ENOD40 encodes a peptide growth factor

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ENOD40 encodes a peptide growth factor

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Stellingen

1. Zonder ENOD40 is normale ontwikkeling van een plant niet mogelijk.

2. De tolerantie voor hoge auxine en cytokinine concentraties in tabaksprotoplasten, veroorzaakt door lipo-chitooligosacchariden, is toe te schrijven aan inductie van ENOD40 expressie.

3. Het phenotype van de soja autoregulatie mutant NOD1-3 kan verklaard worden met productie van een component die knolvorming stimuleert.
   Francisco and Harper (1995) Plant Science 107, 167-176.

4. Het is voorbarig om te concluderen dat een sterk allel het phenotype van de *Arabidopsis* Landsberg *erecta* mutant veroorzaakt.
   Torri *et al.* (1996) Plant Cell 8, 735-746.

5. Niet ongevoeligheid voor ethyleen maar ongevoeligheid voor andere groeifactoren is de oorzaak van hypernodulatie van de mutant *sickle* van *Medicago truncatula.*
   Penmetsa and Cook (1997) Science 275, 527-530.

6. Door het bepalen van de positie van een mutatie op de kaart van het *Arabidopsis* genoom verplicht te stellen zal het meervoudig publiceren van dezelfde mutatie beperkt kunnen worden.
   Boerjan *et al.* (1995) Plant Cell 7, 1405-1419.
   Celenza *et al.* (1995) Genes and Dev. 9, 2131-2142.
   King *et al.* (1995) Plant Cell 7, 2023-2037.
   Lehman *et al.* (1996) Cell 85, 183-194.

7. De activiteit van een eiwit in vitro komt niet noodzakelijkerwijs overeen met de functie van het eiwit in vivo.

   Estruch *et al.* (1991) EMBO J. 10, 3125-3128.
   Filippine *et al.* (1996) Nature 379, 499-500.
   Estruch *et al.* (1991) EMBO J. 10, 2889-2895.
   Faiss *et al.* (1996) Plant J. 10, 33-46.

8. Okada *et al.* negeren dat de lagere hoeveelheden vrij IAA in de *Arabidopsis* PIN1 mutant veroorzaakt kunnen worden door toegenomen auxine conjugatie.

   Okada *et al.* (1991) Plant Cell 3, 677-684.

9. Een hoger percentage bevruchting door *Fuchsia* pollen moet niet toegeschreven worden aan toename van het aantal pollenkorrels dat kan kiemen, tijdens bewaring, maar aan betere omstandigheden tijdens de bestuiving.

   Godley and Berry (1995) Ann. Misouri Bot. Gard. 82, 473-516.

10. Ten onrechte wordt het spreken met een accent vaak gezien als een handicap in plaats van als een uiting van persoonlijke identiteit.

11. De enige die je ziet, zoals je zelf denkt dat je bent, is je hond.

12. Et is, wiet is.
    Et kütt, wiet kütt.
    Et hätt noch immer jutjejange.

Stellingen behorende bij het proefschrift getiteld
"ENOD40 encodes a peptide growth factor"
Karin van de Sande, Wageningen, 3 september 1997.

CONTENTS

Outline

Rhizobium bacteria induce the formation of nodules on the roots of leguminous plants. The nodules create the right biological niche for the rhizobia to carry out biological nitrogen fixation by which atmospheric nitrogen is reduced to ammonia. The nodule is a new organ that provides the plant with a nitrogen source for its growth and development. The formation of a nitrogen fixing root nodule is the final result of an extensive collaboration between the plant and the bacterium, which starts with the exchange of signals. The plant roots secrete flavonoids, which attract rhizobia and induce the expression of nodulation (*nod*) genes in the rhizobia. Due to the *nod* gene expression, specific lipo-chitooligosaccharide signals are produced, the so-called Nod factors, that induce several responses in the roots as a result of which nodule formation can start. The first plant responses are root hair deformation, expression of several plant genes and the mitotic reactivation of root cortical cells which leads to the formation of a nodule primordium. In chapter 1 a general overview is given of the signal exchange leading to the formation of a functional root nodule.

The aim of the research, described in this thesis, was to analyse the role of the early nodulin gene *ENOD40* in nodule development. To adress this issue, *ENOD40* expression was determined in nodules and its actvity was studied in an *in vitro* model system. First an *ENOD40* clone was isolated from pea using the available soybean *ENOD40* cDNA as a probe. This made it possible to compare the expression pattern of *ENOD40* in a determinate (soybean) and indeterminate (pea) nodule by *in situ* hybridisation using the soybean and pea *ENOD40* clones, respectively, as probes (chapter 2). Chapter 3 describes the isolation and characterisation of the soybean *ENOD40-2* gene. A transcriptional fusion between the *ENOD40-2* promoter and the ß-glucuronidase reporter gene was used in *Agrobacterium rhizogenes* mediated transformation of *Vicia hirsuta*. Root nodules were induced on the transgenic hairy roots by infection with *Rhizobium leguminosarum* bv. *viciae* and activity of the *ENOD40* promoter was analysed using GUS assay.

Expression of the *ENOD40* gene is detectable early after infection in the pericycle of the root, before cortical cell divisions take place. It was assumed that *ENOD40* expression might be required for the induction of cortical cell division, and might function by influencing auxin and/or cytokinin levels which play a role in the induction of cell division. This hypothesis was tested in a model system, the tobacco protoplast cell division assay. With this assay, the interaction of *ENOD40* with auxin and cytokinin was studied, and *ENOD40* was shown to induce tolerance of high auxin and cytokinin concentrations in tobacco protoplasts (chapter 4, 6). Using the tobacco protoplast cell division assay it was demonstrated that an oligopeptide of 10 to 13 amino acids encoded by *ENOD40* is the compound responsible for this effect. In addition, a conserved region in the 3' UTR of *ENOD40* also has an effect (chapter 4).

Tobacco cells are able to respond to a soybean *ENOD40* cDNA clone and to the soybean ENOD40 peptide. This indicates in tobacco homologous genes might be present. The cloning of these genes by PCR based methods is described in chapter 4 and 5. The

presence and activity of *ENOD40* in legumes and a non legume indicates *ENOD40* might play a general role in plant development. Therefore, in the concluding remarks (chapter 7) it is discussed whether peptides can play a more common role in plant development and whether and how the 3' UTR of *ENOD40* mRNA might function.

Chapter 1

Signalling in symbiotic root nodule formation

Karin van de Sande and Ton Bisseling. In: Essays in Biochemistry Volume (D. Bowles, ed.). 1997. The Biochemical Society, Portland Press Ltd, London, UK.

Nodule formation and signal exchange in the *Rhizobium*-legume interaction

Soil bacteria of the genera *Rhizobium*, *Bradyrhizobium* and *Azorhizobium* (together indicated as rhizobia) induce the formation of nodules on the roots of leguminous plants (Figure 2c). The nodules are formed in a symbiotic interaction, of benefit to both the pro- and the eukaryotic partner. In these nodules the bacteria carry out biological dinitrogen fixation [$N_2 + 8\ H^+ \longrightarrow 2\ NH_3 + H_2$], forming a nitrogen source for the plant that gains the capacity to grow on nitrogen poor soils. The plant in turn provides the bacteria with a carbon source and creates in the nodule the correct biological niche enabling the rhizobia to carry out the energy consuming nitrogen fixation process. The rhizobia multiply in the root nodules, from which they are released into the soil in large amounts after senescence of the nodules. Interestingly, during successful symbiotic interactions of plant and bacterium no obvious defence mechanisms are activated that might prevent the entry of the bacteria in the plant cells.

Host specificity is a prominent aspect of root nodule formation. Most rhizobia have a narrow host range and only form nodules on a very limited number of plant species. *Rhizobium leguminosarum* bv. *viciae* only forms root nodules with *Pisum* (pea), *Vicia* (vetch), *Lathyrus* and *Lens* species; *Rhizobium meliloti* forms nodules with *Medicago*, *Trigonella* and *Melilotus* species; and *Bradyrhizobium japonicum* forms nodules with *Glycine* and *Vigna* species. There are however exceptional cases, like *Rhizobium* sp. NGR234 that has the broadest host range of any known *Rhizobium*. It can nodulate different legume species of more than 70 genera, like *Vigna, Arachis, Glycine* and *Psophocarpus.* Moreover, it can induce nodules on the only non-legume known to enter a symbiosis with rhizobia, *Parasponia andersonii* (Mylona *et al.* 1995, Price *et al.* 1992).

During root nodule formation, two processes, infection and nodule organogenesis take place simultaneously (Figure 1). In order to infect the root, rhizobia induce root hair deformation and curling. Curled root hairs form so-called Shepherd's crooks, in which rhizobia are trapped in a small confinement formed by the curls. These rhizobia then enter the root, by local hydrolysis of the root hair cell wall and invagination of the plasma membrane (van Spronsen *et al.* 1994). Deposition by the plant cell of new cell wall material around this invagination leads to the formation of an infection thread (see Kijne *et al.* 1992, Turgeon and Bauer 1985). Concurrent with infection, root cortical cells dedifferentiate and start dividing. This way they form a nodule primordium from which the nodule will develop. By tip growth, the infection thread filled with proliferating rhizobia pushes its way through the root hair and the cortical cell layers, in the direction of the nodule primordium (Bakhuizen 1988). Primordial cells become infected by the rhizobia by endocytosis, in the course of which they become surrounded by a plant derived membrane, the peribacteroid membrane (Kijne 1992, Newcomb 1981).

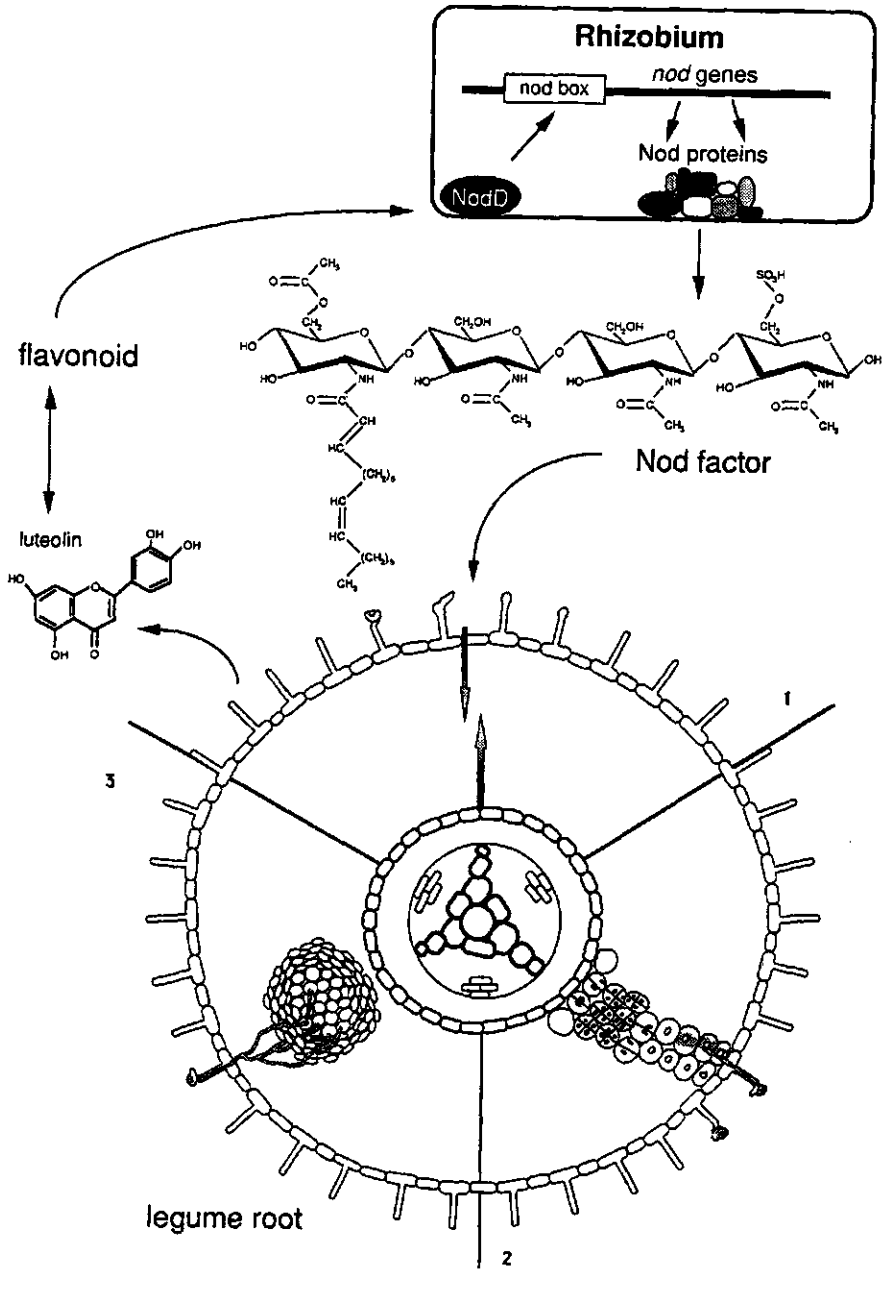


Figure 1. The symbiotic interaction between rhizobia and leguminous plants. The plants exude flavonoids, that activate NodD leading to production of Nod factors. The Nod factors induce root hair deformation (stage 1) and primordium formation (stages 2 and 3), rhizobia initiate infection and the infection thread grows towards the primordium where cells get infected (see text for details).

After infection the primordia develop into dinitrogen fixing root nodules consisting of several tissues, each with a specific function. In Figure 2b, a dinitrogen fixing root nodule with its different tissues is shown. The central tissue consists of the infected cells and the uninfected cells that are dispersed between the infected cells (Figure 2a). The first contain the rhizobia that have differentiated in their symbiotic form, the so-called bacteroids. The peripheral tissues surround the central tissue and consist of the nodule vascular bundles within the nodule parenchyma and the nodule endodermis separating this tissue from the nodule cortex. The uninfected cells and the peripheral tissues contribute to the proper physiological environment allowing rhizobial dinitrogen fixation to occur. (Mylona *et al.* 1995, Vasse *et al.* 1990).

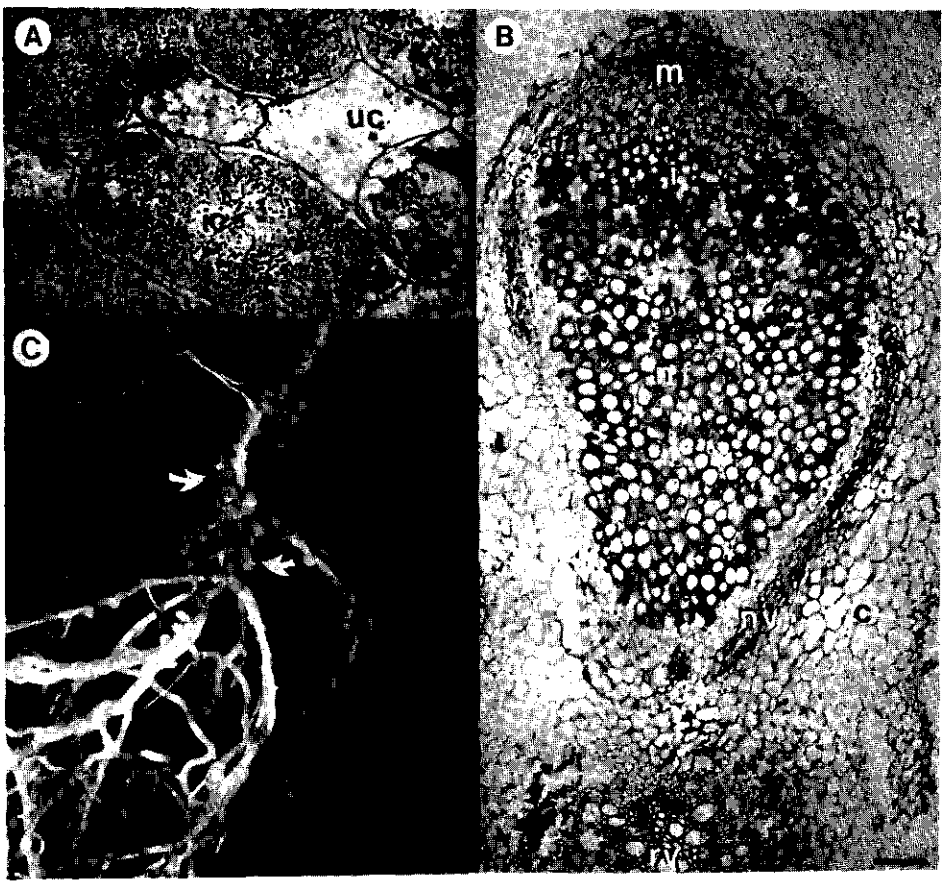


Figure 2. (**a**) Bright field micrograph of infected and uninfected cells from a clover root nodule. ic = infected cell, uc = uninfected cell. Bar = 40 µm. (**b**) Bright field micrograph of a pea nodule showing the central and peripheral tissues. m = meristem, i = infection zone, nf = nitrogen fixation zone, nv = nodule vascular bundle, rv = root vascular bundle, c = cortex. Bar = 250 µm. (**c**) Nodulated pea root system. Arrows indicate nodules.

Plant genes specifically expressed during nodule formation and functioning are called nodulin genes. The early nodulins, indicated with ENOD, are involved in the infection process and the formation of the nodule structure. Late nodulins, indicated with NOD, are generally involved in nodule functioning.

In order to coordinate the responses of both symbiotic partners, extensive signal exchange is required between plant and rhizobium. In addition, the formation of a nodule involves responses in several root tissues of the legume. Therefore signaling between these tissues is required for organogenesis to proceed.

Plant signals influencing rhizobial behaviour

Interactions in the rhizosphere between rhizobia and roots of the host plant are rather complex and our understanding of these processes is yet fragmentary. Some aspects of these interactions are well established and have resulted in the following notions.

Germinating seeds and young growing roots of legumes exude a complex mixture of different organic compounds into the soil. This mixture contains organic acids, (iso)flavonoids, betaines (N methylated derivatives of amino acids and related compounds), amino acids, sugars, biotin and homoserine. The composition of the exudate varies for different host plants (Caetano-Anollés *et al.* 1988, Djordjevic *et al.* 1987, Kape *et al.* 1991). The exuded compounds stimulate growth of rhizobia in the soil, and cause chemotactic movement (Barbour *et al.* 1991, Gulash *et al.* 1984). The chemotactical response to specific compounds varies for different rhizobial species, and several different compounds have the ability to induce chemotactic movement in a rhizobial species. Therefore chemotaxis is not a major factor in determining host specificity (Barbour *et al.* 1991, Caetano-Anollés *et al.* 1988, Kape *et al.* 1991, Parke *et al.* 1985). Several compounds in legume exudates can somehow promote bacterial growth. For example it has been shown that biotin, betaines and flavonoids stimulate growth of *R. meliloti* (Hartwig *et al.* 1991, Phillips *et al.* 1995, Streit *et al.* 1996). At the other hand, flavonoids have also been shown to induce resistance to the soybean phytoalexin glyceolin in the soybean nodulating rhizobia *R. fredii* and *R. japonicum*. Phytoalexins are plant derived anti microbial compounds, which are believed to play an essential part in resistance of the plant against pathogens. In legumes they often are isoflavonoids (Parniske *et al.* 1991). Resistance to phytoalexins might give rhizobia a better chance of survival on the soybean root surface than pathogenic micro-organisms.

Upon colonising the legume root surface, the rhizobia attach to the root hairs. Several compounds have been shown to play a role in attachment. Examples are the rhizobial protein rhicadhesin binding to a plant surface component and plant lectins recognising a rhizobial carbohydrate of unknown nature (Diaz *et al.* 1989, van Eijsden *et al.* 1995, Smit 1988). This initial step of the interaction is followed by exchanging signal molecules setting in motion root nodule formation.

Plant signals inducing *nod* genes

The rhizobial genes that are essential for nodule formation are the nodulation (*nod*) genes. The *nod* genes are divided in the so-called common *nod* genes and the host specific *nod* genes. The common *nod* genes are found in all rhizobial strains and are functionally interchangeable. They are *nodABC* and the regulatory *nodD*, whose protein product regulates the transcription of the other *nod* genes (Mulligan and Long 1985). In several rhizobia, more than one copy of *nodD* is present (Györgypal *et al*. 1988). The regulatory *nodD* genes are the only constitutively expressed *nod* genes. Signals secreted by the plant activate the NodD protein, after which the activated complex induces transcription of the other *nod* genes. The host specific *nod* genes (*hsn*) are not functionally interchangeable and are involved in determining the host range. A mutation in one of these genes changes the host range of the rhizobial species. Examples of host specific *nod* genes are *nodPQH, nodFEL, nodO* and *nodT*.

The *nod* genes encode nodulation (Nod) proteins, most of which are involved in the production of signal molecules called Nod factors (see Nod factor production). Some Nod proteins are membrane proteins, that might be involved in the secretion of Nod factors (e.g. NodI, NodJ and NodT). One Nod protein for which the function is not proven (NodO) is excreted by the rhizobia and might influence Nod factor activated signal transduction since it can compensate for sub-optimal Nod factor structure (Economou *et al.* 1994, see van Rhijn and Vanderleyden 1995).

In the promoter regions of all *nod* operons, except that of *nodD*, a specific highly conserved sequence occurs, called the *nod* box. Most likely, transcription is induced by binding of the activated NodD to the inverted repeats (ATC-N9-GAT) of which two copies in a direct repeat structure are found in the *nod* box (Fisher *et al.* 1988, Goethals *et al.* 1992, Hong *et al.* 1987).

The plant compounds specifically interacting with NodD proteins were identified as (iso)-flavonoids and betaines, types of compounds with a very different chemical structure (Djordjevic *et al.* 1987, Györgypal *et al.* 1991, Hartwig *et al.* 1991, Peters *et al.* 1986, Phillips *et al.* 1992, 1995), some of which also can induce chemotaxis (see Plant signals influencing rhizobial behaviour). Some of the secreted compounds are inducers, activating NodD, whereas others (with similar chemical structure) are inhibitors, blocking the activation of NodD by the inducers (Djordjevic *et al.* 1987, Györgypal *et al*. 1991, Peters *et al.* 1986, Peters and Long 1988, Phillips *et al.* 1992).

The activation of NodD influences host specificity to a certain extent. The root exudate of a certain host plant will activate the NodD proteins of compatible rhizobial species, while the exudate from another legume species might have no or markedly reduced capacities to activate these proteins (Spaink *et al.* 1987, Horvath *et al.* 1987). This is due to the fact that NodD proteins of different species are very specific and that inducers for one rhizobial species can even be inhibitors for another species (Györgypal *et al*. 1991, Phillips

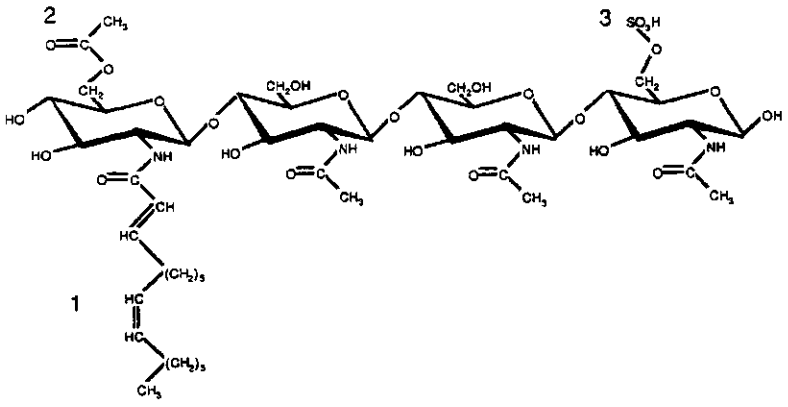
et al. 1995).

If rhizobia express more than one NodD, each one usually has its own specific affinity for inducing compounds. The mixture of compounds excuded by a specific plant determines to what extent different NodD proteins are activated and as a consequence which NodD proteins activate transcription of *nod* genes. Mutations in one, or more, of the *nodD* genes can cause different nodulation phenotypes on different host plants, dependent on which *nodD* gene is mutated (Honma *et al.* 1990). *R. meliloti* for instance, has three *nodD* genes of whose products NodD1 is activated by plant flavonoids such as luteolin (Györgypal *et al*. 1988, Hartwig *et al.* 1990) and NodD2 is activated by betaines as trigonelline and stachydrine (Hartwig *et al.* 1990, Phillips *et al.* 1992). NodD3 is activated by hesperitin, naringenin and genistein but these compounds are inhibitors for NodD1 and NodD2 (Györgypal *et al.* 1991). When NodD1 is inactivated, nodulation on *Melilotus alba* and *Medicago sativa* are delayed, while nodulation on *Medicago truncatula* remains unaffected. NodD2 inactivation delays nodulation on *M. truncatula* and *M. sativa*, while nodulation on *M. alba* is unaffected. *nodD3* deletion influences nodulation on *M. alba* and *M. sativa*, but was not tested on other host plants (Györgypal *et al.* 1988). Thus, NodD activity is important for the host specificity of a rhizobial strain.

Nod factor production

After activation of the *nod* genes by NodD the nodulation proteins are produced, most of which are involved in the biosynthesis of Nod factors. Nod factors are lipo-chitooligosaccharide signal molecules that all have a chitin ß-1,4-linked N-acetyl-D-glucosamine backbone, varying in length between three to six sugar units and a fatty acyl chain on the C-2 position of the non-reducing sugar (Figure 1, 3). Three of the *nod* genes, *nodABC*, are required for synthesising this basic structure. NodC is probably a chitin oligosaccharide synthase, that links N-acetyl-D-glucosamine monomers. NodB is a chitin oligosaccharide deacetylase that removes the acetyl group from the terminal non-reducing sugar, and NodA is an N-acyltransferase that catalyses the addition of a fatty acyl chain (John *et al.* 1993, Röhrig *et al.* 1994, Spaink *et al.* 1994). Host specific *nod* genes, varying in different rhizobial species, are involved in the modification of the fatty acyl chain or the addition of strain specific substitutions that are important in determining host specificity (Carlson *et al.* 1994, Lerouge *et al.* 1990, Roche *et al.* 1991, Spaink *et al.* 1989, see Host specificity in root nodule formation). The modifications of the acyl chain are variations in length and degree of saturation. Strain specific substitutions can be added to the sugar moiety at the reducing and the non-reducing end of the chitin backbone. Examples are the 0-acetyl group at the C6 position of the sugar moiety at the non-reducing end, and the O-sulphate at the C6 position of the sugar moiety at the reducing end of the *R. meliloti* Nod factor (Figures 1,3). Other substitutions at these positions, and/or substitutions at other

positions of the terminal sugar groups lead to a large variety of Nod factors produced by one rhizobial species, and between different rhizobial species. Purified Nod factors can induce early responses of nodulation, like root hair deformation, induction of nodulin genes and nodule primordium formation in a host specific manner (see Nod factor perception). However, it cannot be excluded that some other factors are produced that are also involved in inducing symbiotic responses.



1: fatty acid modifications	2: non reducing end substitutions	3: reducing end substitutions
C18:1-Δ 11Z (all)	O-acetyl (Rm Bj Rv Be)	O-sulphate (Rm Rt)
C18:0 (all)	O-carbamoyl (Ac)	O-acetate (Rvt)
C16:0 (all)		O-arabinose (Ac)
C16:1-Δ 9Z (all)		O-fusose (Be Rf)
C18 to C26 (ω-1)OH (Rm)		O-fucose/ O-acetate (Rl Re)
C16:2 (Δ 2E, 9Z) (Rm)		O-fucose/ O-sulphate (N)
C16:3 (Δ 2E, 4E, 9Z) (Rm)		O-fucose/ O-methyl (Bj N Rf)
C18:3 (Δ 2E, 4E, 6E) (Rt)		O-glycerol (Be)
C18:4 (Δ 2E, 4E, 6E, 9Z) (Rt)		O-mannose (Rtr)
C20:3 (Δ 2E, 4E, 6E) (Rt)		
C20:4 (Δ 2E, 4E, 6E, 8E) (Rt)		

Figure 3. Structure of the main *Rhizobium meliloti* Nod factor, and structural variations for different rhizobial species. Rm = *Rhizobium meliloti*, Rt = *R. leguminosarum* bv. *trifolii*, Rv = *R. leguminosarum* bv. *viciae*, RvT = *R. leguminosarum* bv. *viciae* strain TOM, Bj = *Bradyrhizobium japonicum*, Be = *B. elkanii*, Ac = *Azorhizobium caulinodans*, Rtr = *R. tropici*, Rf = *R. fredii*, Rl = *R. loti*, Re = *R. etli*, N = *R.* NGR 234.

