Early nodulin gene expression and the action of Nod factors in *Vicia sativa*

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Proefschrift
ter verkrijging van de graad van
doctor in de landbouw- en milieuwetenschappen
op gezag van de rector magnificus,
dr C. M. Karssen,
in het openbaar te verdedigen
op dinsdag 9 mei 1995
des namiddags te vier uur in de Aula
van de Landbouwuniversiteit te Wageningen

CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Vijn, Irma

Early nodulin gene expression and the action of Nod factors in *Vicia sativa* /
Irma Vijn. [S.1. : s.n.]
Thesis Wageningen. - With ref. - With summary in Dutch.
ISBN 90-5485-379-4
Subject headings: Nod factors/ *Vicia sativa*/ nodule formation

The investigations described in this thesis were carried out at the department of Molecular Biology, Agricultural University Wageningen, The Netherlands, and were financially supported by a grant from the Foundation for Life Sciences, subsidized by the Dutch Organization for Scientific Research.

NN08201, 1922

Stellingen

1. Onderzoek naar de regulering van de expressie van het vroege noduline gen *ENOD12* leidt niet tot meer inzicht in de weg waarlangs de signaaltransductie van Nod factor naar genexpressie plaatsvindt.

   Dit proefschrift.

2. Crespi *et al.* leveren geen bewijs voor hun conclusie dat het vroege noduline gen *ENOD40* niet wordt vertaald in een eiwit.

   Crespi *et al.* (1994) EMBO J., 13(21), 5099-5112.
   Dit proefschrift.

3. Tomatecelculturen kunnen niet worden gebruikt om de aktiviteit van Nod factoren te testen.

   Staehelin *et al.* (1994) PNAS, 91, 2196-2200.

4. De conclusie van Carsolio *et al.* dat de expressie van een *npv*30-*ß-glucuronidase* gen zich in de wortelknollen van *Lotus corniculatus* beperkt tot de met *Rhizobium* bacteriën geïnfecteerde cellen, is voorbarig.

   Carsolio *et al.* (1994) Plant. Mol. Biol., 26, 1995-2001.

5. Volgens de omschrijving, in het van Dale Groot Woordenboek van de Nederlandse Taal, van het begrip *symbiose* als het verschijnsel dat twee ongelijksoortige organismen leven op, of in elkaar, tot wederzijds voordeel, is het samenlevingsverband tussen vlinderbloemigen en *Rhizobium* bacteriën geen symbiose.

6. Het uitvoeren van promoterstudies in heterologe organismen, die zelf geen gen hebben homoloog aan het gen waarvan de promoter wordt bestudeerd, levert geen informatie op over welke gebieden van belang zijn voor de regulatie van de genexpressie in het homologe organisme.

Szabados *et al.* (1990) Plant Cell, 2, 973-986.
Oommen *et al.* (1994) Plant Cell, 6, 1786-1803.
Fujii *et al.* (1994) Plant Physiol., 104, 1151-1157.

7. De creatie van een nieuw stabiel fenotype door middel van de introduktie van vreemd DNA in planten, lijkt eerder op toevalligheden te berusten dan op wetenschappelijk inzicht.

Finnegan en McElroy (1995) Bio/technology, 12, 883-888.

8. De weerstand tegen de introduktie van transgene produkten wordt hoofdzakelijk veroorzaakt door een gebrek aan communicatie tussen wetenschappers en de media.

9. Het verschil in levensverwachting van vijf jaar rechtvaardigt een beleid gericht op het verkleinen van inkomensverschillen tussen arm en rijk.

Algemeen Dagblad, 31 maart 1995.

Stellingen behorende bij het proefschrift:
"Early nodulin gene expression and the action of Nod factors in *Vicia sativa*"
door Irma Vijn, te verdedigen op 9 mei 1995.

CONTENTS

Outline

Bacteria of the genera *Rhizobium*, *Bradyrhizobium* or *Azorhizobium* secrete lipo-oligosaccharide signal molecules, which play a pivotal role in the induction of early steps of root nodule formation on legumes. In these nodules the bacteria are hosted and a proper environment is provided for the bacteria to fix atmospheric nitrogen into ammonia, making the plant for its growth independent of nitrogen compounds in the soil.

The goal of the research described in this thesis is to provide insight in the mechanism by which the Nod factors secreted by *Rhizobium* bacteria initiate root nodule formation. Such kind of study requires a plant-bacteria system in which both the morphological and molecular changes induced by the Nod factor can be examined. Working on the Nod factors secreted by *Rhizobium leguminosarum* bv. *viciae* the choice of the host plant is limited to the genera *Pisum*, *Lathyrus*, *Lens* and *Vicia*. We decided that *Vicia sativa* (vetch) would be the most useful plant for our studies, since the plant is small and various morphological changes, like root hair deformation and the formation of nodule primordia, that are induced by the Nod factors can easily be observed (Chapter 1).

Nod factors can induce the expression of early nodulin genes. These genes are expressed during different developmental stages of root nodule formation and the expression of these genes can therefore be used as molecular markers of root nodule development and Nod factor induced processes. To study Nod factor induced plant responses in *V. sativa* on the molecular level, the homologues of the early nodulin genes of pea (*Pisum sativum*) were isolated from *V. sativa* and their expression pattern was studied by *in situ* hybridization during root nodule development (Chapter 2).

To obtain information about the pathway from Nod factor to early nodulin gene expression, the mechanisms controlling *ENOD12* expression were studied. With a promoter analysis in transgenic *V. hirsuta* root nodules we have identified that the 200 bp immediately upstream of the transcription start are sufficient to induce nodule specific and Nod factor induced expression (Chapter 3). For the isolation of transcription factors involved in controlling *ENOD12* expression an expression library was screened and a preliminary characterization of cDNA clones encoding polypeptides that bind to the *PsENOD12* promoter is described in Chapter 4.

In Chapter 5 early nodulin gene expression during Nod factor induced morphological changes, like root hair deformation and nodule primordia induction, was studied. Furthermore we examined whether RNA and protein synthesis are required for root hair deformation and for the activation of the early nodulin genes. These studies provided new insights about the mode of action of Nod factors.

In Chapter 6 is discussed to what extent *V. sativa* is a suitable host plant to study the mode of action of Nod factors and in which way the studies reported in this thesis have contributed to elucidate the mechanism by which Nod factors induce a diversity of plant responses.

