23

FUNCTION AND REGULATION OF THE EARLY NODULIN GENE ENOD2

FRANCINE GOVERS, HENK J. FRANSSEN, CORNÉ PIETERSE, JEROEN WILMER and TON BISSELING
Department of Molecular Biology, Agricultural University, Wageningen, The Netherlands

Root nodule formation and nodulin gene expression

The symbiosis between (brady)rhizobia and legumes is known to proceed through a series of characteristic stages. The pre-infection stage includes recognition of two symbiotic partners, attachment of bacteria to the plant roots and deformation of root hairs. In the next stage, deformed root hairs are invaded by bacteria which grow and penetrate within a unique plant-derived infection thread. At the same time cell division is initiated in root cortical cells and this elaborates the formation of meristems. Infection threads grow towards these meristems and a nodule structure is formed wherein differentiation of tissue types occurs. From the growing infection thread bacteria are released into some of the newly formed nodule cells. Finally, the cooperation of nodule cells and differentiated bacteroids leads to a nitrogen fixing plant organ (Vincent, 1980).

The establishment and functioning of such an organ requires the specific expression of a number of genes from both partners. The plant genes, called nodulin genes, are developmentally regulated. Some of these are expressed in stages preceding actual nitrogen fixation when nodule formation is in progress. ENOD2 falls into this category of early nodulin genes. Late nodulin genes, such as the leghaemoglobin genes, are first expressed when the nodule structure is complete and when nitrogen fixation begins.

ENOD2 is an early nodulin

The first ENOD2 cDNA clone to be isolated, pGmENOD2, was selected from a soyabean (*Glycine max*) nodule cDNA library (Franssen *et al.*, 1987). This library was differentially screened with probes made from 10-day-old nodule RNA and root RNA. Most of the clones that hybridized specifically to the nodule probe, appeared to represent the same gene which was named ENOD2 (Early NODulin 2). Northern blot analysis showed that ENOD2 mRNA is first detectable in soyabean roots of 8-day-old plants that were inoculated at day 0 with *Bradyrhizobium japonicum*. At day 10 small nodule bumps are visible on the roots and by then the accumulation of ENOD2 mRNA has reached its maximum. In comparison with the other known soyabean nodulin genes ENOD2 is the earliest

activated nodulin gene. Expression starts at least 4 days prior to the expression of the late nodulin genes.

ENOD2 is extremely proline rich

DNA sequence analyses of the cDNA clone pGmENOD2 and genomic GmENOD2 clones revealed that the encoded protein has a very high proline content (Franssen *et al.*, 1987; 1989). The protein has an *N*-terminal part with the typical features of a signal peptide. Forty-four per cent of the rest of the protein consists of proline residues which are organized in two repeating pentapeptides (Table 23.1).

Table 23.1 COMPARISON OF CHARACTERISTIC AMINO ACID REPEAT UNITS[a] BETWEEN ENOD2 FROM SEVERAL LEGUMES AND TWO OTHER PROLINE-RICH PROTEINS

Soyabean ENOD2[b]	PRO-PRO-GLU-TYR-GLN	PRO-PRO-HIS-GLU-LYS
Pea ENOD2[c]	PRO-PRO-GLU-TYR-GLN	PRO-PRO-HIS-GLU-LYS
Alfalfa ENOD2[d]	PRO-PRO-GLU-TYR-GLN	PRO-PRO-HIS-GLU-LYS
Sesbania rostrata ENOD2[e]	PRO-PRO-TYR-GLU-LYS	PRO-PRO-HIS-GLU-LYS
Soyabean SbPRP1, 1A10[f]	PRO-PRO-VAL-TYR-LYS	PRO-PRO-ILE-TYR-LYS
Carrot p33-PRP[g]	PRO-PRO-VAL-TYR-THR	PRO-PRO-VAL-HIS-LYS

[a] Predicted from DNA sequences.
[b] Franssen *et al.*, 1987.
[c] Van de Wiel *et al.*, 1990.
[d] Dickstein *et al.*, 1988.
[e] Strittmatter *et al.*, 1989.
[f] Hong, Nagao and Key, 1987; Averyhart-Fuller *et al.*, 1988.
[g] Chen and Varner, 1985b.

