Accumulation of *ENOD2*-Like Transcripts in Non-Nodulating Woody Papilionoid Legumes[1]

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Japanese pagodatree (*Styphnolobium japonicum* [L.] Schott) and American yellowwood (*Cladrastis kentukea* Dum.-Cours.) Rudd are the first woody, non-nodulating papilionoid legumes shown to possess putative early nodulin 2 (*ENOD2*) genes. *ENOD2* cDNAs from Japanese pagodatree (807 bp) and American yellowwood (735 bp) have 75% to 79% sequence identity to *ENOD2* sequences and encode deduced proteins that possess conserved *ENOD2* pentapeptides (PPHEK and PPEYQ). Lower percentages of glucose and higher percentages of histidine and valine suggest that *SjENOD2* and *CkENOD2* are different from other *ENOD2*s. Hybridization analyses indicate the clones represent *ENOD2* gene families of two to four genes in Japanese pagodatree and American yellowwood genomes, and *ENOD2*-like transcripts were detected in stems and flowers, as well as roots. Only roots of control species that nodulate, *Maackia amurensis* Rupr. & Maxim. and alfalfa (*Medicago sativa*), produced pseudonodules after treatment with zeatin or 2,3,5-triiodobenzoic acid, an auxin transport inhibitor. Accumulation of *MaENOD2* transcripts was enhanced during the first 10 d of treatment, but 2,3,5-triiodobenzoic acid and zeatin enhanced transcript accumulation after 30 d in roots of Japanese pagodatree and American yellowwood. Characteristics that distinguish *ENOD2* gene families in basal, non-nodulating woody legumes from other *ENOD2* genes may provide new information about the function of these genes during symbiotic and non-symbiotic organ development.

The mutualistic relationship between many legumes (Fabaceae) and bacteria that fix dinitrogen (N_2), such as *Rhizobium* sp., *Bradyrhizobium* sp., *Sinorhizobium* sp., and *Azorhizobium* sp., results in a unique organ, the nodule (Dénarié and Roche, 1992). Nodulation, once considered inherent in legumes, does not occur in all taxa. In subfamilies Caesalpinioideae, Mimosoideae, and Papilionoideae, 71%, 10%, and 3% of the studied species, respectively, do not form root nodules (Bryan et al., 1996). Although most species in the Papilionoideae nodulate, little is known about the molecular biology of nodulation and N_2 fixation in temperate, woody members of this subfamily.

Genes involved in nodule formation and function have been classified into two groups, early (*ENOD*s) and late (*NOD*s) nodulins, based on time of their expression during nodule development. Many *ENOD*s have been identified (Gyorgyey et al., 2000; Jimenez-Zurdo et al., 2000), but few genomes of woody papilionoid species, members of the Caesalpinioideae and Mimosoideae subfamilies, or nodulating non-legumes have been examined (Foster et al., 1998a; Graves et al., 1999; Okubara et al., 2000). Study of *ENOD*s 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; Soltis et al., 1995).

As a conserved early nodulation gene, *ENOD2* has been used as a molecular marker for the early stages of nodule organogenesis. The *ENOD2* sequence predicts that it may encode a Pro-rich protein with a structure similar to hydroxy-Pro-rich cell wall glycoproteins (Franssen et al., 1987). In situ hybridization showed that accumulation of *ENOD2* transcripts occurs in the nodule parenchyma (van de Wiel et al., 1990a; Allen et al., 1991), but transcripts also have been detected in uninoculated, mycorrhizal, and cytokinin-treated roots of herbaceous legumes known to nodulate (Szczyglowski et al., 1997; van Rhijn et al., 1997; Goormachtig et al., 1998). *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 (ATIs) (naphthylphthalamic acid and 2,3,5-triiodobenzoic acid [TIBA]), auxins, and cytokinins induced formation of uninfected nodule-like structures (pseudonodules) on the roots of legumes and non-legumes (Arora et al., 1959; Hirsch et al., 1989; Ridge et al., 1992, 1993). Pseudonodules on roots of *Melilotus alba* Desr., alfalfa (*Medicago sativa*), and pea contained transcripts of *ENOD2*, *ENOD8*, *ENOD12*, and/or *ENOD40* (Hirsch et al., 1989; Scheres et al., 1992; Dickstein et al., 1993; Wu et al., 1996; Fang and Hirsch, 1998). New information about the nature of

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hormone-sensitive *ENOD* genes can be obtained by modifying the endogenous hormone balance of roots of nodulating and non-nodulating legumes.