Host specificity in root nodule formation

As can be concluded from the previous paragraphs, at different stages in the interaction between the host plant and the rhizobia control is exerted. The composition of the mixture of compounds exuded by the host plants, the chemotactic response of the rhizobia, the *nod* gene inducers (flavonoids etc.) produced, the NodD activation and the response of the host plant to specific Nod factors all contribute to achieving host specificity. Usually, the combination of different control levels leads to a host-specific interaction.

In some cases, host specificity is predominantly determined by the type of NodD activators secreted by the host. *R. etli* and *R. loti*, for instance, produce identical Nod factors, but are unable to nodulate each other's host plants, *Phaseolus* and *Lotus*, respectively. The dominant role of flavonoids in controling host specificity in this case, was demonstrated by the introduction of a mutant *nod* gene encoding a constitutively active NodD not dependent on activation by flavonoids. As a result, *R. etli* and *R. loti* were able to nodulate each other's hosts (Cardénas *et al.* 1995, López-Lara *et al* . 1995), showing that the lack of NodD activation was limiting nodulation of heterologous host species.

In other interactions, the structure of the Nod factor is the major host specificity determinant. *R. meliloti* produces Nod factors containing a sulphate substitution at the C6 position of the glucosamine residue at the reducing end (Figure 3). When this sulphate group is absent due to a mutation in *R. meliloti* or is chemically removed, the modified factors no longer induce early responses in *Medicago sativa*. Instead they gain the ability to interact with common vetch that normally is nodulated by *R. leguminosarum* bv. *viciae*, a strain that produces only Nod factors without a sulphate group substitution (Faucher *et al.* 1989, Lerouge *et al.* 1990, Roche *et al.* 1991).

In the described examples, the bacterial compounds determine host specificity, and the effect they induce in the symbiotic partner is clear. However, host specificity can also be influenced by plant compounds. Lectins, the carbohydrate binding proteins on the root hair surface that are implicated in attachment of rhizobia, have also been shown to play a role in controling host range. Upon introduction of a pea lectin gene in clover by *Agrobacterium rhizogenes* mediated transformation, the range of rhizobia able to infect clover broadened: the clover hairy roots could now be nodulated by the normal microsymbiont *R. leguminosarum* bv. *trifolii* as well as by the heterologous *R. leguminosarum* bv. *viciae* (Diaz *et al.* 1989, van Eijsden *et al.* 1995). It is yet unclear whether the role of lectin as host specificity determinant is related to its function in rhizobial attachment or to another step of the interaction, for example Nod factor recognition.

The formation of a root nodule requires quite a number of plant genes, of which the symbiotic or *sym* genes were identified by mutagenesis. For instance, the *sym2* gene has been shown to be involved in *Rhizobium*-host plant interaction and to influence host specificity. The *sym2* allele in Afghanistan pea prevents nodulation by European *R. leguminosarum* bv. *viciae* strains whereas strains from the Middle East, like TOM, form

nodules on pea varieties containing the Afghanistan *sym2* allele. The latter strains contain an additional *nod* gene, *nodX*, that does not occur in European rhizobial strains. NodX is responsible for the addition of an O-acetyl substitution to the C6 position of the glucosamine unit at the reducing end of the pentameric *R. lebuminosarum* bv. *viciae* Nod factors (Firmin *et al.* 1993). Introduction of *nodX* into European rhizobia leads to the production of additional Nod factors which allow nodulation of the *sym2* containing pea varieties. Presumably, *sym2* is involved in the recognition of the NodX modified Nod factors (Davis *et al.* 1988, Kozik *et al.* 1995).

In the given descriptions, host specificity is determined mainly at one stage in the *Rhizobium*-host plant interaction. However, it must be kept in mind that usually the cumulative effect of control exerted at various stages leads to host specific nodulation.

Nod factor perception

Purified Nod factors elicit responses in host plants like root hair deformation, primordium formation and activation of specific nodulin genes. These responses are effected by Nod factors at micro- to picomolar concentration, indicating that high affinity receptors are probably involved in Nod factor recognition. However, the molecular mechanism of Nod factor perception remains to be elucidated.

The epidermis is the outermost cell layer of the root and includes the root hairs. Fast epidermal responses to Nod factors are depolarisation of the plasma membrane potential (Erhardt *et al.* 1992, Felle *et al.* 1995), spiking of cytoplasmic calcium levels in the root hairs (Erhardt *et al.* 1996) and alkalinization of the root hair cytoplasm (Felle *et al.* 1996). These responses are observed within ten minutes after Nod factor application. Root hair deformation first starts one hour after addition of Nod factor, and after three hours the root hairs are fully deformed (Heidstra *et al.* 1994). It is followed by expression of specific early nodulin genes of which *ENOD12* is the best studied example (Journet *et al.* 1994, Vijn *et al.* 1995a, b). The significance of most of these responses in root nodule formation is not clear, but they provide useful tools to gain some insight in Nod factor perception. For a detailed overview of the responses induced by Nod factors in the epidermis we refer to the recent reviews by Dénarié *et al.* (1996), Long (1996) and Spaink (1996).

The major *Rhizobium meliloti* Nod factor has a tetrameric N-acetyl-glucosamine backbone, with a C16:2 acyl chain, an O-acetate substitution at the non-reducing sugar moiety and an O-sulphate substitution at the reducing sugar moiety (Figure 1, 3). By using *R. meliloti* mutants and chemically modified *R. meliloti* Nod factors, the effect of the substitutions and the structure of the acyl chain on the induction of the various epidermal responses have been analysed in alfalfa, as summarised in Table 1.

Based on the demands on the structural properties of *R. meliloti* Nod factors required to induce certain responses in the alfalfa epidermis, these responses can be divided in three

categories. 1) Alkalisation of root hair cytoplasm requires the 'basic Nod factor': a chitin backbone with an acyl chain, but the structure of the fatty acyl chain and the presence of either four or five glucosamine units in the chitin backbone are not important. 2) Deformation of root hairs, induction of *ENOD12* expression and membrane depolarisation require the basic Nod factor, but with the sulphate substitution. Tetrameric Nod factors induce deformation and depolarisation at a thousand fold lower concentration than pentamers; for *ENOD12* expression the importance of backbone length has not been tested. 3) Infection thread formation is the most stringent response since it requires both the sulphate and the acetyl substitutions and the C16:2 unsaturated acyl chain on the Nod factor. Strikingly, the plant responses for which the length of the chitin backbone was shown to be important also involve the sulphate substitution. This indicates that the distance between the non-reducing end and the sulphate group at the reducing end is important, and suggests that the receptor recognises both ends.

The different Nod factor structure requirements of induced responses indicate that more than one receptor may be involved in Nod factor perception. The three distinct groups of Nod factor responses shown in Table 1 suggest that in alfalfa there might even be three

Table 1. Structural requirements of the *R. meliloti* Nod factor for induction of various responses on alfalfa.

1: O-sulphate substitution at position C6 of reducing terminal sugar. 2: *O-acetyl substitution* at position C6 of non reducing terminal sugar. 3: significance of unsaturation of acyl chain. 4: significance of length of chitin backbone. +: the substitution or modification is important. -: the substitution or modification is not important for the plant response. n.d.: not determined.

Plant Response[#]	Nod factor structure			
	1	2	3	4
alkalinization	-	n.d.	-	-
deformation	+	-	-	+
ENOD12 expression	+	-	-	n.d.
depolarisation	+	-[*]	+	+
infection thread formation[≈]	+	+	+	n.d.
primordium formation	+	+	+	+

[*] In the presence of the O-acetyl substitution maximal membrane depolarisation was achieved much faster, the degree of depolarisation reached was the same.

[≈] In addition to specific demands on Nod factor structure, the presence of rhizobia is required, application of purified Nod factors alone is not sufficient. Certain parts of the rhizobial cell wall are essential.

[#] (Ardourel *et al.* 1994, Felle *et al.* 1995, 1996, Journet *et al.* 1994, Kurkdjian 1995, Roche *et al.* 1991, Schultze *et al.* 1992, Truchet *et al.* 1991).

receptors, at least one for non-sulphated and two for sulphated Nod factors. One of the putative receptors for sulphated Nod factors only recognises Nod factors containing the O-sulphate substitution, the O-acetyl substitution and the specific acyl chain (C16:2). Since this receptor seems to be specifically involved in initiation of infection thread growth it has been named entry receptor (Ardourel *et al.* 1994).

The hypothesis that more than one Nod factor receptor is operational gets support from the work of Felle and co-workers (1995, 1996) who demonstrated that application of the sulphated as well as of the not-sulphated Nod factor both resulted in alkalinization of alfalfa root hairs. A second addition of the same Nod factor did not increase the alkalinization response, which was explained as desensitisation of a receptor as a result of saturation. When first non-sulphated Nod factor was added at saturating concentration, followed by sulphated Nod factor, alkalinization took place at each addition of Nod factor. The same response took place when the order of adding Nod factor was reversed. This indicates that the alkalisation response is not just a consequence of differential binding of the Nod factors to the same receptor, but of binding to different receptors (Felle *et al.* 1996). The chemical nature of the possible Nod factor receptors is still unclear. A Nod factor binding protein has been identified in roots of *Medicago truncatula*, binding the tetrameric Nod factor from *R. meliloti*, with the O-acetyl and the sulphate substitutions and the unsaturated fatty acyl chain C16:2, named NodRm-IV(Ac, S, C16:2). Whether this protein is a Nod factor receptor remains to be proven (Bono *et al.* 1995).

Purified Nod factors applied to the root surface do not only induce responses in the epidermis, but also in tissues inside the roots, the pericycle and the cortex that are not in direct physical contact with the medium containing Nod factors. About three hours after Nod factor addition and preceding the induction of cell divisions, *ENOD40* expression is induced in the pericycle opposite the proto-xylem pole (W.-C. Yang, unpublished). About 24 hours after Nod factor application cell divisions are induced in the cortex, leading to the formation of nodule primordia or even nodule like structures (Martinez *et al.* 1993, Spaink *et al.* 1991, Stokkermans and Peters 1994, Truchet *et al.* 1991). In the dividing cells of the primordium nodulin genes are expressed, e.g. *ENOD12* and *ENOD40* (Vijn *et al.* 1993). It is not known whether Nod factors are transported to the inner root layers, and interact directly with cortical and pericycle cells, or whether secondary signal molecules are generated in the epidermis and transported to the inner parts of the root.

The role of plant compounds in nodule organogenesis

On alfalfa roots cytokinin can induce the formation of nodules, resembling rhizobial nodules: cell divisions are induced in the inner cortex, and lead to the formation of a nodule with peripheral vascular tissue, a central region surrounded by a cortex and one or more meristem-like regions. The expression of the early nodulin genes *ENOD2* and *ENOD12* is

induced in these structures (Bauer *et al.* 1996, Cooper and Long 1994). Such mimicking of the effects of Nod factors by cytokinin suggests that Nod factors might induce an increased cytokinin level in the root which subsequently triggers cell division, in line with the general role of cytokinin in plant development. That Nod factors affect the balance between the auxin and cytokinin concentration in the root is also suggested by the effect of the addition of auxin transport inhibitors to legume roots (Jacobs and Rubery 1988). Addition of auxin transport inhibitors results in the formation of nodule-like structures, similar to the ones induced by cytokinin on the roots of several legumes (Hirsch *et al.* 1989, Scheres *et al.* 1992, Wu *et al.* 1996). In addition, alfalfa plants sometimes spontaneously form nodules, in the absence of rhizobia or inducing compounds. These nodules resemble the cytokinin-induced nodules and might be formed due to hormone imbalances in the plant (Joshi *et al.* 1991, Pichon *et al.* 1994, van de Wiel *et al.* 1990).

Strikingly, the infection of legume roots by rhizobia is limited to the part of the root in which root hairs develop. This is the so-called susceptible zone which exudes flavonoids, and other compounds that can induce *nod* gene expression in rhizobia (Djordjevic *et al.* 1987, Peters and Long 1988). Other parts of the root might produce inhibitors which prevent the starting of nodulation (Djordjevic *et al.* 1987). In that way, flavonoids and other NodD activating or inhibiting compounds could contribute to positional information, controling where root nodules are formed.

The position where the nodule primordia are formed in the root cortex, is also strictly regulated. They are practically always formed opposite the protoxylem poles of the root vascular bundle (Libbenga and Harkes 1973). In the positioning of nodule primordia different signaling compounds, acting either positively or negatively, seem to be involved. A compound isolated from the stele, identified as uridine (Smit *et al.* 1995) is able to stimulate phytohormone induced cortical cell division in pea root explants from which the stele was removed even in cortex regions that were originally opposite the phloem. It has been postulated that uridine diffuses from the stele into the cortex in the protoxylem zones of the root and functions locally as a stimulator of cell divisions.

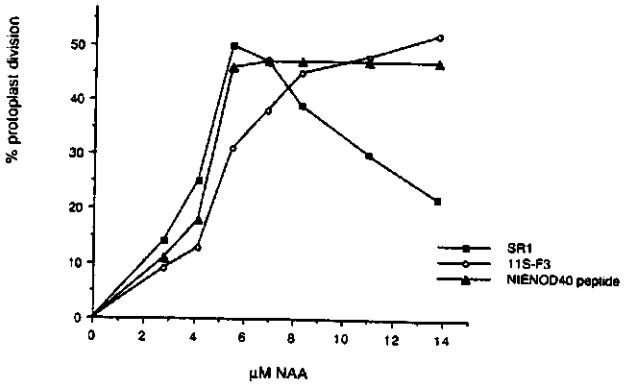
The involvement of ethylene in root nodule formation is clearly shown by two legume mutants. The ethylene-insensitive *Medicago truncatula* mutant *sickle* forms much more nodules in the susceptible zone than wildtype plants (Penmetsa and Cook 1997), while nodulation of low nodulating pea *sym5* mutants can be restored by addition of Ag^+, an inhibitor of ethylene perception (Fearn and LaRue 1991, Guinel and LaRue 1991). The mode of functioning of ethylene in wildtype nodulation is indicated by the work of Heidstra *et al.* (in press). They showed that the expression of ACC oxidase, catalysing the last step of ethylene biosynthesis (Kieber and Ecker 1993), is confined to the pericycle in the phloem sectors of the root (Heidstra *et al.* in press). This leads to localised production of ethylene in the susceptible zone. Addition of AVG and Ag^+, that block ethylene synthesis or perception, respectively (Kieber and Ecker 1993), led to an increased nodule number and the formation of nodule primordia opposite the phloem poles (Heidstra *et al.* in press).

These studies strongly suggest that locally synthesised ethylene diffuses in the cortex and blocks cell division opposite phloem poles most efficiently. In that way, ethylene could act as a signaling compound providing positional information in nodule formation and, in addition, nodule number is controlled.

Recently it was shown that the early nodulin genes *ENOD40* encode peptides of 10-13 amino acids (Figure 4a) that might influence primordium formation. *ENOD40* genes have been found in legumes as well as non-legumes and appear to play a general role in regulating plant development. Expression of soybean *ENOD40* in tobacco resulted in the formation of one or two additional side shoots (van de Sande *et al.* 1996); ectopic expression in alfalfa disturbed embryogenesis and regeneration (Crespi *et al.* 1994). In addition tobacco protoplast division has been used to study the activity of the ENOD40 peptide. Wildtype protoplasts divide with a maximum frequency of about 50 percent at 0.9 μM kinetin and 5.5 μM NAA. Protoplasts treated with the ENOD40 peptide or expressing an *ENOD40* gene were able to divide with maximal frequency at 13.8 μM NAA. Thus the ENOD40 peptide conferred tolerance of high auxin concentration (Figure 4b). The tobacco protoplast studies also revealed that exogenously applied peptide is recognised by protoplasts (van de Sande *et al.* 1996), which strongly suggests that cells contain a

GmENOD40-2	M.ELCWLTTIHGS
PsENOD40	MKFLCWQKSIHGS
MsENOD40	MKLLCWQKSIHGS
NtENOD40	M...QWDEAIHGS

Figure 4. **(a)** Amino acid sequence of the ENOD40 peptide of several legumes and tobacco. Gm = soybean, Ps = pea, Ms = alfalfa and Nt = tobacco.



(b) Protoplast division frequency after 5 days of cultivation in liquid K3 medium supplemented with 0.9 μM kinetin and a concentration range of auxin. o: protoplasts expressing soybean *ENOD40*, Δ: wildtype protoplasts with 10^{-12} M tobacco ENOD40 peptide, wildtype protoplasts.

perception or uptake mechanism at their surface. This makes the ENOD40 peptide an interesting candidate to be involved in cell-cell signaling. Since *ENOD40* transcription in the root pericycle precedes cortical cell divisions it is an attractive hypothesis that after production of the ENOD40 peptide in the pericycle, it can diffuse to the inner cortex. The effect of ENOD40 in the inner cortex might involve a local change of the auxin/cytokinin balance and trigger the onset of cell division.

Autoregulation of nodule formation

Besides the location in which nodules are formed, also the number of nodules is strictly regulated. The regulation of the number of nodules occurs by a systemic feedback mechanism, referred to as autoregulation. Autoregulation is the process in which earlier formed nodules prevent the formation of new nodules in ontogenetically younger parts of the plant root. This causes the formation of a restricted number of nodules in a limited zone of the root (Caetano-Anollés *et al* . 1991, Caetano-Anollés and Gresshoff 1991). Mutants that have been used to elucidate the autoregulatory response are the supernodulation mutants. The supernodulation mutants have a disturbed autoregulation response, which leads to the formation of large amounts of nodules over the entire root system. Supernodulation can have a negative effect on plant growth (Hansen *et al.* 1990) and thus turns *Rhizobium* into a (partially) parasitic organism.

Autoregulation is a process involving a complex exchange of signals between organs. Probably, there is a compound released from the nodules that signals information about the nodulation status of the roots towards the leaves. This compound is released by young primordia and has systemic activity (Caetano-Anollés and Gresshoff 1990). Grafting experiments between wildtype and supernodulation mutant soybean plants revealed that also the shoot plays an important role in autoregulatory control of nodule number (Delves *et al.* 1986). Probably, a leaf signal is formed in response to the nodule signal. The interaction of the leaf signal with root tissues subsequently determines whether root cortical cells will form further root nodules or remain inactive. The signaling between shoot and root is rather complex and probably involves inhibitory and stimulatory compounds (Gresshoff *et al.* 1988, 1989, Francesco and Harper 1995), but their molecular nature remains to be solved.

The autoregulatory nodule signal might be closely related to a signal involved in other steps of plant development since supernodulation inhibits lateral root formation: non-nodulated supernodulator soybean plants form more lateral roots than nodulated mutant plants. Furthermore wildtype soybean root tips appear to produce a compound similar to the nodule signaling molecule involved in autoregulation, since removal of lateral root tips increased nodulation (Caetano-Anollés *et al* . 1991). Therefore the nodule compound might be related to compounds involved in lateral root initiation.

Concluding remarks

Root nodule formation requires extensive communication between the two symbiotic partners, and between different tissues of the legume host. Flavonoids produced by the host plant attract and activate the rhizobia. The Nod factors produced by rhizobia induce several responses in the plant, including the activation of cell-cell signaling between stele and cortical cell layers. This communication involves ethylene, uridine and probably also ENOD40, a novel peptide growth regulator in plants. These compounds play a role in the induction of cell divisions and the location of the primordia to the correct zone of the root, the cortex cells opposite the protoxylem poles. Also systemic signaling between different plant organs is evoked by nodulation, which regulates the nodule number and involves both nodule and shoot signals. From both signaling systems some aspects are known, but large parts and several compounds involved have not been elucidated yet.

Further stages of nodule formation, namely infection thread growth, release of rhizobia from infection threads, formation of a functional root nodule, and nitrogen fixation, probably require more signal exchange. For instance, rhizobial mutants disturbed in the production of surface polysaccharides either cannot form infection threads, or infect primordial cells, but induce defence responses in the host plant. The knowledge about signal exchange at these levels is very limited, and still requires much research. However, the intricate system of information exchange, of which large parts are still unknown, leads to the formation of a functional dinitrogen fixing root nodule, and both partners benefit from this symbiotic interaction by growth and multiplication.

Summary

* Rhizobia form nodules on legume roots in which they carry out biological nitrogen fixation.
* The place and number of nodules is tightly regulated.
* Exchange of signal molecules that activate responses in the symbiotic partner takes place; plant flavonoids induce the production of rhizobial Nod factors, Nod factors induce gene expression and morphogenesis in the legume root.
* *ENOD40* is a plant gene induced by Nod factors that is involved in root nodule formation.
* *ENOD40* encodes a peptide of 12 amino acids in soybean that can induce tolerance of high auxin concentrations in tobacco protoplasts.

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Chapter 2

Comparison of soybean and pea *ENOD40* cDNA clones representing genes expressed during both early and late stages of nodule development

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Comparison of soybean and pea ENOD40 cDNA clones representing genes expressed during both early and late stages of nodule development

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Abstract

A pea cDNA clone representing the homologue of the soybean pGmENOD40-1 was isolated and characterized. At the nucleotide level both clones share 55% homology. Strikingly, the homology between the polypeptides derived from the pea and soybean ENOD40 cDNA sequences is only 14%. Despite this low homology Southern analyses revealed that the isolated pea cDNA clone represents the single pea ENOD40. *In situ* hybridizations showed that at early stages of nodule development and in mature nodules the expression pattern of pea ENOD40 is comparable to that of soybean ENOD40. Although ENOD40 show similar expression patterns in these two nodules, it is questionable whether the putative polypeptides have a similar function, since the homology is very low.

Legume root nodule formation is induced by *rhizobium* and *Bradyrhizobium* bacteria and starts through the induction of mitotic activity in the cortex of legume roots. The dividing cortical cells form a nodule primordium which after infection by the (brady)rhizobia differentiates into a root nodule [5]. The successive steps in root nodule development involve the induction of host genes that are not expressed in uninoculated roots. Of these genes, the genes that are not expressed in any other plant organ are named nodulin genes [7]. However, among the plant genes whose expression during symbiosis is dramatically increased, there are also genes that are expressed (albeit at low levels) in non-symbiotic tissues [1,4] and according to the definition [7] cannot be considered as genuine nodulin genes. Yet, for the sake of convenience, these genes are also called nodulin genes. Recently, Hata and Kouchi as well as our group characterized two homologous cDNA clones representing genes that are induced by *Bradyrhizobium* in dividing root cortical cells of soybean, but they are not expressed in root or shoot meristems [3, 9]. Kouchi and Hata

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X81064.

have named their clones GmN#36a and GmN#36b [3], while our group called our clone pGmENOD40 [9]. To avoid confusion about the nomenclature the two groups have renamed these clones. In future publications, Gm#36a will be called pGmENOD40-1 and pGmENOD40, which is identical to GmN#36b, will be called pGmENOD40-2. These two clones are 95% homologous at the nucleotide level. However, there are some remarkable differences between the putative encoded polypeptides. pGmENOD40-1 contains one long ORF staring with an ATG as a putative start codon, whereas the (comparable) ORF in pGmENOD40-2 does not contain an ATG at all. Furthermore, the two putative polypeptides only have 69% identity due to a few non-triplet insertions.

At early stages of the *Bradyrhizobium*-soybean interaction, GmENOD40-1 and GmENOD40-2 are expressed in the dividing cortical cells forming the nodule primordium, and in addition these genes are induced in a region of the root pericycle facing the nodule primordium. In a mature nodule the GmENOD40s are expressed at a high level in the pericycle of the nodule vascular bundle, and at a low level in the uninfected cells of the central tissue [3, 9].

Soybean nodules have a determinate growth pattern, since the meristem is active only during early stages of development. Therefore, the mature nodule is built up of cells that are more or less at the same stage of development [3].

Temperate legumes, such as pea and alfalfa, form indeterminate nodules. This nodule type has a persistent apical meristem that continuously differentiates proximally into nodule tissue. Therefore, the tissues of these nodules are of graded age; the youngest cells are adjacent to the nodule meristem and the oldest cells near the root attachment point [5].

To study the expression of an ENOD40-like gene during development of an indeterminate nodule, we isolated a pea cDNA clone homologous to pGmENOD40. The sequence of this pea clone was determined and the expression during pea nodule development was studied by *in situ* hybridization.

A pea cv. Sparkle nodule cDNA library was screened with the [^{32}P]-labelled insert of pGmENOD40-2 [9]. Four independent clones were isolated and the clone with the largest insert was named pPsENOD40 and used in further studies. To determine the extent of homology of the pea sequence in this cDNA clone to pGmENOD40-2, the inserts of pPsENOD40 and pGmENOD40-2 were hybridized to Southern blots containing genomic pea DNA digested with *Eco* RI, *Bam* HI or *Hin*d III. Both clones hybridized to a 5 kb *Eco* RI, a 18 kb *Bam* HI and two *Hin*d III fragments of 2.4 and 9.5 kb (Fig. 1). Since the insert of pPsENOD40 contained a *Hin*d III site (Fig. 2A), the 3′ and 5′ *Eco* RI-*Hin*d III subclones of pPsENOD40 were hybridized to *Hin*d III-digested pea DNA. The restriction fragment of 2.4 kb hybridized to the 3′ subclone, while the 9.5 kb fragment hybridized to the 5′ subclone (data not shown). These results show that pPsENOD40 represents the pea sequence most homologous to pGmENOD40 and, moreover, it is likely that the pea genome contains

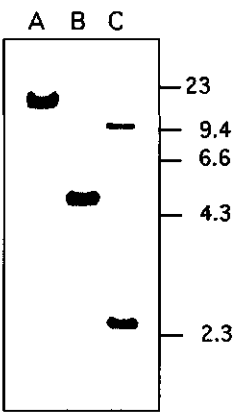

Fig. 1. Autoradiograph of a Southern blot containing pea genomic DNA digested with *Bam* HI (a), *Eco* RI (b) and *Hin*d III (c). Blot was hybridized with [^{32}P]-labelled insert DNA from pGmENOD40-2 or pPsENOD40. The hybridization results were identical for the two probes used and therefore only one set of data has been presented. DNA fragments of lambda DNA cutted with *Hin*d III were used as molecular markers (indicated at the right).

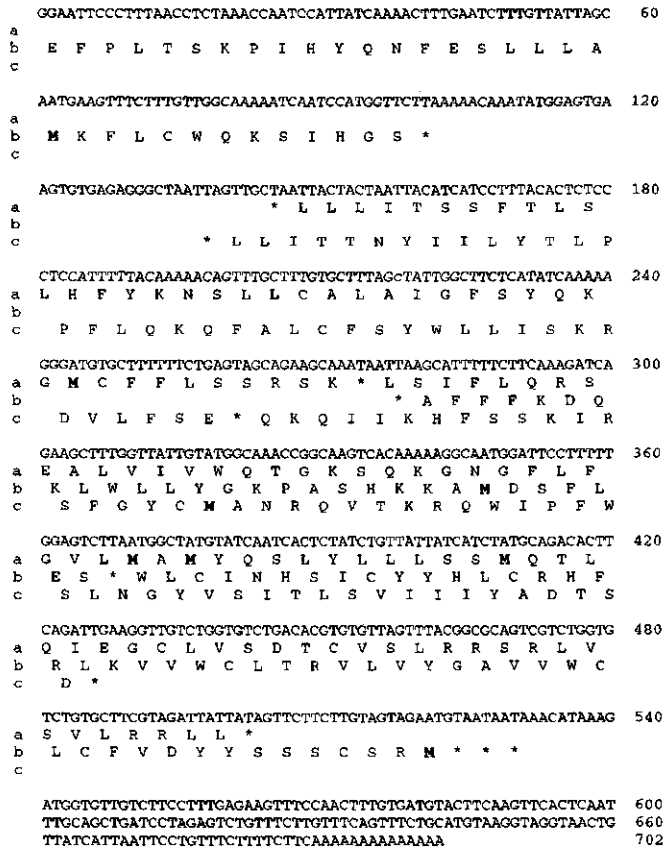

Fig. 2A. Nucleotide sequence of the *Eco* RI insert of pPsENOD40. The predicted amino acid sequences of the ORFs with more than 25 aa are shown in standard single-letter code below the nucleotide sequence.

only one ENOD40. The insert of pPsENOD40 has a length of 702 bp which is similar to the estimated length of 700 bp of the pea ENOD40 mRNA (data not shown). The sequence of the insert of pPsENOD40 is shown in Fig. 2A. Comparison of the sequence of the soybean pGmENOD40-1 and pea pPsENOD40 clone showed that the homology at the nucleotide level is 55%. The proposed coding region of pGmENOD40-1 is 68% homologous to pPsENOD40 (Fig. 2B). To determine the amino acid (aa) sequence of the putative pea ENOD40 polypeptide we searched for ORFs in the sequence of the insert of pPsENOD40. Figure 2A shows all ORFs with more then 25 aa and methionines are indicated with bold type. The longest ORF contains 75 aa (position 277–501) and the first ATG in this ORF is at position 370. If this is the start codon then the pea ENOD40 polypeptide will only be 44 aa long.

