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Nodulation and expression of the early nodulation gene, \textit{ENOD2}, in temperate woody legumes of the Papilionoideae

by

Carol Marie Foster

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Plant Physiology
Major Professors: William R. Graves and Harry T. Horner

Iowa State University
Ames, Iowa
1998

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ABSTRACT

Understanding dinitrogen-fixing symbioses in economically important, temperate, woody legumes requires evaluating their nodulation status and studying molecular mechanisms of nodulation and dinitrogen fixation. *Styphnolobium japonicum* (L.) Schott and *Cladrastis kentukea* (Dum.-Cours.) Rudd were examined for the capacity to form root nodules. Inoculations with various broad-range rhizobia, soil and rhizobia from closely related species of *Sophora*, and soil from *S. japonicum* and *C. kentukea* in Japan, China, and the United States did not elicited nodulation. As an unexpected consequence of these experiments, rhizobia were isolated for the first time from nodules of *Maackia floribunda* Takeda, and most tested isolates also infected *Maackia amurensis* Rupr. & Maxim. An early nodulation gene, ENOD2, was isolated and described in *M. amurensis*, *S. japonicum*, and *C. kentukea*. The cDNAs had 52 to 82% identity to other ENOD2 sequences, and the cDNAs encoded proteins with amino acid compositions and conserved pentapeptides (PPHEK, PPYEK, and PPEYQ) characteristic of ENOD2 proteins. Southern analyses confirmed cDNAs in each species belonged to small gene families. Transcripts were detected in nodules, roots, and flowers from *M. amurensis*, and in roots, stems, and flowers from *S. japonicum* and *C. kentukea*. Four days after inoculation with rhizobia, ENOD2 transcripts were detected in roots of *M. amurensis*, and expression was enhanced as nodules developed. *In situ* hybridization showed that ENOD2
transcripts are restricted to the distribution zone in indeterminant nodules of *M. amurensis*. Pseudonodules developed on roots of *M. amurensis* after treatment with TIBA, an auxin transport inhibitor, but did not form on *S. japonicum* and *C. kentukea*. Zeatin and TIBA enhanced ENOD2 expression in roots of *M. amurensis* only during the first 10 days of a 40-day treatment. Although transcript accumulation in roots of *S. japonicum* and *C. kentukea* was inhibited initially after exposure to TIBA and zeatin, accumulation was enhanced after 30 days. My results suggest ENOD2 activity is not a marker for nodulation in legumes, but instead may be a cellular response to concentrations of plant hormones in diverse tissues. In *M. amurensis*, ENOD2 proteins may be components of cell walls in tissues that regulate nutrient flow to sinks.
CHAPTER 1. GENERAL INTRODUCTION

Dissertation Organization

A general introduction (Chapter 1), five manuscripts (Chapters 2, 3, 4, and 5), and general conclusions (Chapter 6) are the components of this dissertation. An introduction of the project goals and a literature review are included in Chapter 1. An inoculation study examining the nodulation response of woody Papilionoideae species, *Styphnolobium japonicum* (L.) Schott and *Cladrastis kentukea* (Dum.-Cours.) Rudd, after inoculation with rhizobia and soil from Hawaii, Asia, and North America is described in Chapter 2. This copyrighted manuscript has been accepted for publication in *Plant and Soil* In Press and is included in the dissertation with permission from Kluwer Academic Press. Chapter 3 describes the nucleotide and amino acid sequence of *MaENOD2*, a cDNA isolated from nodules of *Maackia amurensis* Rupr. & Maxim. This manuscript has been published in the Plant Gene Register of *Plant Physiology* 116: 1604. Permission has been granted from the American Society of Plant Physiologists to include this copyrighted material in the dissertation. The manuscript presented as Chapter 4 characterizes the spatial, temporal, and *in situ* expression of *MaENOD2* in *M. amurensis*. This manuscript will be submitted to *Plant Physiology*. Chapter 5 is a manuscript documenting the spatial and temporal expression of cloned and sequenced ENOD2-like cDNAs from *S. japonicum* and *C. kentukea*. The effect of cytokinins and auxin transport
inhibitors on pseudonodule development and production of *Sj*\textit{ENOD2} and *Ck*\textit{ENOD2} transcripts was examined also. This manuscript will be submitted to \textit{Plant Physiology}. A summary of the complete dissertation project that examined nodulation and \textit{ENOD2} gene expression in nodulating (*M. amurensis*) and non-nodulating (*S. japonicum*, and *C. kentukea*) woody legumes is included in Chapter 6.

**Project Goals**

Although the capacity to form root nodules is most common in the Papilionoideae, little is known about the biology of nitrogen fixation in temperate woody legumes. A key to establishing or enhancing dinitrogen-fixing symbioses in woody legumes to understand molecular mechanisms that promote or impede optimal nodulation and nitrogen fixation. To facilitate research on molecular barriers to nodule organogenesis and N\textsubscript{2} fixation in non-nodulating woody legumes, a more thorough analysis of the capacity to nodulate is needed to substantiate or refute claims of non-nodulation.

The objectives of this dissertation were: 1) to test the ornamental woody legumes, *Styphnolobium japonicum* (L.) Schott (formerly *Sophora japonica*) and *Cladrastis kentukea* (Dum.-Cours.) Rudd, for the capacity to form root nodules and to characterize any rhizobia isolated from nodules; 2) to isolate and sequence \textit{ENOD2} cDNAs from *Maackia amurensis* Rupr. \& Maxim., a temperate, nodulating, Papilionoid tree of horticultural importance; 3) to determine organ-
specific, temporal, and in situ production of ENOD2 transcripts in Maackia amurensis; 4) to test for the presence of ENOD2 in S. japonicum and C. kentukea; and 5) to study the effect of an auxin transport inhibitor and cytokinin on spatial and temporal production of ENOD2 transcripts, and on the capacity of S. japonicum and C. kentukea roots to form pseudonodules. New data on nodulation capacity and the characteristics of the ENOD2 gene family in woody legumes might provide clues to nodulation patterns in different taxa and will foster a broader understanding of molecular mechanisms associated with nodulation in the Fabaceae.

Literature Review

Benefits of N₂ fixation

Nitrogen (N) is a necessary component of all living things due to its role in protein synthesis. The most abundant form, gaseous dinitrogen (N₂), is incorporated directly by very few organisms (Allen and Allen, 1981). For most plants, soil is the major N source. N can originate from decomposing organic matter, applied fertilizers, or both. N is frequently the limiting nutrient for agronomic plants. Nursery and agricultural practices require large quantities of N fertilizer to support high production levels (Batzli et al., 1992). The mutualistic relationship between rhizobia and legumes results in the formation of a unique plant organ, the nodule. Within nodules, rhizobia reduce N₂ to
ammonia, which is used as a substrate of the ammonia assimilation pathway (Pate and Atkins, 1983). Research regarding nodulation and N\textsubscript{2} fixation is of interest to those promoting sustainable agricultural production (Shantharam and Mattoo, 1997). N\textsubscript{2} fixation may provide N and increased productivity at a low cost. Because the most demanding sink in nodulating legumes is the root nodule, and legumes are very efficient in nitrate uptake from the soil (Imsande and Touraine, 1994), disruption of the balance between cost and demand in the host plant results in inefficient N\textsubscript{2} fixation. As a consequence, there has been limited success in improving nodulation and N\textsubscript{2} fixation in legumes, despite an increased understanding of all aspects of the rhizobium-legume interaction (Shantharam and Mattoo, 1997).

N\textsubscript{2}-fixing symbiosis makes survival on N-poor soil possible for legumes and benefits the surrounding vegetation due to the extra fixed N\textsubscript{2} that is released into the soil. As nursery or crop plants, legumes may have a relatively low requirement for N fertilizer, which reduces production costs and diminishes the amount of damaging nitrate leachate that enters rivers and lakes of the local environment (Batzli et al., 1992). The potential of leguminous trees to nodulate and benefit from fixed N\textsubscript{2} is important for selecting species for reforestation and alley cropping, and affects the management of N applications during tree culture (Brewbaker, 1990). Ecosystems that have little water available and poor soil may be difficult to revegetate, but the woody legumes make good candidates for plant cover due to their rhizobial symbioses (Herrera et al., 1993). Isolation and characterization of rhizobia that fix N\textsubscript{2} with these species could affect methods to

**Nodule Anatomy**

The morphology of nodules from numerous legumes has been described (Bergersen, 1982; Newcomb, 1981), although nodules from additional species continue to be examined (Sutherland et al., 1994). Diverse nodule morphology is determined by the host plant and may reflect variation in interactions between rhizobia and legumes (Corby, 1988; de Faria et al., 1989). But there are four features characteristic of legume nodule anatomy: induction of a meristem in the root cortical cells (Rolfe and Gresshoff, 1988); invasion of nodular tissues and cells by rhizobia (Sprent, 1989); development of a region in the center of the nodule where $O_2$ concentration is reduced (Bergersen, 1982); and presence of vascular tissues inside a nodule endodermis but outside the region of infected cells (Truchet et al., 1989). A diagram of the anatomy of an indeterminant nodule is in Appendix C of this dissertation.

Cellular functions of the nodule require gas exchange, and structural features of the nodule, such as lenticels and intercellular air spaces, facilitate the exchange (Dakora and Atkins, 1989). In contrast to the cells of the boundary layer, the abundant intercellular spaces of the distribution zone form a network of air spaces around the infected region and may promote rapid gas exchange with infected cells. The volume of air space in the distribution zones of nodules of *Phaseolus vulgaris* L. and the indeterminant nodules of *Pisum sativum* L. was
reduced in response to increased $O_2$ concentration (Witty et al., 1987).

Concentration of $O_2$ may be controlled in the nodule parenchyma by antioxidant activity (Dalton et al., 1998). These results suggest that the inner parenchyma, including the boundary layer and distribution zone, may regulate $O_2$ diffusion (Parsons and Day, 1990). Control of $O_2$ diffusion is essential to ensure efficient $N_2$ fixation (Dakora and Atkins, 1989). Factors that inhibit nodule function (limited photosynthesis, combined $N$, and drought stress) may compromise $O_2$ diffusion resistance. Bolanos et al. (1994) observed abnormal nodule development and reduced nitrogen fixation in boron-deficient *Pisum sativum* plants. Although structure of peribacteriod and infection thread membranes was adversely affected, nodule inefficiency was blamed on compromised nodule parenchyma cell walls and presumed elevated levels of $O_2$.

Although the function of uninfected cells in the central region of indeterminant nodules is unknown, Brewin (1991) suggested those of the central tissues facilitate transport of nutrients and organic $N$ compounds between vascular tissues and $N_2$-fixing cells. In the symbiotic region, uninfected cells are smaller than infected cells and may be specialized for exchange of metabolites. In determinant nodules, these uninfected cells contain peroxisomes and nodule-specific uric oxidase for production of ureides (Hirsch, 1992). In *Arachis hypogaea* L. nodules, rays of uninfected cells in the central symbiotic region connect with cells of similar structure in the nodule boundary layer (described as the distribution zone in other species) (VandenBosch et al., 1994). VandenBosch et al. (1994) proposed that synthesis and transport of $N$ assimilates to nodule
parenchyma cells occurs in the interstitial ray cells and fixed nitrogen is stored in nodule parenchyma cells before transport.

Mechanisms of Nodulation

Legumes form mutualistic relationships with bacteria that fix N\textsubscript{2}, like *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, and *Azorhizobium* (Verma et al., 1992). Mechanisms of nodulation are discussed in review articles by Brewin (1991), Hirsch (1992), Sprent and de Faria (1988), and Sprent (1989). Nodulation is divided into three stages: preinfection, nodule formation/infection, and nodule function. Signaling between rhizobia and plant hosts occurs continuously from preinfection through senescence of nodules. Rhizobia are attracted to plant roots by sugars, amino acids, and flavonoid compounds (Caetano-Anolles et al., 1988). Expression of numerous nodulation (*nod*) genes in *Rhizobium* depends upon the release of a plant root exudate or seed exudate into the soil (Zaat et al., 1987). Host recognition and nodule development depend upon bacteria coming in contact with chemical inducers, flavones, and flavanones produced only by legumes (Fleshner, 1988). If rhizobia, with the correct genome, come into contact with the appropriate plant exudate, then the *nod D* gene will be expressed and activate other *nod* genes (Ridge et al., 1992). Rhizobial *nod* genes are activated by flavonoids in conjunction with the *nod D* gene product and the products of these genes generate lipo-chitooligosaccharides or nod factors (Horvath et al., 1987; Spaink et al., 1989; Kondorosi et al., 1989).

Nod factors confer host specificity to the rhizobia and initiate nodule morphogenesis and root hair curling only in compatible plant hosts.
Amplification of plant phenylpropanoid compounds (flavonoids) is induced by rhizobial nod factors (van Brussel et al., 1990) and initiates localized plant responses, such as root hair curling and cortical cell divisions (Lerouge et al., 1990). Concurrently, the rhizobia attach themselves to susceptible regions of the root, form colonies, and initiate formation of infection threads. Upon reaching the developing nodule meristem, rhizobia invade the cortical cells and differentiate into bacterioids with development of the peribacterioid membrane. In a mature nodule, all nodular tissues are formed and nitrogen fixation begins. The physiological status of plant cells (O₂ availability, carbon metabolism, N status, turgor relations, and Ca status) generates signals that affect the success of symbiosis.

Each of these steps plays a role in the specificity of the microbe-host interaction (Verma et al., 1992). Cross-inoculation studies have shown that certain rhizobia are effective with groups of plants (Allen and Allen, 1981). Despite what appears to be a strict recognition system between the plant and the microbe, the level of specificity varies greatly among rhizobia. Variants of *nod D* within one rhizobium confer greater host diversity due to differences in flavonoid recognition. One promiscuous *Rhizobium* species, strain NGR234, nodulates 35 legume genera (Verma et al., 1992). Other rhizobia with a broad host range can be found among the *Bradyrhizobia*. In some cases, host plants exhibit flexibility with signal recognition and form a symbiotic relationship with multiple rhizobia.
Ardourel et al. (1994) proposed a model that explains the role of Nod factors. Two signals are required to stimulate two different pathways. The signaling receptor pathway is responsible for cortical cell activation and helps activate the second pathway. The entry receptor pathway affects infection site and thread formation. Nod factor structural requirements for signaling rhizobium entry are stricter than requirements for induction of developmental responses (Ardourel et al., 1994). Minami et al. (1996) determined that a mixture of at least two nod signals is required to initiate soybean nodule development and to induce nodule parenchyma differentiation and ENOD2 expression. These results suggest that induction of ENOD2 expression requires at least two cellular recognition pathways. Formation of pseudonodules indicates that these pathways are interactive. Nod factors may alter hormone balance, and auxin transport inhibitors may mimick the secondary signals required for nodule development (Hirsch et al., 1989). Infection method and nodule morphology are ultimately controlled by the host plant, indicating that the genetic information necessary for rhizobial symbiosis is possessed by the plant (Verma et al., 1992). Infection occurs through root hairs, between epidermal cells, and through wounds, created primarily by emerging lateral roots (Sprent and de Faria, 1988). Morphological and anatomical homology exists among nodules of the Fabaceae, but differences in modes of infection and tissue organization may reflect legume phylogeny.

**Flavonoids**

Flavonoids are widely distributed in higher plants. The source of flavonoids and isoflavonoids in plants is phenylpropanoid metabolism (Moyano et al.,
1996). Many environmental and developmental changes stimulate the production of flavonoids and other secondary metabolites from the phenylpropanoid pathway. Phenylpropanoids function as pigments, such as anthocyanin, and are involved in pollen viability, lignin biosynthesis, and stress responses. According to Mascarenhas (1990), enzymes vital to phenylpropanoid metabolism are produced by the tapetum and function to produce flavonoids and other phenylpropanoids in the anther loculus. Flavonols stimulate pollen maturation in the anther and later, germination on the stigma (McCormick, 1993). Some defects in pollen development may be due to the absence of the hormone-like flavonols. Before infection, flavonoids are produced by the plant to attract compatible rhizobia to the root surface. Shaw et al. (1997) determined that non-nodulating species retained the capacity to synthesize and release flavonoids and isoflavonoids into the rhizosphere, but composition of soil exudates did not necessarily represent the pool of flavonoids present within root tissues.

An early response to rhizobial inoculation may be localized changes of the flavonoid pool in plants. Some members of the pathway, phenyl-ammonia lyase and chalcone synthase, are symbiosis-enhanced (Hirsch et al., 1993). Jacobs and Rubery (1988) determined that certain flavonoids function as natural auxin transport inhibitors by competing with naphthylphthalamic acid for its membrane-bound receptor. The efflux step of IAA polar transport is blocked, and IAA accumulates in cells, which affects gene expression, ion transport, and cell and organ differentiation. The most common flavonols and flavones in
plants were most effective at inhibiting auxin transport (Jacobs and Rubery, 1988).

Hirsch (1992) proposed that the phenylpropanoid pathway is stimulated by nod factors; specific flavonoids are produced and bound by receptors in specific cells; auxin transport is inhibited, which alters localized hormone balance; and cortical cell divisions are initiated. By creating a hormone imbalance in the root, flavonoids establish the secondary signal for initiation of nodule organogenesis.

**Woody species and nodulation capacity**

Nodulation, once thought to be inherent in legumes, does not occur in all legume species. In the subfamilies Caesalpinioideae, Mimosoideae, and Papilionoideae, 23%, 90%, and 98% of the examined species, respectively, are reported to form root nodules (Allen and Allen, 1981). If proper management and utilization of economically important woody legumes is to be attained, then it is essential to know whether they nodulate, how nodulation takes place, and how efficiently they fix \( \text{N}_2 \) (Sprent, 1994). Interestingly, although many woody legumes are considered non-nodulating (Allen and Allen, 1981), little information is available on mechanisms preventing infection by rhizobia.

Putative non-nodulating woody species, *Cercis canadensis* L. (Eastern redbud), *Gymnocladus dioica* (L.) K. Koch (Kentucky coffeetree), and *Gleditsia triacanthos* L. var. *inermis* Willd. (thornless honey locust), are among the most widely produced ornamental trees in the United States (Brewbaker, 1990).

There are many environmental and organismal factors that affect symbioses between rhizobia and legumes. Environmental factors that restrict nodulation events and influence nodule occupancy by rhizobia are combined \( \text{N} \), soil \( \text{pH} \), soil
temperature, and osmotic stress (Vlassak and Vanderleyden, 1997). Nitrate, light, and ethylene also can inhibit nodulation (Brewin, 1991; Lee and LaRue, 1992). Rhizobia differ in their responses to these factors, which reflects the competitive fitness of the rhizobia. The inability to induce nodules may also indicate the breakdown of early events of symbiosis, such as rhizobial attachment, root hair curling, and thread formation. The most appropriate method to test the capacity for nodulation in legumes is the whole-soil inoculation technique. This involves growing plants in soil presumed to contain rhizobia likely to infect (Somasegaran and Hoben, 1994), but plants also can be inoculated with rhizobia (Caetano-Anollés and Gresshoff, 1991).

Nodulins

Nodule formation after infection of roots by rhizobia requires induction of plant genes involved in growth and development. Nodulin genes, or genes that encode for nodule-enhanced proteins, affect the physical changes that occur during nodule formation (Palacios and Verma, 1988) and the metabolism of N₂ fixation. Nodulin genes have been divided into two categories, early and late, based on time of expression during nodule development. Early nodulin genes (ENOD) are active before N₂ fixation begins in the nodule and are involved primarily with the infection process and nodule organogenesis. Genes involved in nodule function and maintenance are considered late nodulins (NOD). Expression patterns vary depending on whether nodules are determinant or indeterminant. ENOD and NOD gene products are present in both types of nodules, but are regulated to accommodate the different developmental patterns.
Regulation of nodulin genes during development and function of nodules was reviewed by Sánchez et al. (1991). Many nodulins are continuously produced in indeterminant nodules. Meristematic activity ceases after tissue differentiation in determinant nodules and so does the activity of some genes.

Previously, nodulin gene activity was detected only in nodules (Govers et al., 1990; van de Wiel et al., 1990). Later, expression was detected also in stems, suggesting a stem origin of root nodules (Sprent, 1990). Now, nodulin gene expression, having been detected in nodules, uninoculated roots, stems, and flowers (Papadopoulou et al., 1996; Scheres et al., 1990a; Szczyglowski et al., 1997; Yang et al., 1993) are described as symbiosis-enhanced. Examples of symbiosis-enhanced genes are PsENOD12 in stem and flower, and glutamine synthetase in stem and hypocotyls (Scheres et al., 1990b; Cock et al., 1991; Govers et al., 1991). Some nodulins, such as leghemoglobin and ENOD40, are produced also in non-legumes (Bogusz et al., 1990). A hemoglobin promoter from the non-nodulating, non-legume *Trema tomentosa* produced the same pattern of leghemoglobin expression in *Lotus corniculatus* as the native leghemoglobin gene from *L. corniculatus*. Hemoglobin genes may be expressed in all plants in response to microaerobic stresses like lateral root initiation. Multiple transcripts in other organs may indicate that nodulin transcripts detected in nodules were recruited for nodulation. Nodulins have not been studied in species of temperate, woody Papilionoids, members of the Caesalpinioideae and Mimosoideae, or in nodulating nonlegumes.
Rapid divergence of a single ancestor gene may explain sequence similarities among proline-rich nodulins (Verma et al., 1992). Despite sequence similarities, nodulins function in a number of tissues during different developmental events. Gene and sequence duplications, as well as individual nucleotide substitutions, may explain the presence of different nodulins, as these could generate new types and patterns of pentapeptide repeats and variations in codon usage. Subsequent evolution of nodulin genes after the initial gene duplication may have involved random point mutations, exon-shuffling, or transposon-induced rearrangements to produce sequence variation in the coding region and tissue-specific promoters and enhancing elements (Vegh et al., 1990). Sequence comparison of nodulins from *Glycine max* (L.) Merrill revealed that these genes evolved rapidly, providing a larger pool of genes to make nodulation and N$_2$ fixation more efficient (Verma et al., 1992).

**Mutants**

Caetano-Anollés and Gresshoff (1991) suggested studying natural variants (non-nodulators, poor nodulators, or inefficient N$_2$ fixers) of species that nodulate would provide useful information regarding rhizobia-legume symbioses. A non-nodulating variant of *G. max* was reported over 40 years ago (Williams and Lynch, 1954), and genetic studies since have identified 45 mutations in eight species (Sánchez et al., 1991). Mutants exhibiting supernodulation, nitrate tolerance, no root hair curling, and minimal cell divisions have been described (Carroll et al., 1985; Mathews et al., 1987, 1989; Utrup et al., 1993). Csanádi et al. (1994) determined that ENOD12 was not
necessary for nodule formation and N$_2$ fixation in *M. sativa* by using a mutant with a null allele for *ENOD12*. To clarify the roles of other nodulins in nodulating species, loss-of-function mutants and underexpressing transgenic plants were generated (Caetano-Anollés and Gresshoff, 1991). Gene constructs with altered promoter sequences have been introduced into plants to elucidate which regions of the gene promoter control nodule specificity. Mutagenesis of *G. max* has led to isolation of non-nodulating mutants (Carroll et al., 1986). Lack of nodulation in these mutants may be caused by loss of Nod factor perception, production of nod factor hydrolyzing enzymes, or production of ethylene upon infection. Wyss et al. (1990) observed that many of the non-nodulating mutants of *G. max* are still able to establish a normal symbiosis with vesicular-arbuscular mycorrhizas.

