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The early nodulin transcript ENOD2 is located in the nodule parenchyma (inner cortex) of pea and soybean root nodules

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A pea cDNA clone homologous to the soybean early nodulin clone pGmENOD2 that most probably encodes a cell wall protein was isolated. The derived amino acid sequence of the pea ENOD2 protein shows that it contains the same repeating pentapeptides, ProProHisGluLys and ProProGluTyrGln, as the soybean ENOD2 protein. By *in situ* hybridization the expression of the ENOD2 gene was shown to occur only in the inner cortex of the indeterminate pea nodule. The transcription of the pea ENOD2 gene starts when the inner cortical cells develop from the nodule meristem. In the determinate soybean nodule the ENOD2 gene is expressed in the inner cortex as well as in cells surrounding the vascular bundle that connects the nodule with the root central cylinder. The term 'nodule inner cortex' is misleading, as there is no direct homology with the root inner cortex. Therefore, we propose to consider this tissue as nodule parenchyma. A possible role of ENOD2 in a major function of the nodule parenchyma, namely creating an oxygen barrier for the central tissue with the *Rhizobium* containing cells, is discussed.

Key words: early nodulin/*in situ* hybridization/pea/root nodule cortex/soybean

Introduction

Root nodules formed on the roots of leguminous plants are unique organs for symbiotic nitrogen fixation by *Rhizobium* bacteria. Root nodules are organized structures which develop from meristems newly formed in the cortex of the root as a result of interaction with rhizobia. The mature root nodule is made up of a central tissue containing infected and uninfected cells, surrounded by a cortex. The nodule has a common endodermis which divides the cortex into an outer and an inner cortex. The inner cortex is traversed by vascular strands, each surrounded by a bundle endodermis. The strands are connected to the central cylinder of the root (for a review see Newcomb, 1981; Bergersen, 1982).

By their morphology two main categories of leguminous nodules can be recognized: determinate and indeterminate nodules (for discussion see Sprent, 1980). Legumes such as *Pisum* (pea), *Trifolium* (clover) and *Medicago* (alfalfa) species develop indeterminate nodules, whereas determinate nodules are formed on the roots of e.g. *Glycine* (soybean) and *Phaseolus* (bean) species. Indeterminate root nodules have a persistent meristem at the apex from which cells are continuously added to the cortical and central tissues. Consequently all tissues of these nodules are of graded age from the meristem to the root attachment point. The meristem of a determinate nodule ceases to divide 2–3 weeks after inoculation and it differentiates completely into nodular tissue (Newcomb, 1981).

The formation of root nodules involves the differential expression of a series of nodule-specific plant genes, the nodulin genes (van Kammen, 1984). These genes have been divided into early and late nodulin genes. The early nodulin genes are already expressed at early stages of root nodule development, well before the onset of nitrogen fixation. The late nodulin genes are first expressed around the onset of nitrogen fixation, after a complete nodule structure has been formed. Several late nodulins, e.g. leghemoglobin, n-uricase and nodulins present in the peribacteroid membrane, have been located in the central tissue of the nodule (Robertson *et al.*, 1984; Van den Bosch and Newcomb, 1986, 1988; Verma *et al.*, 1986). Involvement of nodulins in the function of the cortical tissues in the nodule has not been defined so far.

Recently we have characterized the product of the early nodulin gene ENOD2 from soybean as a proline-rich protein built up of two repeating pentapeptides (Franssen *et al.*, 1987). In this paper we report the amino acid sequence of a homologous ENOD2 nodulin from pea. Moreover, we demonstrate that the ENOD2 gene is specifically expressed in the inner cortex of the determinate soybean nodule as well as in the inner cortex of the indeterminate pea nodule. In the discussion we suggest that the ENOD2 nodulin has a role in the characteristic morphology of the inner cortex and the function of this tissue as barrier for oxygen diffusion into the root nodules.

Results

Sequence of the pea ENOD2 early nodulin

A cDNA library was prepared against poly(A)$^+$ RNA from 21 day old pea root nodules and several clones were selected that specifically hybridized with the insert from the soybean cDNA clone pGmENOD2 (Franssen *et al.*, 1987). The clone with the largest insert was named pPsENOD2. The insert of pPsENOD2, 558 bp in length, was sequenced and a partial amino acid sequence of the pea ENOD2 nodulin deduced from the cDNA sequence is shown in Figure 1. The sequence contains 336 nucleotides of an open reading frame (ORF) encoding 112 amino acids of the C-terminal end of the ENOD2 protein. The ORF ends with three successive termination codons and is followed by a 3′ non-translated region of ~235 nucleotides in which a potential poly(A) addition signal is present and a short part of a poly(A) tail. The amino acid sequence reveals that the pea

