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Phaseolus ENOD40 is involved in symbiotic and non-symbiotic organogenetic processes: expression during nodule and lateral root development

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Abstract

ENOD40 is an early nodulin gene, recently isolated from legume species forming nodules either after *Rhizobium* infection or spontaneously. ENOD40 cDNAs from *Phaseolus* plants were isolated and nucleotide sequence determination revealed a 85% and 88.5% homology with the reported soybean cDNA clones. The putative polypeptide deduced coincides with the soybean one but a stop codon, almost in the middle of the respective ORF, renders it much shorter. This polypeptide was overexpressed as a fusion protein in *Escherichia coli*. Although the spatial expression pattern of the gene in the root pericycle and nodule primordium at early stages of development as well as in the pericycle of the vascular bundles and uninfected cells in mature nodules is comparable to the gene's expression pattern in soybean, differences in developmental regulation are evident. We have shown that ENOD40 transcripts are also detected at very early stages of lateral root development, in the dividing pericycle cells of the root stele that give rise to the lateral root primordia. The presence of *Rhizobium* causes an enhancement of the gene's expression and also induction of the gene in the vascular tissues of developed lateral roots. Interestingly, a discrimination on the gene's expression level in adventious and acropetal incipient lateral root primordia, emerging in infected and uninfected roots, is observed. This indicates that the gene's product may be involved in the hormonal status of the plant and that ENOD40 may be used as a molecular marker in lateral root initiation.

Introduction

Soil bacteria of the genera *Rhizobium* and *Bradyrhizobium* induce in the roots of leguminous plants the formation of highly organized nodules. In determinant nodules of the 'tropical' legumes, cell divisions in the outer root cortex lead to the formation of the nodule primordium [5, 24]. While the globular primordium enlarges by cell division, the infection thread, filled with proliferating bacteria, ramifies into the central part of the primordium and begins to release rhizobia into the plant

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X86441.

cells. This part of the nodule primordium is the first where cell divisions cease, giving rise to a spherical meristem which will persist for a few more days. The longest time of meristematic activity is observed in the apical periphery of the central tissue but finally it also comes to an end and a further increase in size is caused by cell expansion. Meanwhile, divisions in the pericycle of the root stele and in the cells of the inner root cortex give rise to a vascular bundle, connecting the root stele with the nodule vasculature [2, 5]. The central tissue of the fully developed nodule consists of infected and uninfected cells. A boundary layer of uninfected cells separates the central tissue from the peripheral tissues [10, 23]. The peripheral tissues are divided into an outer and an inner part, the nodule cortex and nodule parenchyma, respectively, separated from each other by the nodule endodermis [36]. The vascular bundles are located in the nodule parenchyma, branching around the central tissue. Around the vascular tissue there is a pericycle of several parenchymatic cell layers and a bundle endodermis.

The plant-bacterium interaction triggers the induction of plant specific genes, which code for early and late nodulins, well before or around the onset of nitrogen fixation, respectively [9, 17, 22, 36]. The early nodulin genes studied so far are proposed either to be involved in the infection process or are expressed in a specific nodule tissue, playing there, most probably, a role in nodule morphogenesis [22, 28, 31].

The isolation of a novel early nodulin gene, named ENOD40, has been reported in soybean [13, 39]. Two different cDNA clones were isolated, p*Gm*ENOD40-1 and p*Gm*ENOD40-2 (nomenclature as defined by Matvienko *et al.* [20]) with a homology of 95% on the nucleotide level. The two encoded putative polypeptides are distinguishable from each other by the absence of a methionine in the sequence of the polypeptide deriving from p*Gm*ENOD40-2. ENOD40 is the first nodulin found to be expressed in the dividing cells of the root outer cortex, where mitotic activity is induced after the infection. In early stages of infection, transcripts are also localized in the region of the root pericycle opposite the site of infection and in the nodule primordium. In later stages, the gene is expressed in the pericycle of the vascular bundles and in the unifected cells of the central tissue, especially the ones that form the boundary layer. Recently, the isolation of ENOD40 genes from *Medicago* spp. [1, 7] and pea [20] was reported, showing basically the same pattern of expression. The most striking feature of this 'nodulin' is that the ENOD40 genes, at least in soybean and *Medicago*, have no coding capacity, which led to the hypothesis that ENOD40 codes for an untranslated RNA [7]. Furthermore, expression of the ENOD40 gene has been recently detected [1] in other meristematic tissues of *Medicago*, like the margins of young leaves and lateral root primordium.

We report here the isolation of ENOD40 cDNA clones from *Phaseolus vulgaris*. The expression pattern of the gene was studied in nodule primordia and nodules at different developmental stages. The results enlarge the impression of the peculiarity of this gene, which seems to be as much alike as disimilar in different legumes. Furthermore, we report the involvement of the gene in a non-symbiotic morphogenetic process since transcripts were localized in incipient lateral root primordia of uninfected roots of *Phaseolus*. The expression pattern during lateral root formation is also examined and the differential expression of the gene in acropetally and adventiously emerging lateral roots is reported.

Materials and methods

Growth conditions for plants

Bean plants (*Phaseolus vulgaris* cvs. Tendergreen and White Bean) were cultured in gravel as previously described [3]. The plants were inoculated at the day of sowing (day 0) with *Rhizobium leguminosarum* biovar *phaseolii*.

*Library screening and sequence analyses of pPv*ENOD40 *clones*

A λgt11 cDNA library of poly(A)$^+$ RNA from 21-day-old root nodules of *P. vulgaris* cv. Tender-

green was generously provided by Dr B.G. Forde (Rothamsted Experimental Station, UK). Screening of the library with the ^{32}P-labelled insert of p*Gm*ENOD40-2, phage purification and subcloning of the inserts of the positive clones into pBluescript KS$^+$ vector (Stratagene Inc.) were done according to standard methods [27]. The sizes of the inserts of the positive recombinant phage were determined by the PCR technique using the forward and reverse λgt11 primers (Promega). Both strands of the inserts of the p*Pv*ENOD40 cDNA clones and PCR fragments were sequenced using the dideoxynucleotide chain termination method [29]. Data were stored and analysed on a micro VAX/VMS computer using programs written by Staden [33].

