescence, a concomitant decrease in sucrose synthase activity is observed (Thummler & Verma, 1987).

A cDNA clone corresponding to N-100 has been isolated in soybean (Fuller et al., 1983). Through Northern blot analysis, the N-100 transcript was found to be 3150 nucleotides long (Fuller and Verma, 1984). The expression of N-100 mRNA is 10-20 fold higher in 3 day old infected root tissue as compared to uninfected root and is 70-fold higher relative to leaf tissue.

Late nodulin genes with unknown functions

A number of other late nodulins have been identified in different legume plants, but the function of these late nodulins is still unknown.

Nodule-specific cDNA clones N-24, N-27 and N-44 were identified by screening a soybean nodule cDNA library with cDNA probes prepared from nodule poly $(A)^+$ RNA from roots and nodules (Fuller et al., 1983). Northern blot analysis revealed that N-27 and N-24 transcripts are 1150 and 770 nucleotides in length, respectively. N-24 mRNA was present in nodule tissue at 50-200 times the level in uninfected root and leaf tissue respectively (Fuller and Verma, 1984). DNA sequence analysis of N-24 revealed that it codes for a polypeptide with a MW of 15,100 daltons and is interrupted by four

introns. The three middle exons and their flanking segments appeared to have been generated by duplications of a unit resembling an insertion sequence. The three exons code for the central hydrophobic domains of the protein. The N-24 gene codes for a polypeptide that is part of the peribacteroid membrane (Katinakis and Verma, 1985).

Expression of N-23 is nodule-specific in soybean. The 5′ region of the N-23 gene contains multiple sequences capable of acting as eukaryotic and prokaryotic promoters (Wong and Verma, 1985). Three conserved regions present in the 5′-flanking regions of N-23 and N-24 genes were present as well in the Lbc3 gene. The conserved regions suggested a potential regulatory role in controlling the coordinate expression of nodulin genes, including Lb, during symbiosis (Mauro et al., 1985). From a soybean nodule cDNA library, two cDNA clones corresponding to N-20 and N-22 genes have been isolated (Sandal et al., 1987). These nodulin genes were related to genes coding for peribacteroid membrane proteins N-23 and N-44. Alignment of the deduced amino acid sequences of all four genes revealed three domains of high homology. The first conserved domain coded for a putative signal peptide, and the other two each contained four cysteine residues that could be arranged in a way similar to the metal-binding domains present in some enzymes and in several DNA-binding proteins (Sandal et. al., 1987).

Pea nodulin genes referred to as "PsNod" genes have been identified by screening a cDNA library prepared from pea nodule poly (A)$^+$ RNA (Govers et al., 1987a). The cDNA library was screened with cDNA probes prepared from root and nodule RNA, respectively. The time course of appearance and accumulation of nodulin mRNAs

corresponding to nodulin clones PsNod 6, 10, 11, 13 and 14 was similar to that of Lb mRNA (Govers et al., 1987a). In ineffective pea root nodules (induced by bacterial *fix*⁻ mutants), expression of PsNod genes was induced but the final accumulation levels of the mRNAs was markedly reduced to varying degrees (Govers et al., 1987a).

In winged bean, a 21 kD protein was specifically expressed in senescing nodules (Manen et al., 1991). Sequence analysis of the cDNA corresponding to the protein revealed that the protein had all the features of legume Kunitz protease inhibitor (Manen et al., 1991). Immunocytochemical studies showed that the protease inhibitor is exclusively localized in infected senescent cells of the nodule (Manen et al., 1991).

**Nodulin gene expression in aberrant nodules**

Pea plants infected by *Agrobacterium tumefaciens* containing the symbiotic plasmid from *R. leguminosarum* formed empty nodules. These empty nodules were devoid of intracellular bacteria, and some structures similar to infection threads were observed. The ENod2 gene was expressed in these empty nodules, indicating that intracellular invasion of the bacteria is not required for expression of the ENod2 gene in pea (Govers et al., 1986).

Dunn et al. (1988) have isolated late nodule-specific cDNA clones including nodule-specific GS and three unidentified nodulins N-32/34, N-14 and N-22 from alfalfa. Northern and dot blot analyses of RNA isolated from wild-type nodules and from defective nodules elicited by a variety of *R. meliloti* mutants revealed that RNAs

corresponding to nodule-specific GS and three unidentified nodulins were coordinately expressed along with Lb during the course of nodule development. These late nodulins were expressed in *fix*$^-$ nodules (induced by mutant rhizobia) that contained infection threads and bacteroids, but were not expressed in nodules that lacked infection threads and intracellular rhizobia (Dunn et al., 1988).

In alfalfa, *fix*$^-$ nodules were induced by a non-N-fixing *R. meliloti nifH* mutant (Dickstein et al., 1988). The *fix*$^-$ nodules contained bacteria and differentiated bacteroids, and Lb gene expression was similar to the wild-type nodules. In empty nodules induced by *R. meliloti exo* and *ndv* mutants (*ndv* refers to nodule development, *ndv* mutants are defective in cyclic glucan production), and by *Agrobacterium tumefaciens* strains carrying *R. meliloti nod* genes, a nodule meristem was present but infection threads were not formed. In these empty nodules only two early nodulins, Nms-30 and a nodulin homologous to soybean ENod2 were detected (Dickstein et al., 1988).

Auxin transport inhibitors (ATI), N-(1-naphthyl)phthalmic acid and 2,3,5-triiodobenzoic acid induced the formation of psuedonodules on alfalfa roots (Hirsch et al., 1989). These pseudonodules were white and devoid of bacteria and resembled nodules elicited by *R. meliloti exo* mutants at both the histological and molecular level. Early nodulin genes ENod2 and Nms-30 were expressed in the pseudonodules, and late nodulin genes such as Lb were not expressed. *In situ* hybridization studies have revealed that ENod2 gene is expressed in the same tissue-specific manner in empty nodules (bacterially or ATI-induced) as in wild-type *R. meliloti*-induced alfalfa nodules (van de

Weil et al., 1990b).

In *Vicia sativa*, an early nodulin Nvs-40 was identified among *in vitro* translation products on 2-D gels (Moerman et al., 1987). Nvs-40 was shown to immunoprecipitate with an antiserum that is specific for pea N-40′ and is therefore structurally and functionally identical to N-40′, an early nodulin of pea (Moerman et al., 1987). An *Agrobacterium* transconjugant harboring an *R. leguminosarum* Sym plasmid induced nodules on *V. sativa* that contained some infected cells and in these nodules two early nodulin genes, ENod2 and Nvs-40 were expressed. In contrast, nodules induced in pea by the same strain were "empty" (devoid of infected cells) and only ENod2 gene was expressed, indicating that expression of *Vicia* Nvs-40 gene is linked to the release of bacteria into the nodule cells (Moerman et al., 1987).

**Host plant nodulation genes**

Plant nodulation or symbiotic genes have been identified as naturally occurring variants or through induced mutation studies in several different legume plants (Rolfe & Gresshoff, 1988; Vance et al., 1988; Caetano-Anollés & Gresshoff, 1991). Unlike nodulin genes, which have been identified using molecular techniques, virtually nothing is known about nodulation genes at the molecular level.

The most extensive mutational analysis of nodulation genes has been in pea. Several pea nodulation mutants referred to as "*sym*" mutants have been isolated through the use of mutagens such as EMS (ethyl methyl sulfonate) or $\gamma$ or neutron radiation

(Kneen & LaRue, 1984, 1988; Duc and Messager, 1989; Kneen et al., 1990).

Extensive work has been done in pea in mapping plant *sym* genes on the pea chromosome (Weeden et al., 1990). Out of 15 *sym* genes, 4 have been mapped to a small region on chromosome 1 near a Lb gene cluster. The other *sym* genes were distributed throughout the pea genome (Weeden et al., 1990).

Several soybean mutants with altered symbiotic properties including non-nodulating, ineffectively nodulating and supernodulating mutants have been identified and characterized at the physiological and genetic level (Caetano-Anollés & Gresshoff, 1991; Carroll et al., 1985a, 1985b; 1986). Among the soybean mutants, the supernodulating (*nts*, nitrate tolerant) mutant has been the subject of molecular investigation. The genetic locus (*nts*) controlling supernodulation has been linked very close to a cloned molecular marker (Landau-Ellis et al., 1991).

Alfalfa plant nodulation mutants have been used in studying the physiological and biochemical parameters associated with symbiotic N-fixation (Groat & Vance, 1982; Egli et al., 1989). Egli et al. (1989) were the first to report the effect of mutations in plant genes on nodule enzyme activities. In ineffective alfalfa nodules under control of the plant gene $in_1$, GS, GOGAT, AAT and PEPC enzyme activities were lower than in normal effective alfalfa nodules (Egli et al., 1989).

Chickpea nodulation genes

Several nodulation mutants have been isolated by selection, following $\gamma$-irradiation

mutagenesis of the highly inbred chickpea line ICC640 (Davis, 1985; Davis et al., 1985). Genetic analyses of mutants PM233, PM665, PM679, PM405, and PM796 resulted in the identification of five recessive genes involved in chickpea symbiosis. These mutations confer either non-nodulating or ineffectively nodulating phenotypes (Table 2). The mutants PM233, PM665, PM679, PM405, and PM796 have been backcrossed twice to ICC640, to produce lines PM233B, PM665B, PM679B, PM405B, and PM796B. The need for backcrossing was to eliminate, through independent assortment, any unlinked secondary mutations that might have occurred in the mutant lines. The backcross-derived lines are isogenic to the wild-type except with respect to single nodulation gene mutations (Davis et al., 1986, 1988).

Information known to date on the chickpea mutants has been summarized in Table 2. Chickpea line ICC435M is a spontaneous mutant derived from its wild-type parent ICC435 (Rupela & Sudharshana, 1986). The non-nodulation phenotype in ICC435M is conferred by a single recessive gene $rn_6$ (Singh et al., 1992). The $rn_6$ and $rn_1$ recessive alleles at two different loci control non-nodulation phenotype in mutants ICC435M and PM233B, respectively (Davis, unpubl.; Singh et al., 1992).

The non-nodulating chickpea mutant PM233B has been characterized by microscopic comparisons with the normally nodulating parental isoline, ICC640 (Matthews & Davis, 1989). Infection threads and cortical cell divisions were observed in serially sectioned root segments of ICC640, but not in PM233B. Scanning electron microscope observations of inoculated root segments revealed that PM233B plants were not defective in their ability to adsorb *Rhizobium* bacteria. The infection process in

**Table 2:** Characteristics of chickpea nodulation mutants

| Chickpea Line | Phenotype | Mutant gene |
|---|---|---|
| ICC640 | Nodulates effectively at root temperatures 24°c and 29°c. | wild-type |
| PM233B | No nodules formed under any conditions tested. Rhizobia bind to root hairs, but no root hair curling, infection threads, or cortical cell divisions. | rn1 |
| PM665B | Forms a few effective nodules at 24°c root temperature, but no nodules at 29°c. Has aberrant epidermal trichomes and abnormal pod shape. | rn2 |
| PM679B | Forms a few, marginally effective nodules at 24°c, but no nodules at 29°c. | rn3 |
| PM405B | Forms numerous, small white nodules which do not fix nitrogen. | rn4 |
| PM638A | Forms numerous, yellowish nodules which do not fix nitrogen. | Dominant[2] |
| PM796B | Forms prematurely senescent nodules which fix very low levels of nitrogen. Tips of nodules are red, remaining tissue is green. Lacks activity of nodule-specific form of aspartate amino transferase (AAT), though AAT protein is present immunologically[1]. | rn5 |

[1] AAT-2 antiserum obtained from C. P. Vance (University of Minnesota).
[2] Establishing mode of inheritance for this mutant was one goal of the present study.

PM233B is blocked prior to the initiation of cortical cell division and infection thread formation. Results from reciprocal grafting between ICC640 and PM233B indicated that non-nodulation in PM233B is determined by the root genotype and not the shoot genotype (Matthews & Davis, 1989).

Chickpea mutants PM665 and PM679 exhibit temperature sensitive non-nodulating phenotype (Davis et al., 1986). At 24°C, mutants PM665 and PM679 form a few effective nodules, but at 29°C nodulation is strongly suppressed in PM665 and is eliminated in PM679. The wild-type parent ICC640 forms effective nodules at temperatures ranging from 24°C to 29°C (Davis et al., 1986).

Chickpea mutants PM405B and PM796B produce ineffective ($nod^+ fix^-$) nodules. The term "ineffective" is used to describe nodules that do not fix any nitrogen or have the ability to fix very little nitrogen. PM405B nodules do not fix any nitrogen whereas PM796B nodules fix very little nitrogen compared to wild-type (Davis, 1988). PM796B nodules differ biochemically from wild-type nodules due to lack of AAT-C2 (nodule-specific isoform in chickpea) activity, although they do contain AAT-C2 mRNA and protein (Heard, 1992).

Chickpea mutant PM638A produces nodules that do not fix any nitrogen. This mutant line is a product of one backcross between PM638 and ICC640. Based on segregation analysis of progeny obtained from crosses between PM638 and ICC640, the genetic determinant conferring the mutant nodule phenotype was shown to be due to a dominant mutation in PM638A (Davis, unpubl.). However, due to the small progeny size, data was insufficient to clearly establish a single gene model for inheritance of the

mutation. Therefore, one objective of the present study was to confirm monogenic inheritance of the dominant mutation in PM638A, and to produce backcross line PM638B.

**Concluding remarks**

From reviewing the literature, it is clear that little is known about the relationships and interactions between molecularly identified nodulin genes and mutationally identified nodulation genes in legume host plants. In chickpea, there have been no reports on identification of nodulin genes or studies on nodulin gene expression. The two approaches that are being explored in our laboratory are 1) analyses of nodulin gene expression in plant nodulation mutants, and 2) linkage mapping of nodulin and/or nodulation genes. The first approach has been used in the research described in (Chapter I). Research presented in this thesis is unique due to the fact that heterologous nodulin cDNA probes were used in the study of plant nodulation mutants with the aim of characterizing the genetically defined mutants at the molecular level. Research of this nature has not been described in any other legume.

# CHAPTER I

## SECTION B - MATERIALS AND METHODS

### Plant material

Chickpea lines used in the experiments include the highly inbred parental line ICC640 and six nodulation mutants derived from ICC640 via γ-irradiation mutagenesis (Davis et al., 1985). Mutant lines PM233B, PM665B, PM679B, PM405B and PM796B were derived by twice back-crossing original mutants PM233, PM665, PM679, PM405 and PM796 to ICC640 to eliminate possible secondary mutations and to obtain isogenic mutant lines that differ from the wild-type parent only with respect to single nodulation gene mutations (Davis, 1988). Mutant line PM638A was derived by a single backcross of the original mutant PM638A to ICC640 (Davis, unpubl.). The characteristics of these mutants (Davis et al., 1986; Davis, 1988; Matthews & Davis, 1989; Heard, 1992) are listed in Table 2. A spontaneous mutant ICC435M derived from the parent ICC435 (Rupela & Sudarshana, 1986) was also included in this study.

### Inheritance study

To establish the mode of inheritance of the mutant PM638A nodule phenotype,

and to advance the mutant line through a second cycle of backcrossing, reciprocal crosses were made between PM638A and ICC640. The process of crossing involved hand emasculation of anthers from the female parent, and transfer of pollen (with the help of tweezers) from the male parent to the stigma of the female parent. Several $F_1$ plants were grown in soil in the greenhouse to produce seeds, by natural self-pollination, for $F_2$ generation testing. Other $F_1$ plants and all $F_2$ and $F_3$ plants were grown under nitrogen-deficient conditions to test their nodulation phenotype. Seeds were planted in 5" diameter pots filled with vermiculite. The pots were inoculated with *Rhizobium ciceri* strain CC1192 at the time of planting and watered daily with nitrogen-free nutrient solution (Davis et al., 1985). Plants were grown in a Root Zone Chamber (Environmental Growth Chambers, Chagrin Falls, OH), which provided a constant root temperature of 26°C. Ten seeds each of PM638A and ICC640 were planted to serve as controls. Observations were made and phenotypic segregation data collected 25 days after planting.

**Growth conditions**

Leaf material for DNA extraction

Plants of wild-type ICC640 and ICC435, nodulation mutants PM233B, PM665B, PM679B, PM405B, PM638A, PM796B and ICC435M, soybean, and alfalfa were grown in the greenhouse. At least 20 seeds of each sample were planted in 6" diameter pots

(4 seeds/pot) filled with gromix. The leaves harvested from 2- to 3-week-old plants were used for DNA extraction.