CHAPTER 1

The vetch (*Vicia*) and *Rhizobium leguminosarum* bv. *viciae* symbiosis: A system to study the activity of *Rhizobium* Nod factors

Adapted from:

Irma Vijn, Anton van Brussel, Albert van Kammen and Ton Bisseling. In: Plant Molecular Biology: Molecular genetic analysis of plant development and metabolism, pp 203-218 (1994).

Introduction

The interactions of leguminous plants and bacteria of the genera *Rhizobium*, *Bradyrhizobium* and *Azorhizobium* - here collectively called rhizobia - result in the formation of root nodules, new organs in which the bacteria are able to fix atmospheric nitrogen into ammonia. The formation of root nodules involves a number of developmental steps (Newcomb, 1976; Newcomb, 1981). First the bacteria attach to the root hairs of the host plant and cause deformation and curling of root hairs. Then the bacteria enter the plant by newly formed tubular structures, the infection threads, starting from the curls of the root hairs. At the same time, cells in the cortex of the root become mitotically active and form a nodule primordium. The infection threads grow towards the nodule primordia, and after penetration of individual primordium cells the bacteria are released in the cytoplasm, by endocytosis. Then the nodule primordium differentiates into a nodule (See also figure 1.1).

During nodule formation several plant genes, called nodulin genes (Van Kammen, 1984), are specifically expressed. The genes of which the expression is induced before nitrogen fixation starts are called early nodulin genes (*ENOD* genes) (Nap and Bisseling, 1989). Different early nodulin genes have been isolated, mainly from pea and soybean, and *in situ* hybridization experiments showed that the expression of individual nodulin genes marks specific stages of nodule development (Franssen *et al.*, 1992).

The first identified early nodulin gene was *ENOD2*. *ENOD2* gene expression is found in the nodule parenchyma (Van de Wiel *et al.*, 1990) and starts at the nodule primordium stage when the nodule peripheral tissues begin to differentiate.

Genes specifically of interest to study initial steps of nodule formation are *ENOD5* and *ENOD12*, because they are already expressed during the first steps of nodule formation. These genes might be infection related, because *ENOD5* gene expression is found in root cells containing the infection thread tip and the *ENOD12* gene is transcribed in cells containing the infection thread, in cells preparing for infection thread penetration and in the dividing root cortical cells (Scheres *et al.*, 1990a; Scheres *et al.*, 1990b). In mature indeterminate nodules, which have a persistent meristem, the *ENOD5* and *ENOD12* genes are expressed in partly overlapping zones of the central tissue. The *ENOD5* gene is expressed in infected cells of the whole infection zone II, whereas *ENOD12* expression is only found in the distal part of pre-fixation zone II (Franssen *et al.*, 1992). The *ENOD5* and *ENOD12* genes were initially identified in pea and homologous genes have been identified in alfalfa (Allison *et al.*, 1993; Hirsch *et al.*, 1991; Pichon *et al.*, 1992) (Barker, pers. comm.) and *Vicia sativa* (vetch) (see Chapter 2). In vetch *ENOD12* expression is detected in the whole pre-fixation zone II.

Another early nodulin gene, *ENOD40*, was first identified in soybean and homologous genes have been isolated from pea (Matvienko *et al.*, 1994), alfalfa (Crespi *et*

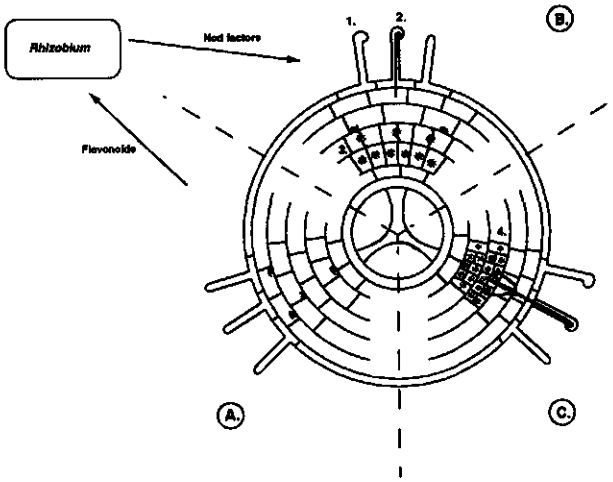

Figure 1.1: How nodulation begins. Changes in the root of a temperate legume induced by *Rhizobium.*

A: The plant secretes flavonoids, which stimulate *Rhizobium* to produce Nod factors.

B: The Nod factors and the bacteria induce root hair deformation (1) and root hair curling (2). From this curl a tubular structure, infection thread, is formed through which the bacteria can enter the plant (2). Concomitantly cells in the inner cortex are mitotically activated and form the nodule primordium (3).

C: The infection threads grow towards this nodule primordium and after penetration of individual primordium cells the bacteria are released in the cytoplasm (4). Then the primordium differentiates into a nodule.

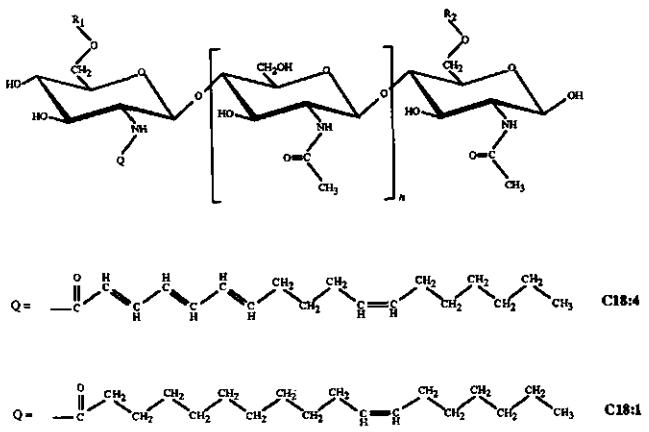

Figure 1.2: Structure of the Nod factors produced by *R. leguminosarum* bv. *viciae* (Spaink *et al.*, 1991).

n = 2 or 3; **Q** = C18:1 or C18:4; $\mathbf{R_1}$ = H or CH_3CO; $\mathbf{R_2}$ = H. When n = 3, Q = C18:4 and R_1= CH_3CO the nomenclature for the factor is: NodRlv-V(Ac, C18:4).

al., 1994) and *V. sativa* (Chapter 2). The expression of the *ENOD40* gene is induced at another step of nodule formation than the *ENOD5* and *ENOD12* genes. In all legumes studied so far, its expression is found in cells of the nodule primordia as well as in the pericycle of the root facing the nodule primordia (Yang *et al.*, 1993). Since the expression of these nodulin genes marks specific early steps or stages of nodule development they lend themselves for studying mechanisms by which *Rhizobium* triggers nodule formation.