Isolation of nucleic acids and blotting

Soybean genomic DNA from *P. vulgaris* cv. Tendergreen was prepared from etiolated hypocotyls according to Rogers and Bendich [26]. Uninfected root segments, stems and leaves were harvested and frozen immediately in liquid nitrogen. Total RNA from these tissues was isolated as described by Kouchi *et al.* [14]. Digested genomic DNA and total RNA denatured in DMSO/glyoxal were electrophoresed on 1% agarose gel by a standard procedure [27] and transferred to GeneScreen Plus filters (New England Nuclear). The blots were hybridized to ^{32}P-labelled insert of p*Pv*ENOD40-a, obtained by random priming, in 6× SSC (1× SSC is 0.15 M NaCl, 0.015 M trisodium citrate pH 7.0), 5× Denhardt's solution, 0.5% SDS and 10 mM Tris.HCl (pH 7.0) at 65 °C for 16 h. The DNA transfer blots were washed for 15 min in 2× SSC/0.1% SDS at room temperature and 4 × 20 min in 1× SSC/0.1% SDS at 65 °C. The RNA transfer blots were washed for 15 min at room temperature in 2× SSC/0.1% SDS and then for 10 min at 60 °C in the same washing buffer.

Genomic PCR

Sequences of synthetic oligonucleotides used as PCR primers are depicted in Fig. 1. The fragment of 381 bp was amplified from genomic DNA (100 ng) using 300 ng of each primer in a stan-

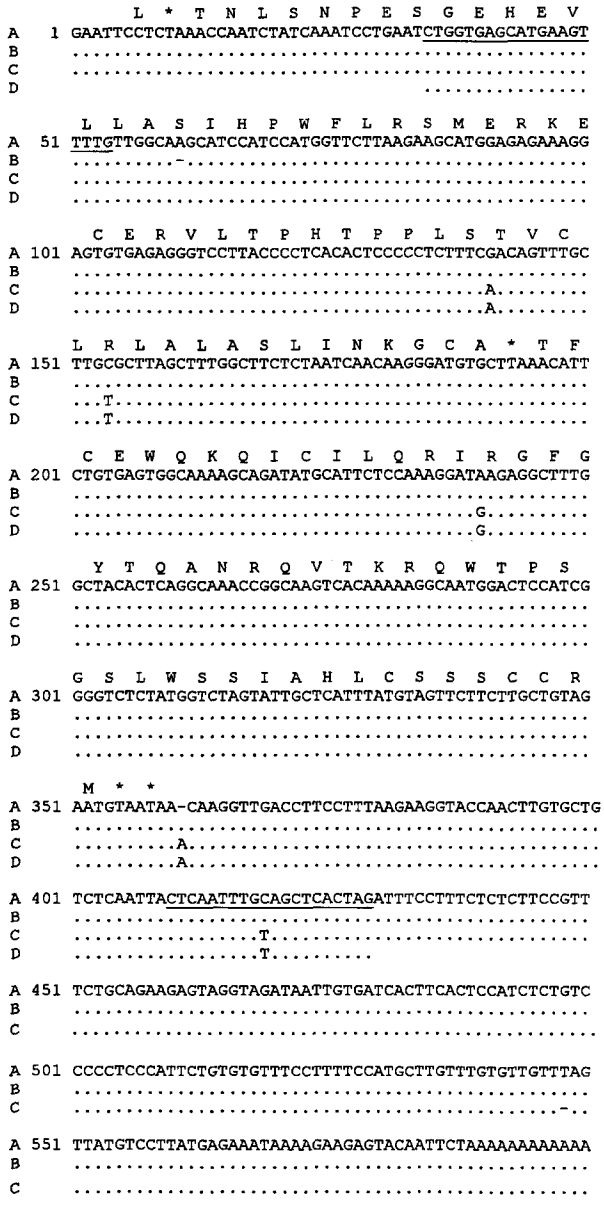

Fig. 1. Nucleotide sequence of p*Pv*ENOD40 cDNA clones. A, p*Pv*ENOD40-a; B, p*Pv*ENOD40-b; C, p*Pv*ENOD40-c; D, genomic PCR-amplified fragments. Nucleotides are numbered to the left of the sequence. Dots represent identical nucleotides, dashes missing nucleotides. The set of primers used in the PCR is underlined. The putative amino acid sequence proposed in the text is depicted in standard single-letter code above the first nucleotide of each triplet. The termination code is indicated with the asterisk.

dard PCR of 30 cycles (1 min at 94 °C, 1 min at 54 °C, 2 min at 72 °C). The amplified fragments were filled in with 4 units of Klenow DNA polymerase in the presence of 0.5 mM dNTPs and were ligated in the *Eco*RV or *Sma*I site of pBluescript KS$^+$ vector.

Expression in E. coli

A PCR product synthesized from the p*Pv*ENOD40-a clone, using by the same set of primers as above, was blunt-ligated into the pBlusecript KS$^+$ vector in the *Sma*I restriction site. This plasmid was digested with *Bam*HI and *Eco*RI and was inserted in pGEX-3X expression vector (Pharmacia) by directed cloning. The putative polypeptide of *Phaseolus* ENOD40 was, thus, in frame with the GST polypeptide. The pGEX-3X plasmid containing the ENOD40 insert, namely pGEX-3X.40, was used for transformation of *E. coli* DH5a cells. As a control the same fragment of p*Pv*ENOD40-a was inserted in the pGEX-3X vector in the inverted orientation (pGEX-3X.40i clone). The sequence and reading frames of clones fragments in both positive and negative orientation were verified by sequencing using the 5′-GST primer (Pharmacia). Growth of transformants and induction with IPTG of the recombinant fusion protein were done according to the manufacturer's (Pharmacia) protocols. The fusion protein was purified by affinity chromatography on immobilized glutathione and the GST carrier was cleaved off the fusion protein by digestion with the site-specific blood coagulation Factor Xa [32]. Antibodies against the fusion protein were obtained from Gentech (Belgium). Five boosts (total protein 3 mg) were performed and antibodies were tested on western blot of fusion and digested with Xa factor protein using the Protoblot kit of Promega.

In situ *hybridization*

Root segments where nodules would appear were cut 5–10 days after infection and 15-, 21- and 35-day-old nodules were picked and were fixed in 4% paraformaldehyde/0.25% glutaraldehyde in 10 mM phosphate buffer at 4 °C overnight. Embedding and sectioning were done as described by van de Wiel *et al.* [36]. Antisense and sense *Pv*ENOD40 RNA was obtained from linearized plasmids by *in vitro* transcription using T3 or T7 RNA polymerase (Promega). The RNA was labelled either with ^{35}S-UTP (Amersham, 1000–1500 Ci/mmol) [30] or with (DIG-11-UTP) according to the Boeringer manual. The probes were partially degraded to an average length of 150 nucleotides. Sections (7–10 μm) were taken and prepared for hybridization according to Scheres *et al.* [30] and hybridized overnight at 42 °C in 50% formamide, 300 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA, 10% dextran sulfate, 60 mM DTT, 500 μg/ml poly(A) RNA and 150 μg/ml yeast tRNA. Sections hybridized to the radioactively labelled probe were coated with Kodak NTB2 nuclear emulsion, exposed for 2 weeks and stained with toluedine blue O. Sections hybridized to the non-radioactive probe were block-stained with 0.5% safranin and the signal detection procedure was performed as described by Kouchi and Hata [13] and De Block and Debrouwer [8].

