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Two Early Nodulation Genes are not Markers for the Capacity of Leguminous Nursery Crops to Form Root Nodules[1]

William R. Graves[2], Carol M. Foster[3], Faye M. Rosin[4], and James A. Schrader[4]
Department of Horticulture
Iowa State University, Ames, IA 50011-1100

Abstract

Little is known about mechanisms that permit or prevent formation of root nodules in which nitrogen gas (N_2) is fixed by rhizobial bacteria in association with nursery crops in the legume family. We tested the hypothesis that two genes associated with the fixation of N_2 could be used as markers of nodulation capacity among legumes because they occur only in species that can form functional nodules. The presence of *ENOD2* and *ENOD12* was tested in genomic DNA from three non-nodulating legumes [*Cercis canadensis* L. (eastern redbud), *Gleditsia triacanthos* L. var. *inermis* Willd. (thornless honeylocust), *Gymnocladus dioicus* (L.) K. Koch (Kentucky coffeetree)] and two nodulating legumes [*Albizia julibrissin* Durazz. (silk-tree), *Laburnum alpinum* (Mill.) Bercht. & Presl. (Scotch laburnum)]. Southern analyses indicated that *ENOD2* is present in thornless honeylocust, Kentucky coffeetree, and Scotch laburnum, and that *ENOD12* is present in eastern redbud, thornless honeylocust, and Scotch laburnum. These results diversify the group of nodulating legumes in which *ENOD2* and *ENOD12* have been found and show these genes also occur in legumes considered incapable of nodulation. We conclude that neither gene can be used to screen existing or new leguminous nursery crops for the capacity to form N_2-fixing nodules.

Index words: nutrition, symbiosis, sustainable production.

Species used in this study: *Cercis canadensis* L. (eastern redbud), *Gleditsia triacanthos* L. var. *inermis* Willd. (thornless honeylocust), *Gymnocladus dioicus* (L.) K. Koch (Kentucky coffeetree), *Albizia julibrissin* Durazz. (silk-tree), *Laburnum alpinum* (Mill.) Bercht. & Presl. (Scotch laburnum), *Maackia amurensis* Rupr. & Maxim. (Amur maackia).

Significance to the Nursery Industry

Efficient management of nitrogen (N) inputs during production of nursery crops is increasingly important as efforts to protect the environment from damage caused by excess N intensify. After nursery crops are installed in the landscape, N often is the only mineral element that limits their growth. Crops that can form root nodules in which bacteria fix N_2 gas from the atmosphere have the potential to be produced with relatively little N fertilizer. Such plants might also be sustained with relative ease in landscapes where soil is of poor nutritive quality and no N is applied. Yet little is known about the genetics and physiology of N_2 fixation in nursery crops that may have this potential, most of which are in the legume family. A broad objective of our research program is to identify and strive to overcome barriers that prevent development of effective nodules in legumes important in the nursery and landscape industries. This paper reports research designed to test for the occurrence of two genes associated with N_2 fixation in several leguminous tree species. Some legumes we studied form root nodules in which N_2 is fixed, whereas others, which never have been observed with nodules, are considered incapable of associating with N_2-fixing bacteria for reasons unknown. Nursery and landscape professionals will benefit from this research by gaining information on the potential value of N_2 fixation and the present understanding of the nodulation capacity of some common nursery crops. Moreover, this research illustrates a facet of fundamental research that eventually may lead to a broader palate of species that can be produced and maintained with minimal inputs of N.

Introduction

Some plants form mutually beneficial relationships with N_2-fixing bacteria that infect their roots. Most of these plants are in the legume family (Fabaceae), while their microbial partners, collectively known as rhizobia, are assigned to several bacterial genera. Infection of plants by rhizobia depends on genetic compatibility of the two organisms and a susceptible infection site, often a root hair of a plant deficient in N. Root nodules, which form after infection, are an obvious sign that an N_2-fixing symbiosis may be established (1). The potential of N_2 fixation to reduce dependence on N fertilizers during production of leguminous food crops has been recognized for many years. Research to compare different rhizobia for their efficiency at fixing N_2 in association with these crops has led to inoculants marketed to crop producers wishing to promote development of functional nodules.