Japanese pagodatree (*Styphnolobium japonicum* Schott) and American yellowwood (*Cladrastis kentukea* Dum.-Cours.) Rudd are commercially important, temperate, woody members of the Papilionoideae that do not nodulate (Wilson, 1939; Batzli, 1991; Graves and van de Poll, 1992; Foster et al., 1998b). Phylogenetic analyses of *rbc*L sequences indicated the genera *Cladrastis* and *Styphnolobium* are sister taxa that represent a basal (primitive) group within the subfamily where nodulation is not common (Doyle et al., 1997). As a first step in determining whether molecular events typical of nodulation of herbaceous legumes occur in these non-nodulating tree species we tested for the presence of *ENOD2* in Japanese pagodatree and American yellowwood and studied spatial and temporal production of transcripts. In the absence of compatible rhizobia to stimulate gene function in Japanese pagodatree and America yellowwood, TIBA and zeatin were used to induce the formation of pseudonodules and production of *ENOD2* transcripts in roots of both species.

RESULTS

Isolation of PCR-Generated Sequences

Conserved *ENOD2* sequences encoding 5′- and 3′-translated regions and Pro-rich pentapeptide repeats were used to design degenerate primers for PCR. DNA fragments from Japanese pagodatree (555 and 807 bp) and American yellowwood (387 and 735 bp) were amplified by using the primers and genomic DNA or first-strand cDNA generated from total RNA from roots. Fasta analysis of the 807-bp cDNA sequence from Japanese pagodatree (*SjENOD2*; accession no. AF289097) showed greatest identity (64%, 792-bp overlap) to *MaENOD2* (Foster et al., 1998a). The 735-bp cDNA sequence from American yellowwood (*CkENOD2*; accession no. AF289098) showed greatest identity (67%, 755-bp overlap) to *GmENOD2B* (Franssen et al., 1989). Deduced *ENOD2* amino acid sequences from *SjENOD2* and *CkENOD2* are shown in Figure 1. These results indicated that the PCR-generated fragments were partial *ENOD2* sequences.

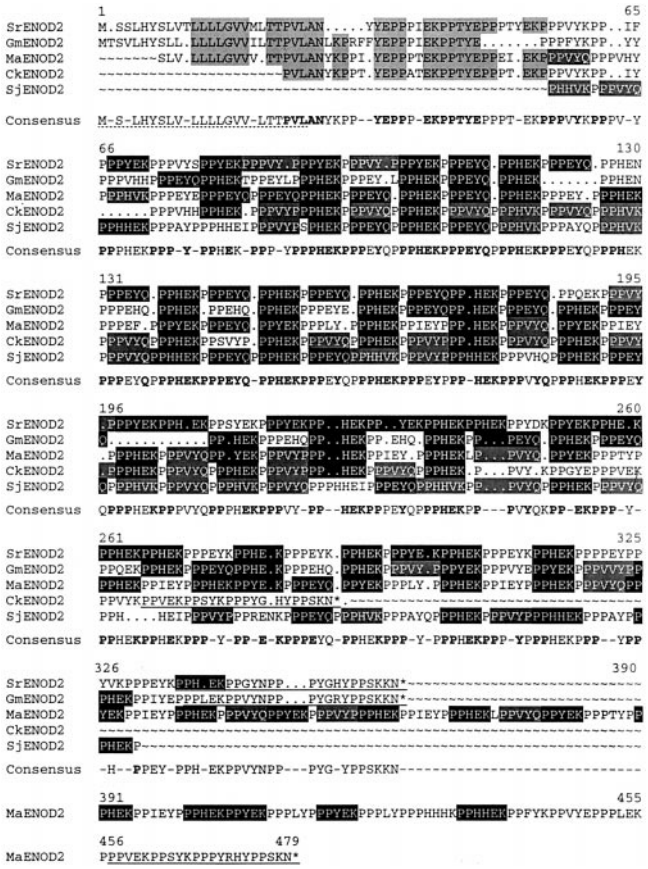

Figure 1. Comparison of deduced *ENOD2* amino acid sequences from Japanese pagodatree (SjENOD2), American yellowwood (CkENOD2), *M. amurensis* (MaENOD2), soybean (GmENOD2), and *Sesbania rostrata* (SrENOD2). Alignments were obtained with GCG computer programs Lineup and Pileup. Pentapeptide repeats conserved in *ENOD2* proteins are shaded in black, whereas repeats common to *ENOD2* proteins are shaded in dark gray. Light gray shading indicates conserved regions in the N-terminal domain. Conserved sequences for C-terminal domains that are not aligned because of differences in sequence length are underlined with a solid line. The consensus line identifies conserved amino acids in four and five presented sequences (bold) or in two and three of the sequences. The putative signal peptide sequence is underlined with a dotted line in the consensus sequence. Asterisks represent presence of stop codons. Dashes (approximately) indicate no sequence data available. Dots represent gaps in the sequence.