**ENOD2 genes**

Nodulin genes highly homologous to the *G. max* ENOD2 gene have been found in all legumes tested so far (Nap and Bisseling, 1990). ENOD2 may not play a role in oxygen regulation by the nodule parenchyma of the nodule (James et al., 1991; van Rhijn et al., 1997; Wycoff et al., 1998). Function of ENOD2 genes may be determined by molecular mapping, but cosegregation with mutant loci is required (Caetano-Anollés and Gresshoff, 1996; Ghassemi and Gresshoff, 1998). Based on mapping information from *Pisum sativum*, it is hypothesized that genes integral to nodulation are clustered. Positional cloning has linked *enod2b* in *G. max* to the seed coat color gene, *I*, and *Rhg4*, a cyst nematode resistance gene (Ghassemi and Gresshoff, 1998). Other genes in the region may be cell wall
proteins involved with seed hardness and pod filling. Franssen et al. (1987, 1990) suggested that the proline-rich protein encoded by ENOD2 is a cell wall protein with a structure similar to hydroxyproline-rich cell wall glycoproteins. Sequences of N- and carboxy-termini of ENOD2 cDNAs differ markedly from one another, and deduced ENOD2 proteins vary in length, pattern of pentapeptide repeats (Dehio and de Bruijn, 1992), and codon usage. Function of ENOD2 proteins does not appear to depend on a conserved length or linear order of proline-rich repeats. It is speculated that ENOD2 protein cannot cross-link with carbohydrate groups on polysaccharides or glycoproteins of the cell wall without boron (Brewin, 1991). Nodules deficient in boron possess defective nodule meristems, abnormal infection threads, and altered parenchyma regions with no ENOD2 protein incorporated into the parenchyma cell walls (Bonilla et al., 1997).

In situ hybridization showed that the outer cortex of uninfected root primordia on the stems of Sesbania rostrata Brem. & Oberm. possessed ENOD2 transcripts, and expression in the cortex was enhanced by infection (Goormachtig et al., 1998). ENOD2 protein in the cortex may protect root primordia or developing nodules on the stem from dessication. Another possible role could be to provide structural support to the inner parenchyma as the central region of the nodule develops. Transcript accumulation of SrENOD2 in the nodule parenchyma of transgenic Lotus corniculatus is directed by the 3’ untranslated region of the SrENOD2 gene (Chen et al., 1998). In G. max, the 5’ upstream region of GmENOD2 gene controls nodule-specific transcript production (Lauridsen et al., 1993).
SrENOD2 transcripts have been detected in Agrobacterium-induced tumors on S. rostrata (Vlachova et al., 1987). Agrobacteria possess cytokinin biosynthesis genes, and strains with a cytokinin mutation do not induce SrENOD2 expression in tumors. ENOD2 genes in hairy roots of L. corniculatus and Melilotus alba Desr. are expressed also when infected with Agrobacterium rhizogenes (Govers et al., 1990). These results show that induction of ENOD2 results from a hormone imbalance or sensitivity, and suggests that ENOD2 expression is a marker for cellular activity influenced by hormonal fluxuations. Dehio and de Bruijn (1992) showed that applications of cytokinin, such as zeatin, 2iP(dimethyl- alyl-amino purine), kinetin, and BAP (6-benzylamino-9-methyl purine), to the roots of S. rostrata result in production of ENOD2 transcripts. SrENOD2 transcripts accumulated posttranscriptionally in the cytoplasm of root cells of S. rostrata in response to exogenously applied cytokinin (Silver et al., 1996) but inhibition of protein synthesis prevented enhancement of ENOD2 transcript production by cytokinins. This suggests that protein synthesis are required for regulation of ENOD2 by cytokinins in S. rostrata.

Proline-rich proteins

Hydroxyproline-rich glycoproteins are cell wall proteins that function to maintain cell wall integrity, accumulate upon wounding and pathogen attack, and participate in growth and development (Showalter, 1993). Reviews regarding plant cell wall protein structure, function, and gene expression are available by José and Puigdomènech (1993), Showalter (1993), and Cassab (1998). Functions of developmentally regulated glycoproteins found in extracellular
spaces, cell walls, and plasma membrane glycocalyx (VandenBosch et al., 1994; Govers et al., 1990; Scheres et al., 1990b; Rae et al., 1991) of nodules are not known. Preliminary evidence suggests that cell wall proteins may interact to facilitate cell signaling and nodule morphogenesis. Cassab (1998) proposed that association of certain cell wall proteins to plasmodesmata may indicate roles in cell-cell recognition, signaling in morphogenesis, or cell-to-cell transport via interactions with plasmodesmata regulators. Proline-rich proteins occur in xylem, epidermis, aleurone, and nodule parenchyma, in association with developmental events of the plant, such as nodulation, gametophyte maturation, and germination (Cassab, 1998). Transcripts encoding glycine- and proline-rich polypeptides with properties of cell wall proteins are anther-specific and localized in nonreproductive tissues such as the tapetum and endothecium (Goldberg et al., 1993). Few pistil-specifically expressed genes encoding proline-rich and extensin-like proteins have been isolated and seem to be expressed in the transmitting tissues of the style. Because these proteins possess putative signal peptides for excretion from the cell and their homologs are induced in response to pathogen attack or wounding, it has been suggested that they may protect the ovary against fungal or bacterial infection (Gasser and Robinson-Beers, 1993).

Although proline-rich proteins are characterized by high percentages of certain amino acids and pentapeptide repeats, amino acid compositions and motifs of proline-rich proteins are actually quite variable (José and Puigdomènech, 1993; Showalter, 1993). Deduced polypeptides can be divided into
two domains, an N-terminal putative signal peptide and a protein consisting of proline-rich pentapeptide repeats. The pattern found in all signal peptides is a hydrophobic core flanked by charged residues, followed by a recognition motif for signal peptide cleavage. Presence of such a sequence indicates probable secretion from the cell and localization in the extracellular matrix. An amino acid composition rich in Tyr and Lys suggests that proline-rich proteins are covalently cross-linked with other proline-rich proteins, glycine-rich proteins, and extensins, and also form ionic interactions with acidic pectins within the cell wall (Showalter, 1993).

Sequence repeats, localization in the plant, and expression during organogenesis are the characteristics used to speculate on possible functions for cell wall proteins (Cassab, 1998). Regulation and expression of genes encoding proline-rich proteins during cellular differentiation and tissue development may be influenced by different structural properties that result from sequence variations in the proline-rich proteins. Although interactions between cell wall proteins and other cell wall components are unknown, a review by Fry (1986) addressed the topic of cross-linking of matrix polymers. Cross-linking of wall components may render cell wall proteins insoluble and enhanced wall integrity. Amino acid composition and protein structure play a role in the extent and nature of interactions between cell wall proteins. Showalter (1993) suggested that future research into the function of proline-rich proteins should include localization of the proteins to specific organs and tissues, production of cell wall
protein mutants, examination of cell wall protein interactions in the cell wall, and isolation of more sequences, especially from more primitive plants.

**Plant growth regulators**

RNA studies using auxins and cytokinins to induce gene expression have shown that these plant growth regulators initiate rapid and specific changes in mRNA accumulation (Hagen, 1995). Genes that are regulated by auxins or cytokinins can facilitate the study of growth and development in plants. Cytokinins affect cell division and morphogenesis, and auxin levels affect lateral root initiation and floral organ development (Davies, 1995). Concentrations of plant hormones are generally the highest in developing seeds (Rock and Quatrano, 1995). Cytokinin levels are high during the liquid endosperm stage of early seed growth. Syono et al. (1976) found that, of the nodular tissues of *Pisum sativum*, meristematic cells possess the highest concentration of cytokinins. Zeatin compounds, produced by the plant meristem, and rhizobia-synthesized 2iP (isopentenyladenine) derivatives are present in *Pisum sativum* nodules. Although auxins and cytokinins have been detected in nodule extracts and are produced by infecting rhizobia (Sturtevant and Taller, 1989), as well as the plant host, their role in early nodule development has not been established.

Hormone balance changed by signals from the plant and rhizobia plays an important role in nodule organogenesis (Verma et al., 1992). Plant hormones can elicit nodulin gene expression, yet roles for particular hormones have not been determined. Possible roles of phytohormones in nodulation are discussed by Hirsch and Fang (1994) and Hirsch et al. (1997). Roles of plant hormones in
nodulation can be elucidated by studying gene expression in roots that are exposed to exogenous plant hormones (Hirsch, 1992). Long and Cooper (1988) proposed that a *Rhizobium*-induced event in root epidermal cells initiates signal transduction and production of a secondary signal in plants that is responsible for cortical cell divisions. Hirsch (1992) and de Bruijn et al. (1994) suggested that plant hormones are such a secondary signal for nodule morphogenesis. Nodule-specific plant chalcone synthase (CHS) genes (Estabrook and Sengupta-Gopalan, 1991; Yang et al., 1992) may be involved in nodule development by producing flavonoids that function as auxin transport inhibitors (Jacobs and Rubery, 1988; Hirsch, 1992).

High photosynthesis rates in plants may affect activity of plant hormones required for nodule initiation and starch deposition (Bauer et al., 1996). Plant hormones might have an indirect effect on assimilate partitioning by regulating cell division and differentiation in developing sinks, such as pollen grains and seeds (Brenner and Cheikh, 1995). Coordination of metabolic activities and mobilization of storage products during the development of different sinks may involve cytokinins. Reduction of sink strength may occur if altered cytokinin levels prevent development of amyloplasts in sink tissues (Brenner and Cheikh, 1995). Nitrate inhibition of nodulation may be mediated by ethylene (Ligero et al., 1991) and can be overcome with ethylene inhibitors. Lee and LaRue (1992) reported that inhibition of nodulation by light may be a consequence of increased ethylene production.
Pseudonodules

Pseudonodules are nodule-like structures possessing nodular tissue differentiation, but no symbiotic region. Pseudonodules have been induced on rhizobia-inoculated roots of nonlegumes, *Oryza sativa* and *Brassica napus*, by applying enzymes that degrade cell walls (Al-Mallah et al., 1989, 1990) and on inoculated roots of *B. napus*, *O. sativa*, and *Triticum aestivum* without enzyme treatment (Cocking, 1990; Cocking et al., 1992). False nodules form also on roots treated with hormones without the presence of rhizobia (Dénaire et al., 1992). Pseudonodules were induced on the roots of *G. max* with 2-bromo-3, 5-dichlorobenzoic acid, a synthetic benzoic acid derivative (Allen et al, 1953). Although NPA and zeatin did not induce pseudonodules on *T. aestivum* and *O. sativa*, roots treated with 2,4-D did form pseudonodules at the same rate as lateral roots developed on untreated plants (Ridge et al., 1992, 1993). Species that produce pseudonodules in response to cytokinin include: *Arachis hypogaea* L., *Sesbania grandiflora* Poir (Allen and Allen, 1940), *Cicer arietinum* L. (Arora, 1956), *Nicotiana tabacum* L. (Arora et al., 1959), and *Macroptilium atropurpureum* Urb. (Relic et al., 1994). Stimulation of spontaneously developed pseudonodules on the roots of alfalfa suggests that cortical cells are signaled to divide by a component independent of nod factor (Hirsch, 1992). Exogenously applied cytokinins induced pseudonodules on the roots of *Alnus glutinosa* (L.) Gaertn., an actinorhizal plant (Rodriguez-Barreuco and Bermudez De Castro, 1973). Detection of nodulin genes in pseudonodules indicated that, at the molecular level, false nodules are similar to symbiotic nodules (Bauer et al.,
1996) and implies that gene expression is under developmental control, not symbiotic control (Hirsch, 1992).

**Metabolism**

N assimilation pathways are compartmentalized in infected and uninfected cells; this influences the regulation of key enzymes and increases efficiency of N₂ fixation (Newcomb and Tandon, 1981; Nguyen et al., 1985). Although involvement of uninfected cells in events that generate and maintain a functional nodule is not known, the formation of exportable nitrogenous solutes in determinant nodules requires cooperation between various organelles in infected and uninfected cells of the nodule (Atkins, 1987). Little information is available regarding transport of assimilates from the symbiotic region of the nodule to the host plant. Flow of sucrose to the nodule is important to fuel N₂ fixation. Uninfected cells have been observed to contain numerous starch grains presumably to provide N₂-fixing cells with a carbon source at times of reduced photosynthetic activity. Some nodulins may be under metabolic control, as well as developmental control (Nguyen et al., 1985). Nodule-specific sucrose synthase, encoded in part by nodulin-100, converts sucrose to usable units for nodule metabolism (Reibach and Streeter, 1983; Thummler and Verma, 1987). Evidence suggests that low O₂ levels control expression of sucrose synthase in nodules (Yang and Russell, 1990). In *Zea mays* L. roots, low O₂ levels induced sucrose synthase.

Bauer et al. (1996) proposed a model which links nod factor, cytokinin actions and carbon/nitrogen metabolism during nodule initiation. N starvation of
plants with an elevated photosynthetic rate sensitizes root cells to nod factors and cytokinins. Roots respond to nod factors and cytokinins by initiating cortical cell divisions, increasing sink strength, starch deposition, and inducing ENOD genes. This indicates that signal transduction elements that respond to lipochitooligosaccharides and cytokinins may be linked for nodule initiation and starch accumulation. The model suggests that, during organogenesis in leguminous and non-leguminous plants, metabolic activities, sink capacities, and the mobilization of storage products may be coordinated by cytokinins. Application of pure cytokinins activated cortical cell divisions in M. sativa with amyloplast deposition and expression of MsENOD12, a gene that might be involved in modifying cell walls for rapid cell division and growth (Bauer et al., 1996). Rhizobial inoculation elicited host cell division and starch deposition primarily in the region of the root initiating root hairs, but amyloplast formation occurred in other areas of the root, indicating that these cells were competent to respond also. Two receptors might be required for rhizobial invasion and cortical cell division. Cytokinin might be part of the pathway downstream of the cell division receptor (Bauer et al., 1996). N inhibits host response to nod factors and cytokinins. Photosynthesis is required for induction of host cell division by cytokinins and nod factors. Bauer et al. (1996) suggest that auxin transport inhibition is probably not a major factor in nodulation, but local inhibition and its effects on cytokinin and nod factor can not be excluded. Auxin-induced formation of lateral root primordia, MsENOD12 expression, and starch accumulation were not influenced by N availability.
Evolution of nodulation

Swensen and Mullin (1997) suggested that predisposition for root nodule symbiosis has a single origin among angiosperms and subsequent occurrences within this group result from multiple origins. Phylogenetic analysis of \textit{rbcL} sequences from the ten angiosperm families with N\textsubscript{2}-fixing symbioses grouped the families in a single clade. This indicates only one lineage of closely related taxa possesses the genetics necessary to generate root nodules and subsequent N\textsubscript{2} fixation (Soltis et al., 1995). Nodulation may have evolved two or three times independently in the Fabaceae (Doyle, 1994; Doyle et al., 1997; Sprent, 1994). Despite sequence data suggesting one to three separate evolutionary events were responsible for symbiotic N\textsubscript{2} fixation and nodulation, development of spontaneous and hormone-induced pseudonodules indicates that genes for induction of nodulation and \textit{ENOD} gene expression exist in all plants (Dehio and de Bruijn, 1992).

Doyle (1994) suggests that specific variations in a nucleotide sequence, resulting from exon shuffling, duplication, and/or recombination, might provide clues to nodulation patterns in different taxa. Analyzing nodulin sequences in plants from various positions on the legume phylogenetic tree, such as Papilionoid model plants that share a phylogenetic cluster positioned near the Mimosoideae and the Caesalpinoideae, will help answer questions about origins of rhizobia-legume symbiosis (Doyle, 1994).
Literature Cited

Allen ON, Allen EK (1940) Response of the peanut plant to inoculation with rhizobia, with special reference to morphological development of the nodules. Bot Gazette 102: 121-142


Dehio C, de Bruijn FJ (1992) The early nodulin gene *SrENOD2* from *Sesbania rostrata* is inducible by cytokinin. Plant J 2:117-128


Shaw JE, Reynolds T, Sprent JI (1997) A study of the symbiotic importance and location of \textit{nod} gene inducing compounds in two widely nodulating and two non-nodulating tropical tree species. Plant Soil \textbf{188}: 77-82


Wyss P, Mellor RB, Wiemken A (1990) Vesicular-arbuscular mycorrhizas of wild-type soybean and non-nodulating mutants with Glomus mosseae contain symbiosis-specific polypeptides (mycorrhizins), immunologically cross-reactive with nodulins. Planta 182: 22-26


CHAPTER 2. NODULATION RESPONSE OF WOODY PAPILIONOID SPECIES AFTER INOCULATION WITH RHIZOBIA AND SOIL FROM HAWAII, ASIA, AND NORTH AMERICA

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Abstract

Among subfamilies in the Fabaceae, the capacity to form root nodules is most common in the Papilionoideae. Yet nodules have never been observed on species of Cladrastis, and there are conflicting reports of the capacity of species in the genus Styphnolobium to nodulate. Our objectives were to evaluate \textit{Styphnolobium japonicum} (formerly \textit{Sophora japonica}) and \textit{Cladrastis kentukea} for the capacity to nodulate and to characterize any isolated rhizobia. N-deficient plants were inoculated with rhizobia chosen for their low host specificity or for their symbiotic potential with indigenous and introduced trees and shrubs of

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\textit{Key words:} Cladrastis, inoculation, leguminous trees, \textit{Maackia}, non-nodulating, \textit{Styphnolobium}
Sophora species in Hawaii, Japan, and China. Soil samples from the root zones of mature S. japonicum, C. kentukea, and other woody legumes, introduced or indigenous to Hawaii, Japan, China, and the continental USA, also were used as inocula. Inoculation did not elicit nodulation of C. kentukea or S. japonicum, despite that N concentrations of shoots of S. japonicum (1.6%) and C. kentukea (1.5%) fell below the highest shoot N percentage that previously was associated with well-nodulated plants of Maackia amurensis (1.8%). In addition to these analyses, rhizobia were isolated from nodules on the roots of a tree reported to us as S. japonicum. Nine of the 10 isolates selected as representatives of similarity groups were capable of nodulating M. amurensis, which led to the identification of the putative S. japonicum as Maackia floribunda. We also found that broad-range Bradyrhizobium USDA 6, USDA 3384, and USDA 3456 induce nodules on R. pseudoacacia and M. amurensis, which were used as control species during inoculation trials with S. japonicum and C. kentukea. Our conclusion that S. japonicum and C. kentukea lack the capacity to nodulate is based on the most thorough analysis of the nodulation capacity of these species to date. Previous reports of nodulation of S. japonicum may have been due to inaccurate plant or nodule identification.

Introduction

Knowing whether leguminous trees can nodulate after infection by rhizobia is important for selecting species for reforestation and alley cropping, managing N
applications during tree culture, and for clarifying molecular signals and evolutionary relationships between legumes and their rhizobial symbionts. But nodulation, once thought to be inherent in legumes, does not occur in all members of the Fabaceae. In subfamilies Caesalpinioideae, Mimosoideae, and Papilionoideae, 60, 17, and 5% of the genera, respectively, are reported not to form root nodules (Allen and Allen, 1981). Putative non-nodulating woody species include several important agricultural and nursery crops (Brewbaker, 1990).

Although 98% of studied species in the Papilionoideae nodulate, the nodulation status of woody ornamental species in two genera, *Cladrastis* and *Styphnolobium*, is uncertain. Of the 8 species in the genus *Cladrastis*, seven are indigenous to Japan or China (Andrews, 1997). Only *C. kentukea* (Dum.-Cours.) Rudd (American yellowwood) is native to North America. No *Cladrastis* species has been observed to nodulate (Allen and Allen, 1981); efforts to induce nodulation of *C. kentukea* have included inoculations with rhizobia (Wilson, 1939) and soil from one of the isolated areas where trees are indigenous (Graves and van de Poll, 1992).

The newly formed genus *Styphnolobium* consists of 9 species (Sousa and Rudd, 1993). Eight species are indigenous to USA, Mexico, or Central America, but *S. japonicum* (L.) Schott (Japanese pagodatree), formerly *Sophora japonica* L., is native to China and Korea. Nodules were reported on *S. japonicum* in Hawaii and Japan in the 1940's (Allen and Allen, 1981; Asai, 1944), but compatible rhizobia reportedly isolated (Ishizawa, 1953 and 1954) and held at the National
Institute of Agrobiological Resources, Tsukuba, Japan (Allen and Hamatová, 1973) are no longer available (T Hiroi, Forestry and Forest Products Research Institute, Kukizaki, Japan, pers. comm.). Soil samples from the USA and China that led to nodulation of *Maackia amurensis* Rupr. & Maxim. and *Robinia pseudoacacia* L. did not elicit nodules on *S. japonicum* (Batzli, 1991). Although nodules reportedly from *S. japonicum* were examined by Sutherland et al. (1994), no nodules have been observed on other *Styphnolobium* species, which led some to conclude that *Styphnolobium* is a non-nodulating genus (Santamour and Riedel, 1997).

A phylogenetic analysis of *rbcL* sequences by Doyle et al. (1997) indicates that *Cladrastis* and *Styphnolobium* are sister taxa that hold positions nearer to the Mimosoideae and Caesalpinioideae subfamilies than do nodulating woody species of the Papilionoideae, such as *R. pseudoacacia* and *M. amurensis*. Sousa and Rudd (1993) and Palomino et al. (1993) used chromosome number (x=14) and morphological characteristics to separate the genus *Styphnolobium* from nodulating species in *Sophora*. *Cladrastis* and *Styphnolobium* share the haploid chromosome number of x=14. The capacity to nodulate has been correlated with chromosome number (Allen and Allen, 1981) and self-pollination (Wilson, 1939). Given the discrepancy between reports of nodulation (Allen and Allen, 1981; Asai, 1944; Sutherland et al., 1994) and the lack of nodulation of *S. japonicum* (Batzli, 1991; Santamour and Riedel, 1997), and because previous attempts to elicit nodulation of *C. kentukea* have been limited (Graves and van de Poll, 1992; Wilson, 1939), a more thorough analysis of the nodulation status of
Styphnolobium and Cladrastis to nodulate is needed to substantiate or refute claims that they are non-nodulating genera.

