

ENOD8, a Novel Early Nodule-Specific Gene, Is Expressed in Empty Alfalfa Nodules

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The alfalfa ENOD8 nodule-specific gene is expressed in empty nodules elicited by exopolysaccharide-deficient *Rhizobium meliloti*, and it is expressed early in nodule development. An ENOD8 cDNA was sequenced and found to encode a novel product. Its deduced polypeptide sequence was found to be similar to the non-proline-rich domains of the putative polypeptides encoded by a class of anther-specific genes from *Arabidopsis thaliana* and *Brassica napus*. The role of the ENOD8 gene product is predicted to be in nodule organogenesis. ENOD8 is expressed in a developmental pathway triggered as a result of *Rhizobium* nodulation signal transduction.

Additional keywords: *Medicago sativa*, nodulin genes.

Alfalfa, *Medicago sativa*, is able to enter into a mutually beneficial relationship with the soil bacterium, *Rhizobium meliloti*, resulting in nitrogen-fixing root nodules. In a complex developmental interplay, rhizobia induce plant cells in the root cortex to differentiate into a new organ, the root nodule. Rhizobia invade the developing nodule through plant-derived infection threads, which begin to form in the curled root hairs. Eventually, the rhizobia are deposited into plant-derived membrane vesicles in the cytoplasm of nodule cells. There they differentiate into bacteroids, capable of reducing atmospheric nitrogen to ammonia, a nitrogen source that can be assimilated by the plant. Genes from both the plant host and the bacterial symbiont are required for proper nodule development and function. Questions about the coordinated expression of symbiotic genes have begun to be addressed using genetic markers for different stages of both plant and bacterial development.

Research has identified many *Rhizobium* genes that are required for nodulation, nodule invasion, and nitrogen fixation. In *R. meliloti*, the nodulation (*nod*) genes control the inducible synthesis of a diffusible lipooligosaccharide compound, NodRmIV(S), which is able to curl root hairs and induce nodule meristem development (Lerouge *et al.* 1990; Truchet *et al.* 1991; Schultze *et al.* 1992; reviewed in Fisher and Long 1992). Nodule invasion is dependent on the exopolysaccha-

ride (*exo*) (Leigh *et al.* 1985) and nodule development (*ndv*) genes (Dylan *et al.* 1986). Nitrogen fixation is controlled by the *nif* and *fix* genes (reviewed in Ditta 1989). Other *R. meliloti* genes have been implicated in nodule development, blocking it at intermediate stages: *hemA* mutants elicit defective nodules in which bacteria are not released from aberrant infection threads (Dickstein *et al.* 1991) and a *bacA* mutant produces nodules in which the intracellular rhizobia fail to develop into mature bacteroids (Allen *et al.* 1992).

Progress has also been made on plant genes and their role in symbiosis. Nodule-specific plant proteins, or nodulins, are defined by their presence in root nodules and absence from other plant tissue, specifically roots (reviewed in Sanchez *et al.* 1991; Franssen *et al.* 1992). Nodulins and their genes have been categorized according to when (early or late) in development they are expressed. Late nodulin gene (NOD) products are generally involved with nodule function and maintenance and include leghemoglobin, an abundant oxygen transporter; glutamine synthetase, an ammonia assimilatory enzyme; and proteins involved with peribacteroid membrane function/formation, as well as proteins of unknown functions. Early nodulins (ENODs) have been isolated only as *in vitro* translation products and cDNAs and include genes associated with nodule organogenesis and with the nodule invasion process.

In alfalfa, nodule organ formation can also be elicited by rhizobia defective in infection properties (Finan *et al.* 1985; Leigh *et al.* 1985; Dylan *et al.* 1986); by auxin transport inhibitors (Hirsch *et al.* 1989, van de Wiel *et al.* 1991); by bacteria overexpressing cytokinins (Long and Cooper 1988); by flavonoids (Hirsch *et al.* 1991); spontaneously at a low frequency (Truchet *et al.* 1989; Caetano-Anolles and Gresshoff 1992); by *Agrobacterium tumefaciens* strains harboring *Rhizobium* nod genes (Truchet *et al.* 1985); and by the NodRmIV(S) signal itself (Truchet *et al.* 1991). The resulting structures, called pseudonodules or empty nodules, are arrested at an early stage of nodule development. Expression of two nodulin genes, and the absence of expression of other nodulin genes, have been used as diagnostic criteria to identify empty nodules: ENOD2 and Nms-30, known only as an *in vitro* translation product (Dickstein *et al.* 1988; Norris *et al.* 1988; van de Wiel *et al.* 1991). ENOD2 encodes a proline-rich polypeptide that is expressed in the nodule parenchyma and is predicted to be localized to the plant cell wall (Franssen *et al.* 1987; van de Wiel *et al.* 1990; Govers *et al.* 1990; Allen *et al.* 1991). Recently, several new cDNA fragments were found to be expressed in empty nodules (Dickstein *et al.* 1991). These cDNAs are candidates for new

early nodulin genes. The goal of the work reported here was to characterize in detail one of these cDNAs, ENOD8.

