

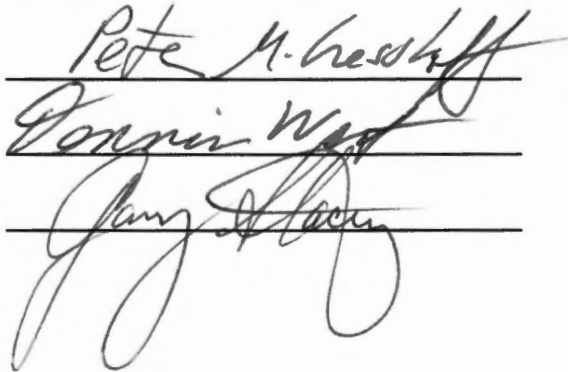
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I am submitting herewith a dissertation written by Daqi Qian entitled "Identification and Molecular Mapping a Gene Restricting Nodulation in Soybean." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Plant and Soil Science.

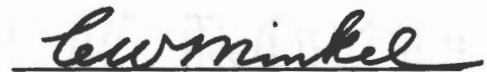


Fred L. Allen, Major Professor

We have read this dissertation
and recommend its acceptance:



Accepted for the Council:



Associate Vice Chancellor
and Dean of The Graduate School

**Identification and Molecular Mapping of a Gene
Restricting Nodulation in Soybean**

**A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville**

**Daqi Qian
December 1993**

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Thesis
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DEDICATION

This dissertation is dedicated to all teachers in my life.

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ABSTRACT

Successful establishment in soybean (*Glycine max* L. Merr.) nodules of *Bradyrhizobium japonicum* strains with improved nitrogen-fixing ability over indigenous strains has been suggested as an approach to enhance soybean productivity. A major obstacle to this achievement has been the superior competitive ability of indigenous strains over improved strains for developing nodules. The objectives of this study were to: (1) search for genes which restrict nodulation with certain strains of *B. japonicum*, (2) study the genetic pattern of restricted nodulation, (3) investigate at which stage nodulation development is blocked, and (4) use RFLP technology to map the gene restricting nodulation in soybean. Preliminary screenings indicated that 'TN 4-86', BARC-2(*Rj4*), and PI 468.397 (*G. soja*) showed restricted nodulation with *B. japonicum* strain 61A101C. Study of F₁, F₂, and F₃ families of Essex X PI 468.397 indicated that restricted nodulation in PI 468.397 on strain 61A101C is controlled by a recessive gene. In contrast, the restricted nodulation in TN 4-86 is controlled by a dominant gene which is allelic to gene *Rj4*. The kinetic study of nodule development indicated that the blockage of nodule development in BARC-2(*Rj4*), TN 4-86, and PI 468.397 do not occur during nodule initiation, but during further development of initiated infection. Unlike non-nodulation, restriction of nodulation in TN 4-86 and PI 468.397 is not 100%. Molecular mapping of the restricted nodulation gene in PI 468.397 was attempted. Seventy enzyme/probe combinations were tested on Essex and PI 468.397. Eighteen polymorphisms were

detected. Some probes were very useful and others were relatively uninformative. Enzymes *HindIII* and *EcoRV* were more effective than *DraI* in producing polymorphisms between Essex and PI 468.397. Sixteen enzyme/probe combinations were tested on F₂ plants that expressed the restricted nodulation phenotype. These RFLP loci covered linkage groups A to H. No statistically significant linkage was detected between RFLP loci and the gene controlling restricted nodulation based on the data obtained. Although the segregation ratios of marker patterns in some enzyme/probe combinations are significantly different from those of independent segregation, the number of heterozygote patterns is more than that expected. This could have been due to small sample size.

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PART I

Introduction and Literature Review

CHAPTER 1

Introduction

Biological nitrogen fixation is confined to microorganisms. Only prokaryotes, such as bacteria, blue-green algae, and actinomycetes can reduce nitrogen to ammonia. Such bacteria can be either free-living, such as *Azotobacter* (Hill, 1992), or can form symbiotic associations with higher plants, like the (*Brady*)*rhizobium*-legume system (Long, 1989). The latter group is very important agriculturally. In exchange for fixed nitrogen supplied by the bacterium, the legume supplies energy in the form of carbohydrate by photosynthesis. Thus, renewable solar energy powers this fertilizer production system in contrast to the non-renewable energy sources used commercially. Additional advantage of biological nitrogen fixation systems is that the fixed nitrogen is produced directly at the site of need, thus reducing nitrogen losses and contamination.

In the last several decades, studies of the symbiotic relationship between legumes and (*Brady*)*rhizobium* have been intensified. The legumes include temperate and tropical plants, ranging from small plants, like clovers (*Trifolium*, sp.), to large trees, like *Acacia*. The best known associations occur with soybean (*Glycine max*), clover, and alfalfa (*Medicago sativa*) etc.. Nodulation and nitrogen fixation of non-legumes is restricted to nodulation of plants within the *Parasponia* genus, a woody

member of the dicotyledonous elm family (*Ulmaceae*) (Trinick, 1982). Currently, an increasing amount of work has been done on the non-legume crops such as rice (*Oryza sativa*) and wheat (*Triticum aestivum*) (Al-Mallah et al., 1989; Jain and Patriquin, 1985; Ridge et al., 1992).

When effective nitrogen-fixing strains of (*Brady*)*rhizobium* are used as inoculants on legume seeds, the plants are often in fact nodulated by inferior nitrogen-fixing strains or even by ineffective rhizobia from the soil population. Even if (*Brady*)*rhizobium*, used as an inoculant, effectively nodulates the host in the first growing season, it may not persist to form nodules on plants in the following seasons (Dudman and Brockwell, 1968; Weaver and Frederick, 1974a; Ikram and Broughton, 1981). In the last several decades, host genotypes and genes which control non-nodulation, ineffective nodulation, and restricted nodulation have been reported (Cregan and Keyser, 1986; Cregan et al., 1989; also see review by Caetano-Anollés and Gresshoff, 1991). These findings suggest that it should be possible to manipulate the genetic control of nodulation in the host. Indeed, legume breeding programs showed promise of developing plants capable of enhanced nitrogen fixation under field conditions (Attewell and Bliss, 1985; Barnes et al., 1985; Teuber et al., 1984).

To incorporate the host genes controlling nodulation into breeding programs, efficient methods for isolation, identification, and transformation are needed. Genetic markers provide an excellent tool to map and characterize systematically genes that are important in conferring traits. Isozyme markers have been used very successfully in certain aspects of plant breeding and genetics as nearly-neutral markers.

Unfortunately, the number of genetic markers provided by isozyme assays is insufficient for many applications in plant breeding. Restriction fragment length polymorphisms (RFLPs) have been used in chromosome mapping. RFLPs are genetic markers based on cloned fragments of DNA. Using RFLPs, high density linkage maps can be constructed and used to locate genes of economic importance (Donis-Keller et al., 1987; Landry et al., 1987; Landau-Ellis et al., 1991). Once a gene of interest is associated with a linked RFLP marker, selection for that gene can be based on the genotype of the RFLP rather than the trait phenotype. This can be extremely valuable if a trait phenotype is difficult or expensive to score. RFLPs can also be used to select against unwanted genomic segments, which is important if one of the parents in a breeding program is uncultivated and carries undesirable characters (Young and Tanksley, 1989a). Finally, RFLPs can act as starting points for map-based cloning or positional cloning, a strategy for cloning genes known only by their genetic map location, not their biochemical product (Young, 1990; Bond et al., 1992; Gresshoff, 1993).

The objectives of this research were to: (1) search for the genes in soybean which restrict certain strain(s) of *B. japonicum*, especially those which restrict a group of strains or specific strain; (2) study the genetic pattern of those genes that restrict nodulation by specific strains; (3) investigate the initiation to cessation of nodulation caused by the plant gene(s); and (4) use RFLP technology to map the gene(s).

CHAPTER 2

Literature Review

Rhizobia are gram-negative, soil-inhabiting bacteria that form root nodules on various legumes. The symbiotic relationship is genetically controlled by both plant and bacterium and proceeds through a sequence of developmental stages that result in the establishment of a effective, nitrogen-fixing symbiosis. Soybean improvement programs are searching for soybean-*Bradyrhizobium* genotype combinations that optimize nitrogen fixation. The first step in such a program is to identify genetic variation in the host and the symbiont. This includes a search for resistance or exclusion and coevolved, effective symbiosis.

Nodule Development in Legumes

Temperate legumes, such as alfalfa, develop indeterminate nodules characterized by a defined meristem during nodule growth and an open vascular system connecting the root vascular system with the nodule meristem. The nodule is cylindrical in shape (Dudley et al., 1987). In mature nodules, the central tissue can be divided into four distinct regions, meristem, early symbiotic zone, late symbiotic zone, and senescent zone. Tropical legumes, such as soybean, form spherical,

determinate nodules, which arise from the outer cortical cell regions just below the epidermis (Turgeon and Bauer, 1982). Determinate nodules do not have a persistent meristem. Although there are differences in the structure between determinate and indeterminate nodules, the establishment of symbiosis in both cases involves a series of steps of nodule development (Calvert et al., 1984; Dudley et al., 1987).

Multiplication and Colonization (Roc): In order to colonize the roots (*Brady*)*rhizobia* must first reach the roots. Two possible mechanisms, chemotaxis and electrotaxis, have been suggested to account for the presence of rhizobia near the host roots (Gotz and Schmitt, 1987; Miller et al., 1986). By the process of chemotaxis, rhizobia, like many other bacteria, may move towards certain compounds, often potential nutrients. *Rhizobium* responds to flavonoids which are part of the nodulation signalling system (Kosslak et al., 1988; Lerouge et al., 1990). Recent studies have indicated that isoflavones were not chemoattractants (Kape et al., 1991) or not principal attractants in exudates (Barbour et al., 1991). Glutamate, aspartate, and dicarboxylic acid were natural attractants for *B. japonicum* (Barbour et al., 1991). The major function of isoflavones is in the regulation of *nod* genes (Banfalvi et al., 1988; Trese and Pueppke, 1990). Miller et al. (1986) found that, in plant developmental processes, plants generate electrical fields in which current consistently enters the main elongation zone of the roots and exits from mature, elongated tissue. They speculated that this extracellular current loop could act as a 'fine-tuning' device, acting on bacteria close to the root surface.

Attachment to Roots (Roa): Attachment to roots and root hairs proceeds in stages with increasing levels of specificity and decreasing levels of reversibility, the later stages involving recognition. Attachment of (*Brady*)*rhizobium* to the root hair is considered to be a two-step process (Smit et al., 1989a). In the first step, single rhizobial cells adhere to the surface of the root hair. The adhering of other rhizobia to the root hair-bound bacterial cells is considered as second step. Two types of substances usually appear to be involved in both recognition and polar attachment (Diaz et al., 1989). These are lectins on the plant side and surface polysaccharides on the rhizobial side. Lectins (non-catalytic proteins produced by the host) may recognize particular carbohydrate residues in rhizobia in a specific way (Stacey et al., 1980; Dazzo et al., 1983). Confirmation of this suggestion has been obtained by using the plant pathogen *Agrobacterium rhizogenes* to carry the gene for pea lectin to clover roots. The latter could then be nodulated by a pea rhizobial strain (Diaz et al., 1989). The carbohydrate residues involved are on the extracellular (surface) polysaccharides (EPS). However, others could not demonstrate attachment to be host specific, mediated by lectins (Smit et al., 1986; Stacey et al., 1991). In addition to EPS, there are three types of surface polysaccharides, Lipopolysaccharide (LPS), Capsular polysaccharides (CPS), and Cyclic beta 1,2-glucans, which may be involved in infection (Carlson, 1982; Halverson and Stacey, 1986a,b; Puvanesarasah et al., 1987). In serial papers, Smit et al. (1987, 1989a,b) demonstrated that Ca^{2+} - dependent adhesin, named rhicadhesin (a soluble surface protein of bacteria), was involved in the first step in attachment of bacteria to root hair tips. Preincubation of

pea roots with rhicadhesin inhibited attachment of *Rhizobiaceae* to the root hair tips. Light microscope observations revealed that *R. japonicum* (now named *B. japonicum*) cells attached to the root epidermal and root hair cells at all stages of development in less than 1 min after inoculation. The number of attached bacteria increased substantially during the first hour following inoculation (Turgeon and Bauer, 1982).

Nod Gene Induction (Ngi, Ini): In *Rhizobium* spp., the *nodD* gene is constitutively expressed without need of induction and essential for the expression of the other *nod* genes. The expression of *nod* genes, except *nodD*, is regulated by the host plant or host plant exudates (Long, 1989). The *nod* gene-inducing compounds from several species are usually flavonoids (Peters et al., 1986; Maxwell et al., 1989). The mechanism by which these compounds induce *nod* gene expression is not completely understood. In *B. japonicum* the *nodD₁* gene is not constitutively expressed to an appreciable level and its expression is dependent on the presence of plant-produced isoflavones genistein and daidzein (Banfalvi et al., 1988; Göttfert et al., 1988; Kossalak et al., 1988). A proposed model for *nod* gene activation is that NodD protein interacts with the inducer molecule and binds to a conserved DNA region (*nod* box) 5' of each *nod* gene transcript to stimulate transcription (Hong et al., 1987; Gerhold et al., 1989). *NodD₁* also has its own *nod* box (Wang and Stacey, 1991). Each plant host produces a distinctive mixture of inducer compounds (Trese and Pueppke, 1990), thus to a certain extent, *nod* gene induction is host specific. Smit et al. (1992) reported that *nodD₁* of *B. japonicum* can be specifically induced by some soybean

flavonoids which cannot induce the *nodYABCSUIJ* operon. The flavonoids were identified to be derivatives of genistein, glycitein, and daidzein which can induce the *nodYABCSUIJ* operon. They proposed that the specific induction of *nodD*₁ by those flavonoids led to an increased level of NodD₁ protein which subsequently leads to an enhanced expression of *nodYABCSUIJ* operon under suboptimal genistein concentration. *NodD*₂, a second copy of *nodD*, is not required for induction of *nodYABCSUIJ* operon in *B. japonicum*. However, a *B. japonicum* deleted for both copies of *nodD* still showed delayed nodulation on soybean, but nodule number was strongly reduced on mung bean and siratro (Göttfert et al., 1992).

Root Hair Deformation, Curling and Branching (*Had*, *Hac*, *Hab*): Like attachment, root hair curling appears to be associated with physiological conditions and surface polysaccharides in (*Brady*)*rhizobium* (Halverson and Stacey, 1986a). The marked root hair curling which is an apparent prerequisite for hair infection requires the presence of living rhizobia. Curling may result from a redirection of hair growth by rhizobia (Batenburg et al., 1986). In soybean, curls are very close to the root surface (Calvert et al., 1984). Such hairs do not usually branch. In other species, including clovers and peas, hairs grow quite long before they curl and they may also branch. A number of reports demonstrated that (*Brady*)*rhizobium* release signal molecular which can cause root hair deformation (Lerouge et al., 1990; Spaink et al., 1992). In *R. meliloti*, common *nodABC* genes as well as host specific *nodH* and *nodQ* are involved in the production of extracellular Nod signal, NodRm-1 (Lerouge et al., 1990). In

Rhizobium leguminosarum biovar *viceae*, the *nodABC* genes, the *nodFE* genes, and *nodL* gene are involved in the production of Nod metabolites (Spaink et al., 1992).

Bradyrhizobium japonicum Nod factor [NodBj-V (C_{18:1}, Me-Fuc)] for soybean was also isolated and characterized which is similar to the lipo-oligosaccharide signal from *Rhizobium* species (Sanjuan et al., 1992).

Infection (*Inf*, *Inb*): Infection in roots may be by hairs, wounds or cracks, or between cells of the intact epidermis. Hair infection occurs in the most widely studied crop plants and has frequently been reviewed (e.g. Bauer, 1981). Detailed studies on wound infection have been made for *Arachis* and *Stylosanthes* (Chandler, 1978; Chandler et al., 1982). In both genera, rhizobia gained entry at the point where lateral roots emerged. Nodules appeared, macroscopically, to be in the axiles of lateral roots. There have been sporadic reports of direct infection through undamaged epidermis of legumes normally infected *via* hairs (Dart, 1977). Many nodulating legumes, especially woody species, do not regularly produce hairs, nor do they have nodules associated with lateral roots. Since nodules develop on young and apparently undamaged roots, epidermal infection seems likely and was confirmed in *Mimosa scabrella* by Faria et al. (1988).

Cortical Cell Division and Pericycle Cell Division (*Ccd*, *Pcd*): Initiation of cell division is not consequent upon infection. Cortical cell division may precede infection. In soybean, the cortical cells underneath curled root hairs divided first. The

central mass of infected tissue is derived mainly from the outer layer of cortical cells. In contrast, mitoses occur in the inner cortical cell of the root in development of indeterminate nodule (e.g., pea nodule, Newcomb et al., 1979). The inner cortical cells contribute extensively to the infected tissue of the pea nodules (Newcomb et al., 1979). If these divisions are to be organized into a nodule, some of the daughter cells must become infected and others must differentiate into one of several specialized cell types (Newcomb, 1976). There are at least two ways in which cells may become infected. The first way is that each individual cell in a young nodule becomes infected by a branch of the infection thread while some cells, called interstitial cells, remain uninfected. The cessation of mitotic activity of host cell coincides with the release of rhizobia in it (Newcomb, 1976). This pattern is seen in nodules of indeterminate growth such as those of alfalfa. The infection process continues, throughout the life of the nodule, in cells newly formed by the nodule meristem. The second way is that a limited number of cells may become infected by the infection thread. These cells and the bacteria contained within them divide and then enlarge, forming a soybean-type nodule of limited growth (Turgeon and Bauer, 1982). This is considered a method for distribution rhizobia when only a few infection threads are present.

Bacterial Release and Differentiation (Bar, Bad): Bacteria invade the root hair cells and then the root cortex through a tube-like structure, the infection thread. Before the formation of infection thread, the cortical cells underneath curled root hair undergo morphological changes which lead to the formation of cytoplasmic bridge. Infection

thread grows through the cytoplasmic bridge (Kijne, 1992). There are two types of infection thread, the broad infection thread like that in alfalfa and the narrow infection thread like that in soybean. Surface polysaccharides play an important role in the development of either broad or narrow infection threads (Dylan et al., 1990; Noel et al., 1986). Infection threads grow toward meristematic centers so that rhizobia are released from infection threads into the cytoplasm of the plant cells. This release is an endocytotic process in which the bacteria become enclosed by a membrane, the so-called peribacteroid membrane which is initially derived from the host plasma membrane (Bassett et al., 1977). The term 'symbiosome' was proposed by Roth et al. (1988) to describe the symbiotic unit which includes the bacteroid, the symbiosome space, and the peribacteroid (symbiosome) membrane. Until the structure of infected cells of *Parasponia* was described by Trinick and Galbraith (1976), it was thought that release of bacteria from infected threads was a necessary prerequisite for development of functional nitrogen-fixing nodules. However, release of bacteria does not occur in the nodules of the non-legume *Parasponia*, although these can show high rates of nitrogen fixation (Price et al., 1984; Trinick and Hadobas, 1988).

Autoregulation (Aut): Once a certain number of nodules have formed on soybean roots, development of additional nodules is blocked (Bhuvaneswari et al., 1980, 1981; Pierce and Bauer, 1983). The split-root studies in soybean showed that inoculation of one half-side suppressed subsequent development of nodules on the opposite side (Kosslak and Bohloul, 1984; Olsson et al., 1989). Similar results were reported in

alfalfa (Caetano-Anollés and Bauer, 1988). In contrast, there is no suppression of nodulation in the supernodulation mutant (nts382) (Olsson et al., 1989). Graft studies between a number of nodulation mutants of 'Bragg' soybean, 'Bragg', and *G. soja* indicated that suppression of nodulation is shoot-controlled (Delves et al., 1986, 1987a, 1987b). The model for the regulation of nodule formation in legumes proposed by Caetano-Anollés and Gresshoff (1991) involves two systemic messengers. One is the root synthesized inducer and the other is the shoot-derived inhibitor which will finally arrest development of further infections or block nodule initiation. The blockage of nodule development occurs relatively early during nodule ontogeny. The maturation of nodule meristems into emergent nodules appears to be the target of the regulatory suppressive response (Gerahty et al., 1992)

Anatomical and Cytological Analysis of Nodule Development

The processes of infection and nodule development have been studied anatomically and cytologically in several legume species. Early infection events in *G. max* was reported (Bhuvaneswari et al., 1980; Turgeon and Bauer, 1982). Calvert et al. (1984) arbitrarily divided the process of nodule development in soybean infected by *B. japonicum* into twenty stages. Stages I to VIII describe the events of the various stages of cortical cell division, formation of meristem, and differentiation of the vascular nodule. Stages IX through XX are generally characterized by a steady

increase in the nodule mass and differentiation of the infected cortex and surrounding vascular bundles. They found the first cell division in hypodermal cells occurred approximately 12 h post-inoculation. Infection proceeded to stage I within 24 h of inoculation, to stage II within approximately 36 h, and progressed to stage III and IV within 48 and 60 h of inoculation, respectively.