Sequences of *SjENOD2* and *CkENOD2* Are Similar to Other *ENOD2* Sequences

Nucleotide and predicted amino acid sequences of *SjENOD2* and *CkENOD2* were compared with sequences from GenBank by using GCG Gap program (Genetics Computer Group, Madison, WI; Table I). Three *ENOD2*s, a Pro-rich protein, *MtPRP4* (Wilson et al., 1994), and an extensin, *HRGPnt3* (Keller and Lamb, 1989) were selected for comparison based on best sequence match to *SjENOD2* and *CkENOD2* with GCG Fasta program. Values for percentage similarity for *SjENOD2* and *CkENOD2* were highest with other ENOD2s (75% to 83%) and lowest with *MtPRP4* and *HRGPnt3* (50% to 70%). Nucleotide (76%) and amino acid (79%) sequence similarities between *SjENOD2* and *CkENOD2* were consistent with the values determined for other *ENOD2* sequences.

In Table II, amino acid compositions of SjENOD2 and CkENOD2 are compared with ENOD2s, MtPRP4, and HRGPnt3. Six amino acids (Pro, Glu, Tyr, Lys, His, and Val) made up more than 97% and 92% of the SjENOD2 and CkENOD2 polypeptides, respectively. Although Pro, Lys, and Tyr levels in SjENOD2 and CkENOD2 are consistent with those of other *ENOD2* proteins, Glu content is markedly lower, and

Table I. *Percentage similarity of SjENOD2 and CkENOD2 with ENOD2 and Pro-rich protein sequences encoding translated regions of primary transcripts*

Percentages for nucleotide and deduced amino acid sequences are left and right of the diagonal line, respectively.

Sequence (kb)[a]	*SjENOD2* (0.807)	*CkENOD2* (0.735)
GmENOD2[b] (1.203)	77/81	75/81
SrENOD2[c] (1.274)	76/82	79/83
MaENOD2[d] (1.345)	76/83	75/80
MtPRP4[e] (1.750)	54/60	62/70
HRGPnt3[f] (1.862)	54/60	50/62

[a] Length of the complete or partial sequence used in the analysis. [b] Soybean (accession no. X16876; Franssen et al., 1989). [c] *Sesbania rostrata* (accession no. X63339; Dehio and de Bruijn, 1992). [d] *Maackia amurensis* (accession no. AF039708; Foster et al., 1998a). [e] *Medicago truncatula* (accession no. L23504; Wilson et al., 1994). [f] Tobacco (accession no. X13885; Keller and Lamb, 1989).

Val content in CkENOD2 and His content in SjENOD2 are higher. In general, the amino acid compositions of SjENOD2 and CkENOD2 are more similar to ENOD2s than to MtPRP4 and HRGPnt3.

Deduced *ENOD2* proteins consist of two domains, a signal peptide and a sequence of Pro-rich pentapeptide repeats (Govers et al., 1990). The deduced *SjENOD2* and *CkENOD2* proteins (Fig. 1) consist primarily of five pentapeptide motifs, PPHEK, PPEYQ, PPVYQ, PPHVK, and PPVYP. PPHEK and PPEYQ are conserved and highly repeated in *ENOD2* proteins (Wycoff et al., 1992; Table III). The other motifs are found in *ENOD2* proteins as well, especially in the deduced *MaENOD2* protein from another temperate woody species, *Maackia amurensis* (Foster et al., 1998a). Pentapeptide repeats conserved in PRPs (PPVEK and PPVYK; Wycoff et al., 1992) also were found in CkENOD2, MaENOD2, and SrENOD2, but to a much lesser extent. These sequence analyses (percentage similarity, amino acid composition, and conserved motifs) indicate 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 Japanese pagodatree and American yellowwood, high stringency Southern hybridizations of genomic DNA were performed with *SjENOD2*, *CkENOD2*, and *GmENOD2* cDNAs. No *Eco*RI, *Bam*HI, or *Xho*I restriction sites were found in *SjENOD2* and *CkENOD2* cDNA sequences. Two hybridizing fragments were detected in each lane of the blot for Japanese pagodatree (Fig. 2), and three or four fragments were detected for American yellowwood. *SjENOD2* and *CkENOD2* probes hybridized to a 4.5-kb *Eco*RI fragment from soybean (*Glycine max* [L.] Merrill subsp. Marcus BC). At lower stringency (40% formamide), a 10.5-kb *Eco*RI fragment was detected (data not shown). These are the same *Eco*RI fragment sizes to which the *GmENOD2* cDNA hybridized (data not shown; Franssen et al., 1989). No DNA fragments from maize (*Zea mays* subsp. *mays* B73; 1 PI 550473) were detected with *SjENOD2* or *CkENOD2*. These results suggest that the cloned *SjENOD2* and *CkENOD2* PCR products may be members of *ENOD2* gene families consisting of two and four genes, respectively.

Accumulation of *SjENOD2* and *CkENOD2* Transcripts Is Organ-Dependent

To determine whether *SjENOD2* and *CkENOD2* were organ-dependent, 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 Japanese pagodatree, and 1.2 and 0.9 kb in America yellowwood. Size of transcripts was estimated according to Lambda/*Hin*dIII and ØX174/*Hae*III DNA markers.