Roots/nodules for RNA extraction

The chickpea lines used for RNA extraction included ICC640, PM233B, PM405B, PM638A and PM796B. Surface-sterilized seeds were planted in vermiculite contained in Leonard jars (Matthews & Davis, 1989), in order to maintain aseptic conditions. The Leonard jars were autoclaved before planting seeds. The Leonard jars, except those containing ICC640 seeds used as controls, were inoculated with a slurry of chickpea *Rhizobium* strain CC1192 prepared from freshly harvested root nodules. The inoculum was prepared by mixing 5 g ground nodules in 0.5 l nutrient solution (Davis et al., 1985) lacking inorganic nitrogen. The inoculum was filtered through cheese cloth and transferred to a dispenser bottle, and approximately 10 ml of the inoculum was dispensed into each Leonard jar. The plants were grown in the root zone chamber with the root temperature maintained at 26°C, and watered daily with autoclaved nutrient solution. The root temperature was maintained at 26°C, the optimum temperature for nodulation (Davis et al., 1986).

Roots and/or nodules were harvested at different time points after planting, quickly frozen in liquid nitrogen and stored at -70°C until ready for RNA extraction.

## Nodulin cDNA clones

Nodulin cDNA clones were obtained from different sources for use as probes for Southern and Northern analysis. A list of the nodulin cDNA clones, their sources and vector descriptions are summarized in Table 3.

## Bacterial culture and storage of cDNA clones

*E. coli* bacteria containing plasmids were grown on plates or in liquid suspension of LB medium (Appendix 1) containing the appropriate antibiotic (for antiobiotic resistance markers, see Table 3; Appendix 2).

In order to obtain bacteria for plasmid isolation, or for long-term storage of cDNA clones, plates containing LB medium were streaked with a loop of bacterial culture and incubated at 37°C overnight. Single colonies of the bacteria were used to inoculate 5 ml of liquid LB medium which was then incubated at 37°C overnight. For long term storage of cDNA clones, 150 $\mu$l of sterile glycerol and 850 $\mu$l of overnight bacterial culture were combined in an eppendorf tube and stored at -70°C. For plasmid isolation, 1 ml from the overnight culture was used to inoculate 150 ml of liquid medium, which was then incubated overnight at 37°C.

**Table 3:** Information on nodulin cDNA clones in plasmid vectors.

| cDNA clone | plasmid | selection marker | restriction site | insert size (bp) |
|---|---|---|---|---|
| **I. alfalfa (K. Dunn, Boston College)** | | | | |
| A2ENOD2 | Bluescript | amp$^r$ | *Eco*RI | 292 |
| Lb | pGEM | amp$^r$ | *Pst*I | 400 |
| **II. soybean (D.P.S. Verma, Ohio State University)** | | | | |
| Lb | pBR322 | tet$^r$ | *Pst*I | 510 |

$^r$= antibiotic (ampicillin/tetracycline) resistance

**Plasmid isolation**

Plasmid DNA was isolated from an overnight bacterial culture using the modified alkaline-lysis method (Maniatis et al., 1982). Bacterial cells were pelleted by spinning 150 ml overnight bacterial culture in 50 ml Oakridge tubes at 3,020 g for 10 min. The pelleted cells were resuspended in 2 ml wash buffer (Appendix 3) and mixed with 400 $\mu$l of freshly prepared lysozyme (Appendix 4) and incubated on ice for 30 min. The cells were lysed with 4 ml SDS-NaOH (Appendix 5) and placed for 5 min on ice. Then, 3 ml of 3 M sodium acetate pH 5.2 was added and mixed. The tubes were held on ice for 1 hr, then centrifuged at 17,400 g for 5 min at 4°C. The supernatant fluid was removed to fresh Oakridge tubes, mixed with 20 ml of 95% ethanol, and incubated at -20°C overnight.

The tubes were then centrifuged at 12,100 g for 15 min and the supernate discarded. The pellet was washed with 95% ethanol, and resuspended in 300 $\mu$l TE (Appendix 6). The DNA solution was mixed with 10 $\mu$l RNase (Appendix 7) and incubated at 37°C for 1 hr followed by phenol/chloroform-isoamyl alcohol (Appendix 8) extraction. The aqueous solution was mixed with 0.1 volume of 3 M sodium acetate and 2 volumes of 95% ethanol and the tubes were kept at -20°C overnight.

Plasmid DNA was pelleted by spinning the tubes for 15 min at 12,100 g in a microfuge. The supernate was discarded and the pellet dried in a speedvac. The dried pellet was resuspended in TE.

Absorbance values of the samples at 260 nm and 280 nm as measured on a

spectrophotometer were used to determine the concentration of plasmid DNA samples. Two $\mu$g plasmid DNA samples were electrophoresed on 0.8% agarose gels in 1X TBE (Appendix 9) buffer at 40 V for 2-3 hr and stained with ethidium bromide (Appendix 10).

**Removal of cDNA insert from plasmid vector**

Insert DNA was isolated from 30 $\mu$g plasmid DNA cut with the appropriate restriction enzyme (Table 3). The digested DNA fragments were separated by agarose gel electrophoresis at 40 V for 2-3 hr on 0.8% low melting Nusieve GTG agarose gels containing ethidium bromide, after which the gels were viewed on a UV light box. The DNA inserts were cut out of gels and placed in Eppendorf tubes, and heated to 65°C to melt the agarose. Following phenol/chloroform-isoamyl alcohol extraction, the aqueous phase was removed and mixed with 0.1 volume sodium acetate and 2 volumes of 100% ethanol, then precipitated overnight at -20°C. The tubes were centrifuged at full speed in a microfuge for 30 min to pellet the insert DNA, dried in a speedvac and resuspended in 10 $\mu$l TE. The insert DNAs were run on 0.8% agarose gels and stained with ethidium bromide to confirm the presence of the DNA and to estimate its concentration as compared to lambda-*Hind*III marker DNA.

**Sequencing of A2ENod2 cDNA insert**

Bluescribe plasmid containing the A2ENod2 cDNA was isolated and purified from an overnight culture of *E. coli*, using the alkaline lysis/ PEG precipitation method (as described in manual, Applied Biosystems, Inc., Foster City, California). For the purpose of sequencing, 1 $\mu$g (concentration of 200-250 ng/$\mu$l) of the purified plasmid dissolved in sterile, deionised water, was submitted to the UNH DNA sequencing laboratory, where the DNA sequence of A2ENod2 cDNA was determined by using the method of "DyeDeoxy Cycle Sequencing" (as described in manual, Applied Biosystems, Inc.). Both M13 forward and reverse primers were used to read the sequence from both ends of the cDNA insert.

**Genomic DNA extraction**

The procedure followed for DNA extraction was as described by Dellaporta et al. (1983). Fresh leaf tissue samples weighing 0.75 g were quickly frozen in liquid $N_2$, ground to a fine powder and then transferred into 50 ml Oakridge tubes containing 15 ml extraction buffer (Appendix 11). After addition of 2 ml of 10% SDS, the contents of the tubes were mixed thoroughly and incubated at 65°C for 10 min. Next, 5 ml of 5 M potassium acetate was added, the tubes shaken vigorously and incubated at 0°C for 20 min, followed by centrifugation at 25,000 g for 20 min. The supernate was separated through miracloth filters into clean Oakridge tubes, mixed with 10 ml cold isopropanol,

and incubated at -20°C overnight. Genomic DNA was pelleted by spinning the tubes at 20,000 g for 15 min, then re-dissolved in 0.7 ml of 50 mM Tris, 10 mM EDTA, pH 8.0. DNA solution was transferred to eppendorf tubes and spun at full speed in a microfuge for 10 min to remove insoluble debris. The supernate was transferred into fresh eppendorf tubes, mixed with 0.5 volume of 7.5 M ammonium acetate, and 500 $\mu$l of cold isopropanol, then incubated at -20°C overnight.

DNA was precipitated by spinning the tubes for 15 min in a microfuge. The pellet was washed with 80% ethanol, dried in a speedvac and redissolved in 100 $\mu$l TE. To 100 $\mu$l DNA samples, 3 $\mu$l of RNase (Appendix 7) solution was added and incubated at 37°C for 1 hr. The concentration of DNA samples was determined by measuring absorbance values at 260 and 280 nm. The quality of DNA was tested by visual examination of 2 $\mu$g DNA samples separated on 0.8% agarose gels.

DNA samples that did not show any degradation were digested separately with different restriction enzymes - *Eco*RI, *Hind*III, *Eco*RV and *Xba*I. Ten $\mu$g digested DNA samples mixed with loading buffer (Appendix 12) were loaded in separate wells in 1% agarose gels. Lambda DNA cut with HindIII was used as the DNA molecular size standard. As a positive control, DNA extracted from soybean and alfalfa was also digested with restriction enzymes and loaded similarly. The stained gels were viewed on a UV light box to confirm the presence of restriction fragments in each lane. Later, the gels were prepared for Southern blotting.

**Southern blotting and hybridization**

The procedure followed for Southern blotting was as described by Maniatis et al. (1982). The gels containing digested DNA samples were soaked in 0.25 M HCl for 5 min, followed by 1 hr incubation in 1.5 M NaCl and 0.5 M NaOH. The gels were then neutralized in 1 M Tris and 1.5 M NaCl for 1 hr. About 600 ml of 10X SSC (Appendix 13) was used as the transfer solution. DNA transfer from gel to nylon membrane (Hoefer Nylon 66 Plus) was allowed to take place overnight. The nylon membranes were baked for 2 hr at 80°C under vacuum and stored at room temperature until ready for probing.

Nodulin cDNA inserts were labeled with $\alpha$-d[CT$^{32}$P] by the procedure of random priming (Boehringer Mannheim booklet). Nylon membranes (Hoefer Nylon 66 Plus) were prehybridized for 8 hr at 42°C. The prehybridization solution contained 4X SSC, 3X Denhardt's solution, 0.5% SDS, 0.2 mg/l salmon sperm DNA, and 50% formamide. The Southern blots were hybridized at 40°C with denatured labeled cDNA probes (sp. ac. 1.0 x $10^8$cpm/$\mu$g DNA). Following hybridization, when ENod2 probe was used the blot was washed sequentially for 30 min, at 55°C in each wash solution containing 2X SSC, 0.1% SDS; 1X SSC, 0.1% SDS; and 0.5X SSC, 0.1% SDS. When Lb probe was used, the Southern blots were washed twice for 30 min in 2X SSC, 0.1% SDS, at 35°C and 40°C, followed by two washes for 30 min in 1X SSC, 0.1% SDS, at 50°C and 55°C. The blots were exposed to X-ray film at -70°C, for 1 to 3 d. Autoradiograms were developed and examined for cross hybridization of the specific nodulin cDNA probes to

chickpea and positive control DNAs.

**Total RNA extraction**

All solutions used for RNA work were prepared using 0.1% diethylpyrocarbonate (DEPC)-treated double distilled water (Appendix 16).

Total RNA was extracted from roots and/or nodules as described by Dunn et al. (1988). Harvested tissue weighing 0.5 to 5 g was quickly frozen in liquid nitrogen. The frozen tissue was ground to a fine powder and 0.5 g samples were added to eppendorf tubes containing 400 $\mu$l phenol, 100 $\mu$l chloroform-isoamyl alcohol and 500 $\mu$l extraction buffer (Appendix 17). The tubes were vortexed, then spun in a microfuge for 5 min. The clear supernate was removed to a fresh tube followed by a second phenol/chloroform-isoamyl alcohol extraction. The aqueous phase was removed, 1/3 vol. of 8 M LiCl was added, and the tubes were incubated at -20°C overnight.

The tubes were centrifuged for 15 min in a microfuge. The pellet was resuspended in 100 $\mu$l DEPC-treated water. The solution was mixed with 10 $\mu$l of 3 M sodium acetate and 2 volumes of 95% ethanol and incubated at -20°C overnight.

The procedure followed for isolating total RNA from *R. ciceri*, a Gram negative bacterium was as described by Ausubel et al., (1989). To begin with, a frozen culture of *R. ciceri* was streaked on LMB (Appendix 18) agar plates and incubated at 28°C. From the fresh bacterial culture, a loopful of the bacteria was used to inoculate liquid LMB medium and incubated at 28°C, to obtain a liquid suspension of the bacteria.

Bacterial cells were collected by centrifuging 10 ml culture in 30 ml Oakridge tubes. The cells were resuspended in 10 ml protoplasting buffer (Appendix 19) and 80 $\mu$l (from 50 mg/ml stock) of lysozyme was used to lyse the cells. The bacterial spheroplasts were collected in the tube by centrifugation and resuspended in Gram negative lysing buffer (Appendix 20). Saturated NaCl (Appendix 21) was added to precipitate protein and DNA. The clear supernate was transferred to eppendorf tubes and precipitated overnight in ethanol and total RNA was recovered by centrifugation. The RNA pellet was resuspended in DEPC water and stored at -70°C.

**Northern blotting and hybridization**

Two $\mu$g samples of total root and/or nodule RNA were electrophoresed on 1.2% formaldehyde gels to test for RNA quality. A suspension of 0.6 g Seakem GTG agarose in 42.5 ml double distilled water was heated to 90°C to dissolve the agarose. After the solution cooled to 60°C, 2.7 ml formaldehyde and 5 ml 10X MOPS (Appendix 22) buffer were added, mixed, and poured into the gel mold. RNA samples containing 10X MOPS buffer, formaldehyde, and formamide (Appendix 23) were incubated at 55°C for 15 min, then mixed with 2 $\mu$l loading buffer and loaded in the gel wells. The relative size of RNA was calculated using Lambda DNA cut with HindIII and RNA markers as molecular size standards.

Similarly, 30 $\mu$g samples of total RNA were electrophoresed on formaldehyde gels and prepared for Northern blotting. The procedure for Northern blotting as

described by Maniatis et al. (1982) was followed.

cDNA probes were labeled in a similar fashion as described previously for probing Southern blots. Nylon membranes (Hoefer Nylon 66 Plus) were prehybridized for 8 hr at 42°C. The prehybridization solution contained 4X SSC, 3X Denhardt's solution, 0.5% SDS, 0.2 mg/l salmon sperm DNA, and 50% formamide. The Northern blots were hybridized at 40°C with denatured labeled cDNA probes (sp. ac. 1.0 x $10^8$cpm/$\mu$g DNA). Following hybridization, when ENod2 probe was used, the Northern blots were washed twice for 30 min, in 2X SSC, 0.1% SDS, at 45°C, and once sequentially for 30 min in 1X SSC, 0.1% SDS; 0.5X SSC, 0.1% SDS and in 0.1X SSC, 0.1% SDS at 55°C. Northern blots hybridized with Lb probe were washed twice for 30 min in 2X SSC, 0.1% SDS, at 35°C and 40°C; followed by two washes in 1X SSC, 0.1% SDS, at 50°C and 55°C. The blots were exposed to X-ray film at -70°C, for 1 to 3 d. Autoradiograms were developed and examined for evidence of cross-hybridization of the specific nodulin cDNA probes to total RNA from root and/or nodule tissue from wild-type and chickpea nodulation mutants.

**Light microscopy**

Nodules were harvested from chickpea lines ICC640, PM405B and PM638A and fixed overnight in 3% glutaraldehyde (v/v) and 0.2 M sodium cacodylate buffer, pH 7.2. After overnight incubation, the samples were washed twice in cacodylate buffer.

The fixed samples were dehydrated by incubating sequentially for 15 min each

in 50%, 70% and 90% ethanol. After the 90% ethanol wash, the samples were washed for 15 min twice in 100% acetone and then incubated overnight in a 1:1 acetone/epoxy resin mixture.

Samples were then transferred to gelatin capsules containing epoxy resin (4-5 nodules/capsule). The capsules were kept in a vacuum dessicator for 2 hr. The nodules were individually embedded in the epoxy resin molds and incubated overnight at 60°C.

The embedded samples were trimmed and sectioned with a glass knife on a microtome (Reichart Ultracut E). Sections 2 microns thick were stained in toluidine blue and basic fuchsin stain for 10 min and observed under light microscope.

# CHAPTER I

## SECTION C - RESULTS

### Inheritance of the defective symbiosis phenotype in chickpea mutant line PM638A

When grown in the absence of mineral nitrogen and inoculated with *R. ciceri*, PM638A plants were easily recognised by their chlorotic leaves and yellowish nodules. This is in contrast to ICC640 plants which produce dark green leaves and reddish-brown nodules when grown under the same nitrogen-deficient conditions. Similarly, wild-type and/or mutant phenotypic characteristics were easily distinguishable in all the $F_1$, $F_2$ and $F_3$ plants that were used in the inheritance study.