Rhizobium Genetics of Symbiotic Nitrogen Fixation

Genes Involved in Nodulation: The *Rhizobium*-legume symbiosis is a complex association between two partners that results in the formation of root nodules. The process requires the expression of a number of plant and bacterial genes that are critical for maintenance of the symbiotic interaction. An interesting feature of the interaction is the specificity of (*Brady*)*rhizobium* species for a particular set of host plants. Establishment of the bacterial symbiont within the host root nodule requires the coordinate expression of at least two sets of bacterial genes. One set, common *nod* genes (Table 1-1), appear to be structurally and functionally conserved among *Bradyrhizobium* and *Rhizobium* strains (Djordjevic et al., 1985; Downie and Johnston, 1986; Downie et al., 1985; Jacobs et al., 1985; Long et al., 1982; Russell et al., 1985; Hahn and Hennecke, 1987a). Mutations in *nodABC* prevent marked root hair curling and formation of infection threads in clovers (Djordjevic et al., 1986) and in soybean (Nieuwkoop et al., 1987). The number of copy of *nodD* is different among *Rhizobium* spp. and *Bradyrhizobium* (Honma et al., 1986; Göttfert et al., 1989). The

Table 1-1. *Nod*, *nif*, and *fix* genes of *Bradyrhizobium* and their functions

Gene	Species	Proposed function
<i>nodABC</i>	<i>B. japonicum</i>	Synthesis of a low-molecular-weight factor essential for root hair curling and cortical cell division, NodC protein is homologous to chitin synthetase†
	<i>B. sp.(Parasponia)</i>	
<i>nodD₁</i>	<i>B. japonicum</i>	Positive transcriptional regulator required for <i>nod</i> gene expression
	<i>B. sp.(Parasponia)</i>	
<i>nodD₂</i>	<i>B. japonicum</i>	Unknown function; mutation causes delayed nodulation
	<i>B. sp.(Parasponia)</i>	
<i>nodI</i>	<i>B. japonicum</i>	Unknown function; sequence similarity to ATP-dependent transport proteins
	<i>B. sp.(Parasponia)</i>	
<i>nodJ</i>	<i>B. japonicum</i>	Unknown function
	<i>B. sp.(Parasponia)</i>	
<i>nodK</i>	<i>B. sp.(Parasponia)</i>	Unknown function
<i>nodLMN</i>	<i>B. sp.(Parasponia)</i>	Found by hybridization; host range determination in <i>Rhizobium</i>
<i>nodSU</i>	<i>B. japonicum</i>	Unknown function
<i>nodV</i>	<i>B. japonicum</i>	Host range determination; sequence similarity to membrane sensor family
<i>nodW</i>	<i>B. japonicum</i>	Host range determination; sequence similarity to transcriptional regulatory family
<i>nodY</i>	<i>B. japonicum</i>	Unknown function
<i>nodZ</i>	<i>B. japonicum</i>	Host range determination
<i>nolA</i>	<i>B. japonicum</i>	Genotype specific nodulation
<i>nifA</i>	<i>B. japonicum</i>	Transcriptional regulator of <i>nif</i> and <i>fix</i> expression
	<i>B. sp.(Parasponia)</i>	
<i>nifH</i>	<i>B. japonicum</i>	Structural gene for dinitrogenase reductase
	<i>B. sp.(Parasponia)</i>	
	<i>B. sp.(cowpea)</i>	
<i>nifDK</i>	<i>B. japonicum</i>	Structural gene for dinitrogenase
	<i>B. sp.(Parasponia)</i>	
	<i>B. sp.(cowpea)</i>	
<i>nifB</i>	<i>B. japonicum</i>	FeMoco‡ synthesis
	<i>B. sp.(Parasponia)</i>	
<i>nifE</i>	<i>B. japonicum</i>	FeMoco‡ synthesis
	<i>B. sp.(Parasponia)</i>	
<i>nifN</i>		FeMoco‡ synthesis
<i>nifS</i>	<i>B. japonicum</i>	Maturation of nitrogenase
<i>fixA</i>	<i>B. japonicum</i>	Electron transport to nitrogenase
<i>fixBC</i>	<i>B. japonicum</i>	Electron transport to nitrogenase
	<i>B. sp.(Parasponia)</i>	
<i>fixIJ</i>	<i>B. japonicum</i>	Sequence similarity to family of two-component regulatory proteins
<i>fixR</i>	<i>B. japonicum</i>	Function unknown; sequence similarity to dehydrogenase

†: Debelle et al., 1992.

‡: Co-factor of nitrogenase.

Source: Barbour et al., 1992.

nodII region is not absolutely required for symbiosis, since mutations cause only a marginally defective nodulation phenotype (Evans and Downie, 1986). The second set of genes (for example, *nodEFGH*) appears to be involved in host range recognition and, at least functionally, is limited to specific host-bacterium combinations (Horvath et al., 1986; Shearman et al., 1986). The latter genes are commonly referred to as host-specific nodulation (*hsn*) genes (Horvath et al., 1986). Host-specific nodulation genes from several genera of *Rhizobiaceae* have been identified and characterized (Horvath et al., 1986; Lewin et al., 1987; Shearman et al., 1986; Surin and Downie, 1988). Genes involved in host-specific nodulation in *Bradyrhizobium* were identified (Nieuwkoop et al., 1987; Deshmane and Stacey, 1989; Wilson et al., 1987).

A third set of genes are involved in the recognition of symbiotic association in the genotype level called genotype-specific nodulation gene. Genotype-specific nodulation gene involved in several legume species have been discovered (Davis et al., 1988; Heron et al., 1989; Sadowsky et al., 1991). The case in which modified Nod metabolite produced by mutant strain overcome the genotype-specific nodulation of *Rj4* soybean was reported (Stokkermans et al., 1992).

Genetic studies involving plasmid transfer in *Rhizobium* have been done almost exclusively with the fast-growing species, such as *R. meliloti* (Djordjevic et al., 1983). This may in part be due to the fact that nitrogen fixation genes and some of the symbiotic genes are plasmid-borne in this group (Beynon et al., 1980; Hombrecher et al., 1981; Hooykaas et al., 1981). Large plasmids (molecular weight > 100x10⁶ dalton) were found in 10 fast-growing *R. fredii* strains (Sadowsky and

Bohloul, 1983). Removal of one of these plasmids resulted in the loss of nodulating ability by the mutant, which suggests its direct involvement in the symbiosis. Several others researchers have shown plasmid-linked *nif* and *nod* genes in fast-growing *Rhizobium* (Broughton et al., 1984; Masterson and Atherly, 1986; Mathis et al., 1985). Shantharam et al. (1988) classified the mutants of *R. fredii*, USDA 191, as five distinct phenotype classes by using several deletion mutants in the symbiotic plasmid of *R. fredii*, USDA 191, which is defective in the early and advanced stages of root nodule development. The classes were: (1) mutants that do not adhere to the root hair surface; (2) mutants that do not cause root hair curling; (3) mutants that do not form an infection thread; (4) mutants that are not surrounded by a peribacteroid membrane envelope; and (5) mutants in which the bacteria are released only into the intercellular spaces in the cortex. Sadowsky et al. (1988) found two host-inducible genes of *R. fredii* from a library of total plasmid DNA of one of the mutants of USDA 201, 3F1. Their results suggested that, in addition to common nodulation genes, several other genes appear to be specifically induced by compounds in the root exudate of the host plants. The genetics of root nodule bacteria of such an important crop as soybean has been neglected due to difficulties in identifying the location of the symbiosis-related genes. In *Bradyrhizobium*, large plasmids were found in some strains, but they do not carry Sym genes as the major symbiosis-related genes are located on the chromosome. The physical and genetic maps of *B. japonicum* were reported by Kündig et al. (1993).

Genes Involved in Nitrogen Fixation. In addition to the *nod* genes, *nif* and *fix* genes are required to fix nitrogen (Table 1-1). *Nif* genes are responsible for the synthesis of the nitrogenase enzyme complex and are classified according to sequence similarity with *nif* genes in non-symbiotic bacteria such as *Klebsiella pneumoniae*. *Fix* genes are required for nitrogen fixation. Mutations in *fix* genes result in nodules incapable of nitrogen fixation. *Nif* genes have been identified through two main approaches, homology with the identified *nif* genes of *K. pneumoniae* and sequence analysis of DNA regions believed to carry *nif* gene. *Fix* gene have been identified in several ways, random mutagenesis and screening and sequencing of DNA adjacent to the other *nif* genes etc. (David et al., 1987; also see reviews by Long, 1989, and Barbour et al., 1992).

In *Rhizobium*, *nif* and *fix* genes are located on plasmids. Masterson et al.(1982) reported that the structural nitrogen fixation genes (*nifD*, *nifH*, and *nifK*) are located on the large plasmid in several of the fast-growing *R. fredii* isolates. In contrast, *nif* and *fix* genes are located on the chromosome in *Bradyrhizobium* located in two clusters (Hahn and Hennecke, 1987a,b; Russell et al., 1985). NifA plays a central role in *nif-fix* gene regulation. NifA-dependent activation of *nif* and *fix* genes in *B. japonicum* also involves the recognition of promoters by the alternative sigma factor (Ebeling et al., 1987). The ability of *nifA* to regulate other *nif* genes is decreased by oxygen, but the effect seems to occur as a result of direct oxygen inactivation of the NifA protein itself (Fischer and Hennecke, 1987). In structure, *nif* and *fix* genes in *B.*

japonicum have typical upstream -24 and -12 consensus sequences and some also have an upstream activator sequence (UAS).

Soybean Genetics of Symbiotic Nitrogen Fixation

Nutman first reported a recessive gene responsible for non-nodulation in red clover (Nutman, 1949). Since then, the number of host genes involved in nodulation has steadily increased. These occur in various legumes and are either naturally occurring or induced by chemical or physical mutagenesis. Some mutant phenotypes are specific for certain rhizobial strains, (e.g., *Rj2* in soybean; Caldwell, 1966); whereas other genes can control non-nodulation with a broad range of strains, (e.g., *rj1* in soybean; Caldwell, 1966). Although some non-nodulating phenotypes have been characterized morphologically at the cellular level, (e.g., *nod49* in soybean, Mathews et al., 1989a), none have been fully explained at biochemical or molecular level.

The first recessive soybean gene controlling non-nodulation (*no*) was reported by Williams and Lynch (1954). Later, this gene was renamed as *rj1* (Caldwell, 1966). This gene conditions non-nodulation with a broad range of *B. japonicum*. The dominant gene *Rj2* conditions ineffective nodulation with rhizobial strains of the c1 and 122 serogroup (Caldwell, 1966). The ineffective nodulation refers to the development of cortical proliferation or small nodule-like structures with white, chlorotic nitrogen deficiency symptoms (Vest et al., 1973). The dominant gene *Rj3*

conditions ineffective nodulation with rhizobial strain USDA 33 (Vest, 1970), and the dominant gene *Rj4* conditions ineffective nodulation with rhizobial strain USDA 61 (Vest and Caldwell, 1972). Devine (1984) identified a dominant allele, segregating in a Mendelian fashion, which conditions the ineffective nodulation response of 'Kent' soybean with *R. fredii* strain, USDA 205. A recessive allele carried by 'Peking' conditions effective nodulation. However, Devine pointed out that his discovery of that gene does not imply that the total incompatibility (including both effectiveness of nodulation and efficiency of fixation) of Kent or other US-bred soybean lines with the fast-growing strains is controlled by a single gene. Other genes may condition incompatibility, perhaps at other stages of nodule ontogeny.

Several other genes involved in soybean nodulation symbiosis with *B. japonicum* have been reported. A nitrate-tolerant-symbiosis (*nts*) trait in soybean was discovered by Carroll et al. (1985). Mathews et al. (1989b) found a recessive non-nodulation gene in nod139 which is not allelic to *rj1*. This new gene was later officially named *rj6* and a dominant gene identified in 'Harosoy 63' conditioning normal nodulation with *B. japonicum* was named as *Rj5* (Pracht et al., 1993).

(*Brady*)rhizobium-Soybean Interaction

Individual (*Brady*)rhizobium strains are able to form nodules on only a limited number of host legumes. Similarly, leguminous plants are each nodulated by a limited

number of strains. The specificity in this interaction can occur at different stages. For example, when non-nodulation soybean (*rj1rj1*) is inoculated with *B. japonicum*, only a few or no root hair curlings develop (Mathews et al., 1987; Suganuma et al., 1990). In this instance, recognition between two partners terminates at the very beginning of symbiosis. In addition to the specific recognition between the host plant and bacteria, competition between bacteria has been reported in *Rhizobium* and *Bradyrhizobium* (McLoughlin et al., 1985). A number of studies have demonstrated that even at high inoculation rates, the proportion of nodules formed by an inoculum strain is generally less than 20% (Caldwell and Vest, 1970; Kuykendall and Weber, 1978). Although Johnson et al. (1965) recovered an inoculum strain in 82% of the nodules of 'Kent' soybean, the inoculum was applied at 700 times the recommended commercial rate and at least 12% of the indigenous population was serologically indistinguishable from the inoculum strain. Similarly, Kapusta and Rouwenhorst (1973) recovered an inoculum strain in up to 60% of the nodules after application of ultra-high rates of inoculum. Weaver and Frederick (1974b) indicated that in order to establish an inoculum strain in 50% of the nodules of a soybean plant, the rate of inoculum application would have to be at least 1000 times greater than the indigenous population. Cregan and Keyser (1986) and Cregan et al. (1989) suggested using certain soybean genotypes to restrict the nodulation by indigenous strains. They found several PI soybean genotypes that restrict the nodulation by USDA 123, which is the predominant strain in the Midwest of the United States and less effective in nitrogen fixation (Ham, 1980). Another approach is to manipulation interstrain competition for

nodulation using certain chemicals. Application of methyl bromide substantially decreased the number of nodules containing native rhizobial strains in peanut (*Arachis hypogaea*) (Kishinevsky et al., 1992). Similar results of chemical treatment was reported in soybean (Cunningham et al., 1991). However, the practical problem is that any one chemical would be effective against a diverse indigenous *B. japonicum* and also bacteria could develop resistance to a particular chemical inhibitor.

Genetic Linkage Maps Based on RFLP Markers

To incorporate the genes controlling restricted nodulation into soybean varieties, efficient methods for isolation and transformation of those genes are needed. As with many aspects of biology, recombinant DNA technology has an enormous impact on plant science. Many plant genes have already been cloned, but only a few of these have been directly associated with characters of major agricultural importance, such as yield, product quality or nitrogen fixation. This is hardly surprising since these important characters are often under complex genetic control, involving the joint action of a number of genes. In the case of nitrogen fixation, the genetic control of symbiotic bacteria, host plants, and the interaction between the two partners are necessary. In the absence of much understanding of genes controlling characters of economic interest, plant breeders develop new varieties by selecting plants with desirable phenotypes. However, a plant phenotype is determined, not only

by its genetic composition, but also by numerous environmental factors. In many cases the effects of environment mask those of the genotype, so the phenotype provides an imperfect measure of a plant genetic potential. To deal with this problem, elaborate plant breeding techniques, based on statistical inference, have been developed. These techniques have been very useful in producing new crop varieties, but at the cost of much time and effort. Scientists have long theorized about the use of genetic maps and markers to speed up the process of plant and animal breeding. As early as in 1923, Sax (1923) proposed identifying and selecting for minor genes of interest by linkage with major genes, which could be scored more easily. Isozymes have been used very successfully in certain aspects of plant breeding and genetics as nearly-neutral markers. Unfortunately, the number of genetic markers provided by isozyme assays is insufficient for many applications in plant breeding. Restriction fragment length polymorphism (RFLP) (Grodzicker et al., 1974), random amplified polymorphic DNA (RAPD) (Williams et al., 1990; Welsh et al., 1991), and DNA amplification fingerprinting (DAF) (Caetano-Anollés et al., 1991) provide unlimited markers and have been used in chromosome mapping.

Restriction fragment length polymorphisms were first used as a tool for genetic analysis in 1974. Linkage of temperature-sensitive mutations of adenovirus to specific restriction fragment length differences was used to locate the mutations on a physical map of the restriction fragments (Grodzicker et al., 1974). The potential impact of RFLP mapping on eukaryotic genetics was first described in 1980 by researchers in human genetics (Botstein et al., 1980; Wyman and White, 1980). Restriction fragment

length polymorphisms now serve a major role in human genetic research. The detailed genetic linkage map of the human genome, based on RFLP markers, has been published in 1987 (Donis-Keller et al., 1987). In addition to their role in human genome mapping, RFLPs are also being utilized for genome mapping of crop species such as pepper (*Capsicum annuum*), tomato (*Lycopersicon lycopersicum*) (Tanksley et al., 1988a), lettuce (*Lactuca sativa*) (Landry et al., 1987; Kesseli et al., 1992), rice (McCouch et al., 1988), *Arabidopsis thaliana* (Chang et al., 1988), maize (*Zea mays*) (Helentjaris, 1987), potato (*Solanum tuberosum*) (Bonierbale et al., 1988), soybean (Apuya et al., 1988; Roth et al., 1988; Keim et al., 1989), barley (*Hordeum vulgare*) (Heun et al., 1991), and common bean (*Phaseolus vulgaris*) (Nodari et al., 1993).

Mapping with RFLPs involves the application of molecular biological techniques to the basic concepts of transmission genetics. Single copy DNA is cloned from a species of interest and used as a probe to follow the segregation of homologous but polymorphic regions of the genome in individuals from segregating populations such as a F₂ or backcross (Landau-Ellis et al., 1991). Since many different copy clones can be tested, genetic linkage maps can be constructed which contains a very large number of markers at close intervals. The primary requirements for developing a RFLP-based linkage map of an organism are sexual reproduction and a source of single copy DNA clones, i.e., genomic DNA clones or cDNA clones. Single copy DNA can be found in any organism, and a number of methods have been devised for selectively cloning this fraction of plant genomes (Tanksley et al., 1988b). As markers for use in plant breeding programs, RFLPs differ from morphological

markers in several important ways: (1) the level of allelic variation for RFLP markers in natural plant population is much greater than that for morphological markers (e.g., Shoemaker et al., 1992); (2) the majority of RFLP markers are believed to be phenotype-neutral; and (3) RFLPs are apparently free of epistatic and environmental effects and a virtually limitless number can be monitored in a single population (Young and Tanksley, 1989a). Since RFLP markers are screened directly at the DNA level, they normally behave in a codominant manner, allowing the genotype of a locus to be determined in plants derived from any mating system (Diers et al., 1992).

Detecting Major Genes by Linkage with RFLP Markers

The utility of RFLP markers in plant breeding is based on finding linkages between these markers and genes of interest. There are a number of single gene traits that are frequently transferred from one genetic background to another by breeders. Genes conferring resistance to pathogens are classic examples. Traditionally, progeny are screened for the presence of disease resistance genes by inoculation with the pathogen. However, simultaneous or even sequential screening of plants with several different pathogens can be difficult or impractical. In contrast, detecting disease resistance genes by their linkage to RFLP markers makes it practical to screen for many different disease resistance genes simultaneously without the need to inoculate the population. Restriction fragment length polymorphism markers have been

identified that are tightly linked to genes for resistance to tomato mosaic virus (Young et al., 1988), bacterial speck, root knot nematodes, major genes controlling plant habit, fruit ripening properties (*u*) in tomato (Paterson et al., 1988), and mildew resistance locus in barley (Schuller et al., 1992). In lettuce, researchers have established linkages between RFLP markers and genes for resistance to downy mildew (Landry et al., 1987). Several other genes with qualitative effects have been mapped using near-isogenic lines combined with RFLP markers. Young et al. (1992) mapped a bruchid resistance gene in mungbean. In soybean, RFLP markers have been linked to genes such as nitrate tolerant symbiotic gene (*ns*) (Landau-Ellis et al., 1991), brown seed with black stripes (*r*), pentafoliate leaves (*Lf₁*) (Muehlbauer et al., 1991), *Phytophthora* resistance (*Rps*) loci, and ineffective nodulation gene (*Rj2*) (Diers et al., 1992).

The technique of backcross breeding was developed as a method to recover the more desirable genotype of the recurrent parent after the introduction of gene(s) from the donor plant. Introduction of RFLP techniques promises to overcome some limitations of backcross breeding. If the gene(s) to be transferred are tightly linked with RFLP markers, segregating populations of plants can be screened at the seedling stage. Even several donor parents can be used and the progeny screened at one time. In tomato, for example, genes that effect slight changes in fruit weight, pH, and soluble solids levels have been marked with RFLPs and are amenable to rapid transfer between varieties by the backcross method (Paterson et al., 1988).

Segregating progeny contain chromosomes that are mosaics of chromosomal pieces derived from parental varieties (Young and Tanksley, 1989a). It is important to consider the complete genome of individuals, as well as target genes, especially when the parents have large variation in other agronomic characters. Young and Tanksley (1989b) first used the concept of "graphical" genotype (numerical data expressed in graphical form) based on the RFLP data from backcross and F_2 populations in tomato, in which the segments of chromosome are referred to the parental origin.

Another important application of RFLP is map-based gene cloning (Young, 1990) or positional cloning (Bond et al., 1992; Gresshoff, 1993). Map-based cloning offers an independent method for gene isolation. Once tightly-linked RFLP markers are identified, the physical map then can be constructed by technique such as pulsed-field gel electrophoresis. Chromosome walking from one of the flanking RFLPs to the RFLP on the other side will result in cloning all the DNA between two RFLPs, including the target gene. Taking advantage of DNA transfer techniques, cloned DNA can be inserted into plants. In this sense, RFLPs makes it possible to use traits from other species, genera and even other kingdoms.