This high proline content has a severe impact on the migration behaviour of ENOD2 on SDS-polyacrylamide gels. In hybrid released translation experiments (Franssen *et al.*, 1987), RNA selected by pGmENOD2 and translated in a cell free system with [^{35}S]-methionine as radioactive precursor gives rise to a protein with an apparent molecular weight of 75 000 and an isoeletric point of 6.5. With [^{3}H]-leucine in the translation mixture two proteins are visualized, one of which is identical to the ^{35}S-labelled protein whereas the other, more prominent, polypeptide is more basic but has the same molecular weight. The two ^{3}H-labelled proteins were previously, in independent experiments, identified as early nodulins on two-dimensional gels of *in vitro* translation products of soyabean nodule RNA. They were named Ngm-75 (Gloudemans *et al.*, 1987). Calculation of the molecular weight of ENOD2 from the deduced amino acid sequence results in a protein of 45 000 molecular weight and this is in agreement with the coding capacity of the mRNA which is 1200 nucleotides in length.

In all proline-rich plant proteins that have been analysed to date, one-half or more of the proline residues are hydroxylated and the overall protein is usually glycosylated. Moreover, all known hydroxyproline-rich glycoproteins (HRGPs) are cell wall components. As yet no conclusive evidence has been presented

demonstrating that ENOD2 is also such a HRGP. However, there are at least three reasons to assume that ENOD2 is a cell wall protein which is hydroxylated and glycosylated *in vivo*.

First, the pentapeptide repeats in ENOD2 have a striking similarity with pentapeptide repeats found in a proline-rich cell wall protein designated 1A10 that was isolated from soyabean by Averyhart-Fuller, Datta and Marcus (1988) (*see* Table 23.1). The deduced amino acid sequence from a partial cDNA clone p1A10 was compared with the amino acid distribution of the isolated cell wall protein and it was shown that one-half of the prolines in 1A10 are hydroxylated and that 1A10 is a glycoprotein. A soyabean gene designated SbPRP1 and encoding a protein with pentapeptide repeats homologous to those found in 1A10 has been analysed by Hong, Nagao and Key (1987). As with ENOD2, SbPRP1 has an *N*-terminal hydrophobic region which is a putative signal peptide, it has no serines but a relatively high content of basic amino acids and the region with the repeats is very hydrophilic.

Second, the hydrophobic signal peptide and the hydrophilic regions with hydroxyprolines organized in pentapeptide repeats are also found in the cell wall proteins extensin and the carrot p33-PRP (Chen and Varner, 1985a,b). A signal peptide is necessary for transport of the newly formed proteins through the cell membrane to reach the cell wall. For extensin, which is the best studied cell wall protein, the post-translational processing pathways resulting in a secreted HRGP have been elucidated (reviewed by Cooper, 1988). Subsequently Varner and coworkers (Cooper, 1988) have shown that soluble extensins are slowly rendered insoluble following excretion into the cell wall. Intra- and intermolecular crosslinks are formed due to the presence of tyrosine derivatives and carbohydrate sidechains on the hydroxyproline residues. This results in a three-dimensional glycoprotein network wherein cell wall polysaccharides are entangled. ENOD2, SbPRP1 and carrot p33-PRP have the same basic components as extensin. Therefore these proteins may be post-translationally modified and translocated in the same way as extensin.

The third plea in support of a hydroxyproline-rich ENOD2 is the report of Cassab (1986) who showed that the cortex of soyabean root nodules has a very high hydroxyproline content which is mainly localized in the cell wall. Recently it was shown by hybridization *in situ* that the ENOD2 mRNA is specifically localized in the inner cortex of the root nodule (Van de Wiel *et al.*, 1990) and this supports the idea that at least a part of the cell wall hydroxyproline is derived from ENOD2.