Besides specific gene expression in the host plant, several genes are specifically expressed in rhizobia during nodule formation. Genes that play a pivotal role in different steps of nodulation are the *nod* genes. In fast growing *Rhizobium* species the *nod* genes are located on a large plasmid (pSym), whereas in the slow growing *Bradyrhizobium* species these genes are present on the chromosome (Long, 1989). In free-living bacteria the *nod* genes are not expressed, except the *nodD* gene. Flavonoids secreted by the root of the plants most likely bind to the NodD protein, and the complex acts as a transcriptional activator (Goethals *et al.*, 1992; Long, 1989), which induces the transcription of the other *nod* genes (Peters *et al.*, 1986; Redmond *et al.*, 1986). These *nod* genes encode for proteins that are essential for the biosynthesis of the so-called Nod factors. The Nod factors are secreted by the bacteria and are able to achieve in the plant, even in the absence of the bacteria, the first steps of nodule formation, like root hair deformation, cytoplasmic rearrangements in the root outer cortex and cortical cell divisions in the inner cortex (Fisher and Long, 1992; Lerouge *et al.*, 1990; Spaink *et al.*, 1991; Van Brussel *et al.*, 1992).

The legume-*Rhizobium* symbiosis has a host specific nature, which implies that a distinct bacterial strain can only nodulate a particular set of host plants. Some bacterial strains have a narrow host range of one or a few plant species, others have a broad host range including many different plant species. The bacterial *nod* genes involved in determining the bacterial host range are the so-called host specific *nod* genes. The class of *nod* genes that are functionally exchangeable between *Rhizobium* species are called the common *nod* genes. The common Nod proteins accomplish the formation of the core molecule of the Nod factor, while the products of the host specific *nod* genes decorate this core molecule and make them most likely host plant specific (Lerouge *et al.*, 1990; Spaink *et al.*, 1991). The Nod factors of several *Rhizobium* species have been purified and their structure has been elucidated. The core molecule of all Nod factors is a chitin oligomer, containing a N-acyl moiety at the non-reducing terminal sugar, but the decoration of these core molecules varies in the different species (Dénarié *et al.*, 1992). The Nod factors produced by *R. leguminosarum* bv. *viciae* consist of a tetra- or pentamer of N-acetyl-D-glucosamine with an unsaturated C18 fatty acid at the non-reducing terminal sugar. The acyl chain may either have one or four unsaturated bonds (Spaink *et al.*, 1991). Furthermore the molecules contain an O-acetyl group at the non-reducing terminal sugar

(Figure 1.2).

Thus different *Rhizobium* species produce structurally different Nod factors, but the process of nodule formation induced by the various Nod factors on their host plants is quite similar. The availability of purified Nod factors makes it possible to study the action of each of the Nod factors in different early steps of nodulation. Such a study requires a plant system in which the different steps of nodule formation can be readily induced with purified Nod factors and easily observed. We find that plants of the genus *Vicia* are most suitable host plants to study the effect of the Nod factors produced by *R. leguminosarum* bv. *viciae* as will be described below.

Vetch (*Vicia*): general aspects

Since the symbiosis between legume plants and rhizobia is a host specific interaction, as outlined in the introduction, the choice of the test plant is limited with each *Rhizobium* species. Working with *R. leguminosarum* bv. *viciae* gives the possibility to use plants of the genera *Pisum, Vicia, Lathyrus* and *Lens* as host plant. The interaction between pea (*Pisum sativum*) and *R. leguminosarum* bv. *viciae* has been studied in considerable detail in view of the agricultural and economic importance of this crop plant. Furthermore, the genetics of pea has been studied for a long time (Mendel, 1866) and pea has been often used as a model plant in plant physiological and biochemical research. For studying the different developmental steps of nodule formation on the other hand, pea is not always such a suitable host plant. It is for example quite a large plant and therefore not easily usable for nodulation tests with large numbers of different *Rhizobium* strains. Furthermore, the phenotypic changes characteristic for the first steps of the interaction, as root hair deformation and nodule primordium induction or the formation of short thick roots can not be easily observed in pea after application of Nod factors produced by *R. leguminosarum* bv. *viciae*.

Plants from the genus *Vicia* turned out not to have such disadvantages. The genus *Vicia* contains a number of plant species varying in size from very small (*V. tetrasperma*) to large (*V. narbonensis*). Especially the small species *V. hirsuta, V. tetrasperma, V. sativa* and *V. lathyroides* were found to be very suitable for nodulation tests with many different *Rhizobium* strains (Van Brussel *et al.*, 1982). Such tests can easily be performed in test tubes. Nodules are formed four to seven days after inoculation with *R. leguminosarum* bv. *viciae*. Furthermore we have found that *V. sativa* (vetch) is very useful in testing different biological activities of Nod factors, like root hair deformation, the formation of short thick roots or the induction of nodule primordia. With these assays it is possible to check separate steps in nodule formation and examine how each step depends on specific structural

features of Nod factors.

Root hair deformation

One of the first steps during the interaction between plant and rhizobia is root hair deformation. A very useful method to study deformation of root hairs was developed by Bhuvaneswari (Bhuvaneswari and Solheim, 1985). In her procedure white clover (*Trifolium repens* L.) seeds were germinated and seedlings were transferred to Fåhraeus slides. These are microscope slides covered with a coverglass by applying drops of silicon glue at the corners, which served as spacers as well. The slides can be filled with liquid plant culture medium and are incubated upright in a staining dish for 24 hours in a growth cabinet. Then the medium is decanted and the chamber filled with the solution to be tested for deformation activity. The slides are incubated in a moist chamber at room temperature and root hair deformation can be observed microscopically.

We have used this system with *V. sativa* and examined root hair deformation induced by Nod factors produced by *R. leguminosarum* bv. *viciae*. Four different Nod factors are produced by *R. leguminosarum* bv. *viciae* (NodRlv factors)(see Figure 1.2). The factors have four or five glucosamine residues and can contain then either the highly unsaturated C18:4 fatty acid chain or the C18:1 chain. Root hair deforming capacity of these Nod factors was tested by adding only one of the purified Nod factors to the medium within the slides. All Nod factors were added in a concentration range of 10^{-8} - 10^{-13} M to the culture medium and root hair deformation could be observed within three hours with all four factors at concentrations of 10^{-12} or higher (Heidstra *et al.*, 1994). Not all the root hairs on the root deformed after adding the Nod factors. Only the hairs that had just obtained their mature size showed deformation (Figure 1.3A).