Less attention has been given to N_2 fixation by rhizobia that associate with woody legumes used for landscaping. Previous research suggests that numerous woody species important in the nursery industry are incapable of forming N_2-fixing symbioses because nodules never have been observed on their roots (1). Species in this category include *Cercis canadensis* L. (eastern redbud), *Gleditsia triacanthos* L. var. *inermis* Willd. (thornless honeylocust), *Gymnocladus dioicus* (L.) K. Koch (Kentucky coffeetree), and *Styphnolobium japonicum* (L.) Schott (Japanese pagodatree;

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[2]Professor, Department of Horticulture.

[3]Postdoctoral Research Associate, Department of Horticulture.

[4]Graduate Research Assistant, Department of Horticulture.

formerly *Sophora japonica* L.). *Cladrastis kentukea* (Dum.-Cours.) Rudd (American yellowwood), a fifth species in this category, has received considerable attention recently because *Maackia amurensis* Rupr. & Maxim. (Amur maackia), a species some have considered closely related to American yellowwood (1), was induced to form functional nodules (2). Efforts with American yellowwood, however, have not elicited nodulation (7, 10), which strengthens previous evidence (15) that this species lacks the capacity to associate with N_2-fixing bacteria.

Why these legumes apparently cannot become infected by rhizobia and nodulate is not known. Legumes contain numerous genes that may play a role in the formation of functional nodules. *ENOD* genes, which are associated with early stages of nodulation, have been found in all the nodulating legumes that have been studied. Occurrence of these genes in non-nodulating species has not been evaluated. Our hypothesis was that at least one *ENOD* gene could serve as a marker for the capacity of a species to form root nodules. Genetic markers in DNA extracted from woody plants are becoming widely used tools for plant breeding, determining taxonomic relationships, and cultivar identification (3). Researchers and horticulturists could benefit from a DNA marker for screening legumes for the capacity to nodulate. Use of such a marker with species new to the industry might be particularly valuable. For example, *Cladrastis sinensis* Hemsl. (Chinese yellowwood) has outstanding ornamental potential yet is virtually unknown in American nurseries and landscapes. It is one of many woody legumes that never have been evaluated for the capacity to associate with N_2-fixing bacteria. Moreover, the nodulation status of only about 16% of the nearly 20,000 species of legumes has been reported (1). A gene present in legumes that nodulate but absent in all other taxa would be a reliable marker to screen unexamined species rapidly for the capacity to nodulate.

The objective of this research was to test the hypothesis that at least one *ENOD* gene could serve as a marker for the capacity to nodulate by examining woody legumes for the presence of two *ENOD* genes, *ENOD2* and *ENOD12*. This analysis involved two species known to form root nodules, *Albizia julibrissin* Durazz. (silk-tree) and *Laburnum alpinum* (Mill.) Bercht. & Presl. (Scotch laburnum), and three species considered incapable of nodulating, eastern redbud, thornless honeylocust, and Kentucky coffeetree. Although none of these species had been evaluated previously for the presence of either gene, we anticipated their occurrence in the nodulating species based on studies of other nodulating legumes. If either gene were found exclusively in legumes known to nodulate, experiments with a broader array of nodulating and non-nodulating species would be warranted to test the reliability of one or both genes as a marker of the capacity to nodulate.

Materials and Methods

Leaves were collected from one tree each of eastern redbud, Kentucky coffeetree, and thornless honeylocust on the campus of Iowa State University on June 5, 1997. The next day, leaves were collected from seedlings of silk-tree and Scotch laburnum that had been grown from seed scarified 30 and 45 min, respectively, in sulfuric acid and sown in containers of perlite on May 15, 1997. Seeds of silk-tree were collected at the U.S. National Arboretum in Washington, DC. Seeds of Scotch laburnum were purchased from Lawyer Nursery in Plains, MT. The seedlings were grown in a greenhouse at 23C (73F) ±3C (5F) under ambient radiation and irrigated with tap water.

