Plant Molecular Biology **28**: 1111–1119, 1995.

Short communication

VsENOD5, *VsENOD12* and *VsENOD40* expression during *Rhizobium*-induced nodule formation on *Vicia sativa* roots

Irma Vijn [1], Wei-Cai Yang [1], Niels Pallisgård [2], Eric Østergaard Jensen [2], Ab van Kammen [1] and Ton Bisseling [1,*]

[1] *Department of Molecular Biology, Agricultural University, Dreijenlaan 3, 6703 HA Wageningen, Netherlands (*author for correspondence);* [2] *Laboratory of Gene Expression, Department of Molecular Biology, University of Aarhus, Gustav Wieds vej 10, DK-8000 Aarhus C, Denmark*

Received 8 January 1995; accepted in revised form 30 May 1995

Key words: early nodulins (ENOD), *Rhizobium*, root nodule development, *Vicia sativa*

Abstract

We isolated *ENOD5*, *ENOD12* and *ENOD40* homologues from *Vicia sativa* and studied their expression pattern during *Rhizobium*-induced nodule formation. Comparison of the *VsENOD40* nucleotide sequence with the pea, soybean and alfalfa *ENOD40* sequences showed that the sequences contain two conserved regions, called region I and region II. Comparison of all the potential open reading frames (ORFs) showed that all the five different *ENOD40* clones encode a highly conserved small polypeptide of 12 or 13 amino acids encoded by an ORF located in region I. Furthermore we studied with *in situ* hybridization the expression pattern of *VsENOD5*, *VsENOD12* and *VsENOD40* during *Rhizobium*-induced nodule formation. Although the expression of these genes is largely similar to that of the pea counterparts, differences where found for the expression of *VsENOD12* and *VsENOD40* in *Vicia*. *VsENOD12* is expressed in the whole prefixation zone II, whereas in pea *ENOD12* is only expressed in the distal part of this zone. *VsENOD40* is expressed in the uninfected cells of interzone II-III, while in pea *ENOD40* is expressed in both the uninfected and infected cells of this zone.

Rhizobium and *Bradyrhizobium* bacteria elicit the formation of nodules on the roots of leguminous plants. The formation of these organs involves different developmental steps, which have been described in several recent reviews [2, 7]. The bacterial genes that play a key role in the induction of the early steps of nodulation are the *nod* genes. Flavonoids, secreted by the plant into the rhizosphere, elicit the expression of these genes and the activity of the Nod proteins results in the production of specific lipo-oligosaccharides, called Nod factors. Purified Nod factors can induce the first steps of nodule formation including the induction of some early nodulin genes [8, 18, 22] and on some legumes even genuine nodules are induced [19, 20]. Therefore it is of great in-

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers X83681, X83682 and X83683.

terest to study the mode of action of Nod factors.

We are studying the *Rhizobium leguminosarum* bv. *viciae*-legume interaction. Host plants that can interact with *R. leguminosarum* bv. *viciae* include *Vicia* and pea. For *Vicia*, several bioassays to study Nod factor-induced root hair deformation and the induction of cortical cell division have been developed [6, 18] while, on the other hand, early nodulins have been cloned from pea but not from *Vicia*. Therefore it has not been possible to correlate Nod factor-induced processes with the induction of early nodulin gene expression. We decided to isolate from *Vicia sativa* early nodulin genes that are induced by *R. leguminosarum* bv. *viciae* Nod factors. Previously, we showed that the early nodulin genes *ENOD5* and *ENOD12* can be induced by purified Nod factors in root hairs of pea plants treated with Nod factor [8]. Furthermore we reported that the early nodulin genes *ENOD5*, *ENOD12* and *ENOD40* are active in *Rhizobium*-induced pea nodule primordia [15, 16, 24]. For this reason we cloned the *ENOD5*, *ENOD12* and *ENOD40* homologues of *Vicia sativa* and studied their expression during *Rhizobium* induced root nodule formation. These comparative *in situ* hybridization studies revealed differences between pea and *Vicia* for the expression patterns of *ENOD12* and *ENOD40* which may ultimately help to understand the mode of action of the corresponding gene products. Furthermore, comparison of the nucleotide sequences reveals functional motifs within the early nodulin genes and has led to the identification of a conserved ORF in *ENOD40* encoding an oligopeptide.