Using this bioassay, the relation between the structure of the Nod factors and the induction of root hair deformation can be studied, particularly now Nod factors and their derivatives can be chemically synthesized (Nicolaou *et al.*, 1992). Since all four NodRlv factors induced root hair deformation on *V. sativa* the total number of glucosamine residues (4 or 5) makes no difference for the induction of root hair deformation nor does the saturation level of the fatty acid chain. However, the presence of a N-acyl group, different from acetate, is a necessity since chitin oligomers are unable to induce root hair deformation (Heidstra *et al.*, 1994; Spaink *et al.*, 1991). This suggests that also Nod factors produced by other *Rhizobium* strains, consisting of a glucosamine backbone and a fatty acid chain would be able to induce root hair deformation on vetch. This has in fact been observed since also the Nod factors produced by *R. leguminosarum* bv. *trifolii* are able to induce root hair deformation on vetch (Spaink *et al.*, 1991). However, an exception has been found for

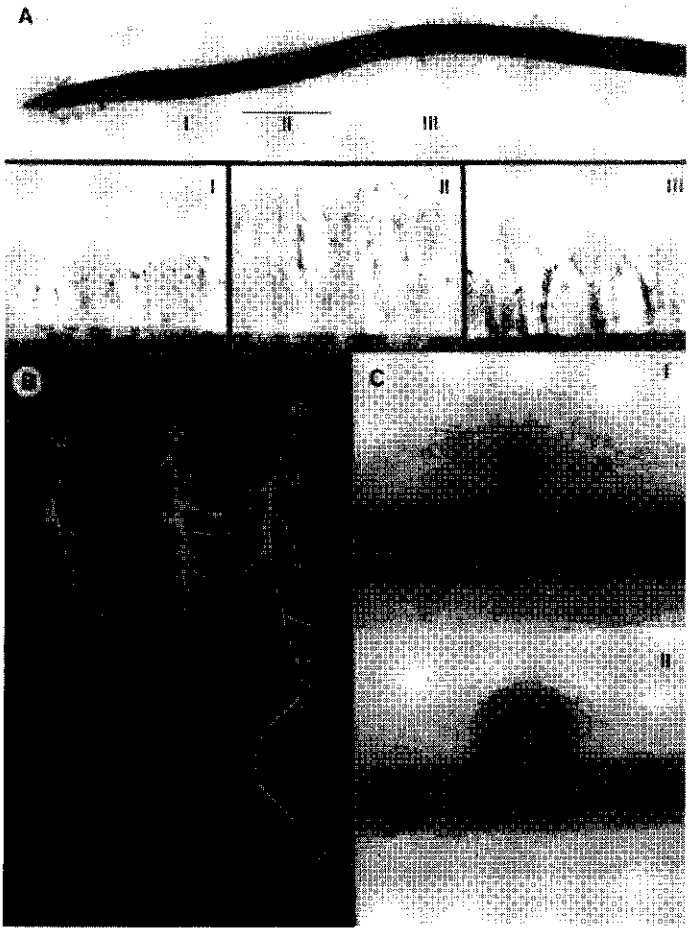

Figure 1.3: Nod factor induced processes on *Vicia sativa* roots.

A: Root hair deformation. 3 h after applying NodRlv factor root hairs present in the susceptible zone (zone II, indicated by the line) show deformation. The susceptible zone (II) of a vetch root is about 2 mm long and encompasses only young root hairs that have almost reached their mature state. The young elongating root hairs (I) and the older mature root hairs (III) do not show deformation (Heidstra *et al.*, 1994).

B: Tsr formation after inoculation with *Rhizobium*. (I) *Vicia sativa* root inoculated with a low inoculum of *Rhizobium* (0.5-5 bacteria/ml). Nodules are formed on the primary root; (II) *Vicia sativa* root inoculated with a high inoculum of *Rhizobium* (4.9 x 10^5/ml). Thick short roots (Tsr) are formed and root nodules are formed on the lateral roots; (III) *Vicia sativa* root not inoculated.

C: Nod factor induced nodule primordium formation. (I) primordium formed after Nod factor addition; (II) lateral root formation.

the Nod factors produced by *R. meliloti*. The major Nod factor produced by *R. meliloti* (NodRm factors) consists of four glucosamine residues, containing an C16:2 fatty acid chain (Q) on the non-reducing terminal sugar and the reducing sugar is substituted with a sulphate group ($R_2 = SO_3H$, Figure 1.2). The NodRm factors induce root hair deformation on its own host plant alfalfa, but these factors are not able to induce root hair deformation on vetch. After removal of the sulphate group the NodRm factors loose the ability to induce root hair deformation on alfalfa but they gain the ability to induce root hair deformation on vetch (Roche *et al.*, 1991). In this case the induction of root hair deformation is a host specific reaction, in which the additional groups added to the core molecule, here the sulphate group, are important. However, since many different Nod factors from rhizobia of several cross-inoculation groups induce root hair deformation on vetch, root hair deformation can not be called host specific in general.

Thick Short Root formation (Tsr)

A phenomenon that can be observed with *V. sativa* roots after inoculation with *R. leguminosarum* bv. *viciae* is the formation of Thick short roots (Tsr) (Van Brussel *et al.*, 1982)(Figure 1.3B). This Tsr phenotype is not observed in *V. hirsuta* roots after inoculation with *R. leguminosarum* bv. *viciae*. After inoculation of *V. sativa* roots with a very low inoculum concentration of *R. leguminosarum* bv. *viciae* (0.5-5 bacteria/ml) the roots do not develop the Tsr phenotype and the nodules are formed on the primary root. Inoculation with a high inoculum concentration (4.9×10^5/ml) causes the formation of aberrant roots and nodulation is delayed for approximately three days, because the nodules are not formed on the primary root but on the sites of lateral-root emergence (Zaat *et al.*, 1989). The formation of Tsr roots on *V. sativa* is not found when the plants are inoculated with strains mutated in the *nodD* gene or in any of the *nodA, B* or *C* genes. Apparently the proteins encoded by these genes are essential for the formation of Nod factors, showing that these compounds are involved in the induction of Tsr formation (Zaat *et al.*, 1987).