For each species, genomic DNA extracted from 5 g (0.18 oz) of leaf blade tissue (6) was subjected to Southern hybridization analysis. DNA (10 µg [3.53×10^{-7} oz]) was digested by using the restriction enzymes *Eco*RI, *Bam*HI, and *Hin*dIII. The fragments were separated by electrophoresis, blotted onto a nylon membrane, and hybridized with a ^{32}P-labeled probe. The probe used to assess the presence of *ENOD2* was a 0.565-kb *Eco*RI insert in the pCRII vector of an *ENOD2* clone of Amur maackia (8), an ornamental woody legume confirmed to form N_2-fixing root nodules (2). The probe used to detect *ENOD12* was a 0.266-kb *Hin*dIII/*Eco*RI insert from an *ENOD12* clone from pea (*Pisum sativum* L.) (13) in the pTz19R vector. Autoradiography of the washed membranes lasted 14 to 17 days. Hybridizations of gene sequences from the digested DNA of each species and the *ENOD2* and *ENOD12* probes were seen as bands on the autoradiography film.

Results and Discussion

Gene sequences from silk-tree, Scotch laburnum, thornless honeylocust, and Kentucky coffeetree hybridized with the *ENOD2* probe, and gene sequences from Scotch laburnum, eastern redbud, and thornless honeylocust hybridized with the *ENOD12* probe (Fig. 1). Evidence of *ENOD2* and *ENOD12* genes was not surprising for the two nursery crops that nodulate, silk-tree and Scotch laburnum, because, unless they have been altered specifically not to (4), the genomes of all other nodulating legumes evaluated to date contain sequences with the characteristics of *ENOD* genes (9). The analogs of at least one of these genes in eastern redbud, thornless honeylocust, and Kentucky coffeetree indicate that *ENOD2* and *ENOD12* also occur in non-nodulating species. Weak bands (Fig. 1) may have resulted from using probes derived from the genomes of species other than those we studied. Evidence of even faint bands in at least one species known to form nodules and one species considered incapable of nodulating was sufficient to meet our objective. Therefore, we did not attempt to enhance the quality of the Southern analyses nor to test further for *ENOD2* in eastern redbud and *ENOD12* in silk-tree and Kentucky coffeetree.

Previous studies of *ENOD2* and *ENOD12* have focused on members of the Papilionoideae subfamily of legumes. Containing some 14,000 species, this is the largest subfamily of legumes and the one in which nodulation and N_2 fixation are most prevalent (1). Doyle (5) has emphasized the need to learn more about genes related to nodulation in diverse species in all three subfamilies of the legume family. All three non-nodulating species we studied are members of the Caesalpinioideae subfamily, which contains about 2,800 species (1). This is the first report of *ENOD2* and *ENOD12* in this subfamily. Silk-tree, the nodulating species we found to contain *ENOD2* (Fig. 1), belongs to the third subfamily of legumes, the Mimosoideae (1). None of the 2,900 species of this subfamily has been reported previously to possess *ENOD2*.

Confirmation of *ENOD2* and *ENOD12* genes in these leguminous nursery crops may lead to additional research on their function, particularly in non-nodulating species. Of immediate value is the impact of our results on horticulturists who seek to know whether a nursery crop can associate