At the other hand, the ORF identified in pGmENOD40-2 lacks an ATG as start codon, but still is translatable in an *in vitro* translation system [9]. So, it is possible that also in

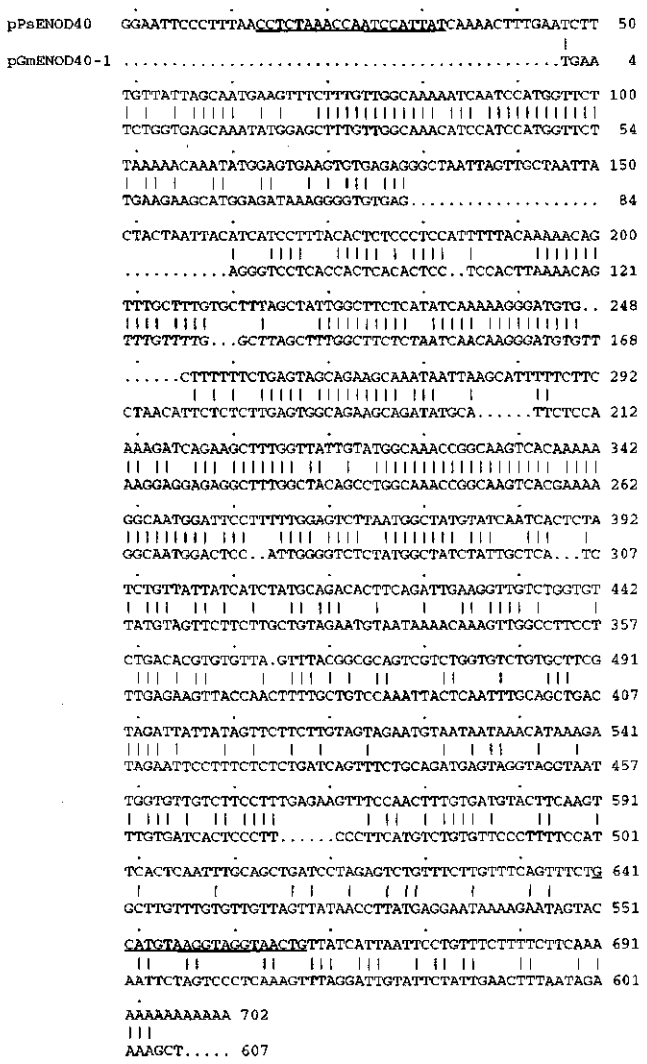

Fig. 2B. Comparison of the nucleotide sequences of the inserts of pPsENOD40 and pGmENOD40-1. Primers used in the PCR experiments are underlined.

pPsENOD40 an ORF does not start with an ATG. Therefore, we will refer to the 75 aa long ORF as the putative pea ENOD40 polypeptide, although we do not have any evidence that this ORF is indeed translated.

Since the pea and soybean ENOD40 cDNA clones are homologous we expected the derived soybean and pea ENOD40 polypeptides to show significant homology. Surprisingly, they showed only 14% homology and 39% similarity. This low

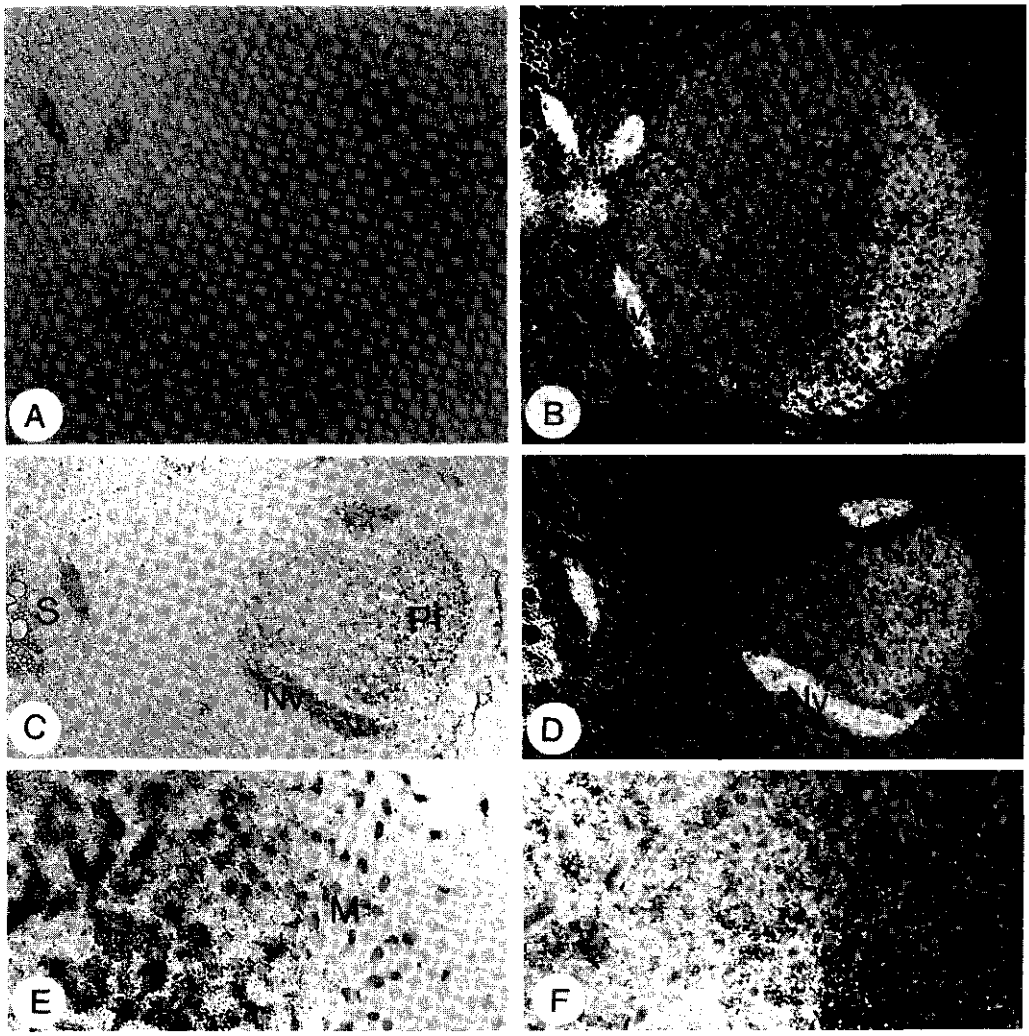

Fig. 3. In situ localization of PsENOD40 mRNA in the full-grown pea root nodule. A, C and E are bright-field micrographs in which the signal is represented by black dots. B, D and F are dark-field micrographs with white dots as signal. The sections are hybridized with [^{35}S]-labelled antisense PsENOD40 RNA. The intense light observed in the phloem and xylem elements in the vascular bundle is the result of scattering of light on the solid cell walls and not of hybridization signals. A and B. Cross section through a pea root containing an indeterminate nodule, harvested 18 days after sowing, showing a high level of PsENOD40 mRNA in the nodule vascular bundle pericycle and in cells of the infection zone compared to the low level in cells in the interzone and nitrogen fixing zone. The arrow points to the start of amyloplast accumulation. E and F. Detail of the distal part of a 18-day-old root nodule showing the absence and presence of PsENOD40 mRNA in the root nodule meristem and in the infection zone, respectively. C and D. As A, but now focusing on the peripheral part of the nodule. M, nodule meristem; Nv, nodule vascular bundle; Pf, pre-fixing zone; S, stele of the root.

level of homology, due to the presence of several non-triplet insertions and deletions, prompted us to determine whether pPsENOD40 contains sequence abnormalities due to cloning artefacts. Hence we amplified on genomic DNA by PCR, using the primer sequences indicated in Fig. 2B, a part of ENOD40 of pea cultivar Sparkle and of two wild pea lines, Afghanistan and Cyprus. In all three cases the obtained PCR fragments had a length of 650 bp and after cloning their nucleotide sequences were determined. The sequence of the cloned region of Sparkle ENOD40 was identical to the Sparkle pPsENOD40 cDNA clone with the exception of one substitution (C→T) at position 133. The same substitution also occurred in the sequence of the ENOD40s of both wild pea lines. Therefore we expected that the C at position 133 of the cDNA clone was due to a transcriptional mistake. The ENOD40 sequence of the Cyprus pea contained one basepair deletion at position 230 and a two basepair deletion at position 468–469, of which the latter also occurred in the Afghanistan pea ENOD40 sequence. The deletion at position 468–469 resulted in a frame shift that led to a shorter ORF by which the putative polypeptides were missing the four N-terminal amino acids present in the Sparkle ENOD40 polypeptide. Since the sequence of the Sparkle ENOD40 was (almost) identical to the sequence of pPsENOD40, indeed the putative ENOD40 polypeptides of soybean and pea have only little homology.

The very low level of homology of the putative soybean and pea ENOD40 proteins suggests that the proteins might have different functions. Therefore, we compared the expression pattern of pea ENOD40 with that of soybean ENOD40 [3, 9]. We studied the expression of pea ENOD40 by northern blot analyses and *in situ* hybridization experiments. Northern blot analyses showed that PsENOD40 RNA is present at a high level in nodules and at a low level in uninoculated roots, stems and flowers (data not shown). This is similar to the expression pattern of GmENOD40 [3], although PsENOD40 is expressed in uninoculated roots at a higher level than the GmENOD40s since the expression of the latter genes could only be detected with PCR-based methods [3].

In situ hybridization studies showed that in a pea root at 3 days after inoculation PsENOD40 is expressed in a globular nodule primordium that has been formed in the root inner cortex as well as in the region of the root pericycle opposite the primordium [9]. To obtain a complete picture of the expression of PsENOD40 during nodule formation, we studied the *in situ* expression of this gene in a nodule 18 days after inoculation. Sections (Fig. 3) of a mature nodule hydridized to anti-sense ENOD40 RNA showed that PsENOD40 is predominantly expressed in the pericycle of the nodule vascular bundle (Fig. 3A, B). In addition, PsENOD40 is expressed in the prefixing zone of the nodule [1, 8], but not in the nodule meristem (Fig. 3C, D; see Fig. 3E, F for a detail). In the pre-fixing zone the infected cells contain a higher level of ENOD40 RNA than the uninfected cells. In the nitrogen-fixing zone [1, 8] a low level of ENOD40 RNA is detectable. Thus, the *in situ* expression pattern of pea ENOD40 is very similar to that of soybean ENOD40s. In both species the ENOD40s are induced in the nodule primordium and the region of the root pericycle opposite the primordium, and in mature nodules these genes are expressed in the pericycle of the vascular bundle. The major difference is the expression in the infected cells in the pre-fixing zone in the indeterminate nodule, whereas in the determinate nodule no expression has been observed in infected cells. The similarity of these relatively complex expression patterns strongly suggests that the ENOD40 genes have a comparable function in pea and soybean. However, despite the significant homology at the

nucleotide level between the pea and soybean clones, there was hardly any homology between the derived polypeptide sequences. There are some explanations that sequence homology is greater at the nucleotide level than at the polypeptide level.

1. The nucleotide sequence contains errors, so that the derived (poly)peptide sequences are out of frame. Since the sequence has been obtained from a cDNA clone and 3 independently obtained PCR fragments, we feel that this trivial explanation can be eliminated.

2. The correct ORF has not been recognized. In our search for the putative ORF in pPsENOD40, we had two criteria: the ORF should consist of at least 25 residues and it should be homologous to the ORF identified in pGmENOD40-2. However, it may well be possible that the ORF in pGmENOD40-2, which is translated *in vitro* although lacking an ATG, does not represent the ORF used *in vivo*. In this case the level of homology between the identified ORFs does not necessarily have to be high. It is also possible that one of the ORFs consisting of less than 25 amino acids, which have not been included initially in our homology search between the ORFs of the pea and soybean cDNA clones, represents the polypeptide, most homologous between pea and soybean. In this respect, it is noteworthy that an ORF staring at nucleotide 62 in pPsENOD40 and consisting of 13 amino acids shows 61.5% identity to a putative 12 amino acid long peptide encoded by pGmENOD40-2 starting at nucleotide 65 [9].

3. Another possibility is that gene evolution by natural selection is acting (primarily) at the DNA/RNA level rather than at the protein level. As a result of this the putative polypeptide products may not even be active at all and the RNA may be the active molecule.

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Chapter 3

Characterization of the soybean gene *GmENOD40-2*

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Characterization of the soybean gene *Gm*ENOD40-2

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Abstract

The *Gm*ENOD40-2 gene was isolated from a soybean genomic library and the nucleotide sequence of a 3 kb EcoRI/HindIII fragment was determined. A 1.7 kb fragment of the *Gm*ENOD40-2 promoter region was used in a transcriptional fusion construct with GUS. In transgenic *Vicia* nodules containing this construct, β-glucuronidase activity was detected in the pericycle of the nodule vascular bundles as well as in the root pericycle at the attachment point of the nodule.

Key words: *Glycine max*, soybean, early nodulin, p*Gm*ENOD40-2.

Introduction

Leguminous plants are capable of utilizing atmospheric nitrogen resources through a symbiosis with soil bacteria of the genera *Azorhizobium*, *Bradyrhizobium* or *Rhizobium*. The *Rhizobium*–legume interaction leads to the formation of a morphologically defined plant organ, termed nodule, which is located on the legume root. During nodule formation, the expression of several plant genes is specifically induced. The proteins that these genes code for are known as 'nodulins' (van Kammen, 1984; Nap and Bisseling, 1990) and they are categorized as early and late nodulins depending upon their timing of expression (Nap and Bisseling, 1990).

Until now, several late nodulin genes have been identified and characterized (Sanchez *et al.*, 1991). Some of these genes code for proteins involved in carbon (Thummler and Verma, 1987) and nitrogen (Nguyen *et al.*, 1985; Sengupta-Gopalan and Pitas, 1986) metabolism in the nodule, or proteins specifically targetted to peribacteroid membrane (Katinakis and Verma, 19 Fortin *et al.*, 1987; Miao *et al.*, 1992). On the c trary, very few early nodulin cDNA clones have b isolated so far, like *Gm*ENOD2 (Franssen *et* 1989), *Ps*ENOD5, *Ps*ENOD12, *Ps*ENOD3, *Ps*ENOL (Scheres *et al.*, 1990*a*, *b*), and *Mt*ENOD10 (Lobler a Hirsch, 1993).

Recently, two early nodulin ENOD40 cDNA clones soybean, p*Gm*ENOD40-1 and p*Gm*ENOD40-2 that 95% identical on the nucleotide level, as well as a ENOD40 clone have been described (Kouchi and Ha 1993; Yang *et al.*, 1993; Matvienko *et al.*, 1994). T putative polypeptides of the longest open reading fra of the soybean clones, are 67.5% identical. The putat *Gm*ENOD40-1 polypeptide starts with a methioni while that of *Gm*ENOD40-2 does not contain a methi ine at all. The expression of the *Gm*ENOD40 genes duri the soybean–*Bradyrhizobium* interaction has been studi by *in situ* hybridization. *Gm*ENOD40 is already induc in the first outer cortical cells that are mitotically activat by *Bradyrhizobium* (Yang *et al.*, 1993) as well as in t region of the root pericycle facing the nodule primordiu (Kouchi and Hata, 1993; Yang *et al.*, 1993). In matu nodules, these genes are transcribed at a high level in t pericycle of the nodule vascular bundles as well as a relatively low level in the boundary layer and the uninf ted cells of the central tissue (Yang *et al.*, 1993).

In this paper we present the isolation and characteriz tion of the *Gm*ENOD40-2 gene. Furthermore, we use construct containing a transcriptional fusion of a p moter fragment of this gene to a GUS reporter gene, order to study its expression in transgenic *Vicia* nodul

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Abbreviations: GUS: β-glucuronidase.

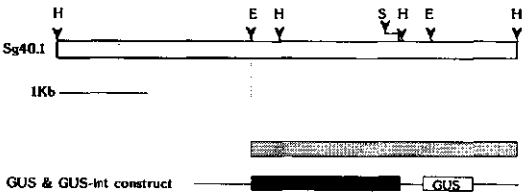


Fig. 1. Physical map of a soybean genomic DNA fragment, of the recombinant phage Sg40.1, hybridizing to p*Gm*ENOD40-2. The complete nucleotide sequence of the fragment indicated by the vertical dotted lines has been determined. E, EcoRI; H, HindIII; S, SstI. The SstI/EcoRI fragment denoted by the solid black line was used for the transcriptional fusions to GUS and GUS-intron.

Materials and methods

Isolation of genomic clone, sequence analysis and DNA manipulations

A soybean genomic library constructed in the Charon40 vector (kindly provided by Dr J. Slightom) was screened by plaque hybridization at 65 °C, using the insert of p*Gm*ENOD40-2 (Yang *et al.*, 1993) as a probe. Only one positive clone was recovered. Phage DNA was isolated and the restriction map of the clone was constructed according to standard methods (Sambrook *et al.*, 1989).

Overlapping genomic fragments of the clone were subcloned into pBluescript KS$^+$ (Stratagene Inc.) and were sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977), using Sequenase v2.0 (U.S. Biochemicals). Both strands were entirely sequenced. The sequence data were analysed by a program written by Staden (1984) on a micro VAX/VMS computer.

To determine the transcription start site of the *Gm*ENOD40-2 gene, nodule RNA was annealed to the specific primer 5'-$^{+163}$GAGGGAGTGTGAGGAGTGAGCAC^{+150}-3', complementary to the 5'-end of the p*Gm*ENOD40-2 cDNA. The size of the extension product was determined according to Scheres *et al.* (1990*a*). Soybean genomic DNA was prepared from etiolated hypocotyls, digested and analysed on agarose gel electrophoresis by standard methods.

The cointegration vector PIV20 (Bak Ramlov, 1993) containing the GUS A, and the binary vector pBI101.2 from which the GUS A gene was replaced by the GUS A-int gene (Vijn *et al.*, 1995) were used for the transcriptional fusions of the SstI/EcoRI fragment of the *Gm*ENOD40-2 promoter region. The constructs were introduced in *Agrobacterium rhizogenes* by direct transformation.

Plant material and induction of transgenic nodules

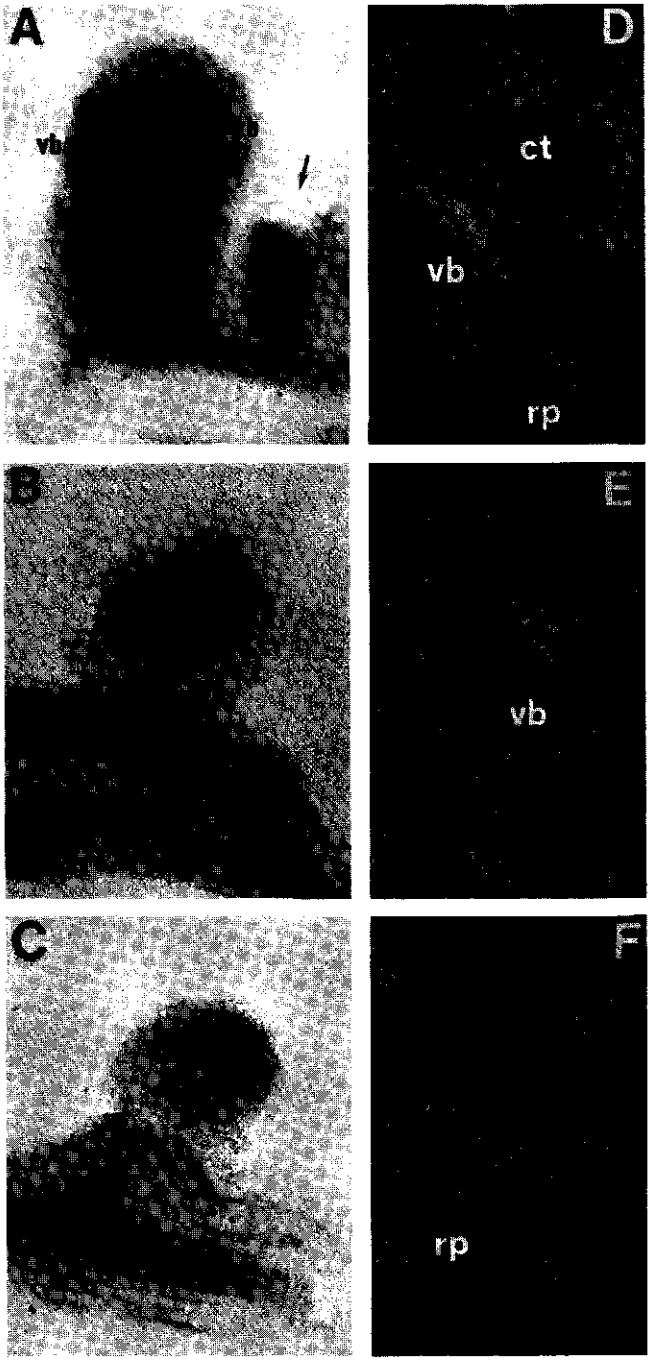
Agrobacterium rhizogenes strain par. 14 and *Rhizobium leguminosarum* biovar *viciae* were grown on LB and YEM media, respectively, at 30 °C. Single colonies of *Agrobacterium rhizogenes* containing the chimeric *Gm*ENOD40-2/GUS clone were selected on media containing appropriate antibiotics. *Vicia hirsuta* seeds were surface-sterilized and cultivated according to Quandt *et al.* (1993) with some modifications (Vijn *et al.*, 1995). Generation of composite transformed plants was performed by *Agrobacterium rhizogenes* puncture innoculation of the seedlings. Transgenic hairy roots were obtained and inoculated by *Rhizobium leguminosarum*. Histochemical staining of plant material was performed according to Jefferson *et al.* (1987) with the modifications described by Quandt *et al.* (1993).

Results and discussion

A recombinant phage was isolated from a soybean genomic library constructed in Charon40 using as a probe the cDNA clone p*Gm*ENOD40-2 and it was designated Sg40.1. The genomic DNA cloned in this phage contains one EcoRI restriction fragment of 2.0 kb and two HindIII fragments of 1.2 and 1.4 kb, respectively, that hybridize to the insert of p*Gm*ENOD40-2 (Fig. 1). Southern blot analyses showed that genomic EcoRI and HindIII fragments with the same length hybridize with p*Gm*ENOD40-2 (A. Roussis, unpublished results).

The nucleotide sequence of a 3.0 kb EcoRI/HindIII fragment of the recombinant genomic clone Sg40.1 was determined (Fig. 1). This sequence starts 1743 bp upstream of the transcription start, as this was determined by primer extension (data not shown). The sequence from nucleotide +29 to +723 has a 100% similarity with the insert of p*Gm*ENOD40-2 (Yang *et al.*, 1993) (Fig. 2). The putative TATA (−44/−41) and CAT (−55/−52) boxes are indicated in Fig. 2. Two conserved sequence motifs 5'-AAAGAT-3' and 5'-CTCTT-3' have been identified in several nodulin genes (Sandal *et al.*, 1987; Miao and Verma, 1993). In some cases, like *lbc*3 and nodulin-26, it has been proven by deletion analysis and site-specific mutagenesis, that these sequences are required for nodule specific expression (Stougaard *et al.*, 1990; Miao and Verma, 1993; Bak Ramlov *et al.*, 1993; Szcyzglowski *et al.*, 1994). Such sequence motifs are present in the *Gm*ENOD40-2 promoter (Fig. 2). However, whether these motifs are involved in nodule-specific expression of *Gm*ENOD40-2 gene is unknown. Furthermore, three groups of direct repeats are located in the p*Gm*ENOD40-2 promoter: a 12 bp repeat (AGGTTAAGGTTA)

Plate 1. Histochemical analysis of GUS activity in transgenic *Vicia hirsuta* root nodules, transformed with *Agrobacterium rhizogenes* carrying the PIV20/40-GUS construct. For the pBI101.2/GUS-int construct, the results are identical. Intact (A) or half (B, C) nodules were treated with x-Gluc. The blue precipitate indicates GUS activity. The expression is seen in more detail in dark field micrographs (D, E, F) of longitudinal sections of transgenic *Vicia hirsuta* mature nodules, where GUS expression is visible as bright pink colour; vb: vascular bundle, ct: central tissue, rs: root stele, rp: root pericycle. (A) Strong GUS activity can be observed in the pericycle of the root and in the nodule vascular bundles. In a young nodule (indicated by an arrow) where the vascular bundles are not fully developed yet, GUS expression is restricted only to the pericycle of the root stele opposite one of the xylem poles where the provascular strands will be initiated. (B, C) A nodule cut longitudinally in two parts. GUS activity is present in the vascular bundle branching around the central tissue (B— arrowhead) and also in the root pericycle facing the nodule (C— arrowhead). (D) Longitudinal section of a transgenic *Vicia hirsuta* nodule. (E, F: Detail of D). GUS activity is localized in the root pericycle and adjacent cortical layers (F) and in the nodule vascular bundle (E). No expression of the chimeric gene is detectable in the uninfected cells of the central tissue of the nodule.


A
vb
D
ct
vb
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B
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starting at positions −282 and −240, a 10 bp repeat (TTATTTTAAT) starting at positions −206 and −188 and a 7 bp repeat (AAATGGA) starting at positions −117 and −96. The significance of these repeats is not known as yet, although the 10 bp repeat is similar with AT-rich protein binding sites identified in other nodulin and non-nodulin genes (Forde, 1994).

Yang *et al.* (1993), have additionally reported the isolation of p*Gm*ENOD40-3, a cDNA clone homologous with p*Gm*ENOD40-2, but 47 bp shorter at its 3′ end. This is consistent with the presence of two putative polyadenylation signals ($^{+593}$AATAAA, $^{+690}$GAATAAT) in a region of 28–125 bp upstream of the polyadenylation site of *Gm*ENOD40-2 gene (Fig. 2). Therefore, we propose that p*Gm*ENOD40-2 and p*Gm*ENOD40-3 are transcribed from the same gene.

The complicated expression pattern of *Gm*ENOD40 raised our interest in the promoter sequences that are responsible for the developmentally regulated and tissue specific expression of this gene.