RESULTS

Isolation and sequence analysis of ENOD8 cDNA.

An alfalfa nodule cDNA library, enriched for nodule-specific cDNAs (Dunn *et al.* 1988) and made in pBR322, was screened by testing individual putative cDNA inserts as hybridization probes against Northern blots containing root, empty nodule, and wild-type nodule RNA. Several cDNAs were found that hybridized to empty as well as wild-type nodule RNA, while not hybridizing to root RNA. One of these was cDNA 11A (Dickstein *et al.* 1991), which was subsequently

found to cross-hybridize to a second small cDNA from this same cDNA library under moderate stringency conditions. The second cDNA clone also hybridized to nodule and empty-nodule RNA and not to root RNA. Both small cDNAs had similar hybridization patterns when used as probes against genomic Southern blots of the closely related species *Medicago truncatula* genomic DNA (not shown).

Both cDNAs were used to screen a second alfalfa cDNA library, constructed in λ ZAPII (Stratagene), generously provided as an amplified library by K. Dunn. An average of two in 10,000 plaques gave positive hybridization signals when probed with either of the two small cDNAs. Several clones were selected from this, plaque purified, and converted to plasmid form. One, now called ENOD8, was selected for extensive further analysis because it was found to have an almost full-length insert.

To ascertain that ENOD8 was of plant, and not bacterial, origin, genomic DNA was isolated from alfalfa, *M. truncatula*, and *R. meliloti*. The DNA was cut with restriction endonuclease *Eco*R1 and analyzed by Southern blot, using ENOD8 cDNA as a probe. The results (not shown) demonstrate that ENOD8 is a plant gene, present in multiple copies in two species of *Medicago*.

The ENOD8 cDNA insert is 1.32 kbp in size; whereas the original study found that the hybridizing mRNA was 1.4 kb (Dickstein *et al.* 1991). Our recent results show that the mRNA is slightly larger: 1.45 kb (not shown). We compared the size of ENOD8 mRNA to that of ENOD2, a control in many of our experiments. ENOD2 mRNA has been previously estimated at 1.3 kb by one of us (R.D.), as well as by other investigators, with a larger cross-hybridizing species at 2.1 kb (Dickstein *et al.* 1988, 1991). In the present study, the ENOD2 mRNA that is 2.0–2.1 kb was the only ENOD2 hybridizing mRNA species that could be detected by Northern hybridization analysis using the A2ENOD2 alfalfa cDNA (Dickstein *et al.* 1988) as a probe.

The ENOD8 cDNA was subcloned using restriction enzymes and sequenced by dideoxy sequencing (Sanger *et al.* 1977). The sequencing strategy and the sequence are shown in Figure 1. The cDNA was found to encode one large open reading frame of 381 amino acids, having a hypothetical molecular weight of 41,797 Da with an isoelectric point of 8.4. There are two potential ATG start sites for translation initiation in the first 45 nucleotides of the cDNA. The first one, shown as the start site in Figure 2, is in a context that compares well with the plant consensus translation initiation sequence of AACAAATGGC (Lutcke *et al.* 1987). At the 3' end of the putative polypeptide coding region, there are two in-frame translation stop codons. A hydropathy plot of the ENOD8 amino acid sequence revealed a hydrophobic N-terminal region (data not shown). Using the predictions of Von Heijne (1983), a potential signal peptide can be found in the ENOD8 polypeptide, which can be cleaved after the alanine residue at amino acid 28 (arrow in Fig. 1B). Additionally, there are five potential N-glycosylation sites present in the ENOD8 predicted polypeptide. These are starred in Figure 1B.