ENOD2 is highly conserved in legumes

The soyabean cDNA clone pGmENOD2 has been used as a probe to screen nodule RNA from other legumes. All legumes studied to date express a nodulin gene that is highly homologous to the soyabean ENOD2 gene. In nodule RNA isolated from pea (*Pisum sativum*, Govers *et al.*, 1986), white clover (*Trifolium repens*), bird's foot trefoil (*Lotus corniculatus*), vetch (*Vicia sativa*, Moerman *et al.*, 1987), alfalfa (*Medicago sativa*, Dickstein *et al.*, 1988), *Medicago truncatula* (D. Barker, personal communication), common bean (*Phaseolus vulgaris*, Sanchez *et al.*, 1988) and *Sesbania rostrata* (Strittmatter *et al.*, 1989) ENOD2 mRNAs have been detected. The striking difference between ENOD2 mRNAs in different plant species is their length. The smallest ENOD2 mRNA, ± 1200 bases, is found in soyabean, whereas

the largest ENOD2 mRNA, ± 2500 bases, is observed in white clover and bird's foot trefoil. If this increase in length is only due to an increase in the number of repeats or to larger non-coding regions then this will probably not affect the functioning of ENOD2.

ENOD2 cDNA clones have been isolated and sequenced from pea (Van de Wiel *et al.*, 1990), alfalfa (Dickstein *et al.*, 1988) and *Sesbania rostrata* (Strittmatter *et al.*, 1989). As shown in Table 23.1 their predicted amino acid sequences have characteristic pentapeptide repeats which always begin with two proline residues and strongly resemble the pentapeptide repeats from soyabean ENOD2. The strong conservation of ENOD2 genes in legumes is restricted to the region where the pentapeptide repeats are encoded. Sequence comparison has shown that there is no significant homology between the 3' non-coding region of soyabean and pea ENOD2 (Van de Wiel *et al.*, 1990), and soyabean and *Sesbania rostrata* ENOD2 (Strittmatter *et al.*, 1989). On the contrary, two soyabean ENOD2 genes are exactly identical in the 3' non-coding region (Franssen, 1989) and the degree of homology between two *Sesbania rostrata* ENOD2 cDNA clones is even higher in the 3' non-coding than in the coding region (Strittmatter *et al.*, 1989).

The function of ENOD2

To obtain further insight into the function of ENOD2, ENOD2 gene expression has been analysed in a variety of aberrant nodules or nodule-like structures. In general, rhizobia mutated in *nif* or *fix* genes induce the formation of nodules which are morphologically indistinguishable from wild-type nodules. *Rhizobium meliloti exo⁻* mutants and some *Agrobacterium* transconjugants carrying the *sym* plasmid or the *nod* region of the *sym* plasmid induce the formation of so-called empty nodules (Finan *et al.*, 1985; Hirsch *et al.*, 1985; Govers *et al.*, 1986). Empty nodules have most of the morphological characteristics of wild-type nodules, i.e. vascular bundles in the periphery of the nodule, cortical cell layers and a central region. However, in the central region the plant cells lack bacteria and mostly no infection threads are found. Nodule-like structures do not have infected cells nor peripherally located vascular bundles. These structures, which are induced on soyabean upon inoculation with *R. fredii* USDA257, arise from a combination of cell swellings and randomly orientated cortical cell divisions (Franssen *et al.*, 1987). In all *fix⁻* nodules, empty nodules and nodule-like structures induced on a variety of plants, ENOD2 genes are expressed (pea, Govers *et al.*, 1986, 1987; alfalfa, Dickstein *et al.*, 1988; *Vicia sativa*, Moerman *et al.*, 1987; soyabean, Franssen *et al.*, 1987 and common bean, Sanchez *et al.*, 1988). All these different nodule types have one factor in common, they all passed through one of the first developmental steps in root nodule organogenesis, that is cell division in root cortical cells and development in certain tissue types of the nodule. Because ENOD2 mRNA is specifically localized in the inner cortical cells, the function of ENOD2 might be related to the morphogenesis of the inner cortex. The observation that ENOD2 mRNA is first detectable in cells that differentiate from meristematic cells to inner cortex cells supports this suggestion (Van de Wiel *et al.*, 1990). Moreover, ENOD2 has the characteristics of a cell wall protein and it is likely that it represents a specific cell wall protein in the inner cortex.

What then is the function of ENOD2 in the inner cortex of root nodules? A root nodule is a plant organ wherein a strict regulation of the O_2 concentration is a

prerequisite for proper functioning of the enzyme nitrogenase. The plant manages to regulate the O_2 concentration in nodules by raising a barrier against O_2 diffusion from the atmosphere to the bacteroids. Within this barrier the O_2 concentration is low and the oxygen binding protein leghaemoglobin is thought to facilitate the transport of O_2 at low O_2 concentration. Measurements with O_2-specific microelectrodes have shown that the inner cortex is the major site of resistance to gaseous diffusion (Witty *et al.*, 1986). Inner cortical cells are more densely packed in comparison with the loosely packed cells of the outer cortex. Dense cell packing in the inner cortex might require extra or other cell wall material of which, possibly, ENOD2 is a specific component. The function of ENOD2 is thus related to the function of the inner cortex.