When $F_1$ plants resulting from reciprocal backcrosses between wild-type parent ICC640 and mutant line PM638A were tested for nodulation phenotype, all $F_1$ plants had PM638A-type mutant nodules, confirming that the mutant phenotype was dominant to wild-type. Segregation into two phenotypic classes was observed in the $F_2$ generation and in some $F_3$ families. Chi-Square analysis was used to test the goodness-of-fit of $F_2$ segregation data to single gene and two gene models (Table 4), and of $F_3$ segregation data to a single gene model. Based on convention, "P" (probability that the deviation between observed and expected values is due to chance) values greater than 0.05 are considered statistically non-significant. This criterion was used in analysing the data presented in

Tables 4 & 5.

The $F_2$ segregation data from reciprocal crosses between ICC640 and PM638A fit a monohybrid (single gene) phenotypic ratio of 3 mutants: 1 wild-type, but deviated significantly from the expectation associated with two-gene models involving complementary factors (9:7 ratio) or duplicate dominance (15:1 ratio) (Table 4). Thus, the $F_2$ segregation data (Table 4) show that the mutant phenotype seen in PM638A is due to a single dominant gene mutation. Similar results from the reciprocal crosses, which show a monohybrid segregation pattern, indicate that the gene involved is nuclear in origin and thereby rule out maternal (cytoplasmic) inheritance of the mutation.

Results of segregation analysis of $F_3$ progeny were consistent with the single dominant gene model (Table 5). All the $F_2$ plants with wild-type phenotype were true breeding; only plants with wild-type phenotype appeared in their $F_3$ progenies. In contrast, the $F_3$ families derived from $F_2$ plants with mutant nodulation phenotype showed a 1(mutant, non-segregating) : 2(segregating) ratio (Table 5). In the segregating $F_3$ families, the overall segregation of 66 mutant versus 31 wild-type also fit a 3:1 monohybrid ratio ($X^2 = 2.51$; $0.20 > P > 0.10$).

The above mentioned results provide sufficient data to establish that the ineffective nodulation phenotye seen in chickpea line PM638A is due to a monogenic dominant, nuclear mutation.

The non-segregating $F_3$ families provided a source for a homozygous mutant line, thus achieving the goal of establishing the double backcross line PM638B. Mutant line PM638B is a product of two backcrosses and has the same wild-type genetic background

**Table 4:** Analysis of $F_2$ segregation data in reciprocal crosses between PM638A and ICC640. All plants were inoculated with *R. ciceri*, and grown under conditions described for testing nodulation phenotype.

| Cross | Phenotype | | $X^2$ (ratio) | P |
|---|---|---|---|---|
| | mutant | wild-type | | |
| ICC640 x PM638A | # of plants | | | |
| observed | 74.0 | 19.0 | | |
| expected (3:1) | 69.8 | 23.2 | 1.04 (3:1) | 0.50>P>0.30 |
| expected (9:7) | 52.3 | 40.7 | 20.57 (9:7) | 0.001>P |
| expected (15:1) | 87.2 | 5.8 | 32.04 (15:1) | 0.001>P |
| PM638A x ICC640 | | | | |
| observed | 59.0 | 18.0 | | |
| expected (3:1) | 57.8 | 19.2 | 0.11 (3:1) | 0.80>P>0.70 |
| expected (9:7) | 43.3 | 33.7 | 13.00 (9:7) | 0.001>P |
| expected (15:1) | 72.2 | 4.8 | 38.71 (15:1) | 0.001>P |

**Table 5:** Determination of $F_2$ genotype by segregation analysis of $F_3$ families from PM638A x ICC640 crosses. All plants were inoculated with *R. ciceri*, and grown under conditions described for testing nodulation phenotype.

| $F_2$ nodulation phenotype | # of $F_3$ families[a] non-segr. | segr.[b] | $X^2$ (1:2) | P |
|---|---|---|---|---|
| mutant | 5[c] | 11 | 0.03 | 0.90>P>0.70 |
| wild-type | 11 | 0 | ---- | ---- |

[a] each $F_3$ family is comprised of 10 plants.
[b] segr. = segregating.
[c] source of PM638B.

as the other chickpea nodulation mutants PM233B, PM665B, PM679B, PM405B and PM796B (Davis et al., 1986; Davis, 1988).

**Molecular biology of host symbiotic genes involved in chickpea-*Rhizobium* symbiosis**

Evaluation of clones used as heterologous probes in Southern and Northern analyses

Early (ENod2) and late (Lb) nodulin cDNAs were used as heterologous probes to identify and study the expression of corresponding nodulin genes in chickpea. In order to ensure that results of molecular analyses using these probes would be valid, the following control measures were undertaken:

(a) The identity of the alfalfa A2ENod2 cDNA (Fig. 2) used as a probe was confirmed by determining its sequence and comparing it (Figs. 3 & 4) to the published sequence (Dickstein et al., 1988). The cDNA probe is 292 bp in size (Fig. 2). Using the method of "cycle sequencing", both ends of the probe (a total of 189 bases) were sequenced (Fig. 2). With the exception of five nucleotide mismatches (Figs. 3 & 4), an exact match was found between the two sequences indicating that the cDNA probe used in the study was indeed A2ENod2.

(b) The proper identity of alfalfa and soybean Lb cDNA clones obtained from two different investigators (Materials & Methods, Table 3) and used as heterologous probes was ensured by using both clones to probe a Northern blot containing chickpea root and nodule RNA (Fig. 6; corresponding gel shown in Fig. 5). Both

probes hybridized to a 0.7 kb transcript expressed in nodules but not in roots. The known size for Lb transcripts in other legume species including soybean, alfalfa, and pea is also 0.7 Kb (Gloudemans et al., 1987; Lullien et al., 1987; Scheres et al., 1990). These results confirmed that the probes were truly Lb clones.

(c) Hybridization and washing conditions as described in Materials and Methods were arrived at by experimentation with the aim of minimising background and at the same time maintaining optimum stringencies required for hybridization of heterologous probes to the target sequences on Southern and Northern blots.

(d) Alfalfa DNA and nodule RNA were used as positive controls on Southern and Northern blots, respectively, probed with the alfalfa A2ENod2 probe. Soybean DNA and nodule RNA were used as positive controls on Southern and Northern blots, respectively, probed with the soybean Lb probe. Uninoculated chickpea root RNA was used as a negative control on all Northerns. RNA from *R. ciceri* was used as a negative control on a Northern blot probed with A2ENod2.

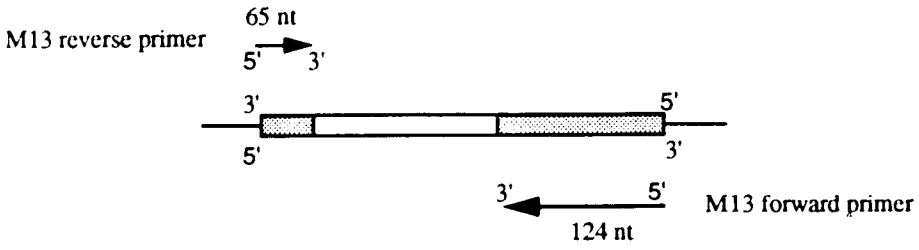



**Fig. 2 :** Schematic representation of sequenced regions of the A2ENod2 cDNA insert used as a probe.

: 292 bp cDNA insert

: Hatched areas represent the parts of cDNA insert that were sequenced

: Bluescribe plasmid vector sequences

```
                                        *                       *
sequence of probe       1   CGANCACATGTGNATCCACCGTCAGAGNATCAACCACCTCTTGATCATCC   50
published sequence      1   CGACCACATGTGCATCCACCGCCAGAGCATCAACCACCTCTTGAACATCC   50
                            .       .       .       .       .       .

                              *
sequence of probe       51  ANCGTCAGAGTACCA                                     65
published sequence      51  ACCGCCAGAGTACCA                                     65
                                .
```

**Fig. 3:** Comparison of the first 65 nt of the cDNA probe used in the study to the published sequence (Dickstein et al., 1988) of alfalfa A2ENOD2 cDNA.
* = disagreement in sequence

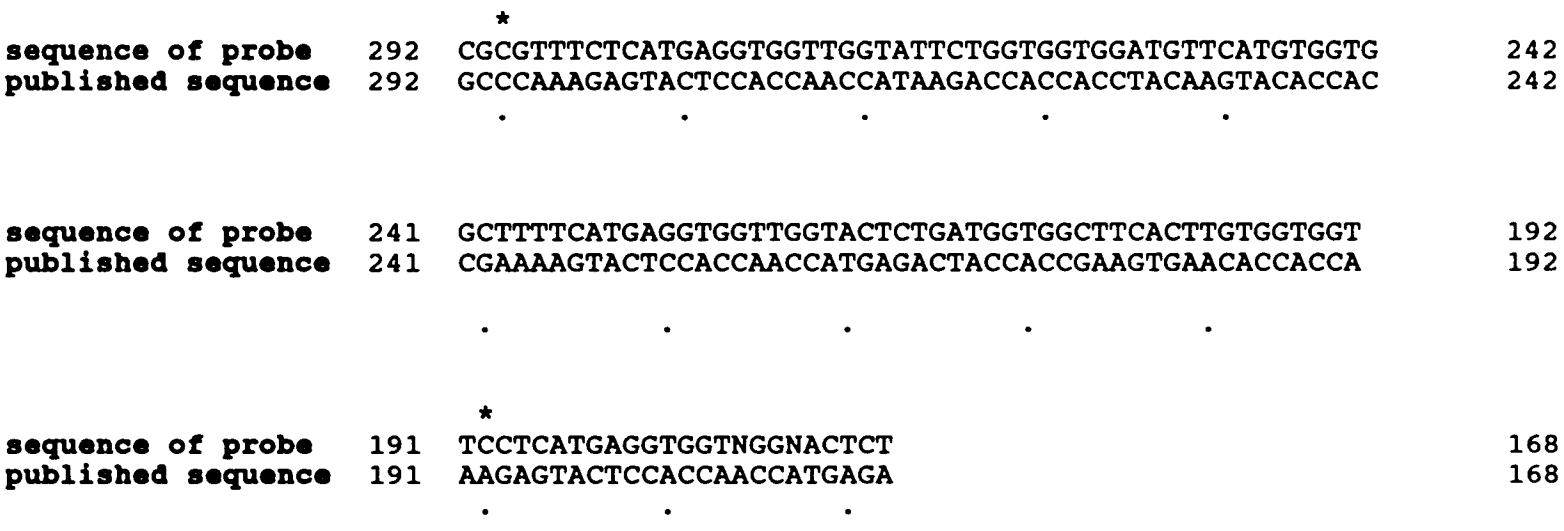



**Fig. 4:** Comparison of DNA sequences of a portion of cDNA probe (124 nt long), which is complementary to the published sequence (Dickstein et al., 1988) of A2ENOD2 cDNA from nt # 292 to # 168.
* = disagreement in sequence

**Fig. 5:** Ethidium bromide stained formaldehyde gel containing total RNA extracted from root and nodule samples of chickpea line ICC640. U, uninoculated; I, inoculated; R, root; N, nodule.

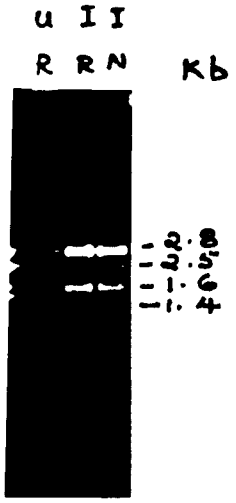
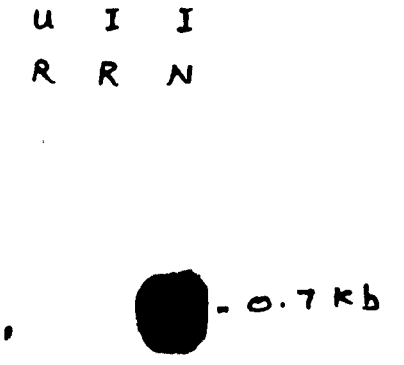


**Fig. 6:** Chickpea mRNA with homology to alfalfa and soybean Lb cDNAs. Total RNA extracted from root and nodule samples (Fig. 5) of chickpea line ICC640 was run on formaldehyde gel, blotted and subjected to Northern hybridization with (a) alfalfa, and (b) soybean Lb cDNA probes. U, uninoculated; I, inoculated; R, root; N, nodule.




(a)


(b)

Detection of ENod2- and Lb-homologous DNA sequences in chickpea

Genomic DNA was extracted from leaves of chickpea wild-type and mutant lines, alfalfa and soybean. Alfalfa and soybean DNAs were included in Southern analysis as positive controls. For Southern analysis, DNA samples were digested separately with various restriction enzymes and separated on 1.0% agarose gels. After electrophoresis, the gels were stained with ethidium bromide to confirm complete digestion of the DNA samples (Figs. 7 & 9).

For detection of ENod2 homologous sequences, genomic DNAs of ICC435, ICC435M, ICC640, PM233B, PM665B, PM679B, PM405B, PM796B, and alfalfa (homologous DNA control) were digested with *Eco*RV. Wild-type ICC640 DNA was also digested with *Eco*RI and *Xba*I. The size fractionated DNAs were transferred from the gels to nylon membranes and probed with $^{32}$P-labeled alfalfa A2ENod2 cDNA.

DNA fragments homologous to A2ENod2 cDNA were found in chickpea wild-type and mutants, and in alfalfa (Fig. 8). No differences were found between wild-type and mutant lines. A difference in number of bands or change in band mobility for a mutant chickpea line would have constituted an RFLP (restriction fragment length polymorphism), and would have suggested that the mutant carried a mutation in the chickpea ENod2 gene itself. However, no such RFLP was found.

A single band 11.0 Kb in size was identified in *Eco*RV digests of all chickpea DNAs (Fig. 8). Single bands of 5.0 Kb and 11.0 Kb were detected in *Eco*RI and *Xba*I digests, respectively, of ICC640 (data not shown). In contrast to single band

**Fig. 7:** Ethidium bromide stained agarose gel containing *Eco*RV restriction enzyme digests of chickpea and alfalfa genomic DNA.
DNA from chickpea lines (lanes 1 to 8) ICC640, PM233B, PM405B, PM665B, PM679B, PM796B, ICC435 and ICC435M, respectively; and (lane 9) alfalfa. M = λ-*Hind*III DNA molecular weight size standard. DNA marker band sizes are indicated in Kb.

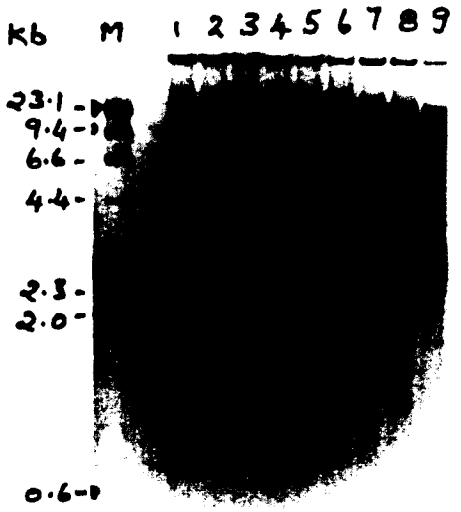
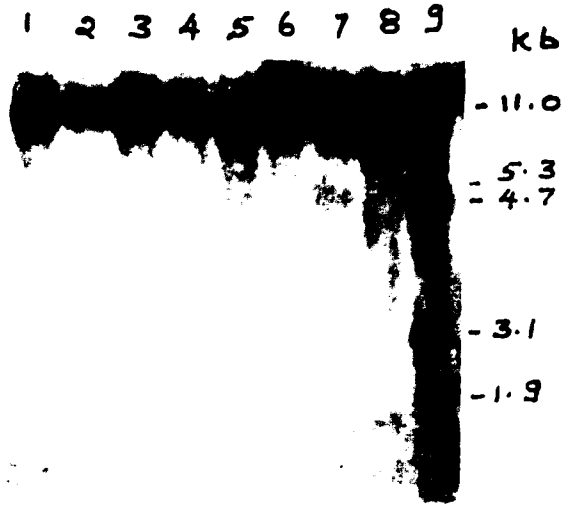


**Fig. 8:** Identification of chickpea and alfalfa DNA restriction fragments with homology to alfalfa early nodulin cDNA A2ENod2.
*Eco*RV-digested DNA from chickpea lines (lanes 1 to 8) ICC640, PM233B, PM405B, PM665B, PM679B, PM796B, ICC435 and ICC435M, respectively; and alfalfa (lane 9), was electrophoresed on agarose gel (Fig. 7), blotted and subjected to Southern hybridization with A2ENod2 probe.



hybridization seen in chickpea DNA, multiple bands homologous to A2ENod2 cDNA were detected in *Eco*RV digested alfalfa DNA. The alfalfa bands corresponded to 5.3, 4.7, 3.1, and 1.9 Kb in size (Fig. 8). Multiple bands were also observed in *Eco*RI- and *Hind*III-alfalfa genomic digests (data not shown). Because alfalfa is a highly heterozygous autotetraploid, multiple bands of different sizes can result from allelic heterozygosity. Multiple bands have been reported in *Eco*RI- and *Hind*III-alfalfa Southerns; however, alfalfa ENod2 band sizes obtained in this study do not match with those published (Barker et al., 1990). This discrepancy may have been due to the considerable allelic polymorphism that exists both within and between alfalfa cultivars.