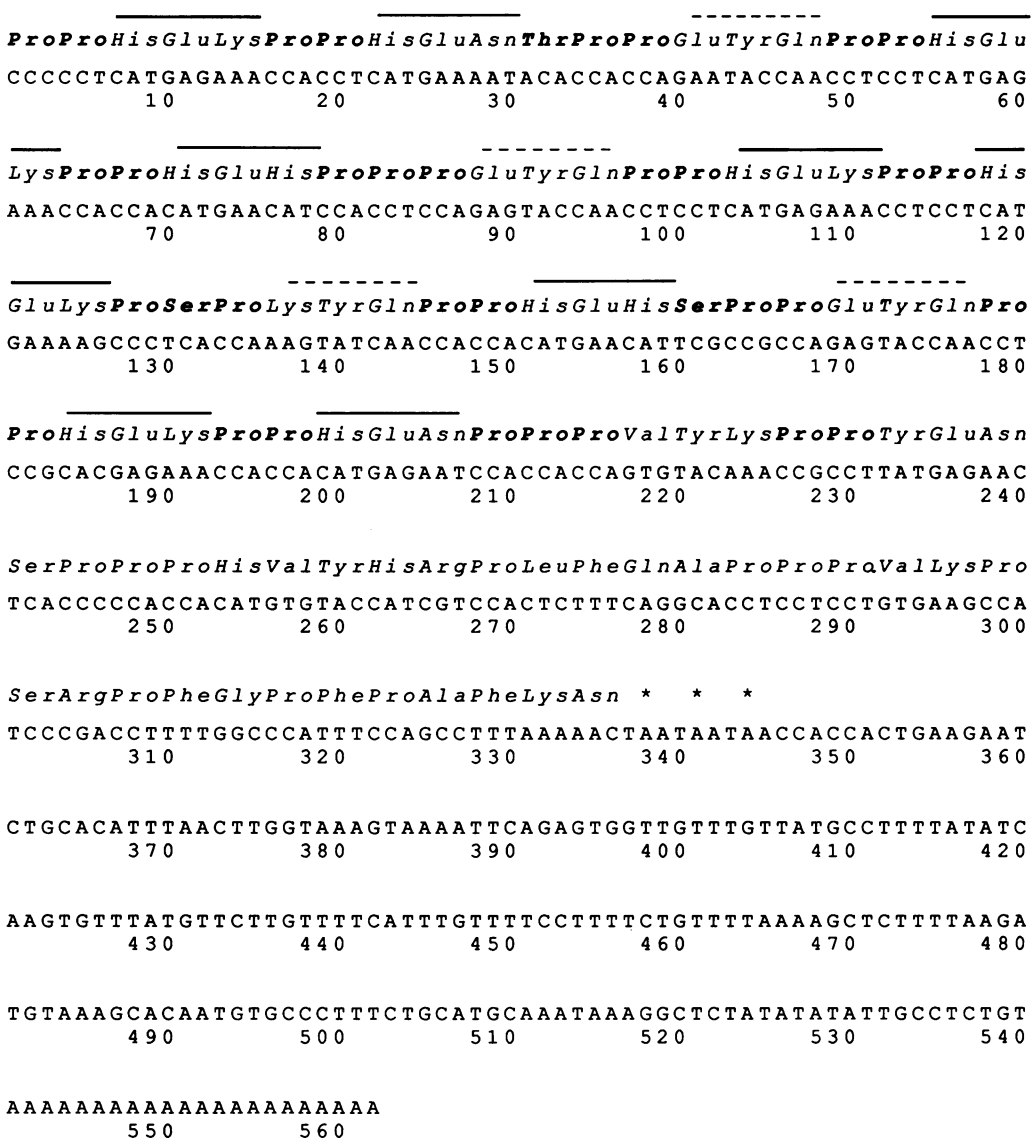


Fig. 1. cDNA and predicted amino acid sequence of the pPsENOD2 insert. Nucleotides 1–562 are determined from the pPsENOD2 insert. The sequence of nucleotides 34–562 is confirmed by analysis of an independently obtained ENOD2 cDNA clone. The amino acid sequence of the only long ORF is displayed over the nucleotide sequence. The amino acid triplets characteristic for the different types of pentapeptide repeats described in the text are overlined with unbroken and dashed bars respectively. The three termination codons ending the reading frame are marked by asterisks. Nucleotides 511–517 encompass the polyadenylation signal.

ENOD2 protein is very proline-rich and is mainly composed of two repeating pentapeptides, ProProHisGluLys and ProProGluTyrGln. Two ProProHisGluLys repeats alternate with one ProProGluTyrGln element. Southern blots containing pea genomic DNA digested with *Eco*RI or *Sph*I and a dilution series of pPsENOD2 were hybridized with the insert of pPsENOD2. A 7.2 kb *Eco*RI fragment and a 4.6 kb *Sph*I fragment hybridized to pPsENOD2. Moreover, comparison of the levels of hybridization of the pPsENOD2 dilution series and the pea genomic fragments respectively, indicated that only one ENOD2 gene is present in the pea genome (data not shown).