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PART II

Plant Genetic Study of Nodulation in Soybean

Abstract

Establishing an inoculum strain of *Bradyrhizobium japonicum* in the nodules of soybean (*Glycine max* L. Merr.) grown in soils populated by indigenous rhizobia has been a critical problem. The displacement of indigenous *B. japonicum* in soybean nodules in favor of a more effective inoculant strain has been suggested as an approach to enhance soybean productivity (Cregan and Keyser, 1986). The objectives of this study were to: 1) screen additional soybean genotypes for ones which restrict nodulation by certain *B. japonicum* stains; 2) study the genetic pattern of restricted nodulation; and 3) investigate at which stage the nodule development is blocked. Two preliminary screenings of twenty three soybean accessions, including *Glycine soja* (PI 468.397), indicated that 'TN 4-86', BARC-2(Rj4), and PI 468.397 showed restricted nodulation with *B. japonicum* strain 61A101C. Cultivar Essex gave effective nodulation. Average number of nodules per plant for TN 4-86, BARC-2(Rj4), PI 468.397, and Essex were 0, 0.08, 0, and 10.5, respectively. The F₁s of Essex X TN 4-86 and TN 4-86 X BARC-2(Rj4) showed an inability to nodulate with strain 61A101C; whereas the F₁ of Essex X PI 468.397 showed a normal nodulation response. Segregation of nodulation to restricted nodulation in the F₂ of Essex X PI 468.397 showed no significant difference ($P \leq 0.05$) from a 3 nodulation : 1 restricted nodulation ratio. All of the eighteen F₃ families from restricted nodulation F₂ plants showed restricted nodulation phenotype. The results indicate that the nodulation response in PI 468.397 on strain 61A101C is controlled by a single

recessive gene. In contrast, the results of the complementation test between TN 4-86 and BARC-2(*Rj4*) confirmed that the restricted nodulation response in TN 4-86 is controlled by a dominant gene which is allelic to gene *Rj4*. Investigation of the pedigree of TN 4-86 indicated that Peking might contribute the gene controlling restricted nodulation to TN 4-86. The study of kinetics of nodule initiation and development over a 14 d period after inoculation with strain 61A101C was conducted on primary roots of TN 4-86, BARC-2(*Rj4*), PI 468.397, and Essex (used as nodulation control and uninoculated control) using a modified fast clearing and staining method. There was no significant difference among the number of markedly curled root hairs (MC) on the primary roots of the four accessions 2 d after inoculation. The development of cortical cell division foci (CDF) beyond stage III was rare in TN 4-86 and BARC-2(*Rj4*), but a few stage IV developments were observed in PI 468.397. The average number of nodules per plant 34 d after inoculation for TN 4-86, BARC-2(*Rj4*), PI 468.397, and Essex was 0.03, 0, 0.04, and 6.8, respectively. The kinetic study of nodule development indicated that the blockage of nodule development in BARC-2(*Rj4*), TN 4-86, and PI 468.397 did not occur during nodule initiation, but during the stage of cortical cell division. The small number of nodules that developed on TN 4-86, BARC-2(*Rj4*), and PI 468.397, was located on lateral roots. Bacteria were isolated from the nodules that developed on TN 4-86, BARC-2(*Rj4*), and PI 468.397 and was inoculated back to TN 4-86, BARC-2(*Rj4*), and PI 468.397. No nodules developed on any of the three accessions from the back inoculation.

CHAPTER 1

Introduction and Literature Review

A major emphasis of recent research in the field of nitrogen fixation has been the development and application of genetic engineering techniques for the creation of rhizobial strains with enhanced nitrogen fixation. However, inoculation of legumes with improved (*Brady*)*rhizobium* is usually not enough to bring about large yield increases on its own. The major problems are highly competitive indigenous (*Brady*)*rhizobium* in the soil and interaction between (*Brady*)*rhizobium* strains and host genotypes. In the major soybean production regions of the United States, those indigenous rhizobia belong to certain serogroups of *Bradyrhizobium* and have become established as dominant soybean nodule occupants. A number of studies have demonstrated that even at high inoculation rates, the proportion of nodules formed by an inoculum strain is generally low (Johnson et al., 1965; Caldwell and Vest, 1970; Kapusta and Rouwenhorst, 1973; Weaver and Frederick, 1974; Kuykendall and Weber, 1978; Reyes and Schmidt, 1979; Miller, 1979).

In a competition study by Ames-Gottfred and Christie (1989) between indigenous *Rhizobium leguminosarum* biovar *trifolii* strains and inoculum strains or

between mixture of inoculum strains, the authors showed that inoculum strains occupied 17.5% to 85% of the nodules. None of the strains were capable of completely overcoming resident rhizobia. They also noted no relationship between strain effectiveness and competitive ability or between strain recovery and host cultivar.

Caldwell and Vest (1970) compared the yield of five different soybean cultivars when inoculated separately with 30 soybean rhizobia strains in four field environments, three of these environments were free of *B. japonicum*. The yield of soybean cultivars inoculated with USDA 123 ranked sixteenth among the 30 strains. Calculations from data presented by Ham (1980) indicated that, averaged across soybean cultivars grown in soil free of indigenous soybean rhizobia, seed yield with USDA 123 ranked last among seven strains tested. These reports suggested that the displacement of serogroup 123 in favor of more efficient strains should result in increased soybean production.

One method to alter the strain of *Rhizobium* found in the nodules of soybean would be the development of cultivars in which nodulation by indigenous rhizobia is eliminated or substantially reduced. In soybean, genotypes exhibiting restricted nodulation with specific strains or serogroups of rhizobia have been reported. The first recessive soybean gene (*no*) controlling non-nodulation was reported by Williams and Lynch (1954). Later, this gene was renamed as *rj1* (Caldwell, 1966). A single dominant gene, *Rj2* (Caldwell, 1966) conditions ineffective nodulation with all available strains of the USDA 122 and c1 serogroups (Caldwell et al., 1966). The

single dominant gene, *Rj4*, conditions ineffective nodulation with USDA 61 (Vest and Caldwell, 1972). Devine (1984) found a dominant allele, segregating in a Mendelian fashion that conditions the ineffective nodulation response of 'Kent' soybean with *R. fredii* strain USDA 205. Mathews et al. (1989) discovered a recessive non-nodulation gene in nod139 which is not allelic to *rj1*. It was named as *rj6* (Pracht et al., 1993). Cregan and Keyser (1986) and Cregan et al. (1989) found that some soybean PI genotypes in maturity Group IV and V excluded strain USDA 123 in favor of the inoculum strains. When inoculated with USDA 110, those PI genotypes showed similar nodulation and nitrogen accumulation to 'Williams'. Cregan and Keyser (1988) reported that *G. soja* (PI 468.397) substantially reduced the competitive ability of *B. japonicum* strain USDA 123 in relation to *R. fredii* strain USDA 193. Their results suggested that the trait identified in the PI genotypes could positively impact soybean productivity by excluding all or part of the indigenous serogroup 123 population in favor of more effective strains of *B. japonicum*.

Anatomical analysis of nodule initiation and development indicated that soybean roots are infected and nodulated through root hair cells on the epidermal surface of the root (Bhuvaneswari et al., 1980; Turgeon and Bauer, 1982). The susceptibility of soybean root hairs to infection and nodulation is developmentally restricted and transient (Bhuvaneswari et al., 1980). Infections and nodules on the primary root develop most frequently in the region just below the smallest emerging root hairs present at the time of inoculation. Root hairs which are already emerged at the time of inoculation are less susceptible to infection (Calvert et al., 1984). Mature,

fully elongated root hairs are not normally susceptible to infection. Acropetal development of the root causes root hairs to acquire and then lose their susceptibility to *Rhizobium* within a period of 4 to 6 h (Bhuvaneswari et al., 1980) or suppresses development of initiated infections into nodules (Calvert et al., 1984). Anatomical and physiological studies in soybean indicate that nodule formation in younger regions of the root is blocked during infection development rather than during infection initiation (Gerahty et al., 1992). Caetano-Anollés et al. (1991) found that both mature nodules and root tips elicit feedback regulation of nodule formation. Caetano-Anollés and Gresshoff (1991) proposed that nodules develop a suppressive field around themselves. Removal of mature nodules and lateral root tips releases the suppression over that region, thus allowing the arrested infection foci to develop into nodules. Furthermore, they proposed that there are two systemic messengers involved in the autoregulation. However, the mechanism of non-nodulation or restricted nodulation still is mysterious. There have been only a few studies on nodulation blockage in non-nodulation soybeans (Mathews et al., 1987; Suganuma et al., 1990).

Soybean genotypes which restrict nodulation can be divided into three categories: (1) those that restrict nodulation with a broad range or all strains of *Bradyrhizobium*, [e.g. the soybean with gene *rj1* (Caldwell, 1966), *nod1* and *nod3* (Carroll et al., 1986)], (2) those that carry a number of alleles conditioning restricted nodulation by specific strains, [e.g. the soybean with *Rj3* (Vest, 1970)], and (3) those that restrict nodulation with the strains from the same serogroup, [e.g. the soybean with *Rj2*, *Rj4* (Caldwell, 1966; Vest and Caldwell, 1972)]. Soybeans genotypes in the

first category are useless in terms of replacing indigenous strains by inoculum strains. The second category of soybeans will only be useful in replacing the one or limited indigenous strains or old commercial inoculum by newly released one if restriction of nodulation is under the control of a single gene. Soybeans in the third category may be the most valuable in replacing certain group strains by an improved inoculant strain.

In order to replace the indigenous or old commercial strains by more effectively nitrogen fixing strains, the first step is to search for the genes conditioning restricted nodulation with certain strains. Studies on the frequency of ineffective nodulation response in plant introduction lines of *G. max* and *G. soja* with strains USDA 7 (defining the ineffective nodulation response of *Rj2* phenotype) and USDA 61 (defining the ineffective nodulation response of *Rj4* phenotype) indicated that there was clear variation in the frequency of ineffective nodulation based on geographic origin of the PIs (Devine and Breithaupt, 1981; Devine, 1987). Ineffective nodulation with strain USDA 61 was found most frequently in PIs from southeast Asia; whereas, ineffective nodulation with strain USDA 7 was found most frequently in accessions from the vicinity of the Chinese cities of Nanking and Hangchow. They also found that the frequency of ineffective nodulation in *G. soja* with both USDA 7 and USDA 61 was higher than in *G. max*. It is not clear whether *G. soja* has a higher frequency in other ineffective or restricted nodulation genes.

The objectives of this research were to: (1) screen additional soybean genotypes for restricted nodulation by certain *B. japonicum* strains; (2) study the

genetic pattern of restricted nodulation; and (3) investigate at which stage the nodulation development is blocked.

CHAPTER 2

Materials and Methods

Preliminary Screening

***Bradyrhizobium* Cultures:** The strains of *B. japonicum*, USDA 94, USDA 135, USDA 110, and 61A101C were obtained from Dr. Gary Stacey, Department of Microbiology, the University of Tennessee, Knoxville. These strains are wild type *B. japonicum* and can nodulate on *G. max* cultivars. The strains USDA 94 and 61A101C belong to DNA homology group II. Strain 61A101C is a commercial strain (Nitragen Co., Milwaukee, WI). Strains in DNA homology group II have been designated as *B. elkanii* (Kuykendall et al., 1992). USDA 135 is in the DNA homology group I. USDA 110 is a member of DNA homology group Ia. Stock cultures were maintained in yeast extract-mannitol-gluconate (YEMG) semisoft medium. Bacteria were grown in YEMG liquid medium to a stationary phase and then subcultured after 100-fold dilution in 30 ml of fresh medium in a rotary shaker at 150 rpm and 27° C. Bacteria from early exponential-phase culture ($OD_{620}=0.3-0.4$) were diluted with YEMG medium to a concentration of 10^8 cells ml⁻¹.

Preliminary Screenings: Soybean accessions [*G. max* (L.) Merr. and *G. soja* Sieb. & Zucc.] used in this study are listed in the Table 2-1. Seeds of *G. soja* (PI 468.397) were scarified by soaking in concentrated sulfuric acid for 10 min. The seeds were then surface sterilized for 5 min with 2.6% sodium hypochlorite, followed by five rinses with deionized water. Seeds of *G. max* accessions were sterilized by soaking in the 2.6% sodium hypochlorite for 5 min, followed by five rinses with deionized water. The sterilized seeds were germinated in the dark at room temperature (about 24° C) on sterile cheesecloth saturated with modified Herridge's plant nutrient solution (Delves et al., 1986).

First Preliminary Screening: After two days, five seedlings of each accession, at a similar stage of emerging radicle, were planted in rows in growth trays containing sterile vermiculite. Five or six accessions were planted per tray and placed in a greenhouse. Each accession was replicated three times. Prior to planting, the floor of greenhouse and benches were sterilized with commercial sodium hypochlorite bleach (5.25%). Each row of seedlings was inoculated with 5 ml of bacterial dilution (about 10^8 cells ml⁻¹). The plants inoculated with different strains were kept separately. Water was supplied during first 10 d, then half-strength nutrient solution was supplied to the plants. The temperature in the greenhouse was maintained between 20 to 30° C. The plants were grown under a 14 h photoperiod. At 28 d post-inoculation, the roots of each plant were cleaned and the number of nodules of each plant was counted.

Table 2-1. Name, characteristics, and maturity group of soybean genotypes used in the study

Entry name	Characteristics	Maturity Group
<i>Glycine max</i>		
1138-2†	cultivar	V
BARC-4(<i>Rj2</i>)‡	ineffective nodulation (<i>Rj2</i>)	IV
BARC-5(<i>rj2</i>)‡	nodulating	IV
BARC-2(<i>Rj4</i>)‡	ineffective nodulation (<i>Rj4</i>)	IV
BARC-3(<i>rj4</i>)‡	nodulating	IV
CNS	cultivar	
Essex	cultivar	IV
Fayette	cultivar	V
Hutcheson	cultivar	V
Manchu	cultivar	
Mandarin	cultivar	
McCall	cultivar	IV
N79-2077	breeding line	V
nod49§	non-nodulation mutant (<i>rj1</i>)	VI
Peking	cultivar	IV
Pershing	cultivar	IV
PI 88.788	plant introduction	
PI 437.654	plant introduction	
S100	cultivar	
Suxie I†	cultivar	V
TN 4-86	cultivar	IV
TN83-26	breeding line	V
<i>Glycine soja</i>		
PI 468.397§	plant introduction	not determined

†: Seed provided by Professor Junyi Gai, Nanjing Agricultural University, Nanjing, Peoples' Republic of China

‡: Isolines of 'Clark' (Devine and O'Neill, 1986, 1987). Seed provided by Dr. Tom Devine, USDA/ARS, Beltsville, MD.

§: Seed provided by Professor Peter M. Gresshoff, Racheff Chair of Excellence, Plant Molecular Genetics, University of Tennessee

Second Preliminary Screening: Uniform seedlings of each of above soybean accessions were transferred to plastic growth pouches (Vaughan's Seed Co., Downers Grove, IL) containing 10 ml of nutrient solution for *G. max* accessions and 7 ml of nutrient solution for *G. soja* (PI 468.397). Pouches were grouped together by *B. japonicum* strains and maintained in the plastic containers separately. Containers were covered with aluminum foil until inoculation. Each soybean plant was inoculated with 250 μ l of bacterial dilution 4 d after seed germination. The pouches were covered by aluminum foil to protect the plant roots from light. After inoculation the seedlings were cultured in a growth chamber with light intensity of 350 μ E M⁻² sec⁻¹ with a 24° C day/22° C night temperature cycle and a 16 h photoperiod. Additional plant nutrient solution was added as required. After 21 d post-inoculation, the roots of each plant were cleaned and the number of nodules of each plant was counted.

Hybridization and Screening of F₁, F₂, and F₃

According to the results of two preliminary screenings, efforts were focused on the bacterial strain 61A101C. TN 4-86, BARC-2(*Rj4*), and PI 468.397 were chosen to represent restricted nodulation parents and Essex was chosen as a normal nodulation parent to study their genetic pattern. All possible combinations among four accessions except Essex X BARC-2(*Rj4*) were made at the Knoxville Plant Science Unit, in 1990 and 1991. The crosses of TN 4-86 X BARC-2(*Rj4*) and Essex X TN 4-86 were made to study the genetic pattern of restricted nodulation of TN 4-86

and the crosses of BARC-2(*Rj4*) X PI 468.397, Essex X PI 468.397, and TN 4-86 X PI 468.397 were made to study the genetic pattern of restricted nodulation of PI 468.397. Essex has grey pubescence, purple flower, yellow seed coat, and erect growth habit. TN 4-86 and BARC-2(*Rj4*) have tawny pubescence, purple flowers, and erect growth habit. PI 468.397 has tawny pubescence, purple flowers, black seed coat, small seed size, and viney growth habit. In the crosses of Essex X TN 4-86 and Essex X PI 468.397, Essex was chosen as the female parent so that any F_1 plant with grey pubescence (selfing) were discarded. Also in the combination of Essex X PI 468.397, the F_1 showed intermediate viney growth habit. Verification of F_1 hybrids was confirmed by flower color, pubescence color, seed coat color, and plant growth habit. Also, RFLP makers were used to verify F_1 hybrid (data not shown). The F_1 s were planted in the greenhouse and after 2 weeks, were transplanted to the field. The confirmed F_1 plants were harvested separately.

The same methods utilized in the second preliminary screening were used to screen the F_1 , F_2 , and F_3 plants for nodulation response to strain 61A101C. Because of limited seeds, only part of the F_3 families from F_2 which showed restricted nodulation phenotype were screened to confirm the genotypes of F_2 s. In order to have enough leaves for DNA extraction, the photoperiod in the greenhouse was controlled at 24 h per day to lengthen the vegetative growth. Under this condition, only part of the F_2 plants set seed.

Blockage Study on Strain 61A101C

Observation of Nodule Development: Bacterial strain 61A101C and soybean PI 468.397, TN 4-86, BARC-2(Rj4), and Essex were chosen for the nodulation blockage study. The methods of bacterial and plant growth were the same as those used in the second preliminary screening. The position of the root tip (RT) and the smallest emerging root hair (EH) were observed via a stereo zoom dissection microscope at 12 X magnification and were marked on the pouches at the time of inoculation. The position of the root tip was marked 2, 4, 6, and 8 d following inoculation.

Five roots of each soybean accession were cut off 2 cm above the EH mark. Roots were cut at an angle and taped so that orientation of the root during observation could be maintained. The length (cm) of root growth in the 2-d increments were measured before the roots were cut off. The roots were cleaned using the hypochlorite clearing method of Truchet et al. (1989). The roots were soaked in a commercial sodium hypochlorite bleach (5.25%) for a total of 15 to 25 min in a vacuum desiccator, depending on the time of observation after inoculation and genotypes. After hypochlorite clearing, the roots were rinsed with deionized water, and stained for 15-30 min with a 0.002% solution of methylene blue. Observation was made at 96 X magnification with a stereo zoom dissection microscope. Each root was placed on a glass plate in the same orientation as found in the growth pouch, and was not rotated to view the underside. Counting of the nodule development stages started 2 cm above

the EH and their locations were expressed as relative distance units (RDU). One RDU is equivalent to the distance from EH to RT at the time of inoculation. The advantage of using RDU is to minimize the growth variation among plants (Bhuvaneswari et al. 1980).

The nodule development stages described here are after Calvert et al. (1984) with minor modification. By definition, a markedly curled root hair (MC) was one that curled back to the surface of the epidermal cells and stained dark blue. At stage I, the hypodermal cells beneath a curled root hair have divided anticlinally and the cell division activity has reached the center of the root cortex. At stage II, cell division activity has reached the inner cortex. The formation of the nodule meristem is the symbol of stage III. At stage IV, expression of the nodule meristem has caused a localized swelling on the root. This is the stage of nodule development called emergence. At stage V, the vascular differentiation is obvious between the nodule meristem and the root stele. At stage VI, the young nodule is visible at the root surface. Stage VII is defined as a fully developed nodule.

The number of markedly curled root hairs and cortical cell division centers in each 2 mm viewing field were counted on the primary roots.

Isolation of Bacteria Inoculated with Strain 61A101C from Nodules: Among the plants of TN 4-86, BARC-2(Rj4), and PI 468.397, a few plants developed nodules. Most of the nodules were located on the lateral roots. In order to confirm whether

these nodules were results of contamination, the nodules were cut from the plants in such a way that the nodules were not damaged. The nodules were washed with deionized water and immersed in 95% ethanol for 10 sec. The nodules were then transferred to 5.25% sodium hypochlorite for 4 min, followed by five rinses in deionized water. The sterilized nodules were crushed in deionized water to give a turbid suspension and a drop of suspension was transferred to the YEMG semisoft medium. The drop of suspension was stroked onto the medium surface. The plates were incubated in an inverted position at the 27° C for a week. The smooth surface single colony from each plate was cultured in the liquid medium according to the same conditions as mentioned above and inoculated back to the three soybean genotypes. The exponential-phase bacteria were frozen in glycerol and stored at -70° C.