A homology search using the BLASTP algorithm (Altschul *et al.* 1990) revealed significant similarity of ENOD8's predicted gene product with two proline-rich proteins, the APG protein of *Arabidopsis thaliana* (PIR accession number S21961) and the homologous APG protein of *Brassica napus*

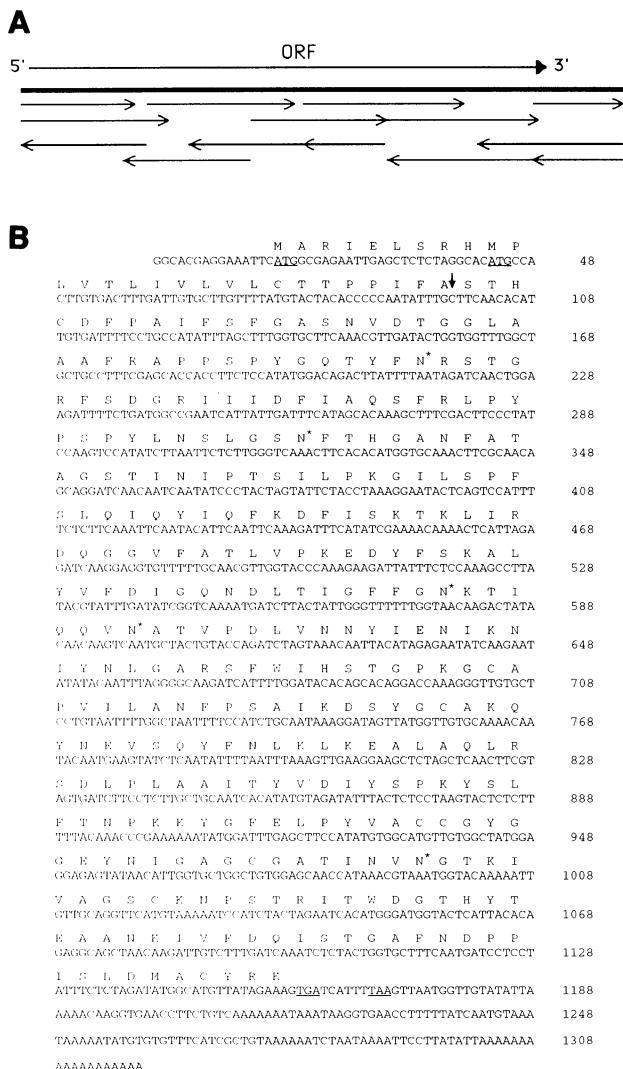


Fig. 1. Sequence of ENOD8 cDNA. A, Schematic showing the position of the putative ENOD8 polypeptide open reading frame (ORF), the ENOD8 cDNA (thick line), and the position and direction of the sequencing reactions (directional thin lines underneath the thick line). **B,** ENOD8 cDNA and deduced putative polypeptide sequences. Asterisks indicate the asparagine residues that are potential N-glycosylation sites. Arrow points to the position of the potential signal sequence cleavage site. The two translational start codons at the N-terminal end and the two translational stop codons at the C-terminal end of the ENOD8 amino acid sequence are underlined.

(PIR accession number S16748). Both APG genes are specifically expressed in anthers (Roberts *et al.* 1993); the acronym APG has not been defined. For both matches, the BLASTP score was 98, with a smallest Poisson probability of 4.6×10^{-6} . Six regions of ENOD8 were scored as having a high (52–75%) similarity to the *A. thaliana* APG polypeptide: ENOD8 amino acid residues 35–48, 66–84, 177–188, 212–232, 248–308, and 336–358 (Fig. 2). In the *A. thaliana* APG polypeptide, only one third of the protein is proline rich: the proline-rich region extends from amino acid 45 through amino acid 205. When ENOD8 polypeptide and the APG protein of *A. thaliana* were compared using the ALIGN program (Pearson 1990), we found that the *A. thaliana* APG polypeptide (534 amino acids long) shares 20.5% sequence identity and 48.5% similarity overall with the ENOD8 polypeptide. The similarity between the two coding regions begins at amino acid 205 of the APG polypeptide and amino acid 35 of the ENOD8 putative protein and extends to the carboxy terminal of both polypeptides (Fig. 2). Similar results were found when the *B. napus* APG protein was aligned with ENOD8, with the region of similarity between the two polypeptides beginning at the carboxy terminal side of the *B. napus* APG proline-rich domain and extending to the carboxy terminus of both putative polypeptides.

Tissue-specific expression of ENOD8.