Localization of the ENOD2 transcript in indeterminate pea nodules

We used the *in situ* hybridization technique to examine in which nodular tissue the pea ENOD2 gene is expressed. Longitudinal sections of pea nodules from 20 day old plants were hybridized with ^{35}S-labeled sense and antisense RNA transcribed from the insert of pPsENOD2. After autoradiography the antisense RNA probe appeared to hybridize with RNA present in the sections whereas the sense RNA probe did not (result not shown). The antisense RNA probe only hybridized with RNA present in the inner cortex of the nodule, suggesting that the pea ENOD2 gene is exclusively expressed in this nodular tissue (Figure 2A and B). The ENOD2 gene is expressed throughout the whole inner cortex: from the youngest cells directly adjacent to the meristem up to the oldest cells near the root attachment point. The vascular tissue traversing the nodule inner cortex does not contain detectable levels of the ENOD2 transcript.

The presence of the ENOD2 transcript in the inner cortical cells close to the nodule meristem indicated that expression of the ENOD2 gene is induced at a relatively early stage of development. To test this we also hybridized serial sections of nodule primordia of 7, 8 and 10 day old roots

to antisense RNA from pPsENOD2. The pea nodule primordia are initiated in the inner cell layers of the root cortex. At day seven no ENOD2 messenger was detectable in the nodule primordia (data not shown). The ENOD2 transcript is first detectable in nodule primordia of an 8 day old pea plant (Figure 3A and B). At this stage the infection thread, which transports the rhizobia from an infected root hair to the nodule primordium, has already reached the primordium and branched off into different cells of the central part of the primordium. Moreover, the first differentiation of procambial strands (not shown in Figure 3A, but visible in consecutive sections of the same primordium) and the formation of an apical meristem have taken place (Figure 3A). The ENOD2 messenger is present in a few inner cortical cells at the base of the nodule primordium (Figure 3B). In nodules of 10 day old pea plants, which is 3 days before the onset of nitrogen fixation, infected cells filled with bacteroids can be seen at the base of the nodule (Figure 3C). The ENOD2 transcript is now present throughout the inner cortex as in the 20 day old nodule (cf. Figure 3C,D with Figure 2A,B).

Localization of the ENOD2 transcript in determinate soybean nodules

Sections of soybean nodules of 21 day old plants were hybridized with ^{35}S-labeled antisense RNA made from the insert of pGmENOD2. Figure 2C and D shows that as in pea nodules the soybean ENOD2 messenger is located in the nodule inner cortex and in the tissue surrounding the vascular bundle connecting the nodule to the central cylinder of the root. Since there is no persistent meristem in this type of nodule, the inner cortical tissue completely surrounds the central tissue of the mature nodule (cf. Figure 2C,D with Figure 2A,B of pea). The distribution of the silver grains in the different nodule tissues is better shown in Figure 2E and F, which represents magnifications of a section through a 21 day old soybean nodule hybridized with ENOD2 antisense RNA. In order to obtain a good impression of the various tissues, a similar part of a section of a soybean nodule from a 21 day old plant embedded in glycolmethacrylate resin is shown in Figure 2G. Here the tissue morphology is better preserved than in paraffin. The inner cortical cells have fewer and smaller intercellular spaces than the outer cortical cells. The endodermis that separates the inner and outer cortex mainly consists of large sclerenchymatic cells at this stage (Figure 2G). Figure 2E and F shows that the vast majority of ENOD2 transcript is found in the inner cortex, but low levels of this messenger are also present in the endodermis and the outer cortical cell layer directly adjacent to it. The boundary cell layers of uninfected cells between the cortex and the central tissue, like the central tissue itself, appear to contain no ENOD2 transcript (Figure 2E and F).

We also studied the appearance of ENOD2 transcript during nodule development in soybean. The earliest stage that we investigated was 6 days after sowing and inoculation. At this stage small bumps just become visible on the main root indicating the presence of nodule primordia. The primordia of the determinate nodule type originate in the outer cell layers of the root cortex. At 6 days cell divisions have also been induced in the inner cell layers of the root cortex and the central part of these dividing cells is developing into vascular tissue that connects the root nodule with the central cylinder of the root (Figure 4A and C). At 6 days the soybean ENOD2 messenger is detectable in the newly formed tissue surrounding the procambial strand between the primordium and the root central cylinder and in inner cortical cells at the proximal and lateral sides of the nodule primordium (Figure 4B and D). In a 10 day old plant the globular meristem has further developed into a central and a cortical tissue (Figure 4E). The ENOD2 gene is expressed in the nodule inner cortex as well as in the tissue surrounding the vascular strand that connects the nodule with the central cylinder (Figure 4E and F). At this stage the inner cortex at the distal part of the nodule already contains the ENOD2 messenger, albeit still at a lower level than in the proximal part of the nodule (Figure 4E and F). In nodules from 21 day old plants similar amounts of the ENOD2 transcript are present in all parts of the nodule inner cortex (cf. Figure 2C,D).