CHAPTER 3

Results and Discussion

Genetic Study of Nodulation in Soybean

Preliminary Screenings: As expected, nod49 restricted nodulation to all four strains because it harbors a gene allelic to *rj1* (Table 2-2). The latter can restrict nodulation with a broad range of rhizobia (Williams and Lynch, 1954). TN 4-86, BARC-2(*Rj4*), Peking, and PI 468.397 showed restricted nodulation with strain 61A101C. BARC-2(*Rj4*) and Peking were expected to show the restricted nodulation with strain 61A101C because BARC-2(*Rj4*) and Peking carry the *Rj4* gene which restricts the nodulation by part of strains in *B. japonicum* DNA homology group II, for example, USDA 61, USDA 62, USDA 83, USDA 94, USDA 238, and USDA 259 (Devine and O'Neill, 1989) and 61A101C is a group II strain. Cultivar Essex gave effective nodulation. The average number of nodules per plant for TN 4-86, BARC-2(*Rj4*), PI 468.397, Peking, and Essex were 0, 0.08, 0, 0, and 10.5, respectively. Even though BARC-2(*Rj4*) has a dominant gene (*Rj4*) restricting nodulation with *B. japonicum* strains in DNA homology group II, it still has a few nodules when

Table 2-2. Average number† of nodules formed per plant for 23 soybean entries inoculated with four strains of *Bradyrhizobium japonicum*

Genotype	<i>B. japonicum</i> strain			
	USDA 94	USDA 110	61A101C	USDA 135
<i>Glycine max</i>	Nodules/plant and standard error			
1138-2	2.8±0.6(6.5±1.2)	2.5±0.7(7.1±1.1)	3.0±0‡(9.3±0.6)	1.0±0.4(1.6±0.5)
BARC-4(Rj2)	4.8±1.1(5.9±0.9)	7.2±0.8(3.5±1.6)	4.8±1.0(6±1.0)	2.3±0.6(2.8±1.2)
BARC-5(rj2)	5.0±1.1(4.6±0.7)	1.0±0.5(4±0.7)	1.5±0.2(3.8±0.8)	1.2±0.6(1.3±0.7)
BARC-2(Rj4)	0.3±0.2(0)	2.3±1.0(8.5±0.7)	0(0.1±0.2)	1.1±0.3(2.6±0.7)
BARC-3(rj4)	7.2±1.6(8.4±1.6)	4.0±0.6(7±0.7)	2.0±1.0(7.2±1.3)	1.4±0.6(1.9±0.5)
CNS	9.8±4.8(7.7±1.1)	2.3±0.7(7.1±0.5)	3.0±1.0(10.5±1.5)	1.6±0.8(1.7±0.3)
Essex	31.9±2.7(6.8±1.2)	11.3±1.2(5.8±1.0)	13.5±3.1(9.4±1.3)	9.7±1.4(1.6±1.0)
Fayette	3.3±1.1(4.4±0.6)	0(6.6±1.6)	3.0±2.0(5.8±1.5)	0.2±0.2(3±1.4)
Hutcheson	4.2±0.7(8.3±1.2)	4.8±1.7(9.4±1.3)	4.2±1.6(9.3±0.8)	3.1±0.8(0.8±0.3)
Manchu	1.0±0(5.4±0.6)	2.0±0‡(5.3±0.9)	2.5±1.6(5.7±1.1)	5.7±3.2(2.4±0.6)
Mandarin	9.8±2.0(6.6±1.5)	4.0±0.6(4.4±0.7)	4.6±1.1(6.8±1.4)	6.5±0.7(1.4±0.5)
McCall	5.6±1.7(5.9±0.8)	6.0±0.7(10.7±0.9)	2.3±0.8(8.2±1.1)	2.4±0.4(2.5±0.7)
N79-2077	4.6±0.8(8.6±1.8)	6.9±1.5(6.9±1.1)	2.4±0.2(8.4±1.6)	2.5±1.2(0.3±0.2)
nod49	0(0)	0(0)	0(0)	0(0)
Peking	0(0.1±0.1)	2.4±0.6(5.1±0.8)	0(0)	1.2±0.5(1±0.4)
Pershing	3.0±0.9(6±0.8)	6.2±1.3(8.8±1.5)	5.5±1.5(5±1.1)	0.3±0.2(0.1±0.1)
PI 88.788	3.4±1.2(5.3±0.8)	2.9±0.5(5.1±1.1)	2.5±0.7(6.3±0.6)	2.0±0.7(1±0.4)
PI 437.654	3.6±0.5(5.8±0.5)	6.7±0.7(6.8±0.6)	5.0±1.2(5.8±1.1)	2.8±0.9(0.1±0.1)
S100	8.0±1.3(4.8±1.3)	3.7±0.9(9‡)	5.0±0.5(4.5±1.0)	2.8±0.7(1.9±0.7)
Suxie I	5.7±0.6(9.4±1.3)	4.0±1.9(8±0.9)	6.3±0.7(10.1±1.5)	5.5±1.1(0.8±0.6)
TN 4-86	5.0±1.0(0)	3.3±0.3(4.3±0.6)	0(0)	3.7±0.8(2.5±0.5)
TN83-26	6.0±1.0(10.7±2.9)	7.7±2.2(7.5±3.5)	4.8±1.4(11.2±2.3)	2.8±0.6(0.4±0.2)
<i>Glycine soja</i>				
PI 468.397	3.5±0.4(0.1±0.1)	3.5±1.5(2.2±0.7)	0(0)	2.9±0.96(0)
Uninoculated controls	0(0)	0(0)	0(0)	0(0)

† Number outside the parentheses are from the first preliminary screening in the greenhouse and those inside parentheses are from the second preliminary screening in growth pouches in growth chamber.

‡ only one plant.

inoculated with 61A101C. Whether the nodules formed on BARC-2(*Rj4*) were caused by contamination will be discussed later. The preliminary screenings indicated that TN 4-86 and PI 468.397 carry a gene(s) which restricts nodulation by strain 61A101C. The difference in results between two screenings on certain soybean accession and strain combinations (e.g., TN 4-86/USDA 94) might be due to the different methods used in screenings. There could be two reasons for this phenomenon. The first is that the plants grown in the plastic trays became contaminated with bacteria from dust in the air. However, this does not appear to be the cause, since the uninoculated controls did not develop nodules. The second possible reason is that plants had more conducive environmental conditions for nodulation when growing in vermiculite than in pouches.

Hybridization and Screening of F_1 , F_2 , and F_3 : The results of two preliminary screenings indicated that TN 4-86 and PI 468.397 carry gene(s) restricting nodulation with strain 61A101C. The F_1 of Essex X TN 4-86 showed an inability to nodulate with strain 61A101C; whereas the F_1 of Essex X PI 468.397 showed an effective nodulation response (Table 2-3). One F_1 plant from Essex X PI 468.397 did not show nodulation, but most likely because it was a small weak plant. The above results indicate that TN 4-86 carries a dominant gene controlling restricted nodulation. In contrast, PI 468.397 appears to carry a recessive gene controlling restricted nodulation. The segregation ratio of nodulation to restricted nodulation in F_2 of Essex X PI 468.397 showed no significant difference ($P \leq 0.05$) from 3:1 ratio (Table 2-3).

Table 2-3. Nodulation response of parents, F₁, F₂, and selected F₃ families to strain 61A101C.

Parents	Uninoculated	Inoculated
	Nodules/plant and standard error	
BARC-2(<i>Rj4</i>)	0	0.06±0.03
TN 4-86	0	0.04±0.01
PI 468.397	0	0
Essex	0	11.5±1.0

F ₁	Nodulation	Restricted nodulation		
	No. of F ₁ plant	Nodules/plant and standard error		
BARC-2(<i>Rj4</i>) X PI 468.397	3†	10	0.2±0.1	
Essex X PI 468.397	7	1	8.8±1.8	
TN 4-86 X PI 468.397	3†	10	0.2±0.1	
TN 4-86 X BARC-2(<i>Rj4</i>)	3†	7	0.3±0.2	
Essex X TN 4-86	2†	12	0.1±0.1	

F ₂	No. of F ₂ plants		Ratio	P>χ ²
Essex X PI 468.397	150	66	3:1	0.08
TN 4-86 X BARC-2(<i>Rj4</i>)	1‡	140	0:1	0.995

F ₃ families	No. of families	
Essex X PI 468.397	0	18§

† One nodule per plant.

‡ One F₂ plant had one nodule on a lateral root.§ These 18 families came from 18 F₂ plants in second screening and are a subset of the total 66 restricted nodulation plants.

All of eighteen F₃ families from restricted nodulating F₂ plants tested showed restricted nodulation phenotype. This indicates that the nodulation response in PI 468.397 is controlled by a single recessive gene. In contrast, the F₁ of TN 4-86 X BARC-2(*Rj4*) showed similar phenotype as that of TN 4-86 and BARC-2(*Rj4*). Among 141 F₂ plants of TN 4-86 X BARC-2(*Rj4*), one plant had one nodule on a lateral root. The rest showed restricted nodulation phenotype. The same phenomenon was observed on TN 4-86 and BARC-2(*Rj4*) in preliminary screenings. These results indicated that the restricted nodulation response in TN 4-86 is controlled by one dominant gene which is the same or allelic to *Rj4*. Otherwise, if the gene(s) controlling restricted nodulation to strain 61A101C in TN 4-86 is different from *Rj4*, the phenotype of F₂ population should segregate at the ratio of 15 restricted nodulation : 1 nodulation (one gene difference) or 63:1 (two gene difference) etc.. From the study of the pedigree of TN 4-86, it was found that TN 4-86 descended from the cultivar Peking (Allen and Bhardwaj, 1987; Allen et al., 1988) which harbors the *Rj4* gene (Devine and O'Neill, 1989). It is possible that TN 4-86 received the *Rj4* gene from Peking. TN 4-86 is a commercial variety developed by the Tennessee Agricultural Experimental Station. The selection and yield evaluation of TN 4-86 was performed at the Knoxville Plant Science Unit and throughout the southern region (Allen et al., 1988). The USDA 94 and USDA 76 are indigenous *B. japonicum* strains in the Tennessee region and belong to DNA homology group II. These strains are poorly adapted to efficient symbiosis with the soybean and cause foliar chlorosis (Devine et al., 1990). Elimination or reduction of nodules formed by

group II strains on TN 4-86 by gene *Rj4* would allow other strains to form nodules which fix nitrogen more efficiently and thus may be a partial explanation of how TN 4-86 produces high yield.

G. soja is considered to be the wild progenitor species from which *G. max* was domesticated in eastern Asia (Hymowitz, 1970). *G. soja* is interfertile with *G. max* and, therefore, is a potential source for introgression of genes with agricultural value, such as disease, insect, and nematode resistance, into *G. max*. However, if *G. soja* is used in breeding programs as a source of agronomically desirable alleles, potential linkage or random chance could lead to indiscriminate introgression of genes controlling undesirable traits from *G. soja* to *G. max* in derived lines. On the other hand, the higher frequency of ineffective or restricted nodulating gene was found in *G. soja*. Thus, *G. soja* might give a better chance to find restricted nodulation genes. In both cases, efficient methods are needed for isolation and transformation of those genes to avoid incorporation of the undesirable traits from *G. soja* into *G. max*.

Nodulation Blockage Studies

Several genes involved in conditioning non-nodulation and ineffective nodulation have been discovered. A precise evaluation of the interaction between a given rhizobia strain and a given legume cultivar is essential for the genetic analysis of the nodulation process. The stages of nodulation development have been defined in several legume species (Calvert et al. 1984, Dudley et al. 1987). A few studies have

been carried out to detect at which stage nodulation development is blocked by host genes (Mathews et al. 1987, Suganuma et al. 1990). The studies reported herein focus on the nodulation process in four soybean genotypes inoculated with *B. japonicum* strain 61A101C.

Nodule Development Stages on Four Genotypes: Visual inspection of nodulation is not sufficient for the study of the nodule development process. On the other hand, microscopic evaluation of embedded and sectioned infection root tissue, such as described by Turgeon and Bauer (1982) and Calvert et al. (1984), is too time-consuming to be used for examining the stages of nodulation on a large number of samples. To facilitate such a screening, faster methods which allow the visualization of the inner histology of the nodule have been developed and used in the study of *Rhizobium*-legume symbiosis (Vasse and Truchet 1984; Truchet et al., 1989).

The examples of nodule development stages of Essex when inoculated with bacterial strain 61A101C are shown in Fig. 2-1. At 2, 4, 6, 10, and 14 d following inoculation five plants of each accession were harvested and their roots prepared for infection study. At 2 d after inoculation, curled root hairs were observed (Fig. 2-1A). At 4 d after inoculation the hypodermal cell beneath a curled root hair divided anticlinally and reached the center of the root cortex (Fig. 2-1B). The cell division continued and reached the inner cortex. At the same time, some cells of the outer cortex have divided obliquely and formed young meristem (Fig. 2-1C). The activity of the nodule meristem then caused a localized swelling of the root (Fig. 2-1D). This

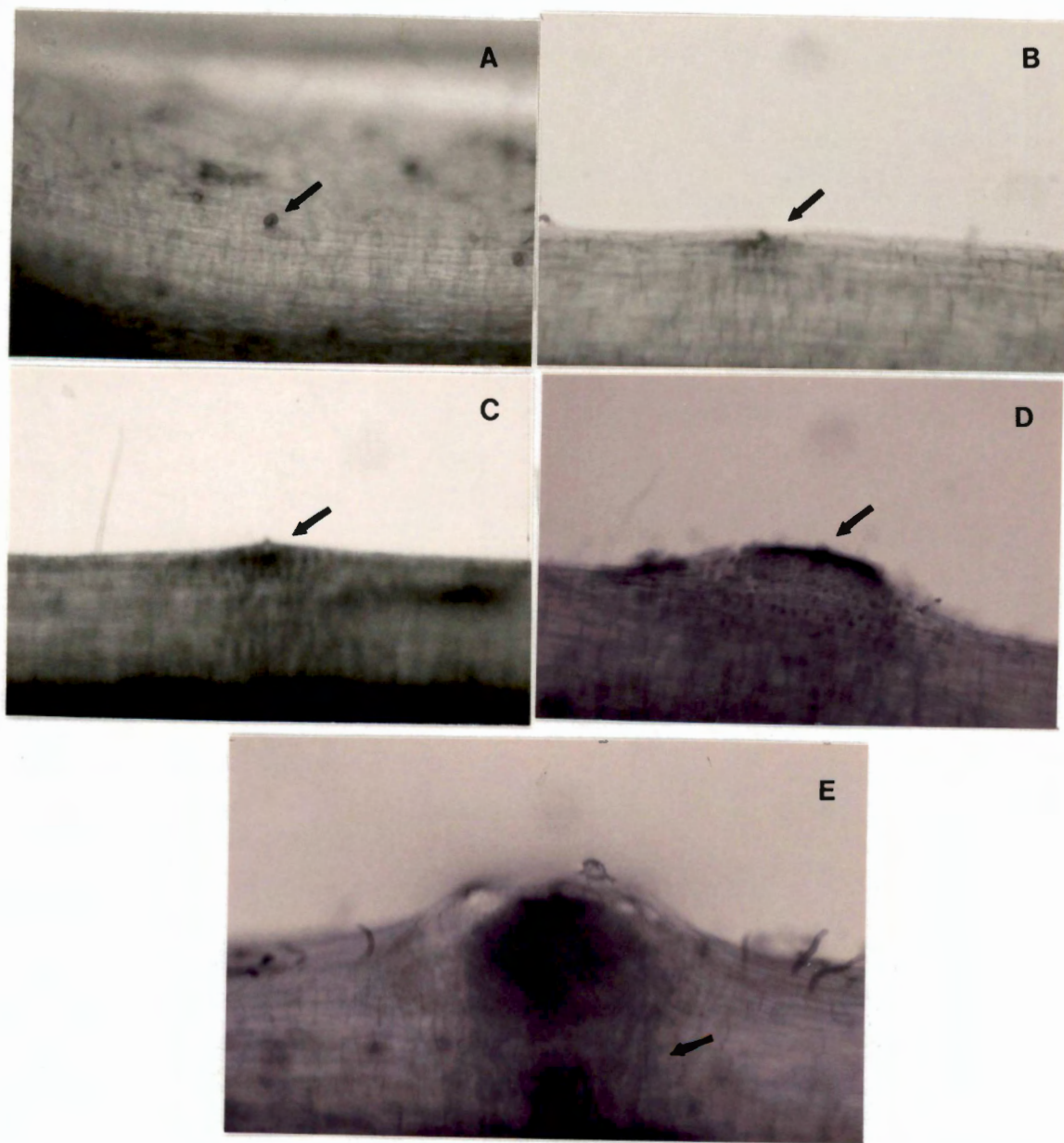


Fig. 2-1. Examples of nodule development stages in Essex. Root hair curling (A), cortical cell division (B), young meristem (C), nodule emergence (D), and nodule vascular branch (E).

is the stage of nodule development called emergence. Subsequently, the vascular branch formed, connecting the nodule vascular system with the root stele (Fig. 2-1E). A few cortical cell division centers without curled root hairs were also observed on Essex roots. The same phenomenon was observed by Calvert et al. (1984). These cortical cell division centers might be caused by Nod factor produced by bacteria without bacterial infection.

The markedly curled root hairs were observed on the primary roots of four genotypes inoculated with strain 61A101C. Most markedly curled root hairs appeared between the smallest emerging root hairs (EH) and root tip (RT). The average distances between EH and RT were 10.9 ± 1.3 , 10.1 ± 1.5 , 8.5 ± 1.0 , and 7.5 ± 1.6 mm for Essex, TN 4-86, BARC-2(*Rj4*) and PI 468.397, respectively. Root hairs were not present on this part of the root at the time of inoculation, but they developed acropetally throughout this region after inoculation. However, a few markedly curled root hairs above the EH were observed. This suggests that some young root hairs above EH can be infected by the rhizobia. Quantitatively, there were no significant differences in the number and distribution of markedly curled root hairs in the primary roots of Essex, TN 4-86, BARC-2(*Rj4*), and PI 468.397 2 d after inoculation (Fig.2-2). The total number of MC were 42, 52, 56, and 37 for BARC-2(*Rj4*), Essex, TN 4-86, and PI 468.397, respectively. Thus, the blockage of nodulation in TN 4-86, BARC-2(*Rj4*), and PI 468.397 occurs after MC stage. Suganuma et al. (1990) showed that the number of bacteria detected in the roots of *rj1* and wild type *Rj1* soybean isolines were similar, but the percentage of root hair curling was reduced in roots of

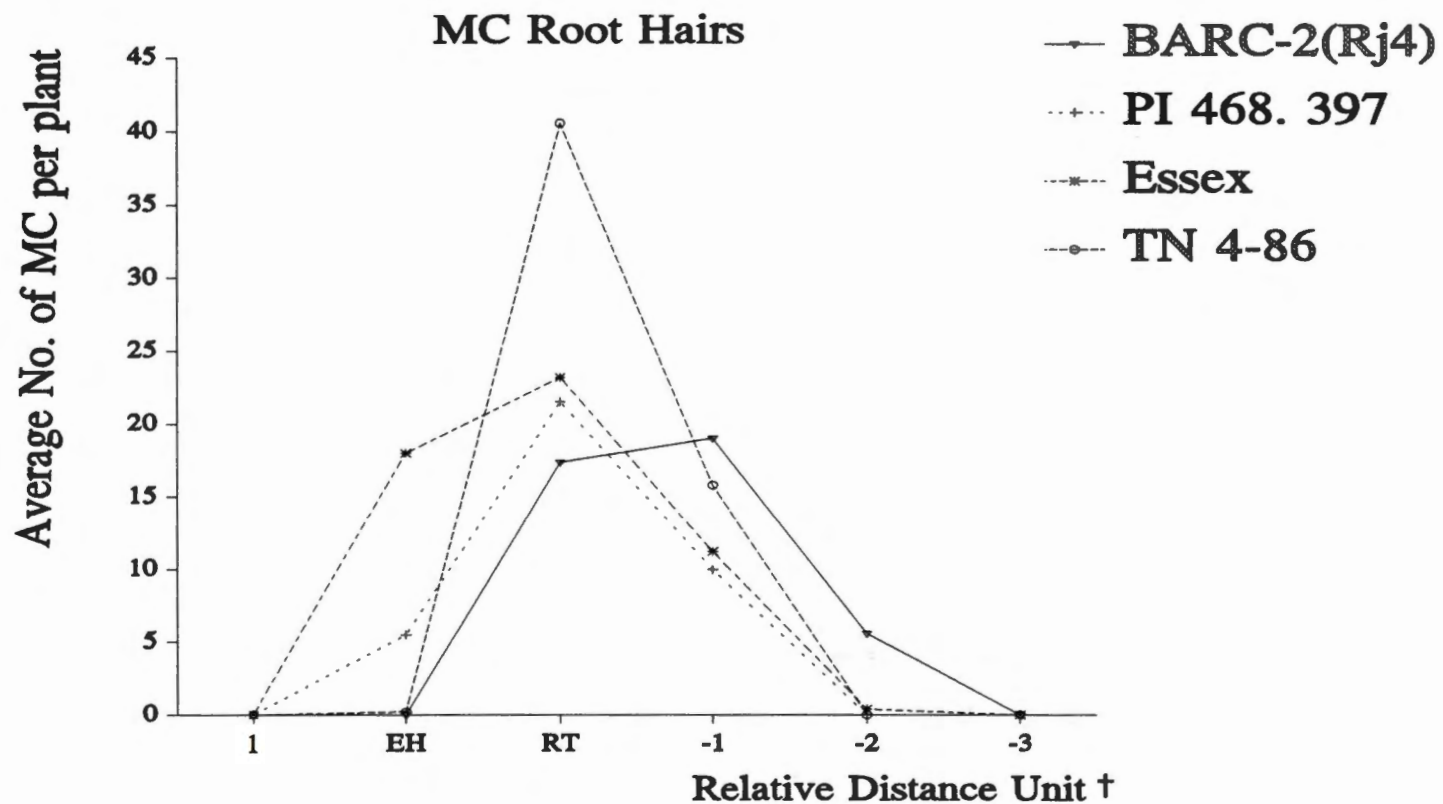


Fig. 2-2. The distribution of markedly curled root hairs (MC) along the primary roots 2 d after inoculation.
 †: Distance from smallest emerging root hair (EH) on a primary root to the root tip (RT) at the time of inoculation.

rj1 soybean, as compared with *Rj1* soybean. Their results indicated that in the *rj1* soybean blockage occurs at the stage of root hair curling and not at the stage of bacterial adsorption. In contrast, Mathews et al. (1987) reported that there was no root hair curling in the *rj1* soybean mutant (nod49). However, a few nodules were formed on nod49 with high dose of bacteria. The difference in results might be due to the different genetic backgrounds, the bacterial strain, and the concentration of bacteria used in the studies.

By 4 d after inoculation nodule development had progressed to stage II in Essex, BARC-2(*Rj4*), and TN 4-86 but not PI 468.397 (Table 2-4). The number of stage I developments was lower in PI 468.397 and BARC-2(*Rj4*) than in Essex. PI 468.397 had lowest number of stage I developments partly because of its smaller root surface and slower development of nodules compared to the genotypes of *G. max*.

Six d after inoculation, Essex had the highest and PI 468.397 the lowest number of MC. Furthermore, about 22% of the infections had developed to stage I or stage II in Essex and PI 468.397. In contrast, less than 1% of infections had developed to stage I or stage II in BARC-2(*Rj4*) and TN 4-86 (Table 2-4). Stage VI developments were observed on the primary roots of Essex 10 d after inoculation. No developments beyond stage III were observed in TN 4-86 and BARC-2(*Rj4*) at 10 d after inoculation (Table 2-4). A few stage IV were detected on the roots of PI 468.397, but no further stages were observed at 10 d post-inoculation.

The distribution of nodules on the primary roots of Essex was around the

Table 2-4. The number of nodule development stages on the primary roots of four genotypes 4, 6, 10, and 14 days after inoculation.