Results

*Isolation of p*Pv*ENOD40 clones and sequence analysis*

Phaseolus vulgaris ENOD40 clones were isolated from a λgt11 cDNA library prepared from nodules of plants 21 days after inoculation with rhizobia. The insert of the heterologous cDNA clone p*Gm*ENOD40-2 from soybean was used as a probe. Positive hybridization signals were obtained, corresponding to three different cDNA clones, namely p*Pv*ENOD40-a, p*Pv*ENOD40-b and p*Pv*ENOD40-c. The length of the clones was 581 bp, not including the poly(A) tail. Sequence determination showed that p*Pv*ENOD40–a and p*Pv*ENOD40-b differ only in an additional

nucleotide at position 61 in p*Pv*ENOD40-a. p*Pv*ENOD40-c exhibits a higher divergence, i.e. four single nucleotide substitutions at positions 141 (G→A), 154 (C→T), 240 (A→G), 419 (C→T), a deletion of one nucleotide at position 548 and an addition of an extra one at position 362, when compared with p*Pv*ENOD40-a (Fig. 1).

Phaseolus genomic DNA, digested with four enzymes, was Southern blotted and hybridized with the insert of p*Pv*ENOD40-a clone (Fig. 2a). Multiple hybridizing bands suggest for a small gene family. The presence of more than one closely related genes or alleles is further supported by the isolation of two different cDNAs (p*Pv*ENOD40-a and p*Pv*ENOD40-c) coding for ENOD40 in *Phaseolus*. Furthermore, the presence of another ENOD40 transcript, especially in younger nodules cannot be excluded, since the above clones were isolated from a 21-day-old nodule library.

The *Phaseolus* ENOD40 clones exhibit a very high sequence identity at the nucleotide level with the reported soybean clones, i.e. 88.5% with p*Gm*ENOD40-1 and 85% with p*Gm*ENOD40-2. The alignment of the *Phaseolus* and soybean cDNA clones showed that p*Pv*ENOD40-a is shorter by 51 bp and 75 bp at its 3′ end compared to p*Gm*ENOD40-1 and p*Gm*ENOD40-2, respectively, while its 5′ end is 28 bp longer than p*Gm*ENOD40-1. Two putative polypeptides have been proposed as being encoded from the two soybean clones. Striking features of these polypeptides are the absence of a methioniine in the one encoded by p*Gm*ENOD40-2 and a similarity of only 79%, although the two cDNA clones share a 96% nucleotide sequence identity between them. The ORF of the *Phaseolus* clone encoding the respective putative polypeptide of ENOD40 is 60 amino acids long and the first ATG resides on the 26th codon (Fig. 1). If this ATG serves as the translation start site, then the polypeptide is only 35 amino acids long. A stop codon present in the respective ORF renders the *Phaseolus* polypeptide much shorter and disrupts the continuing similarity between the *Phaseolus* and the two soybean polypeptides for the following 60

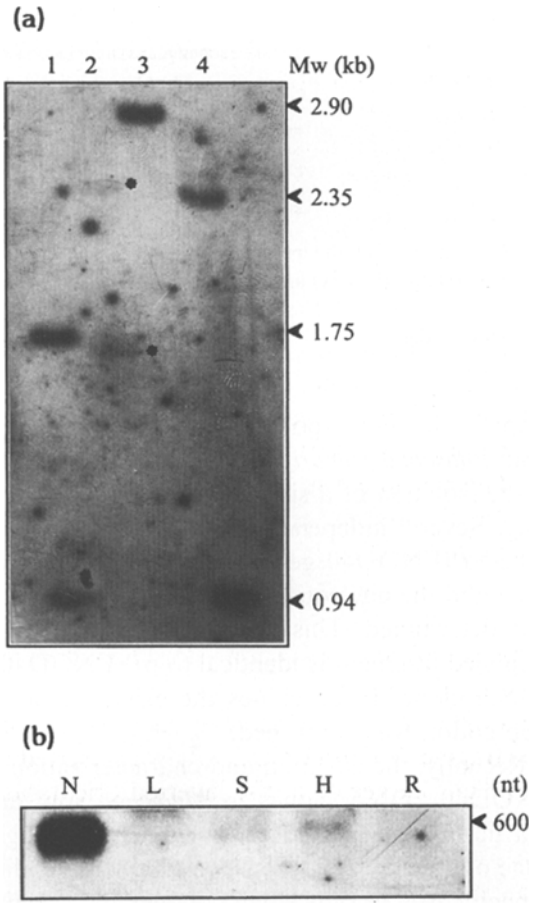

Fig. 2. Southern and Northern analyses of *Phaseolus* ENOD40. (a) Southern blot analysis of total genomic DNA (20 μg) isolated from *Phaseolus* plants digested with *Pst*I (lane 1), *Bam*HI (lane 2), *Hin*dIII (lane 3) and *Eco*RI (lane 4). Asterisks denote the position of the more weakly hybridizing bands. (b) RNA transfer blot of total RNA (10 μg) isolated from uninfected roots (R), hypocotyls (H), stem (S), leaves (L) of *Phaseolus* plants and 21-day-old nodules (N). Both blots were hybridized to the insert of p*Pv*ENOD40-a.

amino acids. Thus, the expected similarity (with the stop codon excluded) of 87% and 62% with the derived polypeptides of p*Gm*ENOD40-1 and p*Gm*ENOD40-2, respectively, changes dramatically (Fig. 3). Nevertheless, the homology in the overlap region of the first 35 amino acids is significant.