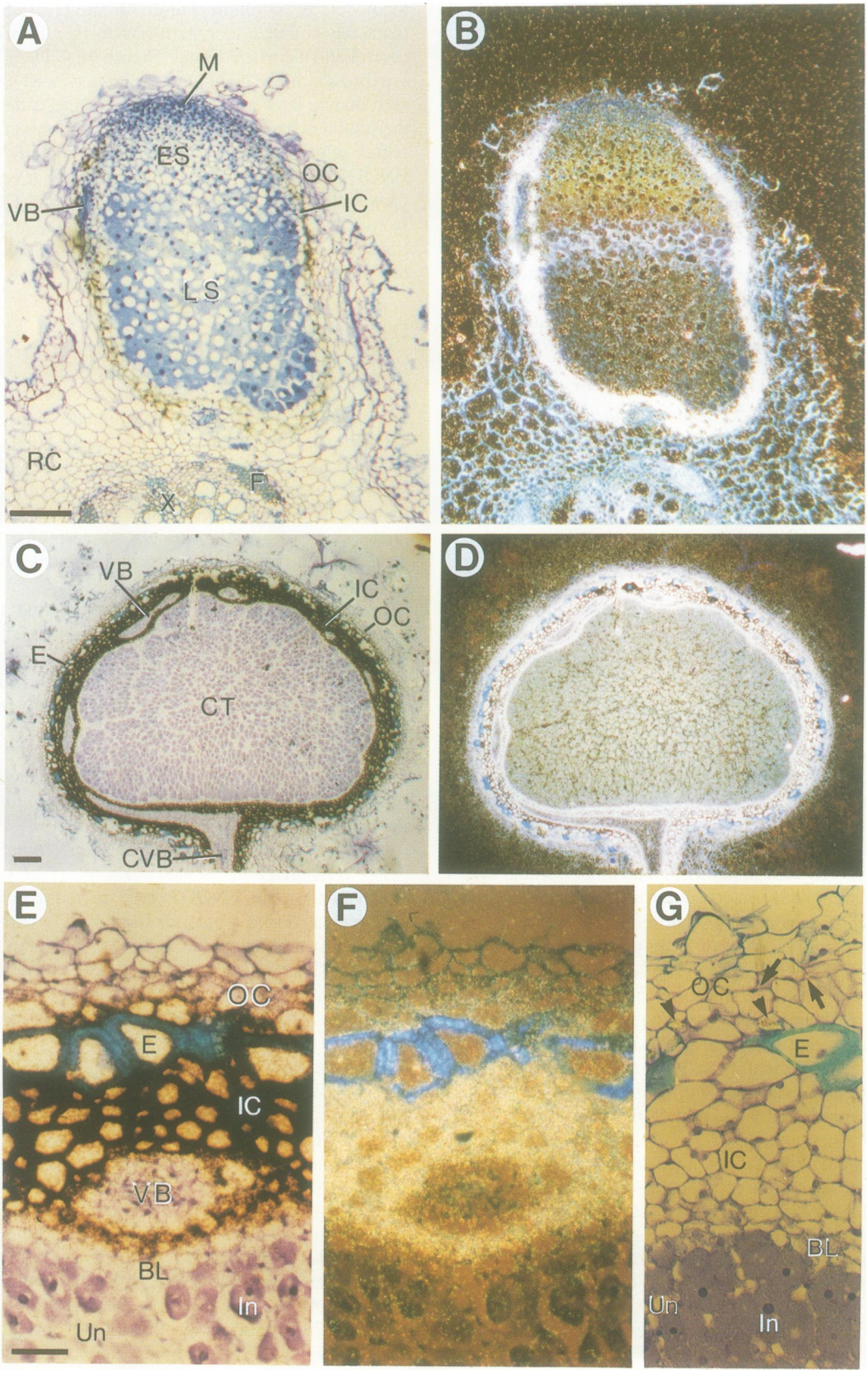
Discussion

In this paper we have presented evidence that the early nodulin gene ENOD2 is specifically expressed during the formation of the tissue in determinate as well as in indeterminate root nodules that has so far been described as the inner cortex. Moreover, the occurrence of homologous ENOD2 genes encoding polypeptides with a conserved structure in different legume species (Franssen *et al.*, 1987; Dickstein *et al.*, 1988) strongly suggests that the ENOD2 protein has a role in the function of this root nodule tissue (Govers *et al.*, 1989).