Cloning and sequencing

A *V. sativa* λ-ZapII cDNA library was prepared from mRNA of young root nodules. Total RNA was isolated from nodules harvested 7, 9, 12 and 17 days after inoculation with *Rhizobium leguminosarum* bv. *viciae* [14]. Equal amounts of these RNAs were mixed and poly(A)$^+$ RNA was obtained by oligo-dT purification. First-strand cDNA synthesis [11] was performed by using an oligo(dT)-*Not* I primer (Promega). After second-strand cDNA synthesis according to Gubler [5], double-stranded cDNA fragments larger than 400 bp were purified on a sepharose CL-4B column. These cDNA fragments were provided with *Eco* RI adaptors (Promega), digested with *Not* I and ligated into *Eco* RI-*Not* I digested λ-ZAPII arms (Stratagene). The DNA was packed by using a Gigapack Gold II kit (Stratagene), as described by the manufacturer. Starting from 5 μg poly(A)$^+$ RNA, 500 ng of double-stranded cDNA, larger than 400 bp, was obtained. From 75 ng of cDNA ligated to λ-ZAPII arms, 8×10^5 independent recombinant phage were obtained. The library was amplified once and this amplified library was screened with ^{32}P-labelled inserts of pPsENOD5, pPsENOD12 and pPsENOD40, which had been isolated previously [13, 15, 16]. From each screening 4 independent clones were isolated. The clones containing the longest inserts were named pVsENOD5, pVsENOD12 and pVsENOD40, respectively.

pVsENOD5 has an insert of 538 bp and contains a poly(A) stretch at the 3′ end. The cloned *V. sativa* cDNA starts with the coding sequence of 12 aa which are highly conserved between pea and vetch and form, in pea, the second half of a putative signal peptide. This suggest that also the vetch ENOD5 polypeptide will contain a signal peptide and the sequence coding for the 11 amino acids, including the AUG start codon, at the beginning of the open reading frame are missing in the cloned VsENOD5 cDNA (Fig. 1A). Comparison with the PsENOD5 polypeptide showed 76% identity (Fig. 1A). Like PsENOD5, VsENOD5 contains a high percentage of Pro, Ser, Gly, and Ala residues and a short sequence of 5 amino acids consisting of alternating Pro and Ala occurs in this protein. Both features are considered to be characteristics of arabinogalactan proteins [17] and so the vetch ENOD5 sequence supports the hypothesis that ENOD5 might be an arabinogalactan protein.

pVsENOD12 has an insert of 548 bp that contains a complete open reading frame (ORF) coding for a 100 amino acid long polypeptide. Like the pea ENOD12 polypeptides it contains a pu-

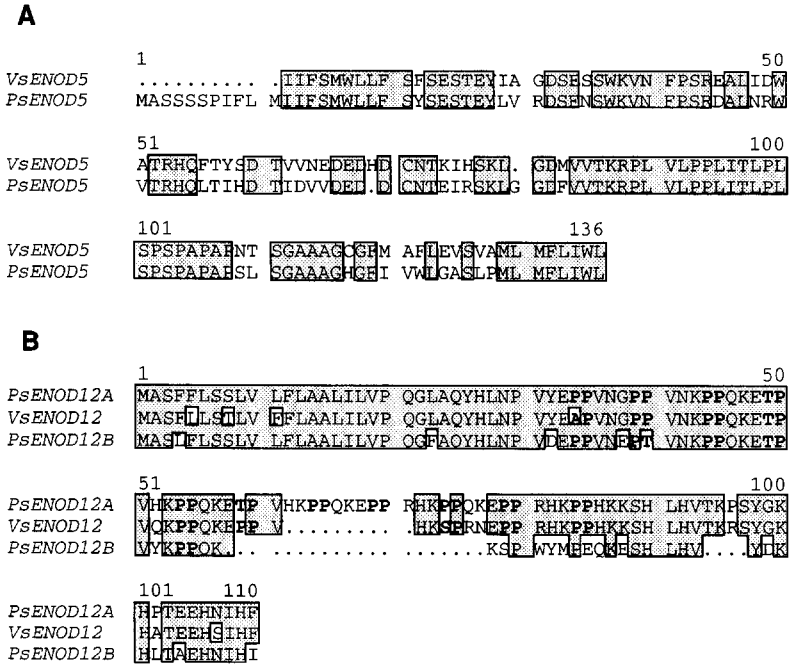

Fig. 1. Alignment of *Vicia* and pea ENOD5 (A) and ENOD12 (B) amino acid sequences. Homologous amino acids are boxed. Dots represent deletions.