We extracted RNA from alfalfa nodules induced by wild-type *R. meliloti* and by exopolysaccharide-deficient (*exoA*⁻) *R. meliloti*. The RNA was subjected to Northern blot analysis using ENOD8 as a probe. As can be seen in Figure 3, ENOD8 is expressed in the empty nodules elicited by *exoA*⁻ *R. meliloti* as well as in wild-type alfalfa nodules. This was expected, as

the small cDNA 11A hybridizes to an mRNA found in empty nodules or wild-type nodules but not in roots (Dickstein *et al.* 1991). We also determined that an ENOD8 homologue was expressed in wild-type nodules but not in roots in *M. truncatula* (not shown).

To find out whether ENOD8 is expressed in other tissues of the plant, we extracted RNA from leaves, flowers, and seed-pods of *M. truncatula* and analyzed it by Northern blotting and hybridization. As can be seen in Figure 4, we were unable to detect ENOD8 expression in any tissue of the plant besides nodules. The control, a probe for large subunit ribosomal RNA, demonstrates that intact RNA was present in each of the lanes.

Temporal course of ENOD8 expression.

To determine whether ENOD8 was expressed early in nodule development, nodules were harvested from the time of nodule emergence at 5 days postinoculation, at 7 days and 9 days postinoculation, and at maturity (4 wk). Total RNA was isolated from the nodules and subjected to Northern blot analysis. We found that ENOD8 expression was barely detectable at 5 days postinoculation, and clearly visible at 7 days, 9 days, and 4 wk postinoculation. When the same blot

APG2	MKRSLVDSVCSYRIFRSIFCLLSFCIFLTTTNAQVMHRLWPLWPRYPQPMNP	60
ENOD8	MARIEL-----SRHMP-----LVTLLVLVL-----	20
APG2	PTFDFSPKPVAPFGSPKPVAPFGSPCPSPKPKPPAPSPSPSPKPKPV	120
ENOD8	-----CTTPPI-----F	27
APG2	PPPACPTPPKPKKPAKPPAPKPAKPPAPKPPVPCSPKPPAPTPKPVPHGPPKPA	180
ENOD8	ASTHC-----	32
APG2	APTAPSPKPAKPPKPKPKTIPAVFFFGDSVFDGNNNLETKIKSNYRPGMDKFRV	240
ENOD8	-----DFPATEFGASNVVGG-----LAAAFRAPSPYQTY-FNR	68
APG2	ATGRFSNGMVASDYLAKYMGVKEIVPAIYDLPKIQPNLLTVGSFASQAGYNPTTSEAN	300
ENOD8	STGRFSDGRIITIDFLAQSRPLP-YPSPLANS--LGSNFTGAFATAGSTINIPSTILPK	125
APG2	AI--PMLDQTY--FDYIEKVNRLVRQHSQYKLAGLEKTNQLSKGVAIVVGGNDLI	356
ENOD8	GILSPFSLQIQYIQKDFISKI-KLIRDQGVF--ATLVKEDYFSKALYVFDIGQNDLT	182
APG2	ITYFGSGAQRKNDISYTTIADSAASFVLQLYGYGARRIGVIGTPPLGCVF-----S	410
ENOD8	IGFFGN--KTIQVNVATVPDLVNNYIENIKNTYHLCARFVHSTGPKGCAEVILANFP	239
APG2	QRLKKKKICNEELNYASQFLNSKLLILGLQSKTLNPNSTFVYMDIYTIISQMLETPAAYG	470
ENOD8	SAIKDSYGCARQYNEVSOYFNILKEALQLESDEPLAATVVDIVSPKYSLFTNPKYK	299
APG2	FEETKKPCK--TGLLSAGALC---KKSTKI---CPTSSYLFWDGVHPTQRAYKTI-	520
ENOD8	FELPYVACCGGGEYNIGAGGATINVTGKIVAGSKNPNSTRITWDGTHYTEANKILVF	359
APG2	NKVLKEY-----LHV--LSK	534
ENOD8	DQISTGAFNDPPISLDMACYRK	381

Fig. 2. Similarity between *A. thaliana* APG and *Medicago sativa* ENOD8 polypeptides. The sequence of amino acids (aa) of APG (534 aa long, top) was aligned with the ENOD8 sequence (381 aa, bottom) as described in the text. Identical aa are indicated with a line and conserved exchanges by colons. Gaps, indicated by a dash, have been introduced to maximize the alignment. The six regions of high similarity that do not contain introduced gaps for alignment are underlined in the ENOD8 sequence.