For detection of Lb homologous sequences, genomic DNAs of ICC640 and PM796B, and soybean (homologous DNA control) were digested separately with *Eco*RV, *Xba*I, and *Eco*RI. The size fractionated DNAs were transferred from the gel to nylon membrane and probed with $^{32}$P-labeled soybean Lb cDNA.

DNA sequences homologous to the soybean Lb probe were found in chickpea wild-type and mutant, and in soybean (Fig. 10). No differences were found in the hybridized DNA band sizes between ICC640 and PM796B. A difference, constituting an RFLP, would have indicated a possible mutation in a leghemoglobin gene in PM796B. However, no such RFLP was found. A single band, 8.9 Kb in size, was detected in *Eco*RV- and *Xba*I-digests of chickpea genomic DNA. Two bands, 7.0 and 3.0 Kb in size, were detected in *Eco*RI chickpea digests, suggesting that an *Eco*RI site was present in the Lb homologous sequence. In contrast, a total of eight bands, 11.5, 8.6, 7.0, 6.0, 5.9, 4.2, 3.0 and 1.4 Kb in size were detected in *Eco*RI digests of the soybean genome

(Fig. 10). The banding pattern seen in soybean genomic Southern is the same as in the published report (Sullivan et al., 1981).

**Fig. 9:** Ethidium bromide stained agarose gels containing DNA from (a) chickpea lines ICC640 (lanes 1, 3 & 5), PM796B (lanes 2, 4 & 6), and (b) soybean digested with different restriction enzymes. The relative position of λ-*Hind*III DNA marker bands is indicated in Kb. V, *Eco*RV; X, *Xba*I; R, *Eco*RI.

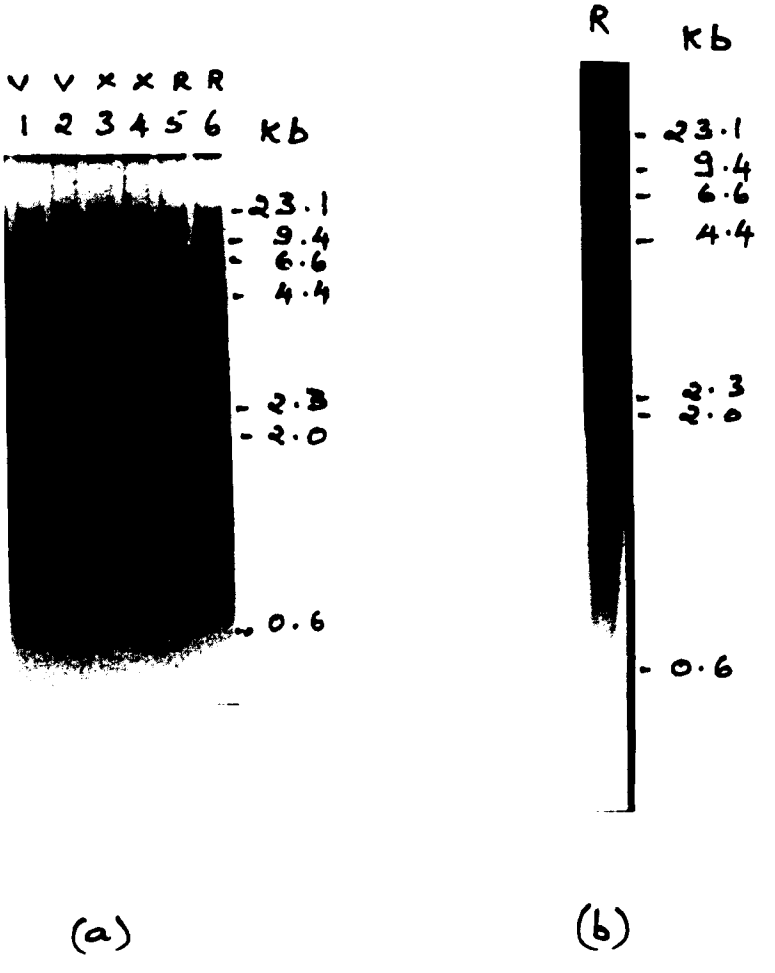
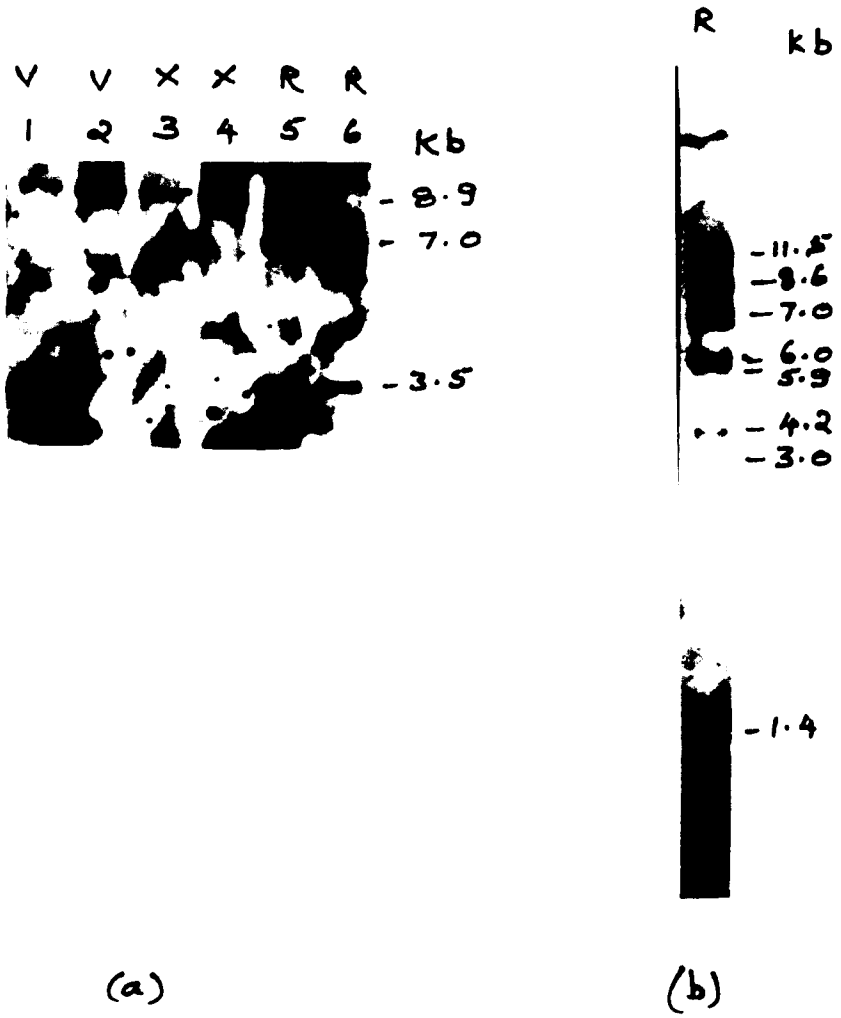


**Fig. 10:** Identification of chickpea and soybean DNA restriction fragments with homology to soybean Lb cDNA.
Genomic DNA from (a) chickpea lines ICC640 (lanes 1, 3 & 5), PM796B (lanes 2, 4 & 6), and (b) soybean was digested with different restriction enzyme(s), run on agarose gel (Fig. 9), blotted and subjected to Southern hybridization with Lb cDNA probe. V, *Eco*RV; X, *Xba*I; R, *Eco*RI.



Nodulin gene expression during chickpea nodule development

In order to define the patterns of ENod2 and Lb gene expression during normal chickpea nodule development, total RNA was extracted from roots and nodules of inoculated chickpea line ICC640, harvested at 8, 12, 16, 20, 30, 40 d after planting. Total RNA from uninoculated chickpea root served as a negative control.

Chickpea root and nodule RNA samples as visualized on ethidium bromide-stained gel

RNA was separated on a 1.2% formaldehyde gel, stained with ethidium bromide, and photographed before RNA transfer onto the nylon membrane (Fig. 11). The sharpness of rRNA bands was an indication of the quality of RNA samples prepared (Fig. 11). The two expected plant rRNA bands (2.8 and 1.6 Kb) were present in all the sample lanes. Their similar staining intensities (Fig. 11) indicate that similar amounts of plant RNA were present in the different lanes.

In lanes 1-3 (Fig. 11), only the two plant rRNA bands are seen. Bacterial rRNA bands, as expected, were absent in uninoculated root RNA (lane 1). Bacterial rRNA bands were also absent in inoculated 8-day-old root (in which the developing nodule is in the form of protrusions on the root), and in 12-day-old developing nodules (lanes 2 & 3, respectively). In lanes 4-8, the two additional bands of 2.5 and 1.4 Kb in size are bacterial rRNA. Bacterial rRNA bands were present thereafter in 16 to 40-day-old nodules (lanes 4-7), as well as in alfalfa nodule RNA (lane 8) indicating the presence of bacteria in these nodules.

ENod2 gene expression during chickpea nodule development

The alfalfa A2ENod2 clone was used as a probe to detect ENod2 expression; therefore, alfalfa total RNA (obtained from K. Dunn, Boston College, MA) was used as a positive control on the Northern blot. RNA was transferred from the gel (Fig. 11) to nylon membrane and probed with $^{32}$P-labeled A2ENod2 cDNA. The following observations were made by analyzing the autoradiograph (Fig. 12).

A 1.8 Kb chickpea transcript homologous to A2ENod2 first appeared faintly in 12-day-old nodules (Fig. 12, lane 3). This transcript was detected at a much higher intensity in 16- and 20-day-old nodules (Fig. 12, lanes 4 & 5) and decreased in intensity in 30- and 40-day-old nodules (Fig. 12, lanes 6 & 7). This transcript was absent in uninoculated root and in 8-day-old inoculated root. Thus, the chickpea ENod2 transcript was expressed in a developmentally regulated manner in response to inoculation with *R. ciceri*. The chickpea ENod2 transcript size was 1.8 Kb as compared to the 1.6 Kb ENod2 transcript in alfalfa. The expected size of alfalfa ENod2 transcript, although initially described as 1.3 Kb (Dickstein et al., 1991), is uncertain (R. Dickstein, personal communication).

Lb gene expression during chickpea nodule development

The soybean Lb cDNA clone was used as a probe to detect Lb gene expression. Therefore, total RNA isolated from soybean nodules (obtained from M. L. Guerinot, Dartmouth College, NH) was used as a positive control on the Northern blot. The RNA was transferred from the gel (Fig. 13) to nylon membrane and probed with $^{32}$P-labeled

**Fig. 11:** Ethidium bromide stained formaldehyde gel containing total RNA extracted from chickpea and alfalfa roots and/or nodules.
**Lane 1:** ICC640, uninoculated, root.
**Lane 2:** ICC640, inoculated root, 8 days after planting (DAP).
**Lane 3:** ICC640, nodule, 12 DAP.
**Lane 4:** ICC640, nodule, 16 DAP.
**Lane 5:** ICC640, nodule, 20 DAP.
**Lane 6:** ICC640, nodule, 30 DAP.
**Lane 7:** ICC640, nodule, 40 DAP.
**Lane 8:** alfalfa, nodule.

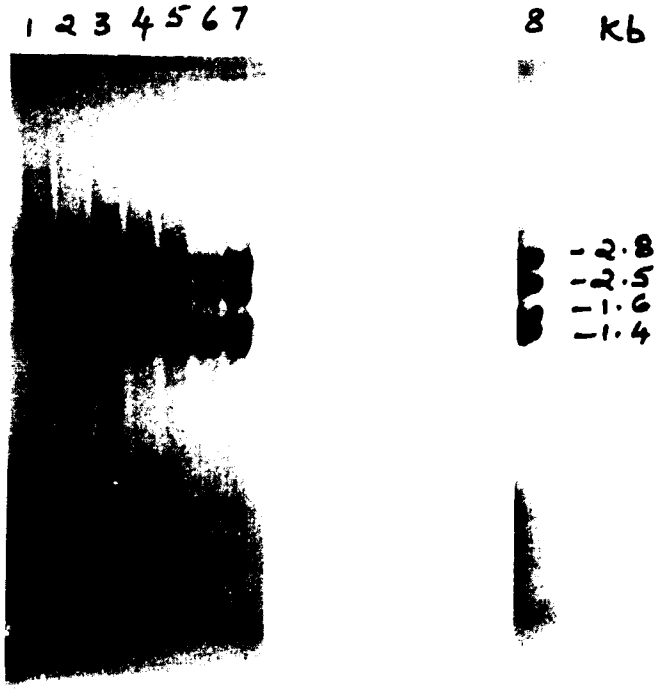
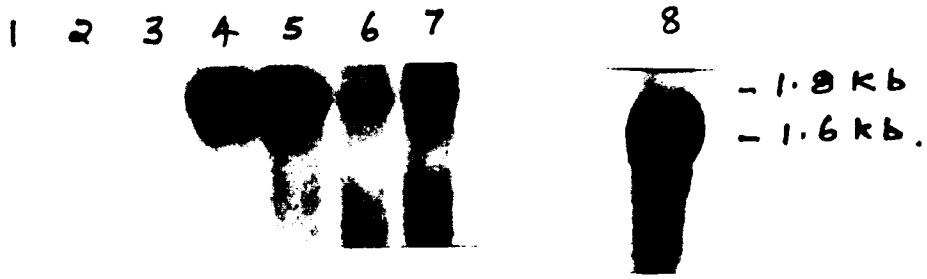


**Fig. 12:** ENod2 gene expression at different time points during chickpea nodule development and in alfalfa nodules.
Total RNA extracted from roots and/ or nodules was run on formaldehyde gel (Fig. 11), blotted and subjected to hybridization with alfalfa A2ENod2 cDNA.
**Lane 1:** ICC640, uninoculated, root.
**Lane 2:** ICC640, inoculated root, 8 days after planting (DAP).
**Lane 3:** ICC640, nodule, 12 DAP.
**Lane 4:** ICC640, nodule, 16 DAP.
**Lane 5:** ICC640, nodule, 20 DAP.
**Lane 6:** ICC640, nodule, 30 DAP.
**Lane 7:** ICC640, nodule, 40 DAP.
**Lane 8:** alfalfa, nodule.

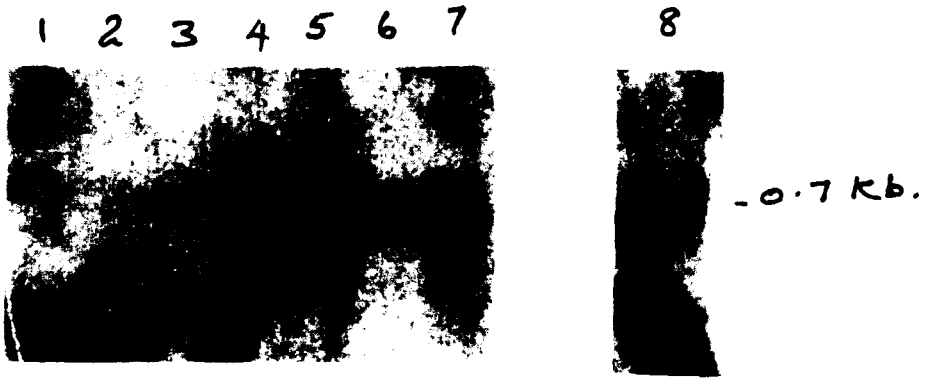


soybean Lb cDNA (Fig. 14).