tative N-terminal signal peptide of 24 amino acids and the major part of the protein is composed of two proline-rich pentapeptide repeating units. The unit consisting of the pentapeptide Pro-Pro-Gln-Lys-Glu is repeated three times but the third time the unit is changed into Ser-Pro-Arg-Asn-Glu. The other unit starts as Pro-Pro-Val-Asn-Gly but towards the carboxy terminus of the polypeptide gradually every amino acid except the prolines is changed to a final Pro-Pro-His-Lys pentapeptide. At three positions the first proline codon is changed into an alanine, threonine or serine codon by a single-basepair substitution. Comparison with the PsENOD12A polypeptide showed 88% identity, with a deletion of 10 amino acids located in the proline-rich pentapeptide repeat region, whereas the PsENOD12B polypeptide shows 73% identity. PsENOD12B contains fewer and less conserved proline-rich pentapeptide repeats, causing the lower homology (Fig. 1B).

pVsENOD40 has an cDNA insert of 718 bp and on the nucleotide level this sequence is 93% homologous to the pea ENOD40 cDNA clone recently cloned by Matvienko *et al.* [13]. A comparison of the pea ENOD40 cDNA with the previously cloned soybean ENOD40 cDNAs [9, 24] showed that the long ORF present in the soybean ENOD40 cDNAs does not occur in the pea ENOD40 cDNA due to several non-triplet insertions and deletions [13]. To identify the conserved regions of *ENOD40* we compared the *Vicia* ENOD40 cDNA clone with the pea, soybean and the recently cloned alfalfa [3] ENOD40 sequence (Fig. 2A). Comparison on the nucleotide level of the complete cDNA clones showed that the *Vicia* and pea cDNA clone are 87% homologous to the alfalfa cDNA clone, and that the *Vicia*, pea and alfalfa clones are 55% homologous to the soybean cDNA clones. However, the comparison showed that the sequences contain two conserved regions, indicated in Fig. 2A as region I and region II. The homology of region I between *Vicia*, pea and alfalfa is 98%, whereas the homology of these sequences with soybean is 82%. The homology of region II is 91% between *Vicia*, pea and alfalfa and compared to soybean the sequences show a homology of 76%. Comparison