Days after inoculation	Genotype	Stages							
		MC	I	II	III	IV	V	VI	VII
4	Essex	56±5.3†	11.4±7.2	1.2±0.7					
	BARC-2(Rj4)	46±10.6	6.2±4.0	1.4±1.0					
	TN 4-86	65±6.0	21.8±3.8	2.8±1.0					
	PI 468.397	32.8±7.4	1.7±0.9						
6	Essex	130±19	17±1.1	20±16.2	0.8±0.3				
	BARC-2(Rj4)	58±15.6	0.3±0.3						
	TN 4-86	79±38	0.5±0.5	0.3±0.3					
	PI 468.397	10±4.0	1.8±0.7	1.6±0.7					
10	Essex	25±8.3	44±14.5	61±12.4	7.6±4.3	1±0.6	0.6±0.6	0.4±0.4	
	BARC-2(Rj4)	39±14.3	4±2.4	2.3±1.3	0.5±0.5				
	TN 4-86	53±13.4	0.2±0.2						
	PI 468.397	19±4.3	3.4±1.7	0.4±0.2	0	0.2±0.2			
14	Essex	154.6±34	33±9.8	18.8±6.0	11.8±4.2	1.4±0.5	1.4±1.0	0	0.6±0.6
	BARC-2(Rj4)	51.6±15.2	6.2±2.2	3.6±1.2					
	TN 4-86	55.2±14.8	0.2±0.2	0	0.2±0.2				
	PI 468.397	8±3.6	2.6±0.8	0.8±0.4	0.6±0.4	0.2±0.2			

†: Average number and standard error from five primary roots of five different plants at each sampling stage.

region between EH and RT at the time of inoculation. Most of the nodules were located within 3 RDU beyond EH (Fig. 2-3). One nodule was found on lateral root of one plant in both TN 4-86 and PI 468.397 among 31 and 28 plants, respectively. No nodules formed on the roots of any of the BARC-2(*Rj4*) plants. The average number of nodules per plant 34 days after inoculation for TN 4-86, BARC-2(*Rj4*), PI 468.397, and Essex were 0.03, 0, 0.04, and 6.8, respectively. Uninoculated controls of Essex did not develop nodules.

The mechanism by which the non-nodulation or restricted nodulation genes alter the nodulation process is still unknown. Mathews et al. (1987) reported that the *rj1* soybean mutant did not show curled root hairs after inoculation. Suganuma et al. (1990) did find curled root hairs in *rj1* soybean mutant (T201), but the number of curled root hairs was much less than that in wild type isolate T202. These studies indicated that in the *rj1* soybean nodulation blockage occurs at the stage of root curling. Our study here showed that in TN 4-86 and BARC-2(*Rj4*) blockage occurs between the stage III and stage IV. In contrast, the blockage of nodulation in PI 468.397 occurs between the stage IV and stage V. A high percentage of the initial infection events, even in Essex, were arrested at the early stages of nodulation development (Table 2-4). In Essex, only 0.3% of infections reached the stage VI 14 d after inoculation. About 69.5% of infection was still at the MC stage. It should be noted that the observations were performed on different plants. Due to the fact that single infection can not be monitored throughout whole nodule development process. The results reported here are nodule development trends in these accessions. Calvert

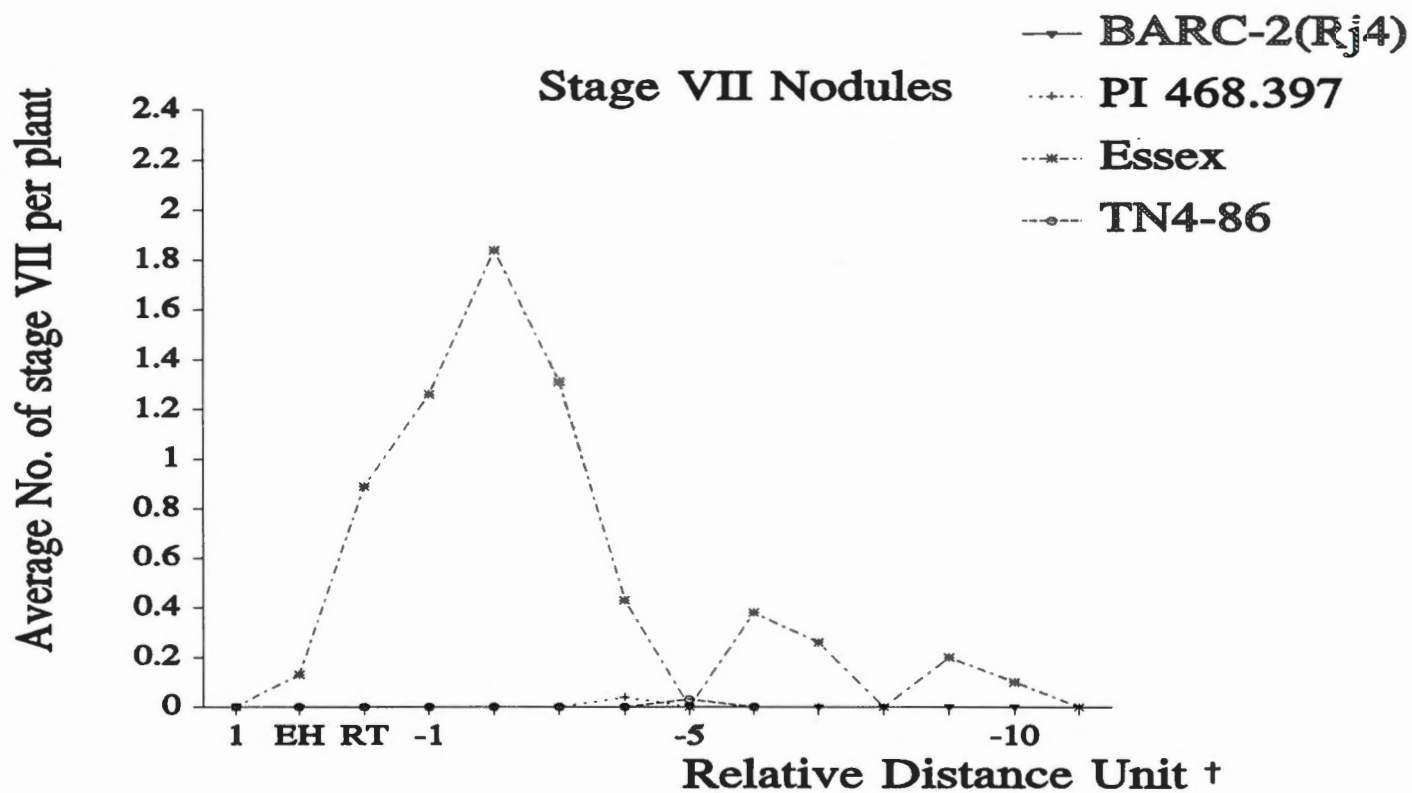


Fig. 2-3. The distribution of fully developed nodules (stage VII) 34 d after inoculation.
 †: Distance from smallest root hair (EH) to the root tip (RT) on a primary root at the time of inoculation.

et al. (1984) reported that the blocked infections were observed at all stages of development on 'Williams' 10 d after inoculation; whereas Gerahty et al. (1992) indicated that the blockage occurs relatively early during nodule ontogeny. They concluded that the maturation of nodule meristems into emergent nodules appears to be the target of the regulatory suppressive response. These results indicated that the different bacterial strains, plant genotypes, and experimental conditions affect nodule development. In a study of feedback control of nodulation, sufficient evidence confirmed that nodulation in legumes is controlled by a signal-and-response mechanism that suppresses nodule emergence in younger parts of the root system (Caetano-Anollés and Gresshoff, 1991). The model for the regulation of nodule formation in legumes proposed by Caetano-Anollés and Gresshoff (1991) involves two systemic messengers. One is the root synthesized inducer and the other is the shoot-derived inhibitor which arrests development of further infections or blocks nodule initiation.

Isolation of Bacteria from Nodules and Reinoculation: The nodules that formed on the roots of TN 4-86, BARC-2(*Rj4*), and PI 468.397 were removed and isolated and bacteria were used to reinoculated TN 4-86, BARC-2(*Rj4*), and PI 468.397. Concurrently, the three genotypes and Essex were also inoculated with strains 61A101C and USDA 110. No nodules formed on the roots of TN 4-86, BARC-2(*Rj4*), and PI 468.397 when inoculated with bacteria isolated from them. Furthermore, no nodules were formed on TN 4-86, BARC-2(*Rj4*), and PI 468.397

plant roots inoculated with 61A101C; however, an average of 8.8 nodules per plant formed on Essex (17 plants). When inoculated with USDA 110, the average number of nodules formed per plant was 4, 4, 1.9, and 7.5 for TN 4-86, BARC-2(*Rj4*), PI 468.397, and Essex, respectively (Table 2-5). Uninoculated control plants of each soybean genotype produced no nodules. These results indicated that the nodules found on TN 4-86, BARC-2(*Rj4*), and PI 468.397 were not the results of contamination. The restriction of nodulation by host is not 100%. This phenomenon was also observed by Cregan and Keyser (1989). It should be emphasized that the plants were tested in plastic pouches only for 3 to 4 weeks. Whether those plants will develop nodules needs further study if they are tested in soil and allowed to grow longer. The phenomenon of tolerance of a pathogen from the host's genetic control is common in the pathogen-host interaction (McCarter, 1993; Muyolo et al., 1993). For example, in the Jerusalem artichoke resistance to rust (McCarter, 1993), several genotypes were scored for resistance to *Puccinia helianthi* from 0.25 to 3.56. A zero indicated no disease. A 1-5 indicated an increasing percent of leaf area covered with fungal growth, with a 5 rating indicating near 100% coverage. These results indicated that the resistance of plant to *Puccinia helianthi* is not complete. The one possible explanation of tolerance of the pathogen from the control of plant resistance gene(s) is that the genetic background of plant modifies the effect of those resistance genes.

Table 2-5. The average number of nodules per plant of four soybean genotypes following inoculation with different source strains of *B. japonicum*.

Strain	Plant genotype			
	TN 4-86	PI 468.397	BARC-2(<i>Rj4</i>)	Essex
USDA 110	4±0.8(4)‡	1.9±0.4(8)	4±0(1)	7.5±0.9(16)
61A101C	0.25±0.16(8)	0(8)	0(8)	8.8±0.8(17)
Isolated from nodule† on BARC-2(<i>Rj4</i>)	0(12)	0(4)	0(4)	nd§
Isolated from nodule† on PI 468.397	0(4)	0(11)	0(4)	nd
Isolated from nodule† on TN 4-86	0(4)	0(4)	0(16)	nd
Uninoculated controls	0(8)	0(8)	0(8)	0(8)

†: Originally inoculated with strain 61A101C.

‡: The number inside the parentheses is the number of plants observed.

§: Not determined.

CHAPTER 4

Conclusion

In the last several decades, studies of symbiotic nitrogen fixation on legume plants have been intensified. Currently, an increasing amount of work has been done on the non-legume crops such as rice and wheat. One of the major problems involving symbiotic nitrogen fixation is the competition of nodulation from indigenous (*Brady*)*rhizobium*. One approach to alter the strains found in the nodules of legume would be development of cultivars in which nodulation by indigenous rhizobia is eliminated or substantially reduced. In soybean, genotypes exhibiting different nodulation response to strains of *Bradyrhizobium* have been reported. Several soybean genes involving nodulation have been identified. Those genes are either naturally occurring or induced. The limited nodulation genes identified so far in soybean are not enough to meet the need in soybean breeding program.

Diversity of nodulation response to strains of (*Brady*)*rhizobium* exists in the soybean population. Most of those gene are in exotic germplasm of soybean and wild soybean. A new gene controlling restricted nodulation in PI 468.397 (*G. soja*) with

strain 61A101C of *B. japonicum* was identified in this research. High frequency of nodulation genes found in *G. soja* by several reports (Devine and Breithaupt, 1981; Devine, 1987) and this study indicates that it would be easy to find restricted nodulation genes in *G. soja* population. These results also remind people the importance of collecting and maintaining these germplasm. Cultivar TN 4-86 is found carrying a dominant gene controlling restricted nodulation with 61A101C, which is allelic to *Rj4*. It is possible that incorporation of restricted nodulating gene, *Rj4*, helps TN 4-86 to increase yield by eliminating less inefficient nitrogen fixing strains and allowing more efficient nitrogen fixing strains to form nodules. But, this possibility needs to be confirmed by investigating composition of nodules on TN 4-86 and its sister lines under field condition. In study of pea nodulation, it was discovered that wild pea genotypes are genetically highly variable with regard to the response to *Rhizobium* in the gene center of pea plant. Indigenous *Rhizobium* strains are strongly adapted to the indigenous pea plant. However, *Rhizobium* strains result in incompatibility of symbiosis or non-nodulation with pea genotypes from other regions (Lie, 1978, 1981, 1984). If this is also true in soybean, it should be possible to find restricted nodulation genes from the soybean population originated from other regions.

Most studies on restricted nodulation on soybean were carried out in controlled environmental conditions, including this study. The nodulation is largely affected by the conditions in which plant grows. In both preliminary screenings, the number of nodules per plant was low. Whether these restricted genotypes will still restrict corresponding strains under field condition needs to be confirmed.

The anatomical study indicated that nodulation was blocked at infection development, not at infection initiation in TN 4-86 and PI 468.397. In TN 4-86, nodulation appeared to be blocked between Stage III and Stage VI. In contrast, nodulation seems to be blocked between Stage V and Stage VI in PI 468.397. Even in Essex, a normal nodulation genotype, most of initial infection events remained in the early nodule development stages, which indicates that plants can optimize the number of nodules. Unlike non-nodulation genotypes, the restriction of nodulation on both TN 4-86 and PI 468.397 with 61A101C is not 100%. The concentration of bacteria applied to plants could affect the result of nodulation.

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PART III

Use of RFLP Technology to Map a Gene Controlling Restricted Nodulation in Soybean

Abstract

The symbiotic relationship between legume and (*Brady*)*rhizobium* is genetically controlled by both plant and bacterium and proceeds through a sequence of developmental stages that result in the establishment of an effective, nitrogen-fixing symbiosis. The objective of this study is to use RFLP technology to map the gene controlling restricted nodulation in *Glycine soja*, PI 468.397. Seventy enzyme/probe combinations were tested on cultivar Essex (*Glycine max*, L. Merr.) and PI 468.397. Eighteen polymorphisms were detected. Some probes were very useful and others were relatively uninformative. The enzymes *Hind*III and *Eco*RV were more effective than *Dra*I in producing polymorphisms between Essex and PI 468.397. Sixteen enzyme/probe combinations were tested on recessive homozygous restricted nodulation genotype F₂ plants. These RFLP loci covered linkage groups A to H. No linkage was detected between RFLP marker loci and the gene controlling restricted nodulation. Although the segregation ratios of marker patterns in some enzyme/probe combinations are significantly different from those of independent segregation, the number of heterozygote patterns is more than that expected. This could have been due to small sample size.

CHAPTER 1

Introduction and Literature Review

The nitrogen fixing symbiotic relationship between soybean and *Bradyrhizobium* is genetically controlled by both plant and bacterium and proceeds through a sequence of developmental stages (Calvert et al., 1984). Individual (*Brady*)*rhizobium* strains are able to form nodules on only a limited number of host legumes (Caldwell, 1966). Competitions between or among bacteria has been reported in *Rhizobium* and *Bradyrhizobium* (McLoughlin et al., 1985). A number of studies have demonstrated that even at high introduced inoculation rates, the proportion of nodules formed by a non-resident inoculum strain is generally less than 20% (Caldwell and Vest, 1970; Kuykendall and Weber, 1978). Although Johnson et al. (1965) recovered an inoculum strain in 82% of the nodules of 'Kent' soybean, the inoculum was applied at 700 times the recommended commercial rate and at least 12% of the indigenous population was serologically indistinguishable from the inoculum strain. Weaver and Frederick (1974) indicated that in order to establish an introduced inoculum strain in 50% of the nodules of a soybean plant, the rate of inoculum application would have to be at least 1000 times greater than the indigenous population. Recently, Cregan and Keyser (1986) and Cregan et al. (1989) suggested

using certain soybean genotypes to restrict the nodulation by indigenous strains. They found several PI soybean genotypes that restrict nodulation by USDA 123, which is the predominant strain in the midwest of the United States and less effective than other strains in nitrogen fixation (Ham, 1980).

As early as in 1923, Sax (1923) proposed identifying and selecting for minor genes of interest by linkage with major genes, which could be scored more easily. This concept has been extensively utilized in human, animal, and plant genetic research. For example, isozymes have been used very successfully in certain aspects of plant breeding and genetics as nearly-neutral markers. They have been used to estimate genetic diversity of germplasm components, cultivar identification, confirmation of hybrids, marking of monogenic traits, and dissection of quantitative traits (Weeden, 1989). Unfortunately, the number of genetic markers provided by isozyme assays is insufficient for many applications in plant breeding. DNA fragments referred to as restriction fragment length polymorphism (RFLP), has been developed and widely used as markers in DNA analysis of humans, animals, plants, and bacteria. RFLPs are genetic markers based on cloned fragments of DNA. Using RFLPs, high density linkage maps can be constructed and used to locate genes of economic importance. Once a gene of interest is associated with a linked RFLP marker, selection for that gene can be based on the genotype of the RFLP rather than the phenotype itself. This is extremely valuable if a phenotype is difficult or expensive to score. RFLP was first used as a tool for genetic analysis in 1974. Linkage of temperature-sensitive mutations of adenovirus to specific restriction

fragment length differences was used to locate the mutations on a physical map of the restriction fragments (Grodzicker et al., 1974). The potential impact of RFLP mapping on eukaryotic genetics was first described in 1980 by researchers in human genetics (Botstein et al., 1980; Wyman and White, 1980). RFLPs now occupy a major role in human genetic research and the first genetic linkage map of the human genome, based on RFLP markers, has been published (Donis-Keller et al., 1987).

Over the past several years, plant geneticists and breeders have continued to develop RFLP maps for important crop species. Partial maps are now available for several different plant species, including tomato, potato, rice, maize, soybean, barley, lettuce, *Arabidopsis thaliana*, and common bean (Tanksley et al., 1988 a,b; McCouch et al., 1988; Helentjaris, 1987; Landry et al., 1987; Bonierbale et al., 1988; Heun et al., 1991; Kesseli et al., 1992; Chang et al. 1988; Nodari et al. 1993).

Restriction fragment length polymorphism markers have been identified which are tightly linked to genes for resistance to mosaic virus (Young et al. 1988), bacterial speck, root knot nematodes, major genes controlling plant habit and fruit ripening properties in tomato (Paterson et al., 1988), and mildew resistance in barley (Schuller et al. 1992). In lettuce, researchers have established linkages between RFLP markers and a gene for resistance to downy mildew (Landry et al., 1987). Several other genes with qualitative effects have been mapped using near-isogenic lines combined with RFLP markers. Young et al. (1992) mapped a bruchid resistance gene in mungbean. In soybean, molecular markers have been linked to genes such as nitrate tolerant symbiosis (*nts*) (Landau-Ellis et al., 1991), brown seed with black stripes (*r*) and

pentafoliolate leaves (*Lf₁*) (Muehlbauer et al., 1991), and *Phytophthora* resistance (*Rps*) loci and ineffective nodulation (*Rj2*) (Diers et al., 1992). The objective of this research was to screen RFLP markers for linkage with the restricted nodulation gene in PI 468.397 (*G. soja*) reported in Part II.

CHAPTER 2

Materials and Methods

Bradyrhizobium Cultures: The strain 61A101C of *Bradyrhizobium japonicum* was obtained from Dr. Gary Stacey, Department of Microbiology, the University of Tennessee, Knoxville. This strain is wild-type *B. japonicum* of DNA homology group II and can cause nodulation on *G. max* cultivars. Stock cultures were maintained in yeast extract-mannitol-gluconate (YEMG) semisoft medium. Bacteria were grown in YEMG liquid medium to a stationary phase and then subcultured after 100-fold dilution in 30 ml of fresh medium in a rotary shaker at 150 rpm and 27° C. Bacteria from early exponential-phase culture ($OD_{620}=0.3-0.4$) were diluted with YEMG medium to a concentration of 10^8 cells ml^{-1} .

Plant Hybridization and Screening: Soybean genotypes PI468.397 (*G. soja*, restricted nodulation phenotype) and Essex (*G. max*, nodulation phenotype) were chosen as the parents. The cross of Essex X PI468.397 was made at the Knoxville Plant Science Field Unit, in 1990 and 1991. Verification of F_1 hybrids was confirmed by F_1 plant growth habit and RFLP pattern, and the seed color of F_2 plants. Seeds of PI 468.397 were scarified by soaking in concentrated sulfuric acid for 10 min followed by sterilization for 5 min in 2.6% sodium hypochlorite and five rinses with deionized

water. Seeds of Essex, F₁, F₂, and F₃ were sterilized by soaking in 2.6% sodium hypochlorite for 5 min followed by five rinses with deionized water. The sterilized seeds were germinated at room temperature (about 24° C) on sterile cheesecloth saturated with modified Herridge's plant nutrient solution (Delves et al., 1986). After two days, uniform seedlings were transferred to plastic growth pouches (Vaughan's Seed Co., Downers Grove, IL) containing 10 ml of nutrient solution for Essex and F₁, and 7 ml of nutrient solution for PI 468.397, F₂ and F₃. Pouches were maintained in plastic containers which were covered with aluminum foil until inoculation. Each soybean plant was inoculated with 250 µl of bacterial dilution 4 d after seed germination. The pouches were covered by aluminum foil to protect the plant roots from light. After inoculation the seedlings were cultured in a growth chamber with a 16 h photoperiod and light intensity of 350 µE M⁻² sec⁻¹ with a 24° C day/22° C night temperature cycle. Modified Herridge's plant nutrient solution was added as required. After 21 d of inoculation, the roots of each plant were cleaned and number of nodules per plant was counted.