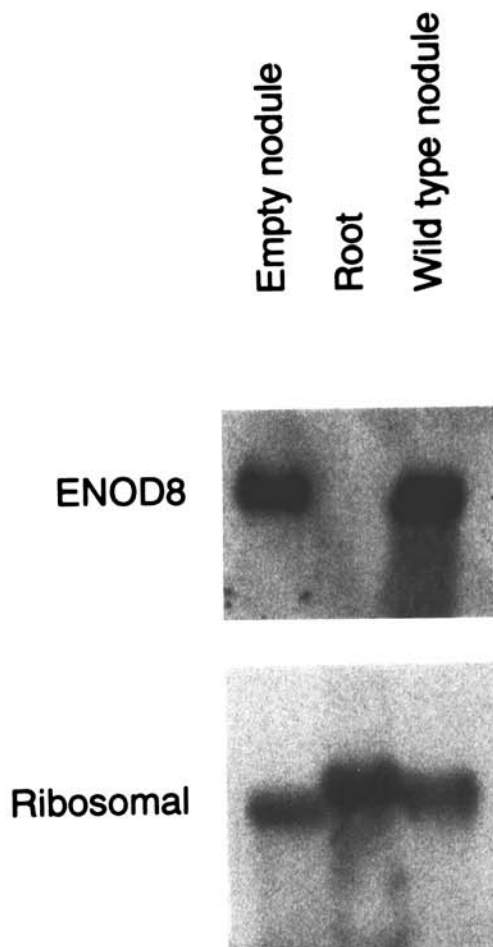


Fig. 3. Northern blot analysis of RNA (10 µg total) from empty alfalfa nodules elicited by exopolysaccharide-deficient *R. meliloti*, uninoculated roots, or wild-type nodules induced by *R. meliloti* strain 1021. Electrophoresis on formaldehyde agarose gels; ENOD8 and ribosomal cDNA used as sequential probes.

was reprobed with ENOD2, we found a similar pattern of expression, although the ENOD2 signal is clearly much stronger (Fig. 5). Not shown is the control, in which the same blot was probed with leghemoglobin cDNA, which controls for induction of late nodulin genes. We found that leghemoglobin mRNA was present in the 7-day postinoculation nodule RNA, as well as in the 9-day and 4-wk postinoculation nodule samples. In a typical Northern blot analysis with cDNA probes of the same approximate specific activity (1×10^9 cpm/ μ g) and using identical hybridization and washing conditions, we were able to just detect ENOD8 mRNA from mature nodules in 1–2 days of autoradiographic exposure, whereas it took only several hours of exposure to fully detect the ENOD2 and leghemoglobin mRNA. When autoradiographic exposure times were lowered for the ENOD2 hybridization, the intensities of the ENOD8 and ENOD2 hybridization signals were comparable during the temporal course of nodule development (data not shown). These experiments were performed on alfalfa grown in growth pouches. Similar results were obtained when alfalfa was grown on 1.2% agar in tubes (data not shown). We also examined root segment tissue earlier in nodule development than at nodule emergence. For this experiment, root tissue from the 1-cm regions flanking the location of the root tip at the time of inoculation of the roots with wild-type *R. meliloti* was isolated at 1, 2, 3, and 4 days after inoculation with *R. meliloti*. RNA was isolated from root segment tissue and subjected to Northern blot analysis. Neither ENOD8 nor ENOD2 expression could be detected, only that of the ribosomal RNA control (data not shown).

DISCUSSION

We have isolated and begun to characterize a novel nodule-specific gene from alfalfa, ENOD8. ENOD8 was cloned as an