A 0.7 Kb Lb transcript was first detected in 16-day-old nodules and continued to be present in 20-, 30-, and 40-day-old chickpea nodules. This transcript was absent in inoculated 8-day-old root, and 12-day-old nodules (Fig. 14, lanes 2 & 3, respectively), and in uninoculated chickpea roots (Fig. 14, lane 1). Thus, the Lb transcript in chickpea was expressed in a developmentally regulated mannner in response to inoculation with *R. ciceri*. The Lb mRNA expressed in soybean nodules was also 0.7 Kb in size (Fig. 14, lane 8), as expected from previous reports (Gloudemans et al., 1987; Lullien et al., 1987; Scheres et al., 1990).

**Fig. 13:** Ethidium bromide stained formaldehyde gel containing total RNA extracted from chickpea and soybean roots and/or nodules.
**Lane 1:** ICC640, uninoculated, root.
**Lane 2:** ICC640, inoculated root, 8 days after planting (DAP).
**Lane 3:** ICC640, nodule, 12 DAP.
**Lane 4:** ICC640, nodule, 16 DAP.
**Lane 5:** ICC640, nodule, 20 DAP.
**Lane 6:** ICC640, nodule, 30 DAP.
**Lane 7:** ICC640, nodule, 40 DAP.
**Lane 8:** soybean, nodule.

**Fig. 14:** Lb gene expression at different time points during chickpea nodule development and in soybean nodules.
Total RNA extracted from roots and/ or nodules was run on formaldehyde gel (Fig. 13), blotted and subjected to hybridization with soybean Lb cDNA.
**Lane 1:** ICC640, uninoculated, root.
**Lane 2:** ICC640, inoculated root, 8 days after planting (DAP).
**Lane 3:** ICC640, nodule, 12 DAP.
**Lane 4:** ICC640, nodule, 16 DAP.
**Lane 5:** ICC640, nodule, 20 DAP.
**Lane 6:** ICC640, nodule, 30 DAP.
**Lane 7:** ICC640, nodule, 40 DAP.
**Lane 8:** soybean, nodule.



Comparison of nodulin gene expression in wild-type and mutant nodules

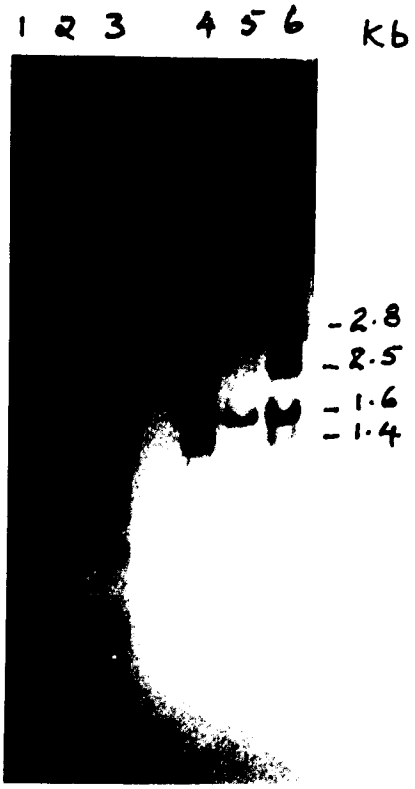
For this study, 3-week-old mature wild-type and mutant nodules were harvested for RNA isolation. At this point in development, the wild-type and mutant (PM405B and PM638A) nodule features were as described in the anatomical study. Mutant PM796B was included in the study of Lb gene expression because, although the mutant nodules have Lb pigmentation, and are similar in size and shape to ICC640 nodules, they fix very little nitrogen. PM796B nodules have a zone of distinct reddish-brown pigmentation at the distal end of the nodule, indicating the presence of Lb protein. However, the proximal region of these nodules turns green at an early stage as the nodules begin their characteristic pattern of premature senescence (Davis, 1988). It was of interest to test if the reduced N-fixing nature and premature senescence of these nodules was due to abberant Lb gene expression compared to the wild-type.

ENod2 gene expression in wild-type and mutant nodules

Total RNA extracted from nodules of wild-type and mutant lines PM405B and PM638A, two samples of alfalfa nodule total RNA (obtained from K. Dunn, Boston College, MA; and R. Dickstein, Drexel University, PA), and *R. ciceri* total RNA were run on a formaldehyde gel, stained with ethidium bromide (Fig. 15), blotted and probed with A2ENod2 cDNA (Fig. 16).

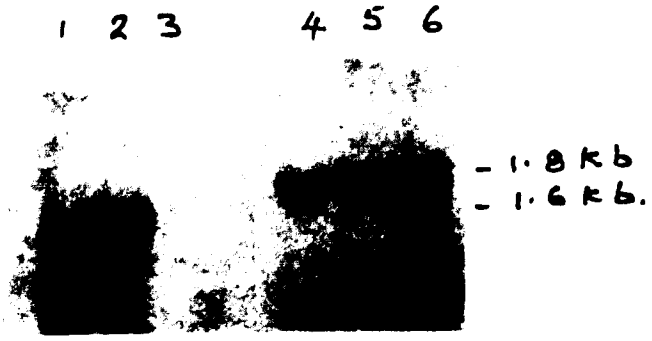
The 1.8 Kb chickpea ENod2 transcript was expressed in wild-type (ICC640), as well as in both mutant nodules (PM405B and PM638A). Thus, the mutant genes in

**Fig. 15:** Ethidium bromide stained formaldehyde gel containing total RNA extracted from chickpea and alfalfa nodules, and from *Rhizobium ciceri*. **Lanes: 1 & 2,** alfalfa[1] nodules; **3,** *R. ciceri*; **4 to 6,** ICC640, PM405B, and PM638A nodules, respectively.




[1] Alfalfa nodule total RNA obtained from R. Dickstein (lane 1) and K. Dunn (lane 2).

**Fig. 16:** ENod2 gene expression in nodules of wild-type and mutant chickpea lines, and in alfalfa.
Total RNA extracted from chickpea and alfalfa nodules, and from *R.ciceri* was electrophoresed on formaldehyde gel (Fig. 15), blotted and subjected to Northern hybridization with alfalfa A2ENod2 cDNA. **Lanes: 1 & 2,** alfalfa nodules; **3,** *R. ciceri*; **4 to 6,** ICC640, PM405B, and PM638A nodules, respectively.



PM405B ($rn_4$) and PM638A ($Rn_?$) did not prevent expression of the ENod2 gene in these mutant nodules. The absence of bands in the *R. ciceri* lane demonstrated that A2ENod2 probe did not hybridize non-specifically to bacterial rRNA (Fig. 16). In alfalfa, a transcript of 1.6 Kb was detected in nodule RNA from both sources, demonstrating consistent detection of the same ENod2 band as in previous blots.

Lb gene expression in wild-type and mutant nodules

Total RNA was extracted from nodules of inoculated wild-type ICC640 and mutant lines PM405B, PM638A, PM796B, and from roots of uninoculated ICC640 plants. The RNA samples were run on formaldehyde gel, blotted and probed with soybean Lb cDNA (Fig. 18).

The 0.7 Kb Lb mRNA was present at similar levels in ICC640 and PM796B, and at a markedly reduced level in PM638A. Lb mRNA was not detected in PM405B nodules, or in uninoculated ICC640 root. The results show that $rn_5$ mutation (PM796B) did not affect the transcript size or relative level of Lb expression. The dominant mutation in PM638A did not affect transcript size, but did reduce expression of the Lb gene. In contrast, the $rn_4$ (PM405B) mutation resulted in elimination of detectible Lb mRNA.

**Fig. 17:** Ethidium bromide stained formaldehyde gel containing total RNA extracted from root and/or nodule samples of different chickpea lines.
**Lane 1:** λ-*Hind*III molecular weight size standard.
**Lane 2:** ICC640, uninoculated, root.
**Lane 3:** ICC640, nodule.
**Lane 4:** PM233B, root.
**Lane 5:** PM405B, nodule.
**Lane 6:** PM638A, nodule.
**Lane 7:** PM796B, nodule.

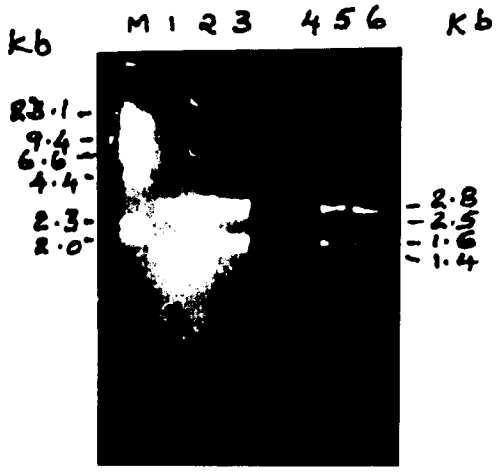
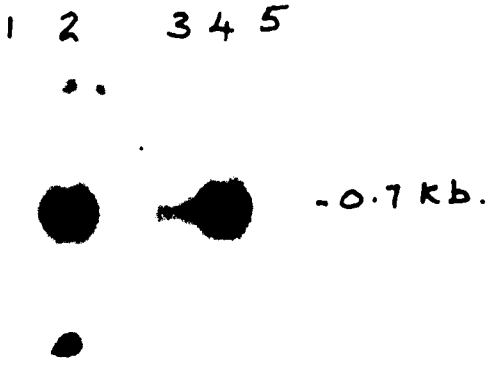


**Fig. 18:** Lb gene expression in wild-type and mutant chickpea nodules.
Total RNA extracted from root and/or nodule samples of different chickpea lines was run on formaldehyde gel, blotted and subjected to Northern hybridization with soybean Lb cDNA probe. A gel different from that shown in Fig. 17 was used for this blot; however, the relative RNA amounts for each sample shown in Fig. 17 are the same as for the respective samples used in this blot.
**Lane 1:** ICC640, uninoculated, root.
**Lane 2:** ICC640, nodule.
**Lane 3:** PM638A, nodule.
**Lane 4:** PM796B, nodule.
**Lane 5:** PM405B, nodule.



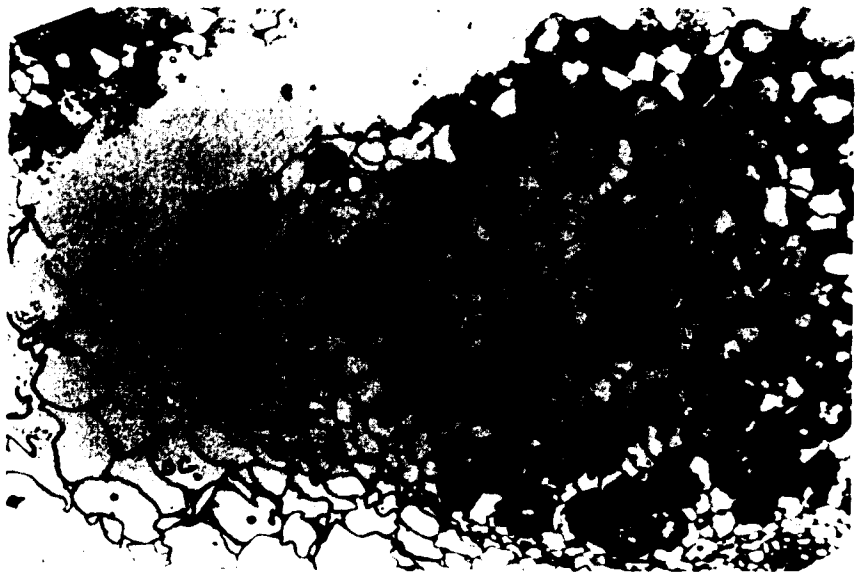
**Anatomy of wild-type and mutant ineffective nodules**

Description of visual features of nodules given below is based on general observations made during the course of the developmental study on the chickpea-*Rhizobium* symbiosis. ICC640 nodules are effective with respect to symbiotic N-fixation, as reflected by normally vigorous growth and dark green leaves of nodulated plants in N-free soil. The nodules exhibit an indeterminate growth pattern, as seen in pea and alfalfa nodules. Indeterminate growth is due to the presence of a persistent, actively growing meristem located at the distal end of the nodule.

ICC640 nodules have a characteristic reddish-brown pigmentation, which is a visible marker for the presence of substantial amounts of Lb protein. Wild-type nodules contain both infected and uninfected cells (Fig. 19). The infected cells are filled with bacteroids which carry out the process of N-fixation. The uninfected cells are interspersed among infected cells and do not contain any bacteria.

PM405B and PM638A mutant nodules are similar to each other with respect to their inability to fix any nitrogen (Davis, 1988; Davis, unpubl). Due to their non-N-fixing nature, the mutant plants are less vigorous, and have chlorotic light green leaves when grown in N-free soil. In addition, the mutant nodules are devoid of the reddish-brown pigmentation seen in ICC640 nodules. The lack of reddish-brown pigmentation indicates the absence of visually detectable amounts of Lb protein in these mutant nodules. PM638A nodules are similar in size to the wild-type nodules, whereas PM405B nodules are relatively much smaller.

**Fig. 19:** Nodule anatomy of wild-type chickpea line ICC640, as viewed through compound microscope.
Bar= 55 $\mu$m; I, infected cell; U, uninfected cell; VB, vascular bundle; OC, outer cortex.



The absence of any Lb pigmentation and lack of N-fixation by mutant nodules raised the question of whether these nodules were "empty" (devoid of infected cells), a phenomenon seen in alfalfa and pea mutant nodules induced by certain bacterial mutants (Dickstein et al., 1988; Govers et al., 1986). Examination of the nodule partial cross-sections revealed that the mutant (PM405B and PM638A) nodules were not "empty"; they contained both infected (with intracellular bacteria) and uninfected cells (Figs. 20 & 21). However, although a systematic investigation of many nodule samples was not made, both mutants appear to have fewer infected nodule cells as compared to the wild-type. The fact that mutant nodules have infected cells (Figs. 20 & 21) but do not fix nitrogen, demonstrates that some plant determined factor(s) required for N-fixation is(are) lacking in these mutants.

**Fig. 20:** Nodule anatomy of chickpea mutant line PM405B, as viewed through compound microscope.
Bar= 44 $\mu$m; I, infected cell; U, uninfected cell; OC, outer cortex.

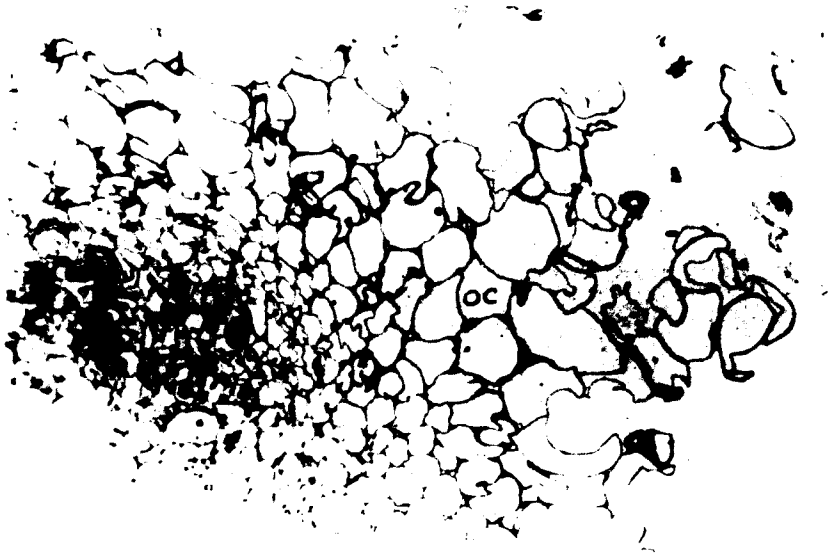
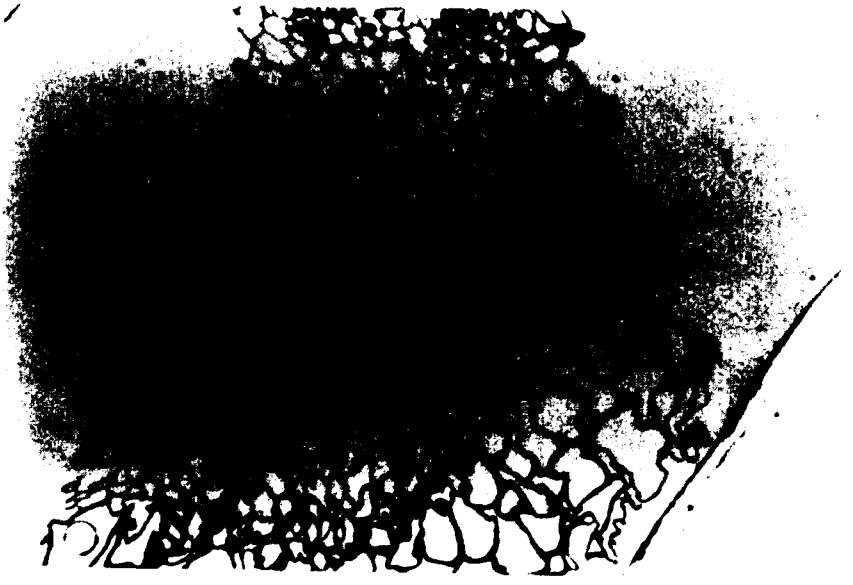


**Fig. 21:** Nodule anatomy of chickpea mutant line PM638A, as viewed through compound microscope.
Bar = 55 $\mu$m; I, infected cell; U, uninfected cell; OC, nodule cortex.