Table II. *Amino acid compositions predicted from DNA sequence data of early nodulins and Pro-rich proteins*[a]

Amino Acid	SjENOD2	CkENOD2	MaENOD2[b]	GmENOD2[c]	SrENOD2[d]	MtPRP4[e]	HRGPnt3[f]
Pro	48.0	47.1	45.0	40.8	41.8	33.7	42.9
Glu	8.0	7.8	12.8	14.2	13.6	9.8	0.3
Tyr	8.0	9.8	11.6	8.4	11.2	4.6	6.1
Lys	7.0	9.8	9.0	8.4	11.8	18.4	0.2
His	12.0	7.0	5.8	8.4	6.1	6.6	5.1
Val	7.0	9.0	4.0	3.2	3.0	15.8	1.6
Ser	0.4	1.2	0.7	1.0	1.8	0.9	12.4
Others	3.0	7.3	11.1	15.6	10.7	10.2	31.4

[a] Presented as mole percentages. [b] *M. amurensis* (accession no. AF039708; Foster et al., 1998a). [c] Soybean (accession no. X16876; Franssen et al., 1989). [d] *S. rostrata* (accession no. X63339; Dehio and de Bruijn, 1992). [e] *M. truncatula* (accession no. L23504; Wilson et al., 1994). [f] Tobacco (accession no. X13885; Keller and Lamb, 1989).

Table III. *Repetitive pentapeptide motifs in early nodulins and Pro-rich proteins*

Motif	SjENOD2	CkENOD2	MaENOD2[a]	GmENOD2[b]	SrENOD2[c]	MtPRP4[d]	HRGPnt3[e]
PPHEK	6x[f]	12x	18x	17x	17x	–[g]	–
PPEYQ	7x	–	6x	8x	7x	–	–
PPYEK	–	–	12x	2x	9x	–	–
PPVYQ	6x	8x	7x	–	–	–	–
PPVYP	4x	4x	2x	1x	3x	–	–
PPHVK	5x	2x	1x	–	–	–	–
PPVYK	–	3x	–	–	1x	8x	–
PPVEK	–	2x	1x	–	–	32x	–
PPVHK	–	–	–	–	–	20x	–
SPPPP	–	–	–	–	–	–	26x

[a] *M. amurensis* (accession no. AF039708; Foster et al., 1998a). [b] Soybean (accession no. X16876; Franssen et al., 1989). [c] *S. rostrata* (accession no. X63339; Dehio and de Bruijn, 1992). [d] *M. truncatula* (accession no. L23504; Wilson et al., 1994). [e] Tobacco (accession no. X13885; Keller and Lamb, 1989). [f] x, Number of times motifs is repeated. [g] –, Motif not present.

ENOD2 Transcript Production Responds to TIBA and Zeatin

Exogenously supplied ATIs and cytokinins induce the formation of pseudonodules that may contain *ENOD2* transcripts (Hirsch et al., 1989). Roots of Japanese pagodatree, American yellowwood, alfalfa, and *M. amurensis* were treated with TIBA and zeatin to determine whether morphogenesis of pseudonodules 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 alfalfa (Fig. 4B). Zeatin induced similar pseudonodules in alfalfa (data not shown). Root tips of Japanese pagodatree (Fig. 4C) and American yellowwood (Fig. 4D) swelled when exposed to TIBA and zeatin, but did not produce pseudonodules.

Total RNA from these experiments was extracted from roots after 0, 10, 20, 30, and 40 d of treatment and subjected to RNA-blot analysis (Fig. 5). TIBA inhibited accumulation of *SjENOD2* transcripts on d 10 and 20 in Japanese pagodatree, but transcripts increased on d 30 and 40. Zeatin did not affect transcript accumulation in Japanese pagodatree until d 40 when accumulation was enhanced. Accumulation of *CkENOD2* transcripts in the roots of American yellowwood was enhanced by TIBA on d 30 and 40.

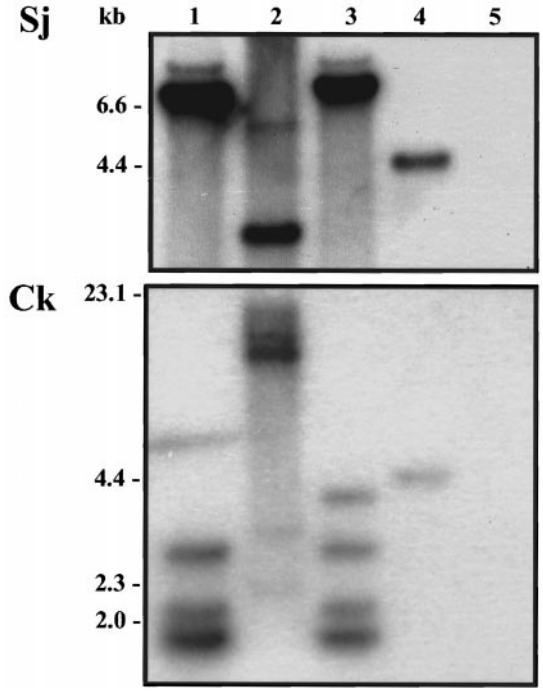

Figure 2. Southern hybridizations for Japanese pagodatree (Sj) and American yellowwood (Ck). Genomic DNA was digested with *Eco*RI (lane 1), *Bam*HI (lane 2), and *Eco*RI/*Xho*I (lane 3). Genomic DNA from soybean and maize, digested with *Eco*RI, was used as positive (lane 4) and negative (lane 5) controls, respectively. Ten micrograms of DNA was loaded in each lane. ^{32}P-labeled *SjENOD2* and *CkENOD2* PCR fragments were hybridized to blots for Japanese pagodatree and American yellowwood, respectively.