In order to exclude the possibility of cloning and sequencing errors in the p*Pv*ENOD40 clones and verify the existence of the stop codon in this particular position, a set of primers was used to

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                                MEIKGCERVLTTHTPPLKTVCFGLALASLINKGCVLTFSL   A
                                || | |||||| ||||| ||| ||||||||||| ||
TNLSNPESGEHEVLLASIHPWFLRSMERKECERVLTPHTPPLSTVCLRLALASLINKGCA*TFC.   B
                                         |||||||||  |||  ||||||||||   |
                                         ERVLTPHTPSKLTVCFGLALASLINKGCVLTFFL   C

EWQKQICILQRRRGFGYSLANRQVTKRQWTPLGSLWLSIAHLCSSSCCRM   A
|||||||||||| |||||  |||||||||||| |||| ||||||||||||
EWQKQICILQRIRGFGYTQANRQVTKRQWTPSGSLWSSIAHLCSSSCCRM   B
|| ||| || | ||           ||||||| ||||   ||
EWRKQIHILRRRRGLATAWQTGKSQKRQWTPLGSLWLCSAHVVLLAVECN   C
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Fig. 3. Alignment of the putative polypeptide of *Phaseolus* ENOD40 (lane B) with the respective polypeptides encoded by p*Gm*ENOD40-1 (lane A) and p*Gm*ENOD40-2 (lane C). Identical amino acids are depicted by vertical lines (|), the termination codons by asterisks (*). The part of the *Phaseolus* polypeptide fused to the GST is in bold and the region conserved in all legumes studied is underlined.

amplify the corresponding genomic fragments from *Phaseolus vulgaris* total DNA. This resulted in the isolation of a single PCR product, 381 bp long. Several independent subclones of the amplified *Pv*ENOD40 genomic fragment were isolated and the nucleotide sequence of five of them was determined. This analysis showed that the amplified fragment is identical to p*Pv*ENOD40-c cDNA clone. In all clones the existence of the stop codon was confirmed.

Recently, the isolation and characterization of ENOD40 cDNA clones in *Medicago* spp. and pea has been reported [1, 7, 20]. Interestingly, none of the above clones has a significant coding capacity; that is, only small polypeptides, lacking a methionine in most cases, can be deduced from the putative reading frames. Furthermore, the soybean and *Medicago* clones were tested by Crespi *et al.* [7] on a computer analysis program for RNA stability and the results led them to propose for ENOD40 a function as a riboregulator. To elucidate the type of active molecule encoded by the ENOD40 gene, a translational fusion of *Phaseolus* ENOD40 and the *Schistosoma japonicum* glutathione *S*-transferase (GST) in the pGEX expression vector, which allows overex-

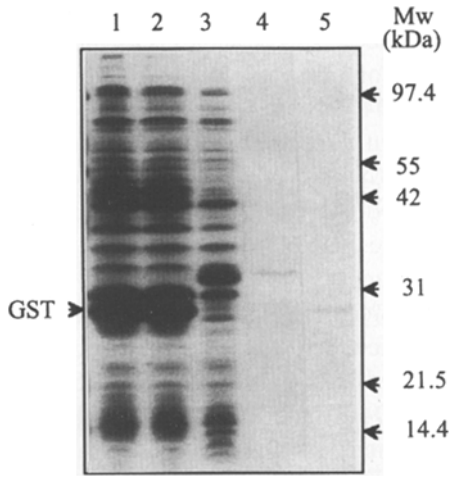
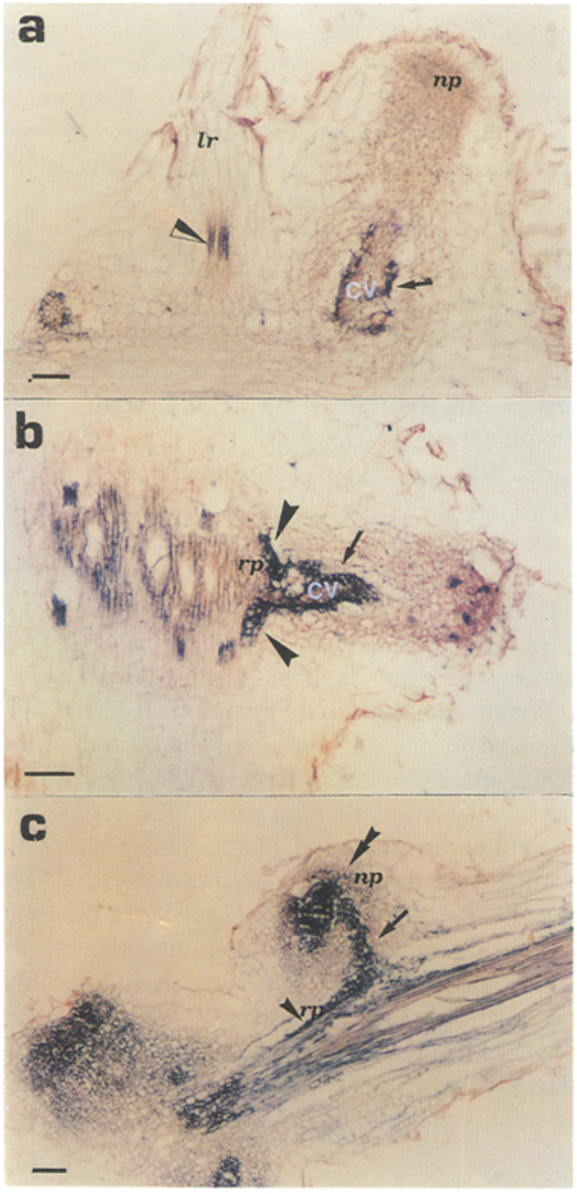


Fig. 4. Expression and purification of ENOD40-GST fusion protein. Total protein of *E. coli* cells transformed with pGEX-3X vector (lane 1), with pGEX-3X.40i (lane 2) and with pGEX-3X.40 (lane 3) after induction with IPTG, were analysed on a 10% SDS-PAGE (10 μg/lane). Purified ENOD40-GST fusion protein (lane 4) was digested with Factor Xa (lane 5).

Fig. 5. In situ hybridization of early stages of nodule development (up to 6 days post inoculation). Sections were hybridized to DIG-11-rUTP-labelled antisense RNA, *in vitro* transcribed from p*Pv*ENOD40-a. Signal is detected as a blue colour precipitate in the pericycle cells of the root stele (arrowheads) and of the developing connecting vascular bundle (arrows) as well as in the nodule primordium (double arrowheads in c). In the fully developed lateral root, signal is detected in the vascular traces (half-darkened arrowhead in a). Bar represents 100 μm in a, c and 50 μm in b. **cv**, connecting vascular bundle; **rp**, root pericycle; **lr**, lateral root; **np**, nodule primordium.