Regulation of ENOD2 gene expression

A fascinating aspect of the *Rhizobium*-legume symbiosis is the coordination of gene regulation of two interacting symbionts. This is undoubtedly accompanied by an exchange of signals between the two organisms. Since nodule formation is a multistep process, it is conceivable that there is a continuous flow of different signals in successive stages of the symbiosis. In the elucidation of the regulation of nodulin gene expression by signals from *Rhizobium*, bacterial mutants are essential. In all empty nodules induced by *Rhizobium exo⁻* mutants and *Agrobacterium* transconjugants ENOD2 genes are expressed (*see* previous section) and this led to the conclusion that genes located on the *nod* region of the *sym* plasmid are responsible for the induction of ENOD2 genes (Nap, Van Kammen and Bisseling, 1987). It is well established that the *nod* genes are also required for the earliest stages of nodule development, i.e. root hair curling and cortical cell divisions. It is, however, unknown how the *nod* gene products induce these processes. Although the functions of the *nod* gene products have yet to be determined it seems plausible that the *nod* genes either produce one or more signals or are involved in the production of signals that trigger nodule development in the plant. With regard to the induction and regulation of ENOD2 genes, the question arises whether substances produced by *Rhizobium* directly activate ENOD2 genes or whether ENOD2 gene expression is one of the targets of a signal transduction pathway that in the earliest stages of nodule development is initiated by *Rhizobium* signals. Another question is whether the same activation or the same initiation of a pathway can be achieved by certain compounds in the absence of rhizobia.

Recently Hirsch *et al.* (1989) presented evidence that the latter is indeed possible. They repeated experiments described four decades ago in which substituted benzoic acids were used to induce the formation of nodule-like structures on legume roots. *N*-1-naphthylphthalamic acid (NPA) and 2,3,5-triidobenzoic acid (TIBA) are compounds that presumably modify hormone levels in the plant by inhibiting the polar transport of auxins. Hirsch *et al.* (1989) found that nodules which are formed upon adding either TIBA or NPA to alfalfa roots are morphologically similar to empty nodules that are induced by *R. meliloti exo⁻* mutants. Moreover, expression of ENOD2 genes is observed in the TIBA- or NPA-elicited nodules and the ENOD2 mRNA concentration is more or less the same as the concentration present in *exo⁻* nodules. The expression of ENOD2 genes in the absence of *Rhizobium* suggests that ENOD2 genes are developmentally regulated and that *Rhizobium* is able to control the plant's developmental

programme. The resemblance between *Rhizobium exo⁻* nodules and TIBA- or NPA-induced nodules leads to the speculation that *Rhizobium* initiates nodule development by changing the auxin/cytokinin ratio in root cortical cells which then causes cell division, followed by differentiation of cells and ENOD2 gene expression in inner cortical cells. Whether endogenous auxins and cytokinins are indeed present in a specific ratio in those cells where ENOD2 genes are activated, and whether this by itself is sufficient for the induction of ENOD2 gene expression are still open questions.

ENOD2 gene expression in transgenic plants

Another approach which has been successfully used in elucidating the regulation of various plant genes is making use of transgenic plants. For analysing ENOD2 gene expression we have transformed leguminous and non-leguminous plants with a soyabean ENOD2 gene. In our laboratory, two soyabean ENOD2 genes have been isolated from a genomic library and the genes, designated GmENOD2A and GmENOD2B, have been analysed (Franssen, *et al.*, 1989). The 5′ non-coding region up to 600 bp upstream of the start codon, the coding region and 400 bp 3′ non-coding region are exactly identical in both genes. Moreover, the nucleotide sequence of the coding region is 100% homologous to the cDNA clone pGmENOD2. On Southern blots of genomic soyabean DNA two fragments of the same length as those found in the two genomic clones hybridize to pGmENOD2. It was concluded that the soyabean genome contains two ENOD2 genes and that both genes have been cloned. A 4.5 kb soyabean genomic DNA fragment with the 1 kb coding region of GmENOD2A in the middle was introduced into a binary Ti-plasmid and conjugated to *Agrobacterium tumefaciens* LBA4404 and *Agrobacterium rhizogenes* LBA9402. The disarmed *A. tumefaciens* transconjugants were used to transform tomato, whereas the *A. rhizogenes* transconjugants, which have both a wild-type Ri-plasmid and the disarmed binary Ti-plasmid, were used to obtain hairy root cultures from white clover (*Trifolium repens*) and bird's foot trefoil (*Lotus corniculatus*).