Plant DNA Extraction, Restriction, and Blotting: The F₂ plants showing restricted nodulation (homozygous recessive) response were transplanted to plastic trays with synthetic growth medium in the greenhouse. The photoperiod was controlled at 24 h d⁻¹ in order to obtain enough leaves from each plant for DNA extraction. Young leaves from each selected homozygous recessive F₂ plant were harvested and stored at

-70° C. DNA extractions were performed according to the protocol described by Dellaporta et al. (1983), with the following procedure added for further purification of the DNA. After DNA was precipitated and resuspended in water, one-half volume of 8 M ammonium acetate was added. The solution was mixed and incubated at -70° C for 1 h and then centrifuged for 15 min at 15,000 rpm. The supernatant was carefully poured into new tubes and the precipitate was discarded. The DNA in the supernatant was precipitated with 2.5 times supernatant volume of 95% ethanol and centrifuged. The supernatant was discarded and the precipitate was washed with 80% ethanol. The DNA was resuspended in the water. Purified DNA of the two parents, F₁, and the homozygous recessive F₂ was digested with *EcoRI*, *EcoRV*, *HindIII*, *DraI*, and *TaqI* according to the manufacturer's specification. Agarose gel electrophoresis of digested DNA was run to confirm the full digestion of DNA and the concentration of DNA after digestion was measured using a fluorometer (TKO 100, Hoefer Instrument). Digested DNA was separated by 0.9% agarose gel electrophoresis at 35 voltage for 15 h and then transferred onto Zeta Probe Nylon membrane by vacuum blotting (LKB). Each blot contained DNA from F₂, F₁, and two parents digested with a single enzyme.

DNA Clones and Amplification of Fragments: The polymorphic clones were provided by Dr. R. C. Shoemaker, Iowa State University, Ames, Iowa. The clones are part of the *PstI* library of soybean line A81-356022 in the pBS+ plasmid from Stratagene. The *E. coli* with plasmid was first cultured in LB solid medium with

ampicillin ($40 \mu\text{g ml}^{-1}$) overnight. The inserted fragments were amplified by the polymerase chain reaction (PCR). A single colony of bacteria were scraped off the plate and fluxed into a 0.5 ml Eppendorf tube with $35 \mu\text{l}$ sterile water. The solution was incubated at 105°C for 2 min to denature the DNA. The $2 \mu\text{l}$ denatured DNA was added into $18 \mu\text{l}$ reaction mixture which includes PCR buffer [10 mM tris:HCl (pH 8.3), 50 mM KCl and 1.5 mM MgCl_2], $1.0 \mu\text{M}$ of primers, $200 \mu\text{M}$ of each dNTP, 1.4 M DMSO and 1 unit of *Taq* polymerase. The reaction mix was overlaid with 2 drops of mineral oil and amplified in an Ericomp thermocycler connected to running water. The PCR program was controlled at 2 min at 93°C , then 41 cycles of 2 min at 60°C , 3 min at 72°C , 45 sec at 93°C , 1 cycle of 2 min at 60°C , 7.5 min at 72°C , and 16 hrs at 4°C . The lower layer of amplified products was removed to a fresh tube and stored at -20°C . The amplified fragments were analyzed by agarose gel electrophoresis. The actual molecular size was compared to the original molecular size of that obtained from Dr. R. C. Shoemaker, Iowa State University, Ames, Iowa.

DNA Hybridization: Approximately 20-100 ng of each amplified genomic sequence was radiolabeled with [^{32}P]dATP by Boehringer Mannheim Random Primer Labeling Kit and probed to membranes. Hybridization and washes were performed at 60° and 55°C respectively according to Zeta Probe (BioRad) recommendations. Membranes were exposed to Kodak X-Omat AR film (E.I. DuPont De Nomours & Co. Inc.).

After autoradiography, hybridized probes were stripped from the membranes by washing with 0.1N NaOH and 0.5% sodium dodecyl sulfate at room temperature for 5 min.

CHAPTER 3

Results and Discussion

Polymorphisms Between Parents: Seventy enzyme/probe combinations were screened for polymorphism between Essex and PI 468.397, which included 5 enzymes and 21 probes. Eighteen polymorphisms were found. Table 3-1 summarizes the frequency of polymorphism detected by probes. Some probes were very useful and others were relatively uninformative. For example, pA-584 and pA-486 each resulted in three polymorphisms between Essex and PI 468.397 with five enzymes. The three probes pA-112, pK-493, and pA-885 each resulted in two polymorphisms with five enzymes. Six probes did not result in any polymorphism with five enzymes. Of the five enzymes tested, *HindIII* and *EcoRV* resulted in the highest percentage of polymorphisms between Essex and PI 468.397 (Table 3-2). No polymorphisms were detected between Essex and PI 468.397 using *DraI* with 14 probes. It is important to keep in mind that the results came from a small sample. With an increase in the number of probes, the frequency of polymorphisms between Essex and PI 468.397 detected with an individual enzyme could change. The data reported by Shoemaker et al. (1992) indicated that the probability of detecting genotypic diversity in soybean

Table 3-1. The frequency of polymorphisms between Essex and PI 468.397 detected by twenty-one probes with five different enzymes.

Probe	<i>EcoRI</i>	<i>EcoRV</i>	<i>HindIII</i>	<i>TaqI</i>	<i>DraI</i>	polymorphisms
pA-584	+†	+	+	-‡	-	3
pA-96	nd§	nd	+	nd	nd	1
pA-486	-	+	+	+	-	3
pK-493	-	+	+	-	-	2
pA-112	+	-	+	+	-	3
pA-381	-	nd	+	nd	nd	1
pA-63	nd	nd	nd	+	nd	1
pA-885	+	+	-	-	-	2
pA-86	nd	nd	+	nd	nd	1
pA-111	nd	nd	-	nd	nd	0
pA-505	-	-	-	-	-	0
pA-711	-	-	-	-	-	0
pA-384	-	-	-	-	-	0
pA-73	-	-	-	-	-	0
pA-85	-	-	-	-	-	0
pA-235	-	-	-	+	-	1
pK-390	nd	nd	nd	-	nd	0
pK-14	-	nd	nd	nd	nd	0
pA-89	nd	nd	nd	nd	-	0
pA-397	nd	nd	nd	nd	-	0
pA-572	-	-	-	-	-	0

† polymorphism.

‡ no polymorphism.

§ not determined.

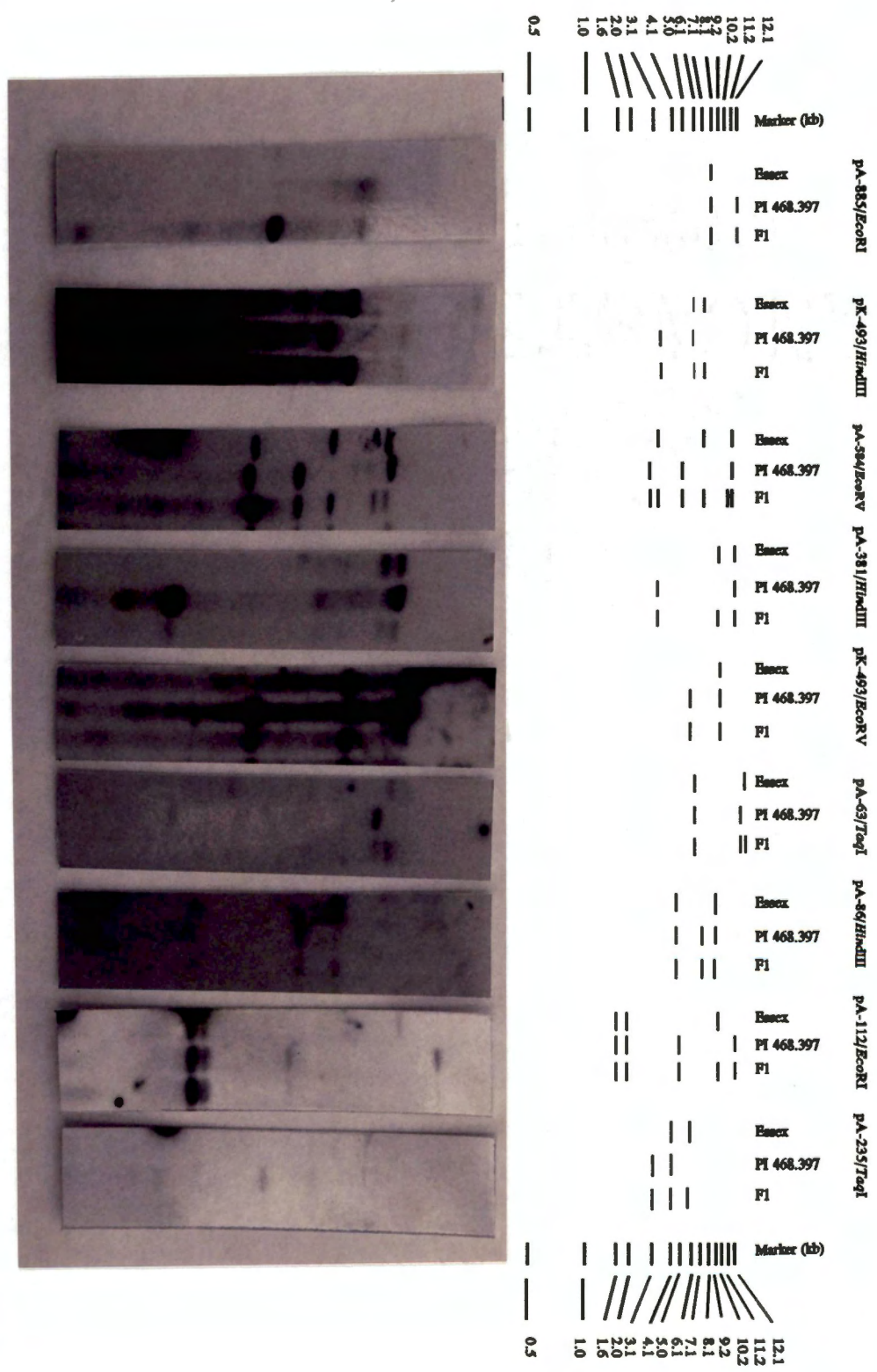
Table 3-2. The frequency of polymorphisms between Essex and PI 468.397 detected by five enzymes with different probe.

Enzymes	Probes tested	Polymorphism	Percentage (%)
<i>HindIII</i>	16	7	44a†
<i>EcoRI</i>	14	3	21a
<i>EcoRV</i>	12	4	33a
<i>TaqI</i>	14	4	29a
<i>DraI</i>	14	0	0b

†: Percentages followed by the same letter are not significant different at the 5% level according to comparison of proportion using *t* test.

with the same five enzymes ranged from 22% to 27% when more than 30,000 comparisons were made among 32 molecular probes, and 51 soybean genotypes (*G. max*). They also found that some probes are very productive, e.g., pA-112 and pA-486, and others less efficient in resulting in polymorphisms, e.g., pA-505 and pA-711. In this study, pA-112 and pA-486 produced three polymorphisms between Essex and PI 468.397 with five enzymes; whereas pA-505 and pA-711 did not produce any polymorphism between Essex and PI 468.397 with five enzymes. The polymorphisms observed between Essex and PI 468.397 were of two types (Fig. 3-1). The first type is that each parent only had one band but in different locations, for example, pA-86/*HindIII*. The second type is that one parent had two or more bands and the other had a fewer number, for example, pA-885/*EcoRI*. The first type of polymorphisms are more valuable than those of the second type in terms of mapping because the former can distinguish the heterozygote from the parents and give more accurate estimate of genetic distance between markers. Conversely, with the second type, the heterozygote cannot be distinguished from the parent, since they have the same molecular marker pattern. Nine enzyme/probe combinations, which detected polymorphisms between the soybean strains, A81-356022 and PI 468.916 (Keim et al., 1990), did not result in polymorphisms between Essex and PI 468.397 in this study, which could be due to the difference of genotypes used. Two probe/enzyme combinations among them (pA-89/*DraI* and pA-111/*HindIII*) also did not result in polymorphisms between PI 468.397 and Bragg (Landau-Ellis, personal communication). These results point to the need for a highly saturated RFLP map that

Fig. 3-1. The RFLP loci between Essex and PI 468.397. Since polymorphisms were analyzed on different gels, the diagram shows the fragments aligned with molecular markers.



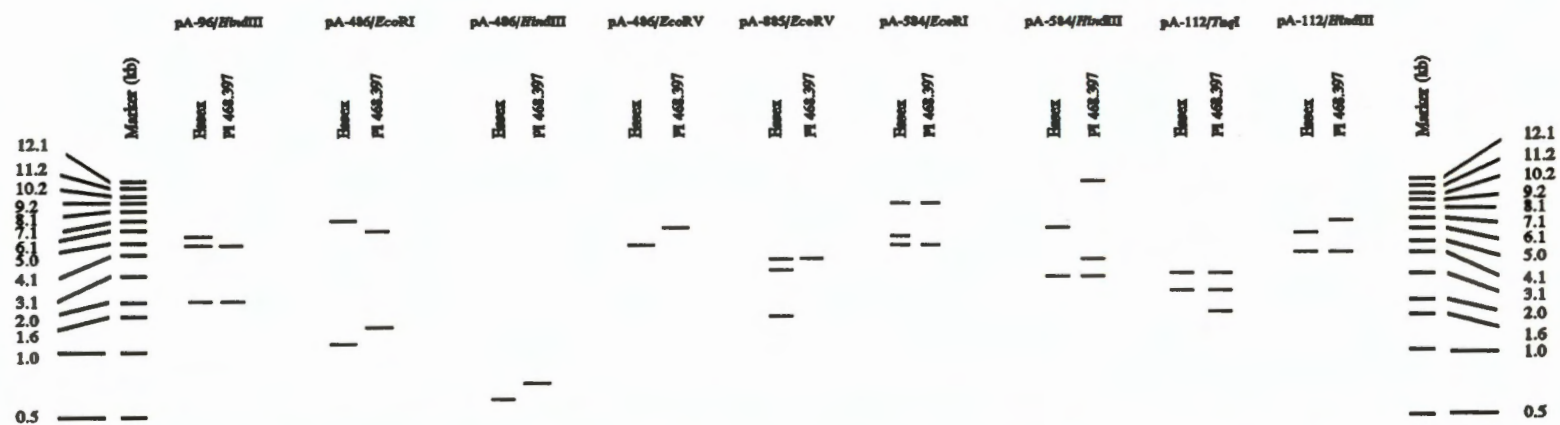


Fig. 3-1. Continued.

will cover populations with different genetic backgrounds.

The RFLP marker patterns between PI 468.397 and Essex of nine probe/enzyme combinations in this study were compared to those between PI 468.397 and Bragg in *nts* study (Landau-Ellis, personal communication) detected with same probe/enzyme combinations (Fig. 3-2). All nine marker patterns of PI 468.397 were the same except that there was a extra thin band at 7.1 Kb in pA-63/*TaqI* combination and at 3.1 Kb in pK-390/*TaqI* combination. There were only two polymorphisms between Essex and Bragg with nine probe/enzyme combinations, which indicated that it would be more difficult to detect polymorphism among *G. max* than between *G. max* and *G. soja*.

Mapping of a Restricted Nodulation Gene in PI 468.397: Sixteen enzyme/probe combinations were tested only on F₂ plants that expressed restricted nodulation homozygous recessive phenotype. The segregation of RFLP markers and segregation ratio among F₂ plants which restrict nodulation with *B. japonicum* strain 61A101C were listed in the Table 3-3 and Table 3-4, respectively. The locations of these RFLP loci on linkage map (Shoemaker and Olson, 1993) are listed in the appendix. These RFLP loci covered linkage groups A to H. When pA-885 hybridized to F₂ plants digested with *EcoRI*, one plant showed the Essex marker pattern (Fig. 3-3). Nine F₂ plants showed PI 468.397 or heterozygote marker pattern. One nodulated plant showed heterozygote marker pattern (Fig. 3-3). It was difficult to distinguish the PI 468.397 marker pattern from the heterozygote pattern because they were exactly

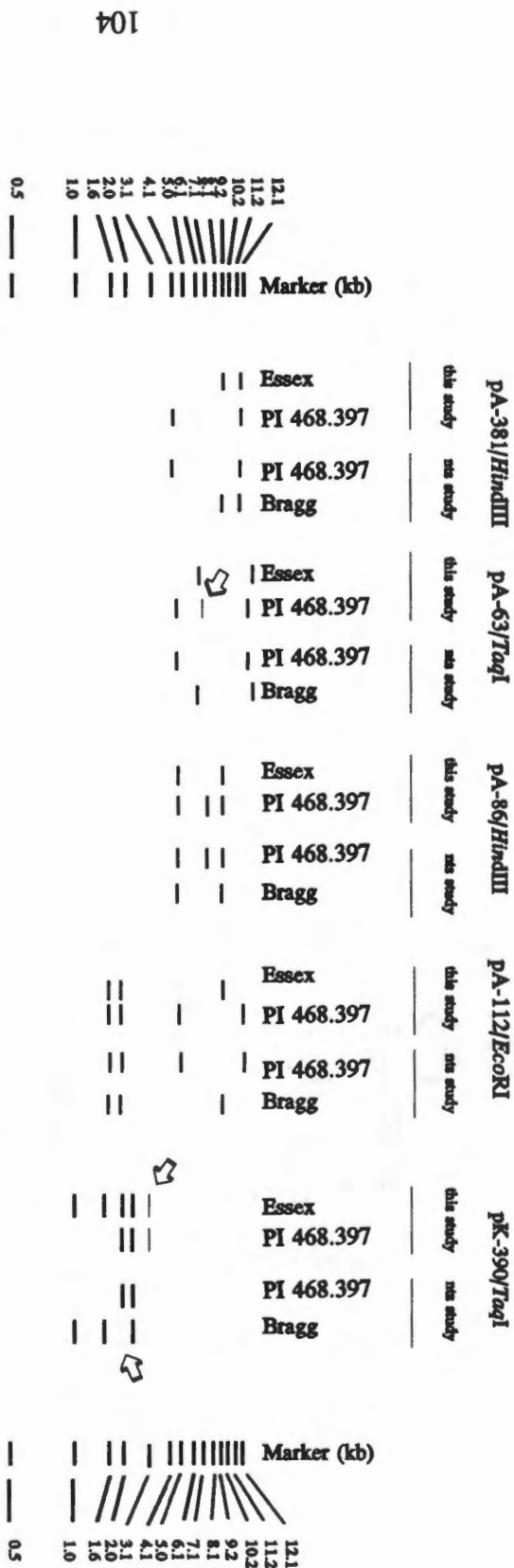


Fig 3-2. The comparison of RFLP marker patterns detected between Essex and PI 468.397 in this study with the marker patterns between Bragg and PI 468.397 in nts study which are provided by Deborah Landau-Ellis, Plant Molecular Genetics, University of Tennessee.

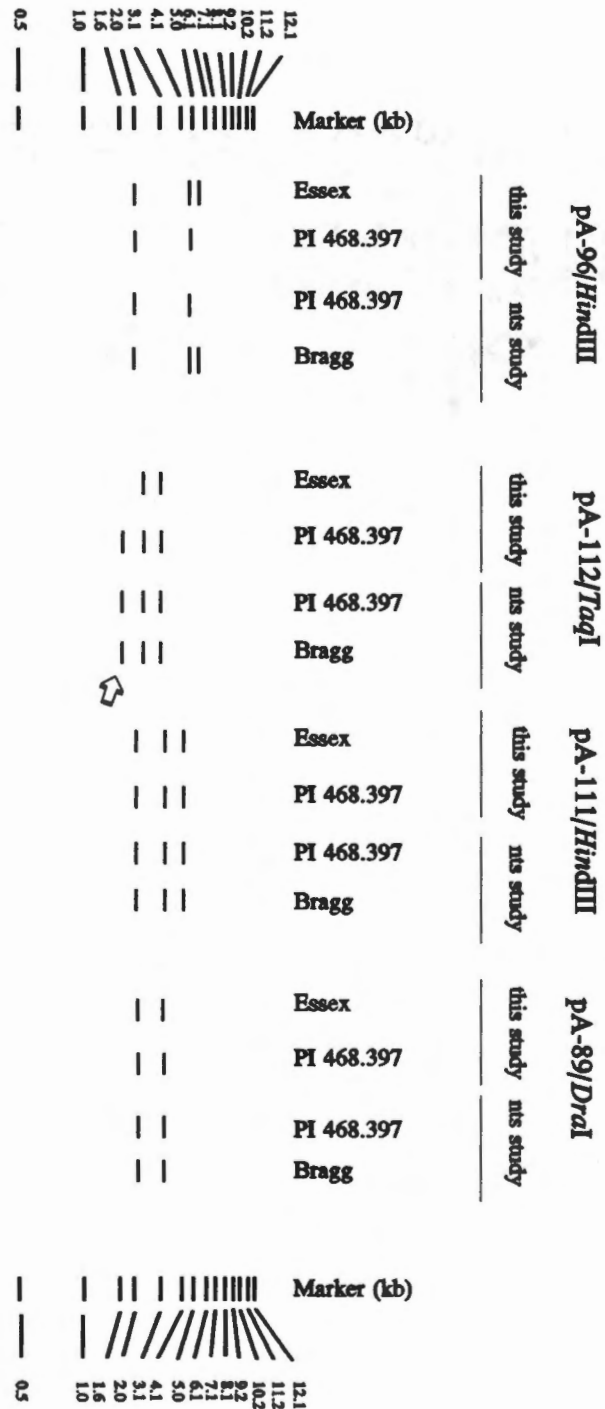


Fig. 3-2. Continued.

Table 3-3. Segregation of RFLP markers in F₂ plants restricting nodulation with *B. japonicum* strain 61A101C.