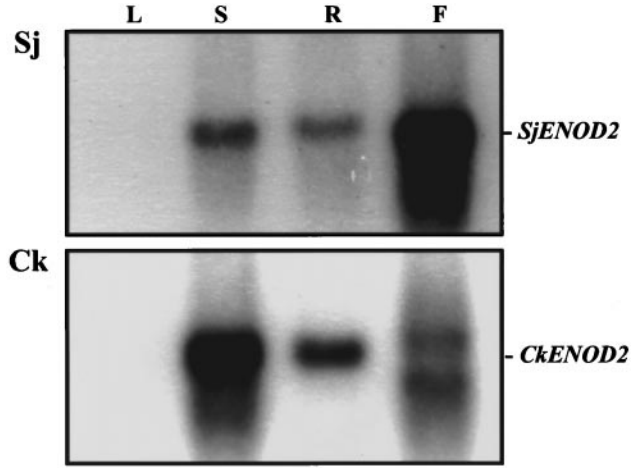

Figure 3. Northern blots of poly(A$^+$) transcripts from leaves (L), stems (S), roots (R), and flowers (F) of Japanese pagodatree (Sj) and American yellowwood (Ck). Each lane contained 2.5 μg of mRNA. ^{32}P-labeled *SjENOD2* and *CkENOD2* PCR fragments were hybridized to blots of Japanese pagodatree and American yellowwood RNA, respectively.

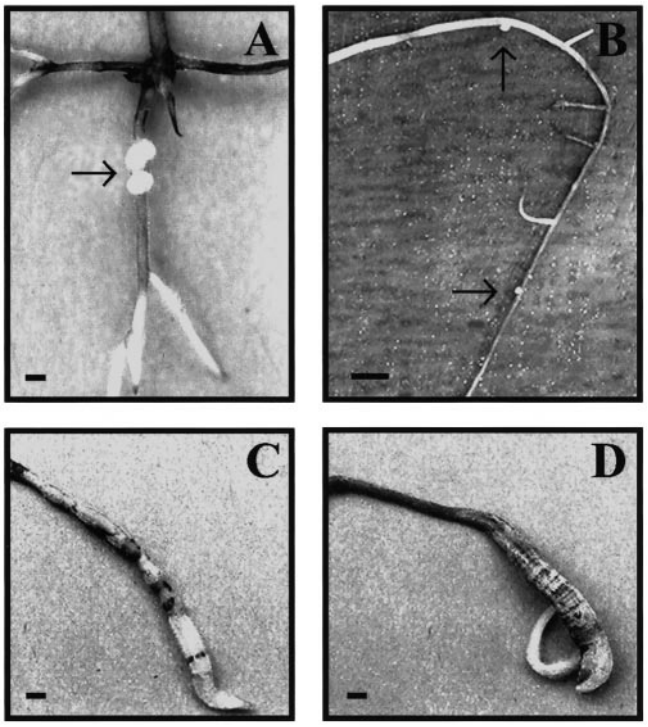

Figure 4. TIBA-treated roots of *M. amurensis*, alfalfa, Japanese pagodatree, and American yellowwood. Plants were grown in nutrient solution with 50 μM TIBA for 40 d. Presumed pseudonodules are indicated by arrows in the positive controls, *M. amurensis* (A) and alfalfa (B). Root tips of Japanese pagodatree (C) and American yellowwood (D) swelled, but no pseudonodules were observed on root systems. Bar = 2 mm.

Zeatin inhibited accumulation of transcripts in American yellowwood on d 30, but transcripts increased on d 40. Transcript accumulation in *M. amurensis* was enhanced on d 10 by TIBA and zeatin. Equal loading of samples was monitored by rehydridizing membranes with an 18S rDNA probe. These results suggest that *ENOD2* transcript accumulation in Japanese pagodatree, American yellowwood, and *M. amurensis* varies with concentrations of plant growth regulators.

DISCUSSION

To determine whether molecular events characterized during nodulation of herbaceous legumes occur in non-nodulating leguminous trees, we have identified putative *ENOD2* genes in Japanese pagodatree and American yellowwood. *SjENOD2* and *CkENOD2* are the first *ENOD2* homologs isolated from non-nodulating, temperate species from basal taxa of the Papilionoideae. Members of small gene families, SjENOD2 and CkENOD2 share sequence similarity and repetitive motifs with other *ENOD2* proteins and respond to fluctuations in concentrations of plant growth regulators. However, differences in spatial and temporal production of transcripts distinguish *SjENOD2* and *CkENOD2* from *ENOD2* genes in previously studied legumes.