Transgenic tomato plants, genotype MsK93 and MsK9, were regenerated from transformed leaf discs (Koornneef *et al.*, 1986). From five transgenic plants for which it was shown that one or more copies of the GmENOD2A gene are stably integrated in the genome, RNA was isolated from shoots, roots and callus derived from transgenic leaf or root segments. Northern blot analyses showed that the GmENOD2A gene is expressed in all tissues analysed (Figure 23.1). The length of the ENOD2 mRNA is the same in soyabean nodules and transgenic tomato plants, and primer extension analysis has shown that the startpoint of transcription is also identical. The amount of ENOD2 mRNA accumulating in transgenic roots, shoots and callus appears to be correlated to the number of integrated GmENOD2A genes, indicating that all introduced genes are constitutively expressed. Moreover, the overall ENOD2 mRNA concentration is higher in roots compared with shoots. If this indeed mirrors the transcription level of the GmENOD2A gene in the two different organs, then it is tempting to speculate that the expression is influenced by the endogenous auxin/cytokinin ratio which is different in roots and shoots.

Why the GmENOD2A gene is constitutively expressed in plants which do not interact with *Rhizobium* is not clear. One possible explanation is that in soyabean, expression of ENOD2 genes is normally repressed and that the repressor is
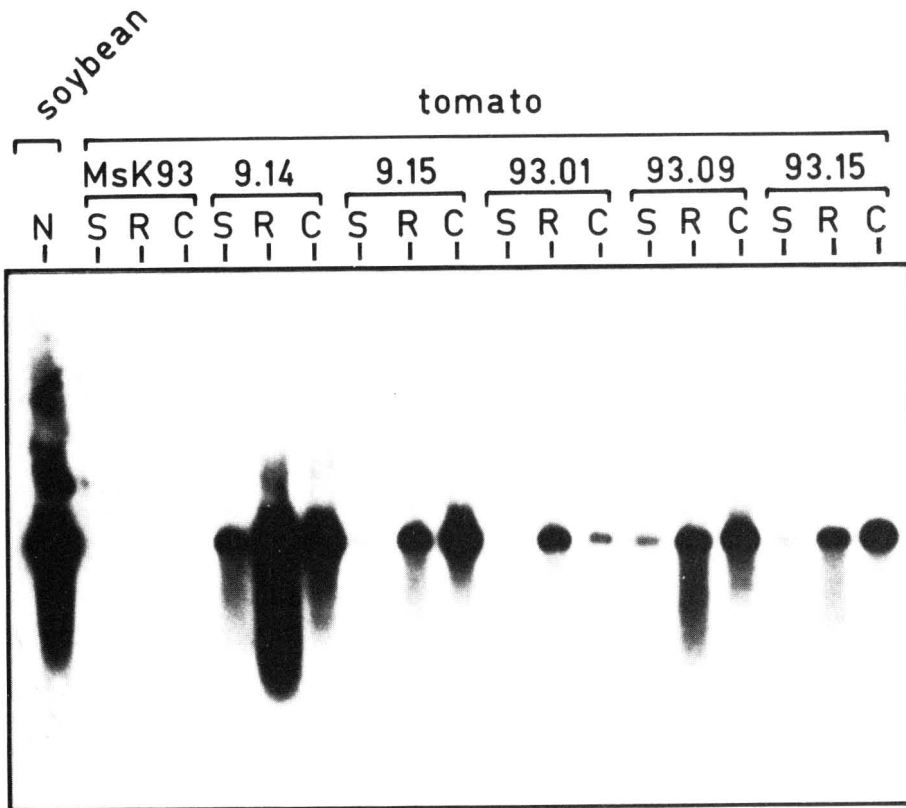
Figure 23.1 Expression of the GmENOD2A gene in transgenic tomato plants. Autoradiograph of a Northern blot containing RNA from 14-day-old soyabean nodules (N) and from shoots (S), roots (R) and callus (C) from wild-type tomato (genotype MsK93) and transgenic tomato plants (9.14, 9.15, 93.01, 93.09, 93.15). The blot was hybridized with the cDNA clone pGmENOD2. The length of the ENOD2 mRNA is 1200 bases