Nodulation of species once considered non-nodulating has been achieved after using soil (Batzli et al., 1992) and rhizobia (Faria et al., 1984; Faria et al., 1987) as inocula. Our objectives were to test S. japonicum and C. kentukea for the capacity to form root nodules after inoculation with various rhizobia and soils and to characterize any symbiotic rhizobia isolated from nodules. We used two approaches for testing the nodulation capacity of Styphnolobium and Cladrastis. First, N-deficient plants of S. japonicum, C. kentukea, and control species were inoculated with rhizobia chosen for their symbiotic potential with indigenous and introduced Sophora species from Hawaii, Japan, and China, or for their low host specificity. Second, soil samples from the root zones of mature S. japonicum, C. kentukea, and other leguminous species, introduced or indigenous to Hawaii, Japan, China, and continental USA, were used to inoculate N-deficient plants of S. japonicum, C. kentukea, and control species. We hypothesized that soils from near the roots of trees in native stands and in regions where nodulation reportedly was observed are most likely to contain compatible microsymbionts. Screening soil from indigenous sites for compatible rhizobia, the whole-soil inoculation technique, is a promising method for isolating effective strains for legumes lacking rhizobial symbionts (Somasegaran and Hoben, 1994). We have broadened the previous efforts of Wilson (1939) and Graves and van de Poll (1992) by inoculating plants with rhizobial isolates that
may be more likely to infect and with soil from a greater number of sites where trees are indigenous and where nodulation has been reported previously.

Materials and methods

Sources of plant material

Seeds of *Styphnolobium japonicum* (L.) Schott were purchased from F. W. Schumacher Co., Inc. (Plains, MT, USA) and Lawyer Nursery, Inc. (Sandwich, MA, USA). We collected seeds of *Cladrastis kentukea* (Dum.-Cours.) Rudd and *Robinia pseudoacacia* L. from trees at Iowa State University, Ames, IA, USA. Half-sib seeds of *Maackia amurensis* Rupr. & Maxim., described as 15-5 by Pai and Graves (1995b), were obtained from the National Arboretum, Washington, DC, USA. *Styphnolobium affine* (Torr. & A. Gray) Walp. seeds were obtained from the Texas Agriculture Research and Extension Center, Dallas, TX, USA. Seed sources for *Sophora flavescens* Ait., previously known as *Sophora angustifolia* Sieb. & Zucc., were the Botanic Garden of Toyama, Toyama; Fukushima University, Fukushima; Tohoku University, Sendai; and University of Tokyo Botanical Gardens, Tokyo, in Japan. The National Tropical Botanical Garden, Kauai, HI, USA, provided seeds of *Sophora chrysophylla* (Salisb.) Seem. and *Sophora tomentosa* L. Seeds of *Sophora tomentosa* also were supplied by Heng-Chun Tropical Botanic Garden, Heng-Chun, Taiwan, and USDA-National Resources Conservation Service, Guam. Our seed source for *Sesbania tomentosa* H & A was the Honolulu Botanical Garden, Oahu, HI, USA. Control species
used to verify the viability of soil inoculants from Asia, Hawaii, and the continental USA were *M. amurensis*, *S. chrysophylla* and *Sesbania tomentosa*, and *R. pseudoacacia* and *S. affine*, respectively. *S. flavescens*, *Sophora tomentosa*, *S. chrysophylla*, *M. amurensis*, and *R. pseudoacacia* were used as control species for rhizobial inoculation.

**Rhizobial strains and culture conditions**

Isolates confirmed compatible with *S. chrysophylla*, *S. angustifolia*, and *S. tomentosa*, and broad range rhizobia were used as inocula (Table 1). Cells from a single colony of each isolate were used to inoculate 3 mL of liquid arabinose-gluconate (AG) medium (Kuykendall and Weber, 1978). Inoculated medium was shaken at 150 rpm for 5 days at 28 °C. A sample (100 μL) of a 5-day-old culture was used to inoculate 100 mL of AG medium. Cultures were shaken at 150 rpm for 72 h at 28 °C to a cell density of $10^8$ to $10^9$ cells mL$^{-1}$.

**Sources and collection of soil samples**

Forty-six samples of soil from root zones of indigenous and introduced trees or shrubs in the USA, Japan, and China were used as inoculum (Table 2). Characteristics of the collection sites are listed in Table 2. Samples were collected within the dripline of the canopy and 10 to 20 cm below the soil surface, placed in sterile plastic bags, and stored in the dark at 4 °C up to 3 weeks before use.
Experimental design and statistical analysis

For all inoculation experiments and for the analysis of N content, treatments were replicated 3 times and arranged in 3 completely randomized blocks. Analysis of variance (ANOVA) was performed on the data by using the SAS general linear model (GLM) (SAS Institute, Cary, NC), and least significant difference (LSD) at \( p = 0.05 \) was determined for treatment means.

Plant inoculation and growth

Seeds were scarified (Batzli et al., 1992) and germinated aseptically (Ralston and Imsande, 1983). Glass Leonard jars (Leonard, 1944) with diameters of 9.4 cm and volumes of 700 cm\(^3\), and clay pots and saucers with diameters of 15 cm and volumes of 550 cm\(^3\) were filled with coarse perlite, autoclaved, and used for soil and rhizobial inoculations, respectively. Five-day-old seedlings that were free of N were inoculated with rhizobia or soil. Roots of seedlings were dipped in liquid rhizobial cultures and planted in perlite inoculated with 300 \( \mu \)L of liquid culture. Soil (5 to 10 g) was placed in a well in the center of the perlite in each pot, and seedlings were planted so roots were in direct contact with the soil. Control seedlings were not inoculated with soil or were inoculated in AG medium without rhizobia. Inoculations were done aseptically in a laminar flow hood. Three and 4 seedlings were planted in each container for rhizobial and soil inoculation, respectively. Containers were arranged in a glasshouse at 23.2 °C (SD = 1.1) under 401 \( \mu \)mol·m\(^{-2}·s^{-1} \) (SD = 146) photosynthetically active radiation
(PAR) from the sun and high-pressure sodium lamps that maintained a 16-h photoperiod. Plants were irrigated with sterile, 1/4-strength, N-free nutrient solution (Hoagland and Arnon, 1950) adjusted to pH 6.8. Plants remained under these conditions for 6 to 7 weeks. Roots were then separated from the perlite and examined for nodules.

Isolation, characterization, and authentication of rhizobia

Sixteen nodules were excised from washed roots and surface sterilized by agitating in 95% ethanol and 3% solution of sodium hypochlorite for 15 seconds and 5 minutes, respectively. Nodules were rinsed 5 times in sterile deionized water. Procedures for microbe isolation, preliminary characterization, and presumptive tests were followed as described in Somasegaran and Hoben (1994) and repeated 3 times for all isolates and 2 control strains, Bradyrhizobium japonicum (USDA 6) and Sinorhizobium fredii (USDA 205). Characterization of isolates included growth rate, Gram stain, and descriptions of colony size, shape, and color on AG medium. Relative growth rate [slow (3 to 5 days), fast (2 to 3 days)] to produce a turbid culture (cell density of $10^6$ to $10^9$ cells mL$^{-1}$), capacity to absorb congo red (+, inconclusive, −), capacity to alter the pH of growth medium with bromothymol blue (acidic, neutral, basic), and colony size and color were characteristics used to divide the bacteria into 10 groups with similar traits. One isolate was selected from each group for authentication and was cultured as previously described for rhizobia culture. Cultures were grown for 3 to 5 days. Seeds of S. japonicum and M. amurensis were scarified (Batzli et al., 1992) and
germinated (Ralston and Imsande, 1983) for 3 days. Seedlings were inoculated as previously described for plant inoculation and growth. Eight seedlings of *S. japonicum* or *M. amurensis* were grown in each sterile, 1-L mason jar as described by Ralston and Imsande (1983). Sterile germination paper around the seedlings was inoculated with 500 μL of the same culture. Jars were arranged on a glasshouse bench where natural radiation was supplemented with radiation from high-pressure sodium lamps for a total of 410 μmol·m⁻²·s⁻¹ (SD = 147) PAR for 10 weeks. Photoperiod and temperature were 15 h and 23 °C (SD = 1.2), respectively. Plants were irrigated with sterile, 1/4-strength, N-free Hoagland solution. Roots were examined for nodules every 2 to 3 weeks.

**Analysis of plant total N**

Seeds of *S. japonicum*, *C. kentukea*, and *M. amurensis* were scarified (Batzli et al., 1992) and imbibed water for 24 h. Seed coats were removed from 30 germinated seeds of each species, and their mass was determined after seeds were dried at 67 °C for 48 h. Thirty seeds of a species were planted in each of thirty-six 15-cm-diameter plastic pots (volume = 1.9 L) filled with sterile coarse perlite. A sterile plastic screen was placed inside of each pot to prevent loss of perlite through drainage holes. Pots were placed in open plastic bags to retain moisture and leachate after irrigations. Twelve pots each of *M. amurensis*, *S. japonicum*, and *C. kentukea* were placed on a glasshouse bench under 392 μmol·m⁻²·s⁻¹ (SD = 146) PAR from the sun and high-pressure sodium lamps for 2 (six pots per
species) or 7 (six pots per species) weeks. Temperature and photoperiod were 23.3 °C (SD = 1.0) and 16 h, respectively. Two pots each of *M. amurensis*, *S. japonicum*, and *C. kentukea* in each block were irrigated every 3 to 4 days with 200 ml of sterile, 1/2 strength, Hoagland solution that contained 7.8 mM NO₃⁻. The remaining pots received sterile, 1/4 strength, N-free Hoagland solution (pH 6.8). After 2 and 7 weeks, 20 seedlings were removed from one +N and one −N pot in each block for each species and examined for nodules. Shoots were harvested, dried at 67 °C for 48 h, and weighed. Total N was measured for shoots and seeds by using a Lachat autoanalyzer (Lachat Instruments, Milwaukee, WI).

Results

*Inoculation*

Inoculation did not elicit nodulation of *C. kentukea* or *S. japonicum*. Inoculation with rhizobia in culture (Table 1) and soil (Table 2) did cause nodulation of *Maackia*, *Robinia*, and *Sophora* species used as controls. Uninoculated control plants did not nodulate. Three *Bradyrhizobium* species were effective with *R. pseudoacacia* and *M. amurensis*. NGR 234 produced ineffective nodules on the two control species. The surface of nodules on *R. pseudoacacia* was white, whereas the surface of nodules on *M. amurensis* was black. Inoculation with *S. fredii* induced no nodules on *M. amurensis*, but large (≥ 5 mm) nodules formed on *R. pseudoacacia*. 
N content of seeds and seedlings

Seeds of *C. kentukea*, *S. japonicum*, and *M. amurensis* contained 8.2, 6.9, and 5.6% N, respectively (LSD= 0.1). Differences in total N of shoots from *C. kentukea* (6.7%), *M. amurensis* (5.7%), and *S. japonicum* (5.3%) plants provided no N were found on day 14 (LSD= 0.3), and shoot N of plants provided combined N (6.1%) was higher than plants not provided N (5.7%) (LSD= 0.3) across species. On day 49, species differences were detected in shoot N of plants not supplied N. *S. japonicum* (1.6%) contained more N than *M. amurensis* (1.4%), but not *C. kentukea* (1.5%), and N contents of *M. amurensis* and *C. kentukea* were not different (LSD= 0.1). Mean N of species of plants provided combined N was at least 2.6% on day 49, which was higher (LSD= 0.1) than the N percentages of plants not provided N. Shoots of plants of *S. japonicum*, *M. amurensis*, and *C. kentukea* supplied N for 49 days had 3.3, 2.9, and 2.6% N, respectively (LSD= 0.3).

Authentication

Twenty-six bacterial isolations were made from 12 of 16 nodules (1 to 5 isolates per nodule) of a 5-year-old tree described to us by our cooperator as a *S. japonicum* located at the National Forest Tree Breeding Center, Kumamoto, Japan. Each isolate was treated individually. Presumptive tests indicated that 3 of the 26 isolates were not rhizobia. All remaining isolates were gram-negative and rod-shaped. Twenty-one isolates formed creamy-white, circular, small (≤ 1.5 mm) colonies on AG medium in 3 to 5 days. One produced translucent yellow colonies, and another produced large (≥ 1.5 mm) creamy-white colonies. Isolates
were placed into 10 groups based on similarities in their traits (Table 3). The isolate selected for authentication from each group did not induce nodules on *S. japonicum*. Nine of the 10 tested isolates induced nodules on *M. amurensis*, verifying that they were rhizobia. The only isolate that did not nodulate *M. amurensis* represented the fast, –, acidic group.

Discussion

These results suggest that previous reports of nodulation of *S. japonicum* were erroneous. The most descriptive reports of nodules on *S. japonicum* were made by Allen and Allen (1981) and Asai (1944), who observed nodulated plants in Hawaii and Japan, respectively. *S. japonicum* was introduced to Hawaii by Rock (1920) in the early 1900’s. Neal (1948 and 1965) mentioned *S. japonicum* in her list of garden flora of Hawaii, but a later guide to the native and naturalized flowering plants of Hawaii did not include *S. japonicum* (Wagner et al., 1990). The last known location for *S. japonicum* was in the Waimea Arboretum & Botanical Gardens on Oahu (C Imada, Bishop Museum, HI, USA, pers. comm.). Yet soil samples we obtained from locations where nodulated trees reportedly existed (Table 2) did not induce nodulation, while the viability of the soil as inoculum was verified by our controls.

Ishizawa (1953) reportedly isolated two elongated, rod-shaped rhizobia with peritrichous flagella from *S. japonicum* and characterized them as part of the cowpea group (Ishizawa, 1954). The isolates were stored at the National Institute
of Agricultural Sciences, Tokyo, Japan (Allen and Hamatová, 1973). The fact that these isolates no longer are held in Japanese collections precluded verifying their effectiveness. Nodules reportedly from *S. japonicum* were effective and contained "typical" papilionoid peribacteriod units (Sutherland et al., 1994).

Without herbarium samples, precise locations of the nodulating trees, or other records, questions can be raised about the nature of the nodules and the identity of the *S. japonicum* reported to nodulate in Japan and Hawaii (Allen and Allen, 1981; Asai, 1944) and used by Ishizawa (1953 and 1954) and Sutherland et al. (1994). The misidentification by one of our collaborators of *Maackia floribunda* as *S. japonicum* illustrates what may be long-standing confusion regarding the traits of these species. Although the absence of herbarium samples and the current absence of *S. japonicum* on the Hawaiian islands (C Imada, Bishop Museum, HI, USA, pers. comm.) makes it impossible to determine, we speculate that other nodulating trees were misidentified as *S. japonicum*. It is also possible that structures on the roots of *S. japonicum* were mistaken as nodules.

Inaccurate identification of plant species and structures on their roots previously has led to conflicting reports of nodulation (Allen and Allen, 1940; Faria et al., 1984; Hogberg and Nylund, 1981).

Although conflicting with previous reports, our conclusion that *S. japonicum* lacks the capacity to nodulate is not new (Batzli, 1991; Santamour and Riedel, 1997). Our efforts to achieve nodulation extend previous work (Batzli, 1991) by inoculating N-deficient plants of *S. japonicum* with rhizobia from related taxa and promiscuous rhizobia (Table 1) and with soil from numerous sites where *S.*
japonicum and C. kentukea are indigenous and where nodulation has been reported (Table 2). Differences between rhizobia from S. angustifolia (S. flavescens) and from S. japonicum (Ishizawa, 1953 and 1954) were considered minor by Allen and Allen (1981). Because rhizobia from both hosts were placed in the cowpea miscellany (Ishizawa, 1954), we used a strain from S. angustifolia as inoculum for S. japonicum (Table 1). Rhizobia compatible with indigenous and naturalized Hawaiian species, S. chrysophylla and S. tomentosa, respectively, also were used as inoculum (Table 1). The lack of nodulation is consistent with results from inoculation trials that included rhizobia from the related taxa, Sophora arizonica S. Wats., Sophora microphylla Ait., Sophora secundiflora (Ort.) Lag. ex DC., and Sophora tetrapetra J. Mill. (J Batzli, pers. comm.).

An unplanned consequence of our study was making what may be the first isolation of rhizobia compatible with M. floribunda (Allen and Allen, 1981; Faria et al., 1989) (Table 3). Nine of the 10 isolates from M. floribunda formed effective nodules on M. amurensis, illustrating cross compatibility between the hosts and promiscuous nodulation by slow-growing rhizobia. Although effective nodules on leguminous trees are induced almost exclusively by either Bradyrhizobium or Rhizobium, promiscuous nodulation has been observed on woody species by rhizobia within a genus (Turk and Keyser, 1992). The fast-growing isolate that did not nodulate M. amurensis could be specific for M. floribunda, or it may have been from the nodule surface, despite surface sterilization. We also obtained new information about rhizobia compatible with R. pseudoacacia and M. amurensis. Specificity of R. pseudoacacia for effective nodulation by
Rhizobium was observed by Allen and Allen (1981). But we found that Bradyrhizobium from V. unguiculata, C. paulina, and G. max elicited what appeared to be ineffective nodules on R. pseudoacacia and M. amurensis. No nodules or ineffective nodules resulted from inoculation of M. amurensis and R. pseudoacacia with NGR 234 and S. fredii. Our results are similar to reports of ineffective nodulation on R. pseudoacacia after inoculation with a variety of strains from Bradyrhizobium and Rhizobium (Turk and Keyser, 1992), but little has been reported about rhizobial specificity of M. amurensis.

The absence of nodules on C. kentukea is consistent with results of inoculations of plants with various rhizobia (Wilson, 1939) and USDA 4349 (compatible with M. amurensis) and soil from one location (Graves and van de Poll, 1992). We inoculated plants with rhizobial isolates that are compatible with species in related taxa and that have a broad host range and with soil from seven sites where trees are indigenous. Our results from this approach provide the most convincing evidence to date that C. kentukea lacks the capacity to nodulate.

We used the same inocula on S. japonicum and C. kentukea, because of recent refinements to the Papilionoideae phylogenetic tree. The genus Styphnolobium was separated from Sophora by Sousa and Rudd (1993) and Palomino et al. (1993) based on several characteristics including the haploid chromosome number, x=14, a trait shared by Cladrastis species (Goldblatt, 1981). Doyle et al. (1997) determined that Cladrastis and Styphnolobium are sister taxa based on rbcL sequence analysis. The analysis also placed Cladrastis and Styphnolobium nearer to the Mimosoideae and Caesalpinioideae subfamilies than the nodulating
genera, *Maackia, Robinia*, and *Sophora*. The relatively primitive *S. affine* may be a link between North American members of the genus and the only Asian species, *S. japonicum* (Sousa and Rudd, 1993). Native habitats of *S. affine* and *C. kentukea*, characterized by rocky limestone cliffs or rich, well-drained soils, overlap in the south-central USA (Spongberg and Ma, 1997; Turner, 1959; Wasowski and Wasowski, 1988). Use of the same soil inocula for *C. kentukea* and *S. japonicum*, including soil from sites in Arkansas and Oklahoma where *S. affine* is native (Table 2), was based on the overlapping ranges of the genera in North America and in Asia (Andrews, 1997; Chun, 1921).

Unlike most previous studies of this kind, we have documented the sources of our inocula and the identity and N status of the plants we studied. Faria et al. (1984) suggested that registering plant material in herbariums might resolve discrepancies in nodulation reports. Samples of the plants we used are deposited in the Ada Hayden Herbarium (Department of Botany, Iowa State University, Ames, IA, 50011-1020, USA) and origins of the inocula we used are identified in Table 1 and Table 2. Total N was measured to ensure that inoculated plants were N deficient and to minimize the chance that endogenous N prevented nodulation (Gibson, 1980). The N content of seeds of *C. kentukea* and *S. japonicum* was higher than N in seeds of *M. amurensis*, which nodulates. Although data on seed N content are rare, our findings are consistent with a previous report that nodulating leguminous trees have lower seed N concentrations than non-nodulating species (Bryan et al., 1997). Despite relatively high percentages of N in the seeds of *S. japonicum* and *C. kentukea*, it
is doubtful that N concentration of seedlings prevented nodulation. The N concentrations of shoots of *S. japonicum* (1.6%) and *C. kentukea* (1.5%) in our study were below the highest shoot N percentage at which the most nodulation occurred in *M. amurensis*, 1.8% (Pai and Graves, 1995a).

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References


Knapp, Drewett, & Sons Ltd, London.


Graves W R and van de Poll W 1992 Further evidence that Cladrastis kentukea (Dum.- Cours.) Rudd does not fix nitrogen with rhizobia. HortScience 27, 1137.


Turner B L 1959 The Legumes of Texas. University of Texas Press, Austin.


Table 1. Rhizobial strains used to inoculate seedlings of *Styphnolobium japonicum*, *Cladrastis kentukea*, and control species grown in sterile, N-free medium in Leonard jars for 42 to 49 days

<table>
<thead>
<tr>
<th>Strain (host legume)</th>
<th>Other name(s)</th>
<th>Origin</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bradyrhizobium</em> species</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>USDA 6 [<em>Glycine max</em> (L.) Merrill]</td>
<td><em>B. japonicum</em></td>
<td>Japan</td>
<td>USDA</td>
</tr>
<tr>
<td>USDA 3384 (<em>Crotalaria paulina</em> Schrank)</td>
<td>Nitragin 32H1</td>
<td>Brazil</td>
<td>USDA</td>
</tr>
<tr>
<td>USDA 3456 [<em>Vigna unguiculata</em> (L.) Walp.]</td>
<td>NifTAL 169</td>
<td>Wisconsin, USA</td>
<td>USDA</td>
</tr>
<tr>
<td><em>Rhizobium</em> species</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAL 1442 (<em>Sophora chrysophylla</em>)</td>
<td>Original</td>
<td>Hawaii, USA</td>
<td>NifTAL</td>
</tr>
<tr>
<td>TAL 1444 (<em>S. chrysophylla</em>)</td>
<td>Original</td>
<td>Hawaii, USA</td>
<td>NifTAL</td>
</tr>
<tr>
<td>TAL 1945 (<em>S. chrysophylla</em>)</td>
<td>Original</td>
<td>Hawaii, USA</td>
<td>NifTAL</td>
</tr>
<tr>
<td>TAL 1946 (<em>S. chrysophylla</em>)</td>
<td>Original</td>
<td>Hawaii, USA</td>
<td>NifTAL</td>
</tr>
<tr>
<td>TAL 1947 (<em>S. chrysophylla</em>)</td>
<td>Original</td>
<td>Hawaii, USA</td>
<td>NifTAL</td>
</tr>
<tr>
<td>TAL 1948 (<em>S. chrysophylla</em>)</td>
<td>Original</td>
<td>Hawaii, USA</td>
<td>NifTAL</td>
</tr>
<tr>
<td>TAL 1949 (<em>S. chrysophylla</em>)</td>
<td>Original</td>
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<td>NifTAL</td>
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<tr>
<td>TAL 1950 (<em>S. chrysophylla</em>)</td>
<td>Original</td>
<td>Hawaii, USA</td>
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</tr>
<tr>
<td>ICMP 5377 (<em>Sophora angustifolia</em>)</td>
<td>NZP 5440, J2061</td>
<td>Japan</td>
<td>Landcare</td>
</tr>
<tr>
<td>ICMP 12641 (<em>Sophora tomentosa</em>)</td>
<td>NZP 5171, CB 562</td>
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<td>Landcare</td>
</tr>
<tr>
<td>USDA 4146 [<em>Lablab purpureus</em> (L.) Sweet]</td>
<td>NCR 234, ANU 240</td>
<td>New Guinea</td>
<td>USDA</td>
</tr>
<tr>
<td><em>Sinorhizobium</em> species</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>USDA 205 (<em>G. max</em>)</td>
<td><em>Sinorhizobium fredii</em></td>
<td>China</td>
<td>USDA</td>
</tr>
</tbody>
</table>

* Host legumes were used as controls for TAL and ICMP strains. *Maackia amurensis* and *Robinia pseudoacacia* were controls for USDA *Rhizobium*, *Bradyrhizobium*, and *Sinorhizobium*.