Partial *ENOD2*-like clones were generated with reverse transcriptase (RT)-PCR from total RNA from zeatin- and TIBA-treated roots of Japanese pagodatree (*SjENOD2*) and American yellowwood (*CkENOD2*). Similarity of the coding region of *SjENOD2* and *CkENOD2* at the nucleotide and deduced amino acid levels were as high as 83% with *ENOD2* genes from other taxa (Table I). Pentapeptide repeats found most often in the deduced *SjENOD2* and *CkENOD2* proteins (Table III) and their tandem arrangement (forms of PPHEK and PPHVK followed by variants of PPEYQ, PPVYQ, and PPVYP; Fig. 1) were characteristic of *ENOD2* proteins (van de Wiel et al., 1990b). Based on our sequence analyses we conclude *SjENOD2* and *CkENOD2* are distinct from other Pro-rich proteins and extensins. However, compared with previously described *ENOD2* proteins (Nap and Bisseling, 1990; Wycoff et al., 1992), 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*. Disparity in *ENOD2* sequences may be used to infer genetic distance between Japanese pagodatree and American yellowwood and higher taxa in the Papilionoideae (Soltis et al., 1995) and may reflect the different cell wall properties of the tissue in which they are expressed (Franssen et al., 1988).

Transcripts of the *ENOD2*-like genes in Japanese pagodatree and American yellowwood were identified in roots, and for the first time, in stems and flowers of both species (Fig. 3), which suggests that these genes might have roles in the development of different organs. However, functions of Pro-rich proteins involved in nodule formation and normal plant development have not been identified (Showalter, 1993). As a putative cell wall protein, ENOD2 may play a role in cell wall remodeling during organ development. Although *ENOD2* expression had been detected only in nodules (van de Wiel et al., 1990b), *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* Brem. & Oberm. (Goormachtig et al., 1998), and in mycorrhizal roots of alfalfa (van Rhijn et al., 1997). *ENOD2* mRNA had not been detected previously in stems or flower organs as has mRNA of other *ENOD*s (Scheres et al., 1990; Yang et al., 1993). Synthesis of nodulins in parts of the plant other than nodules does not preclude the utility of nodulins in characterizing events during symbiosis (Sánchez et al., 1991).

Application of purified *nod* factors, ATIs, auxins, and cytokinins that change the concentrations of endogenous hormones can stimulate some early stages of nodule development (Arora et al., 1959; Ridge et al., 1992, 1993; Hirsch et al., 1997) and *ENOD* gene

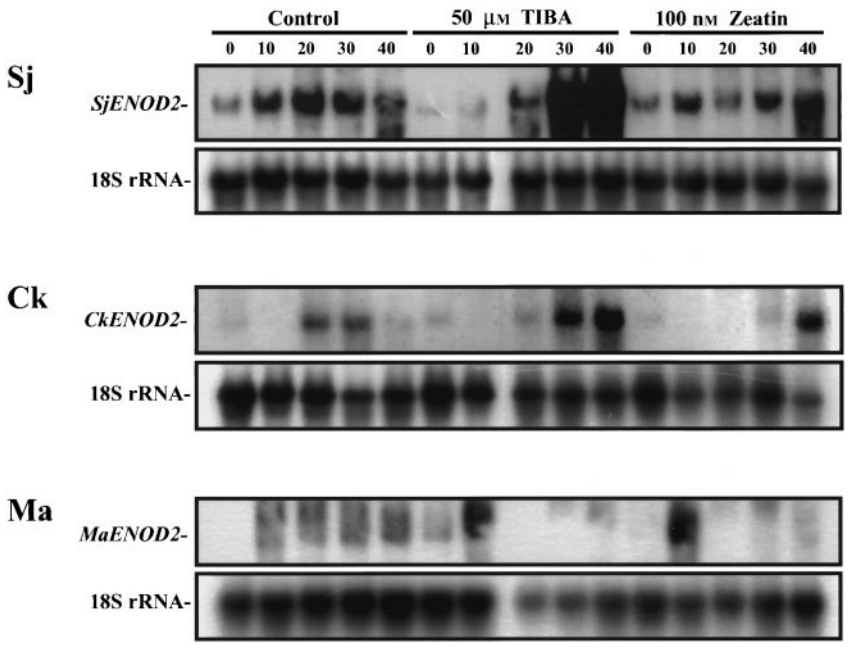

Figure 5. Temporal analysis of putative *ENOD2* transcripts from TIBA- and zeatin-treated roots of Japanese pagodatree (Sj), American yellowwood (Ck), and *M. amurensis* (Ma). Plants were grown in nutrient solution with 50 μM TIBA or 100 nM zeatin for 0 to 40 d. Control plants were grown in nutrient solution without TIBA or zeatin. Total RNA was hybridized to ^{32}P-labeled *SjENOD2* (Sj), *CkENOD2* (Ck), and *MaENOD2* (Ma) PCR fragments. Ten micrograms of RNA was loaded in each lane. Hybridization with a 18S rDNA probe served as a control for loading.