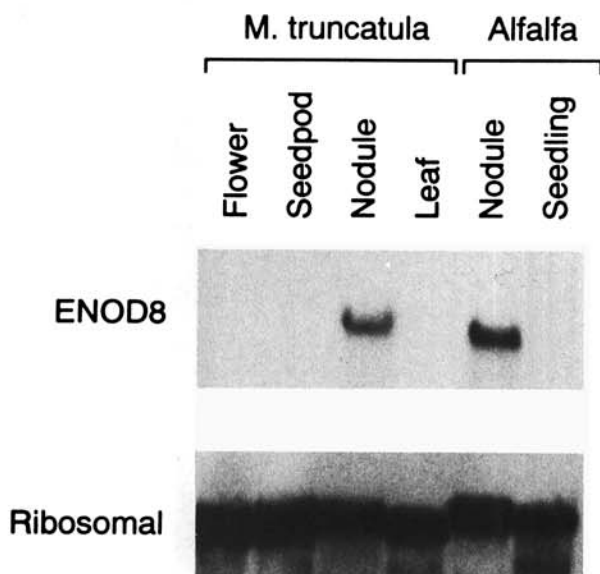


Fig. 4. Tissue distribution of ENOD8 expression. RNA (10 μ g total) from each of the indicated tissues, from either *Medicago truncatula* or alfalfa, were electrophoresed on formaldehyde agarose gels and subjected to Northern blot analysis using ENOD8 and ribosomal cDNA as sequential probes. Expression of ENOD8 was detected only in nodule tissue. The ribosomal control probe demonstrates the presence of approximately equal amounts of RNA in each lane. Note that the *M. truncatula* ENOD8 mRNA is slightly larger than the alfalfa ENOD8 message.

almost full-length cDNA of 1.32 kbp. This compares to the size of the hybridizing mRNA, which was measured at 1.40–1.45 kb. The ENOD8 cDNA was sequenced, and from the sequence information, we are fairly confident that the ENOD8 cDNA represents the entire coding region of the gene. The putative ENOD8 polypeptide begins at an ATG that is an excellent match for the consensus start site for plant protein translation. A potential signal sequence cleavage site is a reasonable distance from the putative N-terminal end of the gene product. This supports the assignment of the translation start site and suggests that the ENOD8 polypeptide is membrane-bound, secreted, or targeted to the vacuole. Additionally, we found five potential sites where N-linked glycosylation could

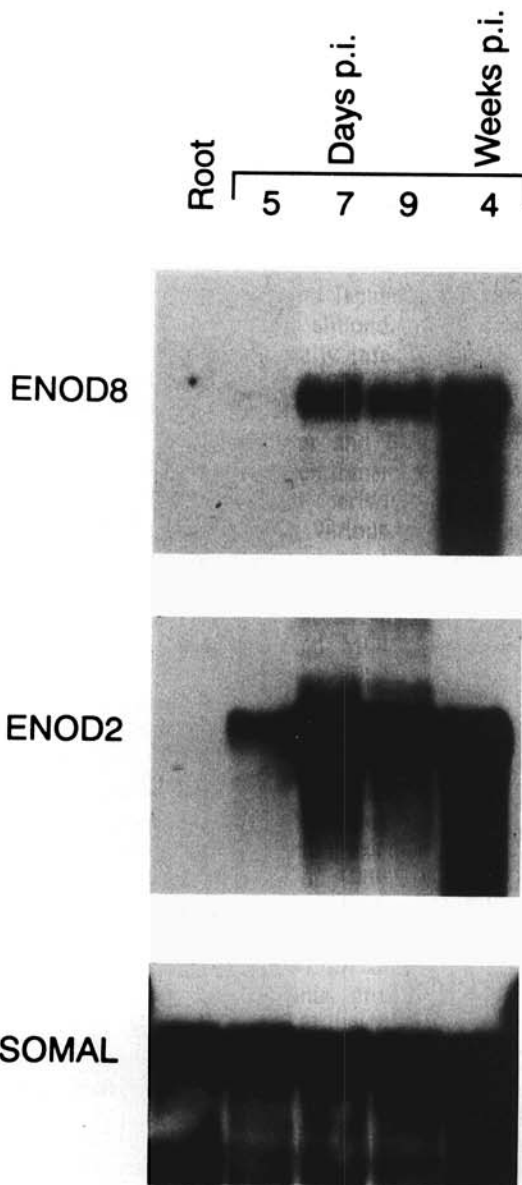


Fig. 5. ENOD8 expression early in alfalfa nodule development. RNA (10 μ g total) from nodules harvested at the indicated times postinoculation (p.i.) from wild-type *R. meliloti* strain 1021 were electrophoresed on formaldehyde agarose gels and subjected to Northern blot analysis using ENOD8, ENOD2, and ribosomal cDNAs as sequential probes. Expression of ENOD8 and ENOD2 is first detectable at five days postinoculation.

take place. Unlike many other early nodulin genes sequenced to date, the polypeptide predicted by the ENOD8 gene is not proline rich.