Fig. 6. In situ hybridization of 9/10-day-old nodules. Sections were hybridized to ^{35}S-labelled ENOD40 antisense RNA. Signal is detected in cells of the last stage of differentiation into infected or uninfected cells of the nodule central tissue (arrows) and in the pericycle of the vascular bundle (arrowheads). Bar represents 50 μm in a, b and 100 μm in c, d. a and c are bright-field micrographs, b and d are dark-field micrographs. **ct**, central tissue; **vb**, vascular bundle; **df**, differentiating cells.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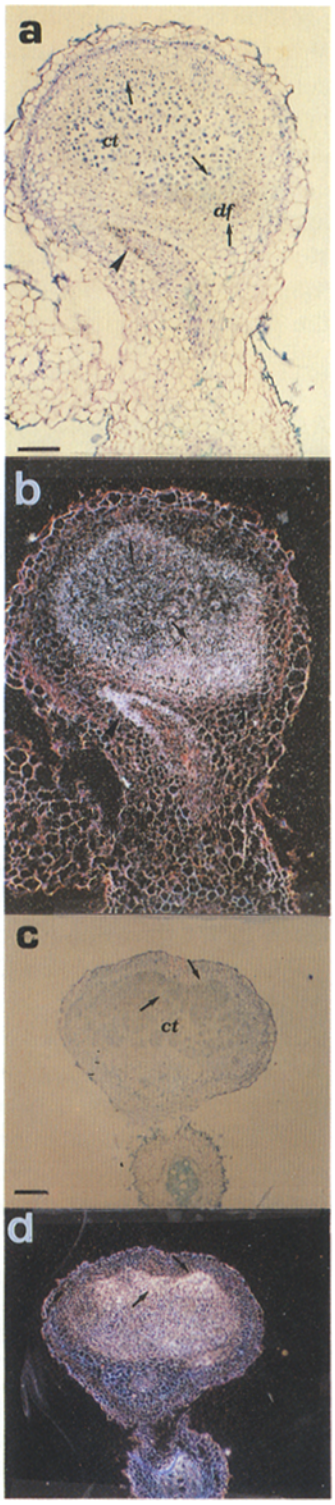
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pression of the fusion product in *E. coli*, was constructed. For cloning purposes the first 7 amino acids were omitted and after induction with IPTG in transformed cells, a polypeptide of ca. 50 amino acids (in bold in Fig. 3) was obtained as a carboxy-terminal extension of GST (Fig. 4). The length of the polypeptide produced shows that the stop codon, present in the respective reading frame of the *Phaseolus* clone, is recognizable by the *E. coli* translation machinery.

RNA analysis and in situ *hybridization experiments in nodules*

The insert of p*Pv*ENOD40-a was used as a probe in transfer blots of total RNA isolated from 21-day-old nodules of *Phaseolus* plants and was found to hybridize strongly to an RNA band of ca. 600 bases. To find out whether ENOD40 gene is expressed in other tissues of the plant, RNA from uninfected roots, hypocotyls, stem and leaves was extracted and subjected to northern blot analysis. As shown in Fig. 2b, the presence of ENOD40 homologous transcripts in roots, hypocotyls and stems of the uninfected plants was determined, although expression is considerably lower than expression in nodules.

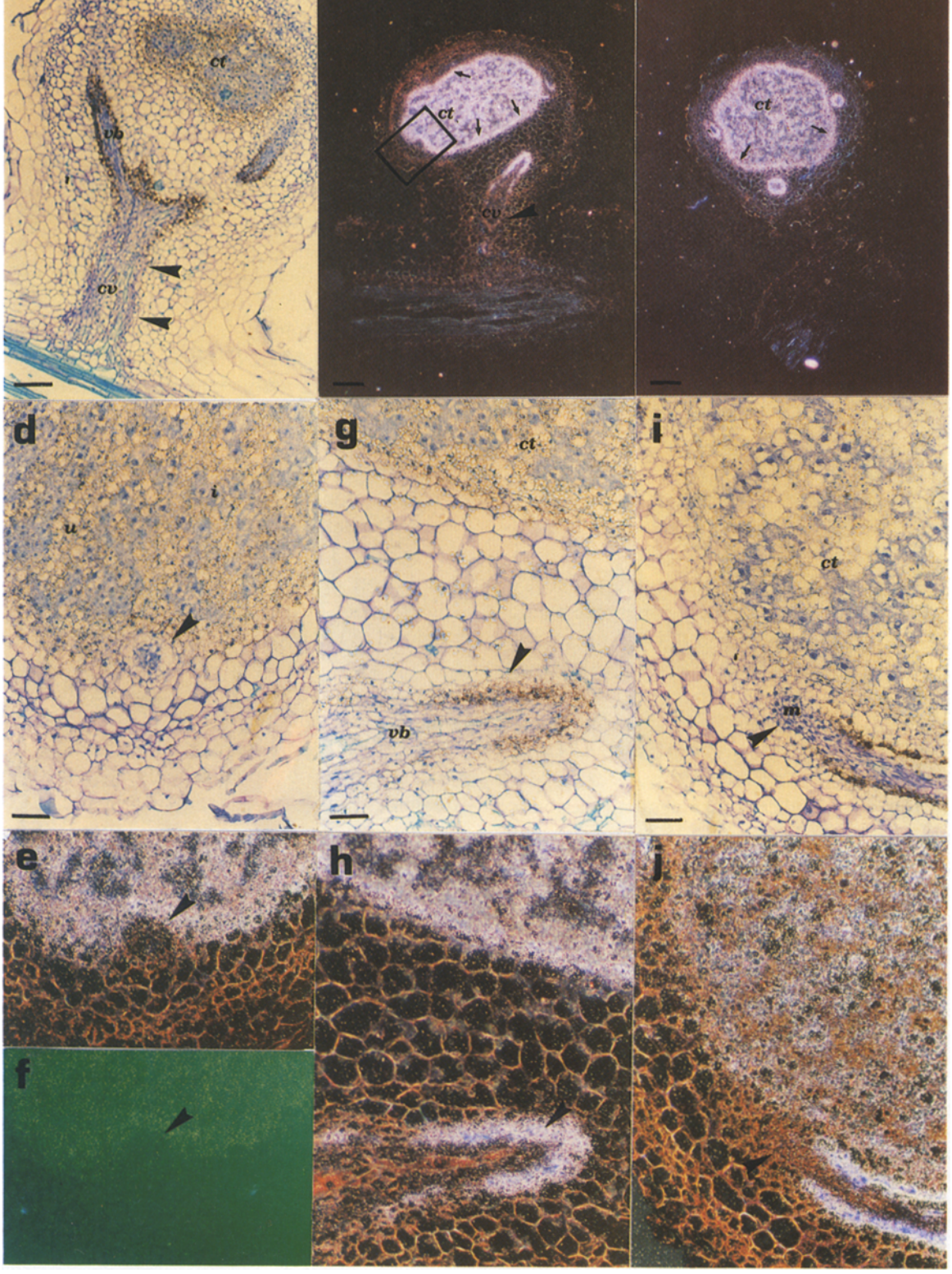
The expression of *Pv*ENOD40 gene in different developmental stages of nodular tissue was further examined by *in situ* hybridization.