expression (Govers et al., 1990; van de Wiel et al., 1990a). Similarities between pseudonodules and nodules induced by rhizobial infection at the molecular level have been evaluated by examining expression of *ENOD* genes (Bauer et al., 1996). In this study we sought to elicit formation of pseudonodules on Japanese pagodatree and American yellowwood 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 and ATIs (Allen et al., 1953; Hirsch et al., 1989, 1993; Scheres et al., 1992).

Temporal changes in *ENOD2* transcript levels during development of pseudonodules are similar to transcript accumulation during symbiotic nodule development. After inoculation with rhizobia or treatment with ATIs or cytokinins, nodules and pseudonodules become visible on roots in 7 to 14 d, and *ENOD2* transcripts can be detected in 4 to 10 d (Hirsch et al., 1989, 1993, 1997; Govers et al., 1990; Dehio and de Bruijn, 1992; Scheres et al., 1992; Cooper and Long, 1994; Wu et al., 1996). In our experiment pseudonodules developed on the TIBA-treated roots of the nodulating controls, alfalfa and *M. amurensis*, and zeatin induced pseudonodules in alfalfa (Fig. 4). Accumulation of *MaENOD2* transcripts was enhanced during the first 10 d of treatment with TIBA and zeatin (Fig. 5), which is consistent with the response of other *ENOD2* genes to ATIs and cytokinins. However, *SrENOD2* RNA is present in roots of *S. rostrata* 2 to 48 h after exposure to benzylaminopurine even though no pseudonodules develop (Dehio and de Bruijn, 1992).

Despite the lack of pseudonodule development on uninoculated roots of Japanese pagodatree and American yellowwood, *ENOD2* transcript levels were affected by exogenously supplied ATIs and cytokinins. But in contrast to the response of *ENOD2* genes in nodulating legumes, enhanced *ENOD2* transcript accumulation was delayed considerably in roots of Japanese pagodatree and American yellowwood when phytohormone balance was changed. Accumulation of *SjENOD2* and *CkENOD2* transcripts in roots was inhibited initially by TIBA, but by d 30, expression was enhanced (Fig. 5) concomitant with swelling of the root tip (Fig. 4). Although zeatin did not affect *SjENOD2* transcript accumulation until d 40, *CkENOD2* gene expression was delayed and then later enhanced by zeatin (Fig. 5). *SrENOD2* genes in *S. rostrata* are similar to *SjENOD2* in that they have a specific response to one treatment, cytokinin (Dehio and de Bruijn, 1992), whereas, *SjENOD2* responds to TIBA.

Sprent (1994) and others have questioned whether legumes considered non-nodulators lost the capacity to nodulate over time or never acquired it. 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). It is tempting to speculate that Japanese pagodatree and American yellowwood lack a component of the signal transduction pathway leading to localized cortical cell division and nodule organogenesis. But other factors, such as autoregulation by a local signal (ethylene) or a global signal (shoot-derived inhibitor; Schultze and Kondorosi, 1998) may influence nodule initiation in Japanese pagodatree and American yellowwood. We now know that Japanese pagodatree and American yellowwood possess *ENOD2*-like genes, and their transcripts have 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, 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 demonstrate that *ENOD2* homologs are present in non-nodulating, temperate tree species from basal papilionoid taxa (Doyle et al., 1997) and add to the growing body of evidence that genes involved with nodulation have been recruited from other developmental pathways in the plant (Gualtieri and Bisseling, 2000). Characteristics that distinguish *ENOD2* genes in woody legumes from *ENOD2* genes in herbaceous legumes may provide insight into their role in symbiotic and non-symbiotic organ development. A broader understanding of the evolution of nodulation in plants may be fostered as well (Soltis et al., 1995).

MATERIALS AND METHODS

Plant Material and Treatment

Seeds of Japanese pagodatree (*Styphnolobium japonicum* [L.] Schott) were obtained from F.W. Schumacher Co. (Plains, MT) and Lawyer Nursery (Sandwich, MA). Half-sib seeds of *Maackia amurensis* Rupr. & Maxim. (seed source 15–5 by Pai and Graves, 1995) were obtained from the U.S. National Arboretum (Washington, DC). Seeds of American yellowwood (*Cladrastis kentukea* Dum.-Cours.) Rudd, alfalfa (*Medicago sativa*), soybean (*Glycine max* [L.] Merrill subsp. Marcus BC), and maize (*Zea mays* subsp. *mays* B73; 1 PI 550473) were obtained at Iowa State University (Ames).