When the predicted ENOD8 protein was compared to genes and proteins previously sequenced, a similarity was found to two homologous APG proteins, the predicted products of the APG genes from *A. thaliana* and *B. napus*. These are a class of anther-specific genes of unknown function. APG transcription has been determined to be confined to the anther during the period of microspore development. The APG gene promoter directs the expression of reporter gene fusions in a number of anther cell types in transformed plants, including both gametophytic and sporophytic cell types (Roberts *et al.* 1993). The APG gene products are proline rich, but the proline-rich domains of both APG proteins are confined to the amino-terminal ends of the polypeptides; the similarity of APG to ENOD8 starts at the end of the proline-rich domains and extends to the carboxy terminus of both the APG and ENOD8 putative proteins, with six high-similarity subregions. Although the activity and functional domains of APG are still unknown and the significance of the similarity between ENOD8 and APG is unclear, it is tempting to speculate that the ENOD8 gene product carries out a function similar to that of the APG gene product, but without the proline-rich domain. The ENOD8 gene may encode part of a multi-subunit protein of which there is also a proline-rich part. Or, perhaps the ENOD8 gene is equivalent to a *Medicago* APG gene that could be differentially spliced to produce either an APG-like transcript in non-nodule tissue or a nodule-specific ENOD8 transcript.

Because of the similarity between ENOD8 and APG, one might expect that ENOD8 would be expressed in anthers of *Medicago*. We tested to find out whether ENOD8 was expressed in *Medicago* flowers and were unable to detect expression. However, our experiments do not rule out the possibility that ENOD8 is expressed in *Medicago* anthers. We extracted RNA from a population of flowers that included mature flowers, with a small proportion of immature buds. Because the anthers at the proper stage of development for APG expression represented a small fraction of this tissue sample, the RNA from this particular developmental stage might be too diluted in our flower RNA sample for detection. Also, this experiment was performed using *M. truncatula* flower tissue and the heterologous *M. sativa* ENOD8 cDNA as a probe. Although a clear homologue of ENOD8 is easily detectable in *M. truncatula* nodules using the alfalfa cDNA probe, it is possible that the ENOD8 genes in the two *Medicago* species are too divergent for a possible ENOD8-like transcript present in *M. truncatula* flower RNA to be detected with the alfalfa probe.

By Northern blot analysis, ENOD8 was found to be first expressed early in nodule development, at the time when nodules first become visible, which is at the fifth day postinoculation with wild-type *R. meliloti* under our experimental conditions. ENOD8 gene expression was not detectable in root segments harvested before nodule emergence from the region surrounding the position of the root tip at the time of *R. meliloti* inoculation. It is still possible that ENOD8 is expressed before visible nodule emergence. The root segment tissue that was harvested from inoculated alfalfa roots contains a high proportion of root tissue to nodule primordia. If ENOD8 is expressed in nodule primordia, it may be too diluted

by root RNA to be detected on Northern blots. For a similar reason, it is difficult to quantitatively estimate the amount of ENOD8 expression at early time points in nodule development. The amount of ENOD8 expression is very low at 5 days postinoculation, but the proportion of nodule tissue present in the tissue sample from which the RNA is extracted is lower than at any other assayed time point. Because young nodules are small, it is difficult to completely dissect the small nodules away from the surrounding root tissue; consequently there is an inversely proportional relationship between nodule age and contamination of root tissue in the nodule tissue harvested. It is useful to compare the expression of ENOD8 to that of another nodulin gene expressed early in nodule development. We found that ENOD8 was expressed concurrently with ENOD2 and estimated that the relative amount of ENOD8 expression early in development as compared to later in development approximated that of ENOD2. However, when we attempted to compare the expression of these "early" nodulins to that of a "late" nodulin, the leghemoglobin gene, we found that leghemoglobin is expressed as early as 7 days postinoculation in alfalfa in our hands, which is different from what others have observed (Allen *et al.* 1992). Thus, our assignment of ENOD8 as an early nodulin gene is based on a single time point difference between expression of early and late nodulin genes, with the ENOD8 signal at the earlier time point being weak but distinguishable. The conclusion that the ENOD8 gene is an early nodulin gene in the temporal sense may have to be modified once the nodule tissue distribution of ENOD8 mRNA is taken into account or an ENOD8 homologue is found and its temporal course of gene expression determined in a legume with determinate nodules. Alfalfa is an outcrossing tetraploid with indeterminate nodules. Both properties complicate the analysis of nodule development. Each alfalfa plant is genetically unique, and alfalfa nodules persistently have a meristem and nodule cells at early stages of development.