# CHAPTER I

## SECTION D - DISCUSSION

### A dominant, single gene mutation blocks the symbiotic process in chickpea mutant PM638A

Chickpea mutant PM638A was shown to carry a single, dominant, nuclear gene mutation. This is the first dominant mutation known to affect the chickpea-*Rhizobium* symbiotic process. Ineffective nodulation caused by single gene dominant mutations has been reported in soybean (Caldwell, 1966; Caldwell et al., 1966; Devine, 1984; Vest, 1970; Vest & Caldwell, 1972). The other nodulation gene mutations that impair the symbiotic process in chickpea (Table 2), as well as most mutations that impair the symbiotic process in other legumes (Caetano-Anollés & Gresshoff, 1991; Vance et al., 1988), are recessive.

Although mutant PM638A has a unique phenotype as compared to other chickpea nodulation mutants, it is not known for certain whether the dominant mutant gene it carries is allelic or non-allelic to any of the other five root nodulation genes that have been identified (Davis et al., 1986; Davis, 1988). Allelic relationships among the $rn_1$ through $rn_5$ genes (Table 2) were determined by $F_1$ complementation tests (Davis et al., 1986; Davis, 1988). However, direct complementation tests cannot be carried out for

mutant line PM638B because the mutation is dominant to the wild-type phenotype and therefore complementation between two mutations cannot be detected in $F_1$ hybrids. One way to address the issue of allelism will be to determine genome map positions of all chickpea nodulation genes. If the PM638A mutation is in a unique gene it would map to a different locus compared to the other nodulation gene mutations, but if it is a second mutation in one of the five known mutant nodulation genes it would map to the same position as one of these five "rn" genes. Results of ongoing investigations (Davis, unpubl.) indicate that the PM638A mutation is closely linked but not allelic to the $rn_1$ mutation, and is unlinked to the other mutant nodulation genes. Thus, the PM638A mutation appears to be in a new nodulation gene.

Once the dominant PM638A mutation is rigorously shown to be non-allelic to other chickpea nodulation genes, it will be named. Until then, the tentatively assigned gene symbol for the dominant mutation will be "$Rn_7$". The nomenclature for the gene symbol is in accordance with other chickpea nodulation gene mutations identified to date (Davis et al., 1986; Davis, 1988; Singh et al., 1992); indicating that it is the seventh gene in the series of identified genes that have a role in the symbiotic process.

**Chickpea nodulin genes**

This is the first report of nodulin genes and nodulin gene expression in chickpea. Furthermore, this is the first documentation of analysing legume nodulation mutants at the molecular level.

Rationale for using heterologous nodulin cDNA probes

In the experiments described here, alfalfa A2ENod2 and soybean Lb clones were used as heterologous probes to detect corresponding sequences in chickpea DNA and in RNA. This approach was necessitated by the fact that no chickpea nodulin genes have been cloned.

The approach of using heterologous DNA probes to analyze legume DNA and RNA has been employed by other researchers to analyze nodulin genes in different legumes. An ENod2-homologous DNA sequence was first identified in pea by probing pea genomic DNA digests with soybean pENod2 cDNA (Govers et al., 1987a). Soybean pENod2 cDNA was also used to study the genetic complexity of the tetraploid alfalfa species *M. sativa* compared to its diploid relative *M. truncatula* (Barker et al., 1990). Leghemoglobin-like sequences were identified in the DNA of actinorhizal plant species using soybean Lb cDNA as the probe (Roberts et al., 1985). Actinorhizal plants are non-legume species that have a N-fixing symbiotic relationship with actinomycetes of the genus *Frankia*.

Heterologous probes have also been used in studies of nodulin gene expression. Soybean pENod2 cDNA strongly cross-hybridized to mRNA from normal nodules of pea, alfalfa, and vetch (*Vicia sativa*), indicating that ENod2-homologous nodulin genes are expressed in these legume species (Govers et al., 1987b). Soybean pENod2 cDNA was used as a probe to investigate ENod2-homologous gene expression in nodule-like structures induced on alfalfa by auxin transport inhibitors N-(1-naphthyl)phthalamic acid

(NPA) and 2,3,5-triiodobenzoic acid (TIBA) (Hirsch et al., 1989). Temporal Lb gene expression during nodule development in *Phaseolus vulgaris* was studied by Northern analysis using soybean Lb cDNA as the probe (Campos et al., 1987).

Since ENod2 and Lb gene sequences are highly conserved in the Leguminoseae family and their corresponding cDNAs have been used to analyze plant genes in other legume-*Rhizobium* systems, it was reasonable to expect that heterologous cDNAs coding for ENod2 and Lb nodulins would work as probes for Southern and Northern analyses in chickpea.

Though ENod2 and Lb genes are highly conserved, and their corresponding cDNAs have been widely used as heterologous probes, precautionary measures were taken to ensure the validity of the results and conclusions. These measures included confirmation of the identity of the clones used as probes, and inclusion of appropriate positive and negative controls when these probes were used to analyze chickpea DNA and RNA. The above mentioned considerations and control measures provide a sufficient basis for confidence that the DNA sequences identified through Southern analysis correspond to chickpea ENod2 and Lb sequences and the RNA transcripts and expression patterns documented do in fact correspond to the presence and expression of chickpea ENod2 and Lb genes.

Presence of ENod2 and Lb sequences in the chickpea genome

Results from Southern analysis demonstrate the presence of ENod2 and Lb

sequences in the chickpea genome. In each case, results indicated the presence of a single target sequence in the chickpea genome. The A2ENod2 probe detected a single band in each of the three restriction digests. The Lb probe detected a single band with each of the two restriction enzyme digests and two smaller bands with the third enzyme (*Eco*RI), suggesting the presence of an *Eco*RI site within a single target sequence. In contrast, multiple bands were identified in alfalfa genomic digests probed with A2ENod2 cDNA, and in soybean genomic digests probed with Lb, as has also been described by other workers (Barker et al, 1990; Sullivan et al., 1981).

There have been several reports relating to the question of number of genes coding for ENod2 in different legumes. Single band hybridization was observed in pea genomic DNA digests probed with soybean pENod2 cDNA (Govers et al., 1987a). It has been previously reported that soybean pENod2 cDNA hybridized to one band on Southerns containing genomic digests from *Medicago truncatula* (a diploid) and to at least four bands in its autotetraploid relative, *M. sativa* (alfalfa) (Barker et al., 1990). In cowpea, a cDNA clone VuA homologous to soybean pENod2 has been isolated. Southern analysis of cowpea genomic DNA digests with VuA (cowpea ENod2) cDNA indicated that the ENod2 gene in cowpea may belong to a small gene family (Trese & Pueppke, 1991). In soybean, two different genomic clones corresponding to pENod2 cDNA have been isolated. Since the sequences of the two ENod2 genomic clones were identical over a considerable part of their length, the authors suggested that the two ENod2 genes might have arisen due to a recent gene duplication event (Franssen et al., 1989). In this context, it is worth mentioning that soybean genome has undergone

genome duplication and has a diploid number, 2n=40 (Hadley & Hymowitz, 1983).

From several published reports, it is known that in legumes such as alfalfa and soybean, Lb is encoded by a multi-gene family (Barker et al., 1988; Brisson et al., 1982; Brisson & Verma, 1982). In *M. truncatula*, (with a genome half the size of alfalfa), two analogous Lb genes are known to exist. *Eco*RI-digests of *M. truncatula* DNA probed with two different alfalfa Lb cDNA probes, gave one- and two-band hybridization patterns, respectively. This is in contrast to multiple bands seen in alfalfa DNA using the same probes (Barker et al., 1988).

Since multiple bands were not detected on chickpea genomic Southerns, it suggests that in chickpea, ENod2 and Lb could be encoded by single copy genes rather than by a small gene family. This condition would not be surprising, given the low diploid chromosome number and relatively small genome size of chickpea. However, the issue of copy number of chickpea ENod2 and Lb genes can be best resolved by using homologous probes.

Chickpea ENod2 and Lb gene expression during nodule development

In the evaluation of results from Northern analysis, it is important to recognize that there is ample precedence for the use of total RNA to analyze ENod2 and Lb gene expression in other legume species, including cowpea and alfalfa (Trese & Pueppke, 1991; Hirsch et al., 1989). The use of total RNA provided advantages of comparing relative levels of RNA in different lanes based on the intensity of rRNA bands on

ethidium bromide-stained gels. In addition, total RNA was relatively easy to isolate as compared to mRNA. Hence, total RNA was used in the present study.

The ENod2 gene in chickpea behaves as a nodulin gene since it is specifically expressed in nodules in response to infection by *Rhizobium*. The same tissue specificity (present in nodules, absent in roots) is seen in the expression of ENod2 genes in other legumes (Franssen et al., 1987; Scheres et al., 1990).

Chickpea ENod2 gene expression is also similar to that seen in other legumes with respect to the timing of expression during the symbiotic process. The ENod2 gene is first expressed in chickpea during the early stages of the symbiotic process when the nodules are still developing, prior to the expression of Lb gene, and therefore fits the description of an early nodulin gene. The detection of ENod2 mRNA first in 12-day-old nodules prior to the initial appearance of bacterial rRNA in 16-day-old nodules, indicates that the detected band did not result from non-specific hybridization of the A2ENod2 probe to a bacterial rRNA band of similar molecular weight. Probing of a Northern blot which contained a lane of *R. ciceri* RNA confirmed the lack of non-specific hybridization to bacterial rRNA.

Chickpea Lb gene behaves as a nodulin gene because it is expressed specifically in the nodules and in response to infection by *Rhizobium*. This result is consistent with reported results in other legumes (Govers et al., 1987a; Campos et al., 1987). Chickpea Lb gene expression was first detected in 16-day-old nodules, which was four days after the initial expression of ENod2 gene and coincident with the detection of abundant quantities of bacterial rRNA. Hence, chickpea Lb gene behaves as a late nodulin gene

with respect to the timing of expression during the symbiotic process.

Comparison of ENod2 and Lb gene expression in wild-type and mutants

Although numerous nodulation mutants have been isolated in several legume species (Caetano-Anollés & Gresshoff, 1991; Vance et al., 1988), there have been no published comparisons of nodulin gene expression in mutants and their respective wild-type parents. In the present study, nodulin gene expression was examined in three chickpea mutants and their common wild-type parent.

No differences were found with respect to presence and size of ENod2 gene transcripts in wild-type and mutant (PM405B and PM638A) non-N-fixing nodules. The expression of ENod2 in these two mutant nodules is consistent with the possible role of ENod2 in early stages of nodule development rather than nodule function (Sánchez et al., 1991), in that both mutants do form nodule structures, but that the nitrogen fixation function is lacking.

Lb mRNA was detected, although at subnormal level, in PM638A nodules, but was not detected in PM405B nodules. Because Lb is a late nodulin, the presence of Lb mRNA in PM638A and not in PM405B implies that the nodule developmental process in PM638A is blocked at a slightly more advanced developmental stage as compared to that in PM405B. The normal Lb transcript size and expression level in PM796B provides no indication that the mutant phenotype is due to abnormal Lb expression. Rather, the results indicate that the reduced N-fixation and premature senescence seen

in this mutant are probably due to other factors that affect nodule function, such as carbon supply or assimilation of fixed nitrogen.

**Effects of $rn_4$ mutation on the symbiotic process in mutant PM405B**

The nod$^+$fix$^-$ PM405B nodules have infected cells, and therefore are not "empty" (devoid of intracellular bacteria) like the nod$^+$fix$^-$ alfalfa nodules induced by *R. meliloti exo*$^-$ mutants (Dickstein et al., 1988) or *Agrobacterium tumefaciens* transconjugants carrying *R. meliloti* nodulation genes (van de Wiel et al., 1990a). In the empty nodules seen in alfalfa, the ENod2 gene was expressed but Lb gene was not expressed (Dickstein et al., 1988). Similarly, in PM405B nodules, the ENod2 gene was expressed but there was no detectible Lb mRNA. The fact that ENod2 is expressed in both "empty" alfalfa nodules and in PM405B nodules (with intracellular bacteria), is consistent with the putative structural role of ENod2 protein in nodule morphogenesis. The presumed role of ENod2 gene product is based on the finding that ENod2 gene is expressed in the non-infected nodule parenchyma cells that surround the central tissue (comprised of infected and uninfected cells) of pea and soybean nodules, and that the ENod2 amino acid sequence reveals a likely role as a cell wall protein (Nap & Bisseling, 1990; van de Wiel et al., 1990).

The PM405B mutant nodules appear white and do not have any reddish-brown pigmentation normally indicative of the presence of Lb protein. The absence of detectible Lb gene expression in PM405B nodules may be attributable simply to the

paucity of infected cells, because Lb gene expression is localized in the infected cells of the nodule (Scheres et al., 1990). The formation of small nodules indicates that the process of formation of nodule structure is in some way incomplete, and this could account for the absence of detectible levels of Lb mRNA, which accumulate in nodules with normal structure development. Since the Lb gene is not expressed, it is to be expected that the nodules would lack Lb protein. Without Lb protein, there is no regulation of oxygen flux in the root nodules and under such conditions N-fixation does not take place.

The fact that PM405B mutant forms nodules which contain infected cells implies that wild-type $Rn_4$ gene expression does not play a role in early symbiotic events of recognition, nodule initiation and infection, but probably has a role in later stages of development of nodule structure and nodule function.

**Effects of a dominant mutation on the symbiotic process in PM638A**

The $nod^+fix^-$ phenotype in PM638A chickpea line is conferred by a single dominant mutation. The dominant mutation in PM638A has been tentatively designated as "$Rn_7$".

Since ENod2 is expressed in PM638A nodules, $Rn_7$ mutation does not block the expression of ENod2 gene. Like PM405B nodules, PM638A nodules have few infected cells. PM638A nodules lack the reddish-brown pigmentation which is indicative of the presence of Lb protein. But, the fact that PM638A nodules appear yellowish and not

white (like PM405B nodules) suggests that these nodules may not be totally devoid of Lb protein. The $Rn_7$ mutation in PM638A does reduce the level of Lb transcripts detectible in total nodule RNA. The reduced amount of Lb transcripts could be due to presence of few infected nodule cells. Alternatively, the absence of Lb pigmentation despite the expression of Lb gene could have other causes. It is possible that the Lb transcripts get degraded and therefore apoleghemoglobin protein is not synthesized. Apoleghemoblogin is the product of translation of Lb transcripts. Apoleghemoglobin combines with heme prosthetic group to form the functional Lb protein which imparts the reddish-brown color to the nodules. It is also conceivable that apoleghemoglobin may be synthesized in PM638A nodules but the functional Lb protein is not formed and therefore the mutant nodules do not fix nitrogen.

The presence of infected nodule cells and the detection of Lb gene transcripts in these nodules suggest that $Rn_7$ mutation does not affect symbiotic events related to recognition, nodule initiaton and nodule development, but that the mutation could possibly be in a gene that has a role in nodule function. The dominant effect of the mutation could be due to the presence of a mutant protein (encoded by the mutant gene) with a regulatory function. This regulatory protein could behave as a *trans* factor that controls gene expression associated with the symbiotic process. *Trans*-acting factors involved in regulation of Lb gene expression have been identified in legumes including alfalfa, *Phaseolus vulgaris* and *Sesbania* (de Bruijn & Schell, 1993).