Seeds were scarified (Batzli et al., 1992) or surface-sterilized (Ralston and Imsande, 1983) and germinated aseptically for 5 d. Roots of 5-d-old seedlings not provided combined nitrogen were harvested for DNA extraction. Seven-day-old seedlings were irrigated with a sterile nutrient solution (Hoagland and Arnon, 1950; pH 6.8) that contained 10 μM Fe-EDDHA (with 30 μM NO_3^-) and 0.42 pM $CoCl_2$, or with the same solution containing 50 μM TIBA or 100 nM zeatin (Sigma, St. Louis). Plants were grown in sterile, 1-L mason jars (Ralston and Imsande, 1983). Jars were arranged randomly in a growth chamber at 24.0°C ± 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 d 0, 10, 20, 30, and 40, roots were harvested for a temporal study of *ENOD2* expression. To study organ-dependent accumulation of *ENOD2* transcripts, leaves, stems, and roots from untreated 12-d-old seedlings were harvested. Inflorescences from mature American yellowwood and Japanese pagodatree at Iowa State University were harvested in June and August, respectively, when >50% of the flowers in the panicle were open and before any had senesced. All samples were frozen in liquid N_2 immediately after harvest and stored at −80°C.

PCR Amplification of DNA Probes

Putative *ENOD2* DNA fragments were generated with GeneAmp (PCR System 2400, Perkin Elmer, Norwalk, CT) using oligonucleotide primers and genomic DNA of Japanese pagodatree and American yellowwood. Primer sequences were derived from Pro-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, Foster City, CA). Primer pairs used to amplify *ENOD2* fragments from Japanese pagodatree and American yellowwood were 5′-CCACCTCATGA(G/-A)AAACCA-3′ and 5′-TTGATA(T/C)TCTGGTGGTGG-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).

DNA Extraction and Southern-Blot Analysis

Genomic DNA was extracted from freshly harvested roots of 5-d-old seedlings of Japanese pagodatree, American yellowwood, soybean, and maize using the cetyltrimethyl-ammonium bromide method (Doyle and Doyle, 1987). Ten micrograms of genomic DNA was digested with restriction enzymes *Eco*RI, *Bam*HI, and *Xho*I (Promega, Madison, WI), subjected to electrophoresis in a 0.7% (w/v) agarose gel with Tris [tris(hydroxymethyl)-aminomethane]-acetate-EDTA buffer, and blotted onto a nylon membrane (MSI, Westboro, MA) with 25 mM sodium phosphate buffer. Prehybridization and hybridization were done at 42°C in 50% (w/v) formamide, 6.7× SSC, 3.3× Denhardt's solution, 0.4% (w/v) 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 Japanese pagodatree, American yellowwood, and soybean (Franssen et al., 1989) were labeled with [^{32}P]dCTP and [^{32}P]dATP by nick translation (Nick Translation System, Promega) and used as probes. After hybridization, washed membranes were subjected to autoradiography.

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 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% (w/v) denaturing agarose gels by using 10 mM methyl mercury hydroxide in Tris-borate buffer (Maniatis et al., 1982). Gels were blotted onto nylon membranes with 10× sodium chloride/sodium phosphate/EDTA buffer. Prehybridization and hybridization of the membranes were done as described for Southern-blot analysis. Membranes were hybridized with gel-purified ^{32}P-labeled *ENOD2* cDNAs from Japanese pagodatree (555 bp), American yellowwood (387 bp), and *M. amurensis* (561 bp). The sequence of the 561-bp PCR clone from *M. amurensis* was identical to nucleotides 235 to 796 of the coding region of

MaENOD2 (Foster et al., 1998a). Washes were followed by autoradiography of the membranes. To confirm equal loading of RNA per lane, membranes were stripped of the former probe and rehybridized with a wheat 18S rDNA probe.

RT-PCR

To amplify more complete putative *ENOD2* sequences from Japanese pagodatree and American yellowwood for sequence analysis, RT-PCR was performed with components and protocols from RETROscript Kit (Ambion, Austin, TX). First-strand cDNA was synthesized from 1 μg of total RNA from zeatin- and TIBA-treated roots using M-MLV reverse transcriptase and an 18-mer oligo(dT)-primer. PCR amplification of cDNAs was achieved by using a 5-μL aliquot of the RT-PCR reaction and primers derived from 5′- and 3′-translated regions and Pro-rich pentapeptide repeats of *MaENOD2* (Foster et al., 1998a). Primer combinations were 5′-CCAGTGTTGGCAAATTAC-AA-3′and 5′-TTAATTTTTGGAAGGTGGATA-3′ for American yellowwood and 5′-CCACCACCAGA(G/A)TATCAA-3′ and 5′-TGGTTT(C/T)TCATGAGGTGG-3′ for Japanese pagodatree. Amplified cDNAs were cloned into the pCRII vector using the TA cloning kit (Invitrogen).

DNA Sequencing and Analysis

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

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