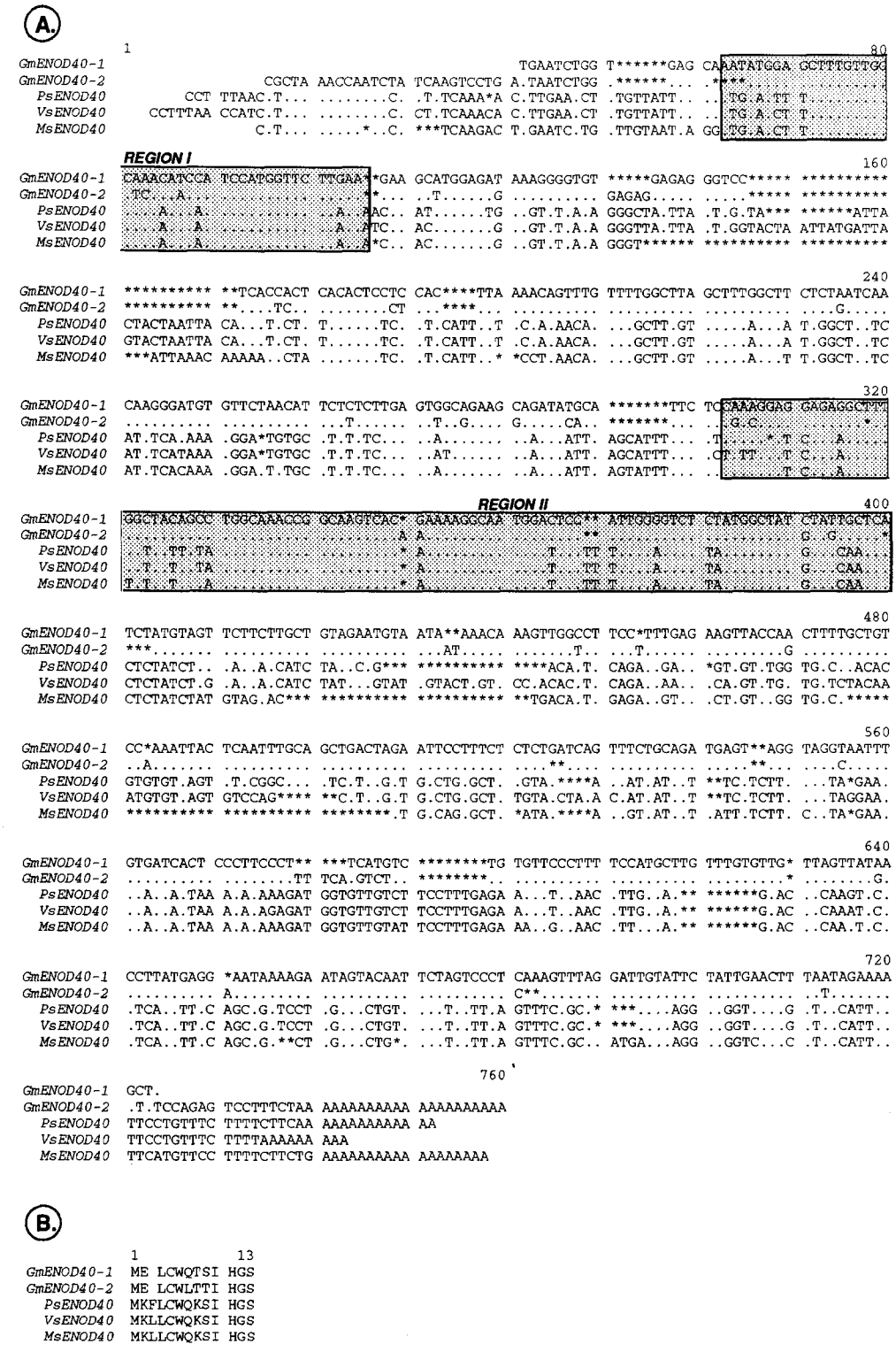

Fig. 2. Alignment of nucleotide and amino acid sequences of *ENOD40*. A. Alignment of nucleotide sequences of different ENOD40 sequences. *GmENOD40-1* and *GmENOD40-2* are isolated from soybean by Kouchi and Hata [9] and Yang *et al.* [24], respectively; *PsENOD40* has been isolated from pea by Matvienko *et al.* [13]; *MsENOD40* has been isolated from alfalfa by Crespi *et al.* [3] and *VsENOD40* is isolated from *Vicia sativa* as described in this paper. The conserved regions I and II are boxed. Dots represent identical nucleotides, stars represent deletions. B. Alignment of the conserved polypeptide encoded by region I of all *ENOD40* genes.

of all potential ORFs, that means sequences starting with an AUG start codon, showed that all five different clones encode a highly conserved small polypeptide of 12 or 13 amino acids (Fig. 2B), encoded by an ORF located in region I. Furthermore, the AUG start codon of this ORF is flanked by an adenine at the −3 position in all *ENOD40* sequences in which the A of the AUG is numbered +1 and bases 5′ of this are numbered negatively. At position +4 the soybean AUG codon is flanked by a guanine whereas the AUG codon in the other *ENOD40* sequences are flanked at this position by an adenine. According to Kozak [10] and Lutcke *et al.* [12] AUG codons flanked by these bases are most efficient as an initiation codon. This could mean that *ENOD40* encodes a short polypeptide. The second conserved region does not encode a polypeptide.

Matvienko *et al.* [13], Crespi *et al.* [3] and Asad *et al.* [1] suggested that *ENOD40* might be active as a RNA transcript, because of the absence of significant ORFs and the tendency of ENOD40 RNA to form secondary structures. However, the methods used by Crespi *et al.* [3] to show that *ENOD40* codes for a non-translatable RNA might not be applicable for messengers encoding small proteins. The Test Code program (GCG software package) was designed for detection of coding regions longer than 200 bp, so small polypeptides are overlooked and for a good comparison of the free energies of folding between coding and non-coding RNAs also RNAs encoding small polypeptides should have been used. Therefore these studies do not exclude that *ENOD40* encodes an unusual small conserved protein and further studies are essential to reveal which *ENOD40* product is biologically active.