**Prospects for future research in the study of plant genes involved in chickpea-*Rhizobium* symbiosis**

To further pursue research on chickpea nodulin genes and to avoid future reliance on heterologous probes, nodulin cDNA clones should be isolated from chickpea. These nodulin cDNA clones should be used as homologous probes to further characterize the chickpea nodulation mutants with the goal of identifying the similarities and differences as compared to the wild-type. The characterization should be done with respect to the structure and expression of different nodulin genes. This kind of research will help in understanding the role of plant genes in the symbiotic process.

The technique of Northern analysis enables identification of specific gene transcripts in tissues that contain a considerable amount of the target mRNA species. If a particular RNA transcript is expressed only in a few cells, it may go undetected by Northern analysis. The method of *in situ* hybridization is more sensitive than Northern analysis since it is possible to detect cell-specific gene messages in complex tissues such as the nodule. *In situ* hybridization studies using antisense RNA probes have revealed the presence of nodulin gene transcripts in specific cell types within nodules (van de Wiel et al., 1990; Scheres et al., 1990). *In situ* hybridization studies using anti-sense RNA probes would be useful for determining the cellular expression of chickpea nodulin genes. Similar experiments should be conducted in mutant nodules to determine if there is any defect in the cellular localization of nodulin gene transcripts.

Research should be directed at developing a transformation system for chickpea.

Techniques such as *Agrobacterium*-mediated transformation or particle bombardment can be tried. Once the techniques of transformation and regeneration are optimized, they could be used for studying the expression of *in vitro* modified nodulin genes. By introducing *in vitro* modified nodulin gene constructs into mutant lines and thereby correcting or partially compensating for particular mutations, the role and relationships of chickpea nodulin and nodulation genes in the symbiotic process could be elucidated.

## Conclusions

The main conclusions of the study described in chapter I are listed below -

1) A single dominant mutation, tentatively designated as "$Rn_?$" confers ineffective nodule phenotype in chickpea mutant PM638A.
2) DNA sequences corresponding to ENod2 and Lb heterologous probes are present in the chickpea genome.
3) Chickpea ENod2 and Lb genes behave as early and late nodulin genes, respectively, and are expressed in a developmentally regulated nodule-specific manner.
4) The $rn_4$ (PM405B) and $Rn_7$ (PM638A) mutations do not prevent the expression of chickpea ENod2 gene.
5) The $rn_4$ mutation results in elimination of detectible levels of Lb mRNA. In contrast, the $Rn_7$ mutation results in reduced levels of detectible Lb mRNA. The $rn_5$ mutation did not detectibly reduce Lb gene expression

in chickpea mutant PM796B.

6) The symbiotic process in PM638A ($nod^+fix^-$) is blocked at a later developmental stage as compared to that in PM405B ($nod^+fix^-$).

# CHAPTER II

An *in vitro* regeneration system for chickpea (*Cicer arietinum* L.)

# CHAPTER II

## SECTION A - LITERATURE REVIEW

The Leguminoseae family includes many crop species that are agronomically important. A major factor for the ecological success of legume plants is their ability to have a symbiotic relationship with *Rhizobium* bacteria. Nodulated legume plants supplement their nitrogen nutrition with ammonia formed as a product of N-fixation by the bacterial nitrogenase enzyme complex. Some of the important legume crops are soybean (*Glycine max*), pea (*Pisum sativum*), common bean (*Phaseolus vulgaris*), vetch (*Vicia sativa*), alfalfa (*Medicago sativa*), and chickpea (*Cicer arietinum*).

Genetic engineering of legume plants has been limited because of the difficulty of regenerating plants *in vitro*. The importance of having an *in vitro* regeneration system for crop plants has been well documentated (Goodman et al., 1987). The existence of an *in vitro* regeneration system enables crop improvement through the application of non-conventional genetic engineering techniques.

Legume plants have been difficult to regenerate through tissue culture. However, continued research in several legume systems in recent years has resulted in much success. Regeneration of legume plants has been achieved using different kinds of explants including shoot tips, leaves, embryos, and cotyledons. The mode of regeneration has been through organogenesis or somatic embryogenesis. In

organogenesis, shoots and/or roots and whole plants are produced from explants cultured in the appropriate media. In somatic embrogenesis, first there is formation of somatic embryos (resembling zygotic embryos) from explants, and these somatic embryos develop to form plantlets.

**Legume regeneration through organogenesis**

The mode of regeneration through organogenesis can be direct or indirect (with an intermediate callus stage). Peanut (*Arachis hypogaea*) plants have been regenerated directly from de-embryonated cotyledons in the absence of any growth regulators (Illingworth, 1968; 1974). Frequency of shoot regeneration from peanut cotyledons was increased with the addition of 0.05 ppm BA (benzyladenine) (Bhatia et al., 1985). Plants have been regenerated from peanut embryo axes and cotyledon segments cultured on MS medium supplemented with NAA ($\alpha$-naphthaleneacetic acid) and/or BA (Atreya et al., 1984). Adventitious shoots developed from the proximal region of mature de-embryonated cotyledons cultured in medium containing BA alone or in combination with IBA (indolebutyric acid) (Mante et al., 1989). The presence of the embryo axis was found to inhibit shoot morphogenesis (Mante et al., 1989). Several legume plants including soybean, cowpea, peanut, and bean have been regenerated from shoot meristems through organogenesis (Kartha et al., 1981).

Soybean plants have been regenerated from immature embryos through organogenesis following an intermediate callus formation stage. Immature embryos

cultured on medium containing 13.3 $\mu$M BA and 0.2 $\mu$M NAA produced organogenic callus that gave rise to shoots and roots (Barwale et al., 1986). Pea plants have been regenerated from primary leaf tissue (Wright et al., 1987). The auxin 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) induced callus and leaf formation from the leaf explants, and BA was required to enhance regeneration (Wright et al., 1987).

**Legume regeneration through somatic embryogenesis**

The development of somatic embryos can follow one of two patterns - 1) direct embryogenesis in which embryos originate directly from explant tissue without any callus proliferation, or 2) indirect embryogenesis in which callus proliferation occurs prior to embryo development (Sharp & Evans, 1982).

Direct somatic embryogenesis has been observed from immature embryos and cotyledons of several legume plants. Whole plants were recovered by direct somatic embryogenesis from immature embryos of *Trifolium repens* (white clover) cultured on medium containing BA (Maheswaran & Williams, 1984). A reliable system for regeneration of soybean from immature coytledon explants through somatic embryogenesis using auxins 2,4-D (2,4-dichlorophenoxyacetic acid) and NAA has been reported (Lazzeri et al., 1985). Somatic embryos were induced on immature zygotic embryos cultured on medium containing 2,4-D. Whole plants were also recovered in pea through somatic embryogenesis (Kysely et al., 1987).

Legume plants have been regenerated via indirect somatic embryogenesis. When

soybean cotyledonary tissue was cultured on media containing 2,4-D, there was formation of smooth-shiny callus. Somatic embryos that developed from the smooth-shiny callus gave rise to whole plants (Ghazi et al., 1986).

Factors affecting somatic embryo formation

In soybean, the effect of auxin type and concentration on the induction of somatic embryos from cultures of embryos and cotyledons has been investigated. Modified MS (Murashige & Skoog) medium supplemented with 23 and 25 $\mu$M 2,4-D produced larger numbers of somatic embryos whereas higher levels of NAA (100 to 150 $\mu$M) were needed to efficiently obtain embryogenesis (Lazzeri et al., 1987).

Somatic embryo formation is dependent on the age of the explant. The ability of soybean cotyledon explants to form embryogenic tissue and embryoids was dependent on the developmental stage of the embryos that served as explant source (Lippmann & Lippmann, 1984). The embryogenic efficiency of the explant is also dependent on the genotype of the explant source (Komatsuda & Ohyama, 1988; Parrott et al, 1989).

The importance of orientation of the cotyledon explant on the agar medium has been documented. Peanut cotyledons cultured with the adaxial surface facing the medium gave a higher frequency of somatic embryos than did the abaxially oriented cotyledons (Ozias-Akins, 1989). In soybean, somatic embryogenesis on immature cotyledons was found to be an interactive function of explant orientation and type of auxin. The greatest numbers of embryos were initiated on 2,4-D regardless of explant orientation, whereas

when NAA was used the maximum number of embryos were produced in the abaxial orientation (Hartweck et al., 1988).

Somatic embryos with clearly defined cotyledons, shoot apex, root apex, hypocotyl and radicle were found to germinate easily and form whole plantlets, whereas abnormal embryos often required long periods of incubation or culture manipulations (Lazzeri et al., 1985; Ranch et al., 1985). Lazzeri et al., (1985) observed that NAA-induced embryos had a more-normal morphology than the 2,4-D-induced embryos on soybean cotyledon explants. Somatic embryos induced on soybean cotyledons, lacking a distinct shoot apex, without cotyledons or with fused cotyledons have been termed abnormal embryos (Lazzeri et al., 1987). Soybean cotyledon explants cultured on medium with NAA gave rise to somatic embryos that had a more normal morphology than 2,4-D-induced embryos (Lazzeri et al., 1987). NAA-induced embryos typically exhibited bipolarity, with distinct radicle and hypocotyl regions, well-defined cotyledons and a shoot apex that was visible from an early stage of development. The abnormalities seen in NAA-induced embryos included the loss of one or more cotyledons, and fusion to the parental cotyledon tissue (Lazzeri et al., 1987). Somatic embryos induced by 2,4-D and originating from soybean cotyledon tissue, were abnormal with indistinct or fused cotyledons, and the shoot apex was underdeveloped (Lazzeri et al., 1987).

Histological studies have revealed that somatic embryos from soybean cotyledon explants were of surface (Finer, 1988; Barwale et al., 1986) or subsurface origin (Finer, 1988) or originated from meristematic centers in the mesophyll (Lippmann & Lippmann, 1984).

**Chickpea regeneration**

Little progress has been made towards developing an *in vitro* regeneration system for chickpea, an important food legume crop. To date, chickpea has been regenerated only from pre-existing shoot meristems (Sharma et al., 1979; Kartha et al., 1981). There have been no reports on regeneration of chickpea from cotyledon explants or through the process of somatic embryogenesis.

In the present study, the morphogenic response of immature chickpea cotyledons to various growth regulator regimes, including a number of media reported to give morphogenic responses from immature cotyledon explants in other large-seeded legumes, was investigated. The experiments described in this chapter were done with the objective of establishing a reproducible regeneration system and ultimately an *in vitro* genetic transformation system for chickpea line ICC640. Line ICC640 is the wild-type parent of several nodulation mutants (Davis et al., 1985) being used in our lab for genetic studies on symbiotic N-fixation in chickpea and therefore was the genotype of choice for developing a regeneration protocol for chickpea.

## CHAPTER II

## SECTION B - MATERIALS AND METHODS

Seeds from chickpea line ICC640 were planted in 10 cm diameter pots filled with potting soil, and plants were grown to the pod-fill stage in the greenhouse. Green pods containing partially mature seeds were carefully harvested to avoid puncturing pods. Pods were agitated in soapy (Ivory dishwashing liquid) water for 5 min followed by three rinses in distilled water. Under aseptic conditions, the washed pods were immersed in 1.05% sodium hypochlorite (20% commercial bleach) for 15 min with frequent agitation, then rinsed twice in sterile distilled water. The disinfected pods were cut open and the seeds carefully removed, avoiding damage to the seedcoat. Seeds were soaked in 0.52% sodium hypochlorite for 5 min, then immediately rinsed in sterile distilled water. After seedcoats were removed, cotyledons were separated from the embryo axis with forceps.

Immature cotyledons approximately 5 mm in length were placed, four per 100 x 15 mm plastic petri plate, on basal medium [Gamborg's B5 salts and vitamins (Gamborg et al., 1968), 1.5% sucrose, pH 5.8, 8 g/l Phytagar (Gibco)] supplemented with various concentrations and combinations of the growth regulators 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), α-naphthalene acetic acid (NAA), indole-3-acetic acid (IAA), 6-benzyladenine (BA), kinetin, zeatin and abscisic acid (ABA) (see Results, Table 6). IAA and ABA were filter-sterilized and added to cooled,

autoclaved media; all other growth regulators were added to media before autoclaving. Growth regulator combinations 1 through 8 were patterned after published reports of legume somatic embryogenesis as listed in Table 6. Cotyledon explants were always placed with adaxial surface facing the medium except in one experiment, where the influence of explant orientation on CLS formation was examined by placing immature cotyledons adaxial versus abaxial side down on basal medium containing 13.7 $\mu$M zeatin. In certain experiments, detached embryo axes were also cultured. All cultures were incubated at $25 \pm 3$°C under cool-white flourescent bulbs alone or in combination with Plant Lights® (General Electric, #F20T12.PL) (65 $\mu$mol $m^{-2}s^{-1}$) with a 16-hr photoperiod.

In treatments where shoot formation was evident, morphogenic portions of the explants were excised and subcultured onto basal media containing 4.4 $\mu$M BA or 4.6 $\mu$M kinetin to stimulate shoot development and proliferation. In an effort to induce root formation, regenerated shoots were sub-cultured on B5 medium alone or supplemented with 4.9 $\mu$M IBA or 5.4 $\mu$M NAA.

## CHAPTER II

## SECTION C - RESULTS AND DISCUSSION[1]

The responses of immature chickpea cotyledons to media containing various growth regulators are summarized in Table 6. The response frequencies shown (Table 6) are the pooled results of several experiments utilizing different source plants and separately prepared media. All media gave consistent results over all experiments except as discussed below for the effects of supplementing zeatin-based media with 0.2 $\mu$M IAA.

On media (Table 6) containing auxin alone (media 1, 2, 3, 4, 6, 7, 8), or supplemented with ABA (medium 5), callus formed at the wound site produced by separating cotyledons from the embryo axis. Callus then proliferated over the entire proximal end of the cotyledons. Callus texture varied among explants within and between growth regulator treatments, ranging from very loose and friable to hard and nodular; however, no clearly recognizable embryogenic structures were observed on media 1 through 8 up to 40 days after culture initiation.

The results obtained with immature chickpea cotyledons differed from reported responses of other legumes to high auxin media. For instance, clearly defined, trumpet-shaped embryos formed on excised, immature cotyledons of soybean

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[1]the results presented in this section have been published (Shri & Davis, 1992).

**Table 6:** Responses of immature chickpea cotyledons to growth regulator combinations after 30 days culture. Formulations 1 through 8 were patterned after published protocols for soybean regeneration.

| Medium No. | Growth regulator concentration (μM) | Reference[a] | Type of response | Response frequency[b] |
|---|---|---|---|---|
| 1. | 11.3 μM 2,4-D | i, iii | CA[d] | 20/20 |
| 2. | 22.6 μM 2,4-D | i, ii, iii | CA | 20/20 |
| 3. | 45.2 μM 2,4-D | i, ii, iii | CA | 44/44 |
| 4. | 90.5 μM 2,4-D | i, ii, iii | CA | 28/28 |
| 5. | 45.2 μM 2,4-D + 0.7 μM ABA | i | CA | 28/28 |
| 6. | 53.7 μM NAA | ii, iii | CA | 40/40 |
| 7. | 3.9 μM 2,4,5-T | iii | CA | 64/64 |
| 8. | 19.6 μM 2,4,5-T | iii | CA | 8/8 |
| 9. | 4.6 μM zeatin | _____ | CLS[c] | 5/31 |
| 10. | 13.7 μM zeatin | | CLS | 68/133 |
| 11. | 45.6 μM zeatin | | CLS | 14/26 |
| 12. | 13.7 μM zeatin, 0.2μM IAA | | CLS | 71/111 |
| 13. | 13.7 μM zeatin, 1.1uM IAA | | FCLS[e],CA | 37/70 |
| 14. | 13.7 μM zeatin, 5.7uM IAA | | FCLS, CA | 29/53 |
| 15. | 13.7 μM zeatin, 22.8uM IAA | | FCLS, CA | 8/38 |
| 16. | 4.4 μM BA | | FCLS | 2/26 |
| 17. | 13.3 μM BA | | FCLS | 12/59 |
| 18. | 4.6 μM kinetin | | NM[f] | 24/24 |
| 19. | 13.9 μM kinetin | | NM | 25/25 |
| 20. | No growth regulators | | NM | 35/35 |

[a]: (i) Ghazi et al., 1986: (ii) Lazzeri et al., 1987: (iii) Lippmann and Lippmann, 1984.
[b]: Ratio: no. of cotyledon explants showing response / no. of explants tested
[c]: CLS= cotyledon-like-structure
[d]: CA= callus
[e]: FCLS= fused, deformed CLS
[f]: NM= no morphogenesis

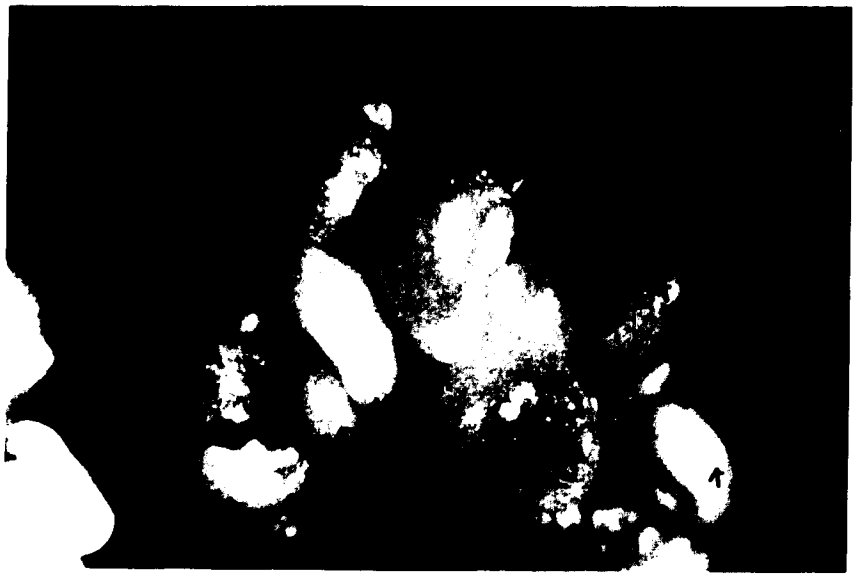
(Hartweck et al., 1988; Lippmann and Lippmann, 1984; Lazzeri et al., 1985) and peanut (Ozias-Akins, 1989) within 30 to 40 days of plating on auxin-supplemented media. With chickpea cotyledons, no such structures were observed on any of the growth regulator regimes tested.