To determine whether the isolated *Gm*ENOD40-2 clone contains the sequences required for nodule specific expression, transcriptional fusions with GUS reporter genes (Jefferson *et al.*, 1987) were made; a 1731 bp SstI-EcoRI fragment of the 5′ untranscribed region of *Gm*ENOD40-2 was cloned in the cointegration vector PIV20 (Bak Ramlov *et al.*, 1993) and the binary vector pBI101.2 GUS-int (Fig. 1). This promoter fragment encompasses the sequence between nucleotides +12 and −1743. *Vicia hirsuta* was transformed with the *Agrobacterium rhizogenes* strains, containing these constructs according to the system of Quandt *et al.* (1993). The hairy roots were inoculated with *Rhizobium leguminosarum* bv. *viciae* and, 14 d after inoculation, the expression of the chimaeric *Gm*ENOD40-GUS gene and the *Gm*ENOD40-GUS-int gene was histochemically localized in roots and nodules of transgenic plants. GUS activity, expressed from the *Gm*ENOD40-2 promoter using both constructs, was located in the vascular tissues surrounding the nodule and in the pericycle of the root stele, just opposite the nodule (Plate 1A, B, C). In addition, in younger, not fully-developed nodules, the characteristic blue colour is exclusively detected in the root pericycle, where the provascular strands of the nodule will be initiated (Plate 1A). This expression pattern is observed in intact nodules or half nodules cut longitudinally and is in agreement with performed *in situ* hybridization experiments, which showed

-1743 GAATTCATTT TTCATAATTT ACGTTCTTCC ATTTATCTTA GGGACTAATT
-1693 TGATTTTTCA CCCTTCTTTG ACTAGGTTAG TTAAATGGGT TTGTTGTTTC
-1643 ATACCAATGG CCACAAAGAA AAGTTCATAA AAGAACGTAT GAATTGTGAT
-1593 CTCATATCCA GTGTAATATA ATAGGCAATG CTGATGACAT GTACAAATAA
-1543 TAAGTGGCTA GGAACAGTAA TAAATAAACA GCATAGAGAG ATTCCAAAAG
-1493 GGGACTTAAA AGCCATAAAA ATATATTGGT TTCTTGCTTG AAGAAGAGGA
-1443 AAACCAAAAG CTTGAATGGA TTGTCTAGCT GCTATGATTG TGCGCATATC
-1393 TGAGAAGGAG ATCACGCAAT CTTTCGTAAA ATTACCAAAG AGTGTTACAG
-1343 GAATTTTGGT TAATCTATAA AGCCGGCTAG GAAAGAAAAA TGACTCTTGG
-1293 ATATAAGTTC AAGCTATTGT ATTGACTGCT TCTCTTCTTT TTTTTTTTTA
-1243 ATATATAAAA TAGAAAACTT CACTTCTTGG TGATGTGATG ACTAATCACT
-1193 CTTTAATTTC AAAGGAAAGT TGAATTGCCC CCCTAGAAAT TATGAAAAGA
-1143 AGACACATGA TAGGGATTAA CAACAACTAA GCTAAACAGT ACAGTATGCT
-1093 ACATACACGG CCAAGATTAT TTTTTAAGCT CTGAAATCAA ATCATGTATT
-1043 TGACATTATA TTAAAAGAAG AAACAAAGTG GTTTGCTGGC ATGGCAGGAT
-993 GGTGGGGCAC AGAACGAAAT TAGTGGATAG TAAGGAATTT AAAATAATAT
-943 ATCATTGATG TTTAAATTTC TTTTAACAAA TCCCTCGTAA CATTTTCTAA
-893 TTATTTCAAA CAGAAAATGT AAGCATGTTT AATATCTTGG AGGCTAAAAC
-843 ATATGTGTTT AATGTTTTTT TTTATTCAAT ACTTAAGAGG ATTATTTAAT
-793 TGGTTCATTA AGAATATATA AAATTATTTT GAAAGCAAAG TGGGGTTGAG
-743 TCTCAGTAAG TGGATTCACT GCTGCAATAA AAGCCTCCTT CTATCAAAAG
-693 TCAAGCACGA ATGAAAAAGT AAAGAGTGGG TGCCCATGTT TTAAAGTTTA
-643 TACATATTAC ACTTTGACAT TCATATCCTT TGTATTTCAA TTAGATTAAG
-593 ATATCTTCTA TGGACACTAT TTAGTATTTA ATTTTAAATT CCCTAAAATG
-543 CCTTTGTGAC CTAATATAAT ATGTAGTGTA GTGTATGTCT TTGGTAAAAA
-493 AAAAAAAAAC TAAGCTGAAA TTTGGCGAAG GACAAGATAG AGGTCACAAA
-443 TTCAATATGA ACATATTATA ACTAAGTACT GCTTAAATAG TAGAAAAAAA
-393 CAGAATGAGA TAATCAAATT AGGTTAGGGA TCTCAAAACT TCATTCCATG
-343 TAAACCAGTA GAAGAAAAAT GTGTAAACAG ATCAATTAGG TCAGGTCATA
-293 TTGCTGGTTA AGGTTAAGGT TAAGTGAAGT TCAAGAAAAG TTAGGCCGGT
-243 ATTAGGTTAA GGTTAGATTC AATAAACCAA AGAAATGTTA TTTTAATCCA
-193 TTTAATTATT TTAATGAGCG CAGTACAATT TTCTTTCTTG AGTTTTCTAT
-143 ATTGCAACTT TTGATGATAG TTTTTTAAAT GGAAAAAAGT AATAAGCAAA
-93 TGGATAATGA AAAGATGATG ATAGCACTTC TTAGTTCTCA ATCATCAACT
-43 ATTTAAAACA ATGGTCAGAG GCTAACTTCT CCACTAGTTT TCTGTGTAGA
+8 GAGCTCTTTG GACAACACCC TCTAAACCAA TCTATCAAGT CCTGATTAAT
+58 CTGGTGAGCA TGGAGCTTTG TTGGCTCACA ACCATCCATG GTTCTTGAAG
+108 AAGCTTGGAG AGAAAGGGGT GTGAGAGGAG AGGGTGCTCA CTCCTCACAC
+158 TCCCTCACTT AAAACAGTTT GTTTTGGCTT AGCTTTGGCT TCTCTGATCA
+208 ACAAGGGATG TGTTCTAACA TTCTTTCTTG AGTGGCGGAA CGAGATACAC
+258 ATTCTCCGAC GGAGGAGAGG CTTGGCTACA GCCTGGCAAA CCGGCAAGTC
+308 ACAAAAAAGG CAATGGACTC CATTGGGGTC TCTATGGCTA TGTAGTGCTC
+358 ATGTAGTTCT TCTTGCTGTA GAATGTAATA ATAAACAAAG TTGGTCTTCC
+408 TTTTGAGAAG TTACCAGCTT TTGCTGTCCA AAATTACTCA ATTTGCAGCT
+458 GACTAGAATT CCTTTCTCTC TTCAGTTTCT GCAGATGAGT AGGTAGGCAA
+508 TTTGTGATCA CTCCCTTCCC TTTTCATGTC TTCTGTGTTC CCTTTTCCAT
+558 GCTTGTTTGT GTTGTTAGTT ATGACCTTAT GAGGAAATAA AAGAATAGTA
+608 CAATTCTAGT CCCTCAGTTT AGGATTGTAT TCTATTGAAC TTTATTAGAA
+658 AAGTTTCCAG AGTCCTTTCT AAGGTTGGAG TCGAATAATG TTCATGATCC
+708 CTCACCCTTT CCCTTTATAC TGCTGCATTT CTGATTTTGC TAACACTTGA
+758 AATTGTTCAG GGGACTAAAT GGTAAACTAG TTTCTTTATC CTCTTCTTAT
+808 GGCTTCCTAA GAAAGAAATA TTTCATGTCC CAAAAGTGAT AGAAAAGAGA
+858 AATGAACTTC TTAAGTAGGA ACGGTATACA TAAGACTAAG ACACCTGATA
+908 TATACAAGTG AGTCTTATTA AAAGTACATT TATTTCTAAA AGAATACTCC
+958 TTAGATATAC TAACTTGTTT CTTATAAATA GTCTCGAGGT AGTATCCATT
+1008 ATTGATTTTT AGTCATATAG ATAAAACATG CTAGCTTAAA GTTTATTCAA
+1058 GTCTATAGTC AAAAACCAAT TTCATCCAAG GCCAACGTTT CTCCTTATCA
+1108 TCATAAGAAA CATGTTGAAG GTTTACTTC CATTCATCTT AGTTAAACAA
+1158 AAGAGATTTC TTCTACATTT TTTACATTGG AAAGTAGCAA ATATCCAGTT
+1208 TAACAAGCTT

Fig. 2. Nucleotide sequence of the genomic clone Sg40.1 containing the *Gm*ENOD40-2 gene. The sequence is numbered from the transcription start indicated in the figure by +1 which was determined by primer extension. Putative TATA and CAAT boxes are highlighted by rectangles. The 'nodule specific motifs' AAAGAT and CTCTT are in bold. The direct repeats found in the promoter region are double underlined. The underlined sequence from nucleotide +29 to +724 corresponds to the cDNA sequence of p*Gm*ENOD40-2.

that the ENOD40-2 transcripts were localized in the same tissues (Yang *et al.*, 1993; Kouchi and Hata, 1993). However, Yang *et al.* (1993) had also reported the expression of this gene in the uninfected cells of the central tissue of mature nodules. Therefore, sections of transgenic *Vicia hirsuta* nodules were taken and stained for β-glucuronidase activity. Microscopical analysis showed that the expression of the *Gm*ENOD40-2-GUS gene is restricted to the vascular bundles of the nodules and the region of the root pericycle, while no GUS expression at all was detected in the central tissues (Plate 1D, E, F). Thus, it can not be excluded that sequences located either upstream from nucleotide −1743 of the promoter region or downstream from the 3' end of the coding region of *Gm*ENOD40-2 gene, are imperative for the expression in this cell type.

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Chapter 4

Modification of phytohormone response by a peptide encoded by *ENOD40* of legumes and a non legume

Karin van de Sande, Katharina Pawlowski, Inge Czaja, Ursula Wieneke, Jeff Schell, Jürgen Schmidt, Richard Walden, Martha Matvienko, Joan Wellink, Ab van Kammen, Henk Franssen and Ton Bisseling. Based on Science, Vol. 273, 370-373, 1996.

Abstract

Legume nodule formation is initiated by Nod factor induced root cortical cell divisions. These divisions are most probably triggered by local changes in, or altered responses to, phytohormone ratios. The early nodulin gene *ENOD40* is expressed in the root pericycle prior to these cell divisions, opposite the site in the cortex where they occur. A possible role for *ENOD40* in modulating the activity of auxin is indicated by the facts that *ENOD40* transformed tobacco plants exhibit reduced apical dominance and *ENOD40* overexpressing tobacco protoplasts are capable of dividing in high auxin concentrations.
A homologous clone was isolated from tobacco, and this, as does *ENOD40* from soybean, encodes an oligopeptide of 10 and 12 aa in tobacco and soybean, respectively. Using fusions with the Green Fluorescence Protein (GFP), as well as an antiserum against the soybean peptide, it was shown that this peptide is made *in vivo*. In tobacco protoplasts this peptide changes the respons to auxin at concentrations as low as $10^{-12}/10^{-16}$ M. The peptide encoded by *ENOD40* appears to act as a new type of growth regulator in plants.

Legume nodule organogenesis is initiated by local dedifferentiation of root cortical cells. Specific lipo-chitooligosaccharides (*1*) - the so-called Nod factors - secreted by *Rhizobium* bacteria trigger cell divisions in the cortex of the root (*2*). Nod factors probably are responsible for the induction of a local change of the auxin/cytokinin ratio, or of a modified phytohormone response resulting in induction of the cortical cell divisions (*3*). In these dividing cortical cells, the expression of a few nodule specific plant (nodulin) genes is induced by the Nod factors (*4, 5*). One of these nodulin genes, *ENOD40*, is first expressed in the region of the root pericycle opposite the nodule primordium (*4, 6, 7*). The expression of *ENOD40* in the pericycle precedes the induction of cell divisions (*8*) which suggests that *ENOD40* may play a role in changing the cells response to phytohormones and this may be involved in the induction of division. To test this assumption, we used tobacco as a model system. Tobacco, though a non-legume, provides an excellent model to test phytohormone responses, first because of the ease of transformation and the ability to test the effect of transgene expression on internal levels or ratios of phytohormones (*9*); and second because tobacco protoplasts provide a simple assay of phytohormone activity (*10*) since callus growth requires a defined ratio of auxin to cytokinin (*11*). Therefore, protoplast division under defined culture conditions can be used to study the effects of the expression of genes either directly modifying levels of active phytohormones or their signal transduction (*9, 15*).

In order to investigate ENOD40 action we transformed tobacco plants (*12*) with the construct 40-2/1-448 representing 448 basepairs (bp) of soybean *GmENOD40-2* under the control of the Cauliflower Mosaic Virus (CaMV) 35S promoter (Fig. 1; *13, 14*). Thirty percent of the transgenic F1 plants had one or two adventitious shoots at the base of the main shoot, as compared with none in untransformed tobacco plants (Fig. 2A). Side shoots at the base of the

main shoot suggest reduced apical dominance, raising the possibility that the transgenic plants were changed in terms of auxin metabolism or perception. One homozygous plant line, 11S-F2, containing a single copy of the *ENOD40* transgene and displaying a similar phenotype (data not shown), was used for further studies.

We assayed division of 11S-F3 protoplasts in the presence of different hormone concentrations to study the effects of *GmENOD40* expression at the cellular level. We found that wild type tobacco leaf mesophyll protoplasts display optimal division (about 50-60%) at 5.5 μM 1-Napthalene Acetic Acid (NAA), while they divide poorly (about 20-25%) at high auxin concentrations (13.8 μM NAA; *15*; Fig. 3A). At NAA concentrations up to 5.5 μM, 11S-F3 protoplasts divided at a frequency similat to that of wild type protoplasts (Fig. 3A). However, at higher concentrations of auxin, at which the division frequencies of wild-type protoplasts declined (to 20-25%), 11S-F3 protoplasts displayed undiminished division frequencies (Fig. 3A). Apparently, *ENOD40* expression removes the sensitivity of dividing protoplasts to high levels of auxin.

To confirm that tolerance of high auxin concentrations resulted from *ENOD40* expression, wild type tobacco protoplasts were transfected (*15*) with *GmENOD40* linked to the 35S promoter (*13*), and the frequency of cell division was scored in the presence of either 5.5, or 13.8 μM NAA (Fig. 1). Protoplasts transiently expressing *GmENOD40* (40-2/1-448) showed a division frequency (ca. 60%) at 5.5 μM NAA similar to that of untransfected protoplasts, or protoplasts transfected with a control plasmid (the cloning vector pMEX001; *13*). At 13.8 μM NAA, protoplasts transfected with *ENOD40* divided with a similar frequency (ca. 67%) as those at 5.5 μM. In contrast, protoplasts transfected with the control plasmid lacking the *GmENOD40* insert, were equally sensitive to high levels of auxin as untransfected protoplasts (Fig. 1). A similar tolerance to high auxin concentrations was obtained with full length *ENOD40* cDNA clones from alfalfa (Table 1) and soybean (40-2/1-656; Fig. 1) under control of the CaMV 35S RNA promoter (*13*). Thus, transient, as well as stable, overexpression of *ENOD40* confers tolerance of high auxin concentrations to tobacco protoplasts.

The observation that a soybean *ENOD40* sequence is biologically active in tobacco, indicated that nonlegumes contain an *ENOD40*-like gene. Therefore, we attempted to clone a tobacco homolog of *ENOD40*. A comparison of *ENOD40* sequences of different legumes (*4, 6, 7, 17, 18, 19*) revealed two regions with a high degree of sequence conservation (Fig. 5). This enabled us to use a Polymerase Chain Reaction (PCR) based strategy to isolate a tobacco homolog, using primers with sequence homology to the two conserved regions (*13*). Poly(A) RNA from tobacco flowers was used as a template because in pea, apart from the nodules, flowers contain the highest levels of *ENOD40* RNA (*7*). A tobacco *ENOD40* cDNA fragment was obtained from which a full size clone was produced by 3' and 5' racing (Nt40/1-470; Fig. 1; Fig. 5; *13*). To confirm the cDNA sequence, primers homologous to the 3' and 5' ends were used to isolate the corresponding genomic sequence identical to the cDNA by PCR.

When the tobacco *ENOD40* cDNA (*NtENOD40*), linked to the CaMV 35S promoter,

was transfected into tobacco protoplasts, it conferred tolerance of high auxin concentrations in a manner similar to that of soybean *ENOD40* (Fig. 1). Evidently, *ENOD40* activity is conserved between tobacco and legumes.

Sequence comparison of the tobacco and legume *ENOD40* clones revealed that the two conserved areas found in different legume *ENOD40* sequences are present in tobacco as well (Fig. 5). The area conserved at the 5' end of the different cDNAs (region 1) contains a highly conserved small open reading frame (ORF), starting with the first ATG available, encoding a peptide of 10 (in tobacco), 12 (in soybean) or 13 (in pea, alfalfa, vetch) amino acids (Fig. 3B). Sequence comparison suggests that these first ATGs can serve as translational start sites (*20*), consistent with the occurrence of *ENOD40* RNA in monosomes of pea (*21*) and alfalfa root

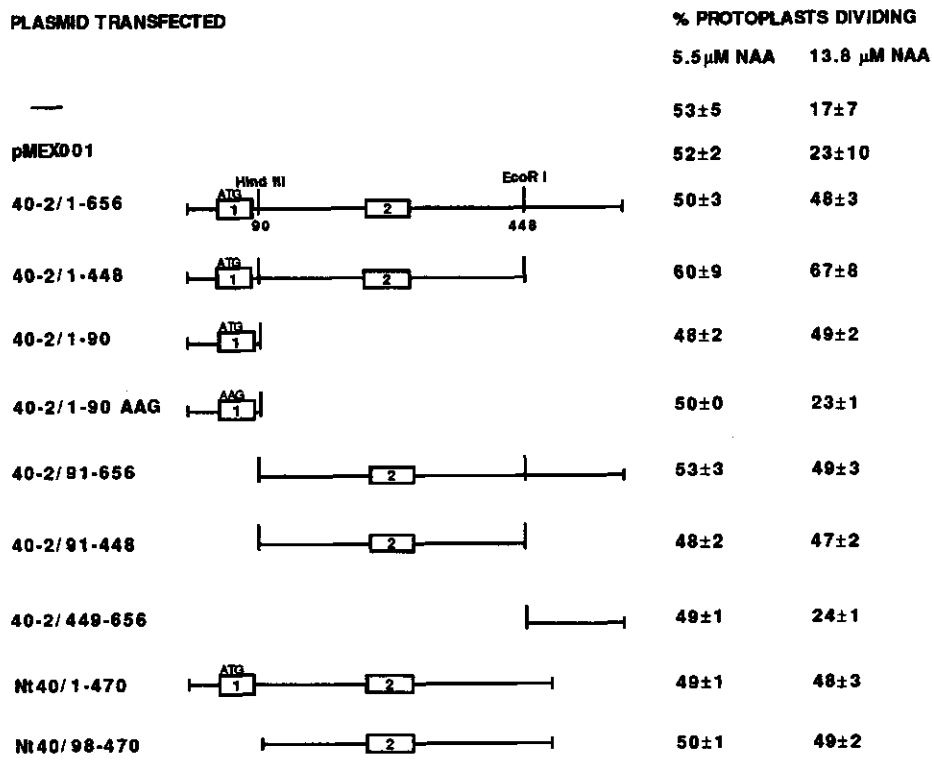
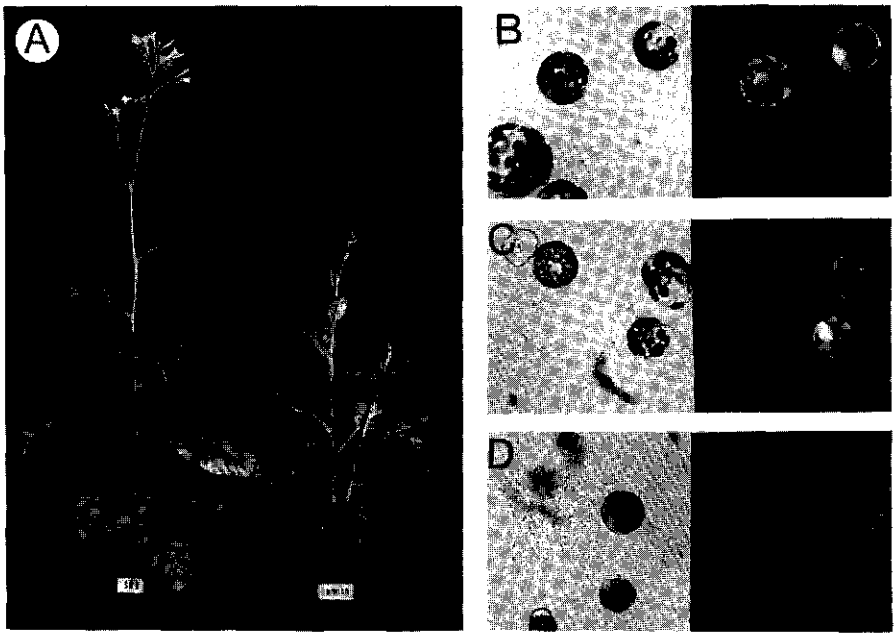


Figure 1. Effect of transient expression of different *ENOD40* constructs on tobacco mesophyll protoplast cell division. *ENOD40* expression vectors 40-2/1-656 and Nt40/1-470 contain full size cDNA clones (*13*). The conserved regions 1 and 2 are denoted as white boxes. The *Hin*dIII and *Eco*RI restriction sites, and their positions within the cDNA sequences are indicated. Transient expression assays were performed as described (*15*). Wild type tobacco protoplasts were cultured at 0.9mM Kinetin and 5.5mM or 13.8mM NAA, respectively, and division frequencies were scored in three independent experiments. The percent of protoplasts dividing is given with the standard deviation.



E

pMON40-GFPΔM (in-frame)

GAATTCCGCTAAACCAATCTATCAAGTCCTGATTAATCTGGTGAGCATGGAGCTTTGTTGGCTCaCAACCATCCATGGTTCTAGAAGTAAAGGAGAAGAACTTTTCACTGGAGT
M E L C W L T T I H G S R S K G E E L F T G V

pMON40-δXbaGFPΔM (out-of-frame)

GAATTCCGCTAAACCAATCTATCAAGTCCTGATTAATCTGGTGAGCATGGAGCTTTGTTGGCTCACAACCATCCATGGTTCCTAGAAGTAAAGGAGAAGAACTTTTCA
M E L C W L T T I H G S *
M V P R S K G E E L F T

Figure 2.(**A**) Phenotype of a heterozygous 35S-*ENOD40* (40-2/1-448) transformed tobacco plant (*28*). In contrast to the wild-type SR1 (left), the plant expressing *ENOD40* (4S-F1, right) has additional side shoots. In most of the transformed plants, the development of the additional shoots stopped after they had reached a length of 5-10 cm.
(**B, C, D**) Detection of GmENOD40-2/GFP fusion products in tobacco mesophyll protoplasts. One day after transfection, expression of *GFP* was scored by looking for fluorescence under a Leica DMBR microscope using the Chromatechnology 41014 GFP filter. Photographs were taken under a bright field (left panels) and with the GFP filter (right panels). **B**, protoplasts transfected with pMON-GFP; **C**, protoplasts transfected with pMON40-GFPΔM; and **D**, protoplasts transfected with pMON40-δXbaGFPΔM. About 20% of protoplasts transfected with the in-frame pMON-40-GFPΔM construct showed fluorescence (**C**) and fluorescence was detectable in none of the protoplasts transfected with the out-of-frame construct (**D**).
(**E**) Sequences of vectors pMON40-GFPΔM and pMON40-δXbaGFPΔM, representing in-frame and out-of-frame translational fusions of the peptide encoding part of *GmENOD40-2* and *GFP* (*22,29*), used for transfection into tobacco protoplasts. (In the out-of-frame construct a new ATG has been introduced into the *GFP* ORF). pMON-GFP was used as a positive control.

nodules (*18*). The second conserved sequence (region 2) is located in the central part of the *ENOD40* clones and lacks a conserved ORF. Because the only conserved ORF in all *ENOD40* genes is located in region 1, we tested whether this small ORF is actually translated in protoplasts by making a translational fusion between the ORF of *GmENOD40* and Green Fluorescent Protein (GFP; pMON40-GFPΔM; *22*). The *GmENOD40-GFP* ORF is preceded by the *ENOD40* 5'-untranslated region (Fig. 2E). As a control, an out-of-frame *GmENOD40-GFP* construct was made with a new ATG in frame with the GFP ORF (pMON40-∂XbaGFPΔM Fig. 2E). Tobacco protoplasts were transfected with the in-, or out-of-, frame construct. For comparison, a plasmid containing the *GFP* gene with its own translation start, driven by the *35S* promoter, was used (pMON-GFP). The *35S-GFP* construct (Fig. 2B) and the in-frame *GmENOD40-GFP* fusion (Fig. 2C) produced similar amounts of GFP. In contrast, the out-of-frame GmENOD40-GFP construct did not produce GFP activity (Fig. 2D), despite the introduction of an in-frame ATG. Thus, the *ENOD40* ORF is indeed translated in protoplasts and its ATG and 5' untranslated region function as a translational start.

To test directly whether the small ORF functions to produce tolerance to high auxin concentrations, a deletion derivative of GmENOD40-2 containing only the ORF driven by the 35S promoter (40-2/1-90) was tested in transfected protoplasts. This induced tolerance of high auxin concentrations in transfected protoplasts resulting in division in the presence of high auxin levels. A clone in which the ATG of the small ORF was replaced by AAG (40-2/1-90 AAG) was unable to induce the same response (Fig. 1). Taken together, these data suggest that the ENOD40 peptide is the biologically active molecule.

The peptides encoded by the small ORFs of *GmENOD40-2,* and *NtENOD40,* were both synthesized *in vitro* (Research Genetics; Fig. 3B). Both the soybean and the tobacco ENOD40 peptide conferred tolerance of high auxin concentrations in wild type tobacco protoplasts when added exogenously (Fig. 3C).

The tobacco peptide appeared to be far more active than the soybean peptide in tobacco protoplasts, since half-maximal activity was reached at approximately 10^{-12}M with the soybean peptide as compared with 10^{-16}M with the tobacco peptide (Fig. 3C). A peptide with 42% sequence identity to the soybean ENOD40 peptide and two unrelated peptides with sequences of Cowpea Mosaic Virus (CPMV) proteins used as control peptides (Fig. 3B) did not stimulate cell division in the presence of high auxin concentrations. Thus, the peptide encoded by *ENOD40* confers tolerance of high auxin.

The frequency with which transfected protoplasts divide at high auxin concentrations (about 50%) was high compared with the normally achieved transfection frequencies of about 20% (*15; 16;* GFP transfections). This was suggestive of the release of a soluble compound confering tolerance to wildtype protoplasts. To test this, 40-2/1-448 transfected protoplasts, or protoplasts from 11S-F3 plants were cultured in medium with 13.8 μM NAA for five days, and 100 μl of the culture supernatant was removed and added to freshly isolated wild type protoplasts. These protoplasts were cultured in either 5.5 μM or 13.8 μM NAA. The culture supernatant from transgenic protoplasts cultured at 13.8 μM NAA conferred tolerance of high

auxin concentrations to wild type protoplasts, while supernatant from wild type SR1 protoplasts did not (Table 1). The active compound was heat labile and sensitive to chymotrypsin (Table 1). Since the soybean ENOD40 peptide has the same characteristics (Table 1), an enzyme linked immuno sorbent assay (ELISA) with an antibody directed against the GmENOD40 peptide was used to check the presence of the peptide in the medium. Concentrated medium (50 fold) of wild type and 11S-F3 protoplasts grown at high auxin concentration was tested by measuring the extent to which the medium can compete for binding of the antibody to the antigen ENOD40 coupled to KLH (Fig. 4). The rate of competition of the 50 fold concentrated 11S-F3 medium and 100 ng of soybean ENOD40 peptide are comparable while medium of wild type protoplasts was unable to compete. It appears that the concentration of the peptide in the medium is around 10^{-8}M. This concentration is in good agreement with the activity found with the synthetic peptide (Fig. 3C). Whether the peptide is actively secreted into the medium, or released by bursting protoplasts is not clear.