Expression of early nodulin genes during Rhizobium-*induced nodule development*

The expression of *ENOD5*, *ENOD12* and *ENOD40* was studied by *in situ* hybridization [23] at different stages of *Rhizobium*-induced nodule development. Four days after spot inoculation with *R. leguminosarum* bv. *viciae* a globular nodule primordium has been formed in the inner cortex of the root. The peripheral tissues have started to form, but the apical meristem has not yet been formed (Fig. 3A). Some branches of the infection thread have penetrated the primordium. One day later (day 5) the nodule meristem is visible at the distal part of the young nodule and at the proximal part infected cells are present, which are larger than the cells at the distal part (Fig. 3B). Twenty days after inoculation a mature nodule is formed (Fig. 3C). Like pea, *V. sativa* forms indeterminate nodules, which have a persistent meristem that continuously differentiates in the different nodule tissues. As a result the central tissue of indeterminate nodules has been divided into zones representing successive stages of development. The following nomenclature for these zones, from the apex to the root attachment point, are used in alfalfa and pea: the meristematic zone I, prefixation zone II, interzone II-III, fixation zone III, and in older nodules a senescent zone IV is present. In both alfalfa and pea the sudden start of amyloplast accumulation in the infected cell type marks the transition of prefixation zone II into interzone II-III [4, 21]. Such a sudden accumulation of amyloplasts in the infected cells was also found in *V. sativa* nodules and this will be considered as the transition of prefixation zone II into interzone II-III (Fig. 3C, arrow). Adjacent cross sections of vetch roots 4, 5 and 20 days after inoculation with *R. leguminosarum* bv. *viciae* were hybridized with ^{35}S-labelled anti-sense RNA transcribed from the three cloned early nodulin cDNAs.

At 4 days after spot inoculation both ENOD12 and ENOD40 mRNA are present in all cells of the centre of the nodule primordium, while ENOD40 mRNA is also present in the region of the root pericycle facing the nodule primordium (Fig. 3G and 3J). Remarkably ENOD12 mRNA also occurs in the root cortical cells containing the infection thread (Fig. 3G, arrowhead). In contrast to the expression pattern of *ENOD12* and *ENOD40* in the primordium, *ENOD5* transcript is only found in a small cluster of cells of the primordium (Fig. 3D). In this cluster infection