In earlier studies we have demonstrated that in both soybean and pea the ENOD2 gene is expressed during early stages of nodule morphogenesis (Govers *et al.*, 1986; Franssen *et al.*, 1987). Besides, it has been shown that in soybean and alfalfa this early nodulin gene is expressed in so-called empty nodules that contain neither infection threads nor intracellular bacteria (Franssen *et al.*, 1987; Dickstein *et al.*, 1988). Such empty nodules are elicited on legume roots by certain *Rhizobium* and *Bradyrhizobium* strains and mutants (Finan *et al.*, 1985; Franssen *et al.*, 1987) and by *Agrobacterium* strains carrying the *Rhizobium meliloti nod* genes (Hirsch *et al.*, 1985; Truchet *et al.*, 1985). The expression of the ENOD2 gene in these empty nodules strongly suggested a role for the ENOD2 early nodulin in the formation of the nodule structure and not in the infection process. This conclusion has now been consolidated by our finding that the ENOD2 gene is specifically expressed upon differentiation of the nodule meristem into inner cortical cells.

Root nodules are organs with a histological organization that is markedly different from roots. Nevertheless since one originates from the other these two organs might share homologous tissues. Thus, the names nodule inner cortex and root inner cortex suggest that these two tissues are closely related. However, by definition, the root cortex is inwardly delimited from the central cylinder by the endodermis. In nodules only what has hitherto been called the outer cortex has a similar position as the root cortex and is also connected with it at the base of the nodule. In contrast, what has hitherto been called the inner cortex has no positional relationship with the root cortex: it is located inside the nodule endodermis and around the vascular strands. In other plant parts, notably the stem, the cortex is also, by definition,

always located outside the vascular system and, to our knowledge, never surrounding individual vascular strands. In addition, the morphology of the nodule inner cortical cells distinguishes this tissue from root cortical tissues. The nodule inner cortical cells have fewer and smaller intercellular spaces than most other cortical cells (Figure 2G, see also Tjepkema and Yocum, 1974 and Witty *et al.*, 1986). Also at the molecular level the nodule inner cortex is different



from the root cortex as we showed that at least one nodulin gene is specifically expressed during the formation of the nodule inner cortex. So both from an anatomical and a molecular point of view the name nodule inner cortex is misleading. Therefore we propose to consider this tissue as nodule parenchyma, while the nodule outer cortex can properly be described as nodule cortex. In determinate nodules the tissue that surrounds the vascular bundle connecting the nodule and the root central cylinder is morphologically very similar to the nodule parenchyma (see below). In addition, the ENOD2 gene is expressed in both tissues. Therefore we propose to also consider the tissue surrounding the connecting vascular bundle as nodule parenchyma.

The determination of the nucleotide sequence of the cloned pea ENOD2 cDNA and the amino acid sequence derived from it allow a comparison with the structures of the soybean and alfalfa ENOD2 proteins that have been determined previously (Franssen *et al.*, 1987; Dickstein *et al.*, 1988 respectively). The pea ENOD2 protein appears to be composed of the same two repeating pentapeptides as the soybean ENOD2 protein or variants of these sequences with one amino acid replacement. However, whereas in the soybean ENOD2 protein the repeating elements occur alternately, in the pea ENOD2 protein two ProProHisGluLys repeats are alternated with the ProProGluTyrGln element. The latter organization also occurs in the alfalfa ENOD2 polypeptide, in which the same pentapeptides are present. This difference in structure between the soybean and pea/alfalfa ENOD2 proteins respectively, suggests that the amino acid composition of the pentapeptides might be the main requirement for the function of the ENOD2 protein. A specific organization of the repeating elements seems less essential. The different distribution of the two pentapeptides in soybean and pea/alfalfa respectively, might indicate that independent duplication events involving different basic polypeptide units gave rise to the different ENOD2 genes during the evolution of these legumes. However, more sequence data from a wider variety of legumes will be needed to substantiate this hypothesis.

The amino acid sequence of both the pea and soybean ENOD2 protein strongly resembles the recently described soybean protein 1A10 that occurs in cell walls of the axis tissue of germinating soybean seeds (Averyhart-Fullard *et al.*, 1988). This glycoprotein consists of at least 40 repeating ProProValTyrLys units and ~50% of the prolines are hydroxylated to hydroxyproline. Because of this similarity in structure it is very likely that ENOD2 is also a hydroxyproline-rich cell wall protein. Together with the carrot P33 protein, the 1A10 and ENOD2 proteins seem to form a new class of cell wall proteins that are composed of pentapeptides containing two prolines. Their low Ser content forms a major difference with another important group of hydroxyproline-rich cell wall proteins, the extensins, which are characterized by (Hyp)4Ser-pentapeptide repeats (Cassab and Varner, 1988). Sequence analysis of two soybean ENOD2 genes revealing that a putative signal peptide is present at the N terminus of the ENOD2 protein lends further support to the hypothesis that ENOD2 represents a cell wall protein (Franssen *et al.*, 1988).