Furthermore, the GmENOD40 peptide is found in nodules, but not in the roots of soybeans (Fig. 4). This demonstrates that the ENOD40 peptide is formed in tobacco protoplasts and root nodules. Because ENOD40 is expressed in dividing cortical cells during nodule formation, and the induction of ENOD40 expression in the pericycle precedes the mitotic activation of cortical cells, it is probable that the ENOD40 peptide plays a role in the

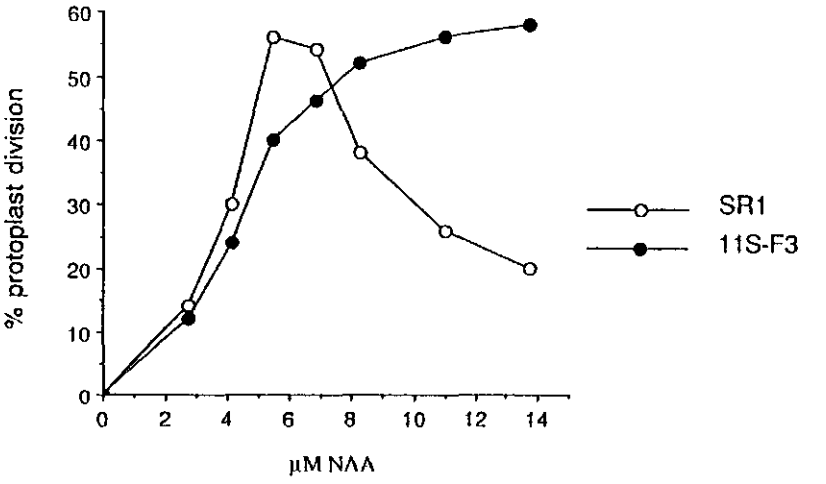


Figure 3:
(A) Tobacco mesophyll protoplast division frequencies of wild-type (SR1) and 35S-*ENOD40* (11S-F3) plants, respectively, in percent of protoplasts dividing after five days of cultivation depending on the NAA concentration.
Protoplasts were isolated (*15*) from leaves of wild type SR1 and transformed (11S-F3) tobacco plants, respectively. They were cultured with 0.9µM cytokinin (Kinetin) and different concentrations of auxin (1-naphtalene acetic acid, NAA, 0 to 13.8µM). Cell division frequency was scored microscopically five days after isolation in three independent experiments. The variation between samples from different repeats was below 10%. The values from one representative experiment are given.

```
GmENOD40-2   M.ELCWLTTIHGS
PsENOD40     MKFLCWQKSIHGS
MsENOD40     MKLLCWQKSIHGS
NtENOD40     M...QWDEAIHGS
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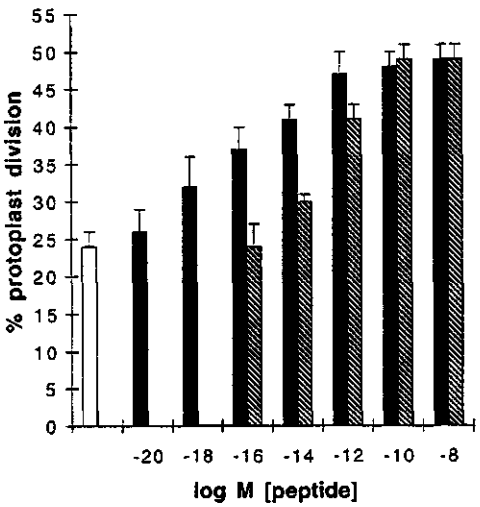
(B) Sequence alignment of the ENOD40 encoded peptides from soybean (*4, 6*), pea (*7*), alfalfa (*17, 18*) and tobacco plants.



(C) Division frequencies of tobacco mesopnyll protoplasts in high auxin concentrations, in the presence of different concentrations of ENOD40 peptides.
Filter sterilized peptides (synthesised by research genetics without the NH_2-terminal methionine residue) were added to freshly isolated wild type tobacco protoplasts. Division frequency was scored microscopically after five days of incubation in the presence of 13.8µM NAA. Averages of three independent experiments are given.
Black bars: tobacco peptide; striped bars: soybean peptide; white bar: background division with a mock addition of H_2O. With the soybean based control peptide MELMFATTARAT (the modified soybean peptide, in which amino acids which are conserved in different ENOD40 peptides are changed into chemically similar ones; amino acids not changed are underlined) and the CPMV control peptides (UFSFTQAKSKISLWTR, where U is norleucine, and an ata-linkage group is present at the N-terminus, and RYLEYEAPTIPEDCGSLVIAHIGGK; *30*) only background levels of cell division were found. At 13.8µM NAA, 21±2 and 22±3% of the protoplasts were dividing, respectively, while at 5.5µM NAA, 49±2 and 49±1% of the protoplasts were dividing.

start of nodule organogenesis (*4, 6-8*). The accumulation of GmENOD40 in the medium and the recognition of exogenously applied peptide by protoplasts suggests that it could be involved in cell-to-cell communication between pericycle and cortex. To answer the question of whether ENOD40 is involved in cell-cell communication, it will be essential to correlate the *in situ* expression pattern of *ENOD40* with the distribution pattern of the peptide.

Several groups have previously reported the absence of a long ORF in *ENOD40*. This, as well as computer analyses predicting that *ENOD40* RNA has a tendency to form

secondary structures such as untranslated regions of mRNAs, led to the hypothesis that *ENOD40* is active as an RNA (*7,17,18*). Our finding that the *ENOD40* small ORF is translated and that the corresponding peptide is functional in causing tolerance of high auxin concentrations eliminates the basis for this hypothesis. Nevertheless, sequence comparison of different *ENOD40* clones shows the highest level of conservation, surprisingly, in region 2, which is located at the 3' untranslated end of *ENOD40* mRNAs and not in region 1 (Fig. 5). This suggests that also this part of the messenger might have a function. Therefore, we tested whether region 2 has biological activity. Constructs derived from *GmENOD40-2*, containing region 1 (40-2/1-90), region 2 (40-2/91-448 and 40-2/91-656) or the non-conserved 3' end (40-2/449-656), as well as a construct derived from the tobacco homolog containing region 2 (Nt40/98-470), were tested by transfecting protoplasts. Of these, only the *GmENOD40-2*

Table 1: Effect of the full size alfalfa *ENOD40* clone, conditioned growth medium or ENOD40 peptide on cell division of wildtype tobacco mesophyll protoplasts.
Medium in which protoplasts had grown for five to six days in the presence of 13.8 µM NAA was treated either by boiling (10 minutes) or by proteolysis: the medium was incubated for one hour at 37°C with 25 µg chymotripsin in 80 mM Tris pH 7.5 and 50 mM $CaCl_2$, and passed through an ultrafiltration membrane (centricon 10, Amicon). From medium treated as described above, the amount corresponding to 1/3 ml was filtersterilized and added to freshly isolated protoplasts in 5 ml medium containing 0.9 µM kinetin and 5.5 or 13.8 µM NAA, respectively. Peptides were heat-treated or subjected to proteolysis at a concentration of 10^{-8} (soybean peptide) or 10^{-12} (tobacco peptide), respectively.

transfected clone	**% of protoplasts dividing on 5.5 µM NAA**	**13.8 µM NAA**
pMsENOD40	51±1	47±3
added to SR1 protoplasts		
SR1* (WT)	49±4	21±4
11S-F3*	49±2	49±1
11S-F3* (100° C)	49±1	22±2
11S-F3* (chymotrypsin)	50±3	23±1
40-2/1-448*	51±1	48±2
40-2/1-448* (chymotrypsin)	49±2	23±2
40-2/91-656*	48±2	49±2
40-2/91-656* (chymotrypsin)	50±2	18±3
GmENOD40-2#	48±2	49±3
GmENOD40-2# (100° C)	50±1	20±4
GmENOD40-2# (chymotrypsin)	48±3	24±1

*: Supernatant of wildtype SR1, transgenic 11S-F3, or transfected protoplasts, cultivated for 5 days and added to freshly isolated wild type protoplasts.

#: *In vitro* synthesised peptide as encoded by the soybean *ENOD40* clone.

construct lacking regions 1 and 2 but containing the 3' non-conserved end did not stimulate cell divisions at 13.8 μM NAA (Fig 1). So an RNA lacking region 1 but containing the untranslated region 2 causes a similar response in transfected protoplasts as does the ENOD40 peptide. Therefore, it seems plausible that region 2 mediates its effect by stimulating the synthesis of the endogenous tobacco ENOD40 peptide. This idea is supported by the observation that the conditioned medium of protoplasts transfected either with 40-2/91-448 or with 40-2/91-656 (containing region 2 alone) contains a chymotrypsin sensitive compound that confers auxin tolerance to wild type tobacco protoplasts (*16* and Table 1, respectively). This compound was not recognized by the antiserum to the GmENOD40 peptide, in contrast to the proteinaceous compound accumulating in the medium of 40-2/1-448 transfected protoplasts, or of protoplasts from 11S-F3 plants (Fig 4). Since the synthetic tobacco ENOD40 peptide is not recognized by this antiserum (Fig 4), it seems likely that the tobacco ENOD40 peptide accumulates in the medium upon transfection with the soybean 40-2/91-448 and 40-2/91-656 constructs. The molecular mechanism by which region 2 may be active remains to be solved. Thus, perhaps the 3' untranslated region has the ability to stimulate expression of the endogenous tobacco *NtENOD40* gene, but a more probable explanation could be that region 2 is a translational regulating sequence. Studies on the maternal Drosophila morphogens Bicoid and Nanos showed that these proteins repress the translation of *caudal* and *hunchback* mRNA, respectively, by binding to a 3' untranslated sequence (*23*). A similar mechanism has been proposed for *tra-2* of *Caenorhabditis elegans* of which the translation is under regulatory control by direct repeats located in the 3' untranslated region of the messenger (*24*). Thus, in protoplasts transfected with region 2, the RNA might titrate out a translational inhibitor that binds to the tobacco *ENOD40* mRNA, thereby removing inhibition of translation.

As shown by the activity of a synthetic peptide (Fig. 3C), the observations that the small ORF of *ENOD40* is translated (Fig. 2C), and that the oligopeptide is synthesized by transfected protoplasts (Fig. 4), *ENOD40* encodes an oligopeptide that is active at concentrations as low as 10^{-16}M. Generally, biologically active peptides are synthesized as inactive precursors, whereas in the case of ENOD40, the primary translation product is the biologically active molecule. Until now, only prokaryotic genes have been identified that encode small active peptides which are not synthesized as precursors, such as microcin C7, a heptapeptide inhibiting protein synthesis in enterobacteriaceae (*25*). Our studies show that also in eukaryotes genes encoding small peptides occur. While in animals small peptides have been shown to have important regulatory functions, in plants up to now only one active peptide, systemin, has been identified. It has been shown to be active at concentrations as low as 10^{-15} M (*26*). In contrast to the ENOD40 peptide, systemin is synthesized as a 200 aa large, inactive precursor and after processing is involved in the systemic induction of proteinase inhibitor genes (*26*).

Our studies show that *ENOD40* encodes a peptide that modulates the action of auxin; transgenic tobacco expressing soybean *ENOD40* displays modified apical dominance and transgenic cells in culture are more tolerant of auxin. Auxin induces a variety of responses in

plants. However, *ENOD40* will not affect all auxin activities, as is obvious from the fact that tobacco protoplasts expressing *ENOD40* behave like wild type protoplasts at NAA concentrations lower than 5.5 mM. Because of its proteinaceous nature and the low concentration at which it is active, ENOD40 could be considered as a new plant growth regulator that alters phytohormone responses.

We find that the nonlegume tobacco contains an *ENOD40* gene and that *ENOD40* genes from both legumes and nonlegumes have the same effect in the tobacco protoplast system. Together with the recent observation that Nod factors can substitute for auxin and trigger auxin related responses in tobacco protoplasts (*27*), this demonstrates that cellular mechanisms of the host used by *Rhizobium* during nodule formation are conserved and are probably important in normal hormone regulated plant growth.

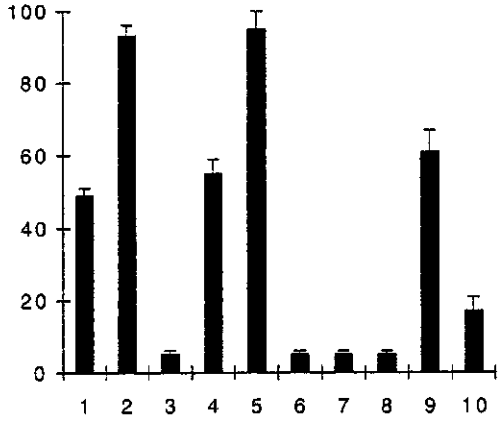


Figure 4: Results of ELISA to detect GmENOD40-2 peptide.
A mouse polyclonal antibody against GmENOD40-2 peptide coupled to keyhole limpet hemocyanin (KLH) was obtained (Eurogentec, Belgium). The specificity of this antibody for GmENOD40-2 peptide was determined in an ELISA using KLH, KLH coupled to GmENOD40-2 peptide, synthetic GmENOD40-2 (sample 1, 25 ng; sample 2, 100 ng) and NtENOD40 (sample 3) peptides in a competition assay for binding of the antibody to antigen (GmENOD40 peptide coupled to KLH) coated microtiter plates. The antibody is specific for the soybean ENOD40 peptide (compare 1, 2 and 3). The presence of antigen in the medium of 40-2/1-448 transfected protoplasts (sample 4; Fig. 1), and of 11S-F3 (sample 5) and wild type (sample 6) protoplasts grown at high auxin concentration was determined in a competition ELISA. To quantify the amount of peptide in the medium the rate of competition for binding to the antibody was compared to the extent of competition by different concentrations of synthesised soybean ENOD40 peptide (1, 25 ng; 2, 100 ng). In a comparable ELISA the absence of a competitive antigen in the medium of protoplasts transfected with 40-2/91-448 (sample 7, Fig.1) or 40-2/91-656 (sample 8, Fig.1) was proven. The rate of competition was determined by assaying the conversion of p-nitrophenyl phosphate by alkaline phosphatase at 405 nm in an ELISA reader (SLT 340 ATTC). Values are corrected for KLH binding to the antibody and are means of three independent experiments. Preimmune serum did not recognise the antigen (data not shown). The *GmENOD40* peptide was shown to be present in extracts of 15-day-old soybean nodules (sample 9) but not in roots (sample 10).

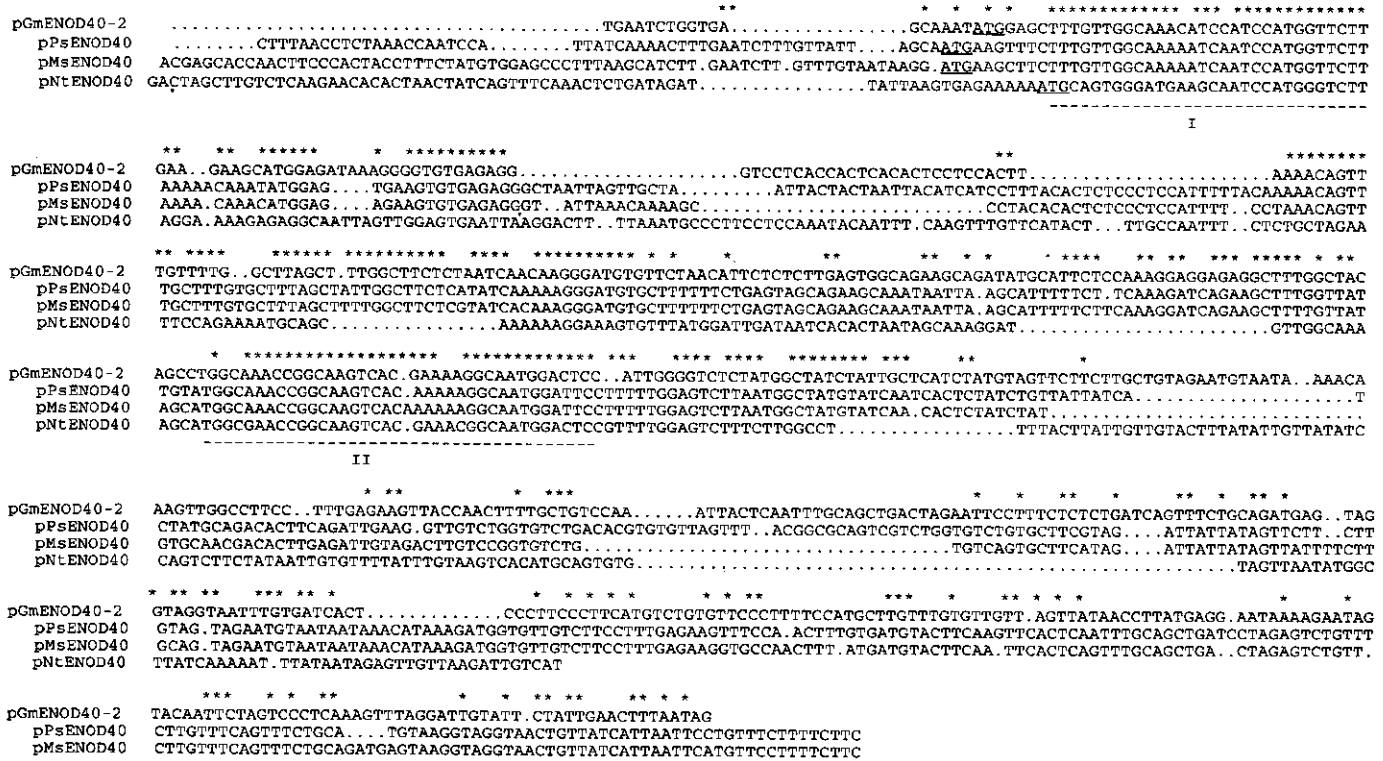


Fig. 5: *ENOD40* cDNA sequences. Sequence alignment of the *ENOD40* clones from soybean (*GmENOD40-2*; *4*), pea (*PsENOD40*; *7*), alfalfa (*MsENOD40; 21*), and tobacco (*NtENOD40*). Nucleotides conserved in all legume sequences are marked by asterisks. The conserved regions 1 (I) and 2 (II) are underlined. A comparison of these *ENOD40* sequences is available on the World Wide Web at http://gcg.tran.wau.nl/MolBio/ENOD40.html.

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13. The *Eco*RI fragment from pGmENOD40-2 (*4*) containing the first 448 bp of the cDNA was blunt-ended and subcloned in the blunt-ended *Bam*HI site of the metothrexate resistance transferring binary vector pMEX001 [Reiß, B., Koncz, C., Moore, I. and Schell, J., (1994) A family of binary gene vectors with low inter-transformant variation. *Plant Physiol. (Life Sci. Adv.)* **13**, 143-149.], yielding 40-2/1-448. Thus, *ENOD40* is expressed under control of the Cauliflower mosaic virus (CaMV) 35S RNA promoter. This promoter is also present in the other expression cassettes used, in pRT105 and pRT106 [Töpfer, R., Maas, C., Höricke-Grandpierre, C., Schell, J. and Steinbiss, H.-H. (1993) Expression vectors for high-level gene expression in dicotyledonous and monocotyledonous plants. *Meth. Enzymol.* **217**, 66-78].

    GmENOD40-2 was amplified by PCR from the original transgenic phage (*4*) using primers 5'-TTTTGGATCCGAATTCCGCTAAACCAATCTATC-3' and 5'-TTTTGTCGACGAA AGGACTCTGGAAACTTTTC-3' and subcloned *Bam*HI/*Sal*I in pBluescript KS^+ and

pRT105. For *GmENOD40-2*, the expression cassette was excised with *Sph*I and subcloned in pHPT, a pUC derivative containing a hygromycin resistance gene (R. Walden, unpublished data) for protoplast transfection. To construct 40-2/1-90, pGmENOD40-2 was digested *Hin*dIII/*Xho*I, blunt ended and selfligated. The 5' 90 bp fragment was excised using *BamHI/Kpn*I and ligated into pRT105. 40-2/91-656 was cloned by digesting pGmENOD40-2 with *Xba*I/*Hin*dIII, blunt ending and selfligation. The *ENOD40* fragment was cloned via *Not*I/*Sal*I in pRT105. 40-2/91-448 was subcloned from 40-2/91-656 by digestion with *Eco*RI and *Sal*I, blunt ending and selfligation. 40-2/449-656 was constructed from the complete *GmENOD40* cDNA in pRT105 via *Eco*RI digestion and self ligation. For construction of 40-2/1-90 AAG, the 5' and 3' parts of 40-2/1-90 were amplified by PCR from a plasmid containing the first 90 bp of the GmENOD40-2 cDNA (forming a *Hin*dIII/*Xho*I deletion derivative of the clone containing the full size cDNA cloned *Bam*HI/*Sal*I in pBluescript KS^+). For the 5' part, M13 forward primer and primer 5'-TTTGGCGCGCCAGATTAATCAGGACTTG-3' were used, for the 3' part M13 reverse primer and primer 5'-TTTCCGCGCGGTGAGCAAGGAGC-3'. The 5' PCR product was digested with *Sst*I and *Asc*I, the 3' PCR product with *Kpn*I and *Asc*I, and both were ligated in pRT105 vector digested with *Sst*I and *Kpn*I. The sequence of the insert was confirmed by sequencing after *Sst*I/*Kpn*I subcloning in pBluescript KS^+.

The tobacco *ENOD40* cDNA clone was amplified by PCR using primers 5'-GGC(A/T)(C/A)(A/G)(C/A)A(A/T)C(C/A)ATCCATGGTTCTT-3' and 5'-GGA(G/A)TCCAT TGCCTTTT-3'. Full length clones were isolated by racing, using for the 5' race two specific primers 5'-GCTTTTGCCAACATCCTTTC-3' and 5'-CTATTAGTGTGATTATCAATC-3' and two universal primers 5'-CTCGAGGATCCGCGGCCGCTTTTTTTTTTTTTTTTT-3' and 5'-GCTCGAGGATCCGCGGC-3'. For the 3' race primers 5'-CAAGTTTGTTCATAC TTTGCC-3' and 5'-GCTAGAATTCCAGAAAATGC-3' were used. For amplifying the genomic clone we used primers 5'-GACTAGCTTGTCTCAAGAAC-3' and 5'-ATGA CAATCTTAACAACTCT-3'. The genomic fragment was cloned in pGEM and from there subcloned to pBluescript KS^+. From there, it was excised using *Kpn*I/*Sst*I and cloned into pRT106.

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28. The 35S-*GmENOD40* construct 40-2/1-448 was used for *Agrobacterium tumefaciens* (strain GV3101(pMP90RK)-mediated leaf disc transformation (*12, 14*) of tobacco [*Nicotiana tabacum* cv. Havana SR1; P. Maliga, A. Sz.-Breznovits, L. Marton, *Nature New Biol.* **244**, 29 (1973)]. Five independent *ENOD40* expressing transformants (shown by Northern blot analysis, data not shown) were self pollinated. To follow the segregation of the T-DNA encoded metothrexate-resistance marker, 100 seeds were germinated on LS medium [Linsmaier, E.L. and Skoog, F. (1965) Organic growth factor requirements of tobacco tissue cultures. *Physiol. Plant.* **18**, 100-127] and the metothrexate-resistant plants were scored. Ten metothrexate resistant descendants of each plant were grown for analysis of the phenotype. About 30% of the transformed plants showed a weak phenotype (one short adventitious shoot) while two plants derived from different lines showed a strong phenotype (2-3 shoots of similar growth). These two plants, called 4S-F1 and 11S-F1, were self-pollinated and their progenies were analysed as described for the F1 generation. The progeny (11S-F3) of one homozygous plant of the F2 of 11S (termed 11S-F2) was used for further analysis (protoplast experiments).
29. To construct in-frame and out-of-frame GmENOD40-2/GFP translational fusions, a DNA fragment containing the 5' leader and the peptide encoding sequences of *GmENOD40-2* were generated by PCR using primers 5'-TTTGAATTCCGCTAAACC-3' and 5'-TTTTTCTAGAACCATGGATGG-3'. The coding part of GFP was obtained by PCR using primers 5'-AAATCTAGAAGTAAAGGAGAAGAACTT-3' and 5'-TTTGAGCTCTTATT TGTATAG-3' on plasmid containing GFP [Haseloff, J. and Amos, B. (1995) Green Fluorescent Protein in plants. *Trends Genet.* **11**, 328-329]. The *GmENOD40-2* DNA fragments were digested with *EcoR*I and *Xba*I and the GFP DNA fragments with *Xba*I and *Sst*I, purified and subsequently cloned into pMON999 [van Bokhoven, H., Verver, J., Wellink, J. and van Kammen, A. (1993) Protoplasts transiently expressing the 200K coding sequence of Cowpea Mosaic Virus B-RNA support replication of M-RNA. *J. Gen. Virol.* **74**, 2233-2241.] digested with *EcoRI* and *SstI*. After transformation of DH5α, DNA was isolated from colonies. The nucleotide sequences of the inserts were determined and two clones pMON40-GFPΔM and pMON40-δXbaGFPΔM, representing in-frame and out-of-frame translational fusions of *GmENOD40-2* and *GFP* (Fig. 5), were used to transfect tobacco protoplasts (*15*). For a positive control, the *GFP* DNA fragment was excised using *Bam*HI/*Sst*I and was cloned into pMON999 using *Bgl*II/*Sst*I.
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31. We thank J. Haseloff for providing a GFP cDNA and Ellen Küsters for help with plant transformation. We are obliged to Renze Heidstra and Gerhard Muster for help with HPLC purifying the tobacco and soybean peptides, to Jochen Wallach for mass spec analysis and to Bernd Reiß for providing the vector pMEX001. I. Kardilsky supplied primers for the 5' race PCR. J. S. was supported by a grant from the Bundesministerium für Forschung und Technologie (Germany). T.B., K.P. and K.v.d.S. were supported by the Dutch Organization for Scientific Research (NWO), K.v.d.S. received a European Commission short term training fellowship.

Chapter 5

Nicotiana tabacum SR1 contains two *ENOD40* homologs

Martha Matvienko, Karin van de Sande, Katharina Pawlowski, Ab van Kammen, Henk Franssen and Ton Bisseling. In: Biology of Plant-Microbe Interactions (G. Stacey, B. Mullin and P.M. Gresshoff eds.). 1996, pp. 387-391, The International Society for Molecular Plant-Microbe Interactions, St. Paul, Minnesota, USA.

Isolation of tobacco *ENOD40*clones

ENOD40 clones have been isolated from several legumes, eg. *Glycine max* (Kouchi and Hata 1993, Yang *et al.* 1993), *Phaseolus vulgaris* (Papadopoulou *et al.* 1996), (forming determinate nodules) and *Vicia sativa* (Vijn *et al.* 1995), *Pisum sativum* (Matvienko *et al.* 1994), *Medicago truncatula* and *M. sativa* (Asad *et al.* 1994, Crespi *et al.* 1994) (forming indeterminate nodules). When *ENOD40* clones from soybean (*GmENOD40-1* and *GmENOD40-2*) and alfalfa (*MsENOD40*, Matvienko unpublished results) where transiently expressed in SR1 protoplasts, this caused division of these protoplasts in the presence of high, normally inhibitory levels of NAA (Van de Sande *et al.* 1996). The activity of the heterologous legume genes in tobacco is indicative of the presence of *ENOD40* homolog(s) in tobacco. Therefore we attempted to isolate tobacco *ENOD40* clones.

The largest homology between the different legume *ENOD40* sequences is found in two small stretches, one located at the 5' end (region 1), the other more centrally (region 2) in the *ENOD40* clones. Region 1 contains a small ORF encoding a peptide of 12 (*Glycine*, *Phaseolus*) or 13 (*Vicia*, *Medicago*, *Pisum*) amino acids (Table 1), while region 2 does not contain a conserved ORF (Fig. 1). Interestingly, the sequence conservation in region 2 is even higher than in region 1 (Fig. 1; van de Sande *et al.* 1996)).

Degenerate primers were designed against region 1 and the 3' half of region 2 of the legume *ENOD40* clones, with the sequences 5'-GGC(A/T)(C/A)(A/G)(C/A)A(A/T)C(C/A) ATCCATGGTTCTT-3' and 5'-GGA(G/A)T CCATTGCCTTTT-3', respectively. This way, the 5' part of region 2 could confirm the identity of the isolated tobacco clones. cDNA clones were obtained via RT-PCR, using poly A RNA from *Nicotiana tabacum* cv. Petit Havana SR1 flowers, and *ENOD40*-like clones were selected by sequencing. Two different cDNA clones were isolated containing the 5' part of region 2. To obtain full length clones named pNtENOD40-1 and pNtENOD40-2, 5' and 3' racing was used. For the 5' race two specific primers, 5'-GCTTTT GCCAACATCCTTTC-3' and 5'-CTATTAGTGTGATTATCAATC-3' and two universal primers 5'-CTCGAGGATCCGCGGCCGCTTTTTTTTTTTTTT TTT-3' and 5'-GCTCGAGGATCCGCGGC-3' were used. For the 3' race primers 5'-CAAGTTTGTTCATACTTTGCC-3' and 5'-GCTAGAATTCCAGAAAATGC-3' were used. The nucleotide sequence of the resulting cDNA clones (Fig. 1) was confirmed by designing primers against their 3' and 5' end and perform PCR on genomic DNA from tobacco. The 5' primer used had the sequence 5'-GACTAGCTTGTCTCAAGAAC-3'. The insert of the cDNA clone pNtENOD40-2 was shorter than that of pNtENOD40-1. So two different 3' primers were designed, 5'-ATGACAATCTTAACAACTCT-3' and 5'-TATTCG GTAATAATTGGTGTG-3', for pNtENOD40-1 and pNtENOD40-2, respectively. The nucleotide sequence of the genomic clones and the cDNA clones was identical, confirming the nucleotide sequence of the cDNA clones and showing that like in legumes, the tobacco *ENOD40* genes do not contain introns.

Table 1: Amino acid sequence comparison of the *ENOD40* encoded peptides of different leguminous plants and from tobacco. IEP = iso-electric point.

peptide from	sequence	IEP
GmENOD40-1	M.ELCWQTSIHGS	5.41
GmENOD40-2	M.ELCWLTTIHGS	5.41
PvENOD40	MKF.CWQASIHGS	8.44
PsENOD40	MKFLCWQKSIHGS	9.62
MsENOD40	MKLLCWQKSIHGS	9.62
MtENOD40	MKLLCWEKSIHGS	8.43
VsENOD40	MKLLCWQKSIHGS	9.62
NtENOD40	M...QWDEAIHGS	4.23

Comparison of tobacco and legume *ENOD40* clones

The overall nucleotide homology between the legume *ENOD40* clones and the tobacco clones is low whereas *NtENOD40-1* and *NtENOD40-2* are 96% identical (Fig. 1). The most striking feature of both tobacco clones, is the presence of two sequence stretches that are highly homologous to the legume region 1 and 2. These are the only regions with significant homology between legume and tobacco *ENOD40* clones. Just like in the legume *ENOD40* clones, the tobacco *ENOD40* region 1 sequences encode an oligo peptide. Region 1 from the tobacco clones is 100% identical at the nucleotide level and so the peptide encoded by *NtENOD40-1* and *NtENOD40-2* is identical. Sequence comparison of the legume and tobacco peptides shows that they are highly homologous (Table 1). The tobacco peptide is ten amino acids in lenght, a bit smaller than the legume peptides. A centrally located tryptophan and the four C-terminal amino acids (ile, his, gly and ser) are conserved in all ENOD40 peptides. However, in iso-electric point (iep) are differences. The peptides from pea, alfalfa and *Vicia* have an iep of 9.62, while those from soybean and tobacco have an IEP of 5.41 and 4.23, respectively.

Region 2 is highly conserved in the legume and tobacco *ENOD40* clones. Between the tobacco clones there is only one basepair substitution, centrally in region 2. Like in the legume clones, region 2 from tobacco *ENOD40* does not contain a conserved ORF (Fig. 1). The already mentioned difference in size between both tobacco *ENOD40* clones is caused by four deletions in *NtENOD40-2*, making it 56 nucleotides shorter. From these deletions, two are located between the conserved regions, and the largest of 32 bp is at the 3' end.

Figure 2: Nucleotide sequence comparison of the *ENOD40* clones from soybean (*GmENOD40-2*), pea (*PsENOD40*), alfalfa (*MsENOD40*), and tobacco (*NtENOD40-1* and *NtENOD40-2*). * indicates nucleotides conserved between all *ENOD40* clones used.

Characterization of tobacco *ENOD40* clones

The expression of *NtENOD40* in tobacco could not be detected with Northern blot hybridization. Using RT-PCR (data not shown) *NtENOD40-1* and *NtENOD40-2* expression was detectable in stems, roots and flowers, and a markedly lower level of expression was detectable in leaves. In the tissues tested, *NtENOD40-1* and *NtENOD40-2* were expressed at similar levels. In legumes *ENOD40* is also expressed in non-symbiotic organs albeit at a markedly lower level than in nodules (Crespi *et al.* 1994, Matvienko *et al.* 1994, Papadopoulou *et al.* 1996, Yang *et al.* 1993)