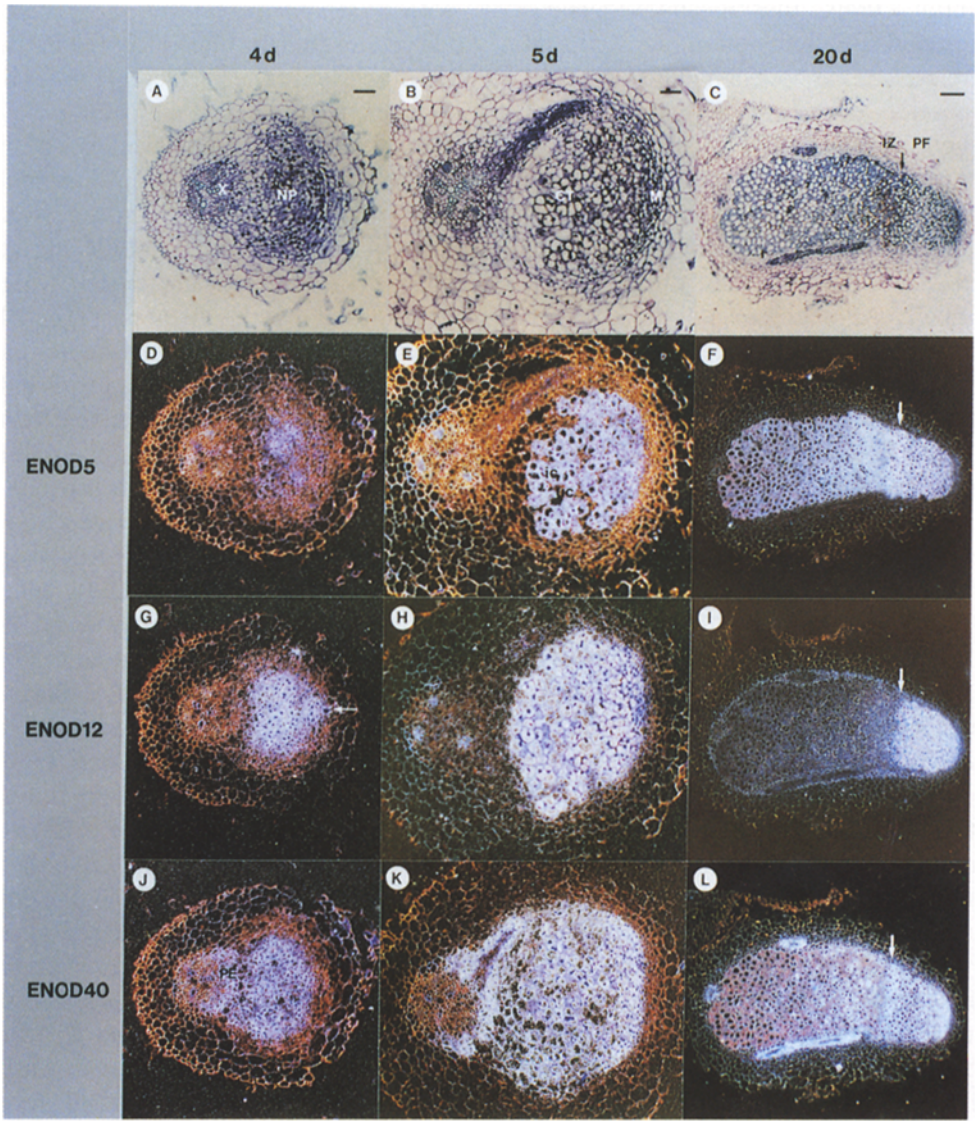

Fig. 3. In situ localization of *VsENOD5*, *VsENOD12* and *VsENOD40* in 4, 5- and 20-day old *Rhizobium*-induced *Vicia sativa* root nodules. NP, nodule primordium; CT, central tissue; PE, pericycle, M, meristem; X, proto-xylem pole; ic, infected cells; uc, uninfected cells; PF, prefixation zone II; IZ, interzone II-III. A, B, C are bright-field micrographs; D, E, F, G, H, I, J, K and L are dark-field micrographs were signals are represented by white dots. The sections were hybridized with ENOD5 (D, E, F), ENOD12 (G, H, I) and ENOD40 (J, K, L) ^{35}S-labelled antisense RNA. A, D, G, J: Serial cross sections of a 4-day old root nodule showing the nodule primordium (NP) formed in the inner root cortex. The sections were hybridized with ENOD5 (D), ENOD12 (G) and ENOD40 (J) ^{35}S-labelled antisense RNA, showing the expression of *ENOD12* and *ENOD40* in all cells of the nodule primordium (NP), whereas ENOD5 mRNA is only detected in a small cluster of cells in the nodule primordium (NP). In addition ENOD40 mRNA is detected in the root pericycle (PE) near one of the xylem poles. Note that *ENOD12* is also expressed in the root cortical cells containing the infection thread (arrow). Bar = 80 μm. B, E, H, K. Serial cross sections of a 5 day old root nodule showing that at the distal part of the nodule a meristem (ME) is visible and at the proximal part infected cells (IC) are present. The sections were hybridized with ENOD5 (E), ENOD12 (H) and ENOD40 (K) ^{35}S-labelled antisense RNA, showing that all three early nodulin genes are expressed in the complete central tissue (CT), but not in the meristem (ME). Bar = 80 μm. C, F, I and L. Serial cross sections of a 20 day old fully developed nodule. The section in C has been stained with a 0.2% I_2 – 2% KI solution to show the accumulation of amyloplasts in the uninfected cells. Amyloplasts are the purple stained material. The sudden accumulation of amyloplasts in the uninfected cells marks the transition of prefixation zone II (PF) into interzone II-III

threads are present (data not shown). At day 5, one day later, all three early nodulin genes are now expressed in the complete central tissue, but in the meristem none of these genes is active (Fig. 3E, 3H and 3K).

The *in situ* expression patterns 4 and 5 days after inoculation are in agreement with the *in situ* hybridization studies in pea, which showed that *ENOD12* and *ENOD40* are already induced in nodule primordia cells before they become infected by *Rhizobium*, whereas ENOD5 mRNA is only present in infected cells [15, 16, 24].

In 20 day old root nodules of *V. sativa* plants *VsENOD5* is expressed in the complete prefixation zone II, where it is only active in the infected cells. Maximal accumulation of ENOD5 mRNA occurred in the proximal part of this zone (Fig. 3F). The level of *VsENOD5* RNA suddenly decreases to a lower level from one cell layer to another at the transition of prefixation zone II into interzone II-III and remains at this reduced level in the fixation zone III. The patterns of *VsENOD5* expression is identical to that of *PsENOD5* in pea nodules [16], although the ENOD5 mRNA, in pea, drops to a lower level at the transition point.