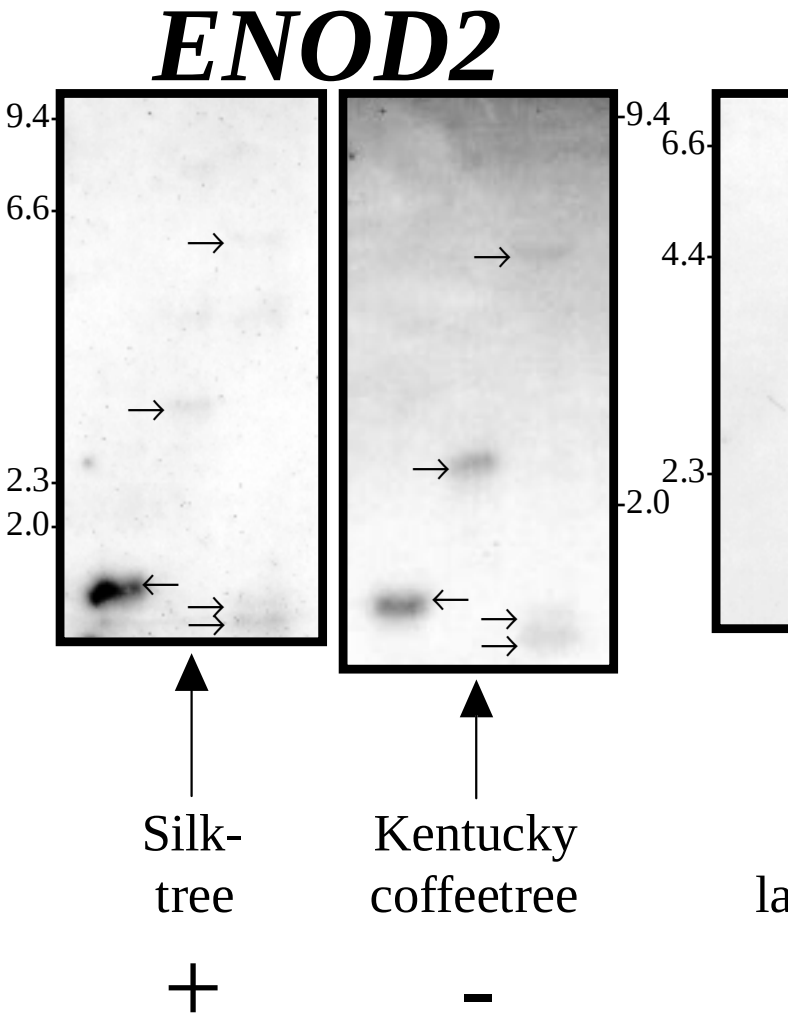

Fig. 1. Representative Southern analyses of genomic DNA extracted from leaves of leguminous trees used for landscaping. Species known to form root nodules in which N_2 is fixed are indicated with a +. Species considered incapable of fixing N_2 in root nodules are indicated with a –. DNA was hybridized with either an *ENOD2* clone of Amur maackia or an *ENOD12* clone from pea. The results for each species include three lanes in which the same amount of DNA was digested with the restriction enzymes *Eco*RI (left), *Bam*HI (middle), and *Hin*dIII (right). Bands are indicated by arrows and show the presence of DNA similar to the *ENOD2* and *ENOD12* probes. At least one band was observed in each lane for *ENOD2*, but only the lane with DNA digested with *Hin*dIII resulted in hybridizations with the probe for *ENOD12*. Molecular size markers in kb are shown to the left or right of each image. Similar evidence was found for *ENOD2* in Scotch laburnum and thornless honeylocust and for *ENOD12* in eastern redbud.

with N_2-fixing bacteria in a container or in soil in the landscape. Because species presumed incapable of forming N_2-fixing nodules contained *ENOD2* and *ENOD12*, we reject our hypothesis that at least one of the genes could serve as a marker of nodulation capacity.

In the absence of such a rapid screening technique, those wishing to assess the capacity of a nursery crop to associate with N_2-fixing bacteria must rely on direct observation of roots. It is critical to observe roots under conditions that would make them most susceptible to infection by bacteria, which leads to nodule development. Because of low susceptibility to infection, it is unlikely that plants grown in a medium with enough N to support near-optimal growth will form nodules even in the presence of compatible bacteria. Likewise, plants grown in a medium that is sterile or soilless are less likely to nodulate than plants grown in untreated soil, particularly soil from areas where the species is native. Growing seedlings or rooted cuttings of nursery crops with sub-optimal N in a medium inoculated with bacteria or soils remains a useful screening method. Cultures of specific bacteria are available commercially and from the U.S. Department of Agriculture in Beltsville, MD. Samples of soil used as inoculant often are from regions where the species is native. Careful management of such inoculation trials (14) can result in nodules visible to the naked eye within a few weeks if the species is capable of nodulation and compatible bacteria are present. During the past decade, inoculation trials with soils from North America and Asia were used to confirm that Amur maackia is capable of forming root nodules in which N_2 is fixed (2). Analysis has begun on how N_2 fixation can be exploited to produce Amur maackia and other nursery crops with less fertilizer and to sustain healthy plants in the landscape (11, 12).

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