The activity of *ENOD40* genes in non symbiotic tissues indicates a function of ENOD40 in common plant development. *ENOD40* genes are expressed in some non-symbiotic organs, for instance in flowers and at a low level in stems (Asad *et al.* 1994, Crespi *et al.* 1994, Kouchi and Hata 1993, Matvienko *et al.* 1994, Papadopoulou *et al.* 1996, Yang *et al.* 1993. The very big difference in *ENOD40* expression levels, between symbiosis associated expression, or common expression is puzzling, but not unique for *ENOD40*. For example *ENOD12* is also expressed in nodule, stem and flower and in the non-symbiotic organs it is also expressed at a markedly reduced level.

Several lines of evidence suggest that signals and genes involved in nodule formation are recruited from common plant development. Several genes that initially where thought to be nodule specific turned out to be expressed at low levels in non-symbiotic organs (e.g. *ENOD12* and *ENOD40*) and are even found in non-legumes (e.g. *ENOD40*). Even the Rhizobial signal molecules, Nod factors, could have their counterparts in common development. Röhrig *et al.* (1995) demonstrated that *in vitro* synthesised lipo chito-oligosaccharides (LCOs), resembling rhizobial Nod factors, could induce cell divisions in tobacco protoplasts in the absence of either auxin or cytokinin in the growth medium. Thus, rhizobial Nod factors might be related to endogenous plant signal molecules and in the *Rhizobium*-legume interaction induce the expression of *ENOD40* from which the gene products appears to be a new kind of plant peptide hormone, influencing the respons to the plant growth regulator auxin (Van de Sande *et al.* 1996).

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Chapter 6

ENOD40 expression modifies plant cell responses to cytokinin

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INTRODUCTION

In the symbiotic interaction between leguminous plants and bacteria from the genera *Rhizobium*, *Azorhizobium* and *Bradyrhizobium* (collectively referred to as rhizobia) root nodules are formed, in which rhizobia carry out biological nitrogen fixation. *ENOD40* is one of the plant genes active during nodule formation and function. *ENOD40* clones have been isolated from various leguminous plants, e.g. soybean (*GmENOD40-1;* Kouchi and Hata 1993, *GmENOD40-2;* Yang *et al.* 1993); pea (*PsENOD40;* Matvienko *et al.* 1994); bean (*PvENOD40;* Papadopoulou *et al.* 1996); vetch (*VsENOD40;* Vijn *et al.* 1995) and alfalfa (*MsENOD40;* Asad *et al.* 1994, Crespi *et al.* 1994). Recently, the first *ENOD40* clones from a non-legume were isolated, the tobacco clones *NtENOD40-1* and *-2* (Matvienko *et al.* 1996, van de Sande *et al.* 1996).

In alfalfa, *Rhizobium* induced *ENOD40* expression is detected hours before the first cell divisions take place, in the root pericycle opposite the protoxylem pole (Yang *et al.* 1997). Later, in early developmental stages of nodule formation, *ENOD40* expression is detected not only in the pericycle, but also in the dividing cells of a *Rhizobium*, or Nod factor induced primordium (Kouchi and Hata 1993, Matvienko *et al.* 1994, Vijn *et al.* 1993, 1995, Yang *et al.* 1993). In spontaneous nodules, formed in the absence of bacteria, expression of *ENOD40* is also detected (Assad *et al.* 1994, Crespi *et al.* 1994) but strikingly, meristems of indeterminate root nodules or lateral root primordia do not show *ENOD40* expression (Matvienko *et al.* 1994, W.-C. Yang unpublished), indicating a specific function for *ENOD40* in nodule formation, rather than a more general role in cell division.

Because of the timing and pattern of *ENOD40* expression during nodule formation it has been postulated that *ENOD40* could play a role in mediating plant hormone effects involved in mitotic reactivation of differentiated root cortical cells. A plant hormone-like effect of *ENOD40* influencing development was suggested by work from Crespi *et al.* (1994), who showed that *ENOD40* overexpression inhibits *Medicago sativa* embryo regeneration. Transgenic tobacco plants overexpressing *ENOD40* showed increased branching of the shoot indicating a disturbed response to, or sensitivity for plant hormones (van de Sande *et al.* 1996). Indeed *ENOD40* was shown to influence the response of plant cells to phytohormones, using both transient and continuous expression in tobacco protoplasts in a cell division assay (van de Sande *et al.* 1996).

The tobacco protoplast cell division assay has been used to test genes implicated in hormone response or metabolism (Brzobohaty *et al.* 1993, Hayashi *et al.* 1992, Walden *et al.* 1993). Under defined culture conditions tobacco protoplast division frequency is determined by the concentration of auxin and cytokinin with which the protoplasts are cultured (Nagata and Takebe 1970, Walden *et al.* 1993). The division frequency can be used to study the influence of either genes or compounds on protoplast division in the absence or in the presence of different concentrations of plant hormones (Brzobohaty *et al.* 1993, Hayashi *et al.* 1992, Röhrig *et al.* 1995, 1996, van de Sande *et al.* 1996). For example, *axi1* identified by tagging

with the insertion of four enhancers of the Cauliflower Mosaic Virus 35S RNA promoter in its vicinity was isolated by selection for growth of transformed protoplasts in the absence of auxin. Interestingly, *axi1* is also able to cause division of tobacco protoplasts in the absence of cytokinin, or in the presence of high concentrations of auxin and cytokinin (Hayashi *et al.* 1992, Walden *et al.* 1994, R. Walden, in preparation). Similarly genes have been isolated following selection for growth of transformed protoplasts in the absence of cytokinin, named *cyi* (Walden *et al.* 1994, 1997). After rescueing the genes and reintroduction in protoplasts they were able to sustain protoplast division in the absence of cytokinin, and some of them also in the absence of auxin (Miklashevichs *et al.* in press).

Transient and stable expression of *ENOD40* in tobacco protoplasts induced tolerance of supra-optimal auxin concentrations. Whereas the division frequency of wildtype protoplasts decreased at supraoptimal NAA concentrations, *ENOD40* expression enabled the protoplasts to divide with unreduced frequency. In addition we showed that a peptide of 12 or 10 amino acids encoded by soybean and tobacco *ENOD40,* respectively, is the active gene product (van de Sande *et al.* 1996). LCOs, rhizobial signalling molecules capable of triggering several plant responses involved in nodulation (Dénarié *et al* . 1996, Vijn *et al.* 1993), have been postulated to influence the plant growth regulator balance in legume roots (Mylona *et al.* 1995). Synthetic lipo-chitooligosaccharides (LCOs) have also been tested for their effect on tobacco protoplast division. They were able to induce tobacco protoplast division in the absence of auxin or cytokinin. Most probably, LCOs activate cell division by a different signal transduction pathway than auxins, since agonists for NAA and LCOs are only able to block divisions induced by their structural analogue. At a certain point both pathways seem to converge, because both LCOs and NAA induce *axi1* expression which precedes cell division. The common part of the signal transduction pathway is influenced by cytokinin since addition of cytokinin was required to obtain maximal *axi1* expression in the presence of either auxin or LCOs. Thus, in protoplasts *axi1* expression is part of the signal transduction pathways started by either auxin or LCOs in cooperation with cytokinin (Röhrig *et al.* 1995, 1996).

Several lines of evidence indicate the involvement of plant hormones in the formation of root nodules. Auxin transport inhibitors, probably leading to a modified auxin distribution in the plant, induce the formation of nodule-like structures, in which early nodulin genes are expressed (Assad *et al.* 1994, Hirsch *et al.* 1989, Scheres *et al.* 1992, Wu *et al.* 1996). Libbenga *et al.* (1973) found that when stele free pea root explants were cultured on medium containing auxin and cytokinin, cell divisions were induced in the inner cortical cell layers, preferentially opposite the former location of the protoxyleme poles. There appeared to be an absolute requirement for the presence of auxin in the medium to induce these cell divisions, but cell divisions were greatly stimulated by the addition of cytokinin.

The role of cytokinin in nodule initiation was shown by Cooper and Long (1994), who spot-inoculated alfalfa roots with an *E. coli* strain containing pTZS (*trans*-zeatin secretion) which encodes an isopentyl transferase from *A. tumefaciens* causing cytokinin production. This cytokinin producing *E. coli* strain induced the formation of bacteria-free nodule-like structures,

just as rhizobial Nod factors do. Cytokinin induces not only cell division in the inner cortex of legume roots, but also expression of *ENOD40* in the part of the pericycle opposite the dividing cells (W.-C. Yang, unpublished).

Studies on *axi1* and LCOs show that auxin and cytokinin signal transduction are linked. Furthermore, cytokinin and LCOs are signal molecules implicated in nodule formation, where both induce cell division and *ENOD40* expression. Therefore, we wanted to know whether in tobacco protoplasts *ENOD40* causes a modification in the response to cytokinin. Thus we tested whether *ENOD40* mediates changes in the cell division frequency of protoplasts in the presence of different kinetin concentrations. The results show that expression of soybean *ENOD40* (in protoplasts from transgenic tobacco plants, containing a 35S-*ENOD40* construct (11S-F3); van de Sande *et al.* 1996) and addition of the ENOD40 peptides of soybean and tobacco to protoplasts from untransformed plants not only leads to tolerance of high NAA concentrations, but also of high levels of kinetin. We also wanted to further characterize the response of tobacco protoplasts toward Nod factor-like LCOs to determine if they can also counteract the negative effect of high auxin or cytokinin concentrations. Therefore we tested the influence of LCOs on protoplast division in the presence of high plant hormone concentrations. We found that LCOs induce tolerance of high plant hormone concentrations, resembling the effect of ENOD40, and we postulate that this tolerance is due to the induction of *ENOD40* expression by LCOs.

MATERIALS AND METHODS

Tissue culture and protoplast isolation

Axenic shoot cultures from *Nicotiana tabacum* cv. Petit Havanna SR1 (Maliga *et al.* 1973), from a soybean *ENOD40* containing transgenic line (11S-F3) (van de Sande *et al.* 1996) and from the tagged line *axi 159* were maintained at 26 °C on agar solidified, modified MS medium (2 mg/l nicotinic acid, 2 mg/l pyridoxin·HCl) with 1% sucrose (MS1) in a 16 h daylight period. In the transgenic line 11S-F3 the soybean *ENOD40-2* cDNA was under control of the cauliflower mosaic virus (CaMV) 35S RNA promoter and polyadenylation sequences (van de Sande *et al.* 1996). The tagged line *axi 159* overexpresses the *axi1* gene driven by a tetramer of enhancers from the CaMV 35S RNA promoter (Hayashi *et al.* 1992).

Protoplast division frequencies were determined in three independent experiments. The average division frequencies with standard deviation are given in the figures. Standard deviation was maximally 10.

Protoplasts were isolated from shoot cultures as described (Walden *et al.* 1993, Negrutiu *et al.* 1987). Briefly, leaf tissue from 6 week old plants was cut in pieces of about 1 cm^2 and digested over night in K3 medium (Nagy and Maliga 1976) pH 5.7, with 0.4 M sucrose, (indicated as 0.4 M K_3), which contained 1.5% cellulase "Onozuka R-10" and 0.5% macerozyme R-10 (Serva) in the absence of plant growth regulators. Protoplasts were released

by gentle shaking for 10 minutes, sieved through a nylon filter (100 µM), concentrated by floating on the medium (six minutes centrifugation at 3000 RPM in a Hettich Rotofix Centrifuge) and washed with 0.4 M K3 medium.

Protoplast transfection, peptide and Nod factor treatment, GUS assay

Freshly isolated protoplasts were pelleted in W5 medium by centrifugation for 3 min in a Hettich Rotofix centrifuge at 800 rpm, and resuspended in Mannitol-$MgCl_2$ medium at a density of 10^6 cells/ml. Aliquots of 3x 10^5 cells were transfected by a heatshock at 45°C followed by addition of DNA and PEG (Negrutiu *et al.* 1987). For culturing, the volume was increased to five ml with 0.4 M K3 medium containing the appropriate amounts of NAA and kinetin. The protoplasts were incubated for two days in the dark, followed by three days in continuous light, at 26 °C. Cell division frequency was determined microscopically in three independent experiments.

ENOD40 peptides were synthesised *in vitro* (Research Genetics, Huntsville, AL) and purified on a reversed phase C18 HPLC column using a gradient of acetonitrile and water, with 0.1% TFA. The soybean peptide stock solution was dissolved in 50% DMSO, the tobacco peptide stock solution in water. To assay cell division in the presence of the ENOD40 peptides, the peptide stocks were diluted in water, filter sterilised (Millex-GS 0.22 µm, Millipore, Molsheim, France) and added to 5x 10^5 SR1 protoplasts in 5 ml 0.4 M K3 medium, with the appropriate amounts of NAA and kinetin, in order to have similar conditions as in the transfection experiments. The peptide concentrations used were 10^{-8} M for the soybean peptide and 10^{-12} M for the tobacco peptide previously determined to provide maximal activity (van de Sande *et al.* 1996). The synthetic Nod factor, or LCO (lipo-chitooligosaccharide), used was (ß-1,4-$GlcNAc_5$)-$C_{18:1}$, 9E, consisting of a pentameric backbone of ß-1,4-linked N-acetylglucosamine residues, N-acylated with *trans*-9-octadecenoic acid, an acyl chain with a *trans* unsaturated bond at the C9 position, and was synthesised as described, dissolved in Jensen medium (Röhrig *et al.* 1995, 1996) and used at 10^{-15} M. The *ENOD40*-GUS fusion contains the 1.7 kb *Eco*R1-*Sst*1 fragment from the *ENOD40-2* promoter (Roussis *et al.* 1995), in a transcriptional fusion with GUS, subcloned in pBluescript KS^+. ß-glucuronidase activity was measured as described (Jefferson *et al.* 1987).

Medium transfer experiments

Transfected, or transformed tobacco protoplasts were cultured for five days in 0.4M K3 medium with plant hormones. After five days, the protoplasts were removed from the medium by centrifugation (for six minutes at 3000 RPM). 100 µl of the medium was added to freshly isolated untransformed SR1 tobacco protoplasts, that in turn were cultured for five days in 0.4 M K3 medium with 0.9 µM kinetin and with 0, 5.5 or 13.8 µM NAA, respectively, after which division frequency was determined. The supplemented medium was either untreated, heat

treated (10 min. 100°C), or chymotrypsin digested (one hour at 37°C, with 2 units chymotrypsin in 80 mM Tris/HCl pH 7, 50 mM $CaCl_2$). After chymotrypsin treatment, the enzyme was removed by ultrafiltration over a centricon 10 membrane (Amicon, Beverly, MA). The treated medium was sterilised by filtration through a 0.22 µm millex-GS filter (Millipore).

RESULTS

Optimal frequencies of untransformed SR1 protoplast division

The division frequency of tobacco leaf mesophyll protoplasts was determined after culturing the protoplasts for five days in liquid K3 medium with different ratios of auxin to cytokinin. The maximum division frequency achieved by wildtype SR1 tobacco protoplasts under our experimental conditions is 50 to 60 %. This is reached at 0.9 µM kinetin and 5.5 µM NAA. The relation between the frequency of cell division and the concentration of kinetin was determined with a fixed NAA concentration of 5.5 µM. The cell division frequency of wildtype SR1 protoplasts is 0 % in the absence of kinetin, increases to about 50 % in the presence of 0.9 µM kinetin concentration, and decreases with higher kinetin concentrations. At 1.8 µM kinetin, the highest concentration tested, the wildtype protoplast division frequency had decreased to about 25 % (Figure 1). In the presence of varying NAA concentrations and a fixed kinetin concentration (0.9 µM kinetin), the division frequency follows the same pattern, namely increases at suboptimal NAA concentrations, and decreases at supraoptimal NAA concentrations. At 13.8 µM NAA, the highest concentration tested, the division frequency had decreased to about 15 to 25 % (Figure 3; van de Sande *et al.* 1996).

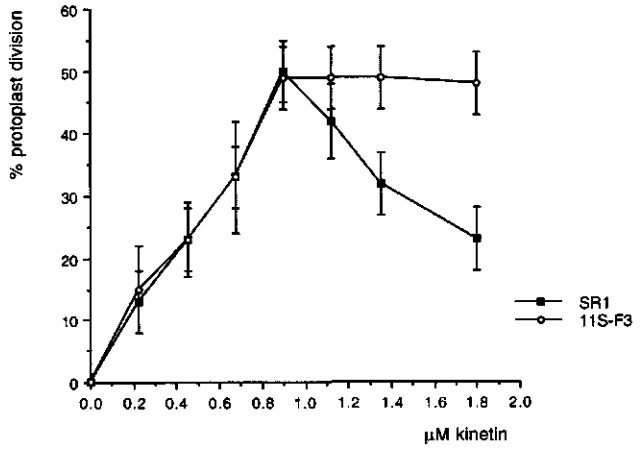

Figure 1: Tobacco mesophyll protoplast division frequencies of untransformed (SR1) and 11S-F3 plants, respectively, in % of protoplasts dividing after 5 days of cultivation, dependent on the kinetin concentration, in the presence of 5.5 µM NAA.

***ENOD40* induces tolerance of high kinetin concentrations.**

To test the effect of expression of soybean *ENOD40* on protoplast division at various kinetin concentrations, protoplasts were isolated from 11S-F3 plants. 11S-F3 expresses the first 448 bp of the soybean *ENOD40* cDNA *GmENOD40-2* containing region 1 which encodes the ENOD40 peptide as well as the conserved region 2 in the 3' UTR, under control of the CaMV 35S RNA promoter (van de Sande *et al.* 1996). The transgenic protoplasts were cultured in parallel with untransformed protoplasts. Cell division frequencies of transgenic protoplasts at suboptimal and optimal kinetin concentrations were similar to division frequencies of untransformed protoplasts. At supraoptimal kinetin concentrations, in contrast to untransformed protoplasts, 11S-F3 cell division frequencies did not decrease (Figure 1). Thus, overexpression of *GmENOD40* induces tolerance of high kinetin concentrations. Addition of the soybean, or of the tobacco ENOD40 peptide caused untransformed SR1 protoplasts to divide with unreduced frequency in the presence of high kinetin concentrations (Figure 2). The cell division frequency of untransformed protoplasts with either of the peptides added in the presence of different kinetin concentrations, was similar to the response of 11S-F3 protoplasts. Furthermore, the ENOD40 peptides were added to protoplasts grown with both high NAA and high kinetin concentrations in the culture medium (Figure 5). The peptides were able to support maximal cell division frequencies, causing tolerance of both hormones simultaneously. Thus besides tolerance of high auxin concentrations (Figure 3 and 4; van de Sande *et al.* 1996), cytokinin tolerance is also caused by the ENOD40 peptide.

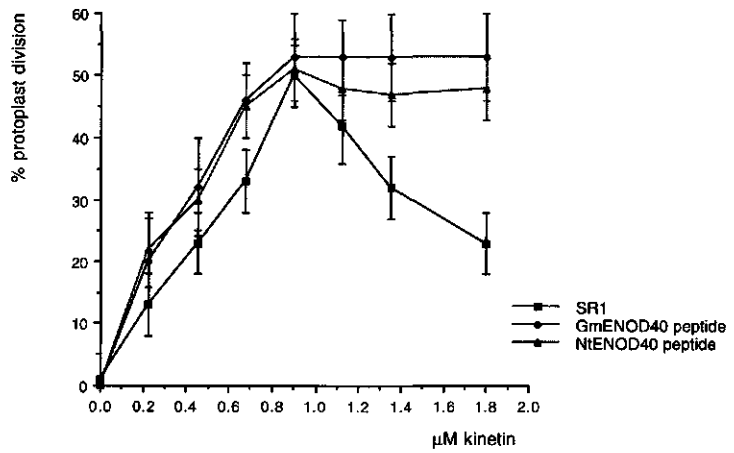

Figure 2: Tobacco mesophyll protoplast division frequencies of untransformed (SR1) protoplasts in the absence or presence of the ENOD40 peptide of soybean (GmENOD40) and tobacco (NtENOD40), respectively, in % of protoplasts dividing after 5 days of cultivation, dependent on the kinetin concentration, in the presence of 5.5 µM NAA.

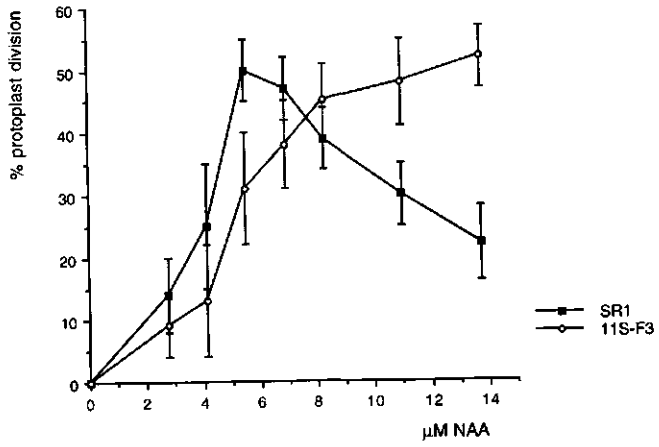

Figure 3: Tobacco mesophyll protoplast division frequencies of untransformed (SR1) and 11S-F3 plants, respectively, in % of protoplasts dividing after 5 days of cultivation, dependent on the NAA concentration, in the presence of 0.9 µM kinetin.

A synthetic LCO allows protoplast division at high NAA and kinetin concentrations.

Synthetic LCOs, which resemble rhizobial Nod factors, enable untransformed tobacco protoplasts to divide in the absence of either auxin or cytokinin (Röhrig *et al.* 1995, 1996). To test the effect of LCOs on protoplast division at different hormone concentrations, the LCO (GlcNAc5)-$C_{18:1}$, 9E was applied in the presence of different concentrations of auxin and cytokinin (Figure 6). The controls (black bars) show the cell division frequencies of wildtype protoplasts, to which 50 µl Jensen medium had been added. These division frequencies were comparable to the frequencies found earlier (Figure 1, 2). Independent of the presence or the concentration of NAA, the LCO (GlcNAc5)-$C_{18:1}$, 9E caused the protoplasts to divide with a frequency of about 50 %. The LCO similarly enabled protoplast division independently of the presence or concentration of kinetin. In the absence of kinetin, the division frequency was about 30 %, for the other kinetin concentrations tested it was about 50 %. In the presence of both high NAA and high kinetin concentrations (the most right gray bar), the LCO also induced about 50% cell division. Thus, just as ENOD40, LCOs can overcome the negative effect of high cytokinin and auxin concentrations on cell division.

***axi1* overexpression causes production of a small proteinaceous compound.**

Protoplasts isolated from *axi159*, a tagged mutant tobacco line overexpressing the *axi1* gene, are able to divide in a phytohormone independent manner (Hayashi *et al.* 1992, Walden *et al.* 1994, R. Walden, in preparation). Nod factors induce *ENOD40* expression in legume roots (Vijn *et al.* 1993), and LCOs induce *axi1* expression in protoplasts, shown both by

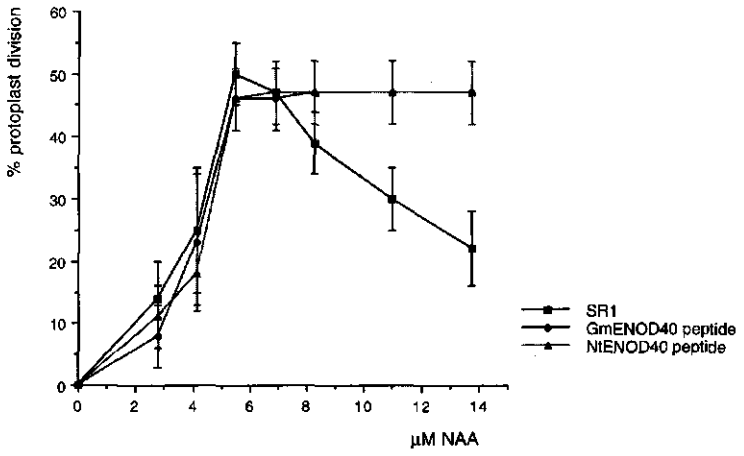

Figure 4: Tobacco mesophyll protoplast division frequencies of untransformed (SR1) protoplasts in the absence or presence of the ENOD40 peptide of soybean (GmENOD40) and tobacco (NtENOD40), respectively, in % of protoplasts dividing after 5 days of cultivation, dependent on the NAA concentration, in the presence of 0.9 µM kinetin.

Northern blot analysis, and by measuring GUS activity driven by the *Arabidopsis thaliana axi1* promoter (Röhrig *et al.* 1995, 1996). Therefore we wondered whether in protoplasts, *axi1* induces tolerance of high auxin concentrations by activating the endogenous *NtENOD40*. To determine this, experiments were carried out to show the presence of the tobacco *ENOD40* peptide by detection of biological activity in culture medium of *axi1* protoplasts (Figure 7). Earlier, medium of protoplasts expressing soybean *ENOD40* was shown to be capable of inducing cell divisions (van de Sande *et al.* 1996). *axi1* protoplasts were cultured for five days in the presence of different concentrations of plant growth regulators, and then the medium was collected and added to freshly isolated untransformed protoplasts. Independently of the hormone concentrations at which the *axi159* protoplasts were cultured, the conditioned medium is capable of inducing cell divisions with a frequency of about 50 % in untransformed SR1 protoplasts cultured in the presence of 13.8 µM NAA (black bar of column 1 in Figure 7, data not shown). In the absence of NAA in the SR1 culture medium, no cell divisions were induced by the *axi159* conditioned medium (data not shown). Thus, addition of *axi1* conditioned medium confers the same cell division properties to untransformed protoplasts as ENOD40 peptide. Previous studies, using conditioned medium of protoplasts transfected with 40.2/91-656, suggest that NtENOD40 can accumulate in the medium of tobacco protoplasts (grey bars in Figure 7; van de Sande *et al.* 1996). Treatment of conditioned medium of *axi159* protoplasts with chymotrypsin or heat (Figure 7, black bars of column 2 and 4) leads to inactivation of the stimulatory factor: the treated medium no longer is able to induce maximal cell division frequencies in the presence of 13.8 µM NAA. These expreriments suggest that *axi1* overexpression leads to the production of a peptide with similar properties as the

ENOD40 peptide. Induction of *ENOD40* by *axi1* can also be shown by measuring the GUS activity of a *GmENOD40* promoter-GUS transcriptional fusion. When *axi 159* protoplasts are transfected with the *GmENOD40*-GUS construct, irrespective of the plant growth regulator concentration with which the protoplasts were grown, GUS activity was detected (data not shown). In wildtype SR1 protoplasts *ENOD40* expression is correlated to *axi1* expression: only

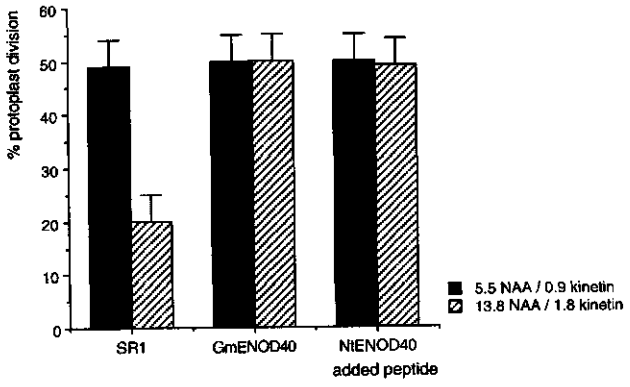

Figure 5: Tobacco mesophyll protoplast division frequencies of untransformed (SR1) protoplasts in the absence or presence of the ENOD40 peptide of soybean (GmENOD40) and tobacco (NtENOD40), respectively, in % of protoplasts dividing after 5 days of cultivation, either with optimal (black bars) or high (grey bars) concentrations of NAA and kinetin.

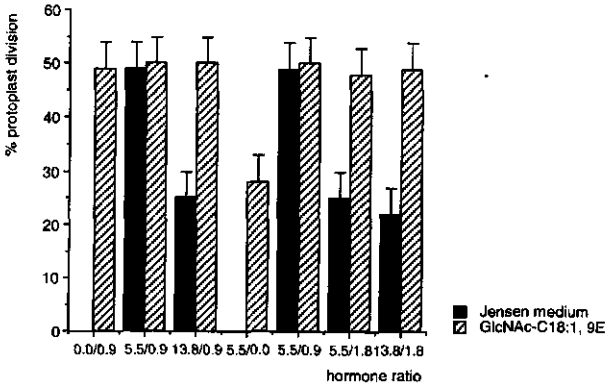

Figure 6: Tobacco mesophyll protoplast division frequencies of untransformed (SR1) protoplasts in the absence (black bars) or presence (grey bars) of the synthetic Nod factor GlcNAc5 - C18:1, 9E at 10^{-15} M, at different concentrations of NAA and kinetin. The three most left grey bars in Figure 6 represent LCO influenced cell division frequency in the presence of 0.9 µM kinetin in the growth medium, without NAA, with 5.5 µM NAA and with 13.8 µM NAA, respectively. The next three grey bars represent LCO influenced cell division frequency in the presence of 5.5 µM NAA and kinetin concentrations of 0, 0.9 and 1.8 µM, respectively. In the presence of both high NAA and high kinetin concentrations (the most right gray bar), the LCO also induced about 50% cell division.

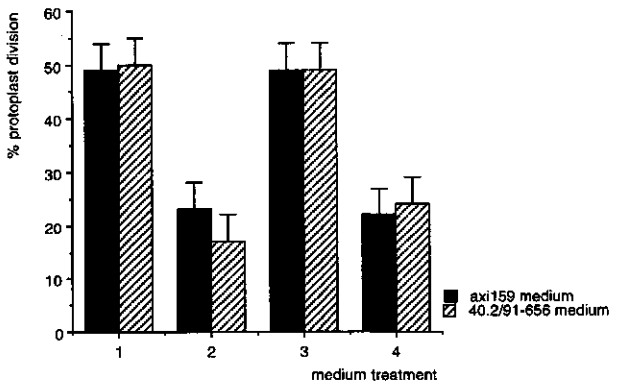

Figure 7: Tobacco mesophyll protoplast division frequencies of untransformed (SR1) protoplasts to which 100 µl medium is added from axi159 protoplasts, overexpressing the *axi1* gene (black bars) or from 40-2/91-656 transfected protoplasts (grey bars). The different medium treatments are 1: untreated medium, 2: chymotrypsin treated medium, 3: medium treated as under 2, without chymotrypsin, 4: medium incubated for ten minutes at 100°C.

under conditions that *axi1* expression could be detected with northern blot analysis (Röhrig *et al.* 1995), could we detect GUS activity driven by the *GmENOD40* promoter (data not shown). Therefore we hypothesise that *axi1* overexpression induces *NtENOD40* expression, which in turn triggers the tolerance of high plant growth regulator concentrations. Thus, the signal transduction cascade activated by synthetic LCOs, inducing tolerance of high plant growth regulator concentrations might involve *axi1* and subsequently *ENOD40* activation (Figure 8).