VsENOD12 is in 20-day old nodules expressed in the complete prefixation zone II as well and its transcript is present at a similar level throughout the whole zone. In the distal part of prefixation zone II *VsENOD12* is probably expressed in all cells, whereas in the more proximal part *VsENOD12* expression in only found in the infected cells. *VsENOD12* expression decreases to an undetectable level at the transition of prefixation zone II into interzone II-III (Fig. 3I). So this drop of *ENOD12* expression exactly coincides with the decrease of the level of *ENOD5* RNA. The expression pattern of *VsENOD12* in *V. sativa* nodules is strikingly different from *PsENOD12* expression in pea nodules, where *ENOD12* is only expressed in the most distal part of prefixation zone II [15]. In the distal part infection threads grow and infect new meristematic cells and for this reason it was proposed that *ENOD12* expression correlates with the infection process [15]. In the proximal part of the prefixation zone the bacteria, surrounded by a plant membrane, together called the symbiosome, proliferate in the cytoplasm of the infected plant cells. This suggests that at least in vetch, the function of *ENOD12* is not strictly linked to the infection process and that *ENOD12* might have an additional function in the proliferation of the bacteria.

Like *VsENOD5* and *VsENOD12*, *VsENOD40* is in 20-day old nodules expressed in the complete prefixation zone II. In the proximal part of this zone ENOD40 mRNA is present in the infected cells but not in the uninfected cells. At the transition of prefixation zone II into interzone II-III the level of *VsENOD40* expression drops markedly in the infected cells, and in the fixation zone III expression of *VsENOD40* is now induced in the uninfected cells. In the proximal (older) part of the fixation zone the expression of *VsENOD40* is not detected anymore. In the pericycle of the nodule vascular bundle *VsENOD40* is expressed at a high level (Fig. 3L). In both pea and vetch nodules *ENOD40* expression is maximal in the pericycle of the nodule vascular bundle and the gene is active in all infected cells of the prefixation zone. However, in the fixation zone of pea nodules *ENOD40* expression is found in both the infected and uninfected cells [13], whereas in vetch *ENOD40* is only expressed in the uninfected cells in this zone.

We showed here that the expression pattern of the *Vicia ENOD5*, *ENOD12* and *ENOD40* genes is largely similar to that of the pea counterparts. The most striking differences are the expression

(IZ) (arrow). The sections were hybridized with ENOD5 (F), ENOD12 (I) and ENOD40 (C, L) ^{35}S-labelled antisense RNA. Note that all three early nodulin genes are expressed in the whole prefixation zone and that their expression pattern is changing at the transition from prefixation zone II (PF) into interzone II-III (IZ) (arrow); the level of ENOD5 mRNA decreases, *ENOD12* expression drops to an undetectable level and the expression of ENOD40 drops in the infected cells and in the fixation zone III *ENOD40* expression is induced in the uninfected cells. ENOD40 mRNA is also detected in the pericycle (PE) of the nodule vascular bundle (arrowhead). Bar = 200 μm.

of *ENOD12* in the whole prefixation zone II in *Vicia*, whereas in pea *ENOD12* is only expressed in the distal part of the zone and secondly the expression of *ENOD40* in the uninfected cells of the interzone II-III in *Vicia*, while in pea *ENOD40* is expressed in both the infected and uninfected cells of this zone. Like in pea *ENOD5*, *ENOD12* and *ENOD40* are induced in nodule primordia. Most likely *ENOD5* is first induced when primordium cells are infected, while *ENOD12* and *ENOD40* are probably already activated prior to infection. Therefore these clones are useful tools to study long- and short-distance signalling in Nod factor-induced nodule formation.

Acknowledgements

This work was supported by grants of the Dutch Organization of Scientific Research (NWO) (IV, TB) and the Human Frontier Science Program (WCY, TB, NP and EØJ).