Adding purified Nod factors produced by *R. leguminosarum* bv. *viciae* to *V. sativa* roots induced indeed the formation of Tsr (Spaink *et al.*, 1991). Like root hair deformation, Tsr formation is induced by each of the four NodRlv factors at concentrations as low as 10^{-10} to 10^{-11} M, but not by the sulphated Nod factors of *R. meliloti*. On the other hand the non-sulphated form of the NodRm factor is capable to induce the Tsr phenotype (Roche *et al.*, 1991), showing that also in this case the presence of a sulphate group on the Nod factor prevents biological activity on vetch.

The induction of the Tsr phenotype is probably a side effect of the Nod factors, because Tsr formation is not a precondition for nodule formation. Most leguminous plants do

not form Tsr roots after inoculation with rhizobia. The formation of Tsr on *V. sativa* can be inhibited with aminoethoxyvinylglycine (AVG), an ethylene synthesis inhibitor. Inhibition of Tsr results in earlier nodulation and the nodules are formed on the primary root (Zaat *et al.*, 1989). Nevertheless, Tsr formation can be used in elucidating structure-function relations of Nod factors, because a specific Nod factor structure is required.

Nodulin gene expression used as a molecular marker

The interaction between *Rhizobium* bacteria and their host plants involves the specific expression of nodulin genes already in an early stage of nodule formation. As mentioned above these *ENOD* genes are expressed in a developmentally and spatially controlled manner.

It has been found that the expression of the *ENOD5* and *ENOD12* genes can be induced after inoculation of the plants with Nod factors (Horvath *et al.*, 1993; Journet *et al.*, 1994; Pichon *et al.*, 1993). In the study performed by Horvath *et al.* (1993) the expression of the *ENOD5* and *ENOD12* genes in root hairs of pea (*Pisum sativum*) plants was followed in time, after adding Nod factors produced by *R. leguminosarum* bv. *viciae* to the roots of the plants. Because the presence of *ENOD5* and *ENOD12* mRNA in root hairs could not be detected on Northern blots after inoculation of the plants with the NodRlv factors, PCR based experiments were used. Both factors NodRlv-V(Ac, C18:1) and NodRlv-V(Ac, C18:4) induce at a concentration of 10^{-8} and 10^{-9} M the expression of the *ENOD5* and *ENOD12* genes in a transient manner between 12 to 48 hours after addition of the factor. Upon adding each factor alone, NodRlv-V(Ac, C18:1) caused a delay of about 12 hours in the induction of gene expression compared to factor NodRlv-V(Ac, C18:4). If an equimolar mixture of all four NodRlv factors at a concentration of 10^{-8} M was added to the roots of the plants, both genes were expressed during the whole period that the plants were assayed. How the mixture of Nod factors can change the period of expression of the *ENOD5* and *ENOD12* genes is not understood.

In contrast to root hair deformation and Tsr formation on vetch that were not induced by the sulphated Nod factor of *R. meliloti*, the expression of both the *ENOD5* and *ENOD12* genes was induced after adding this factor to pea roots. The maximal expression level of each of the two genes was found 48 hours after addition of the NodRm factors at a concentration of 10^{-8} to 10^{-10}, whereas the NodRlv-V(Ac, C18:4) factor induced maximal expression of *ENOD12* at 12 hours and *ENOD5* at 24 hours after inoculation. Thus NodRm factors that do not accomplish root hair deformation on vetch, are able to induce early nodulin gene expression in pea plants. The effect in pea is observed at the same concentration of NodRm factors required for root hair deformation on alfalfa (Schultze *et al.*,

1992).

Induction of expression of the *ENOD12* gene in transgenic alfalfa plants (*Medicago varia* A2), carrying a chimaeric *Medicago truncatula ENOD12* promoter-*ß-glucuronidase* (GUS) gene revealed that already within 3 to 6 hours after inoculation of the plants with *R. meliloti* the *ENOD12* gene is induced (Pichon *et al.*, 1992). GUS expression was found in all epidermal cells in a region of the root that started just above the root tip, continued throughout the zone containing the emerging root hairs and ended at the mature root hair zone. Addition of picomolar concentrations of *R. meliloti* Nod factor (NodRm factor) elicited an identical GUS expression pattern as induced by the bacteria (Journet *et al.*, 1994). Also the non-sulphated NodRm factor is able to induce *ENOD12* expression, but only at concentrations higher than 10^{-9}-10^{-10} M. Saturation of the fatty acid chain or removal of the O-acetyl group from the non-reducing sugar did not influence the *ENOD12* inducing activity of the NodRm factors. These results are consistent with studies on root hair deformation which showed that on alfalfa at low concentrations (10^{-9} - 10^{-12} M) the non-sulphated factors do not elicit root hair deformation but at higher concentration (10^{-8} - 10^{-7} M) they do (Journet *et al.*, 1994). The induction of root hair deformation was not influenced by changes of the degree of fatty acid saturation or the absence of O-acetylation (Schultze *et al.*, 1992).

These results show that *ENOD12* expression and root hair deformation can be induced by non-host Nod factor but only at high concentrations. Since *ENOD12* expression in alfalfa was never induced by *Rhizobium meliloti* carrying a mutated *nodH*, which results in a non-sulphated NodRm factor, Journet *et al.* (1994) suggest that the concentration of Nod factor produced by *Rhizobium* on the root surface will be lower than 10^{-10} M. In that case only the results of the experiments performed at concentrations lower than 10^{-10} M should be taken into account. Those results show then that the structural requirements for the Nod factor for the induction of root hair deformation and *ENOD12* expression are identical, which means that on alfalfa only sulphated Nod factor can induce root hair deformation and *ENOD12* expression, whereas on pea and vetch the Nod factor should be non-sulphated. This suggests that the induction of *ENOD12* expression is in some way a host specific reaction, like root hair deformation and Tsr formation.

Recently also vetch *ENOD5*, *ENOD12* and *ENOD40* cDNA clones have been isolated from a nodule cDNA library (Chapter 2), making it possible to study nodulin gene expression in combination with bioassays like root hair deformation and Tsr formation.