Six days after sowing and inoculation nodule primordia are formed, becoming just visible as small bumps on the lateral roots. The nodule primordium consists of cytoplasmic dense cells in the outer cell layers of the root cortex. Provascular strands are initiated by divisions of the root pericycle while cell divisions have also been induced in the inner cell layers of the root cortex, in the central part of which the provascular strand will differentiate into a vascular bundle, connecting the nodule with the root stele. In sections of nodules at this stage of development, the ENOD40 transcripts are localized in dividing cells of the root pericycle and in the forming connecting vascular bundle (Fig. 5a, 5b). Later on, as the nodule primordium enlarges, positive hybridization signal is also detected in all cells of the nodule primordium (Fig. 5c).

In 9–10 days after inoculation meristematic activity ceases, starting in the centre of the primordium, where cells become infected with bacteria released from the infection threads. Meristematic activity persists for the longest time in the periphery of the central tissue of the nodule. The ENOD40 gene seems to be expressed in exactly those cells that either exhibit meristematic activity or are in the last stage of the differentiation process. In addition, signal is still detected in the cell layer surrounding the vascular bundle but not in the pericycle of the root stele (Fig. 6).

In mature nodules of *Phaseolus* plants 15 days and onward after inoculation, the central region is composed of infected and uninfected cells, surrounded by the peripheral tissues of nodule parenchyma and outer cortex. Fully formed vascular bundles are branching around the nodule and the connecting vascular bundle links the nodule with the central cylinder of the root. ENOD40 mRNAs were *in situ* detected only in the pericycle of the vascular bundle surrounding the central tissue and not in the connecting vascular bundle (Fig. 7a, 7b). It seems that the gene is induced only at a specific stage of the vascular bundle development since no signal is detected in meristematic, procambial cells (Fig. 7i, 7j). Also, the

Fig. 7. In situ hybridization of 15- and 21-day-old nodules. Sections were hybridized to ^{35}S-labelled ENOD40 antisense RNA. Signal is detected in the vascular bundle of the nodule and in uninfected cells of the central tissue. The connecting vascular bundle show no hybridization signal (arrowheads in a, b) while there are traces that do not hybridize at all (box in b; d–f, which are details of b). Only the pericycle cells of the vascular bundle hybridize (arrowheads in g, h, which are details of b) while the meristematic cells show no signal (arrowheads in i, j). Signal in the central tissue appears more intense in the boundary layer (arrows in b, c). Bar represents 100 μm in b, c, 50 μm in a and 25 μm in d–j. b, c, e, h, j are dark-field micrographs; a, d, g, i are bright-field micrographs; f is an epipolarization micrograph. **ct**, central tissue; **vb**, vascular bundle; **m**, meristematic cells; **cv**, connecting vascular bundle; **u**, uninfected cells; **i**, infected cells.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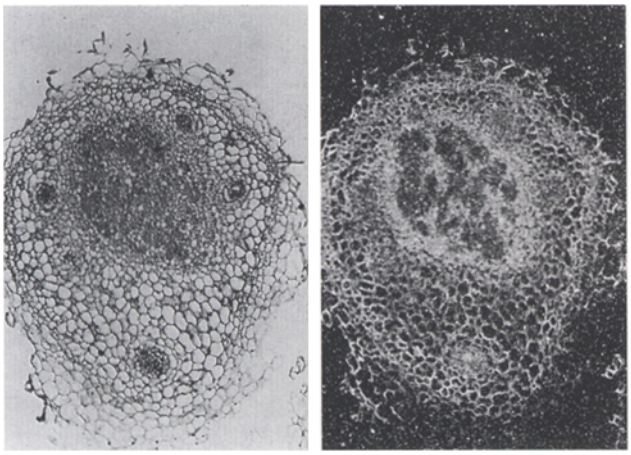


Fig. 8. In situ hybridization of a 15-day-old nodule. Sections were hybridized with ^{35}S-labelled ENOD40 sense RNA. No signal is detected above background level.

gene is switched off in several traces of vascular bundles which do not hybridize at all (Fig. 7b, 7d–f).

The gene is expressed in the uninfected cells of the central tissue and the ones forming the boundary layer. It should be noted that the intensity of the hybridizing signal in the central tissue, with the exception of the cells of the boundary layer, seems to drop in nodules 21-day-old compared to the signal in 15-day-old nodules (compare Fig. 7b and 7c), although all sections were exposed to photographic emulsion for identical period of time. The signal in nodules 35-day-old is completely lost in the central tissue while a signal, though reduced is still visible in the vascular bundle of the nodule (data not shown).

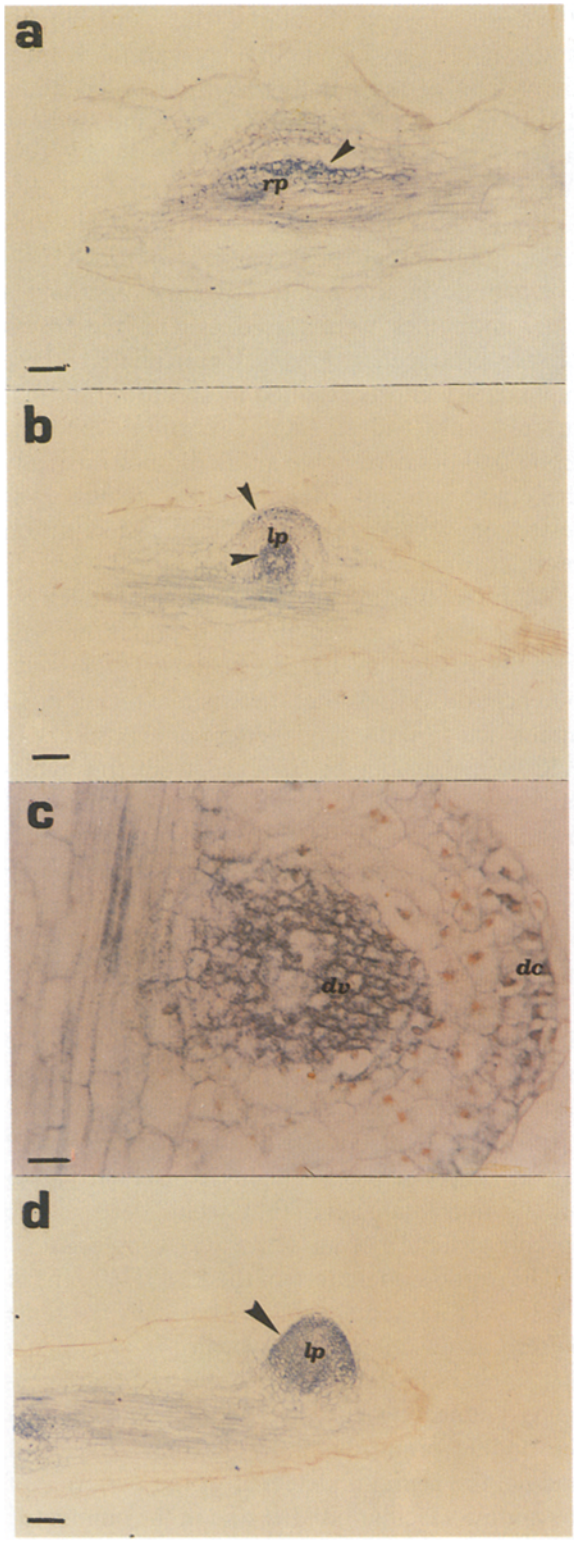
Control nodule sections were hybridized with sense ^{35}S-labelled *Pv*ENOD40 RNA but in any case no signal was detected above background levels (Fig. 8).