Probe/enzyme	Parents		F ₂ plants																												
			From second screening														From first screening														
			F ₁	1	4	8	9	10	16	17	21	22	28	32	38	43	45	47	48	55	65	68	81	85	86	15	29	44	96	109	N
pA-86/ <i>Hind</i> III	E	P	H	PH	-	PH	PH	-	-	-	-	-	nd	-	nd	-	-	nd	-	nd	-	PH	nd	E	E	E	PH	-			
pK-493/ <i>Hind</i> III	E	P	H	H	P	nd	nd	H	nd	nd	H	H	H	P	P	-	H	H	P	H	P	nd	-	-	P						
pA-584a/ <i>Eco</i> RV	E	P	H	H	H	nd	nd	E	nd	nd	H	H	H	-	H	H	H	H	H	H	H	nd	H	H	P						
pA-584b/ <i>Eco</i> RV	E	P	H	P	H	nd	nd	E	nd	nd	E	E	E	-	P	H	H	H	P	P	H	nd	H	H	E						
pA-235/ <i>Taq</i> I	E	P	H	H	H	nd	nd	-	nd	nd	H	H	H	P	-	-	-	-	-	-	nd	-	-	-							
pA-112/ <i>Eco</i> RI	E	P	H	H	P	nd	nd	E	nd	nd	H	H	P	-	-	-	-	-	-	-	nd	-	-	-							
pA-885/ <i>Eco</i> RI	E	P	H	nd	PH	PH	PH	PH	PH	nd	nd	PH	nd	PH	nd	-	nd	nd	-	nd	PH	PH	E	nd							H
pA-63/ <i>Taq</i> I	E	P	H	nd	-	-	H	H	E	nd	nd	-	nd	-	nd	nd	nd	nd	-	nd	-	-	E	H							E
pK-493/ <i>Eco</i> RV	E	P	H	nd	P(H)†	PH	nd	-	-	nd	PH	PH	nd	P(H)†	P(H)†	nd	nd	nd	-	nd	P(H)†	nd	nd	PH	P(H)†						
pA-381/ <i>Hind</i> III	E	P	H	nd	P	-	nd	-	-	nd	nd	-	nd	EH	nd	-	nd	nd	EH	nd	EH	EH	EH	P	nd						H
pA-96/ <i>Hind</i> III	E	P	H	nd	H	-	nd	-	-	nd	nd	-	nd	H	nd	-	nd	nd	-	nd	-	-	H	P	nd						H

Note: E= Essex or Essex marker pattern, P= PI 468.397 or PI 468.397 marker pattern, H= Heterozygote marker pattern, N= nodulated F₂ plant, EH= Essex or heterozygote marker pattern, PH=PI 468.397 marker pattern or heterozygote marker pattern, - = not discernable, nd= not determined. The combinations of pA-486/*Eco*RI, pA-505/*Taq*I, pA-89/*Dra*I, pA-397/*Dra*I, pA-711/*Taq*I, and pK-390/*Taq*I were also tested on F₂ plants, but did not result in polymorphism.

† It was concluded, after comparing with the marker pattern of pK-493/*Hind*III, that this F₂ plant has PI 468.397 marker pattern of pK-493/*Eco*RV at that region.

Table 3-4. Segregation ratio of RFLP markers in F₂ plants restricting nodulation with *B. japonicum* strain 61A101C.

Probe/enzyme	Observed segregation ratio	Expected ratio for independent assortment	P > χ^2
	E:H:P† or EH:P‡ or E:PH§		
pA-86/ <i>Hind</i> III	3:5§	1:3	0.31
pK-493/ <i>Hind</i> III	0:8:6†	1:2:1	0.05
pA-584a/ <i>Eco</i> RV	1:14:1†	1:2:1	0.01
pA-584b/ <i>Eco</i> RV	5:7:4†	1:2:1	0.75
pA-235/ <i>Taq</i> I	0:5:1†	1:2:1	0.10
pA-112/ <i>Eco</i> RI	1:3:2†	1:2:1	0.75
pA-885/ <i>Eco</i> RI	1:9§	1:3	0.50
pA-63/ <i>Taq</i> I	2:3:0†	1:2:1	0.25
pK-493/ <i>Eco</i> RV	0:4:5†	1:2:1	0.05
pA-381/ <i>Hind</i> III	5:2‡	3:1	0.75
pA-96/ <i>Hind</i> III	0:3:1†	1:2:1	0.25

Note: E= Essex marker pattern, P=PI 468.397 marker pattern, H= heterozygote marker pattern, EH= Essex or heterozygote marker pattern, PH= PI 468.397 or heterozygote marker pattern.

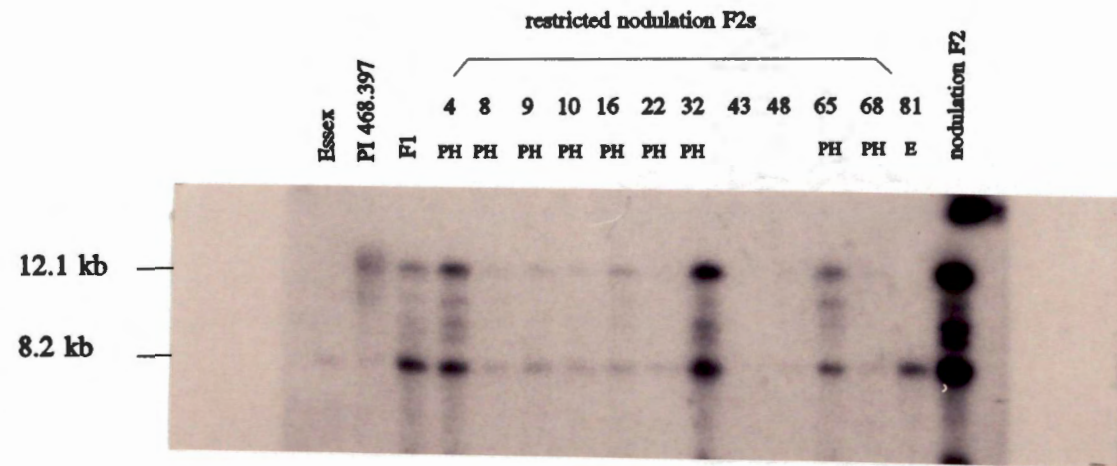


Fig. 3-3. Southern blot hybridization results of parents, F_1 , and only F_2 plants with restricted nodulation phenotype (one normal nodulation F_2 as control) from the cross, Essex X PI 468.397, using pA-885/*Eco*RI probe/enzyme combination. E= Essex marker pattern and PH=PI 468.397 or heterozygote marker pattern. The numbers at the top of picture are F_2 plant number.

same. Although it was difficult to distinguish the heterozygote marker pattern from PI 468.397 marker pattern, there was possible linkage between marker and gene controlling restricted nodulation. Since the marker and the gene controlling restricted nodulation segregate independently, the ratio of PI 468.397 and heterozygote pattern to Essex pattern should be 3:1. The flanking markers of pA-885 were tested to determine if there is a linkage between the marker and the gene controlling restricted nodulation. When pK-493, which is located in the same linkage group as pA-885, hybridized to F₂ plants digested with *Hind*III, eight plants showed the heterozygote pattern, six plants showed PI 468.397 pattern. No Essex pattern was found (Fig. 3-4). If no linkage exists between pK-493 and the gene, the segregation ratio of marker pattern should be 1 Essex pattern : 2 heterozygote pattern : 1 PI 468.397 pattern. Since all of the F₂ plants chosen for test were restricted nodulation phenotype, the Essex marker pattern was eliminated by selection. However, the number of heterozygote pattern was more than that expected if the marker is linked to the restricted nodulation gene. Three more flanking markers were tested. When pA-584 hybridized to F₂s digested with *Eco*RV, two polymorphisms between Essex and PI 468.397 were detected (Fig. 3-5). In pA-584a, one F₂ plant showed Essex pattern and one showed PI 468.397 pattern. The remainder of them showed the heterozygote pattern. The segregation ratio of marker patterns is 1 Essex pattern : 14 heterozygote pattern : 1 PI 468.397 pattern, which is significantly different ($P \leq 0.05$) from independent segregation ratio of 1 Essex pattern : 2 heterozygote pattern : 1 PI 468.397 pattern. However, there were more heterozygote marker pattern than that

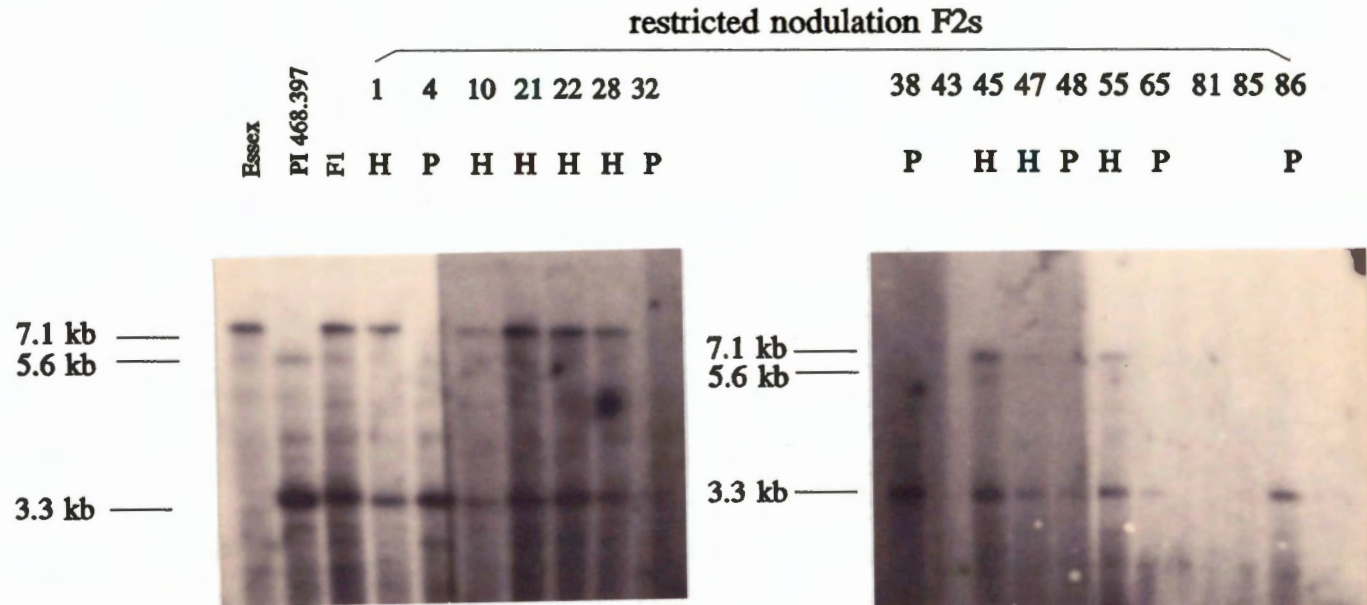


Fig. 3-4. Southern blot hybridization results (on two gels) of parents, F₁, and only F₂ plants with restricted nodulation phenotype from the cross, Essex X PI 468.397, using pK-493/*Hind*III probe/enzyme combination. E= Essex marker pattern, P=PI 468.397 marker pattern, H=heterozygote marker pattern. The numbers at the top of picture are F₂ plant number.

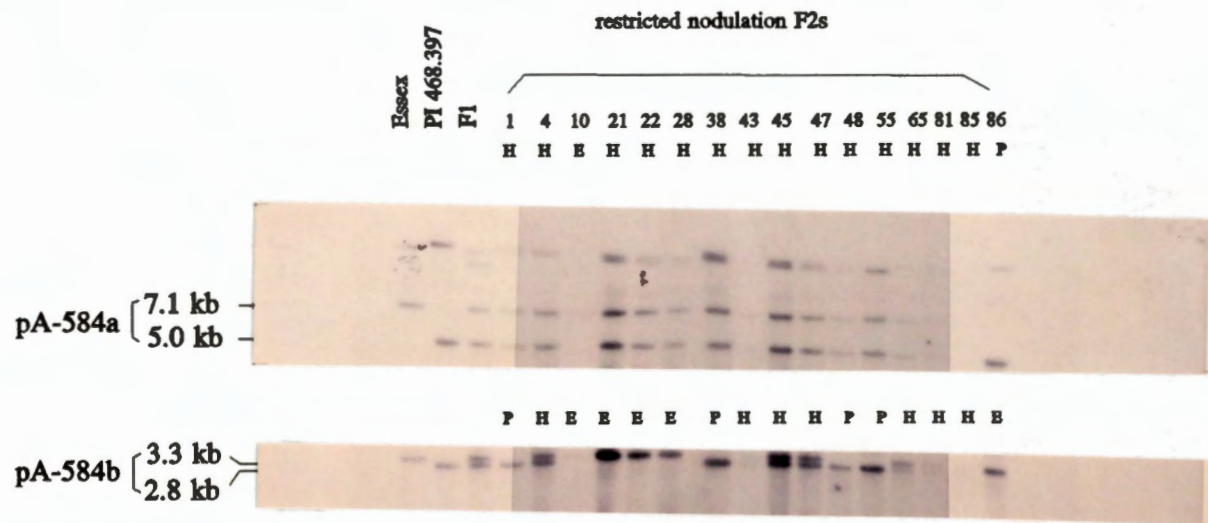


Fig. 3-5. Southern blot hybridization results of parents, F₁, and only F₂ plants with restricted nodulation phenotype from the cross, Essex X PI 468.397, using pA-584/*EcoRV* probe/enzyme combination. E= Essex marker pattern, P=PI 468.397 marker pattern, and H=heterozygote marker pattern, The numbers at the top of picture are F₂ plant number.

expected. The skewed segregation of marker patterns could be explained by small sample size. In pA-584b, seven plants showed heterozygote pattern, four plants PI 468.397 pattern, and five plants Essex pattern, which showed independent segregation. The other two markers also showed independent segregation. These results imply that no statistically significant linkage exists between the RFLP loci tested and the recessive gene controlling restricted nodulation. Since small sample would give large sampling error, the future work should screen whole segregation population, including nodulated F₂ plants, combining random amplified polymorphic DNA (RAPD) or DNA amplification fingerprinting (DAF) with bulked segregation analysis (Michelmoret al., 1991).

CHAPTER 4

Conclusion

One of the major problems involving symbiotic nitrogen fixation is the competition of nodulation from the indigenous (*Brady*)*rhizobium*. Usually those indigenous (*Brady*)*rhizobium* are ineffective or less effective in nitrogen fixation. It was suggested to displace the indigenous (*Brady*)*rhizobium* through the genetic control of host in soybean. However, most restricted nodulation genes are in soybean land race, plant introduction or even in *G. soja*. These types of germplasm usually carry undesirable agronomic traits. In order to efficiently use these genes, efficient methods of isolation, cloning, and integration such as transformation, are required. Mapping of these genes is the first step in the whole process. Once a tight linkage between the target gene and molecular markers is detected. The physical map then can be constructed by the techniques such as pulsed-field gel electrophoresis. Chromosome walking from one of the flanking RFLPs to the RFLP on the other side will result in cloning all the DNA between two RFLPs, including the target gene.

Whether a RFLP map generated from a certain population can cover

populations with different genetic background is major concern in application of those maps. The results of this study indicated that some probe/enzyme combinations results in polymorphism between soybean genotypes, but not other soybean genotypes. These results pointed to the need for a high saturated RFLP map that will cover populations with different genetic background. Some probes were very useful and others were relatively uninformative in producing polymorphisms in this study. Enzymes *HindIII* and *EcoRV* were more effective than *DraI* in producing polymorphisms between Essex and PI 468.397. However, the frequency of polymorphisms between Essex and PI 468.397 detected with individual enzyme could change with an increase of number of probes. Sixteen enzyme/probe combinations were tested on recessive homozygous restricted nodulation F_2 plants. No linkage was detected between RFLP marker and the gene controlling restricted nodulation in PI 468.397. The skewed segregation ratio of marker patterns in some enzyme/probe combinations could have been due to small sample size. As the sample size increases, the possibility of detecting linkage between marker and target gene will increase. Since small sample gave large experimental error, the future work should screen the whole F_2 population, using RADP or DAF combining with bulked segregation analysis.

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PART IV

Overall Summary

CHAPTER 1

Summary of Parts II-III

It has long been recognized that nitrogen is a key to soil fertility. If soil nitrogen levels can be maintained or improved, a stable agricultural system can usually be developed. In the most developed countries, this has been achieved through intensive use of nitrogen fertilizers. As more and more concern has been raised about energy and environmental pollution, biological nitrogen fixation has been considered as an alternative way to supplement the nitrogen required for high production levels. In the biological nitrogen fixation system, at least two approaches have been used. One of them is to use the direct symbiotic relationship between the bacteria and the legume plant to support the need of nitrogen of the crop, for example, soybean-*Bradyrhizobium* symbiosis. The other is to transfer biological fixed nitrogen from legume or non-legume to other crops, for example, transferring biological fixed nitrogen by *Azolla-Anabaena* to rice. Recently, the study of biological nitrogen fixation in rice and wheat has been initiated although it has not provided promising results yet. The legume-(*Brady*)*rhizobium* symbiosis is the best known example of biological nitrogen fixation. However, inoculation of legumes with improved bacteria

is usually not enough to bring about large yield increases on its own. The major problems are highly competitive indigenous rhizobium in the soil and interaction between rhizobium strains and host genotypes. The purposes of this research were to search for restricted nodulation gene(s) in the soybean germplasm and then to manipulate the genetic control of nitrogen fixation through the host. Based on the results described in the preceding papers, it is possible to eliminate or substantially reduce individual or certain groups of *B. japonicum* strains by choosing soybean genotypes which harbor restricted nodulation gene(s).

The first paper of the research reported herein examined the interaction between soybean genotypes and *Bradyrhizobium* strains. Restricted nodulation genes were discovered in both *G. max* and *G. soja*. These results indicated that diversity of nodulation response to *B. japonicum* exists in soybean germplasm. These germplasms are very useful resources which could be used to manipulate the genetic control of nodulation through the host. High frequency of restricted nodulation gene in *G. soja* indicated that it would be easy to find restricted nodulation gene in *G. soja*. The fact that incorporation of a restricted nodulation gene into TN 4-86 might help TN 4-86 increase yield is a good example in which nodulation was manipulated through host gene. A classical genetic study was carried out to examine the genetic pattern of restricted nodulation. Study of F₁, F₂, and part of F₃ families indicated that restricted nodulation in PI 468.397 is controlled by a single recessive gene. In contrast, restricted nodulation in TN 4-86 is controlled by a dominant gene. A complementary study showed that the restricted nodulation gene in TN 4-86 is the same or allelic to

Rj4. An anatomical study was conducted to determine at which stage nodule development was blocked by restricted genes in PI 468.397 and TN 4-86. The results indicated that nodulation was blocked at early nodule development in both cases. In TN 4-86, nodule development was blocked between Stage III and Stage IV, which is from nodule meristem to nodule emergence. However in PI 468.397, a few stage IV developments were observed. These results indicate that different restricted nodulation genes block nodule development at different stages. Even in Essex, a nodulation genotype, a high percentage of the initial infection events remained in the early nodule development stage. Only 0.3% of initial infections reached mature nodule which indicates that plants can optimize the number of nodules. It should not be ignored that the observations were performed on different plants. The single infection can not be monitored throughout whole nodule development process. The results reported here are trends of nodulation response in these accessions. Unlike nod49 which does not form nodules when inoculated with *B. japonicum*, TN 4-86, BARC-2(*Rj4*), and PI 468.397 still have a few nodules when inoculated with restricted strain, even though they carry a restricted nodulation gene. In order to further confirm whether the nodule found in above genotypes is caused by contamination, the nodules on above genotypes were removed and bacteria in these nodules were isolated and reinoculated to the genotypes. Concurrently, strain USDA 110 and 61A101C were used as controls. No nodules developed on any of the three accessions inoculated with bacteria isolated from them. The results indicated that the nodules formed on TN 4-86, PI 468.397, and BARC-2(*Rj4*) were not caused by

contamination. So far, most studies on soybean restricted nodulation have been carried out under controlled conditions. The study of restricted nodulation under field condition should be conducted to finalize the possibility of using these genes.

Studies indicated that most restricted nodulation genes are in soybean land race, plant introductions, and *G. soja*. These type of germplasm usually carry undesirable agronomic traits. In order to efficiently use these genes in a soybean breeding program, efficient methods of isolation and integration are needed, such as transformation. Mapping and isolation of these genes are the important steps in these approaches. The second part of this dissertation dealt with the use of RFLP technology to target the gene controlling restricted nodulation. A total of seventy enzyme/probe combinations were tested. Eighteen polymorphisms between Essex and PI 468.397 were found. Some probes were more productive than others in detecting polymorphism between Essex and PI 468.397. Of the five enzymes tested, *HindIII* and *EcoRV* resulted in the highest percentage polymorphisms. Sixteen enzyme/probe combinations were used to evaluate linkages between the restricted nodulation gene and RFLP markers. These RFLP loci covered linkage groups A to H. No statistically significant linkage was detected between RFLP markers and the gene controlling restricted nodulation in PI 468.397.

PART V

Recommendation for Further Research

CHAPTER 1

Recommendation for Further Research

Lie (1978, 1981, 1984) reported considerable genetic variation among pea accessions having different geographic origins with regard to their response to *Rhizobium*. He found that indigenous *Rhizobium* strains were strongly adapted to the indigenous pea genotypes, but often resulted in incompatibility of symbiosis or non-nodulation with pea genotypes from other regions (Lie, 1978, 1981, 1984). For example, Afghanistan peas restrict nodulation by *Rhizobium leguminosarum* biovar *viciae* strains from Europe (Davis et al., 1988). In soybean, most host genes involving restricted nodulation are in plant introductions and wild soybean (*G. soja*). It should be possible to find restricted nodulation genes from the soybean population originated from other regions. It appears that *G. soja* should give a high chance to find restricted nodulation genes.

Most research on restricted nodulation on soybean have been carried out under controlled conditions. Whether plants with these restricted nodulation genes still restrict corresponding strain under field condition needs to be confirmed. Field investigation of restriction of nodulation by indigenous strains and composition of

nodules on isolines which differ in restricted nodulation genes would give further understanding of the effect of eliminating inefficient indigenous strains on yield of soybean.