Promoter studies of early nodulin genes

Now it has been shown that Nod factors can induce the expression of the early

nodulin genes, *ENOD5* and *ENOD12*, the next question is how Nod factors induce this gene expression. What are the different steps involved in the signal transduction pathway, starting with Nod factor recognition and ending in nodulin gene expression? Studies on the mechanisms controlling specific expression of early nodulin genes encompass promoter analyses, which will result in the identification of cis- and trans-acting elements involved in the induction of expression of these genes. Until now, detailed promoter studies have only been performed with late nodulin genes like the leghemoglobin genes of soybean (*Lbc3*) (Stougaard *et al.*, 1987) and *Sesbania rostrata* (Szabados *et al.*, 1990) and the soybean late nodulin gene N23 (Jørgensen *et al.*, 1988). In these studies chimaeric genes consisting of the promoter of the nodulin gene and a reporter gene were transformed to *Lotus corniculatus* plants with *Agrobacterium rhizogenes* (Petit *et al.*, 1987). These promoter analyses showed that for an efficient expression in nodules at least three different promoter regions are required; a "minimal promoter" containing the CAT and TATAA boxes and, further upstream, an organ specific element (OSE) and positive elements (PE). The OSE of all three genes studied, contained two highly conserved DNA sequences, AAAGAT and CTCTT. So far, several DNA-binding proteins have been identified which interact with positive AT-rich elements in the 5' upstream region of these late nodulin genes (de Bruijn *et al.*, 1989; Jacobsen *et al.*, 1990; Metz *et al.*, 1988). Although it has been shown that these factors form *in vitro* a complex with nodulin promoter regions, their biological role *in vivo* still has to be elucidated.

Lotus corniculatus, the plant used in all these promoter studies, forms determinate root nodules after inoculation with *R. loti*. We are studying the interaction of *R. leguminosarum* bv. *viciae* with its host plants, vetch and pea, which both form indeterminate nodules. Determinate nodules are morphologically different from indeterminate nodules, because they lack a persistent meristem. Especially for promoter studies of genes involved in the early steps of nodulation, like *ENOD5* and *ENOD12*, indeterminate nodules on the transgenic roots will be favourable, since the expression of these genes in such nodules can be studied over a much longer period of time than in determinate nodules. Even in mature indeterminate nodules *ENOD5* and *ENOD12* gene expression can be observed (Franssen *et al.*, 1992; Scheres *et al.*, 1990a; Scheres *et al.*, 1990b). Recently an *A. rhizogenes* based transformation system has been developed for *V. hirsuta* by Quandt *et al.* (1993). In this system *V. hirsuta* plants are stem-inoculated with *A. rhizogenes* resulting in the formation of hairy roots. After inoculation of these hairy roots with *R. leguminosarum* bv. *viciae* transgenic indeterminate nodules were formed. Since transgenic nodules could be obtained within 4 to 6 weeks, this system appears to be suitable for promoter studies of early nodulin genes. Working backwards in the signal transduction pathway, the identification of cis-elements might help in finding trans-acting factors involved in the induction of this specific gene expression and in that way contribute to the elucidation of a

step in the signal transduction pathway elicited by the Nod factors.

Induction of nodule primordia in vetch roots

After inoculation of vetch plants with *R. leguminosarum* bv. *viciae* the bacteria induce cortical cell divisions in the root inner cortex. These dividing cells form the nodule primordium, which will later develop into a nodule. The cells in the inner cortex are fully differentiated and upon infection with *Rhizobium* these cells re-enter the cell cycle. By *in situ* hybridization it has been shown that in these cells genes controlling the cell cycle, like *cdc2* and cyclins, are expressed upon *Rhizobium* infection (Yang *et al.*, 1994). The Nod factors produced by *R. leguminosarum* bv. *viciae* have also the ability to induce cortical cell divisions in vetch roots (Spaink *et al.*, 1991). To study the mitosis inducing activity of the NodRlv factors a special assay has been developed (Spaink *et al.*, 1991). In this system the *V. sativa* roots are grown in amber vials, to shield the roots from the light, because otherwise Tsr formation is induced. If an equimolar mixture of the four NodRlv factors at a concentration of about 10^{-8} M is applied to *V. sativa* plants, cortical cell divisions can be observed after 4 to 6 days (Spaink *et al.*, 1991) (Figure 1.3C). When purified NodRlv-V(Ac, C18:4) at a concentration of about 10^{-8} M is added to *V. sativa* plants these structures are formed as well, whereas NodRlv-V(Ac, C18:1) does not induce cortical cell divisions (Spaink *et al.*, 1991). This suggests that the structure of the acyl chain is important for the induction of cell divisions.

An important question to answer is whether these Nod factor induced clusters of dividing cortical cells have the same characteristics as the primordia induced by the bacteria. The nodule primordia induced by the NodRlv-V(Ac, C18:4) factor or the NodRlv mixture are induced in the inner cortical cell layers preferentially opposite a proto-xylem pole, just as *R. leguminosarum* bv. *viciae* does. Furthermore *in situ* hybridization showed the expression of the early nodulin genes *ENOD12* and *ENOD40*. *ENOD12* gene expression was found in the dividing cortical cells, whereas *ENOD40* gene expression was found in these dividing cortical cells, as well as in the pericycle facing the nodule primordium (Vijn *et al.*, 1993) (Chapter 5). These specific expression patterns are identical to those in nodule primordia induced by the *Rhizobium* bacteria. This shows that for these properties the nodule primordia induced by the bacteria are not distinguishable from the nodule primordia induced by the purified Nod factors. However, in contrast to alfalfa (Truchet *et al.*, 1991), the nodule primordia induced by the NodRlv factors in vetch roots never developed further into a mature nodule. In what stage of development these NodRlv factor induced structures in *V. sativa* are arrested is not known at the moment.

Increase of *nod* gene inducing activity (Ini)

When *V. sativa* roots are inoculated with *R. leguminosarum* bv. *viciae* an increase of *nod* gene inducing activity, called Ini, of the root exudate can be observed after four days of cocultivation (Van Brussel *et al.*, 1990). This is caused by eight additional flavonoids secreted by the plant. All eight flavonoids are able to stimulate the expression of *nod* genes of *R. leguminosarum* bv. *viciae* (Rolfe *et al.*, 1989). The Ini effect is not only observed in the symbiosis between *V. sativa* and *R. leguminosarum* bv. *viciae*, but also in *Trifolium repens* (white clover) inoculated with *R. leguminosarum* bv. *trifolii*. This increase was even seen when white clover seedlings were inoculated with *R. leguminosarum* bv. *viciae* (Recourt *et al.*, 1991). In *V. sativa* Ini seems a host specific reaction, since after four days of cocultivation of *V. sativa* with *R. leguminosarum* bv. *trifolii* this response could not be observed (Van Brussel *et al.*, 1990).