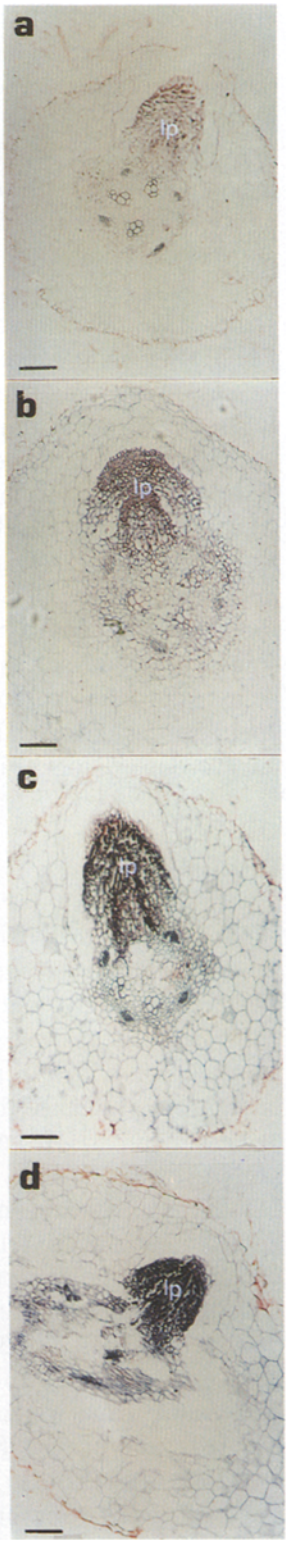
In situ *detection of ENOD40 transcripts in lateral roots*

The detection of ENOD40 transcripts in northern blot analysis of root RNA, prompted us to study ENOD40 gene expression in root development. Since it has been proposed, based on the formation of lateral root-like nodules on *Parasponia* [15, 16, 19], that nodules and lateral roots might share similar compounds and mechanisms involved in their development, expression of ENOD40 gene was studied in lateral root primordia and later stages of development. Sections of uninfected roots of *Phaseolus* containing lateral roots emerging acropetally or later emerging adventitious primordia, developing on older roots, were hybridized with antisense RNA produced by the *Phaseolus* ENOD40 cDNA. Interestingly, in the acropetal lateral roots no signal was detected in any of the developmental stages studied (Fig. 10a). On the contrary, on adventitious lateral roots the gene is strongly expressed in early lateral root development and the signal was localized in the pericycle cells, dividing to give rise to the lateral root primordium (Fig. 9a). Expression is continued in later stages, as the primordium penetrates the cortex of the parent root, where transcripts were localized mainly in the developing root cap and the developing vascular cylinder of the lateral root (Fig. 9b and 9c). At a slightly advanced stage of lateral root emergence, signal is spread all over the developing tissues (Fig. 9d), while no signal is detected in developed lateral roots (>2 mm long above parent root surface). The expression pattern of the gene, mainly in adventitious lateral roots and at specific developmental stages, could also explain the very reduced hybridization signal detected on the north-

Fig. 9. Detection of ENOD40 in uninfected root tissues. Signal is detected in the dividing pericycle cells of root stele (arrowheads in a), in the developing root cap and vascular cylinder of the lateral root (arrowheads in b and c, which is a detail of b), and on the whole lateral root at later stages of development (arrowhead in d). Bar represents 100 μm in a, b, d and 10 μm in c. **rp**, root pericycle; **lp**, lateral root primordium; **dv**, developing root cap; **dc**, developing vascular cylinder.

Fig. 10. In situ hybridization of acropetal (a, b) and adventitious (c, d) lateral roots, emerging on uninfected (a, c) and infected (b, d) roots of *Phaseolus*. Expression is significantly increased in adventitiously emerging lateral roots when compared to acropetal ones. Signal level is enhanced in infected roots, especially in the case of acropetal lateral roots. Bar represents 50 μm. **lp**, lateral root primordium.


a
rp
b
lp
c
dv
dc
d
lp


a
lp
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ern blot of total root RNA. Obviously, the dilution of ENOD40 transcripts is to such an extend that renders the expression of ENOD40 gene in roots barely visible.

In order to test whether the presence of rhizobia influence the expression of the ENOD40 gene in lateral root primordia, sections as described above were taken from infected *Phaseolus* roots. Although it was not possible to obtain exactly the same stage of lateral root development, the ENOD40 gene is expressed in the differentiating tissues of emerging lateral roots (Fig. 10b, 10d). It should be noted that signal in infected roots appeared in an enhanced level, the phenomenon being more evident in acropetal lateral roots, where signal was clearly above the background level (Fig. 10b). Nevertheless, signal intensity never reached the one observed in adventitious roots (Fig. 10, compare b with c, d). In later stages, in contrast with results in uninfected plants, on a fully developed lateral root signal could be detected on the vascular traces of the lateral root cylinder (Fig. 5a).

Discussion

We report here the characterization of ENOD40 in *Phaseolus*. Our results provide evidence that in *Phaseolus* this early 'nodulin' gene has an unclear coding capacity as well as an exceptional expression pattern, which in this case, expands to the lateral root system of the plant.

The comparison of *Phaseolus* ENOD40 cDNAs with the so far reported ENOD40 cDNAs from other leguminous plants adds to the general notion of ENOD40 as a gene with a very conserved homology between different species, which, nevertheless is restricted only to the nucleotide level [7, 13, 20, 39]. On the contrary, the proposed polypeptide for soybean [13, 39], the first legume from which the ENOD40 gene was isolated, can not be found in pea, alfalfa or *Phaseolus*, the reason in each case being different, i.e. a 75 bp insertion in *Medicago* [7] or a stop codon in the middle of the polypeptide in *Phaseolus*. However, the first 35 amino acids of the *Phaseolus* polypeptide starts with a methionine and exhibits very high homology with the respective region of the putative soybean polypeptide. This common part of the *Phaseolus* and soybean polypeptides was overexpressed in *E. coli* as a fusion protein with GST. Certainly, this approach in itself does not enable us to draw conclusions about the actual presence of the ENOD40 polypeptide in the plant. Towards obtaining a clue, antibodies were raised against the fusion protein produced in *E. coli*. Unfortunately, these prelimenary efforts resulted in an antiserum that reacted only with the GST portion while the ENOD40 putative polypeptide did not raise any detectable amount of antibodies, as this was tested on western blots of the digested fusion product (data not shown).