In contrast, chickpea cotyledons gave a striking morphogenic response to the cytokinin zeatin (Table 6). Within 30 days, and sometimes as early as 9 days, of culture initiation on media supplemented with 4.6, 13.7, or 45.6 $\mu$M zeatin, white cotyledon-like structures (CLS) 1 to 4 mm in length formed in clusters of 1 to 20 or more CLS per explant at the proximal end of the cotyledons (Fig. 22). Individual or clustered CLS were infrequently borne on fasciated, petiole-like stalks in a manner suggesting that the CLS were in fact deformed leaflets. However, absence of epidermal trichomes and the thickened, crescent shape of these structures was more suggestive of cotyledons, hence the term "CLS".

CLS were initiated in cotyledonary tissue closely adjacent to, but never within, the wound site left by embryo axis removal. The wound site itself was not morphogenic, but rather formed either a thin callus layer on growth regulator-free and kinetin-supplemented media, or a dense, localized knob of callus on BA- and zeatin-supplemented media. When detached embryo axes were cultured on media containing zeatin, hypocotyl swelling and some shoot development occurred, but no CLS formed. On media containing 4.6 $\mu$M kinetin or no growth regulators, the shoot apices of detached embryo axes developed into normal looking shoots.

The effect of explant orientation on CLS formation was tested on media

**Fig. 22:** Cluster of cotyledon-like structures (CLS: arrows) formed at the proximal end of the explant after 15 days of culture on medium containing 13.7 $\mu$M zeatin + 0.2 $\mu$M IAA.



containing zeatin with the objective of maximizing the frequency of CLS formation. It was found that CLS formation was highly dependent upon explant orientation. In one experiment with 13.7 $\mu$M zeatin, 22 of 35 explants placed with the adaxial surface down produced CLS, while none of the 31 explants placed with the abaxial surface down produced CLS. The importance of cotyledon orientation has been seen in other legume regeneration systems. In peanut, adaxially oriented cotyledons gave a better embryogenic response than explants with the abaxial surface facing the medium (Ozias-Akins, 1989). However, there was more embryo formation in soybean when the abaxial, rather than the adaxial, side of the cotyledon explant was in contact with the medium (Hartweck et al., 1988). Explant orientation was not specified in the earlier reports by Lazzeri et al. (1985, 1987) of soybean embryogenesis from immature cotyledons.

Overall, CLS formation on 13.7 $\mu$M zeatin was more frequent with 0.2 $\mu$M IAA (64%) than without IAA (51%) (Table 6). The apparent beneficial effect of this low concentration of IAA was seen in some individual experiments but not others, leaving it uncertain whether the effect is real. When zeatin was supplemented with 1.1, 5.7, or 22.8 $\mu$M IAA (Media 13, 14, 15; Table 6), hard, green, nodular callus formed around the entire periphery of the explants, and CLS clusters tended to fuse into one or a few large masses, perhaps resembling the "neomorphic" structures described in peanut (Ozias-Akins, 1989) and soybean (Hartweck et al., 1988) cotyledon cultures.

CLS formation also occurred at low frequency (Table 6) on 13.3 $\mu$M BA with or without 0.2 $\mu$M IAA; however, on BA, CLS clusters were commonly fused into one or a few irregular masses and rarely gave rise to shoots. Kinetin induced no discernible

morphogenesis from immature cotyledons.

By 20 to 40 days after culture initiation on media containing zeatin alone or in combination with 0.2 $\mu$M IAA, shoot initiation occurred near the base of CLS (Fig. 23) on greater than 50% of explants bearing CLS. The CLS themselves did not develop into shoots, but commonly turned from white to dark green in color. Glandular epidermal trichomes normally present on chickpea leaves (Lauter & Munns, 1986) were a clearly visible indicator of shoot initiation (Fig. 23). On subsequent transfer of the explants with CLS to 4.4 $\mu$M BA or 4.6 $\mu$M kinetin, multiple shoots (at least 4 shoots / explant) were produced (Fig. 24). The chickpea shoots formed roots after transfer to medium containing 4.9 $\mu$M IBA or 5.4 $\mu$M NAA (Fig. 25). Occasionally there was rooting on medium lacking hormones.

The histogenic origin of the regenerated shoots was not determined. In the absence of sufficient anatomical study, it is not clear if the mode of regeneration was either organogenesis or embryogenesis. Because there was an intermediate stage of CLS formation before the initiation of shoots, as opposed to direct shoot formation from the explants, the shoot formation does not appear to arise via a direct organogenic mechanism. Nor did the CLS and associated shoots have the morphology of normal somatic embryos, which have a pair of cotyledons, a shoot apex, a root apex and a clearly defined hypocotyl region. Rather, they resembled "abnormal embryoids" as seen in soybean (Lazzeri et al., 1985), perhaps with multiple or fused cotyledons. Since shoot formation originated from the bases of the CLS clusters, it is possible that the shoots arose from functional shoot apices associated with "abnormal embryoids".

**Fig. 23:** Leaf formation near the bases of CLS after 21 days of culture on 13.7 $\mu$M zeatin + 0.2 $\mu$M IAA. Note glandular trichomes (arrows) on leaves.

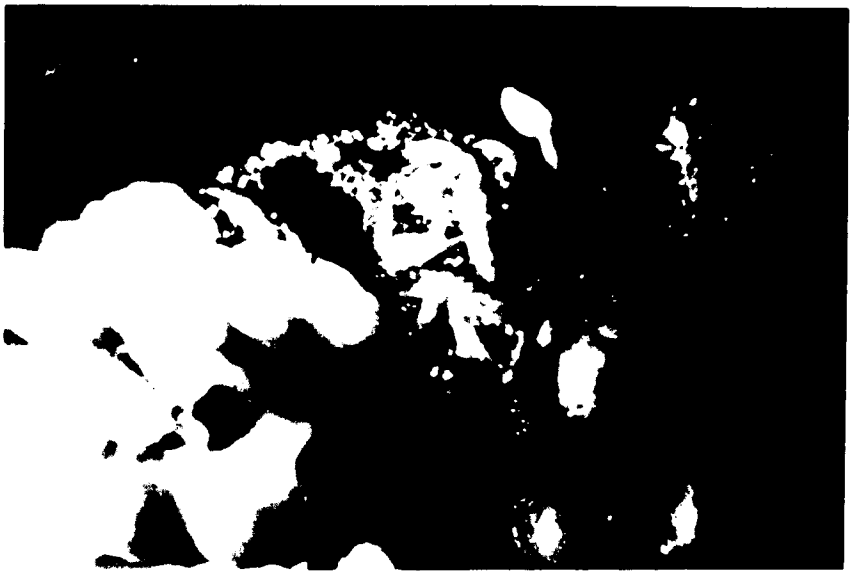


**Fig. 24:** Multiple shoot formation after subculture of morphogenic explant onto 4.4 $\mu$M BA.

**Fig. 25:** Shoots rooted on B5 medium containing 4.9 $\mu$M IBA.

The possibility that CLS and shoot formation resulted from development of axillary buds associated with the detached cotyledons is highly unlikely for the following reasons. First, CLS never formed on detached embryo axes, which obviously contained axillary buds. Second, shoots only emerged from immature cotyledons after the development of CLS, and were always closely associated with CLS. This morphogenic pattern also differentiates the results seen with chickpea from those seen in peanut (Bhatia et al., 1985) where shoots formed directly (with no evidence of CLS formation) from detached mature cotyledons in the presence of BA or no growth regulators. Third, neither CLS nor shoots developed from detached cotyledons on media containing kinetin or no growth regulators, though shoots developed readily from pre-existing buds of detached embryo axes on these same media. Fourth, careful observation of chickpea embryos and seedlings at various stages of development clearly indicated that the axillary buds at the cotyledonary node are not damaged or torn from the embryo axis when cotyledons were excised; these axillary buds remain intact as parts of the embryo axis.

To date, initiation of regenerable embryogenic cultures from immature embryo/cotyledon explants in large-seeded legumes has relied almost exclusively upon high auxin, no cytokinin growth regulator regimes (e.g., Lazzeri et., 1985; Ranch et al., 1985; Ghazi et al., 1986; Parrott et al., 1988). There have been no reports on induction of somatic embryos in grain legumes using zeatin or other cytokinins in the absence of auxins. Lippmann and Lippmann (1984) reported that addition of cytokinins (zeatin, kinetin and BA) to the medium containing 2,4-D inhibited the induction of embryogenic tissue and of embryoids in cotyledon explants. Lazzeri et al. (1987) have shown that

inclusion of BA in auxin-containing media reduced somatic embryo induction from cotyledon tissue or had no effect. On the contrary, Maheshwaran and Williams (1984) have reported embryoid formation directly from immature zygotic embryos of a small-seeded legume, *Trifolium repens* (white clover), on a culture medium containing cytokinin. It is possible that in chickpea as in white clover, CLS and associated shoots (as opposed to normal somatic embryos in clover) arose from predetermined embryogenic cells within the cotyledons, in response to a cytokinin (Sharp et al., 1982).

In the present study, an apparently novel morphogenic response was observed when immature chickpea cotyledons were cultured on media containing zeatin. Detailed histological/ anatomical studies will be required to understand the developmental processes involved. It will also be interesting to learn the responses of other large-seeded legumes, and chickpea genotypes other than line ICC640, to the regeneration protocol described here.

To date, there have been a few reports of successful recovery of transgenic legume plants. Recombinant DNA has been introduced into the genome of legume plants including soybean, peanut and white clover (*Trifolium repens*) using *A. tumefaciens* as the vector for gene transfer (Hinchee et al., 1988; Lacorte et al., 1991; White & Greenwood, 1987), respectively.

Cotyledon explants have been used for *in vitro* regeneration systems in conjunction with genetic transformation of legumes. Transformed soybean plants have been recovered from cotyledons explants through *Agrobacterium*-mediated DNA transfer (Hinchee et al., 1988; Parrott et al. 1989). Stable transformation of soybean from

meristems of immature soybean seeds by particle acceleration has been reported (McCabe et al., 1988). The zeatin-dependent regeneration method described here may be useful for genetic tranformation experiments in chickpea.

# APPENDICES

1) LB medium - g/l: Tryptone, 10; NaCl, 10; Yeast Extract, 5; Agar, 15; pH 7.2.

2) Antibiotics - Stock solutions of ampicillin (50 mg/ml) and tetracycline (10 mg/ml) were filter sterilized and stored at -20°C. To the autoclaved LB medium, 1 $\mu$l of antibiotic stock solution was added / ml of LB medium.

3) Wash buffer - 25 mM Tris, pH 8.0; 10 mM EDTA, 50 mM Glucose.

4) Lysozyme - Aliquots were used from freshly prepared lysozyme stock solution (10 mg/ml).

5) SDS-NaOH - Prepared fresh solution containing 1% SDS and 0.2 M NaOH.

6) TE - 10 mM Tris; 1mM EDTA, pH 8.0.

7) RNase - Prepared a stock solution of 10 mg/ml RNase in 10 mM Tris-Hcl, pH 7.5 and 15 mM NaCl. To remove any DNase contamination, the solution was heated to 100°C for 15 min and allowed to cool at room temperature, and was stored at -20°C.

8) Phenol/chloroform-isoamyl alcohol - To 500 g bottle of crystallized phenol, the following were added -

   1) 100 ml of 2 M Tris, pH 7.8 to 8.0.
   2) 25 ml of double distilled water.
   3) 1 ml of $\beta$-mercaptoethanol.
   4) 500 mg of hydroxyquinoline.

The phenol bottle was warmed to dissolve all the contents. The pH of the aqueous layer was adjusted to 7.8. The equilibrated phenol solution was stored at 4°C.

A mixture of chloroform and isoamyl alcohol (24:1 v/v) was prepared and stored

at 4°C.

9) TBE - TBE (5X) stock solution, pH 8.0 : 1 l of stock solution was prepared by dissolving 54 g Tris base; 27.5 g Boric acid; and 20 ml of 0.5 M EDTA in double distilled water.

10) Ethidium bromide - A stock solution of ethidium bromide (10 mg/ml) was prepared and stored in a dark bottle at 4°C. Care should be taken while preparing and using ethidium bromide since it is a mutagen.

11) DNA extraction buffer - 100 mM Tris, pH 8; 50 mM EDTA, pH 8; 500 mM NaCl; 10 mM mercaptoethanol (added fresh).

12) Loading buffer - contains 0.025% each of bromophenol blue and xylene cyanol; 5-10% sterile glycerol.

13) 20X SSC - 3 M NaCl; 0.3 M trisodium citrate, pH 7.0.

14) Denhardt's solution - A 100 ml stock solution (50X) was prepared by dissolving 1 g each of Ficoll, polyvinylpyrrolidone and bovine serum albumin in double distilled water. The solution was filtered and stored at -20°C.

15) Salmon sperm DNA - A stock solution of ssDNA (10 mg/ml) was prepared in autoclaved double distilled water. The DNA solution was passed through a 18-guage hypodermic needle to shear the DNA. The DNA solution was boiled for 5 min and chilled quickly on ice and stored at -20°C.

16) DEPC-treated water - To 1 l of double distilled water, 1 ml of DEPC was added and the water was stirred vigorously for 30 min using a stir bar, allowed to sit overnight and autoclaved before use.

17) RNA extraction buffer - 3 M sodium acetate; 10% SDS; 0.5 M EDTA, pH 8.0.

18) LMB medium - g/l: Mannitol, 1; Yeast extract, 5; $KH_2PO_4$, 0.3; $Na_2HPO_4$, 0.3; $MgSO_4.7H_2O$, 0.1; $CaCl_2.2H_2O$, 0.05; Agar, 15; pH 6.8.

19) Protoplasting buffer - A stock solution contained 15 mM Tris, 0.45 M sucrose, and 8 mM EDTA; pH 8.0.

20) Gram negative lysing buffer - A stock solution contained 10 mM Tris, 10 mM NaCl, 1 mM sodium citrate, and 1.5 % SDS; pH 8.0.

21) Saturated NaCl - To 100 ml of double distilled water, 40 g of NaCl was added, and stirred until dissolved.

22) MOPS buffer - 200 mM NaMOPS; 50 mM sodium acetate; 10 mM NaEDTA, pH 7.0.

23) Formamide - Deionized formamide was prepared by mixing 50 ml of formamide with 5 g of mixed bed, ion-exchange resin. The mixture was stirred for 30 min at room temperature, filtered through Whatman filter paper, dispensed into aliquots and stored at 4°C.

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