Ini can also be observed after the addition of purified NodRlv factors to *V. sativa* roots, but only factors containing both the O-acetyl group and the highly unsaturated acyl moiety (NodRlv-IV or V(Ac, C18:4)) at the non-reducing sugar were able to produce Ini on *V. sativa* roots (Spaink *et al.*, 1991). The lowest concentration of NodRlv factor that could induce Ini in *V. sativa* roots is $5x10^{-8}$ M. This concentration is higher than the concentration at which the Nod factors are able to induce root hair deformation, Tsr formation, cortical cell divisions and the expression of the early nodulin genes (Spaink *et al.*, 1991; Horvath *et al.*, 1993; Heidstra *et al.*, 1994). In *V. sativa* roots Ini is only induced when the roots are grown in the light, and Ini induction can be inhibited, just like Tsr formation, with aminoethoxyvinylglycine (AVG) (Van Brussel, unpublished). This suggests that the increase of *nod* gene inducing activity of the root exudate induced by *R. leguminosarum* bv. *viciae* or the NodRlv factors is a side effect as well, like Tsr formation.

Concluding remarks

The *V. sativa* and *R. leguminosarum* bv. *viciae* system has several advantages for studying the role of Nod factors in the *Rhizobium*-legume symbiosis. Nod factors induce in *V. sativa* roots various effects that can be easily observed, like root hair deformation, the formation of thick short roots, the formation of nodule primordia and an increase of *nod* gene inducing activity. Assays in which these morphological and physiological changes can be visualized or measured are available. Some of the effects like root hair deformation and formation of nodule primordia correspond to steps in the development of root nodules as achieved by *R. leguminosarum* bv. *viciae* bacteria. Other effects like the formation of thick short roots or the increase of *nod* gene inducing activity seem to be rather side effects. Also

R. leguminosarum bv. *viciae* is able to induce these effects, but the formation of thick short roots is only induced at a high inoculum concentration and the increase of *nod* gene inducing activity is only induced in the light and both effects can be inhibited with aminoethoxyvinylglycine (AVG). However, in all cases the effects are important in studying the structural requirements of Nod factors for the induction of each of the activities.

Results so far showed that for the induction of root hair deformation and Tsr formation the structural requirements are identical. An additional sulphate group at the reducing terminal sugar completely abolishes the induction of both events. However, the structure of the N-acyl group is not important, but the presence of an N-acyl chain, longer that C2, is a necessity. The most striking results from the studies on the induction of early nodulin gene expression are that the sulphated factors produced by *R. meliloti* are able to induce the expression of the *ENOD5* and *ENOD12* genes in pea, suggesting that the signal transduction pathway from Nod factor to root hair deformation is a different pathway than from Nod factor to early nodulin gene expression, but it can not yet be excluded that the sulphate group is removed before induction. Studies on the mitotic activity of the NodRlv factors showed the importance of the acyl chain structure. The NodRlv factors containing the highly unsaturated fatty acid chain (C18:4) were able to induce nodule primordia, in contrast to the NodRlv factors with the C18:1 acyl chain.

To obtain more insight in the complex signal transduction pathways involved in nodule formation, more detailed structure-function analyses of Nod factors have to be performed. The isolation of *V. sativa ENOD5*, *ENOD12* and *ENOD40* cDNA clones provides the opportunity to combine studies on early nodulin gene expression with bioassays in which the biological activity of the Nod factors can be studied, like root hair deformation or the induction of nodule primordia. This will make it possible to obtain an accurate correlation between Nod factor induced phenomena and early nodulin gene expression and might provide additional information on the functions of the proteins encoded by these genes.

The recently developed *A. rhizogenes* transformation system for *V. hirsuta* provides the possibility to work backwards in the signal transduction pathway from Nod factors to the induction of certain early nodulin genes since it might be possible to identify transcription factors that are involved in the expression of these nodulin genes.

In general, vetch can be used in many more bioassays to study Nod factor activity than pea. Pea, however, is probably more suitable for studies aimed at the identification of the function of the plant genes involved in nodule formation, because the pea genetics has been studied extensively and various pea mutants disturbed in the interactions with *Rhizobium* are available. In contrast vetch has not been studied at a genetic level at all. Therefore it will be necessary to use both host plants in unravelling the mechanisms involved in NodRlv factor induced nodule formation.

CHAPTER 2

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

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Abstract

Rhizobium leguminosarum bv. *viciae* secreted Nod factors can induce the first steps of nodulation, like root hair deformation, the induction of cortical cell division and expression of the early nodulin genes *ENOD5*, *ENOD12* and *ENOD40*. To study the first two mentioned processes bioassays were developed for *Vicia sativa*, while early nodulin genes have been cloned from pea (*Pisum sativum*). Therefore early nodulin gene expression during Nod factor induced processes has not yet been studied. To make this possible we isolated *ENOD5*, *ENOD12* and *ENOD40* homologues from *Vicia sativa* and studied their expression pattern during *Rhizobium* induced nodule formation.

Introduction

Rhizobium bacteria elicit the formation of nodules on the roots of leguminous plants. The formation of these organs involves different developmental steps, which have been described in several recent reviews (Brewin, 1991; Hirsch, 1992; Nap and Bisseling, 1990). In short; the first visible step is root hair deformation and curling. Within the curled root hairs an infection thread is formed by which the bacteria enter the plant. At the same time root cortical cells, located opposite a proto-xylem pole, are mitotically activated and form a nodule primordium. The infection thread grows towards the nodule primordium cells and the bacteria are released into the cytoplasm of these cells. Subsequently the primordium differentiates into a nodule.

The bacterial genes that play a key role in the induction of the early steps of nodulation are the *nod* genes. Flavonoids, secreted by the plant into the rhizosphere, elicit the expression of these genes and the activity of the Nod proteins results in the production of specific lipo-oligosaccharides, called Nod factors. Purified Nod factors can induce the first steps of nodule formation including the induction of some early nodulin genes (Horvath *et al.*, 1993; Spaink *et al.*, 1991; Vijn *et al.*, 1993) and on some legumes even genuine nodules are induced (Stokkermans and Peters, 1994; Truchet *et al.*, 1991). Therefore it is of great interest to study the mode of action of Nod factors.