* USDA strains were selected for their broad host ranges.

* NifTAL, NifTAL Project, University of Hawaii, Paia, Maui, HI, USA; Landcare, Landcare Research New Zealand Limited, Auckland, New Zealand; USDA, *Rhizobium* Culture Collection, Beltsville Agricultural Research Center, Beltsville, MD, USA.
Table 2. Location and characteristics of collection sites for soil used to inoculate seedlings of *Styphnolobium japonicum*, *Cladrastis kentukea*, and control species® grown in sterile, N-free medium for 49 days. Soil samples were obtained from root zones of the species at the collection sites.

<table>
<thead>
<tr>
<th>Site</th>
<th>Latitude, longitude</th>
<th>Elevation (m)</th>
<th>Mean annual rainfall (mm)</th>
<th>Species at site</th>
<th>Number of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kunming Botanical Garden, Kunming, China</td>
<td>26° 22' N, 103° 40' E</td>
<td>1891</td>
<td>1035</td>
<td><em>Styphnolobium japonicum</em></td>
<td>1</td>
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<tr>
<td>National Forest Tree Breeding Center, Kumamoto, Japan</td>
<td>32° 52' N, 130° 44' E</td>
<td>85</td>
<td>2004</td>
<td><em>Maackia floribunda</em>, <em>Takeda</em></td>
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<tr>
<td>Uji Campus, Kyoto University, Kyoto, Japan</td>
<td>35° 01' N, 135° 47' E</td>
<td>20</td>
<td>1350</td>
<td><em>Maackia amurensis</em></td>
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</tr>
<tr>
<td>Wood Research Institute, Kyoto University, Kyoto, Japan</td>
<td>35° 00' N, 135° 48' E</td>
<td>20</td>
<td>1350</td>
<td><em>Populus</em> species</td>
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<tr>
<td>Botanical Garden, Osaka City University, Osaka, Japan</td>
<td>34° 45' N, 135° 41' E</td>
<td>50</td>
<td>1413</td>
<td><em>S. japonicum</em></td>
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<td>Hokkaido National Agricultural Station, Sapporo, Japan</td>
<td>43° 11' N, 141° 24' E</td>
<td>120</td>
<td>1130</td>
<td><em>S. japonicum</em></td>
<td>3</td>
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<tr>
<td>Sendai City, Japan</td>
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<td>46</td>
<td>1205</td>
<td><em>S. japonicum</em></td>
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<td>Tohoku University, Aoba, Sendai City, Japan</td>
<td>38° 15' N, 140° 51' E</td>
<td>150</td>
<td>1440</td>
<td><em>S. japonicum</em></td>
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<td>Botanical Garden, University of Tokyo, Tokyo, Japan</td>
<td>35° 43' N, 139° 45' E</td>
<td>24</td>
<td>1560</td>
<td><em>S. japonicum</em></td>
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<td>Red River Basin, Texarkana, AR, USA</td>
<td>33° 26' N, 094° 02' W</td>
<td>101</td>
<td>1063</td>
<td><em>Sophora flavescens</em></td>
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<td>Hualalai, Hawaii, HI, USA</td>
<td>19° 46' N, 155° 56' W</td>
<td>630</td>
<td>625</td>
<td><em>Sophora chrysophylla</em></td>
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<tr>
<td>Kipuka Alala, Hawaii, HI, USA</td>
<td>19° 38' N, 155° 41' W</td>
<td>518</td>
<td>188</td>
<td><em>S. chrysophylla</em></td>
<td>1</td>
</tr>
<tr>
<td>Location</td>
<td>Latitude</td>
<td>Longitude</td>
<td>Soil Depth</td>
<td>Species</td>
<td>Count</td>
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<tr>
<td>---------------------------------------------------</td>
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<td>------------</td>
<td>------------</td>
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<td>Mauna Kea, Hawaii, HI, USA</td>
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<td>1215</td>
<td>2175</td>
<td>S. chrysophylla</td>
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<td>22° 08' N, 159° 39' W</td>
<td>1200</td>
<td>1500</td>
<td>S. chrysophylla</td>
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<td>National Tropical Botanical Garden, Kauai, HI, USA</td>
<td>21° 53' N, 159° 30' W</td>
<td>12</td>
<td>1125</td>
<td>Sophora tomentosa</td>
<td>1</td>
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<tr>
<td>Haleakala National Park, Maui, HI, USA</td>
<td>20° 42' N, 156° 08' W</td>
<td>2040, 2277, 2520, 2730</td>
<td>1000</td>
<td>S. chrysophylla</td>
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<td>Foster Botanical Garden, Honolulu, Oahu, HI</td>
<td>21° 15' N, 157° 51' W</td>
<td>8</td>
<td>600</td>
<td>Sesbania tomentosa</td>
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<td>Waianae Mountain, Oahu, HI, USA</td>
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<td>540, 660, 840</td>
<td>1875</td>
<td>Unsplified species</td>
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<td>Waimea Valley, Oahu, HI, USA</td>
<td>21° 38' N, 158° 03' W</td>
<td>15</td>
<td>1250</td>
<td>S. chrysophylla</td>
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<td></td>
<td>15, 21</td>
<td></td>
<td></td>
<td>Unsplified species</td>
<td>2</td>
</tr>
<tr>
<td>Brown County State Park, IN, USA</td>
<td>39° 06' N, 086° 15' W</td>
<td>270</td>
<td>1025</td>
<td>Cladrastis kentukea</td>
<td>2</td>
</tr>
<tr>
<td>Yellowwood State Forest, IN, USA</td>
<td>39° 12' N, 086° 20' W</td>
<td>263</td>
<td>1075</td>
<td>C. kentukea</td>
<td>1</td>
</tr>
<tr>
<td>Cross Point Camp, Texoma Lake, OK, USA</td>
<td>33° 54' N, 096° 48' W</td>
<td>300</td>
<td>1008</td>
<td>S. affine</td>
<td>1</td>
</tr>
<tr>
<td>Price Falls, Murray County, OK, USA</td>
<td>34° 25' N, 097° 06' W</td>
<td>254</td>
<td>975</td>
<td>S. affine</td>
<td>1</td>
</tr>
</tbody>
</table>

*Control species for Asian, Hawaiian, and continental USA soils were M. amurensis, S. chrysophylla and S. tomentosa, and Robinia pseudoacacia, respectively.*
Table 3. Characteristics of isolates from nodules of a tree located at the National Forest Tree Breeding Center, Kumamoto, Japan. Our cooperator initially described the tree as *Styphnolobium japonicum*. After rhizobia isolated from the nodules were found incompatible with *S. japonicum*, the cooperator concluded the tree had been misidentified and confirmed it to be *Maackia floribunda*.

<table>
<thead>
<tr>
<th>Number of isolates</th>
<th>Relative growth rate</th>
<th>Absorption of congo red</th>
<th>pH change of medium</th>
<th>Colony color</th>
<th>Colony size</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>slow</td>
<td>inconclusive</td>
<td>basic</td>
<td>white</td>
<td>small</td>
</tr>
<tr>
<td>1</td>
<td>slow</td>
<td>+</td>
<td>neutral</td>
<td>white</td>
<td>small</td>
</tr>
<tr>
<td>1</td>
<td>slow</td>
<td>-</td>
<td>slightly basic</td>
<td>yellow</td>
<td>small</td>
</tr>
<tr>
<td>1</td>
<td>slow</td>
<td>-</td>
<td>slightly basic</td>
<td>white</td>
<td>small</td>
</tr>
<tr>
<td>1</td>
<td>slow</td>
<td>+</td>
<td>slightly basic</td>
<td>white</td>
<td>small</td>
</tr>
<tr>
<td>2</td>
<td>slow</td>
<td>-</td>
<td>acidic</td>
<td>white</td>
<td>small</td>
</tr>
<tr>
<td>1</td>
<td>slow</td>
<td>-</td>
<td>acidic</td>
<td>white</td>
<td>large</td>
</tr>
<tr>
<td>1</td>
<td>slow</td>
<td>+</td>
<td>acidic</td>
<td>white</td>
<td>small</td>
</tr>
<tr>
<td>2</td>
<td>fast</td>
<td>inconclusive</td>
<td>acidic</td>
<td>white</td>
<td>small</td>
</tr>
<tr>
<td>12</td>
<td>fast</td>
<td>-</td>
<td>acidic</td>
<td>white</td>
<td>small</td>
</tr>
<tr>
<td><em>Bradyrhizobium</em></td>
<td>slow</td>
<td>inconclusive</td>
<td>slightly basic</td>
<td>white</td>
<td>small</td>
</tr>
<tr>
<td><em>Sinorhizobium</em></td>
<td>fast</td>
<td>+</td>
<td>acidic</td>
<td>white</td>
<td>small</td>
</tr>
</tbody>
</table>

"Slow (3 to 5 days) and fast (2 to 3 days) indicate the relative rates at which the isolates grew to produce turbid cultures (cell density of $10^6$ to $10^8$ cells mL$^{-1}$) in liquid AG medium.

"Small ($\leq$ 1.5 mm) and large ($\geq$ 1.5 mm) colonies of isolates grown on AG medium for 3 to 5 days.

"Rhizobial species used as control for characterization and presumptive tests.
CHAPTER 3. ENOD2 cDNA CLONE FROM NODULES OF MAACKIA AMURENSIS RUPR. & MAXIM. (ACCESSION NO. AF039708) (PGR 98-060)

A paper published in the journal Plant Physiology\(^1\)

Carol M. Foster\(^{2,3,4}\), William R. Graves\(^{2,3,5}\), Harry T. Horner\(^2,4\)

Some legumes have the capacity to form a mutualistic relationship with bacteria that fix dinitrogen, such as *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, and *Azorhizobium* (Dénarié and Roche, 1992). Little is known about the biology of nitrogen fixation in temperate woody legumes. A key to establishing or enhancing dinitrogen-fixing symbioses in woody legumes is to understand molecular mechanisms that promote or impede optimal nodulation and nitrogen fixation. Information on the biochemistry and regulatory mechanisms of nodule organ formation can be derived by isolating and identifying genes involved in nodule morphogenesis (Dickstein et al., 1993). Nodulin genes, or genes that encode for nodule-specific proteins, affect the physical changes that occur during nodule formation. Early nodulin genes (ENOD) have been

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ENOD2, a developmentally regulated early nodulation gene, is conserved among legumes (Nap and Bisseling, 1990), and may be a molecular marker for early events during nodule organogenesis (Hirsch et al., 1991). ENOD2 gene activity has been detected only in nodules, with or without the presence of rhizobia (Govers et al., 1990). The protein encoded by ENOD2 is proline-rich, composed of conserved pentapeptide repeats, and is homologous to hydroxyproline-rich cell wall glycoproteins called extensins. In situ hybridization shows that expression of the gene occurs only in the nodule parenchyma (van de Wiel et al., 1990). Sequence homology and in situ observations suggest that ENOD2 functions in the regulation of oxygen in the inner cortex of the nodule.

Our objective was to isolate and sequence ENOD2 cDNAs in Maackia amurensis. Genomic DNA from M. amurensis and primers, derived from proline-rich pentapeptide repeats of conserved ENOD2 sequences, were used to obtain a 561-bp PCR fragment. This cloned fragment had the greatest identity to lupine ENOD2 (76%, X55371). A 414-bp partial cDNA clone (74% identity to soybean ENOD2, X16876) was isolated from a nodule cDNA library from M. amurensis by using the PCR fragment as a probe. MaENOD2, a cDNA of 1450 bp, was isolated after screening the library with the PCR fragment and the partial cDNA clone. Sequence homology was greatest with ENOD2 nucleotide (72%, 68%, 61%) and...
amino acid (65%, 73%, 74%) sequences from lupine (X55371), soybean (X16876), and *Sesbania rostrata* (X63339), respectively. Nucleotide and amino acid identities were determined by using GCG Gap analysis (Genetics Computer Group, Madison, WI, USA).

Information on the role of *ENOD2* in woody legumes will be beneficial in clarifying the nature of nodulation in these plants, including whether molecular events relevant for nodulation are similar in herbaceous and woody species. New data on the characteristics of nodulin genes from taxa that have not been studied will help to elucidate the evolution of nodulation in legumes (Doyle, 1994).

Acknowledgments

The authors thank Mary Tymeson, Jennifer Hart, Chad Hart, Gary Polking, Clark Ford, and David Hannapel for technical assistance and advice.

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**Table 1.** Characteristics of a cDNA clone encoding *ENOD2* in *Maackia amurensis*.

**Organism:**

*Maackia amurensis* Rupr. & Maxim., Fabaceae

**Source:**

cDNA library constructed in lambda ZapII (Stratagene) from nodules (≤ 1 mm diameter)

**Function:**

Encodes a hydroxyproline-rich, putative structural cell wall protein
Techniques:
The library was screened with a radio-labeled MaENOD2 PCR fragment and a partial MaENOD2 cDNA as probes. Synthetic oligonucleotide primers were used to sequence the 5' and 3' ends of the cDNA. Cloned serial deletions, generated by Exo III nuclease digestion, were used to sequence the coding region of the cDNA. The sequence of both strands was determined by using automated dideoxy sequencing on an ABI 377 sequencer (Applied Biosystems, Inc., Foster City, CA, USA) at the Nucleic Acid Facility, Iowa State University.

Method of Identification:
Sequence similarity with published ENOD2 genes and cDNAs

Features of the cDNA clone:
The partial MaENOD2 cDNA was 1450 bp and consisted of an open reading frame with no start codon of 1342 bp, a stop codon, and a 3' untranslated region of 105 bp that included a polyadenylated tail.

Features of Deduced Protein:
The partial open reading frame encoded a polypeptide of 447 amino acids (51.93 kD) with a pi of 6.39. The N-terminus of the predicted protein was composed of 17 hydrophobic amino acids characteristic of a partial signal sequence. The deduced protein consisted primarily of pentapeptide repeats (PPHEK, PPyEK, PPEYQ) associated with ENOD2 proteins. Proline (45%), glutamic acid (13%), tyrosine (12%), lysine (9%), and histidine (6%) were the dominant amino acids in the predicted protein, and their preferential codons were CCA and CCT, GAG, TAT and TAC, AAG, and CAT, respectively.

Literature Cited


CHAPTER 4. **ENOD2 EXPRESSION IN FLOWERS, ROOTS, AND NODULES OF THE WOODY LEGUME, *MAACKIA AMURENSIS* RUPR. & MAXIM.**

A paper to be submitted to the journal Plant Physiology

Carol M. Foster, David J. Hannapel, Harry T. Horner, and William R. Graves

**ABSTRACT**

A root nodule cDNA library of *Maackia amurensis* Rupr. & Maxim. was screened by using a *MaENOD2* PCR fragment and partial cDNA as probes. Seven cDNAs were isolated, sequenced, and analyzed. cDNA clones had 68-74% identity at the nucleic acid and amino acid levels with *ENOD2* genes. Proline (45%), glutamic acid (13%), tyrosine (12%), lysine (9%), and histidine (6%) dominated the deduced protein encoded by the 1.45-kb *MaENOD2* cDNA. Four conserved pentapeptide repeats (PPHEK, PPEYQ, PPYEK, and PPIEY) characteristic of *ENOD2*s were found in *MaENOD2*. Southern hybridization indicated that *MaENOD2* belongs to a small gene family. Unlike other *ENOD2* genes, *MaENOD2* activity was not nodule specific. Transcripts were detected in non-symbiotic roots and in stamens, carpels, and receptacles of flowers. *MaENOD2* transcripts were detected in roots 4 d after inoculation with rhizobia,
and expression was enhanced as the nodules developed to > 2 mm diameter. *In situ* hybridization showed *MaENOD2* transcripts are restricted to the distribution zone of indeterminant nodules. Our results suggest that *ENOD2* in *M. amurensis* may be a cell wall component of tissues that regulate nutrient flow to sinks, such as developing seeds, pollen grains, and symbiotic regions of a nodule.

**INTRODUCTION**

Many legumes form a unique organ, the nodule, as part of a mutualistic relationship with bacteria that fix dinitrogen (N\textsubscript{2}) in the genera *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, and *Azorhizobium* (Dénarié and Roche, 1992). Nodule morphology is determined by the plant host and is classified as indeterminant or determinant depending upon the presence or absence of a persistent nodule meristem (Nap and Bisseling, 1990a). Temperate species, such as *Pisum sativum* L. and *Medicago sativa* L., possess indeterminate nodules, whereas determinant nodules develop on many tropical legumes, such as *Glycine max* (L.) Merrill, *Phaseolus vulgaris* L., and *Sesbania rostrata* Brem. & Oberm. The central region of a nodule is composed of cells infected with rhizobia and uninfected cells. Within mature indeterminant nodules, the central tissues continue to differentiate during the life of the nodule and are identified according to their stage of development: distal meristem (zone I); invasion region (zone II); early symbiotic region (zone II-III); late symbiotic region (zone III); and proximal senescence region (zone IV). Peripheral tissues surrounding
the symbiotic center of an indeterminant nodule include the epidermis, outer
cortex, endodermis, vascular bundles, and inner parenchyma. In some species,
the innermost layers of the nodule parenchyma are identified as the boundary
layer and the distribution zone (Gresshoff and Delves, 1986; Parsons and Day,
1990).

Among subfamilies in the Fabaceae, the capacity to form root nodules is most
common in the Papilionoideae. Although 98% of studied species in the
Papilionoideae nodulate (Allen and Allen, 1981), the capacity of many members
of this subfamily to nodulate and fix N₂ has yet to be investigated in ways that
link gene expression to nodule development and function. Moreover, little
information regarding genes characterized in herbaceous systems is available for
woody legumes. Because nodule formation and function depend upon an
exchange of molecular signals between the microsymbiont and host plant, a key
to establishing or enhancing N₂-fixing symbioses in woody legumes is to
understand molecular mechanisms that promote or impede optimal nodulation
and N₂ fixation. Information on the biochemistry and regulatory mechanisms of
nodule formation in herbaceous legumes has been derived by isolating and
identifying genes involved in nodule morphogenesis (Dickstein et al., 1993).

Nodulin genes encode plant proteins that accumulate abundantly in nodules
and are classified as early or late based on time of expression during nodule
development (Nap and Bisseling, 1990b). Early nodulin genes (ENODs) are
active before N₂ fixation begins in the nodule and are involved primarily with
the infection process and nodule organogenesis. Genes involved in nodule
function and maintenance are considered late nodulins (NODs). ENODs have been characterized in a limited number of herbaceous systems, such as G. max, M. sativa, P. sativum, P. vulgaris, and S. rostrata but not in genomes of woody species of the Papilionoideae, members of the Caesalpinioideae and Mimosoideae subfamilies, nor nodulating nonlegumes (Doyle, 1994). Consequently, evidence for precise roles of ENODs is not as prevalent as that for NODs, such as leghemoglobin (Brisson and Verma, 1982), nodule-specific glutamine synthetase (Cullimore et al., 1983), and uricase (Bergmann et al., 1983). Study of ENODs in woody species is needed to provide new information about the function of these genes during nodulation and to broaden our understanding of the evolution of nodulation in the Fabaceae (Doyle, 1994).

ENOD2, a developmentally-regulated early nodulin gene, is considered conserved among legumes (Nap and Bisseling, 1990a) and may be useful as a molecular marker for early events during nodule organogenesis (Hirsch et al., 1991). ENOD2 activity had been detected previously only in nodules (Govers et al., 1990; van de Wiel et al., 1990a) and Sánchez et al. (1991) proposed the gene could be used to discern lateral roots and tumors from nodules. However, ENOD2 transcripts since have been detected in total RNA from uninoculated roots of Lotus japonicus (Regel) K. Larsen (Szczyglowski et al., 1997), in root primordia on stem of S. rostrata (Goormachtig et al., 1998), and in mycorrhizal roots of M. sativa (van Rhijn et al., 1997). ENOD2 mRNA has not been detected in stems or flower organs as has mRNA of other ENODs such as ENOD12 (Scheres et al., 1990) and ENOD40 (Yang et al., 1993).
The protein encoded by *ENOD2* is proline-rich, composed of conserved pentapeptide repeats, and has similarity to hydroxyproline-rich cell wall glycoproteins (Franssen et al., 1987). *In situ* hybridization showed that the gene is expressed in the parenchyma of determinant and indeterminant nodules (van de Wiel et al., 1990b; Allen et al., 1991). The nodule parenchyma is considered an O\(_2\) diffusion barrier (Witty et al., 1987). Although sequence similarity and *in situ* observations suggest that *ENOD2* functions in oxygen regulation as part of the nodule parenchyma, increased exogenous oxygen concentrations did not enhance production of *ENOD2* transcripts in alfalfa nodules (Wycoff et al., 1998). Hunt et al. (1995) determined that reductions in *ENOD2* transcripts in antisense, transgenic *M. sativa* did not inhibit nodule formation or the regulation of O\(_2\), but absence of *ENOD2* proteins in boron-deficient nodules from *P. vulgaris* reduced N\(_2\) fixed in the nodule (Bonilla et al., 1997).

The objective of this research was to isolate *ENOD2* cDNAs from *Maackia amurensis* Rupr. & Maxim., a temperate, nodulating, Papilionoid tree of horticultural importance. Our subsequent goals were to determine organ-specific, temporal, and *in situ* production of *ENOD2* transcripts. We characterize *MaENOD2* (Foster et al., 1998), a cDNA from *M. amurensis* that shows homology to the *ENOD2* family of genes based on nucleotide and amino acid sequence analyses, and describe the anatomy of nodules formed on the roots of *M. amurensis*. We hypothesized that *MaENOD2* is an *ENOD2* gene with diverse sites of expression and that the protein has functions other than O\(_2\) regulation in the nodule. Our study will test the hypothesis of Nap and Bisseling (1990b) that
nodulin genes are not novel, but rather are genes expressed in many plant organs that have, over time, become regulated for specific functions in the nodule.

MATERIALS AND METHODS

Rhizobial Culture

Cells from one colony of USDA 4349, rhizobia compatible with *M. amurensis* Rupr. & Maxim., were used to inoculate 3 mL of liquid arabinose-gluconate (AG) medium (Kuykendall and Weber, 1978). Inoculated medium was shaken at 150 rpm for 5 d at 28°C. A sample (100 µL) of 5-d-old culture was used to inoculate 100 mL of AG medium. Cultures were shaken at 150 rpm for 72 h at 28°C to a cell density of $10^8$ to $10^9$ cells mL$^{-1}$.