Additionaly, an alignment of the different polypeptides proposed in the previous reports, together with the herein proposed *Phaseolus* polypeptide, reveals that there is one region of 10 amino acids (in the single-letter code: ANRQVTKRQW), that can be deduced from the nucleotide sequence of all plant species studied until now (underlined in Fig. 3). These amino acids reside towards the carboxy terminus of the soybean polypeptide and in *Phaseolus* on the second half part of the deduced polypeptide, downstream of the stop codon that dissects the respective ORF. Whether this conserved region represents the most necessary, for its function, part of the putative polypeptide is a hypothesis that needs further experimental approaches. Furthermore, it could be argued that the conserved amino acid region simply depicts a highly conserved region on the nucleotide level that would lead to the specific structure of an active RNA molecule.

The expression pattern of the ENOD40 gene in *Phaseolus* root nodules follows basically the same pattern as described for soybean [7, 39]. Signal first appears in the root pericycle and the developing connecting vascular bundles and later in the nodule primordium. In the fully developed nodule, the signal is detected in the pericycle of the nodule vascular bundles and in the uninfected cells of the central tissue. It should be pointed out, though, that unlike the pattern observed in

soybean, *Phaseolus* ENOD40 transcripts are localized on the vascular tissue at specific developmental stages and in most cases it could be suggested that the signal moves onward with the developing vascular bundles as these grow and surround the central tissue. Thus, in the fully formed nodule the connecting vascular bundle shows no hybridization signal while ENOD40 transcripts were present at earlier stages of its development. The presence of ENOD40 transcripts in the pericycle of the vascular bundles has led to the hypothesis that the gene product may play a role in transporting metabolites since the cells of the pericycle have a transport cell morphology [7, 39, 25]. On the other hand, in indeterminate nodules the expression of the ENOD40 gene has been reported to be restricted to the prefixation zone II [1, 7, 20]. This zone consists of cells that are still differentiating [38]. We could speculate that the presence of ENOD40 transcripts in specific cells of the pericycle of the vascular tissue is connected rather to the differentiation process of these cells than to the transport role mentioned. Still, a combined hypothesis could be that the ENOD40 gene product transports specific metabolites or factors necessary for cell development and/or growth. Furthermore, it has been reported that the ENOD40 gene expression in the vascular bundles of the nodule occurs only in the presence of *Rhizobium* [39] indicating that the appearance of the gene in this position is controlled by the bacterium. This is in accordance with our observation that only in infected roots did we manage to localize ENOD40 transcripts in the vascular cylinder of the fully-formed adventitious lateral roots, although several lateral roots of the same size in uninfected roots were checked.

Uninfected cells of mature nodules are believed to be fully differentiated. Therefore, a role of the ENOD40 gene product in the differentiation process may not fit in this case, encouraging a dual function of the gene in early and late stages of nodule development, as has been proposed before [39]. The expression of the ENOD40 gene in the uninfected cells of the central tissue is gradually reduced and eventually 35 days after infection is no longer detectable, indicating that the gene product is not necessary throughout the life of the uninfected cells in a nodule.

The expression of ENOD40 gene in the lateral root primordia of uninfected roots is important since it adds proof to the suggestion [1, 7] that ENOD40 is a plant gene involved in non-symbiotic organogenetic processes. The enhanced signal appearing in lateral root primordia on sections of infected with *Rhizobium* roots as compared to uninfected roots, leads to the suggestion that the same gene is regulated differentially by the bacteria in order to work more intensely. The presence of ENOD40 transcripts in roots and nodules reinforces the notion that the *Rhizobium* uses an already existing machinery of the plant's physiology to trigger nodule organogenesis, if and when other parameters in the form of signal compounds of plant origin are present. The location of the transcripts at the same place in both organs, i.e. the dividing pericycle cells of the root stele and primordia, suggests an analogous function in both organogenetic processes. However, the presence of ENOD40 transcripts in spontaneous nodules of *Medicago* provides evidence that infection with rhizobia is not needed for its expression in nodules, although a different mechanism of regulation of the ENOD40 gene in the developmental processes of different organs, namely nodules, spontaneous nodules and lateral roots, cannot be excluded. A similar case with the ENOD40 gene in *Phaseolus* has been reported for soybean nodulin-26, a gene that meets common regulatory requirements, most probably in the form of *trans*-acting regulatory factors, for expression in soybean nodules and in incipient lateral root primordia, when inserted in a different genetic background, the one in transgenic *Lotus corniculatus* plants [21]. In this respect, the isolation of a *Phaseolus* ENOD40 gene and its detailed promoter analysis is of interest, since it would provide evidence for the regulation of the gene's expression by *cis*- and/or *trans*-acting elements.

The organogenetic potential of the ENOD40 gene product was shown in antisense RNA experiments in *Medicago* explants, where callus growth was arrested, while overexpressing

ENOD40 embryos developed into teratomas [7]. Taking into account that these phenotypes can be reproduced by altering the hormonal ratios [7, 12] and that ENOD40 is a cytokinin-responsive gene [11], a substantial role of interfering with the plant's hormonal status has been attributed to the ENOD40 gene product. Our results further support this hypothesis, especially by the fact that mainly the adventitious lateral root primordia showed expression of ENOD40 gene, while in the acropetally emerging primordia, even in infected roots, ENOD40 gene is expressed in a significantly reduced level. The genetic and environmental factors that root development is based upon are not well defined. Likewise, the pattern and control of lateral root initiation is not fully understood yet. It has been suggested, though, that the acropetally emerging lateral roots are generated by cell marking in the parent root meristem, as part of the root developmental process while adventitious lateral root primordia are initiated from unmarked cells, only if the appropriate conditions were met [6, 34]. This could mean that adventitious lateral root initiation is more sensitive and/or depended on a hormonal change, i.e. an increase in the auxin concentration, which is known to promote formation of lateral roots [4, 18, 35]. The presence of the ENOD40 gene mainly in adventitious lateral root primordia could mean that this hormonal change is caused or mediated by the ENOD40 gene product.

In conclusion, ENOD40 is proven to be an important gene not only because it is involved in nodule development from very early stages, but also because it could serve as a tool in gaining access to mechanisms involved in the determination of other developmental processes in the plant. Furthermore, due to its differential expression in adventitious and acropetal lateral roots, ENOD40 gene could serve as a molecular marker in studies of lateral root initiation.

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