The nodule parenchyma ('inner cortex') appears to be an important tissue in the *Rhizobium*–legume symbiosis. The free oxygen concentration in a nodule shows a sharp decline across the nodule parenchyma to a very low value in the central tissue, which is a necessity to protect the extremely oxygen-sensitive nitrogen-fixing enzyme nitrogenase (Tjepkema and Yocum, 1974; Witty *et al.*, 1986). It was shown that this decline must be due to a high consumption rate of oxygen by the rhizobia in the infected cells of the central tissue combined with a diffusion barrier residing in the nodule parenchyma (Witty *et al.*, 1986). As oxygen diffusion through air is $\sim 10^4$ times faster than through water, it is very likely that in nodules oxygen diffusion occurs through the intercellular spaces. As mentioned above, the nodule parenchyma contains relatively few and small intercellular spaces. In contrast, in both ('outer') cortex and central tissue relatively wide intercellular spaces occur. By this specific morphology the nodule parenchyma will be able to form the oxygen diffusion barrier (Tjepkema and Yocum, 1974; Witty *et al.*, 1986). Since the differentiation of the cell wall will be a factor in determining tissue morphology, we propose that the putative cell wall protein ENOD2 is contributing to this special morphology of the nodule parenchyma.

In soybean the ENOD2 gene also appears to be expressed in the cells that surround the vascular strand connecting the nodule with the central cylinder of the root. In pea such a long connecting vascular bundle is lacking, since here the nodule originates more closely to the central cylinder. The cells surrounding the connecting vascular strand are morphologically similar to the nodule parenchyma of the nodule, i.e. they have relatively few and small intercellular

Fig. 2. Localization of ENOD2 transcripts by *in situ* hybridization in pea (A,B) and soybean (C–G) nodules. (**A**) Bright field micrograph of a longitudinal section through a nodule from a 20 day old pea plant. In the nodule from the top to the base, the apical meristem (M), and early (ES) and late (LS) symbiotic growth stages of the central tissue can be discerned. Over the nodule inner cortex (IC) an autoradiographic signal of black silver grains is present. No signal can be observed over the nodule outer cortex (OC) nor the vascular bundle (VB). The nodule endodermis cannot be easily recognized in this section, since in pea the endodermis does not sclerify like in maturing soybean nodules (Figure 2C and E). At the base of the nodule, part of the root is visible in transversal section. Here the cortex (RC), and a group of phloem fibers (F) and a xylem pole (X) of the central cylinder are indicated. Bar represents 200 μm. (**B**) Dark field micrograph of the same section as in (A) showing the autoradiographic signal as white grains. (**C**) Bright field micrograph of a longitudinal section through a nodule from a 21 day old soybean plant. The central tissue (CT) is completely surrounded by an inner cortex (IC) over which an autoradiographic signal of black silver grains can be observed. This signal continues over the tissue surrounding the vascular bundle (CVB) that connects the nodule to the central cylinder of the root. E, endodermis; other abbreviations as in (A). Bar represents 200 μm. (**D**) Dark field micrograph of the same section as in (C) showing the autoradiographic signal as white grains. (**E**) Bright field micrograph of a detail of a section through the same nodule as in (C). From top to bottom the outer cortex (OC), the sclerified endodermis (E), the inner cortex (IC) with a vascular bundle (VB), the boundary layer (BL), and the infected (In) and uninfected (Un) cells of the central tissue can be discerned. Bar represents 50 μm. (**F**) The same detail as shown in (E), photographed with a combination of bright field and epipolarization illumination. A strong autoradiographic signal of white grains is visible over the inner cortex. A lower signal is present over the endodermis and the adjacent layer of the outer cortex. (**G**) Detail of a glycolmethacrylate section through a 21 day old soybean nodule showing the same tissues at the same magnification as in (E) and (F). Abbreviations as in (E). The arrows indicate intercellular spaces and the arrowheads calcium oxalate crystals in the outer cortex (OC).