Plant Material and Inoculation

Half-sib seeds of *M. amurensis*, described as seed source 15-5 by Pai and Graves (1995), were obtained from the U.S. National Arboretum, Washington, DC. Seeds were scarified (Batzli et al., 1992) and germinated aseptically. Roots of 5-d-old seedlings that were not provided combined N were harvested for DNA extraction or were inoculated with USDA 4349 for temporal and differential RNA analyses. Inoculation was done aseptically by dipping the roots into liquid culture of USDA 4349 at $10^8$ to $10^9$ cells mL$^{-1}$ in a laminar flow hood. Control seedlings were inoculated with AG medium without rhizobia. Seedlings of *M.*
*Amurensis* were grown in sterile, 1-L mason jars (Ralston and Imsande, 1983). Jars were arranged randomly in a growth chamber at 23.5 ± 1.0°C under 145.0 ± 25.0 μm·m⁻²·s⁻¹ photosynthetically active radiation in 16-h photoperiods from incandescent and fluorescent lamps. Plants were irrigated with sterile nutrient solution at pH 6.8 that contained 250 μM K₂SO₄, 500 μM CaCl₂, 50 μM KH₂PO₄, 100 μM MgSO₄, 10 μM Fe-EDDHA (with 30 μM NO₃⁻), 4.6 μM H₃BO₃, 0.9 μM MnCl₂, 76.5 nM ZnSO₄, 32 nM CuSO₄, 11.1 nM H₂MoO₄, and 0.42 pM CoCl₂. Roots were harvested on days 0, 4, 8, and 12, and nodules (< 1 mm, 1 to 2 mm, and > 2 mm diameter) were harvested from inoculated seedlings for a temporal study of *MaENOD1* expression. To study organ-specific *MaENOD2* transcripts, leaves, stems, and roots from inoculated and uninoculated 12-d-old seedlings were harvested. Inflorescences from a *M. amurensis* (MLA-1, #670974) at the Minnesota Landscape Arboretum, Chanhassen, MN, were harvested during the second week of July when > 50% of flowers in the racemes were open and before any had senesced. Pre-anthesis flowers with visible petals and post-anthesis flowers were collected. Samples were frozen in liquid N₂ immediately after harvest and stored at -80°C for RNA extraction or placed in fixative for microscopy.

**PCR Amplification of DNA Probe**

*ENOD2* DNA fragments were generated with PCR by using genomic DNA of *M. amurensis* and oligonucleotide primers. The degenerate primers were
synthesized on an ABI 394 DNA synthesizer (Applied Biosystems, Inc., Foster City, CA). Primer sequences (5' CCACCTCATGA(G/A)AAACCA 3' and 5' TGG(T/C)TTCTCATA(A/T)GGTGG 3') were derived from proline-rich pentapeptide repeats of conserved ENOD2 sequences (Govers et al., 1990). PCR products were cloned into the pCR 2.1 vector from the TA Cloning Kit (Invitrogen, San Diego, CA).

Screening of cDNA Library

A λ ZAP II cDNA library was constructed (Stratagene, La Jolla, CA) from poly (A)* RNA isolated from nodules (≤ 1 mm diameter) of M. amurensis. Approximately 100,000 phage were screened by using the duplicate plaque lift method of Maniatis et al. (1982) and a rinse solution of 0.2 M Tris-HCl (pH 7.5) and 2× SSC. DNA was crosslinked onto filters with a UV Stratalinker 2400 (Stratagene). Prehybridization (1 h) and hybridization (53 h) were done at 42°C in 50% formamide, 6.7× SSC, 3.3× Denhardt’s solution, 0.4% SDS, 25 mM sodium phosphate buffer (pH 7), and salmon sperm DNA at 0.12 μg μL⁻¹. The probe was a gel-purified 561-bp ENOD2 PCR fragment from M. amurensis labeled with [³²P]dCTP and [³²P]dATP by nick translation (Nick Translation System, Promega, Madison, WI). Filters were washed twice in 2× SSC and 0.1% SDS at room temperature for 5 min and three times in 2× SSPE and 0.1% SDS at 65°C for 20 min before autoradiography. Positive plaques were picked and in vivo excision
of the pBluescript phagemid from the λ Zap II vector was done by using the Exassist/Solr System protocol (Stratagene).

**DNA Sequencing and Analysis**

The sequence of both strands was determined by using automated dideoxy sequencing on an ABI 377 sequencer (Applied Biosystems). Fasta, Gap, Pileup, and PepSort computer programs (Genetics Computer Group, Madison, WI) were used to analyze nucleotide and amino acid sequences by determining percentages of identity and similarity, sequence alignments, and amino acid compositions, respectively.

**DNA Extraction and Southern Blot Analysis**

Genomic DNA was extracted from freshly harvested roots from 5-d-old seedlings by using the CTAB method (Doyle and Doyle, 1987). Ten micrograms of genomic DNA were digested with restriction enzyme HindIII (Promega), subjected to electrophoresis in a 0.7% agarose gel with Tris-acetate-EDTA buffer, and blotted onto nylon membrane (MSI, Westboro, MA) with 25 mM sodium phosphate buffer. DNA was crosslinked onto the membrane with UV radiation. The membrane was prehybridized for 1 h and hybridized for 62 h as described for screening of the cDNA library. The 1.45-kb MaENOD2 cDNA (Foster et al., 1998) was labeled as described for screening of the cDNA library and used as the probe. After hybridization, the membrane was rinsed for 5 min at room temperature in
2× SSC and 0.1% SDS. Two washes were done at 65°C, one for 20 min in 1× SSC and 0.1% SDS, and one for 5 min in 0.1× SSC and 0.1% SDS. Washes were followed by autoradiography of the membrane for 14 d.

RNA Extraction and Northern Blot Analysis

Total RNA was extracted from flowers, leaves, stems, roots, and nodules according to Dix and Rawson (1983), and poly(A)^+ RNA was selected by using an oligo(dT)-cellulose column (Maniatis et al., 1982). Total and poly(A)^+ RNA samples of 10 and 2.5 μg, respectively, were denatured for 15 min at 65°C and resolved in 1.0% denaturing agarose gels by using 10 mM methyl mercury hydroxide in Tris-borate buffer (Maniatis et al., 1982). Before transfer, each gel was stained with ethidium bromide and viewed with UV radiation to confirm that equivalent amounts of RNA were loaded in all samples. Gels were blotted onto nylon membranes with 10× SSPE buffer, and RNA was crosslinked to the membranes. Prehybridization of the membranes was done as described for screening of the cDNA library. Membranes were hybridized for 62 h with ^{32}P-labeled MaENOD2 cDNA under the same conditions as prehybridization. After hybridization, membranes were rinsed first for 5 min at room temperature in 2× SSC and 0.1% SDS. A second wash was done at 65°C for 20 min in 1× SSC and 0.1% SDS. The third wash was at 65°C in 0.1× SSC and 0.1% SDS. A final wash only for membranes used to study temporal accumulation of MaENOD2
transcripts was performed for 15 min at 65°C with 5 mM Tris-HCl (pH 8) and 0.1% SDS. Autoradiography of the membranes was conducted for 4 h to 5 d after the washes.

**In Situ Hybridization Analysis**

Tissues were fixed in formaldehyde-acetic acid-alcohol for 48 to 72 h at 4°C, dehydrated through an ethanol/tertiary butyl alcohol series, and embedded in Paraplast (Oxford Labware, St. Louis, MO) as described by Sass (1953). Sections 8 μm thick were cut with a rotary microtome (Spencer Lens Co., Buffalo, NY) and dried onto poly-l-lysine-coated slides (Sigma, St. Louis, MO) at 37°C for at least 12 h.

To prepare for hybridization, slides were dewaxed with xylene, rehydrated through an ethanol series, rinsed with 50 mM Tris-HCl (pH 7.5), treated with proteinase K (1 mg mL⁻¹ in 50 mM Tris, pH 7.5) for 30 min at 37°C, and rinsed in sterile, deionized water. Tissues were acetylated with 0.25% (v/v) acetic anhydride in 80 mM triethanolamine (pH 8.0); rinsed with sterile, deionized water; dehydrated through an ethanol series; and dried under vacuum for 1 h.

Sections were pre-hybridized for 1 h at 45°C with 1 mg mL⁻¹ salmon sperm DNA in 50% formamide, 300 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1.0 mM EDTA, 1× Denhardt’s solution (0.02% Ficoll, 0.02% PVP, 0.02% BSA), 10 mM DTT, and 250 μg mL⁻¹ tRNA and hybridized with digoxigenin-labeled RNA probes for at
least 12 h at 45°C in the same solution, except salmon sperm DNA was replaced with 10% (w/v) dextran sulfate. Probes were produced by \textit{in vitro} transcription of linearized plasmid DNA with T7 polymerase, by using the Riboprobe Combination System- SP6/T7 kit (Promega). The probes were denatured at 70°C for 5 min before use.

After hybridization, slides were washed once with 2× SSC (20 min, room temperature) and three times with 0.2% SSC (25 min, 55°C), treated with RNase A (20 µg mL\(^{-1}\) in 500 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1.0 mM EDTA) for 30 min at 37°C, and washed with sterile deionized water. Components from the DIG Nucleic Acid Detection Kit (Boehringer Mannheim, Indianapolis, IN) were used for immunological detection of the DIG-labeled probes. Sections were blocked for 1 hr with 1.0% (w/v) blocking reagent in 100 mM Tris-HCl (pH 7.5) and 150 mM NaCl; equilibrated for 30 min in 100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% (w/v) BSA, and 0.3% (w/v) Triton X-100 (Fisher, Fair Lawn, NJ); and incubated for 2 h with anti-DIG-AP conjugate (polyclonal sheep anti-digoxigenin Fab-fragments, conjugated with alkaline phosphatase) diluted 1:1000 (0.75 units mL\(^{-1}\)) in the previously described solution. Slides were washed three times, for 20 min each time, in 100 mM Tris-HCl (pH 7.5) and 150 mM NaCl, and equilibrated for 10 min in 100 mM Tris (pH 9.5), 100 mM NaCl, and 50 mM MgCl\(_2\). Alkaline phosphatase activity was detected by incubating sections with color-substrate solution [2.0% (v/v) NBT/BCIP stock solution in 100 mM Tris (pH 9.5),
100 mM NaCl, and 50 mM MgCl₂]. Sections were photographed with bright-field and dark-field optics by using a Leitz Orthoplan microscope.

RESULTS

Isolation of PCR-generated probes

Two pentapeptide repeats (PPHEK and PPYEK) from conserved ENOD2 sequences were chosen to design 18-bp, degenerate oligonucleotides to use as primers to amplify genomic DNA from M. amurensis. Both strands of the cloned 561- and 543-bp PCR products were sequenced. DNA sequences were analyzed with the GCG Fasta program and showed greatest identity to ENOD2 nucleotide sequences of Lupinus luteus (76%) (Szczyglowski and Legocki, 1990) and Glycine max (75%) (Franssen et al., 1990). Identity to other ENOD2 nucleic acid and deduced amino acid sequences ranged from 66 to 74%. Sequences of the PCR products were identical to each other, except for 15 bp that encoded an additional pentapeptide repeat (PPHET) in the 543-bp fragment (data not shown). These results indicated that the PCR-generated fragments were partial ENOD2 sequences.

Isolation of ENOD2 cDNAs

A cDNA library from M. amurensis nodules constructed in λZAP II was screened at high stringency with the 561-bp PCR product. Three clones were
isolated and sequenced completely. Although the 297-, 414-, and 441-bp cDNAs differed in length, overlapping sequences were identical, except for the addition of 15 bp encoding the pentapeptide PPHET in the 414-bp clone. The library was screened again with the 414-bp cDNA and 561-bp PCR fragment, and three clones (0.294, 0.429, and 1.45 kb) were isolated and sequenced. Overlapping sequences of the 294-, 429-, 1.45-bp cDNAs were identical to the other clones, and 294- and 429-bp cDNAs contained the deduced amino acid sequence, PPHET (data not shown).

Comparison of the deduced ENOD2 amino acid sequence of the 1.45-kb MaENOD2 cDNA (Foster et al., 1998) with sequences from G. max (Franssen et al., 1990), Sesbania rostrata (Dehio and de Bruijn, 1992), and L. luteus (Szczyglowski and Legocki, 1990) revealed similarity among putative signal peptide sequences and the repeated pentapeptides conserved in ENOD2 proteins (Fig. 1). A consensus sequence for the five partial cDNAs, two PCR products, and MaENOD2 is underlined in the MaENOD2 sequence, and the position of the pentapeptide PPHET is indicated with an asterisk at position 165 (Fig. 1). This shows that all isolated cDNAs were part of the 5' end of MaENOD2, but MaENOD2 is missing the 15-bp sequence for PPHET found in the 534-bp PCR fragment and 294-, 414-, and 429-bp cDNAs. Sequence variation (PPHET) between the cDNAs suggested the presence of two ENOD2 cDNAs in M. amurensis (Fig. 1).
Sequence of *MaENOD2* is Similar to Other *ENOD2* Sequences

Nucleotide and deduced amino acid sequences of the 1.45-kb *MaENOD2* cDNA were compared to five *ENOD2* sequences available from GenBank by using GCG Gap program (Table I). *MaENOD2* showed high identity and similarity at the nucleotide and deduced amino acid levels with *ENOD2* genes (Table I). Nucleic acid identities ranged from 55% with *Pisum sativum* (van de Wiel et al., 1990) to 78% with *Medicago sativa* (Dickstein et al., 1988) (Table I). At the amino acid level, values for percentage identity ranged from 55% with *P. sativum* to 74% with *S. rostrata*, and sequence similarity was highest with *G. max* (78%) and lowest with *P. sativum* (61%) (Table 1). Percentage identity and similarity of the nucleic acid and amino acid sequences of the five previously identified *ENOD2* cDNAs were determined also (Table 1). Nucleic acid sequence homologies for the five *ENOD2* sequences ranged from 52 to 75%. Ranges for amino acid identity and similarity were 50 to 77% and 55 to 81%, respectively. Sequence identities and similarities for *MaENOD2* were within the range of values determined for the other *ENOD2* sequences in Table I.

In Table II, amino acid composition of the deduced *MaENOD2* protein is compared to the predicted composition of other *ENOD2*s and one *ENOD12*, proline-rich protein, and extensin. Six amino acids (Pro, Glu, Tyr, Lys, His, Val) made up more than 88% of the *MaENOD2* polypeptide (Table II). Most *ENOD*s, *PRPs*, and extensins have a high (hydroxy)proline, Lys, and Tyr content, but *ENOD2*s have elevated Glu as well (Nap and Bisseling, 1990; Wycoff et al., 1992). Comparison of amino acid composition of *ENOD2*s from *M. amurensis*, *G. max*,
S. rostrata, and L. luteus revealed high Pro, Glu, Lys, and Tyr levels characteristic of ENOD2s, but species-specific variation exists (Table II). High Val and Ser contents characterize PRPs and extensins, respectively (Nap and Bisseling, 1990; Wycoff et al., 1992). MaENOD2 differs from PsENOD12, MtPRP4, and VuEXT26 in Glu, His, Val, and Ser content. PsENOD12 and MtPRP4 have higher Val and His content than MaENOD2, but are lower in Glu. VuEXT26 is higher in Ser and lower in Glu and His than MaENOD2 (Table II).

Deduced ENOD2 polypeptides can be divided into two domains, an N-terminal putative signal peptide and a protein consisting of proline-rich pentapeptide repeats (Govers et al., 1990). The 17 hydrophobic amino acids at the N-terminus of the MaENOD2 are characteristic of a signal peptide (Foster et al., 1998), and a recognition site for signal peptide cleavage is present also (Fig 1). Presence of a signal peptide indicates probable secretion from the cell and localization in the extracellular matrix (Nap and Bisseling, 1990). The deduced MaENOD2 protein (Fig. 1) consists primarily of four pentapeptide motifs (PPHEK, PPYEK, PPIEY, PPEYQ) that are repeated 18x, 12x, 8x, and 6x, respectively (Table III). These motifs are conserved in or have been found in other ENOD2 proteins (Wycoff et al., 1992), but were not found in other proline-rich proteins (Table III). Pentapeptide repeats conserved in ENOD12s (PPQKE, PPRHK, PPVNK), PRPs (PPVEK, PPVHK, PPVYK), and extensins (SPPP) (Wycoff et al., 1992) were found in PsENOD12, MtPRP4, and VuEXT26, respectively, but were not present in MaENOD2, LENOD2, GmENOD2, or
SrENOD2 (Table III). Thus, sequence analyses (percentage identity and similarity, amino acid composition, and conserved motifs) are consistent with MaENOD2 belonging to the ENOD2 gene family.

MaENOD2 is Encoded by a Small Gene Family

To evaluate the extent of the ENOD2 gene family in M. amurensis, Southern hybridization of M. amurensis genomic DNA was performed with 1.1-kb GmENOD2 (Franssen et al., 1990) and 1.45-kb MaENOD2 (Foster et al., 1998) cDNAs. The MaENOD2 probe hybridized at high stringency to three fragments of sizes 4.7, 6.3, and 9.4 kb (Fig. 2). The GmENOD2 probe did not hybridize, even at lower stringencies (30 and 40% formamide), nor did the MaENOD2 probe hybridize to genomic DNA from G. max (data not shown). These results suggest that the cloned 1.45-kb MaENOD2 cDNA is a member of a small gene family (Fig. 2). Additional bands suggest the presence of other genes that are or are similar to ENOD2.

Production of MaENOD2 is Organ-specific and Enhanced by Symbiosis

To determine whether MaENOD2 RNA was organ specific, poly A+ RNA from leaves, stems, and roots from uninoculated and inoculated 12-d-old seedlings, and flowers from trees was subjected to RNA blot analysis. Transcripts were detected at low levels in uninoculated roots and flowers but were undetectable in leaves and stems (Fig. 3A and B). The transcript observed in roots and nodules was approximately 1.5 kb (Fig 3A), whereas the flower transcripts were
approximately 1.0, 1.5, and 1.8 kb (Fig. 3B). As seen in Fig. 3C, 1.0- and 1.8-kb RNAs were detected in stamens. Carpels and receptacles/sepalas possessed a 1.0-kb transcript as well. *MaENOD2* transcripts were not detected in petals (Fig. 3C).

The temporal expression pattern of *MaENOD2* during nodulation was determined with a northern analysis of poly A⁺ RNA from roots at three times (0, 4, 8 days) after inoculation with rhizobia and from nodules at three stages of development (<1 mm, 1-2 mm, and >2 mm diameter). During nodule development (Fig. 4), *MaENOD2* mRNA first appeared in roots 4 d after inoculation, and transcript levels increased in nodules.

*MaENOD2* Transcripts Accumulate in the Inner Parenchyma of the Nodule

Tissues of nodules, roots, and flowers from *M. amurensis* were examined by using *in situ* hybridization to determine the location of *MaENOD2* transcripts. Longitudinal sections of nodules at four stages of development (<1 mm, 1-2 mm, >2 mm in diameter, and mature) (Fig. 5), flowers (pre- and post-anthesis), and uninoculated roots were hybridized with DIG-labeled sense and antisense RNAs transcribed from a 602-bp fragment of the coding region of *MaENOD2*. Hybridized probes were localized by using anti-digoxigenin Fab fragments, conjugated with alkaline phosphatase, and viewed as blue precipitate that forms from the reaction between alkaline phosphatase and the substrates BCIP and NBT. Mature nodules from *M. amurensis* possessed a distal meristem (I) and a central infected region consisting of invasion (II), symbiotic (III), and proximal senescence (IV) regions characteristic of indeterminant development (5A).
The antisense probe hybridized with RNA in the distribution zone immediately adjacent to zones II and III of nodules > 2 mm (Fig 5B). Very little MaENOD2 transcript was detected around zone IV of mature nodules (Fig. 5B). In Fig. 5C, peripheral tissues of the nodule (outer cortex, nodule endodermis, and inner parenchyma) were identified and an excessive accumulation of starch grains was observed in inner parenchyma cells. MaENOD2 transcripts were not detected with the antisense probe in meristematic cells (Fig. 5D) where other PRP and ENOD mRNAs occur (Scheres et al., 1990; Wilson et al., 1994; Papadopoulou et al., 1996). Accumulation of MaENOD2 RNA occurred primarily in uninfected cells of the distribution zone, but uninfected cells within the symbiotic region possessed transcripts as well (Fig. 5E). MaENOD2 transcripts were not associated with vascular bundles in nodules > 1 mm diameter (Fig. 5F).

Developing nodules between 1 to 2 mm diameter possessed peripheral tissues and zones I, II, and III of the central region, but no differentiated senescence zone (Fig. 5G). In 1 to 2 mm nodules, accumulation of blue precipitate indicated the presence of transcripts in the inner parenchyma (distribution zone) from the nodule meristem to the point of attachment to the root (Fig. 5H). MaENOD2 transcripts were detected in association with vascular tissues of immature nodules < 1 mm, but not uniformly throughout the inner parenchyma (data not shown). Very low levels of background or alkaline phosphatase activity were produced by the sense probe (Fig. 5C and G). Although MaENOD2 transcripts were detected by northern blot analysis in uninoculated roots (Fig. 4A) and in
floral organs (Fig. 4C), DIG-labeled antisense probes did not detect ENOD2 mRNA in these organs above background (data not shown).

DISCUSSION

ENOD2 cDNA clones from *M. amurensis*, including the 1.45-kb *MaENOD2* (Foster et al., 1998), showed homology to the ENOD2 family of genes based on nucleotide and amino acid sequence analyses. Symbiosis-induced temporal expression of *MaENOD2* mRNA in nodules is consistent with accumulation of ENOD2 genes in indeterminant nodules of herbaceous legumes (Allen et al., 1991; Pichon et al., 1992; van de Wiel et al., 1990b) and supports the hypothesis that *MaENOD2* genes influence development of root nodules. Nodules from *M. amurensis* are indeterminant with morphology and anatomy similar to indeterminant nodules from other temperate species (Fig 5A). Although ENOD2 transcripts were located in the inner layer (distribution zone) of the nodule parenchyma (Fig. 5), ENOD2 activity was not nodule specific in *M. amurensis*. Transcripts were detected in uninoculated roots, stamens, carpels, and receptacles (Fig. 3). Therefore, we conclude *MaENOD2* belongs to an ENOD2 gene family with diverse sites of expression. Identifying different sites of expression for *MaENOD2* through northern and *in situ* analyses led to a new hypothesis concerning the function of ENOD2; *MaENOD2* may play a role in tissues that regulate nutrient flow to sinks, such as developing nodules, seeds, and pollen grains.
Similarity among identified ENOD2 sequences and MaENOD2 confirmed that cDNAs isolated from M. amurensis belonged to the ENOD2 gene family (Table I). A common ancestor gene might explain the structural and presumed functional similarities between ENOD2 genes in different genera (Nap and Bisseling, 1990b). Although sequence homology was high, the lack of cross hybridization between the 1.1-kb GmENOD2 cDNA and M. amurensis genomic DNA, even under conditions of low stringency (data not shown), might reflect variation in codon usage and encoded pentapeptide patterns between species (Dehio and de Bruijn, 1992). Doyle (1994) suggested that specific variations in a nucleotide sequence, resulting from exon shuffling, duplication, and recombination, might provide clues to nodulation in different taxa.