inactivated during nodule formation. In non-leguminous plants such as tomato this repressor might be absent. A second explanation is that the GmENOD2A gene is not the ENOD2 gene which is expressed in soyabean nodules. In soyabean it might be a silent gene because we have never observed ENOD2 gene expression in shoots, roots or callus. Although both genes, GmENOD2A and GmENOD2B are identical over a large region extending beyond the coding region, we have no evidence that both genes are indeed activated in soyabean nodules. Regulatory sequences further upstream from the conserved region might be required for nodule specific expression. The only experimental tool to determine this is analysis of nodule RNA from transgenic leguminous plants which contain the GmENOD2 genes. Therefore we regenerated transgenic *Lotus corniculatus* plants from hairy root cultures that were obtained upon infection of seedlings with *A. rhizogenes* LBA9402. By co-transformation of a binary Ti-plasmid, the GmENOD2A gene and the β-glucuronidase (GUS) gene fused to the cauliflower mosaic virus (CaMV) 35S promoter, were introduced. From GUS positive hairy root cultures transgenic

plants were regenerated according to Petit *et al.* (1987). Nodule, root and shoot RNA isolated from the transgenic plants was hybridized to a subclone of the 3′ non-coding region of pGmENOD2. This clone specifically recognizes soyabean ENOD2 RNA and does not cross-hybridize with ENOD2 RNA of *L. corniculatus*. On Northern blots no expression of GmENOD2A can be detected (Figure 23.2).
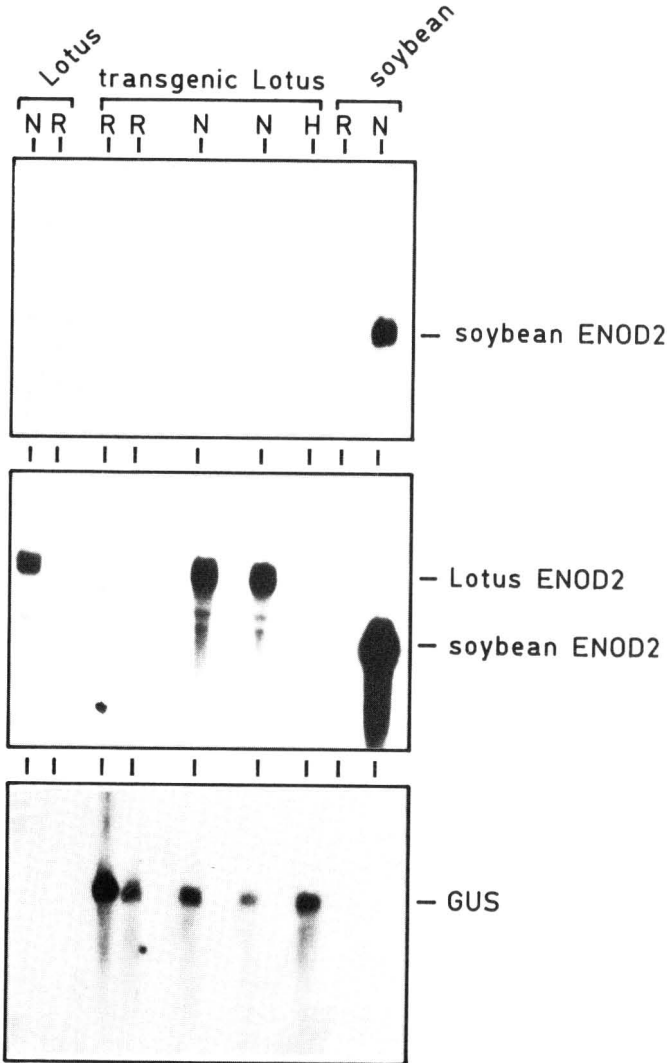
Figure 23.2 Expression analyses of the GmENOD2A gene in transgenic *Lotus corniculatus* plants. Autoradiographs of a Northern blot containing RNA from roots (R) and nodules (N) from wild-type *Lotus corniculatus* plants, transgenic *Lotus corniculatus* plants and wild-type soyabean plants. The lane marked with H contains RNA isolated from the hairy root culture from which the transgenic *Lotus* plants were regenerated. The same blot was hybridized subsequently with a subclone from the 3′ non-coding region of the soyabean cDNA clone pGmENOD2 which specifically recognizes soyabean ENOD2 mRNA (upper panel), with pGmENOD2 which hybridizes with soyabean ENOD2 mRNA (1200 b) and cross-hybridizes with *Lotus* ENOD2 mRNA (2500 b) (middle panel) and with a GUS probe (lower panel)