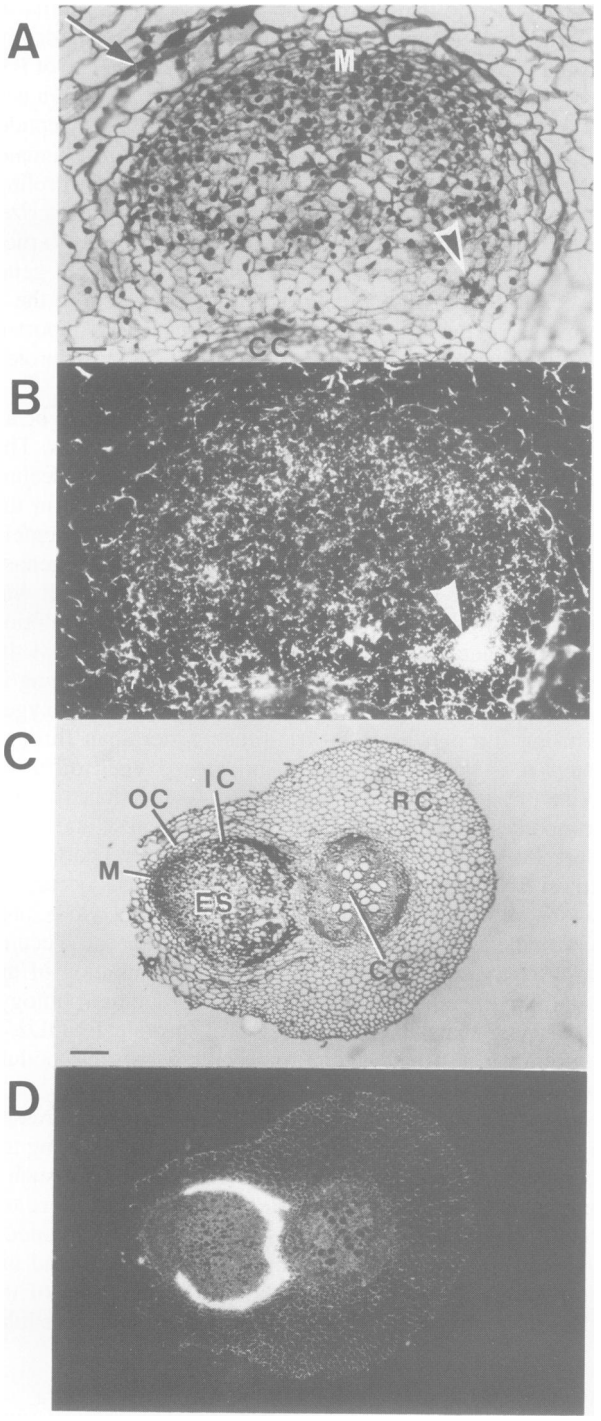


Fig. 3. Localization of ENOD2 transcripts by *in situ* hybridization during nodule development in pea. In the dark field micrographs (B) and (D), which correspond to the bright field micrographs (A) and (C) respectively, the autoradiographic signal is visible as white grains. **(A)** Detail of a transection through an 8 day old root showing a nodule primordium with an apical meristem (M). The arrow points to part of the infection thread that has grown through the root cortex to the primordium. A few inner cortical cells containing an autoradiographic signal of black silver grains are indicated by the large arrowhead. CC, central cylinder of the root. Bar represents 50 μm. **(B)** The autoradiographic signal over the inner cortical cells is indicated by the arrowhead. **(C)** Transection through a root with a 10 day old nodule. The autoradiographic signal of black silver grains is visible over the inner cortex (IC). CC, central cylinder of the root; RC, root cortex; M, apical meristem of the nodule; ES, early symbiotic growth zone of the nodule central tissue; OC, nodule outer cortex. Bar represents 250 μm.