The protein encoded by ENOD2 is homologous to hydroxyproline-rich glycoproteins involved in cell wall synthesis (Jose and Puigdomènech, 1993; Showalter, 1993) and maintenance of cell wall integrity. Among cell wall proteins, ENOD2s are most closely related to proline-rich proteins, which vary in amino acid compositions and motifs (Jose and Puigdomènech, 1993). Overall, pentapeptide motifs (Table III) and amino acid composition (Table II) group the protein deduced from MaENOD2 with other ENOD2s and distinguish it from other proline-rich proteins. A single pentapeptide variation (PPHET) between cloned sequences from M. amurensis indicated at least two ENOD2 transcripts are produced (Fig. 1). The different structural properties that result from sequence variations in proline-rich proteins may influence regulation and expression of PRP genes during cellular differentiation and tissue development.
(José and Puigdomènech, 1993; Showalter, 1993). The PRP gene family exhibits tissue- and cell-specific patterns of expression and has been localized to nodules, stems, roots, flowers, and seeds.

Many nodule-specific genes are expressed in stems but not roots, suggesting a stem origin of root nodules (Sprent, 1990). Previously, ENOD2 gene activity has been detected in nodules (Govers et al., 1990; van de Wiel et al., 1990) and uninoculated roots (Goormachtig et al., 1998; Szczyglowski et al., 1997), but not in stems or flower organs. Our RNA gel blot data showed that expression of different members of the MaENOD2 gene family was specific to nodules, roots, and flowers (Fig. 3 and 4). Expression of MaENOD2 was detected 4 d after inoculation with rhizobia and increased as nodule development continued (Fig. 4), which was consistent with the results of others (Allen et al., 1991; Franssen et al., 1987; Pichon et al., 1992; Strittmatter et al., 1989; van de Wiel et al., 1990b). Despite low levels of MaENOD2 transcripts in uninoculated roots, expression was enhanced during nodule development (Fig. 4), suggesting a restructuring of the cell wall matrix (José and Puigdomènech, 1993). Symbiosis-enhanced expression of MaENOD2 was similar to that of other ENOD2 genes, but detection of transcripts in stamens, carpels, and receptacles (Fig. 3C) was atypical of other ENOD2 genes. Multiple transcripts in flowers (Fig. 3B), including the transcript detected in root and nodule, may indicate that MaENOD2 was recruited for nodulation.

The morphology of nodules from M. amurensis has not been described previously, although thin-walled infection threads were observed by Sutherland
et al. (1994). Indeterminant nodules, such as those on *M. amurensis* (Fig. 5A), elongate from a persistant meristem and possess all stages of symbiotic development in a differentiation gradient in the central tissues (Hirsch, 1992). A specialized layer of uninfected cells that separates the central tissue from the rest of the nodule parenchyma is called the boundary layer (Gresshoff and Delves, 1986). Cells of the boundary layer have small intercellular spaces and radially aligned cell walls, both characteristics that would inhibit diffusion of O$_2$ (Bergersen, 1982; Wycoff et al., 1998). Parsons and Day (1990) described the one or two layers of larger, uninfected cells between the boundary layer and the infected region of a nodule as the distribution zone. In contrast to the cells of the boundary layer, the abundant intercellular spaces of the distribution zone form a network of air spaces around the infected region and may promote rapid gas exchange with infected cells. Witty et al. (1987) established that the inner parenchyma, including the boundary layer and distribution zone, was the O$_2$ diffusion barrier in nodules. Although the function of uninfected cells in the central region of indeterminant nodules is unknown, Brewin (1991) suggested that these cells may facilitate transport of nutrients and organic N compounds between vascular tissues and N$_2$-fixing cells. High concentrations of starch granules in uninfected cells provide assimilates for infected cells at times of limited nutrient availability (Atkins, 1987; Rainbird et al., 1983). In determinant nodules, uninfected cells are a site of assimilation of ammonia into ureides (Hirsch, 1992).
The function of ENOD2 is unknown. Putative protein structure and in situ hybridization data suggested that ENOD2 genes are required for cell wall synthesis in the nodule parenchyma tissues and may help regulate oxygen in nodules (Scheres et al. 1990; van de Wiel et al., 1990). Nodules deficient in boron possessed an altered parenchyma region with no ENOD2 protein incorporated into the parenchyma cell walls (Bonilla et al., 1997). Reduced N₂ fixation by boron-deficient nodules was attributed to an ineffective O₂ barrier. James et al. (1991) reported that cells in the inner parenchyma of G. max nodules respond to an increase in exogenous O₂ by depositing additional glycoprotein in intercellular spaces, but the intercellular spaces of the distribution zone remain unoccluded. Wycoff et al. (1998) determined that increased O₂ concentrations did not induce increased production of ENOD2 mRNA or proteins in M. sativa nodules. ENOD2-antisense plants of M. sativa nodulated normally even in different O₂ concentration (Wycoff et al., 1998). These results suggest that ENOD2 proteins are not involved in short- or long-term O₂ regulation by the tissues of the diffusion barrier, and that ENOD2 expression may not be necessary for nodule development. In addition, ENOD2 transcripts have been detected in roots of alfalfa that are mycorrhizal (van Rhijn et al., 1997), a symbiotic relationship that does not require an O₂ barrier. van Rhijn et al. (1997) proposed that higher concentrations of cytokinins in a mycorrhizal root induced ENOD2 expression and that molecular mechanisms involved with rhizobial and mycorrhizal symbioses are conserved. Expression of ENOD2 and other early nodulins can occur during nodule morphogenesis induced by exogenously applied plant
growth regulators (Dehio and Bruijn, 1992; Govers et al., 1990; Hirsch et al., 1989, 1993, 1997; Scheres et al., 1992; van de Wiel et al., 1990; Wu et al., 1996). These results suggest that \textit{ENOD2} is a molecular marker for cells influenced by hormonal fluxuations.

Bauer et al. (1996) proposed a model that links nod factor, cytokinin actions, and carbon/nitrogen metabolism during nodule initiation. N starvation of plants with an elevated photosynthetic rate sensitizes root cells to nod factors and cytokinins. Roots respond to nod factors and cytokinins by initiating cortical cell divisions, by increasing sink strength which leads to starch deposition, and by inducing \textit{ENOD} genes. This indicates that signal transduction elements for lipo-chitooligo-saccharides and cytokinins may be linked for nodule initiation and starch accumulation. According to this model, metabolic activities, sink capacities, and the mobilization of storage products may be coordinated by cytokinins during organogenesis in leguminous and non-leguminous plants. Plant hormones might have an indirect effect on assimilate partitioning by regulating cell division and differentiation in other developing sinks, such as pollen grains and seeds (Brenner and Cheikh, 1995).

A limited number of anther- and pistil-specific genes encoding glycine-rich, proline-rich, and extensin-like polypeptides with properties of cell wall proteins has been isolated and localized in nonreproductive tissues (Gasser and Robinson-Beers, 1993; Goldberg et al., 1993). Nonreproductive tissue in closest contact and surrounding the developing male gametophyte is the tapetum (Pacini et al., 1985). The tapetum is responsible primarily for the synthesis,
transport, and storage of lipids, proteins, and carbohydrates that facilitate pollen grain development. In many species, the endothelium or integumentary tapetum, a unique cell layer of the ovary similar to the anther tapetum, develops in direct contact with the embryo sac from differentiated inner integument cells (Reiser and Fischer, 1993) and is thought to have a nutritive function similar to the anther tapetum (Gasser and Robinson-Beers, 1993; Pacini et al., 1985). An area of small, dense cells resembling a nectary was observed at the surface of the receptacle from flowers of M. amurensis (data not shown). The tightly packed glandular cells of a nectary are separated from the vascular tissue supplying them by one or more layers of nonglandular or subglandular parenchyma (Durkee, 1983). Nutrients from the vascular tissue must pass through nonglandular cells to reach the metabolically active secretory cells. Similar to the distribution zone in nodules and the tapetum in anthers and ovaries, the nonglandular tissue is considered a pathway along which translocated material moves.

Our in situ experiments showed that MaENOD2 was expressed in the distribution zone in nodules >1 mm (Fig. 5). In mature nodules, MaENOD2 expression was restricted to the distribution zone surrounding areas of the central infected region (zones II and III) that are actively fixing N₂ (Fig. 5B). Although in situ hybridization of flower organs and root tips was inconclusive (data not shown), we speculate, based on our northern analyses (Fig. 3C) and localization of other PRPs (Showalter, 1993; José and Puigdomènech, 1993), that new sites of ENOD2 expression may be root primordia, floral nectaries, and tissues surrounding developing gametophytes. We hypothesize that ENOD2
transcripts will be located in tissues that function in transport of assimilates in the anthers, ovaries, and receptacles. Regulation of nutrients by specialized tissues surrounding a sink may be necessary due to termination of the vascular strands in tissues adjacent to the sink. Starch accumulation and mobilization within the these specialized tissues suggest that flow of nutrients is regulated to ensure continuous sink development (Kuang et al., 1996; Preiss, 1982). Further investigation will clarify whether ENOD2 genes participate in regulation of carbohydrate or organic N distribution to and from sinks, such as symbiotic tissues and developing gametophytes.

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LITERATURE CITED


Dehío C, de Bruijn FJ (1992) The early nodulin gene SrENOD2 from Sesbania rostrata is inducible by cytokinin. Plant J 2:117-128


Sass JE (1953) Botanical Microtechnique. 3rd Ed. Iowa State Univ. Press, Ames, IA.

The *PsENOD12* gene is expressed at two different sites in Afghanistan pea pseudonodules induced by auxin transport inhibitors. Plant Physiol 100: 1649-1655


Vijn I, Christiansen H, Lauridsen P, Kardailsky I, Quandt HJ, Broer I, Drenth J,


Table I. Percentage identity and similarity of ENOD2 sequences

Percentages were determined by using the GCG Gap program (Genetics Computer Group, Madison, WI). Percentages for nucleotide and deduced amino acid sequences are above and below the diagonal line, respectively. Percentage similarities are within parentheses ( ).

<table>
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<th>Lupinus luteus&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Pisum sativum&lt;sup&gt;d&lt;/sup&gt;</th>
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<sup>a</sup>Accession No. X16876 (Franssen et al., 1990). <sup>b</sup>Accession No. X63339 (Dehio and de Bruijn, 1992). <sup>c</sup>Accession No. X55371 (Szczyglowski and Legocki, 1990). <sup>d</sup>Accession No. X51987 (van de Wiel et al., 1990). <sup>e</sup>Accession No. X12580 (Dickstein et al., 1988). <sup>f</sup>Accession No. AF039708 (Foster et al., 1998).
Table II. Amino acid compositions predicted from DNA sequence data of early nodulins and proline-rich proteins

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<sup>a</sup>Maackia amurensis (AF039708) (Foster et al., 1998).  
<sup>b</sup>Lupinus luteus (X55371) (Szczyglowski and Legocki, 1990).  
<sup>c</sup>Glycine max (X16876) (Franssen et al., 1990).  
<sup>d</sup>Sesbania rostrata (X63339) (Dehio and de Bruijn, 1992).  
<sup>e</sup>Medicago truncatula (L23504) (Wilson et al., 1994).  
<sup>f</sup>Pisum sativum (X81366) (Vijn et al., 1995).  
<sup>g</sup>Vigna unguiculata (X91836) (Arsenijevic-Maksimovic et al., 1997).  
<sup>x</sup>x, number of times motif is repeated.  
<sup>—</sup>, motif not present.  
<sup>j</sup>PPVHK, motif found in other ENOD12 proteins.
Figure 1. Comparison of deduced ENOD2 amino acid sequences for *Glycine max* (X16876), *Sesbania rostrata* (X63339), *Maackia amurensis* (AF039708), and *Lupinus luteus* (X55371). Sequences that code for signal peptides are underlined with a dashed line, and the cleavage site is indicated (\(\alpha\)). Conserved pentapeptides (PPHEK, PPEYQ, PPYEK) characteristic of ENOD2 proteins (Wycoff et al., 1992) are bolded in the consensus line. Underlined amino acids represent a consensus sequence for overlapping cDNAs cloned from *M. amurensis*. The position of the unique PPHET pentapeptide is indicated by an asterisk at position 165 in the *M. amurensis* sequence. Periods (\(\cdot\)) represent gaps in sequences. Capital letters indicate consensus between amino acids at individual positions. Dashes (-) indicate no consensus.
Figure 2. Southern hybridization of genomic DNA extracted from \textit{M. amurensis} and $^{32}$P-labeled \textit{MaENOD2} cDNA. Ten micrograms of DNA were digested with \textit{HindIII}, separated by gel electrophoresis, and transferred to a filter. Molecular size markers are on the left.
Figure 3. Northern blot analysis of organ-specific ENOD2 transcripts from *Maackia amurensis*. RNA was separated by electrophoresis in an agarose-methyl mercury gel, transferred to a filter, and hybridized with $^{32}$P-labeled, 1.5-kb *MaENOD2* cDNA. Ten and 2.5 μg of total RNA and poly A* RNA, respectively, were loaded in each lane. A. Poly A* RNA from leaves (L), stems (S), and roots (R) of uninoculated and inoculated 12-d-old seedlings, and from nodulating roots with nodules removed (R-n); B. Poly A* RNA from combined pre- and post-anthesis flowers collected from trees; C. Total RNA from petals (P), stamen (S), carpels (C), and receptacles/sepals (R) from pooled pre- and post-anthesis flowers.
Figure 4. Temporal study of ENOD2 transcript production in inoculated roots and nodules of *Maackia amurensis*. Poly A+ RNA (2.5 μg) was loaded in each lane of an agarose-methyl mercury gel, separated by electrophoresis, blotted on a nylon membrane, and hybridized with 32P-labeled, 1.45-kb *MaENOD2* cDNA.
Figure 5. In situ localization of MaENOD2 transcripts in nodules of Maackia amurensis. Paraffin-embedded root nodules were sectioned and hybridized with digoxigenin-labeled single-stranded MaENOD2 RNA probes. Probes were localized by using alkaline phosphatase-conjugated digoxigenin-binding Fab fragments. Purple precipitate indicated presence of MaENOD2 transcripts in bright-field micrographs of longitudinal sections of indeterminant nodules. A, Mature nodule stained with safranin and fast green. Nodule meristem (I) and invasion (II), symbiotic (III), and senescence (IV) regions of the central tissue are identified. B, Mature nodule hybridized with antisense probe. MaENOD2 expression is in the distribution zone (between arrows) adjacent to regions II and III. C, Mature nodule hybridized with sense probe. Nodular tissues are identified: outer cortex, nodule endodermis, inner parenchyma with starch grains, and symbiotic region. D, Mature nodule hybridized with antisense probe. MaENOD2 transcripts are shown in the distribution zone, but not in the nodule meristem. E, Mature nodule hybridized with antisense probe. MaENOD2 expression extends from the distribution zone into uninfected cells of the symbiotic region. F, >2 mm diameter nodule hybridized with antisense probe. MaENOD2 transcripts are not associated with vascular tissues of the nodule. G, 1-2 mm diameter nodule hybridized with sense probe. H, 1-2 mm diameter nodule hybridized with antisense probe. MaENOD2 expression in the distribution zone surrounds the entire symbiotic region. c, outer cortex; e, nodule endodermis; p, inner parenchyma; sg, starch granules; d, distribution zone; ic, infected cells; uc, uninfected cells; vb, vascular bundle. Bars = 150 μm.
CHAPTER 5. EXPRESSION OF *ENOD2*-LIKE GENES IN ROOTS OF NON-NODULATING AND NODULATING WOODY LEGUMES IS AFFECTED BY TIBA AND ZEATIN

A paper to be submitted to the journal Plant Physiology

Carol M. Foster, Harry T. Horner, and William R. Graves

ABSTRACT

Genomic DNA and primers, derived from proline-rich pentapeptide repeats of conserved *ENOD2* sequences, were used to obtain *ENOD2* PCR fragments from two non-nodulating woody legumes, *Styphnolobium japonicum* (L.) Schott (555 bp) and *Cladrastis kentukea* (Dum.-Cours.) Rudd (387 bp). These cloned fragments had 52 to 82% sequence identity to *ENOD2* sequences and encoded deduced proteins that possessed conserved *ENOD2* pentapeptides (PPHEK and PPEYQ). Lower percentages of Glu and higher percentages of His and Val suggested that *SjENOD2* and *CkENOD2* are different than other *ENOD2*s. Sequence and blot analyses indicated that the clones represented *ENOD2* gene families in *S. japonicum* and *C. kentukea* genomes, and *ENOD2*-like transcripts were detected in stems, roots, and flowers. Only roots of control species that nodulate, *Maackia amurensis* Rupr. & Maxim. and *Medicago sativa* L., produced pseudonodules after treatment with zeatin or TIBA, an auxin transport inhibitor.
MaENOD2 gene expression was enhanced during the first 10 d of treatment, but TIBA and zeatin enhanced transcript accumulation after 30 d and 40 d in roots of *S. japonicum* and *C. kentukea*, respectively. Our results suggested *ENOD2* activity is not a molecular marker for nodulation in legumes but instead may indicate cellular response to concentrations of plant growth regulators.

**INTRODUCTION**

The mutualistic relationship between many legumes and bacteria that fix dinitrogen (N\textsubscript{2}), such as *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, and *Azorhizobium*, results in a unique organ, the nodule (Dénarié and Roche, 1992). Nodulation, once considered inherent in legumes, does not occur in all members of the Fabaceae. In subfamilies Caesalpinioideae, Mimosoideae, and Papilionoideae, 60, 17, and 5% of the genera, respectively, do not form root nodules (Allen and Allen, 1981). Although 98% of studied species in the Papilionoideae nodulate, little is known about the biology of N\textsubscript{2} fixation in temperate, woody members of this subfamily. Considering the uncertainty surrounding the nodulation status of many woody legumes, including several that are important economically (Brewbaker, 1990), it will be useful to characterize the nature of nodulation genes in these species and to determine whether the molecular events of nodulation in herbaceous legumes occur in non-nodulating woody species.
Nodule formation and function depend upon an exchange of molecular signals between the microsymbiont and host plant. Molecular mechanisms that promote or impede optimal nodulation and N$_{2}$ fixation in herbaceous legumes have been studied by identifying genes that encode plant proteins (nodulins) involved in nodule morphogenesis and function (Dickstein et al., 1993).

Nodulin genes have been classified into two groups, early (ENODs) and late (NODs), based on time of their expression during nodule development. ENOD genes can be subdivided into preinfection, infection, and organogenesis classes, while NOD genes may be ascribed to nitrogen fixation, assimilation, and maintenance classes (Nap and Bisseling, 1990a). According to Doyle (1994), few ENODs have been characterized. Of those that have, only a subset has been described in more than a few herbaceous crop species of the Papilionoideae, such as soybean [Glycine max (L.) Merrill], pea (Pisum sativum L.), and alfalfa (Medicago sativa L.). No genomes of woody Papilionoid species, members of the Caesalpinioideae and Mimosoideae subfamilies, nor nodulating nonlegumes have been examined. Study of ENODs in woody species may provide new information about the function of these genes during nodulation and facilitate an understanding of the evolution of nodulation in legumes (Doyle, 1994).

As a conserved early nodulation gene, ENOD2 may be a molecular marker for the early stages of nodule organogenesis (Hirsch et al., 1991) and may encode a proline-rich protein with a structure similar to hydroxyproline-rich cell wall glycoproteins (Franssen et al., 1987). In situ hybridization showed that expression of ENOD2 occurs in the nodule parenchyma (van de Wiel et al., 1990a; Allen et
ENOD2 mRNA has not been detected in stems or flower organs as has mRNA of ENOD12 (Scheres et al., 1990) and ENOD40 (Yang et al., 1993). Expression of ENOD2 and other early nodulins can occur during nodule morphogenesis without the presence of rhizobia (Govers et al., 1990; van de Wiel et al., 1990a). Purified nod factors, auxin transport inhibitors, auxins, and cytokinins induced pseudonodules on the roots of legumes and other plant species (Arora et al., 1959; Ridge et al., 1992, 1993; Hirsch et al., 1997). Pseudonodules on roots of Melilotus alba Desr., M. sativa, and P. sativum, contained transcripts of ENOD2, ENOD8, ENOD12, or ENOD40 (Hirsch et al., 1989; Scheres et al., 1992; Dickstein et al., 1993; Wu et al., 1996; Fang and Hirsch, 1998). Exogenously supplied auxin transport inhibitors [NPA and TIBA (2,3,5-triiodobenzoic acid)], auxins (NAA, IAA, and 2, 4-D), and cytokinins (kinetin, zeatin, and BAP) induced accumulation of ENOD transcripts in uninoculated roots of legumes (Dehio and Bruijn, 1992; Scheres et al., 1992; Hirsch et al., 1993, 1997; Bauer et al., 1996). Regardless of whether pseudonodules develop, new information about the nature of hormone-sensitive ENOD genes can be obtained by modifying the endogenous hormone balance of roots of nodulating and non-nodulating legumes.

Styphnolobium japonicum (L.) Schott (Japanese pagodatree) and Cladrastis kentukea (Dum.-Cours.) Rudd (American yellowwood) are commercially important, temperate, woody members of the Papilionoideae that do not nodulate (Wilson, 1939; Allen and Allen, 1981; Batzli, 1991; Graves and van de Poll, 1992; Santamour and Riedel, 1997; Foster et al., 1998b). As a first step in
determining whether molecular events typical of nodulation of herbaceous legumes occur in these non-nodulating tree species, our overall goals were to test for the presence of ENOD2 in *S. japonicum* and *C. kentukea* and to study spatial and temporal production of transcripts. In the absence of compatible rhizobia to stimulate gene function in *S. japonicum* and *C. kentukea*, TIBA and zeatin were used to induce the formation of pseudonodules and production of ENOD2 transcripts in roots of both species. Two leguminous species known to nodulate, *Medicago sativa* L. and *Maackia amurensis* Rupr. & Maxim., a temperate tree (Batzli et al., 1992), were used as control species for TIBA and zeatin treatments. Here we describe the isolation and sequence analysis of ENOD2-like cDNAs from *S. japonicum* and *C. kentukea*. Organ-specific expression of the genes was detected in stems, roots, and flowers of both species. Root-specific transcript accumulation was inhibited initially by exposure to zeatin and TIBA, but accumulation was enhanced over time despite the absence of pseudonodules. We hypothesized that ENOD2 activity in non-nodulating legumes may indicate a cellular response to plant growth regulators and not the early stages of nodulation. New data on the characteristics of ENOD2 and its gene family in woody legumes will foster a broader understanding of molecular mechanisms associated with nodulation in the Fabaceae.
MATERIALS AND METHODS

Plant Material and Treatment

Seeds of *Styphnolobium japonicum* (L.) Schott were obtained from F. W. Schumacher Co., Inc. (Plains, MT) and Lawyer Nursery, Inc. (Sandwich, MA). Seeds of *Cladrastis kentukea* (Dum.-Cours.) Rudd were collected from trees at Iowa State University, Ames, IA. Half-sib seeds of *Maackia amurensis* Rupr. & Maxim., described as seed source 15-5 by Pai and Graves (1995), were obtained from the U.S. National Arboretum, Washington, DC. The Seed Science Center at Iowa State University, provided seeds of *Medicago sativa* L. Seeds of *Glycine max* (L.) Merrill ssp. Marcus BC were obtained from John Imsande, Department of Agronomy, Iowa State University. USDA-Agricultural Research Service Corn, Insects, and Crop Genetics Research Unit at Iowa State University provided seeds of *Zea mays* L. ssp. mays B73 (1 PI 550473).