When the same Northern blot is hybridized to the complete cDNA clone pGmENOD2, nodule specific expression of the endogenous *Lotus* ENOD2 gene(s) is observed and hybridization with a GUS probe shows that the chimaeric 35S CaMV–GUS gene, which is located on the same T-DNA next to GmENOD2A, is expressed in all tissues of the transgenic plants (Figure 23.2). Apparently the signal that induces expression of *L. corniculatus* ENOD2 gene(s) is not able to induce expression of the soyabean ENOD2A gene. This might be due to the fact that either GmENOD2A, located on a 4.5 kb genomic soyabean DNA fragment, does not contain all the required regulatory sequences for nodule specific expression, or GmENOD2A is also not activated in nodules in soyabean. To investigate these possibilities transgenic *Lotus* plants transformed with the GmENOD2B gene have to be analysed. These experiments are still in progress (in collaboration with K. Marcker and co-workers in Åarhus and F. de Bruyn and co-workers in Cologne).

Because we have used the *A. rhizogenes* co-transformation system to obtain transgenic leguminous plants there is, besides the T-DNA of the binary Ti-plasmid, at least one other T-DNA integrated in the plant genome. That is the T_L-DNA from the *A. rhizogenes* Ri-plasmid. The *rol A, B, C* and *D* loci, which are involved in hairy root formation, are located on the T_L-DNA. Due to the integration of these loci, hairy root cultures can be cultivated on hormone-free medium. The presence of a third T-DNA, the T_R-DNA of the Ri-plasmid, is not essential for the growth of hairy root cultures nor for regeneration. However, this T-DNA, which harbours among others two genes involved in auxin synthesis, may be transferred as well (Birot *et al.*, 1987). It has been shown by Shen *et al.* (1988) that hairy roots of *Lotus corniculatus* are 100–1000 times more sensitive to auxin than normal roots. The most plausible explanation for this is that hairy roots have either an increased number of auxin receptors or an increased efficiency of the transduction system for the auxin signal. For the increased auxin sensitivity only the T_L-DNA-borne genes are responsible and not the T_R-DNA-borne genes. The integration of T_R-DNA and/or T_L-DNA in transgenic plants appears to be a major drawback for studying the regulatory mechanism controlling early nodulin genes. We have found that in several hairy root cultures of white clover and *Lotus corniculatus* endogenous clover or *Lotus* ENOD2 genes are expressed. So far we have only observed this phenomenon in hairy root cultures transformed with wild-type *A. rhizogenes* and co-transformed with micro-Ti-vectors. It will be interesting to see if expression of a transformed ENOD2 gene is coupled to expression of endogenous ENOD2 genes. The observation that ENOD2 genes can be activated in hairy root cultures supports the hypothesis that expression of ENOD2 genes is regulated by phytohormones or changes in the phytohormone balance.

Concluding remarks

We have summarized the current knowledge on the early nodulin gene ENOD2 which is expressed during the formation of legume root nodules. ENOD2 genes are highly conserved in legumes and encode extremely proline rich proteins which most likely belong to the group of hydroxyproline-rich glycoproteins localized in the cell wall. Hybridization *in situ* in wild-type nodules and expression analyses in aberrant nodule types indicate that the function of ENOD2 is related to the function of the inner cortex. Expression of ENOD2 genes seems to be regulated by changes in the phytohormone balance.

Acknowledgements

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