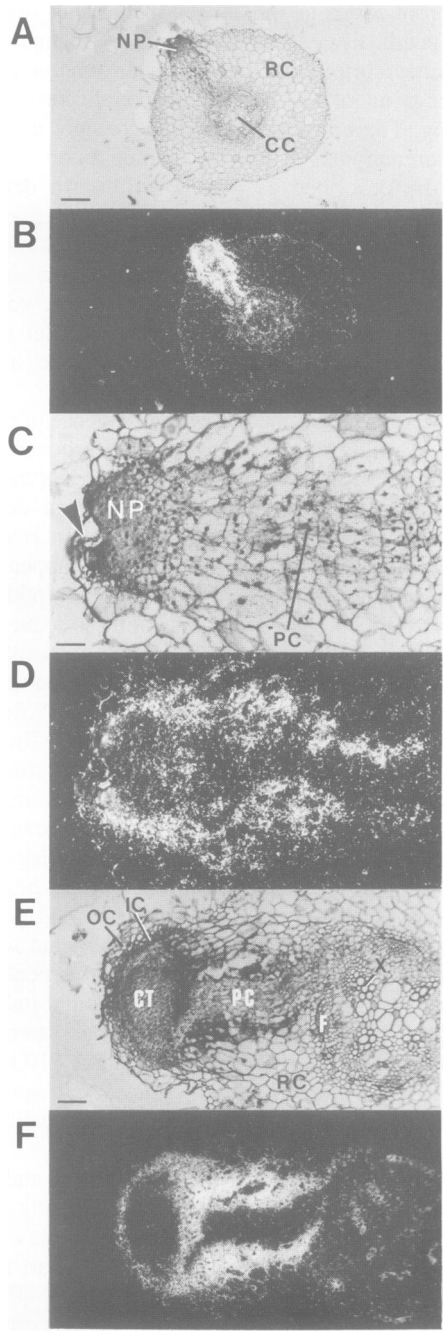


Fig. 4. Localization of ENOD2 transcripts by *in situ* hybridization during nodule development in soybean. In the dark field micrographs (B), (D) and (F), which correspond to the bright field micrographs (A), (C) and (E) respectively, the autoradiographic signal is visible as white grains. **(A)** Transection through a 6 day old root with a nodule primordium (NP); CC, central vascular cylinder; RC, root cortex. Bar represents 250 μm. **(C)** Detail of the root in (A) showing the nodule primordium (NP) and the procambial strand (PC) between the primordium and the central vascular cylinder of the root. The arrowhead indicates an infection thread in the basal part of a root hair cell. Bar represents 50 μm. **(D)** Shows the autoradiographic signal over the newly developed tissue surrounding the procambial strand and over the developing inner cortical cells in the lateral and basal parts of the nodule primordium. **(E)** Detail of a transection through a 10 day old root showing a nodule with the procambial strand (PC) connecting the nodule to one of the xylem poles (X) of the central vascular cylinder. An autoradiographic signal of black silver grains is visible over the inner cortex (IC) and the tissue surrounding the procambial strand. CT, central tissue of the nodule; OC, nodule outer cortex; F, a group of phloem fibers in the central vascular cylinder of the root; RC, root cortex. Bar represents 100 μm.

spaces. This is consistent with the idea that the ENOD2 gene product can contribute to cell morphology. There are, however, no experimental data indicating that this tissue has a function similar to the nodule parenchyma that surrounds the central tissue.

Materials and methods

Growth conditions for plants

Soybean plants [*Glycine max* (L.) Merr. cv. Williams] and pea plants [*Pisum sativum* (L) cv. Rondo] were cultured as described before (Bisseling *et al.*, 1978; Franssen *et al.*, 1987). At the time of sowing the soybean seeds were inoculated with *Bradyrhizobium japonicum* USDA110 and the pea seeds with *Rhizobium leguminosarum* biovar. *viciae* (PRE).

Isolation and sequencing of pPsENOD2

A λgt11 cDNA library against RNA from root nodules of *P.sativum* (L.) cv. Sparkle was kindly provided by Dr G.Corruzi (Tigney *et al.*, 1987). Nitrocellulose replicas from plates containing 2000 plaques were made using standard procedures (Maniatis *et al.*, 1982). The plaques were screened with nick translated (Maniatis *et al.*, 1982) insert from the soybean cDNA clone pGmENOD2 (Franssen *et al.*, 1987). Phage DNA purification, insert isolation and cloning in pUC18 were performed according to standard procedures (Maniatis *et al.*, 1982). Both strands of the pPsENOD2 insert were sequenced using the chemical degradation method (Maxam and Gilbert, 1980).

In situ hybridization

The *in situ* hybridizations were performed essentially as described by Cox *et al.* (1984), following a protocol kindly provided by Drs M.van Montagu and G.Engler. Nodules were fixed with 3% paraformaldehyde and 0.25% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2, at room temperature (RT), dehydrated in graded ethanol and xylene series and embedded in paraplast. Sections, 7 μm thick, were attached to poly-L-lysine-coated slides. Sections were deparaffinized with xylene and rehydrated through a graded ethanol series. They were subsequently pretreated with 1 μg/ml proteinase K in 200 mM Tris−HCl, pH 7.5, 2 mM $CaCl_2$ at 37°C for 30 min and with 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0, at RT for 10 min, dehydrated in a graded ethanol series and air dried. Sections were hybridized with antisense RNA probes, which were made by transcribing pT7 clones (a kind gift from Dr S.Tabor) containing the inserts of pPsENOD2 and pGmENOD2.

The antisense RNA probes were radioactively labeled with [^{35}S]UTP (1000−1500 Ci/mmol, NEN). The probes were partially degraded to a length of 150 nucleotides by heating at 65°C in 0.2 M Na_2CO_3/0.2 M $NaHCO_3$. Sections were hybridized with RNA probes in 50% formamide, 0.3 M NaCl, 10 mM Tris−HCl, pH 7.5, 1 mM EDTA, 10% dextran sulfate, 1 × Denhardt's, 70 mM DTT at 42°C for 16 h. After washing three times in 4 × SSC, 5 mM DTT at 20°C, slides were treated with 20 μg/ml RNase A in 0.5 M NaCl, 10 mM Tris−HCl, pH 7.5, 5 mM EDTA at 37°C for 30 min and washed in the same buffer with 5 mM DTT at 37°C for 30 min. The final wash consisted of two times 2 × SSC, 1 mM DTT at RT. Slides were dehydrated in graded ethanols (each with 300 mM ammonium acetate) and 100% ethanol. After air drying, slides were coated with Kodak NTB2 nuclear emulsion diluted 1:1 with 600 mM ammonium acetate and exposed for 1−3 weeks at 4°C. They were developed in Kodak D19 developer for 3 min and fixed in Kodak Fix. Sections were stained with 0.025% toluidine blue 0 for 5 min and mounted with DPX.

For embedding in glycolmethacrylate resin nodules were fixed with 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2, for 3 h. After dehydration in a graded ethanol series the nodules were embedded in Technovit resin according to the manufacturer's instructions (Kulzer, Friedrichsdorf, FRG). Sections 4 μm thick were stained with 1% toluidine blue 0 for 1 min and mounted with Euparal.

Sections were photographed with a Nikon microscope equipped with dark field and epipolarization optics.

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