Seeds were scarified (Batzli et al., 1992) or surface sterilized (Hirsch et al., 1993; Ralston and Imsande, 1983), and germinated aseptically for 5 d. Roots of 5-d-old seedlings not provided combined nitrogen (N) were harvested for DNA extraction. Seven-day-old seedlings were irrigated with sterile nutrient solution at pH 6.8 that contained 250 μM K₂SO₄, 500 μM CaCl₂, 50 μM KH₂PO₄, 100 μM MgSO₄, 10 μM Fe-EDDHA (with 30 μM NO₃⁻), 4.6 μM H₃BO₃, 0.9 μM MnCl₂, 76.5 nM ZnSO₄, 32 nM CuSO₄, 11.1 nM H₂MoO₄, and 0.42 μM CoCl₂, or with solution with 50 μM 2, 3, 5-triiodobenzoic acid (TIBA) or 100 nM zeatin (Sigma, St. Louis, MO).
Plants were grown in sterile, 1-L mason jars as described by Ralston and Imsande (1983). Jars were arranged randomly in a growth chamber at 24.0 ± 1.0°C under 146.3 ± 23.8 µm·m⁻²·s⁻¹ photosynthetically active radiation in 16-h photoperiods from incandescent and fluorescent lamps. At days 0, 10, 20, 30, and 40, roots were harvested for a temporal study of ENOD2 expression. To study organ-specific ENOD2 transcripts in S. japonicum and C. kentukea, leaves, stems, and roots from untreated 12-d-old seedlings were harvested. Inflorescences from mature S. japonicum and C. kentukea at Iowa State University, were harvested in August and June, respectively, when >50% of the flowers in the panicle were opened and before any had senesced. All samples were frozen in liquid N₂ immediately after harvest and stored at -80°C.

PCR Amplification of DNA Probes

ENOD2 DNA fragments were generated with PCR from genomic DNA of S. japonicum, C. kentukea, and M. amurensis, and oligonucleotide primers. Degenerate primer sequences were derived from proline-rich pentapeptide repeats (PHEKP, PPEYQ, and PPYEK) of conserved ENOD2 sequences (Govers et al., 1990) and were synthesized on an ABI 394 DNA synthesizer (Applied Biosystems, Inc., Foster City, CA). Primer pairs used to amplify ENOD2 fragments from M. amurensis, S. japonicum, and C. kentukea were 5'-CCACCTCATGA(G/A)AAACCA-3' and 5'-TGG(C/T)TTCTCATA-(A/T)GGTGG-3', 5'-CCACCTCATGA(G/A)AAACCA-3' and 5'-TTGATA(T/C)TCT-GGTGGTG-
3', and 5'-CCACCACC(C/A)GA(G/A)TACCAG-3' and 5'-TGG(T/C)TT-(T/C)TCATGAGG(A/T)GG-3', respectively. PCR products were cloned into the pCR 2.1 vector from the TA Cloning Kit (Invitrogen, San Diego, CA).

**DNA Sequencing and Analysis**

The sequence of both strands was determined by using automated dideoxy sequencing on an ABI 377 sequencer (Applied Biosystems). The GCG computer programs (Genetics Computer Group, Madison, WI) Fasta, Gap, Pileup, and PepSort were used to analyze nucleotide and amino acid sequences by determining percentages of identity and similarity, sequence alignments, and amino acid compositions, respectively.

**DNA Extraction and Southern Blot Analysis**

Genomic DNA was extracted from freshly harvested roots from 5-d-old seedlings of *S. japonicum, C. kentukea, G. max,* and *Z. mays* by using the CTAB method (Doyle and Doyle, 1987). Ten micrograms of genomic DNA were digested with restriction enzymes *EcoRI, BamHI,* and *XhoI* (Promega, Madison, WI); subjected to electrophoresis in a 0.7% agarose gel with Tris-acetate-EDTA buffer; and blotted onto nylon membrane (MSI, Westboro, MA) with 25 mM sodium phosphate buffer. DNA was crosslinked onto the membranes with a UV Stratalinker 2400 (Stratagene, La Jolla, CA). Prehybridization (1 h) and hybridization (24 h) were done at 42°C in 50% formamide, 6.7× SSC, 3.3×
Denhardt's solution, 0.4% SDS, 25 mM sodium phosphate buffer (pH 7), and salmon sperm DNA at 0.12 µg µL⁻¹. Gel-purified 0.555-, 0.387-, and 1.1-kb ENOD2 clones from *S. japonicum*, *C. kentukea*, and *G. max* (Franssen et al., 1990) were labeled with [³²P]dCTP and [³²P]dATP by nick translation (Nick Translation System, Promega) and used as probes. After hybridization, membranes were rinsed once in 2x SSC and 0.1% SDS at room temperature for 5 min. Three washes were done at 65°C, one for 20 min in 1x SSC and 0.1% SDS, one for 20 min in 0.1x SSC and 0.1% SDS, and one for 5 min with 5 mM Tris (pH 8) and 0.1% SDS before autoradiography for 14 d.

**RNA Extraction and Northern Blot Analysis**

Total RNA was extracted from flowers, leaves, stems, and roots (Dix and Rawson, 1983), and poly(A)⁺ RNA was selected by using an oligo(dT)-cellulose column (Maniatis et al., 1982). Total and poly(A)⁺ RNA samples of 10 and 2.5 µg, respectively, were denatured for 15 min at 65°C and resolved in 1.0% denaturing agarose gels by using 10 mM methyl mercury hydroxide in Tris-borate buffer (Maniatis et al., 1982). Equal loading of RNA per lane was confirmed by comparing intensities of the rRNAs in an ethidium-bromide-stained agarose gel (data not shown). Gels were blotted onto nylon membranes with 10x SSPE buffer, and RNA was crosslinked to the membranes. Prehybridization of the membranes was done as described for Southern blot analysis. Membranes were
hybridized for 66 h with gel-purified $^{32}$P-labeled ENOD2 cDNAs from S. japonicum (555 bp), C. kentukea (387 bp), and M. amurensis (561 bp) under the same conditions as prehybridization. Membranes were rinsed for 5 min at room temperature in 2x SSC and 0.1% SDS, and washed twice at 65°C for 20 min in 1x SSC and 0.1% SDS. Washes were followed by autoradiography of the membranes for 6 d.

RESULTS

Isolation of PCR-generated Probes

Conserved ENOD2 sequences encoding three pentapeptide repeats (PPHEK, PPEYQ, and PPYEK) were used to design 18-bp, degenerate primers for PCR. Three DNA fragments (555, 387, and 561 bp) were amplified with PCR by using the primers and genomic DNA from S. japonicum, C. kentukea, and M. amurensis, respectively. Both strands of the cloned PCR products were sequenced. The sequence of the 561-bp clone from M. amurensis was identical to nucleotides 235 to 796 of the coding region of MaENOD2 (Foster et al., 1998a). DNA sequences were analyzed with the GCG Fasta program and showed greatest identity (70-75%) to the ENOD2 nucleotide sequence of Glycine max (GmENOD2) (Franssen et al., 1990). Deduced ENOD2 amino acid sequences from S. japonicum (SjENOD2), C. kentukea (CkENOD2), M. amurensus (MaENOD2), and GmENOD2 are compared in Fig. 1. The consensus sequence for the three PCR
products and $GmENOD2$ revealed similarity among repeated pentapeptides conserved in $ENOD2$ proteins (Fig. 1). These results indicated that the PCR generated fragments were partial $ENOD2$ sequences.

**Sequences of $SjENOD2$ and $CkENOD2$ are Similar to Other $ENOD2$ Sequences**

Nucleotide and predicted amino acid sequences of $SjENOD2$ and $CkENOD2$ were compared to six $ENOD2$ sequences available from GenBank by using GCG Gap program (Table I). Percentage nucleotide and amino acid identities were highest between $SjENOD2$ and $CkENOD2$ (90 and 86%, respectively), and amino acid similarity was 86% (Table I). Nucleic acid identities with the other $ENOD2$s ranged from 52% ($Pisum sativum$) (van de Wiel et al., 1990b) to 78% ($Medicago sativa$) (Dickstein et al., 1988) for $SjENOD2$ and from 58% ($P. sativum$) to 81% ($Sesbania rostrata$) (Dehio and de Bruijn, 1992) for $CkENOD2$ (Table I). At the amino acid level, values for percentage identity for $S. japonicum$ ranged from 56% with $P. sativum$ to 76% with $M. amurensis$, and sequence similarity was highest with $M. amurensis$ (81%) and lowest with $P. sativum$ (61%) (Table I).

Amino acid identities for $C. kentukea$ ranged from 61% to 82% for $P. sativum$ and $S. rostrata$, respectively (Table I). Highest and lowest amino acid similarities for $C. kentukea$ were 85% with $S. rostrata$ and 67% with $P. sativum$ (Table I).

Percentage identity and similarity of the nucleic acid and amino acid sequences of the six previously identified $ENOD2$s were determined also (Table I). Nucleic acid sequence homologies for the six $ENOD2$ sequences ranged from 52 to 78%. Ranges for amino acid identity and similarity were 50 to 77% and 55 to 81%,
respectively. Sequence identities and similarities for \textit{SjENOD2} and \textit{CkENOD2} were within the range of values determined for the other \textit{ENOD2} sequences in Table I.

In Table II, amino acid compositions of \textit{SjENOD2} and \textit{CkENOD2} are compared to the composition of other \textit{ENOD2}s, \textit{ENOD12}s, \textit{PRPs}, and extensins. Six amino acids (Pro, Glu, Tyr, Lys, His, Val) made up more than 89% and 92% of the \textit{SjENOD2} and \textit{CkENOD2} polypeptides, respectively (Table II). Most \textit{ENOD}s, \textit{PRPs}, and extensins have a high (hydroxy)proline, Lys, and Tyr content, but Glu content in \textit{ENOD2}s is high as well (Nap and Bisseling, 1990a; Wycoff et al., 1992). Comparison of amino acid composition of \textit{ENOD2}s from \textit{M. amurensis}, \textit{G. max}, \textit{S. rostrata}, and \textit{Lupinus luteus} L. (Szczyglowski and Legocki, 1990) revealed high Pro, Glu, Lys, and Tyr levels characteristic of \textit{ENOD2}s. Although Pro, Lys, and Tyr levels in \textit{SjENOD2} and \textit{CkENOD2} are consistent with those of other \textit{ENOD2} proteins, Glu content is markedly lower (Table II). High His, Val, and Ser contents characterize \textit{ENOD12}s, \textit{PRPs}, and extensins, also (Nap and Bisseling, 1990a; Wycoff et al., 1992). \textit{SjENOD2} differs from \textit{PsENOD12} in Tyr, Lys, and Ser content, but His contents are similar. Although \textit{MtPRP4} has more Val and Lys and less Tyr than \textit{CkENOD2}, Val content in \textit{CkENOD2} is much higher than in other \textit{ENOD2} proteins. \textit{VuEXT26} is higher in Ser and Tyr, and lower in Glu and His, than \textit{SjENOD2} and \textit{CkENOD2} (Table II).

Deduced \textit{ENOD2} proteins consist of two domains, a signal peptide and a sequence of proline-rich pentapeptide repeats (Govers et al., 1990). The deduced \textit{SjENOD2} and \textit{CkENOD2} proteins (Fig. 1) consist primarily of four pentapeptide
motifs, PPHEK, PPEYQ, PPVYQ, and PPVYP (Table III). These motifs are conserved in (Wycoff et al., 1992) or have been found in other ENOD2 proteins (Table III) but were not found in other proline-rich proteins (Table III). Pentapeptide repeats conserved in ENOD12s (PPQKE, PPRHK, PPVNK), PRPs (PPVEK, PPVHK, PPVYK), and extensins (SPPPP) (Wycoff et al., 1992) were found in PsENOD12, MtPRP4, and VuEXT26, respectively, but were not present in SjENOD2 and CkENOD2 (Table III). Results from the sequence analyses (percentage identity and similarity, amino acid composition, and conserved motifs) suggest that SjENOD2 and CkENOD2 belong to the ENOD2 gene family.

**SjENOD2 and CkENOD2 are Encoded by Small Gene Families**

To evaluate the number of ENOD2 genes in *S. japonicum* and *C. kentukea*, Southern hybridizations of genomic DNA were performed with 555-bp *SjENOD2*, 387-bp *CkENOD2*, and 1.1-kb *GmENOD2*. *SjENOD2* and *CkENOD2* probes hybridized at high stringency to blots containing *S. japonicum* and *C. kentukea* DNA digested with EcoRI, BamHI, and EcoRI/XhoI. One or two hybridizing fragments were detected in each lane of the blot for *S. japonicum* (Fig. 2), and three or four fragments were detected for *C. kentukea* (Fig. 2). The *GmENOD2* probe did not hybridize, even at lower stringencies (30 and 40% formamide) (data not shown) to DNA from *S. japonicum* and *C. kentukea*, but *SjENOD2* and *CkENOD2* probes hybridized to genomic DNA from *G. max* (Fig. 2). These results suggest that the cloned *SjENOD2* and *CkENOD2* PCR products
are members of gene families with at least one and three genes, respectively (Fig. 2) and are different than GmENOD2.

Accumulation of SjENOD2 and CkENOD2 Transcripts is Organ-specific

To determine whether SjENOD2 and CkENOD2 were organ specific, poly A\(^{-}\) RNA from leaves, stems, and roots from 12-d-old seedlings and flowers from trees was subjected to RNA blot analysis. Transcripts were detected in stems, roots, and flowers, but were undetectable in leaves (Fig. 3). Transcripts observed in roots and stems were approximately 1.1 kb, whereas flower transcripts were approximately 1.1 kb in S. japonicum (Fig. 3) and 1.2, 1.1, and 0.9 kb in C. kentukea (Fig. 3).

ENOD2 Transcript Production Responds to TIBA and Zeatin

Exogenously supplied auxin transport inhibitors and cytokinins induce the formation of pseudonodules that may contain ENOD2 transcripts (Hirsch et al., 1997). Roots of S. japonicum, C. kentukea, M. sativa, and M. amurensis were treated with TIBA and zeatin to determine whether pseudonodule organogenesis and production of ENOD2 transcripts could be induced. Although TIBA and zeatin inhibited lateral roots in all species (data not shown), presumed pseudonodules developed only on TIBA-treated roots of the positive controls, M. amurensis (Fig. 4A) and M. sativa (Fig. 4B). Zeatin induced similar pseudonodules in M. sativa (data not shown). Root tips of S. japonicum (Fig. 4C)
and *C. kentukea* (Fig. 4D) swelled when exposed to TIBA and zeatin but did not produce pseudonodules.

Total RNA was extracted from roots after 0, 10, 20, 30, 40 d of treatment and subjected to RNA blot analysis (Fig 5). TIBA inhibited production of *SjENOD2* transcripts on days 10 and 20 in *S. japonicum*, but levels increased on days 30 and 40 (Fig. 5). Zeatin did not affect transcript production in *S. japonicum* (Fig. 5). Production of *CkENOD2* transcripts in the roots of *C. kentukea* was inhibited by TIBA on day 20 and enhanced on days 30 and 40 (Fig. 5). Zeatin inhibited production of transcripts in *C. kentukea* on days 20 and 30, but levels increased on day 40 (Fig. 5). Transcript accumulation in *M. amurensis* was inhibited by TIBA and zeatin on days 20, 30, and 40, but *MaENOD2* levels increased on day 10 in both treatments (Fig. 5). Enhanced production of *MaENOD2* transcripts fostered by TIBA and zeatin at day 10 is consistent with the early response of other *ENOD2* genes to auxin transport inhibitors and cytokinins (Fang and Hirsch, 1998). Although treatments with zeatin and TIBA did not induce pseudonodules in *S. japonicum* and *C. kentukea*, *ENOD2* transcript accumulation was altered in roots over time and production was enhanced after 30 d (Fig. 5). These results suggest that *ENOD2* activity in *S. japonicum* and *C. kentukea* varies with concentrations of plant growth regulators.
DISCUSSION

Styphnolobium japonicum and C. kentukea are the first woody non-nodulating legumes shown to possess putative ENOD2 genes, and their transcripts were detected in roots, stems, and flowers. Although zeatin and TIBA did not induce pseudonodules in S. japonicum and C. kentukea, ENOD2 transcript accumulation was altered in roots exposed to both chemicals over time, and transcript production was enhanced after 30 d. These results suggest that ENOD2 activity is not marker for the early stages of nodulation in S. japonicum and C. kentukea. Instead, ENOD2 activity may be a cellular response to fluctuations in concentrations of plant growth regulators in the surrounding environment. Delayed ENOD2 induction in S. japonicum and C. kentukea may indicate an insensitivity to exogenously applied plant growth regulators.

Our first objective was to verify the presence of ENOD2 genes in S. japonicum and C. kentukea. Partial ENOD2-like clones were generated with PCR from the genomic DNA of S. japonicum (SjENOD2) and C. kentukea (CkENOD2). Although identity and similarity at the nucleotide and deduced amino-acid levels were highest between SjENOD2 and CkENOD2, percentages were as high as 81% with ENOD2 genes from other taxa as well (Table I). Pentapeptide repeats found most often in the deduced SjENOD2 and CkENOD2 proteins (PPHEK and PPEYQ) were characteristic of ENOD2 proteins (Table III). Compared to previously described ENOD2 proteins, lower percentages of Glu and higher percentages of His and Val in deduced proteins of SjENOD2 and CkENOD2
(Table II) suggested that these cDNAs are different forms of ENOD2. Based on our sequence and Southern analyses, we conclude SjENOD2 and CkENOD2 are distinct from other proline-rich proteins and extensins, and SjENOD2 and CkENOD2 represent small ENOD2 gene families from the S. japonicum and C. kentukea genomes (Fig. 2). But lack of cross hybridization with GmENOD2 supports the ideal that SjENOD2 and CkENOD2 are previously unidentified forms of ENOD2.

Our second goal was to characterize activity of the ENOD2-like genes in S. japonicum and C. kentukea. Transcripts were produced in stems, roots, and flowers of both species (Fig. 3), which suggests that these genes might have roles in the development of different organs. However, functions of proline-rich proteins involved in nodule formation and normal plant development have not been identified (Showalter, 1993). ENOD2 is expressed in the nodule parenchyma of all species studied to date and has been considered a molecular marker for nodule development (Govers et al., 1990; Hirsch et al., 1991). Although ENOD2 activity had been detected only in nodules (van de Wiel et al., 1990), ENOD2 transcripts have been detected in total RNA from uninoculated roots of Lotus japonicus (Regel) K. Larsen (Szczyglowski et al., 1997), in root primordia on stems of S. rostrata (Goormachtig et al., 1998), and in mycorrhizal roots of M. sativa (van Rhijn et al., 1997). ENOD2 mRNA had not been detected previously in stems or flower organs as has mRNA of other ENODs (Scheres et al., 1990; Yang et al., 1993). Synthesis of nodulins in parts of the plant other than


IN THIS STUDY, WE Sought TO ELICIT FORMATION OF PSEUDONODULES ON S. JAPONICUM AND C. KENTUCEA BY TREATING ROOTS WITH TIBA AND ZEATIN. PSEUDONODULES DID NOT DEVELOP. INSTEAD, TIPS OF THE PRIMARY ROOTS SWELLED (FIG. 4), WHICH HAS OCCURRED IN
other species in response to auxins (Allen et al., 1953). This response raises the possibility that these species lack a component of the signal transduction pathway leading to localized cortical cell division and nodule organogenesis.

Pseudonodules developed on the TIBA-treated roots of the nodulating controls, *M. sativa* and *M. amurensis*, and zeatin-induced pseudonodules in *M. sativa* (Fig. 4). Our temporal analyses indicated expression of *MaENOD2* is constitutive in uninoculated roots and was temporarily enhanced in roots treated with zeatin or TIBA (Fig. 5). Production of *MaENOD2* transcripts in TIBA- and zeatin-treated roots of *M. amurensis*, and subsequent formation of pseudonodules, is consistent with the response of other *ENOD2* genes to auxin transport inhibitors and cytokinins (Dehio and de Bruijn, 1992; Hirsch et al., 1993, 1997; Scheres et al., 1992). Pseudonodules on roots of *Melilotus alba* Desr., *M. sativa*, and *P. sativum* contained transcripts of *ENOD2* (Hirsch et al., 1989; Scheres et al., 1992; Wu et al., 1996), and zeatin produced by rhizobia with a constitutive *trans*-zeatin secretion gene initiated nodule organogenesis and *ENOD2* transcript production in alfalfa (Cooper and Long, 1994). Accumulation of *SjENOD2* and *CkENOD2* transcripts in roots was inhibited initially by TIBA, but over time, expression was enhanced (Fig. 5) concomitant with swelling of the root tip (Fig. 4). Although zeatin did not affect *SjENOD2* transcript accumulation, *CkENOD2* gene expression was delayed and then later enhanced by zeatin (Fig. 5). The *SrENOD2* genes in *S. rostrata* are similar to *SjENOD2* in that they have a specific response to one treatment, cytokinin, and do not respond to exogenously applied TIBA (Dehio
and de Bruijn, 1992). Differences between the species may reflect species-specific responses to hormone changes.

Sprent (1994) and others have questioned whether legumes considered non-nodulators never acquired the capacity to nodulate or lost it over time. In some non-nodulating herbaceous legumes, perception of Nod factors seems uncoupled from expression of ENOD genes (Cooper and Long, 1994; Hirsch et al., 1997). Exposing roots of non-nodulating legumes to exogenously applied auxin transport inhibitors and cytokinins might trigger formation of pseudonodules and induction of ENOD2 expression by amplifying localized hormone imbalances in roots. We now know that S. japonicium and C. kentukea possess ENOD2-like genes, and their activity has been detected, but whether these genes can function in roles ascribed to the ENOD2 genes of other legumes remains unknown. Although ENOD2 expression is specific to certain cell types in nodules, ENOD2 is not required for nodule development (Hunt et al., 1995), and a direct link cannot be made between the induction of ENOD2 genes by exogenously supplied hormones and the capacity of a plant to form symbiotic nodules. Our results suggest that ENOD2 activity is not a useful molecular marker for nodulation in legumes, but instead may indicate a direct cellular response to concentrations of plant growth regulators, or a secondary response to a hormone-induced signal transduction pathway.
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LITERATURE CITED


Batzli JM (1991) Indigenous rhizobial diversity of Robinia pseudoacacia L. and nodulation studies of Maackia amurensis Maxim. & Rupr. and Sophora japonica L. MS thesis. The University of Maryland, College Park


Dehio C, de Bruijn FJ (1992) The early nodulin gene *SrENOD2* from *Sesbania rostrata* is inducible by cytokinin. Plant J 2:117-128


Graves WR, van de Poll W (1992) Further evidence that Cladrastis kentukea (Dum.- Cours.) Rudd does not fix nitrogen with rhizobia. HortScience 27: 1137


**Table I. Percentage identity and similarity of ENOD2 sequences**

Percentages were determined by using the GCG Gap program (Genetics Computer Group, Madison, WI).