Several methods based on DNA composition and structure have been developed for diagnosis of plant, animal, bacteria and human beings at the molecular level. Restriction fragment length polymorphism (RFLP) has been a choice for many applications. However, RFLP assay is time-consuming, labor-intensive, and also involves the use of radioactivity. Polymerase chain reaction-based technology, random amplified polymorphic DNA (RAPD) and DNA amplification fingerprinting (DAF) have been developed to circumvent some of these problem. These methods use single primers to amplify DNA segment. The difference is that the latter uses very short arbitrary primers so that more DNA amplification products will be produced. However, it still is necessary to investigate several hundred primers on whole segregation population of F_2 or backcross to detect the linkage between the gene of interest and molecular marker. Bulkcd segregation analysis was developed as a rapid procedure for identifying markers in a specific region or linkage with certain genes. The method involves comparing two pooled DNA samples of individuals which are different only for the trait or gene of interest but are arbitrary for all other genes. Using bulkcd segregation analysis combined with RFLP, RAPD, or DAF, the very tight linkage (< 15 cM) between a marker and a gene of interest can be detected. Since only restricted nodulation F_2 plants were screened for linkage in this study, the skewed distribution of marker patterns could have been due to small sample size.

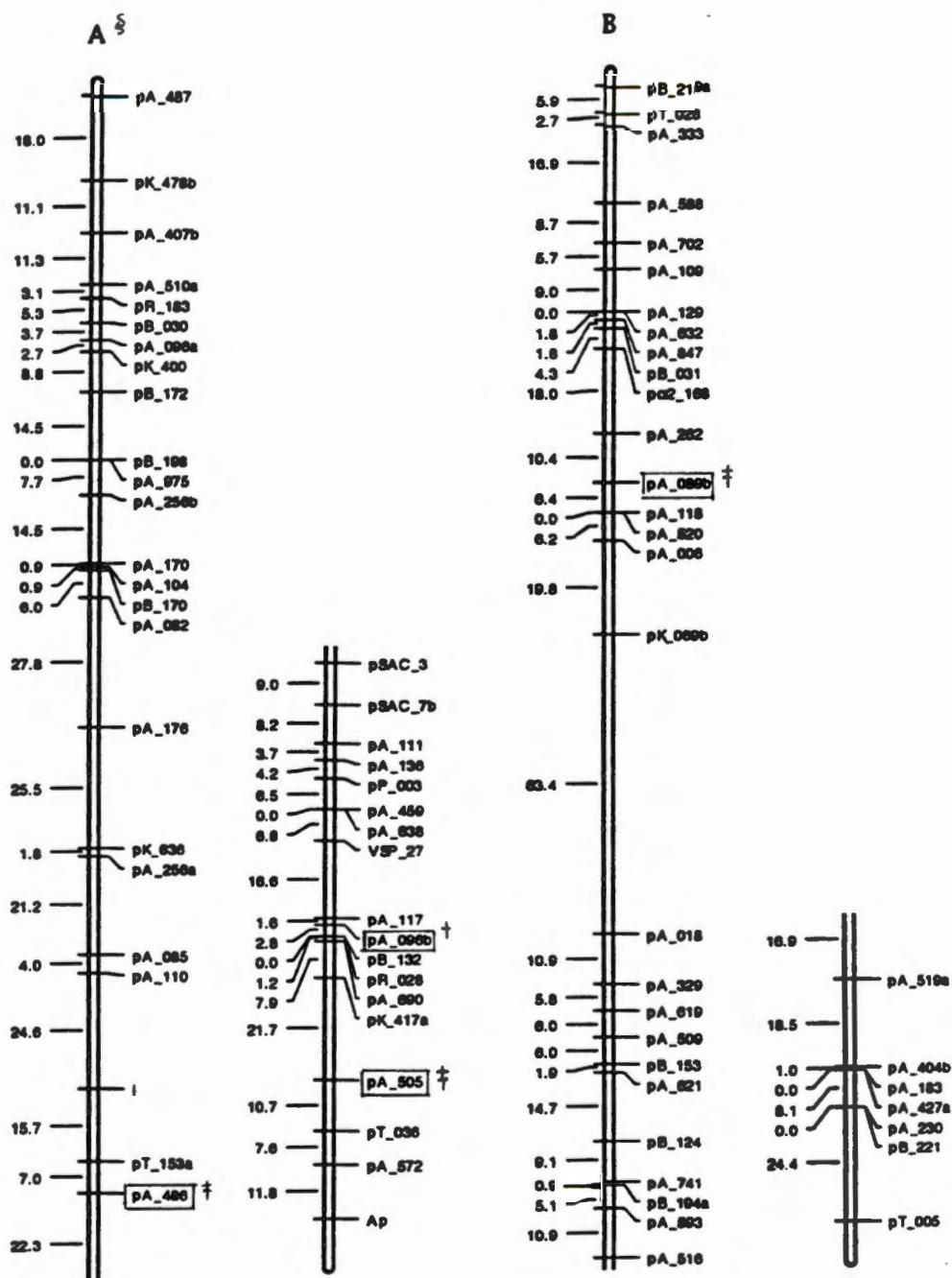
Screening large F_2 segregating populations using bulked segregation analysis combined with RAPD or DAF is suggested for future mapping.

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APPENDIX

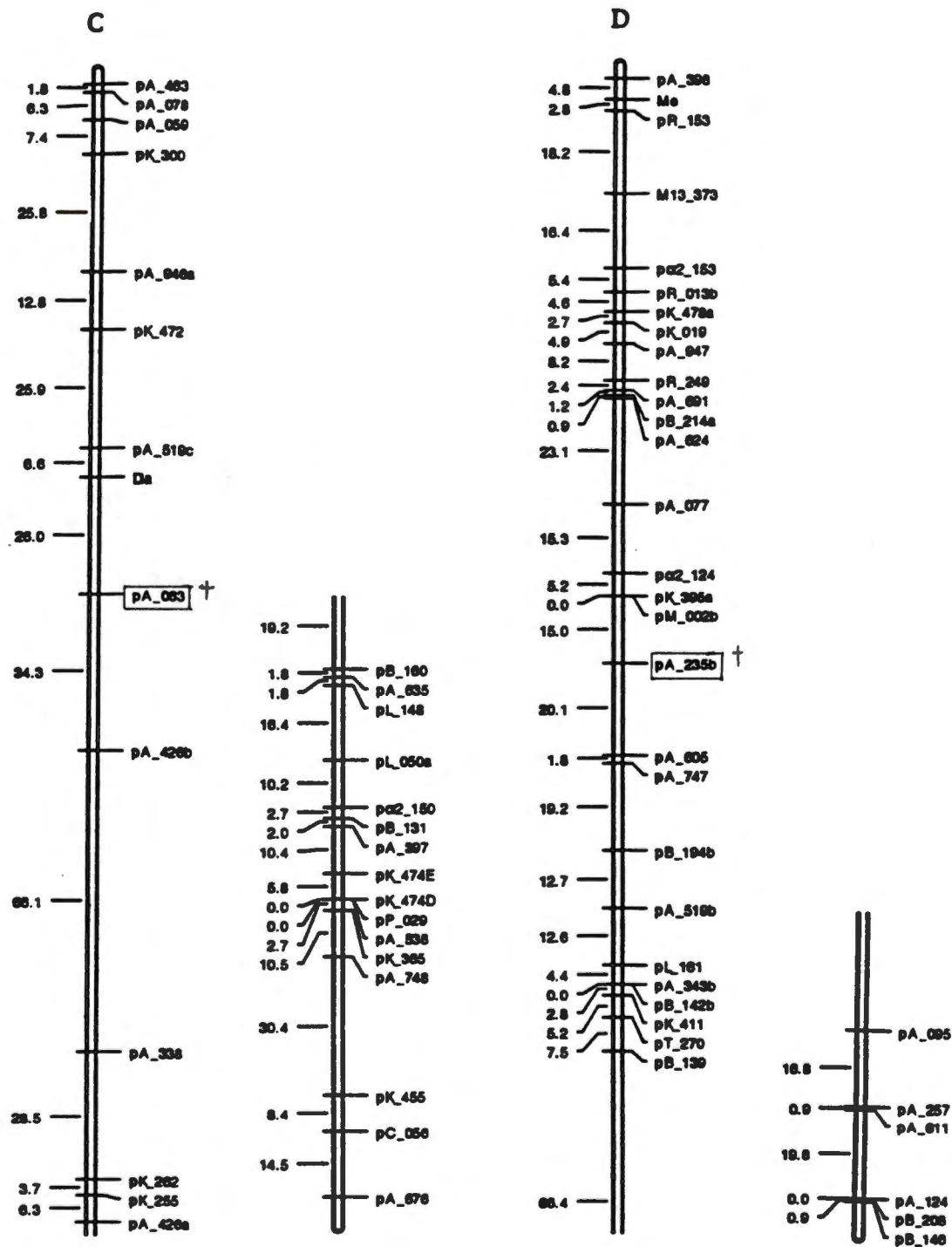
MOLECULAR LINKAGE MAP OF SOYBEAN



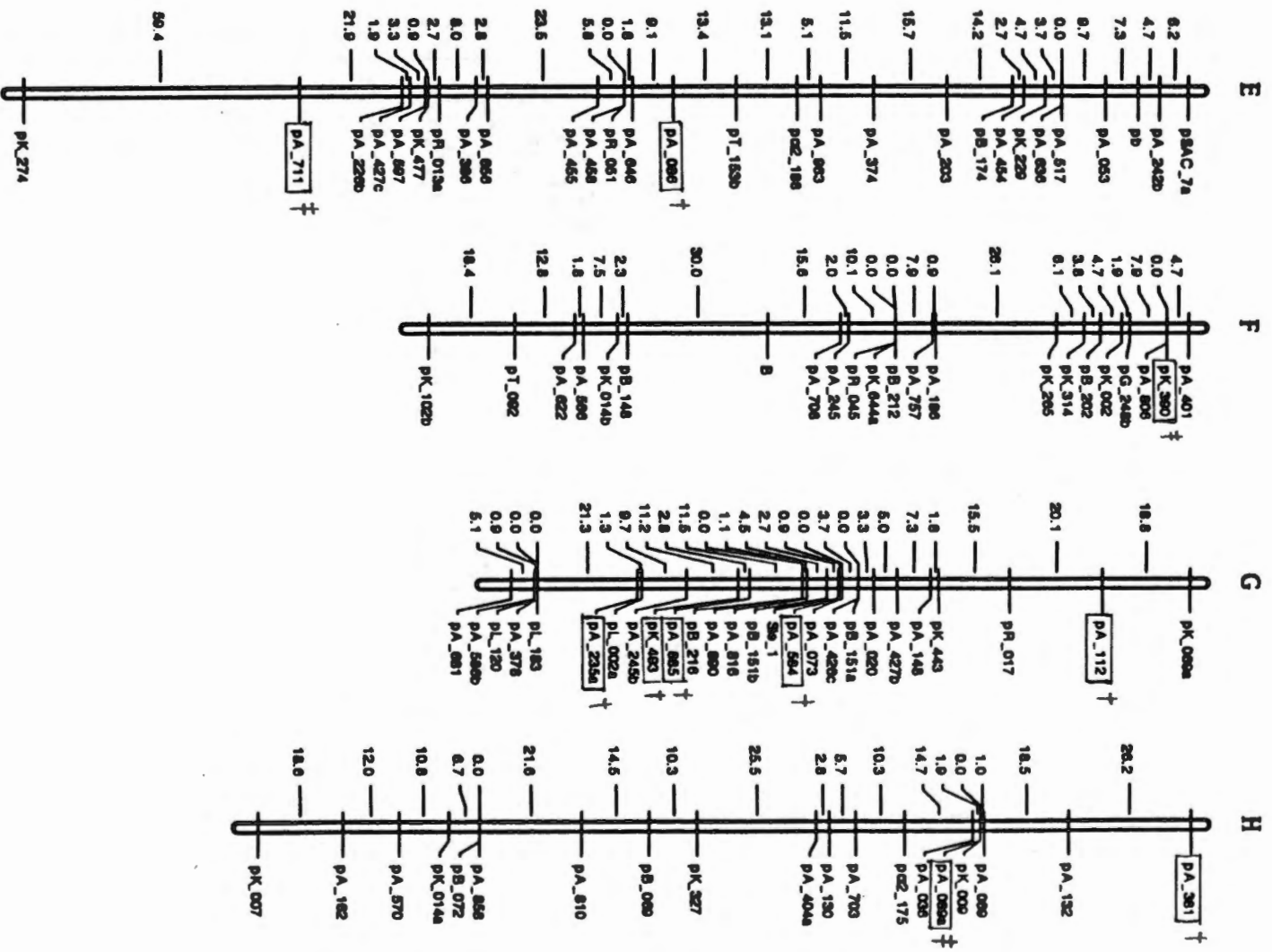
Source: Shoemaker and Olson, 1993

Note: The boxed probes are those used in this study. Probes marked with † resulted in polymorphism between Essex and PI 468.397. Probes marked with ‡ did not result in polymorphism between Essex and PI 468.397.

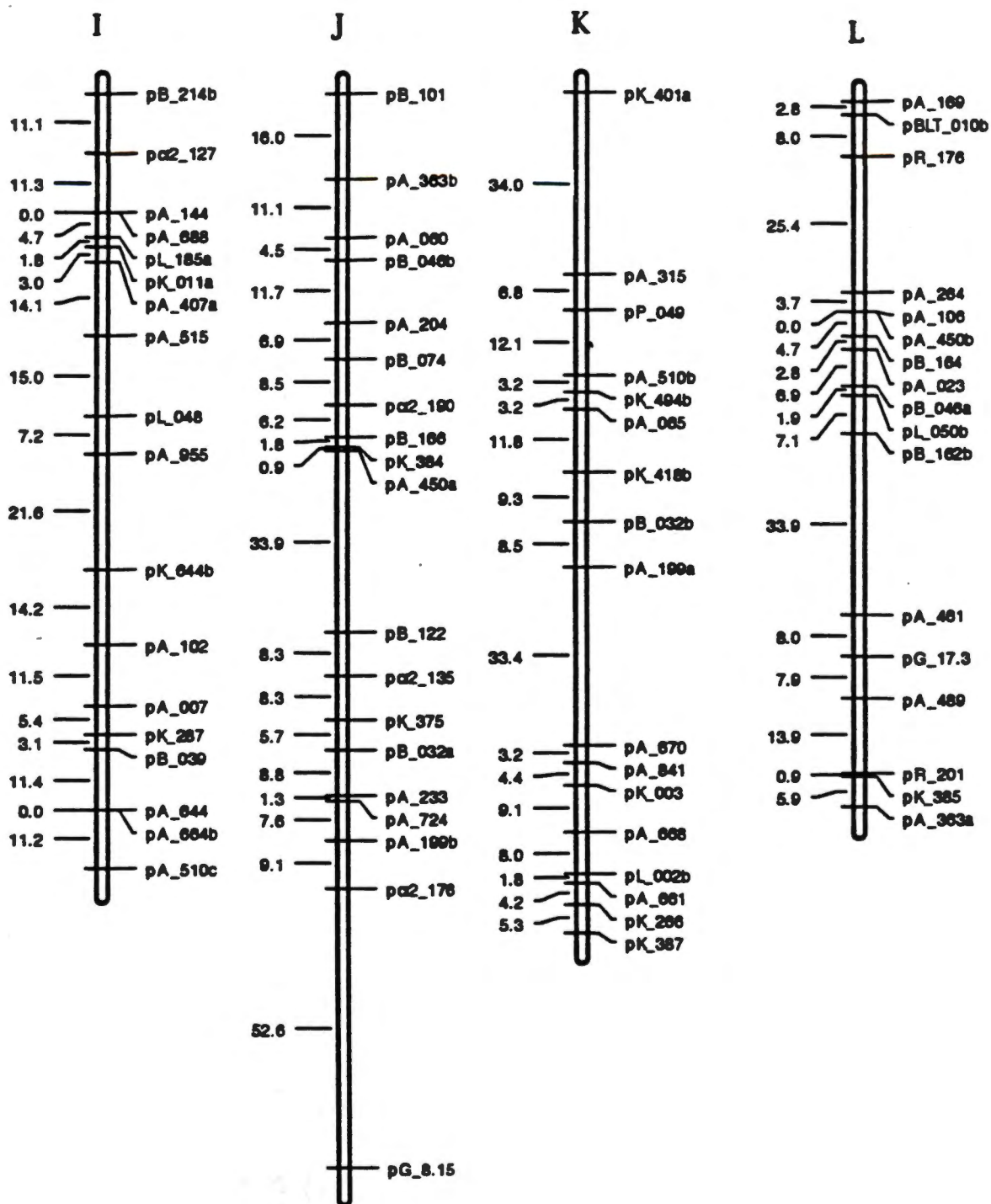
§: Linkage group.



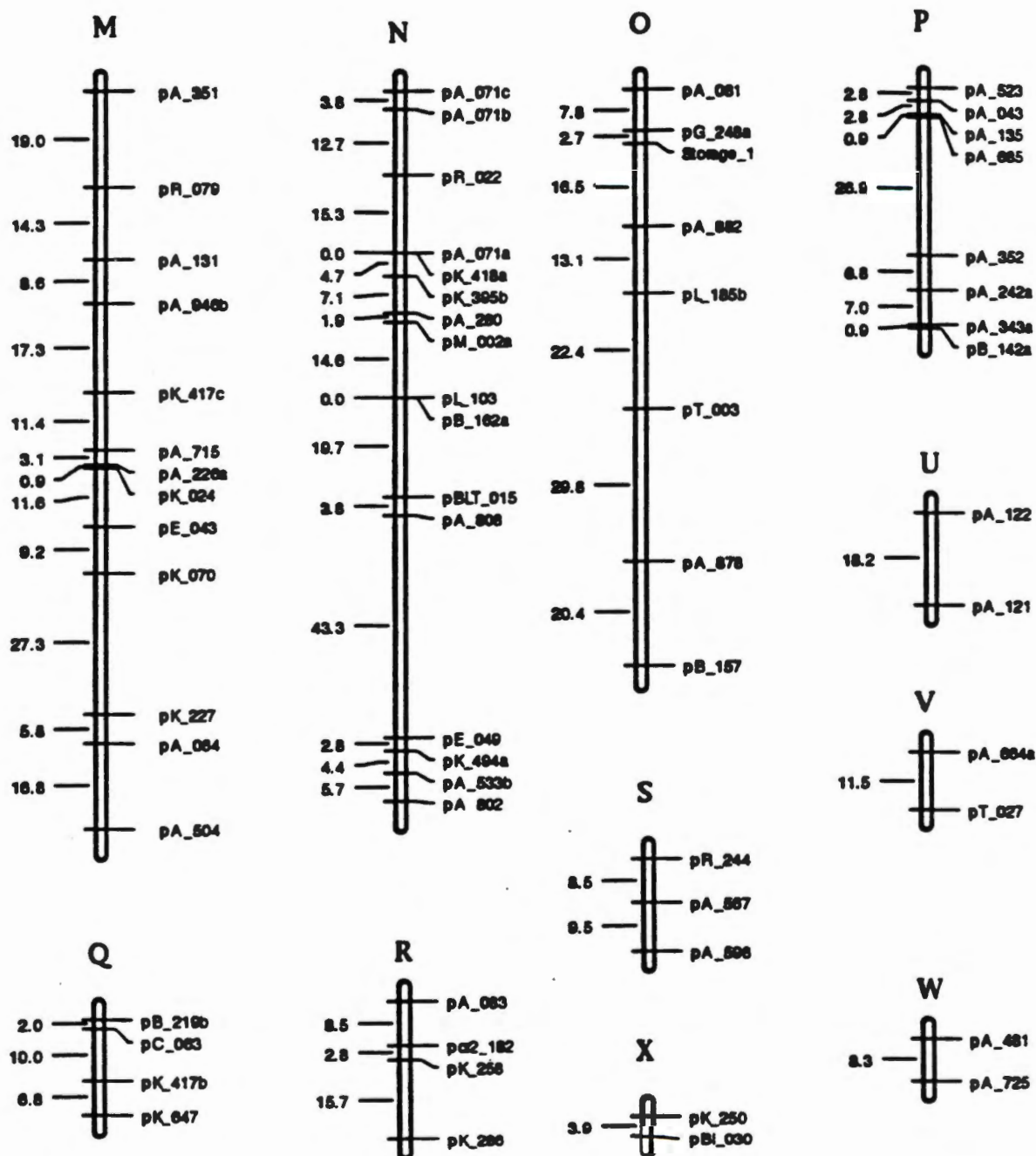
Molecular linkage map of soybean (continued)



Molecular linkage map of soybean (continued)



Molecular linkage map of soybean (continued)



Molecular linkage map of soybean (continued)

Vita

The author, Daqi Qian was born in Wujin, Jiangsu Province, P.R. China, on June 6, 1962. He attended elementary school in Wujin, Jiangsu. He graduated from Minhuang High School, Wujin, Jiangsu, in July 1978. Upon graduate, he enrolled at Jiangsu Agricultural College in October 1978. In July 1979, he was transferred to Nanjing Agricultural University. In July 1982, he received a Bachelor of Science Degree in Agronomy.

In September 1982, he entered the Graduate School of Nanjing Agricultural University and worked in the area of Plant Breeding with emphasis on Statistics and Quantitative Genetics. In December 1985, he was awarded a Master of Science Degree in Plant Breeding. Upon graduation, he accepted a position with Agronomy Department, Nanjing Agricultural University as a research associate in Soybean Breeding and teaching Methods of Statistics for undergraduate students. In July 1988, he was promoted to assistant professor and continued working on soybean breeding and teaching statistics.

In September 1989, he left his lovely and beautiful country to come to the United States of America working toward a doctoral degree in Plant and Soil Science at the University of Tennessee, Knoxville. In December 1993, he was awarded a Doctor of Philosophy in Agriculture, with a major in Plant and Soil Science.

He is married to Hong Yan and has one daughter, Yuetian Jenny Yan.