References

1. Asad S, Fang Y, Wycoff KL, Hirsch AM: Isolation and characterization of cDNA and genomic clones of *MsENOD40*; transcripts are detected in meristematic cells of alfalfa. Protoplasma 183: 10–23 (1994).
2. Brewin NJ: Development of the legume root nodule. Annu Rev Cell Biol 7: 191–226 (1991).
3. Crespi MD, Jurkevitch E, Poiret M, d'Aubenton-Carafa Y, Petrovics G, Kondorosi E, Kondorosi A: ENOD40, a gene expressed during nodule organogenesis, codes for a non-translatable RNA involved in plant growth. EMBO J 13: 5099–5112 (1994).
4. Franssen HJ, Vijn I, Yang WC, Bisseling T: Developmental aspects of the *Rhizobium*-legume symbiosis. Plant Mol Biol 19: 89–107 (1992).
5. Gubler U: Second-strand cDNA synthesis: mRNA fragments as primers. Meth Enzymol 152: 330–335 (1987).
6. Heidstra R, Geurts R, Franssen H, Spaink HP, van Kammen A, Bisseling T: Root hair deformation activity of Nod factors and their fate on *Vicia sativa*. Plant Physiol 105: 787–797 (1994).
7. Hirsch AM: Developmental biology of legume nodulation. New Phytol 122: 211–237 (1992).
8. Horvath B, Heidstra R, Lados M, Moerman M, Spaink HP, Promé J-C, van Kammen A, Bisseling T: Lipo-oligosaccharides of *Rhizobium* induce infection related early nodulin gene expression in pea root hairs. Plant J 4: 727–733 (1993).
9. Kouchi H, Hata S: Isolation and characterization of novel nodulin cDNAs representing genes expressed at early stages of soybean nodule development. Mol Gen Genet 238: 106–119 (1993).
10. Kozak M: Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell 44: 283–292 (1986).
11. Krug MS, Berger SL: First-strand cDNA synthesis primed with oligo(dT). Meth of Enzymol 152: 316–325 (1987).
12. Lutcke HA, Chow KC, Mickel FS, Moss KA, Kern HF, Scheeler GA: Selection of AUG initiation codons differ in plant and animals. EMBO J 6: 43–48 (1987).
13. Matvienko M, van de Sande K, Yang W-C, van Kammen A, Bisseling T, Franssen H: Comparison of soybean and pea ENOD40 cDNA clones representing genes expressed during both early and late stages of nodule development. Plant Mol Biol 26: 487–493 (1994).
14. Pawlowski K, Kunze R, de Vries S, Bisseling T: Isolation of total, poly(A) and polysomal RNA from plant tissues. In: Gelvin, SB, Schilperoort RA (eds) Plant Molecular Biology Manual, pp. D5: 1–13. Kluwer Academic Publishers, Dordrecht (1994).
15. Scheres B, van de Wiel C, Zalensky A, Horvath B, Spaink H, Van Eck H, Zwartkruis F, Wolters AM, Gloudemans T, van Kammen A, Bisseling T: The ENOD12 gene product is involved in the infection process during pea-*Rhizobium* interaction. Cell 60: 281–294 (1990).
16. Scheres B, van Engelen F, van der Knaap E, van de Wiel C, van Kammen A, Bisseling T: Sequential induction of nodulin gene expression in the developing pea nodule. Plant Cell: 8: 687–700 (1990).
17. Showalter AM: Structure and function of plant cell wall proteins. Plant Cell 5: 9–23 (1993).
18. Spaink HP, Sheeley DM, van Brussel AAN, Glushka J, York WS, Tak T, Geiger O, Kennedy EP, Reinhold VN, Lugtenberg BJJ: A novel highly unsaturated fatty acid moiety of lipo-oligosaccharide signals determines host specificity of *Rhizobium*. Nature 354: 125–130 (1991).
19. Stokkermans TJW, Peters NK: *Bradyrhizobium elkanii* lipo-oligosaccharide signals induce complete nodule structures on *Glycine soja* Siebold et Zucc. Planta 193: 413–420 (1994).
20. Truchet G, Roche P, Lerouge P, Vasse J, Camut S, de Billy F, Promé J-C, Dénarié J: Sulphated lipo-oligosaccharide signals of *Rhizobium meliloti* elicit root nodule organogenesis in alfalfa. Nature 351: 670–673 (1991).
21. Vasse J, De Billy F, Camut S, Truchet G: Correlation between ultrastructural differentiation of bacteroids and nitrogen fixation in alfalfa nodules. Bact J 172: 4295–4306 (1990).
22. Vijn I, Christiansen H, Lauridsen P, Kardailsky I, Quandt H-J, Drenth J, Østergaard Jensen E, van Kammen A, Bisseling T: A 200 bp region of the pea *ENOD12*

promoter is sufficient for nodule specific and Nod factor-induced expression. Plant Mol Biol, submitted (1995).
23. Yang W-C, de Blank C, Meskiene I, Hirt H, Bakker J, van Kammen A, Franssen H, Bisseling T: *Rhizobium* Nod factors reactivate the cell cycle during infection and nodule primordium formation, but the cycle is only completed in primordium formation. Plant Cell 6: 1415–1426 (1994).
24. Yang WC, Katinakis P, Hendriks P, Smolders A, de Vries F, Spee J, van Kammen A, Bisseling T, Franssen H: Characterization of GmENOD40, a gene showing novel patterns of cell-specific expression during soybean nodule development. Plant J 3: 573–585 (1993).