Percentages for nucleotide and deduced amino acid sequences are above and below the diagonal line, respectively. Percentage similarities are within parentheses ( ).

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<th>Species</th>
<th>Styphnolobium japonicum</th>
<th>Cladrastis kentukea</th>
<th>Glycine max&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sesbania rostrata&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Lupinus luteus&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Pisum sativum&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Medicago sativa&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Maackia amurensis&lt;sup&gt;f&lt;/sup&gt;</th>
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<sup>a</sup>Accession No. X16876 (Franssen et al., 1990). <sup>b</sup>Accession No. X63339 (Dehio and de Bruijn, 1992).

<sup>c</sup>Accession No. X55371 (Szczyglowski and Legocki, 1990). <sup>d</sup>Accession No. X51987 (van de Wiel et al., 1990).

<sup>e</sup>Accession No. X12580 (Dickstein et al., 1988). <sup>f</sup>Accession No. AF039708 (Foster et al., 1998).
Table II. Amino acid compositions predicted from DNA sequence data of early nodulins and proline-rich proteins

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<th>Amino acid</th>
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<th>LENOD\textsuperscript{c}</th>
<th>GmENOD2\textsuperscript{d}</th>
<th>SrENOD2\textsuperscript{e}</th>
<th>PsENOD12\textsuperscript{f}</th>
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\textsuperscript{a}Presented as mole percentages. \textsuperscript{b}Maackia amurensis (AF039708) (Foster et al., 1998). \textsuperscript{c}Lupinus luteus (X55371) (Szczyglowski and Legocki, 1990). \textsuperscript{d}Glycine max (X16876) (Franssen et al., 1990). \textsuperscript{e}Sesbania rostrata (X63339) (Dehio and de Bruijn, 1992). \textsuperscript{f}Pisum sativum (X81366) (Vijn et al., 1995). \textsuperscript{g}Medicago truncatula (L23504) (Wilson et al., 1994). \textsuperscript{h}Vigna unguiculata (X91836) (Arsenijevic-Maksimovic et al., 1997).
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\(^a\text{Pisum sativum} \text{ (X51987)} \) (van de Wiel et al., 1990). \(^b\text{Medicago sativa} \text{ (X12580)} \) (Dickstein et al., 1988). \(^c\text{Maackia amurensis} \text{ (AF039708)} \) (Foster et al., 1998). \(^d\text{Lupinus luteus} \text{ (X55371)} \) (Szczyglowski and Legocki, 1990). \(^e\text{Glycine max} \text{ (X16876)} \) (Franssen et al., 1990). \(^f\text{Sesbania rostrata} \text{ (X63339)} \) (Dehio and de Bruijn, 1992). \(^g\text{Medicago truncatula} \text{ (L23504)} \) (Wilson et al., 1994). \(^h\text{Pisum sativum} \text{ (X81366)} \) (Vijn et al., 1995). \(^i\text{Vigna unguiculata} \text{ (X91836)} \) (Arsenijevic-Maksimovic et al., 1997). \(^x\) number of times motif is repeated. \(^\sim\) motif not present. \(^\text{PPVHK, motif found in other ENOD12 proteins.} \)
**Figure 1.** Comparison of deduced ENOD2 amino acid sequences for *Cladastis kentukea*, *Styphnolobium japonicum*, *Maackia amurensis* (AF039708) (Foster et al., 1998) and *Glycine max* (X16876) (Franssen et al., 1990).

Sequences that code for PCR primers (PPHEK, PPEYQ, and PPYEK) are underlined with a solid line. Conserved pentapeptides (PPHEK and PPEYQ) characteristic of ENOD2 (Wycoff et al., 1992) are bolded in the consensus line. Periods (.) represent gaps in sequences. Capital letters indicate consensus between amino acids at individual positions. Dashes (-) indicate no consensus.
Figure 2. Southern hybridizations for *Styphnolobium japonicum* (Sj) and *Cladrastis kentukea* (Ck). Genomic DNA was digested with EcoRI (1), BamHI (2), and EcoRI/XhoI (3). Genomic DNA from *G. max* and *Zea mays*, digested with EcoRI, was used as positive (4) and negative (5) controls, respectively. Ten micrograms of DNA were loaded in each lane, separated by gel electrophoresis, and transferred to a filter. $^{32}$P-labeled *SjENOD2* and *CkENOD2* PCR fragments were hybridized to blots for *S. japonicum* and *C. kentukea*, respectively.
Figure 3. Northern blots of poly (A)^+ transcripts from leaves (L), stems (S), roots (R), and flowers (F) of *Styphnolobium japonicum* (Sj) and *Cladrastis kentukea* (Ck). Each lane contained 2.5 μg of mRNA. ^32^P-labeled *SjENOD2* and *CkENOD2* PCR fragments were hybridized to blots of *S. japonicum* and *C. kentukea* RNA, respectively.
Figure 4. TIBA-treated roots of *Maackia amurensis*, *Medicago sativa*, *Styphnolobium japonicum*, and *Cladrastis kentukea*. Plants were grown in nutrient solution with 50 μM TIBA for 40 d. Presumed pseudonodules are indicated by arrows in the positive controls, *M. amurensis* (A) and *M. sativa* (B). Root tips of *S. japonicum* (C) and *C. kentukea* (D) swelled, but no pseudonodules were observed on root systems. Bar = 2 mm.
Figure 5. Temporal analysis of putative ENOD2 transcripts from TIBA- and zeatin-treated roots of *Styphnolobium japonicum* (Sj), *Cladrastis kentukea* (Ck), and *Maackia amurensis* (Ma). Plants were grown in nutrient solution with 50 µM TIBA or 100 nM zeatin for 0 to 40 d. Control plants were grown in nutrient solution without TIBA or zeatin. Total RNA was separated by using electrophoresis in an agarose-methyl mercury gel, blotted onto a filter, and hybridized to ^32^P-labeled *SjENOD2* (Sj), *CkENOD2* (Ck), and *MaENOD2* (Ma) PCR fragments. Ten micrograms of RNA were loaded in each lane.
CHAPTER 6. GENERAL CONCLUSIONS

Summary and Conclusions

I expanded the evidence that *Styphnolobium japonicum* (L.) Schott (formerly *Sophora japonica*) and *Cladrastis kentukea* (Dum.-Cours.) Rudd do not have the capacity to form root nodules (Chapter 2). N-deficient plants were inoculated with rhizobia chosen for their low host specificity or for their symbiotic potential with indigenous and introduced trees and shrubs of *Sophora* species in Hawaii, Japan, and China. Soil samples from the root zones of mature *S. japonicum*, *C. kentukea*, and other woody legumes also were used as inocula. Although inoculation with rhizobia in culture and soil did cause nodulation of species used as controls, inoculation did not elicit nodulation of *C. kentukea* or *S. japonicum*, despite that N concentrations of shoots of *S. japonicum* (1.6%) and *C. kentukea* (1.5%) fell below the highest shoot N percentage that previously was associated with well-nodulated plants of *Maackia amurensis* Rupr. & Maxim. (1.8%). In addition to these analyses, rhizobia were isolated from nodules on the roots of a tree reported to me as *S. japonicum*. Nine of the 10 isolates selected as representatives of similarity groups were capable of nodulating *M. amurensis*, which led to the identification of the putative *S. japonicum* as *Maackia floribunda* Takeda. This was the first isolation of symbionts for *M. floribunda*. I also found that broad-range *Bradyrhizobium* USDA 6, USDA 3384, and USDA 3456 induce nodules on *Robinia pseudoacacia* and *M. amurensis*, which were
used as control species during inoculation trials with \textit{S. japonicum} and \textit{C. kentukea}. My conclusion that \textit{S. japonicum} and \textit{C. kentukea} lack the capacity to nodulate is based on the most thorough analysis of the nodulation capacity of these species to date. Previous reports of nodulation of \textit{S. japonicum} may have been due to inaccurate plant or nodule identification.

In the second manuscript of this dissertation (Chapter 3), \textit{MaENOD2}, a cDNA of 1450 bp, was isolated from \textit{M. amurenensis} after screening a nodule cDNA library with a 561-bp PCR fragment (76\% identity to \textit{LENOD2} from \textit{Lupinus luteus} L.) and a 414-bp partial cDNA clone [74\% identity to \textit{GmENOD2} from \textit{Glycine max} (L.) Merr.]. The partial \textit{MaENOD2} cDNA consisted of a 1342-bp open reading frame with no start codon, a stop codon, and a 3' untranslated region of 105 bp that included a polyadenylated tail. Sequence homology was greatest with \textit{ENOD2} nucleotide (72\%, 68\%, 61\%) and amino acid (65\%, 73\%, 74\%) sequences from \textit{LENOD2} (X55371), \textit{GmENOD2} (X16876), and \textit{SrENOD2} (X63339) from \textit{Sesbania rostrata} Brem. & Oberm., respectively. The partial open reading frame encoded a polypeptide of 447 amino acids (51.93 kD) with a pI of 6.39. The N-terminus of the predicted protein was composed of 17 hydrophobic amino acids characteristic of a partial signal sequence. The deduced protein consisted primarily of pentapeptide repeats (PPHEK, PPyEK, PPEYQ) associated with \textit{ENOD2} proteins. Proline (45\%), glutamic acid (13\%), tyrosine (12\%), lysine (9\%), and histidine (6\%) were the dominant amino acids in the predicted protein, and their preferential codons were CCA and CCT, GAG, TAT and TAC, AAG, and CAT, respectively.
In the third manuscript of this dissertation (Chapter 4), a cDNA library from root nodules of *M. amurensis* was screened by using an ENOD2 PCR fragment and partial ENOD2 cDNA from *M. amurensis* as probes. Seven cDNAs were isolated, sequenced, and analyzed. Similarity among identified ENOD2 sequences and *MaENOD2* confirmed that cDNAs isolated from *M. amurensis* belonged to the ENOD2 gene family. Conserved pentapeptide motifs and amino acid composition grouped the protein deduced from *MaENOD2* with other ENOD2s and distinguished it from other proline-rich proteins. A single pentapeptide variation (PPHET) between cloned sequences indicated at least two ENOD2 transcripts are produced in *M. amurensis*. Southern hybridization indicated that *MaENOD2* belongs to a family of at least three genes. *MaENOD2* activity was not nodule-specific. Transcripts were detected in non-symbiotic roots and in stamens, carpels, and receptacles of flowers. *MaENOD2* transcripts were detected in roots 4 d after inoculation with rhizobia, and expression was enhanced as the nodules developed to > 2 mm diameter. *In situ* hybridization showed *MaENOD2* transcripts are restricted to the distribution zone of indeterminant nodules. My results suggested that ENOD2 in *M. amurensis* may be a cell wall component of tissues that regulate nutrient flow to sinks, such as developing seeds, pollen grains, and symbiotic regions of a nodule.

In the fourth manuscript of this dissertation (Chapter 5), genomic DNA and primers, derived from proline-rich pentapeptide repeats of conserved ENOD2 sequences, were used to obtain ENOD2 PCR fragments from two non-nodulating woody legumes, *S. japonicum* (555-bp *SjENOD2*) and *C. kentukea* (379-bp
These cloned fragments had 52 to 82% sequence identity to \textit{ENOD2} sequences and encoded proteins that possessed conserved \textit{ENOD2} pentapeptides (PPHEK and PPEYQ). Lower percentages of Glu and higher percentages of His and Val in deduced proteins suggested that \textit{SjENOD2} and \textit{CkENOD2} are different \textit{ENOD2}s. Sequence and blot analyses indicated that the clones represented \textit{ENOD2} gene families in \textit{S. japonicum} and \textit{C. kentukea} genomes, and \textit{ENOD2}-like transcripts were detected in stems, roots, and flowers of the two species.

Roots of control species that nodulate, \textit{M. amurensis} and \textit{Medicago sativa} L., produced pseudonodules after treatment with zeatin or TIBA, an auxin transport inhibitor, but pseudonodules did not form on roots of \textit{S. japonicum} and \textit{C. kentukea}. Zeatin and TIBA enhanced \textit{ENOD2} expression in roots of \textit{M. amurensis} during the first 10 days of treatment, but over time accumulation was inhibited. Although transcript accumulation in roots of \textit{S. japonicum} and \textit{C. kentukea} was inhibited initially, production was enhanced after 30 days. My results suggested \textit{ENOD2} activity is not a molecular marker for nodulation in legumes, but instead may indicate cellular response to concentrations of plant growth regulators.

In Appendix A, \textit{Ma1433}, a cDNA from \textit{Maackia amurensis} Rupr. & Maxim., was isolated from a nodule (≤ 1 mm diameter) cDNA library. Sequence analyses indicated that the cDNA encodes a 14-3-3 brain protein homology.
Recommendations for Future Research

Chapter 2:

- Screen soil samples collected in North America and Asia from indigenous stands of *Styphnolobium* and *Cladrastis* species for rhizobia effective with *S. japonicum* and *C. kentukea*.

- Confirm symbiotic compatibility between *Maackia floribunda* and rhizobia isolated from *M. floribunda* nodules, and characterize effective rhizobia.

Chapter 4:

- Localize *ENOD2* transcripts in stamen, carpels, receptacles, and root tips from *Maackia amurensis* with in situ hybridization.

- Isolate and analyze sequences of flower-specific *ENOD2* cDNAs from *M. amurensis* by screening a flower cDNA library or by using RT-PCR with flower RNA.

- Examine *MaENOD2* expression at the protein level using immunocytochemistry and microscopy to localize protein at the tissue and cellular level in nodules, flowers, and roots.

- Isolate genomic clones for nodule- and flower-specific cDNAs and identify regions of the promotors that control organ-specific gene expression.

- Characterize infection and nodule organogenesis of *Maackia amurensis* by examining whole preparations and sections of inoculated roots and developing nodules.
Chapter 5:

- Isolate and analyze full-length ENOD2-like cDNA sequences from *Cladrastis kentukea* and *Styphnolobium japonicum*.
- Localize tissue level production of ENOD2-like mRNAs in *S. japonicum* and *C. kentukea* with *in situ* hybridization.

Overall:

- Determine phylogenetic relationships between nucleotide sequences encoding proline-rich ENODs and other proline-rich proteins.
- Characterize additional symbiosis-enhanced, conserved genes from nodulating and non-nodulating legumes and non-legumes.
APPENDIX A. ISOLATION OF A 14-3-3 BRAIN PROTEIN HOMOLOG FROM NODULES OF MAACKIA AMURENSIS RUPE. & MAXIM. (ACCESSION NO. AF039709) (PGR 98-061)

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The 14-3-3 proteins belong to a heterogeneous family of proteins that are highly conserved among animals, fungi, and plants (Aitken et al., 1992; Ferl, 1996). Found in a broad range of tissues, these 25- to 32-kD proteins are acidic and soluble with various isoelectric points. Dimerization among 14-3-3 proteins results in homodimers and heterodimers of 50 to 60 kD. Isoforms of 14-3-3 proteins possess conserved and unique sequences in their amino- and carboxyl-termini. The conserved regions in the N-terminal half of plant 14-3-3 proteins are essential for dimerization (Wu et al., 1997). Highly variable or unique regions of the N-terminus (Aitken, 1995) and C-terminus (Daugherty et al., 1996) may limit the number of dimer combinations and confer specificity on 14-3-3 isoform function (Ferl, 1996).

The dimeric isoforms of 14-3-3 proteins participate in inter- and intracellular signal transduction through protein-protein interactions and phosphorylation events (Aitken, 1995; Ferl, 1996). Diverse functions of the isoforms include regulation of calcium and protein kinase-related events that involve protein kinase C, activation of tyrosine and tryptophan hydroxylases during the biosynthesis of neurotransmitters, chaperone activities, and calcium-dependent phospholipid binding and exocytosis. The 14-3-3 proteins also play a role in the mitogenic signaling pathway that involves products of proto-oncogenes and oncogenes, such as Raf-1 and Bcr-kinase (Morrison, 1994). ATPase activity, stimulation of ADP ribosylation activity of Exoenzyme S, and DNA damage checkpoint activity are additional biochemical roles for 14-3-3 proteins (Aitken, 1995; Ferl, 1996).

Plant 14-3-3 proteins have been identified in *Arabidopsis*, spinach, maize, barley, rice, tobacco, oat, soybean, broad bean, potato, pea, and tomato. Unique roles for these proteins have been found. Homologs in *Arabidopsis* and maize are associated with the G-box DNA/protein complexes used during transcriptional regulation (Lu et al., 1992; Ferl, 1996). Plant 14-3-3 proteins may regulate phosphorylation events that occur in response to low temperature, hypoxia, salinity and pathogen attack and may act as receptor molecules for the plant phytoxin fusicoccin (Ferl, 1996). Tomato homologs also have been linked to fruit development (Laughner et al., 1995).

A 14-3-3 cDNA from *Maackia amurensis* Rupr. & Maxim., designated Ma1433, was isolated from a nodule (< 1 mm diameter) cDNA library constructed in lambda ZapII (Stratagene). Synthetic oligonucleotide primers were used to sequence the 5' and 3' ends of the cDNA. The sequence of both strands was determined by using automated dideoxy sequencing on an ABI 377 sequencer (Applied Biosystems, Inc., Foster City, CA, USA) at the Nucleic Acid Facility, Iowa State University.

The 1176-bp Ma1433 contained a 5’ untranslated region of 30 bp, an open reading frame of 786 bp with stop codon, and a 3’ untranslated region of 360 bp with polyadenylated tail. Sequence homology was greatest with 14-3-3 brain protein homologs from soybean (87.6%) and broad bean (86.7%) over 1165-bp and 1086-bp overlaps, respectively. Nucleotide identity was determined by using GCG Fasta analysis (Genetics Computer Group, Madison, WI, USA). The deduced polypeptide from Ma1433 was composed of 261 amino acids and had a molecular mass of 29.41 kD and a pI of 4.6. Two highly conserved motifs characteristic of all deduced 14-3-3 amino acid sequences were present in the Ma1433 protein. One peptide sequence (RNLLSVGYKNV) was found in the N-terminal region, and the second peptide sequence (YKDSTLIMQLLRDNLTLWTS) was located in the C-terminal region.

Acknowledgments

The authors thank Mary Tymeson and Jennifer Hart for technical assistance and advice.

Literature Cited


Laughner B, Lawrence SD, Ferl RJ (1995) Two cDNA clones encoding 14-3-3 homologs from tomato fruit. Biochim Biophys Acta 1263: 67-70


Morrison D (1994) 14-3-3: modulators of signaling proteins? Science 266: 56-57

APPENDIX B. DESCRIPTION AND IMAGES OF MAACKIA AMURENSIS, STYPHNOLOBIUM JAPONICUM, AND CLADRASTIS KENTUKEA

Species in *Maackia, Styphnolobium,* and *Cladrastis* are used primarily as ornamental trees, but their wood has been used in carpentry, and dyes have been produced from their wood or seed pods (Allen and Allen, 1981). These trees have been planted also for forage, to sustain bee crops, and to prevent soil erosion.

*Maackia amurensis* Rupr. & Maxim. (Amur maackia) is indigenous to the Russian Far East, Korea, northern China (Andrews, 1997). A variety of *M. amurensis,* *M. amurensis* var. *buergeri* (Maxim.) Kitamura, is native to Hokkaido and Honshu, Japan. Physical attributes that enhance the ornamental value of Amur maackia are silvery pubescence on young vegetative growth, bronze bark that exfoliates slightly, and racemes of creamy-white flowers (Dirr, 1998) (Fig. 1). Disease resistance and cold hardiness improve its survival in the urban landscape.

*Styphnolobium japonicum* (L.) Schott is indigenous to China and Korea. Widely used as a landscape plant, the Japanese pagodatree or Chinese scholaratree possesses desirable ornamental characteristics, such as glossy leaves, creamy-white to pale yellow flowers during late summer, and yellow fall leaf color (Fig. 2) (Chun, 1921; Dirr, 1998). Its survival in urban settings is improved by tolerance to heat, drought, pollution, and cold hardiness (Dirr, 1998).

*Cladrastis kentukea* (Dum.-Cours.) Rudd (American yellowwood), a rare tree native to the United States, is valued as an ornamental tree for its display of white flowers in early June, smooth gray bark, and yellow leaf color in the fall (Fig. 3) (Spongberg and Ma, 1997). In addition, disease resistance and cold hardiness are properties that enhance its survival in the urban landscape (Dirr, 1998).

*Maackia amurensis* Rupr. & Maxim. was considered to be a member of the *Cladrastis* genus, but leaflet position and concealment of lateral buds by petioles
allowed for its distinction (Allen and Allen, 1981). Recent updates to the Papilionoideae phylogenetic tree, based on \textit{rbcL} sequence analysis (Doyle et al., 1997), indicate that \textit{Maackia} is a more advanced genus than \textit{Cladrastis} with a closer association to various members of \textit{Sophora}, other than \textit{S. japonica} L. (now classified as \textit{Styphnolobium japonicum} (L.) Schott) (Sousa and Rudd, 1993). \textit{Cladrastis} and \textit{Styphnolobium} hold positions nearer than \textit{Maackia} to the more primitive Mimosoideae and Caesalpinioideae subfamilies.

**LITERATURE CITED**


Chun WY (1921) Chinese Economic Trees. Commercial Press, Ltd., Shanghai


Figure 1. *Maackia amurensis* Rupr. & Maxim. (Amur maackia). A. Habit (Photo by Jeff Iles, Iowa State University, Department of Horticulture) B. Exfoliating bark (Photo by William Graves, Iowa State University, Department of Horticulture) C. Racemes of creamy-white flowers (Photo by William Graves, Iowa State University, Department of Horticulture) D. Silvery pubescent young leaves (Photo by William Graves, Iowa State University, Department of Horticulture)
Figure 2. *Styphnolobium japonicum* (L.) Schott (Japanese pagodatree). A. Habit and late summer floral display (Photo by Jeff Iles, Iowa State University, Department of Horticulture) B. Panicle of pale yellow flowers (Photo by Jeff Iles, Iowa State University, Department of Horticulture) C. Fall leaf color (Photographer unknown)
Figure 3. *Cladrastis kentukea* (Dum.-Cours.) Rudd (American yellowwood). A. Habit and fall leaf color (Photographer unknown) B. Panicle of pure white flowers (Photo by Jeff Iles, Iowa State University, Department of Horticulture) C. Papilionaceous white flowers (Photo by Carol Foster, Iowa State University, Departments of Horticulture and Botany)
APPENDIX C. DIAGRAM OF THE ANATOMY OF AN INDETERMINANT NODULE*

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