DISCUSSION

The results described in this paper show that expression of *GmENOD40* in tobacco protoplasts induces tolerance of high kinetin as well as high NAA concentrations (Figure 1, 3). Addition of the soybean ENOD40 peptide, as well as the tobacco ENOD40 peptide to untransformed tobacco protoplasts was also effective in inducing tolerance of high kinetin and high auxin concentrations (Figure 2, 4, 5). Strikingly, a synthetic LCO was capable of inducing a similar effect as ENOD40 peptides, sustaining maximal cell division frequencies at supraoptimal auxin and cytokinin concentration (Figure 6).

There are several examples of correlation between the plant growth regulators auxin and cytokinin. The control of apical dominance, transport of metabolites, cell division and morphogenesis in cells and explants cultured *in vitro* are long known (Hobbie *et al.* 1994). Auxin can influence the stability and the metabolism of cytokinin (Palni *et al.* 1988) and auxin and cytokinin can interact, to stimulate gene expression (Dominov *et al.* 1992). Furthermore,

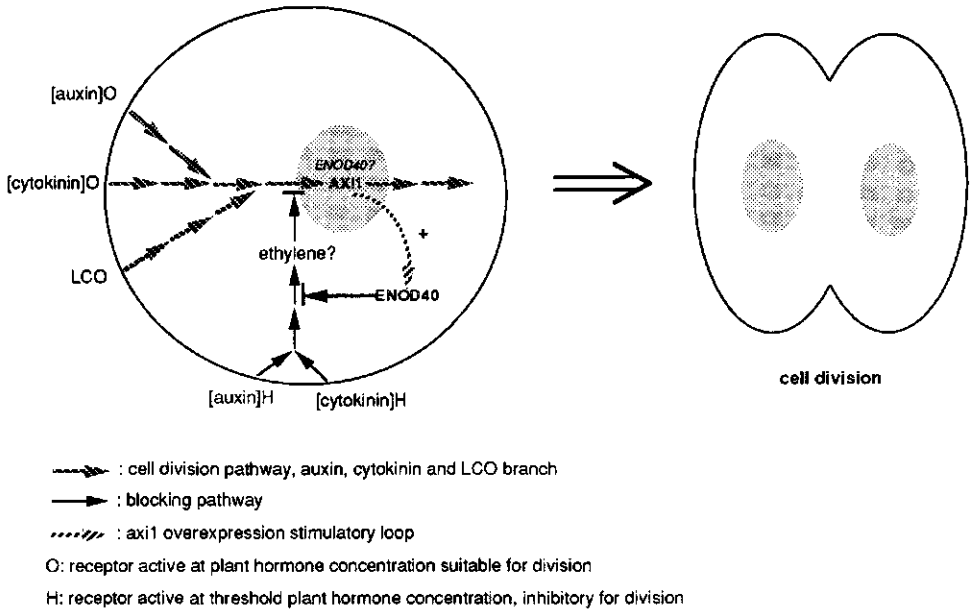

Figure 8: Model for the signal transduction network leading to cell divisions, in ENOD40 influenced or in *axi1* overexpressing tobacco protoplasts, based on Röhrig *et al.* (1996) and results described in this chapter. The network consists of the cell division pathways, blocking pathways and a stimulatory branch formed by the positive regulatory loop from *axi1* to *ENOD40*, that might only be active during *axi1* overexpression. See text for details.

several mutants like the *Arabidopsis* mutant axr4 are resistant to both auxin and cytokinin (Hobbie and Estelle 1995). Some examples more related to the work described here are the tobacco *axi1* gene that when overexpressed confers both auxin and cytokinin independent cell division (Hayashi *et al.* 1992, Walden *et al.* 1994, R. Walden in preparation). The induction of *axi1* expression by auxin is also stimulated by cytokinin (Röhrig *et al*. 1996). LCOs can sustain tobacco protoplast division in the absence of either auxin or cytokinin (Röhrig *et al.* 1996). Therefore the induction of cell divisions by auxin and cytokinin might be achieved through two pathways that converge into one (Figure 8), leading to *axi1* induction and subsequently cell division (the division pathway). LCOs might activate another signal transduction branch, that also converges into this division pathway (Figure 8; Röhrig *et al.* 1996).

In the presence of supraoptimal concentrations of either auxin or cytokinin, wildtype tobacco protoplast division is suppressed. This might happen via an additional signal transduction pathway that would require high levels of plant growth regulators to inhibit cell divisions. This pathway might involve ethylene, since addition of ACC to tobacco protoplasts inhibits division (data not shown). This pathway is hypothesised to block a step of the signalling cascade leading to cell divisions, and will be called the blocking pathway. The

occurrence of two signal transduction pathways involving auxin, one using ethylene as an intermediate, can be inferred from reports describing auxin effects that are directly mediated, or mediated via auxin-induced ethylene (Park and Lee 1994, Romano *et al.* 1993, Smulders and Horton 1991). Especially high auxin concentrations can induce accumulation of ethylene (Smulders and Horton 1991) which makes it possible that also one of the auxin induced pathways leading to protoplast division will involve ethylene.

Overexpression of *ENOD40*, or addition of the peptide, leads to undisturbed cell divisions in the presence of high plant growth regulator concentrations. This might be due to inhibition of the blocking pathway (Figure 8) and thus indirectly the division pathway is stimulated. Although experimental evidence is lacking, it seems plausible that ENOD40 removes the blockage of tobacco protoplast division by influencing one step in the signal transduction network downstream of auxin/cytokinin convergence rather than influencing a step in both the auxin and cytokinin induced pathway. This is because ENOD40 causes a similar response to both high auxin and high cytokinin concentrations, namely the induction of tolerance leading to maximal frequencies of protoplast division in the presence of normally inhibitory concentrations of plant growth regulators.

In our model the activity of a separate blocking pathway would indicate the presence of two sets of binding sites, one with high, and one with low affinity for the plant growth regulators. The putative receptor of the blocking pathway might be active only at a certain threshold concentration of auxin and cytokinin. The LCO activation of the division pathway can still proceed in the presence of high auxin or cytokinin concentrations. Therefore the LCO activated pathway most likely converges with the division pathway at or upstream of the target of blocking. The capability of LCOs to induce protoplast divisions in the absence of both auxin and cytokinin in the growth medium (Röhrig *et al* . 1995), while auxin requires the presence of cytokinin, indicates differences in the signal transduction between auxin and LCOs.

The results described in this paper show, that medium from *axi1* overexpressing protoplasts contains a small proteinaceous compound. This putative peptide shares characteristics with the ENOD40 peptide synthesised *in vitro* or by protoplasts as described by van de Sande *et al.* (1996). Since *axi1* encodes a protein of 569 amino acids, postulated to be a transcription factor (Walden *et al.* in preparation, 1997), it would seem unlikely that the small proteinaceous compound detected in the medium is AXI1. The inability of axi159 medium to induce wildtype protoplast division in the absence of auxin, while axi159 protoplasts can divide in the absence of auxin, confirms this notion. Thus, we postulate that *axi1* overproduction leads to induction of *NtENOD40* expression and production of the tobacco ENOD40 peptide. This notion is supported by the induction of soybean *ENOD40* promoter activity in axi159 protoplasts and in wildtype protoplasts expressing *axi1*. The postulated induction of *ENOD40* expression by *axi1*, and the induction of *axi1* by LCOs shown by Röhrig *et al.* (1995), are in agreement with our hypothesis that tolerance of high plant growth regulator concentrations induced by LCOs or *axi1* overexpression is mediated through *ENOD40* expression.

Possibly, next to influencing protoplast cell divisions under conditions of LCO addition or transgene overexpression ENOD40 also plays a role in the induction of cell divisions under conditions in which wildtype protoplasts normally divide. Indicative of such a role is the activity of the soybean *ENOD40* promoter in wildtype protoplasts in the presence of optimal auxin and cytokinin concentrations, as shown by GUS activity. The blocking pathway could be active during cell division induced by optimal plant growth regulator concentrations, but be inactivated by ENOD40. If the plant growth regulator concentrations increase, downregulation of *ENOD40* expression might take place, or *ENOD40* expression might not be sufficient anymore to block the pathway. *ENOD40* expression, induced by *axi1* overexpression, or driven by the 35S promoter totally blocks a regulatory pathway, but this might be an artefact of the deregulated expression and not reflect a function in 'normal' cell division. Thus, even though the relations are not yet clear, the control of tobacco protoplast cell division in different culture conditions could involve auxin or LCOs, cytokinin and *axi1* and *ENOD40* expression (Figure 8).

Modification of levels of plant growth regulators influences root nodule formation. A local rise in cytokinin level induces cell divisions in the legume root cortex (Bauer *et al.* 1996, Cooper and Long 1994). However, it is not known how cytokinin, or auxin influence root nodule formation. A disturbed ratio between the two of them, or a change in the level of one or both of the plant growth regulators might be the main driving force in nodulation. Expression of the *ENOD40* gene has been shown to be sufficient to induce cell divisions, both in the tobacco protoplasts, in the presence of otherwise inhibitory concentrations of auxin and cytokinin, and in roots of alfalfa plants, where *ENOD40* was transiently expressed after introduction by particle bombardment (Crespi *et al.* 1997). This may indicate that also in legumes, *ENOD40* is involved in the induction of cell divisions by altering the response to phytohormones. However, this remains to be proven since it is not clear whether root cortical cells and leaf protoplasts respond in a similar way to *ENOD40*.

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Chapter 7

Concluding Remarks

The early nodulin *ENOD40*

The early nodulin gene *ENOD40* is involved in root nodule formation in all legumes tested to date (chapter 2, Asad *et al.* 1994, Crespi *et al.* 1994, Kouchi and Hata 1993, Papadopoulou *et al.* 1996, Vijn *et al.* 1995, Yang *et al.* 1993). *ENOD40* is also present in non-legumes like tobacco (chapter 4, 5), indicating it might play a general role in plant development. In all *ENOD40* clones isolated thus far two conserved sequences are present, one being part of an Open Reading Frame (ORF) encoding an oligopeptide (region 1) and the other containing a non-translatable sequence (region 2). We showed that *ENOD40* overexpression induces tolerance of high auxin and cytokinin concentrations in tobacco protoplasts. The same effect was brought about by the peptide encoded by the tobacco or soybean *ENOD40* clones, if added to the tobacco protoplast system, indicating that the ENOD40 peptide is the active gene product while a regulatory role for the 3' UTR cannot be excluded (chapter 4, 6).

The experiments described in this thesis raise questions with regard to four points that will be addressed in the following sections. First the possible role of peptides in plant development will be considered. Next, the function of the conserved region 2 will be the subject of discussion. Then some aspects of the functioning of ENOD40 in protoplasts will be considered and finally, the role of ENOD40 in legume nodule development will be discussed.

Peptides play a common role in plant development

Peptides ranging in size from 3 to 53 amino acids, with various activities are frequently found in bacteria and animals. In bacteria small peptides often have antimicrobial activity, which may function in controling the population of the own species or the population of competitors. Examples of such antimicrobial peptides are for instance microcins such as Microcin C7 from *Escherichia coli* (González-Pastor *et al.* 1994) or lantibiotics such as Nisin (34 amino acids) from *Lactococcus lactis* (de Vos *et al.* 1995). In a few cases a small bacterial peptide has been shown to have a signalling function, like Nisin that is detected via a two-component regulatory system which activates transcription of the nisin biosynthetic cluster (Kuipers *et al.* 1995). It is unknown whether the mode of action of the bacterial peptides is similar to that of the ENOD40 peptide.

In animals, peptides play important roles in regulation of cellular behaviour, growth and differentiation (Culig *et al.* 1996, Massagué and Pandiella 1993, Takahashi *et al.* 1997). Many of these peptides are neurotransmitters that mediate the communication between neurons and other body cells, or cytokines that function as growth factors. Several peptides are involved in long distance communication, and serve as hormones. There are elaborate contacts between the nervous and the endocrine system and some peptides can function in both systems (Argente *et al.*

1996, Berczi *et al.* 1996, Weigent and Blalock 1995).

Some examples of the different classes of signalling peptides are the following: Epidermal Growth Factor (EGF) is a locally working 53 amino acid long peptide with a paracrine or autocrine mode of functioning, involved in stimulation of division of epidermis and other body cells, nutrient transport and induction of embryo development (Culig *et al.* 1996, Massagué and Pandiella 1993). A small, locally active peptide of only four amino acids is Eosinophil chemotactic factor, which is a chemotactic signal for eosinophilic leukocytes (Alberts *et al.* 1983). Enkephalins are neuropeptides of 5 amino acids with morphinelike activity, inhibiting pain pathways in the central nervous system (Alberts *et al.* 1983, Kampa *et al.* 1996) while myosuppressins are decapeptides that inhibit suppression of visceral muscle (Nachman *et al.* 1993). In endocrine signalling, peptides are transported by the bloodstream. Examples are glucagon, a 29 aminoacid peptide involved in regulating blood glucose level by influencing glucose synthesis and release from glycogen in liver (Lodish *et al.* 1995), LH (Luteinising Hormone) releasing factor and somatostatin, peptides of respectively 10 and 14 amino acids, both involved in release of another hormone from the anterior pituitary gland and vasopressin, a 9 amino acid peptide involved in regulation of water resorption by kidneys and of blood pressure, by influencing constriction of small blood vessels (Alberts *et al.* 1983). Other peptides function externally, for instance mastoparans, 14 amino acid toxins from wasp venom (Sukumar and Higashijima 1992) or sodefrin, a 10 amino acid female attracting pheromone from newt (Kikuyama *et al.* 1995).

The activity of signalling peptides is strictly regulated as the active peptides are contained in large precursors, often prepropeptides, from which they are released by proteolytic cleavages (Massagué and Pandiella 1993, Sauer-Ramirez *et al.* 1996). The target cells contain receptors for signalling peptides. Many of these receptors can bind more than one peptide, but the combination of receptors in the cell determines the signals a cell can respond to, and in this way determines the behaviour of the cell (Lodish *et al.* 1995). For animals hundreds of signalling peptides have now been described, what stresses the importance of peptides as signalling compounds (Argente *et al.* 1996, Berczi *et al.* 1996, Weigent and Blalock 1995).

So far, in plants, only a few examples of biologically active peptides are known. The first example is systemin, an oligopeptide of 18 amino acids, that accumulates upon mechanical injury of leaves and is involved in systemic signalling. Systemin behaves like a hormone and brings about a defence response: produced at the site of wounding, it travels via the phloem through the plant and systemically induces proteinase inhibitor genes. In the induction of proteinase inhibitor genes *in vitro* synthesised systemin was active at femtomolar concentrations in tomato and potato (Pearce *et al.* 1991). Active systemin is released by proteolytic processing from prosystemin, a 200 amino acid precursor, just as some peptide hormones in animals (McGurl *et al.* 1992).

The ENOD40 peptide was the second plant peptide identified, with a possible signalling function as described in chapter 4 and 6. Unlike systemin, ENOD40 is not synthesised as an inactive precursor. ENOD40 appeared to be involved in development by interacting with the plant

growth regulators auxin and cytokinin. Recently another signalling peptide interacting with auxin and cytokinin was identified in tobacco. This peptide has a size of about 2 to 3 kDa and is synthesised by dividing tobacco protoplasts; its production is possibly induced by auxin and cytokinin. This peptide is capable of bypassing the block on cell division caused by incubation of protoplasts with the auxin analogue, 2-NAA, and presumably acts by binding to the 1-NAA receptor (Miklashevichs *et al.* 1996).

Other examples of peptides active in plants are found among the elicitors in plant-pathogen interactions. For example the fungus *Cladosporium fulvum* produces an elicitor, AVR9, which is a 28 amino acids peptide. Upon the interaction of AVR9 with the tomato CF9 resistance gene, which is a membrane-anchored extracellular glycoprotein containing leucine-rich repeats, a hypersensitive response is induced (de Wit *et al.* 1997).

The possibility that a more comprehensive role in plant development will be granted to peptides is suggested by the nature of different receptor kinases identified in plants. A large group of receptors which contain leucine rich repeats (LRRs) has now been identified. The resistance genes which have been identified in plant-pathogen interactions often contain LRRs, that might recognise peptide or protein pathogen elicitors. LRRs are for instance found in the tomato Cf-9 protein (Jones 1997, de Wit *et al.* 1997), the Prf cytoplasmic protein which in interaction with the Pto kinase determine resistance to *Pseudomonas syringae* pv. *tomato* expressing the avirulence gene *avrPto* (Jones 1997, Salmeron *et al.* 1996) and the tobacco N protein which induces a hypersensitive response to tobacco mosaic virus (Jones 1997, Whitham *et al.* 1994, 1996).

In addition to resistance genes, genes encoding receptors with extracellular LRRs involved in plant development have been cloned. For instance the *ERECTA* gene of *Arabidopsis thaliana* that encodes a putative receptor protein kinase probably is involved in determining the shape of shoot organs. Mutants at the *erecta* locus have compact inflorescence with the flowers clustered at the top, round leaves with short petioles, and short siliques (Torii *et al.* 1996). The *SERK* (Somatic Embryogenesis Receptor-like Kinase) gene from *Arabidopsis* also encodes a LRR receptor-like kinase and is expressed during early stages of somatic and zygotic embryogenesis (Schmidt *et al.* 1997). The CR4 receptor-like protein kinase from corn, which was isolated from a transposon tagged *crinkly* mutant is involved in epidermis and aleurone differentiation (Becraft *et al.* 1996). It has an extracellular domain with a cysteine-rich region resembling the extracellular domain of the tumor necrosis factor (TNF) receptor, a protein that is involved in regulating inflamatory responses in mammals. Therefore it is possible that CR4 recognises a peptide or protein as ligand (Becraft *et al.* 1996). Since LRRs have been shown to bind peptide hormones and proteins in animal systems (Kobe and Deisenhofer 1995, Svensson *et al.* 1995, Thomas *et al.* 1996), it seems likely that in plants they also recognise these ligands. Taken together these recent findings suggest that, just like in animals, peptides might play a rather common role in plant development. They have probably not yet been detected due to the fact that they are active at very low concentrations and due to the absence of suitable test systems.

The role of the 3' UTR of *ENOD40* mRNA

The ENOD40 mRNA varies in size from about 400 b (tobacco *NtENOD40-2*, chapter 5) to about 600 to 700 b for legumes. Strikingly, there is only one conserved Open Reading Frame present in the different *ENOD40* clones that is limited to about 40 nucleotides and encodes a peptide of 10 to 13 amino acids. Consequently there are very large untranslated regions in the *ENOD40* mRNAs. In addition to the open reading frame, the *ENOD40* mRNAs contain a very conserved region in the large untranslated 3'UTRs, region 2, which probably also is of functional significance. If so, the ENOD40 gene generates two active gene products, a peptide and a transcribed RNA. In chapter 4 we have described the effect of transfection of a soybean *ENOD40-2* deletion clone not encoding the peptide in tobacco protoplasts. If this construct was introduced in tobacco protoplasts, the protoplasts could divide with unreduced frequency, just as with peptide encoding constructs. In the protoplast culture medium the presence of a proteinaceious compound which shared characteristics with the *in vitro* synthesised peptide could be detected. Based on these results we postulated a function for the 3' UTR in translational regulation. In the following we will weight this postulated function of the *ENOD40* RNA against different other activities reported for RNA molecules.

In the transcription and translation machinery many different RNAs play a role. We shall not discuss these RNAs here as there is no resemblance of structure or function between these RNAs and *ENOD40* RNA. Neither shall we consider possible ribozyme activity as the 3' UTR of *ENOD40* does not have any of the features characteristic for ribozyme activity known so far.

There are, however, several other cases of RNA activity reported in the literature. Sometimes the entire RNA is described as the active gene-product. For instance the *H19* transcript that has been isolated from mice and human does not contain a conserved ORF (Brannan *et al.* 1990). *H19* might play a role in development, since it is expressed in several tissues of a pre-natal embryo and is repressed in all tissues except skeletal muscle shortly after birth. Ectopic expression is lethal for embryos, and expression in tumour cell lines inhibits cell division. However, the molecular mode of functioning of *H19* is unclear (Hao *et al.* 1993, Brunkow and Tilghman 1991).

Another example of active RNA molecules is found with gene dosage compensation. In female mammals one of the X chromosomes is inactivated *in cis*, by the non-coding RNA *Xist*. This RNA probably induces remodeling of the chromatin (Herzing *et al.* 1997, Lèe and Jaenisch 1997). In Drosophila the male X chromosome undergoes specific activiaton. Several proteins encoded by the *msl* (male-specific lethal) genes bind to the X chromosome. For changing the chromatin structure probably *roX* (RNA on the X chromosome) RNAs are required, that do not encode proteins and interact in a MSL dependent manner with the X chromosome (Amrein and Axel 1977, Meller *et al.* 1997).

Another RNA molecule implicated in development, is *Pgc* (*Polar granule component*) of

Drosophila, an RNA that is located in polar granules, and which plays a role in localizing these granules during oogenesis and embryogenesis. *Pgc* and polar granule localisation is required for normal differentiation of pole cells into proliferative germ cells. Reduction of *pgc* RNA by antisense technology led to death or no migration of pole cells, and formation of less germ cells in the embryo (Nakamura *et al.* 1996).

In *Xenopus* oocyte development, *Xlsirt* RNA migrates towards the vegetal pole of oocytes early during oogenesis. *Xlsirt* is required for the location at the vegetal pole of specific RNA molecules. E.g.*Vg1*, which encodes a transforming growth-factor-ß-like protein, and is involved in mesoderm formation (Kloc *et al.* 1993, Kloc and Etkin 1994).

Besides there are many reports which describe that the 3'UTR of mRNA molecules may have a role, in *in cis* regulating their own translation. One of the genes that regulates the development of *Caenorhabditis elegans* after hatching of the egg is *lin-14*. The *lin-14* transcript contains seven short imperfect repeat sequences in the 3' UTR that are involved in down regulation of *lin-14* translation by binding of short RNA molecules encoded by *lin-4* , an other gene (Lee *et al.* 1993, Wightman *et al.* 1993).

Translation of *tra-2*, involved in sexual development in *C. elegans*, is regulated by a perfect direct repeat in the 3' UTR , by an unknown mechanism (Goodwin *et al.* 1993). In *Drosophila*, *caudal* mRNA translation is regulated by binding of the Bicoid protein to its 3' UTR, which inhibits translation (Dubnau and Struhl, 1996, Rivera-Pomar *et al.* 1996). By a similar mechanism the translation of *hunchback* mRNA is regulated by Nanos (Murata and Wharton 1995).

3' UTRs might also act *in trans* and influence expression of other genes. The 3' UTR in some mouse mRNAs e.g. α-tropomyosin suppress via an unknown mechanism the proliferation of a differentiation-deficient myogenic cell line (Rastinejad and Blau 1993) and inhibit tumor formation by this cell line (Rastinejad *et al.* 1993) while they induce expression of several muscle-specific genes (Rastinejad and Blau 1993).

So far no indications have been found that the 3'UTR of *ENOD40* can have similar functions as in the different systems described. *ENOD40* does not contain repeats in its 3' UTR, and thus far proteins or RNAs that bind to the *ENOD40* RNA have not been identified. However, the ENOD40 peptide is not released from an inactive precursor, since the primary translation product is active in the protoplast division assay (chapter 4, 6). Regulation of *ENOD40* gene expression, either at the transcriptional or posttranscriptional level therefore must be of great importance, since the presence of a compound involved in the induction of cell division, at developmentally wrong moments might greatly disturb plant growth. This notion together with the conservation of region 2, and the induction of auxin tolerance induced by a soybean *ENOD40* clone not encoding the soybean peptide but containing region 2, makes very conceivable that *ENOD40* regulates its own expression. Further research is required to elucidate the mode of action.

Mode of action of ENOD40 in tobacco protoplasts