ENOD8 is expressed in empty nodules elicited by invasion-deficient *R. meliloti* exopolysaccharide mutants. This was the original basis for our interest in characterizing the ENOD8 gene. Empty nodules have been characterized as being blocked at an early stage of development, and nodule-specific genes expressed in empty nodules are candidates for being early nodulin genes. Genes expressed in empty nodules are likely to be the genes with products and activities that comprise nodule organogenesis. Thus, the function of ENOD8 is likely to be in nodule structure formation. Identification of genes involved in nodule organogenesis is a first step toward understanding the biochemical basis of nodule organ formation as well as mechanisms by which nodule morphogenesis is regulated. Because empty alfalfa nodules can be elicited by a variety of means, including treatment of alfalfa roots with the NodRmIV(S) nodulation signal factor, genes expressed in the empty nodules could be direct or indirect target genes in a developmental pathway set in motion by nodulation signal transduction. However, it remains to be determined how direct the link is between NodRmIV(S) and the expression of ENOD8. The recent finding by Yang *et al.* (1992) demonstrating that empty alfalfa nodules elicited by exopolysaccharide-deficient *R. meliloti* differ in structure from those elicited spontaneously or by the NodRmIV(S) factor suggests that nodule-specific plant genes expressed in nodules induced by

exo⁻ R. meliloti will be a subset of those expressed in nodules elicited by NodRmIV(S) factor. Empty nodules elicited by *exo⁻ R. meliloti* lack a persistent meristem. Thus, it is probable that ENOD8 is not localized to the nodule meristem.

Few of the already-characterized nodulin genes are expressed in empty nodules. Aside from ENOD8, only ENOD2 and Nms-30 have been found to be expressed in empty nodules, and only ENOD2 is cloned. Thus, ENOD8 represents the second cloned and sequenced member of a class of nodulin genes expressed in empty nodules.

MATERIALS AND METHODS

Manipulation of plants.

M. sativa 'Iroquois' seeds were surface sterilized by soaking in sulfuric acid for 10 min, followed by copious rinsing with sterile water and several hours of imbibition. For experiments to obtain empty nodules, imbibed seeds were germinated, placed on Nod media agar or growth pouches watered with Nod media, and inoculated with *R. meliloti* 1021 or *R. meliloti* 7023 (*exoA⁻*) as previously described (Dickstein *et al.* 1988). Nodules were harvested at 4 wk postinoculation, frozen in liquid nitrogen, and stored at -70°C before use. For experiments to determine the temporal course of expression, imbibed seed were placed in the trough of a growth pouch wetted with Nod media. After 2–3 days, when roots were approximately 1 cm long, the plants were inoculated with *R. meliloti* 1021. A mark was placed on the growth pouch to indicate the position of each root tip at the time of inoculation. Root segments were harvested from 1-cm sections flanking the root tip position at daily intervals until 5 days postinoculation, when nodules emerged. From that point on, nodules were harvested. Root segments and nodules were frozen in liquid nitrogen and stored at -70°C .

DNA and RNA isolation.

Leaves from alfalfa or *M. truncatula* 'Jemalong' plants were frozen in liquid nitrogen. DNA was prepared according to the protocol of E. Richards (Ausubel *et al.* 1988). *R. meliloti* DNA was prepared as described by K. Wilson (Ausubel *et al.* 1988). RNA from nodules, roots, and root segments was prepared as described in Dunn *et al.* (1988).

DNA and RNA blot hybridization.

Genomic DNA was cut with *EcoR*I, electrophoresed in 1% agarose gels, and transferred to GeneScreen (Dupont), as described (Sambrook *et al.* 1989). Total RNA was run on 1% agarose gels containing formaldehyde and blotted to GeneScreen, as described (Sambrook *et al.* 1989). The membranes were hybridized to single-stranded cDNA probes radioactively labeled with 32-P using a random priming kit (Boehringer Mannheim, Indianapolis) using standard hybridization protocols (Ausubel *et al.* 1988). High-stringency washing was done at 65°C in $0.2\times$ SSC, 0.1% SDS. The DNA ladder and the RNA ladder (Gibco BRL) were used as molecular weight standards.

DNA sequencing.

cDNA clones were subcloned using restriction sites into the pBluescriptKS (Stratagene, La Jolla, CA) vector. They were sequenced in both directions using the dideoxy Sanger tech-

nique (Sanger *et al.* 1977), with the Sequenase kit (U.S. Biochemical, Cleveland, OH). The data from the sequencing project were handled using the MacMolloy program on a Macintosh computer and the BLAST (Altschul *et al.* 1990) and FASTA (Pearson 1990) programs by electronic mail.

ENOD2, leghemoglobin, and ribosomal cDNA probes.

The probes used were previously described in Dickstein *et al.* (1988) and Dunn *et al.* (1988).

GenBank accession number L18899.

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