In tobacco protoplasts the soybean and tobacco *ENOD40* clones and also *in vitro* synthesised peptides effect tolerance of both high auxin and cytokinin concentrations. This results in cell division at auxin and cytokinin concentrations which would normally be inhibitory. A polyclonal antibody was used to show the *GmENOD40* peptide was made by tobacco protoplasts, indicating the peptide is the *ENOD40* gene product (chapter 4, 6). Probably, the protoplasts secrete the peptide into the culture medium, even though it lacks a signal peptide. The presence of a signal peptide is not always required for secretion, as was found in several cases in animal systems: FGF-9 (member of the fibroblast growth factor family), interleukin-1 ß (mediator of inflammation), thioredoxin (disulfide reducing enzyme) and annexin 1 (function unknown), are secreted without a signal peptide being involved (Miyamoto *et al.* 1993, Rubartelli *et al.* 1990, 1992, Serres *et al.* 1994). This indicates that secretion of proteins does not necessarily require the presence of protein transport routes through the endoplasmic reticulum/Golgi. Possibly a similar pathway exists in plants and is used for ENOD40 secretion. However, it cannot be excluded that the presence of ENOD40 in the culture medium is due to the bursting of protoplasts.

In the induction of auxin tolerance in tobacco protoplasts, the peptides are active at extremely low concentrations. Already at10^{-12} M for the soybean peptide, and 10^{-16} M for the tobacco peptide, they induce half of the maximal response (35 to 40 percent protoplast division at high auxin concentrations vs. a maximum division frequency of 50 to 60 percent). These low concentrations represent for the soybean peptide 8,000 to 23,000 peptide molecules per protoplast and for the tobacco peptide only 1 to 3 peptide molecules per protoplast. The concentrations at which the ENOD40 peptides are active in the stimulation of tobacco protoplast divisions, are much lower than the concentrations in which auxin and cytokinin cause cell divisions. The low concentration required for activity of the peptides is an indication for the presence of a receptor with high binding affinity. Whether such a receptor indeed exists remains to be proven.

Systemin, one of the other signalling peptides active in plants is active in femtomolar concentrations when applied to tomato plants (Pearce *et al.* 1991). Therefore systemin probably also requires a receptor in its activity. The active concentration of systemin is about 100 times higher than that of the tobacco ENOD40 peptide, but since systemin is transported and systemically induces proteinase inhibitor gene activity, there will be dilution of the concentration of the applied systemin and the concentration at the place of functioning might be comparable to that of the ENOD40 peptide. Comparable low concentrations sufficient for activity have been found for lipo-chitooligosaccharides (LCOs). Synthetic LCOs are active in the induction of tobacco protopast division at 10^{-15} M (Röhrig *et al*. 1995, 1996). For Nod factors, several indications exist that receptors are active (Heidstra and Bisseling 1996), and presumably the activity of LCOs in protoplasts will also be mediated by receptors. However, since LCOs are taken up and accumulate in the protoplast membranes (J. Schmidt, personal communication) the

local concentration at the membrane surface will be much higher and we do not know what LCO concentration is required to induce activity.

The structure of ENOD40 has some characteristics that may be required for its functioning. All ENOD40 peptides, known so far, show a regular pattern of alternating hydrophobic and hydrophilic amino acids, and a hydrophillic C-terminus. Based on the sequence of the soybean peptide (MELCWLTTIHGS), a peptide X (MELMFATTARAT) was designed using as much as possible amino acids with side chains with similar chemical properties. In this peptide X, the hydrophobicity pattern is changed. The peptide did not induce protoplast division at high auxin or cytokinin concentration, indicating the hydrophobicity pattern might be important for the peptide functioning (Figure 1). This was confirmed by the design of a peptide Y (MELNIATTARSY) with residues modified in the same positions as in peptide X (chapter 4). This time, however, the amino acids were substituted with amino acids of the same hydrophobicity. The peptide Y therefore retained the same alternating pattern of hydrophobic and hydrophillic amino acids. Interestingly, peptide Y was as active as the original soybean ENOD40 peptide in stimulating protoplast division in the presence of inhibitory auxin concentrations (data not shown). The secondary structure of different ENOD40 peptides might be divers as is derived from the pepplot application from the Genetics Computer Group programs (GCG). Some peptides appear to consist of a ß-sheet, others of an a-helix. Thus it is not likely the secondary structure is a major determinant of ENOD40 action.

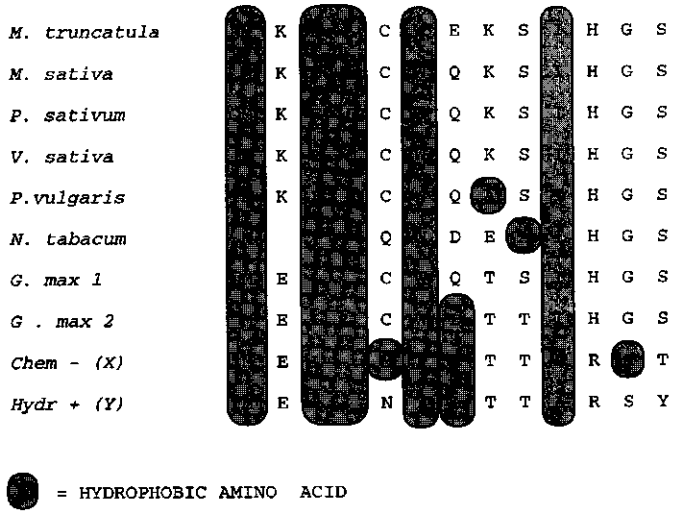

Figure 1: Amino acid sequences of different ENOD40 peptides. Hydrophobic amino acids are indicated with grey boxes.

ENOD40 in nodule development

In tobacco protoplasts ENOD40 affects the role of auxin and cytokinin in cell division. It causes tolerance of high auxin and cytokinin concentrations allowing cell divisions at concentrations of auxin and cytokinin that normally would be inhibitory. Thus, in protoplasts ENOD40 activity is connected with the functioning of plant growth regulators in cell division.

As a first step in determining the function of ENOD40 during root nodule development and in mature nodules the site of *ENOD40* expression was determined using *in situ* hybridisations and GUS reporter constructs. In alfalfa, three hours after inoculation with *Rhizobium, ENOD40* expression was detected in the pericycle of the root vascular bundle. This is long before the first cortical cell divisions take place (W.-C. Yang, unpublished). Also in soybean inoculated with Nod factors, expression in the pericycle was detected before the first corticall cell divisions begin (Minami *et al.* 1996). Once a nodule primordium has been formed in the cortex opposite the *ENOD40* expressing pericycle cells and the protoxylem pole, *ENOD40* expression is also found in all nodule primordium cells (Asad *et al.* 1994, Crespi *et al.* 1994, chapter 2, Vijn *et al.* 1995, Yang *et al.* 1993). When mature, nitrogen fixing nodules have developed *ENOD40* expression is no longer detectable in the root pericycle but high expression of *ENOD40* is found in the pericycle cells of nodule vascular bundles. In addition, *ENOD40* expression is found in the central tissue of root nodules. In the central tissue of determinate soybean root nodules, the highest expression level was found in the boundary layer, i.e. the layer of uninfected cells that separates the central tissue of infected and uninfected cells from the surrounding nodule parenchyma (Yang *et al.* 1993). In indeterminate root nodules of alfalfa, pea and vetch, *ENOD40* is not expressed in the nodule meristem (zone I), but *ENOD40* expression is found in the prefixation zone, where infection takes place (zone II). In alfalfa the *ENOD40* expression is mainly confined to uninfected cells (Crespi *et al.* 1994). In vetch and pea *ENOD40* expression was also detected in infected cells and next to the prefixation zone (II), expression was found at lower levels in the interzone (II-III) and the nitrogen fixation zone (III) (Vijn *et al.* 1995; chapter 2).

GUS expression driven by 1.7 or 3.4 kb of the *ENOD40* promoter strongly resembled the pattern of expression detected by *in situ* hybridisation (chapter 3). GUS expression was detected in the root pericycle opposite nodule primordia. In roots on which mature nodules were present, GUS expression was detected in nodule vascular bundles, mainly in the pericycle and probably due to the stability of the enzyme, still in the zone of the root vascular bundle facing the nodule. In the central tissue of mature nodules, GUS expression could not be detected using the 1.7 kb *ENOD40* promoter fragment, but it was detected using the 3.4 kb promoter fragment (K. van de Sande, G. Zegers and M. Tadege, unpublished results). This could indicate the presence of regulatory sequences, located further upstream than -1.7 kb in the *ENOD40* promoter.

Whereas *ENOD40* expression was found in nodule primordia, it has not been detected in nodule lateral root primordia. This was clearly demonstrated by W.-C. Yang (unpublished data) who showed that in sections containing both a nodule and a lateral root primordium *ENOD40* was

only expressed in the nodule primordium. This indicates that *ENOD40* expression is not related to cell division and primordium formation in general but indeed is specific for nodule formation. This was also demonstrated by the expression pattern in mature nodules. In a few published papers (Asad *et al.* 1994, Papadopoulou *et al.* 1996) it was reported that the authors were unable to demonstrate the absence of*ENOD40* transcripts in nodule meristems and lateral root primordia. The reason could be that meristematic tissues easily give background in *in situ* hybridisation experiments as meristematic tissues are rich in cytoplasm and have high transcription and translation activities.

The pericycle forms the outermost layer of the root stele, and is assumed to perform highly specific functions in the root. This is reflected by specific expression of several genes in the pericycle (Doerner *et al.* 1996, Kanagae *et al.* 1994, W.-C. Yang, personal communication). During legume nodule formation, like in *Vicia, ENOD40* is specifically induced in the pericycle opposite the protoxylem pole (Figure 2; Vijn *et al.* 1995). At the other hand, *ACC oxidase*, encoding the ethylene forming enzyme, is expressed in the pericycle opposite the phloem poles in the susceptible zone of roots (Heidstra *et al.* in press). The expression of a certain gene in a limited part of the pericycle is indicative of distinct roles played by these specific pericycle parts. The heterogeneity of the pericycle is also demonstrated in radish and *Arabidopsis* where pericycle cells in the protoxylem zone are anatomically different from the ones in the phloem zone

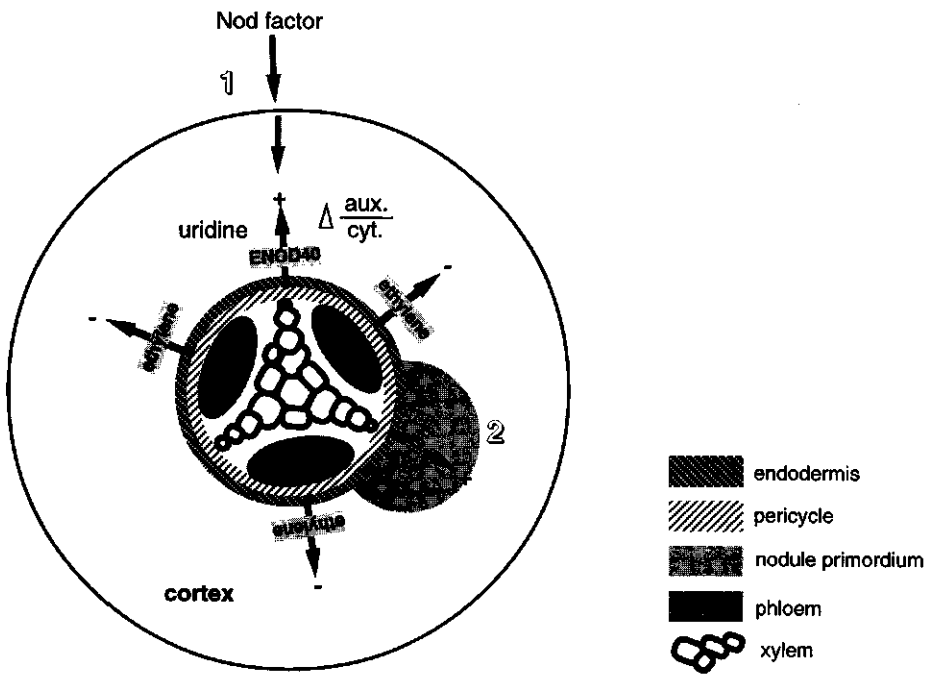

Figure 2: A model for ENOD40 functioning in early stages of legume root nodule formation. See text for details.

(Laskowski *et al.* 1995). In pea, the pericycle consists of one cell layer opposite the protophloem, and of two to five cell layers opposite the protoxylem poles (Lu *et al.* 1991, Popham 1955, Rost *et al.* 1988).

The induction of root nodule formation probably involves changes in the phytohormone balance. When the interaction of ENOD40 with phytohormones in tobacco protoplasts is compared with the legume *ENOD40* expression pattern a possible function for ENOD40 in early developmental stages of nodule formation can be postulated. *ENOD40* expression in the pericycle might cause a change in the transport of plant growth regulators like auxin from the stele, or change the sensitivity (of pericycle and) cortex cells towards plant growth regulators, which then results in cell divisions. This corresponds with the characteristics of ENOD40 activity in protoplasts where ENOD40 activity is connected with the functioning of plant growth regulators in cell division. Auxin or cytokinin antagonism could also be involved in the root nodule formation process. An auxin antagonistic function for ENOD40 is confirmed by another *in vitro* system, embryo formation from carrot suspension culture protoplasts. Here ENOD40 prevents cell elongation in a mannner that is antagonistic to auxin. In addition ENOD40 has an auxin synergistic effect on cell division in this cell suspension culture, possibly resulting in increased embryo formation (Guzzo *et al.* in preparation; chapter4 and 6).

The pericycle is the first tissue that shows *ENOD40* expression after inoculation. The pericycle itself, however, does not respond to *ENOD40* expression with cell division. Perhaps ENOD40 peptides produced in the pericycle are transported to the cortex and there play a role in the induction of the cortical cell divisions. If so, the effect of ENOD40 is outside the cells where the peptide is produced. Later ENOD40 produced in the primordium cells might take over the function. This also corresponds with a characteristic of ENOD40 activity in protoplasts where ENOD40 activity is mediated by a soluble peptide which probably is perceived by a receptor.

The role *ENOD40* plays in root nodule formation requires interaction with several compounds (Figure 2). Nodules are preferentially formed in the root cortex opposite the protoxylem pole from the root vascular bundle (Libbenga and Harkes 1973). This well defined position implies some kind of control of the location where nodule primordia can develop. As recently argued by Heidstra *et al.* (1997), the localisation could be achieved by the interaction of different compounds. One of these compounds might be ethylene, that is proposed to inhibit nodule formation in the phloem sector of the root. As ACC oxidase is expressed in the phloem sector of the pericycle, localised ethylene production can inhibit cell divisions in this sector in the root cortex. Another compound interacting with ENOD40 activity might be the so-called stele factor, identified as uridine by Smit *et al.* (1995). It is unknown whether uridine is secreted from a specific sector of the vascular bundle but since uridine has a positive effect on divisions of inner cortical cells (Smit *et al.* 1995), it might stimulate the induction of cell divisions in cooperation with ENOD40. When Nod factors are applied externally (1) a signal is transmitted to the inner cortex and pericycle. One of the effects is the induction of ENOD40 expression in the pericycle which, as we saw, might lead to a change in the ratio of auxin to cytokinin or in the response to these plant hormones. The ENOD40 peptide produced in pericycle cells would move to the cortex

and in interaction with uridine, cell divisions take place in the inner cortex (2) leading to nodule primordium formation. In the dividing primordium cells *ENOD40* expression is also detected, and is possibly required to maintain cell division.

The mode of functioning of ENOD40 at the molecular level is still an open question. Intriguing observations made in the tobacco protoplast system show that *ENOD40* RNA that lacks the Open Reading Frame encoding the ENOD40 peptide is still able to effect cell divisions at high auxin and cytokinin concentrations that normally are inhibitory. Apparently the RNA transcribed from the *ENOD40* gene can function without being translated. In roots and nodules *ENOD40* is expressed in specific cells and tissues. In these cells the *ENOD40* RNA is present and may exert its effect. In the protoplast system it was striking that the ENOD40 peptide is probably excreted in the culture medium. In very early stages of nodule formation it appears that the visible effect of *ENOD40* expression, cell division, is mainly not in the cells where it is produced but in cells of neighbouring tissues. Therefore it is feasible that in the producing cells a function is performed by the RNA whereas the peptide is active in neighbouring cells. Further research on the nature of action of ENOD40 should resolve these questions.

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Chapter 8

Summary (Dutch)

Rhizobium bacteriën induceren de vorming van wortelknollen op de wortels van vlinderbloemige planten, zoals erwt en klaver. Wortelknollen zijn organen waarbinnen de juiste omstandigheden heersen voor *Rhizobium* bacteriën om biologische stikstof fixatie uit te kunnen voeren. De gebonden stikstof komt beschikbaar voor de plant, die op zijn beurt de rhizobia voorziet van voedingsstoffen zoals suikers. Hierdoor zijn vlinderbloemige planten niet afhankelijk van gebonden stikstof die in de grond voorkomt, en kunnen ze op arme bodems groeien. Om tot een succesvolle symbiotische interactie te komen is een uitgebreide uitwisseling van signalen nodig tijdens alle stappen van de wortelknolvorming. De signaaluitwisseling begint met het uitscheiden van flavonoiden door de wortels van de gastheerplant. Deze secundaire metabolieten worden door de rhizobia herkend, die via een chemotactisch proces naar de plantewortels bewegen. Ook wordt transcriptie van nodulatie (*nod*) genen geïnduceerd door flavonoiden. De nodulatie eiwitten zijn betrokken bij de productie en het uitscheiden van Nod (nodulatie) factoren, dit zijn rhizobiële signaal moleculen. De Nod factoren induceren verschillende responsen in de plant, als wortelhaar deformatie, celdeling waardoor een knol primordium ontstaat, en de expressie van verschillende plantegenen die een rol spelen in de wortelknolvorming. Infectie begint wanneer de rhizobia binden aan wortelharen, die daarop krullen en de rhizobia insluiten in een beperkte ruimte. Hierin wordt plaatselijk de celwand afgebroken en vanaf deze plaats induceren de rhizobia de vorming van een nieuwe buisachtige structuur, de infectiedraad. Deze infectiedraad, waarin de rhizobia zich vermenigvuldigen groeit de wortel in naar de delende cellen van het knolprimordium die worden geinfecteerd door de rhizobia. Het primordium groeit daarna uit tot een stikstof bindende wortelknol, waarbinnen de rhizobia tot bacteroïden zijn gedifferentieerd.

Plantegenen die een rol spelen tijdens het ontstaan van de wortelknol worden vroege noduline genen genoemd. Zij komen tijdens de infectie en de wortelknolvorming tot expressie. Een van deze genen is *ENOD40* (early nodulin 40). De eerste vlinderbloemige plant waaruit *ENOD40* geïsoleerd is, is soja. Soja *ENOD40* is gebruikt om een erwten *ENOD40* kloon te isoleren. Na vergelijking van deze kloons bleek dat de homologie op DNA nivo tussen de soja en erwtenkloon tamelijk laag was, 55 percent. De soja *ENOD40* kloon bevat een groot open leesraam, waarvan gedacht werd dat het voor ENOD40 kodeert. Dit open leesraam werd onderbroken door een stopcodon in de erwten *ENOD40* kloon, en kan dus niet coderen voor het ENOD40 eiwit. Hybridizatie van genomisch DNA van de erwt met de soja en de erwten *ENOD40* kloons bevestigde de identiteit van de erwten kloon. Het expressie patroon van *ENOD40* in de erwt is bestudeerd met *in situ* hybridisatie en bleek vergelijkbaar te zijn met dat van soja. In de delende cellen van het primordium en in de pericycle tegenover dit primordium komt *ENOD40* tot expressie. In volgroeide knollen komt *ENOD40* sterk tot expressie in de pericykel van het knol vaatweefsel, en op een lager nivo in de geïnfecteerde en niet-geïnfecteerde cellen van de prefixatie zone II, maar niet in het meristeem (zone I) van de knol. Op grond van deze gegevens is gepostuleerd dat *ENOD40* of actief is als RNA, of kodeert voor een oligopeptide van 12 aminozuren in soja en 13 in erwt (hoofdstuk 2).

Het soja *GmENOD40-2* gen is geïsoleerd om het gecompliceerde expressie patroon

in knollen via een ander systeem te kunnen bestuderen. De nucleotide sequentie is bepaald van 1.7 kb van de promoter, van het deel waarvan transcriptie plaatsvind en van 500 bp stroomafwaarts van het poly-adenylerings signaal. Het 1.7 kb promoter fragment is in een transcriptionele fusie met het ß-glucuronidase (GUS) reporter gen tot expressie gebracht in transgene *Vicia hirsuta*. Het ß-glucuronidase expressie patroon gereguleerd door de *ENOD40* promoter was vergelijkbaar met het expressie patroon wat met *in situ* hybridisaties gedetecteerd is in erwt of soja: er was sterke expressie in de vaatbundels die de knol omringen, waarschijnlijk in de pericykel, en in de pericykel van de wortel, tegenover de knol. Er werd echter geen GUS expressie waargenomen in het ventrale weefsel van de knol. Dit wordt waarschijnlijk veroorzaakt doordat een aantal regulerende promoter sequenties aanwezig zijn in het gebied stroomopwaarts van de gebruikte 1.7 kb van de *ENOD40* promoter (hoofdstuk 3). In later uitgevoerde experimenten bleek dat dit het geval was: wanneer 3.4 kb van de promoter gebruikt werd in een transcriptionele fusie met het ß-glucuronidase reporter gen, werd ook expressie in primordia en in het centrale weefsel van volgroeide knollen gevonden (hoofdstuk 7).

Gebaseerd op het expressiepatroon tijdens de interactie tussen vlinderbloemigen en rhizobia werd gespeculeerd dat *ENOD40* betrokken zou kunnen zijn bij het induceren van celdelingen, die leiden tot de vorming van een primordium. *ENOD40* zou celdelingen kunnen stimuleren via een interactie met de plantehormonen auxine en cytokinine. Het effect van *ENOD40* overexpressie zou opheldering kunnen verschaffen over de functie die *ENOD40* heeft *in planta*. Daarom is tabak getransformeerd met de soja *GmENOD40* kloon. Transgene tabaksplanten vormden een extra zijscheut (hoofdstuk4), en deze verminderde apicale dominantie wordt waarschijnlijk veroorzaakt door interactie van *ENOD40* met auxine en/of cytokinine. Het bepalen van de functie voor *ENOD40* tijdens de wortelknolvormine en in functionerende wortelknollen, is experimenteel nog niet uitvoerbaar in vlinderbloemigen. Voor een verdere bestudering van de interactie tussen *ENOD40* en de plantehormonen auxine en cytokinine werd gekozen voor een model systeem, de tabak protoplasten delings test (Hoofdstuk 4, 6). De delingsfrequentie van tabaksprotoplasten wordt bepaald door de concentratie auxine en cytokinine in het groeimedium. Protoplasten geïsoleerd van transgene tabaksplanten waarin soja *ENOD40* tot expressie komt zijn tolerant voor hoge auxine en cytokinine concentraties (hoofdstuk 4, 6). Zij delen met onverminderde frequentie in aanwezigheid van auxine en cytokinine concentraties die normaal de celdeling remmen. Na transfectie met soja *ENOD40* dat transient tot expressie komt zijn de protoplasten ook tolerant voor hoge auxine en cytokinine concentraties. Omdat tabak kon reageren op een soja kloon werd aangenomen dat in tabak een homoloog gen aanwezig was. Dit bleek het geval te zijn, en twee tabaks *ENOD40* cDNA kloons zijn geïsoleerd via PCR (hoofdstuk 4, 5). De aanwezigheid en de activiteit van *ENOD40* in vlinderbloemigen en in niet-vlinderbloemigen geeft aan dat *ENOD40* een algemene rol kan spelen in de ontwikkeling van planten. Na vergelijking van de nucleotide volgorde van de verschillende kloons van vlinderbloemigen en tabak bleek

dat er twee geconserveerde gebieden zijn. Het eerste geconserveerde gebied ligt in een open leesraam wat codeert voor een oligopeptide van 10 aminozuren in tabak en 12 aminozuren in soja. Dit peptide is *in vitro* gesynthetiseerd en bleek het *ENOD40* genprodukt te zijn. Een gemuteerde soja *ENOD40* kloon, waarvan het peptide coderende deel verwijderd is, en alleen het tweede geconserveerde gebied aanwezig is, bleek echter ook in staat te zijn protoplasten tolerant te maken voor hoge auxine concentraties. Dit geeft aan dat het 3' niet getransleerde deel van *ENOD40* een rol zou kunnen spelen in de regulatie van *ENOD40* expressie. Een mogelijkheid is dat er een remmer van translatie bindt aan gebied twee van de getransfecteerde kloon. Translatie van het endogene tabaksgen kan dan plaatsvinden waardoor het tabaks ENOD40 peptide geproduceerd wordt. De aanwezigheid van een eiwitachtige component in het medium, die in staat is protoplasten te laten delen bij hoge auxine concentraties lijkt dit te bevestigen (hoofdstuk 4).

Nawoord

Nu dit proefschrift klaar is, wordt een voor mij erg belangrijke periode afgesloten. Bij uitstek is dit het moment om je te realiseren, dat je het nooit allemaal alleen voor elkaar had kunnen krijgen. Daarom wil ik al die mensen, die op wat voor manier dan ook een bijdrage geleverd hebben erg hartelijk bedanken. Ten eerste is daar mijn promotor, Ab van Kammen, die ik wil bedanken voor zijn begeleiding, grote belangstelling en enthousiasme tijdens het onderzoek. Mijn co-promotor, Ton Bisseling, bedankt voor de mogelijkheid dit onderzoek uit te voeren, voor de begeleiding en ondersteuning, maar ook voor het overbrengen van inzicht in het belang en de betekenis van resultaten.

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Curriculum vitae

Karin van de Sande is geboren op 14 november 1964 in Liempde. Na vier jaar HAVO, maakte ze in 1981 de overstap naar het Jacob Roeland Lyceum, waar ze het eindexamen atheneum haalde in 1983. Na wat omzwervingen (staatsexamen wiskunde en natuurkunde op HAVO-nivo in 1985), begon ze met de opleiding tot botanisch analist aan de Internationale Agrarische Hogeschool Larenstein. Het stage onderzoek werd uitgevoerd bij het toenmalige IVT, nu deel uitmakend van het CPRO-DLO, en de vakgroep Plantencytologie en -morfologie onder begeleiding van dr. J. van Tuyl en dr. J. Jansson. Het afstudeervak werd uitgevoerd bij de vakgroep Moleculaire Biologie in de groep van dr. T. Bisseling, onder begeleiding van dr. F. Govers. Het diploma werd behaald in 1989, en in hetzelfde jaar werd begonnen aan het doorstroomprogramma Plantenveredeling aan de Landbouwuniversiteit Wageningen met de afstudeervakken Moleculaire Biologie (prof. dr. A. van Kammen en dr. T. Bisseling) en Genetica (prof. dr. M. Koornneef en dr. T. Peeters). Het diploma werd in 1992 gehaald. In september 1992 werd begonnen met het promotie onderzoek naar de expressie van het vroege noduline gen *ENOD40* en de interactie van ENOD40 met de plantehormonen auxine en cytokinine, wat is uitgevoerd bij de vakgroep Moleculaire Biologie onder leiding van prof. A. van Kammen en dr. T. Bisseling, in samenwerking met dr. R. Walden en dr. J. Schmidt binnen de afdeling van prof. dr. J. Schell op het Max-Planck-Institut für Züchtungsforschung in Keulen. Het onderzoek is gefinancierd door een Pionier subsidie van de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO) toegekend aan dr. T. Bisseling, door een PTP short term training fellowship binnen het BIOTECHNOLOGY programma van de Europese Commissie en een reisbeurs van het fonds 'Stimuleringsgelden voor internationalisering' van het NWO. In de toekomst zal zij werkzaam zijn als onderzoeksmedewerker (post-doc) in de onderzoeksgroep van dr. O. Leyser, aan het Plant Laboratory van de vakgroep Biology van de University of York, met een Marie Curie research training beurs binnen het fourth framework programma Biotechnologie van de Europese Commissie.