We study the *Rhizobium leguminosarum* bv. *viciae*-legume interaction. Host plants that can interact with *R. leguminosarum* bv. *viciae* are, for example, *Vicia* and pea. For *Vicia*, several bioassays to study root hair deformation and the induction of cortical cell division have been developed (Heidstra *et al.*, 1994; Spaink *et al.*, 1991), while on the other hand early nodulins have been cloned from pea but not from *Vicia*. Therefore it has not been possible to correlate Nod factor induced processes with the induction of early nodulin gene expression. We decided to isolate from *Vicia sativa* early nodulin genes that are induced by

R. leguminosarum bv. *viciae* Nod factors during deformation and formation of nodule primordia. Previously, we showed that the early nodulin genes *ENOD5* and *ENOD12* can be induced by purified Nod factors in root hairs of pea plants treated with Nod factor (Horvath *et al.*, 1993). Furthermore we reported that the early nodulin genes *ENOD5, ENOD12* and *ENOD40* are active in *Rhizobium* induced pea nodule primordia (Scheres *et al.*, 1990a; Scheres *et al.*, 1990b; Yang *et al.*, 1993). For this reason we cloned the *ENOD5, ENOD12* and *ENOD40* homologues of *Vicia sativa*. Here we describe the characterization of these clones and their *in situ* hybridization expression pattern during *Rhizobium* induced nodule formation on *Vicia sativa* roots.

Results and Discussion

Isolation of Vicia sativa cDNA clones coding for ENOD5, ENOD12 and ENOD40.

A *V. sativa* cDNA library, prepared from mRNA of young root nodules, was screened with ^{32}P-labelled inserts of the pea nodulin clones pPsENOD5, pPsENOD12 and pPsENOD40, which had been isolated previously (Matvienko *et al.*, 1994; Scheres *et al.*, 1990a; Scheres *et al.*, 1990b). From each screening 4 independent clones were isolated. The clones containing the longest inserts were named pVsENOD5, pVsENOD12 and pVsENOD40, respectively.

pVsENOD5 has an insert of 538 bp and contains a poly (A) stretch at the 3' end. The cloned *V. sativa* cDNA starts with the coding sequence of 12 aa which are highly conserved between pea and vetch and form, in pea, the second half of a putative signal peptide. This suggest that also the vetch ENOD5 polypeptide will contain a signal peptide and the sequence coding for the 11 aa, including the AUG start codon, at the beginning of the open reading frame are missing in the cloned VsENOD5 cDNA (Figure 2.1A). Comparison with the PsENOD5 polypeptide showed 76% identity (Figure 2.1A). Like PsENOD5, VsENOD5 contains a high percentage of pro, ser, gly, and ala residues and a short sequence of 5 aa consisting of alternating pro and ala occurs in this protein. Both features are considered to be characteristics of arabinogalactan proteins (Showalter, 1993) and so the vetch ENOD5 sequence supports the hypothesis that ENOD5 might be an arabinogalactan protein.

pVsENOD12 has an insert of 548 bp that contains a complete open reading frame (ORF) coding for a 100 amino acid long polypeptide. Like the pea ENOD12 polypeptides it contains a putative N-terminal signal peptide of 24 aa and the major part of the protein is composed of two proline rich pentapeptide repeating units. The unit consisting of the pentapeptide Pro-Pro-Gln-Lys-Glu is repeated three times but the third time the unit is

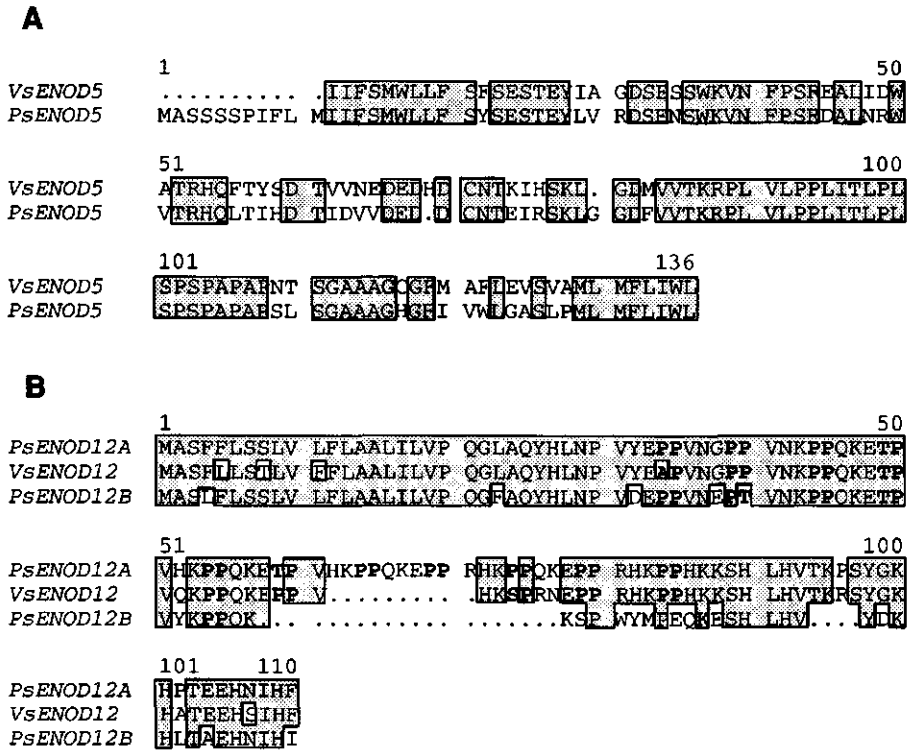


Figure 2.1: Alignment of *Vicia* and pea ENOD5 (A) and ENOD12 (B) amino acid sequences. Homologous amino acids are boxed. Dots represent deletions.

changed into Ser-Pro-Arg-Asn-Glu. The other unit starts as Pro-Pro-Val-Asn-Gly but towards the carboxy terminus of the polypeptide gradually every amino acid except the prolines is changed to a final Pro-Pro-His-Lys-Lys pentapeptide. At three positions the first proline codon is changed into an alanine, threonine or serine codon by a single basepair substitution. Comparison with the PsENOD12A polypeptide showed 88% identity, with a deletion of 10 amino acids located in the proline-rich pentapeptide repeat region, whereas the PsENOD12B polypeptide shows 73% identity. PsENOD12B contains fewer and less conserved proline-rich pentapeptide repeats, causing the lower homology (Figure 2.1B).

pVsENOD40 has an cDNA insert of 718 bp and on the nucleotide level this sequence is 93% homologous to the pea ENOD40 cDNA clone recently cloned by Matvienko *et al.* (1994). A comparison of the pea ENOD40 cDNA with the previously cloned soybean ENOD40 cDNAs (Kouchi and Hata, 1993; Yang *et al.*, 1993) showed that the long ORF present in the soybean ENOD40 cDNAs does not occur in the pea ENOD40 cDNA due to several non-triplet insertions and deletions (Matvienko *et al.*, 1994).

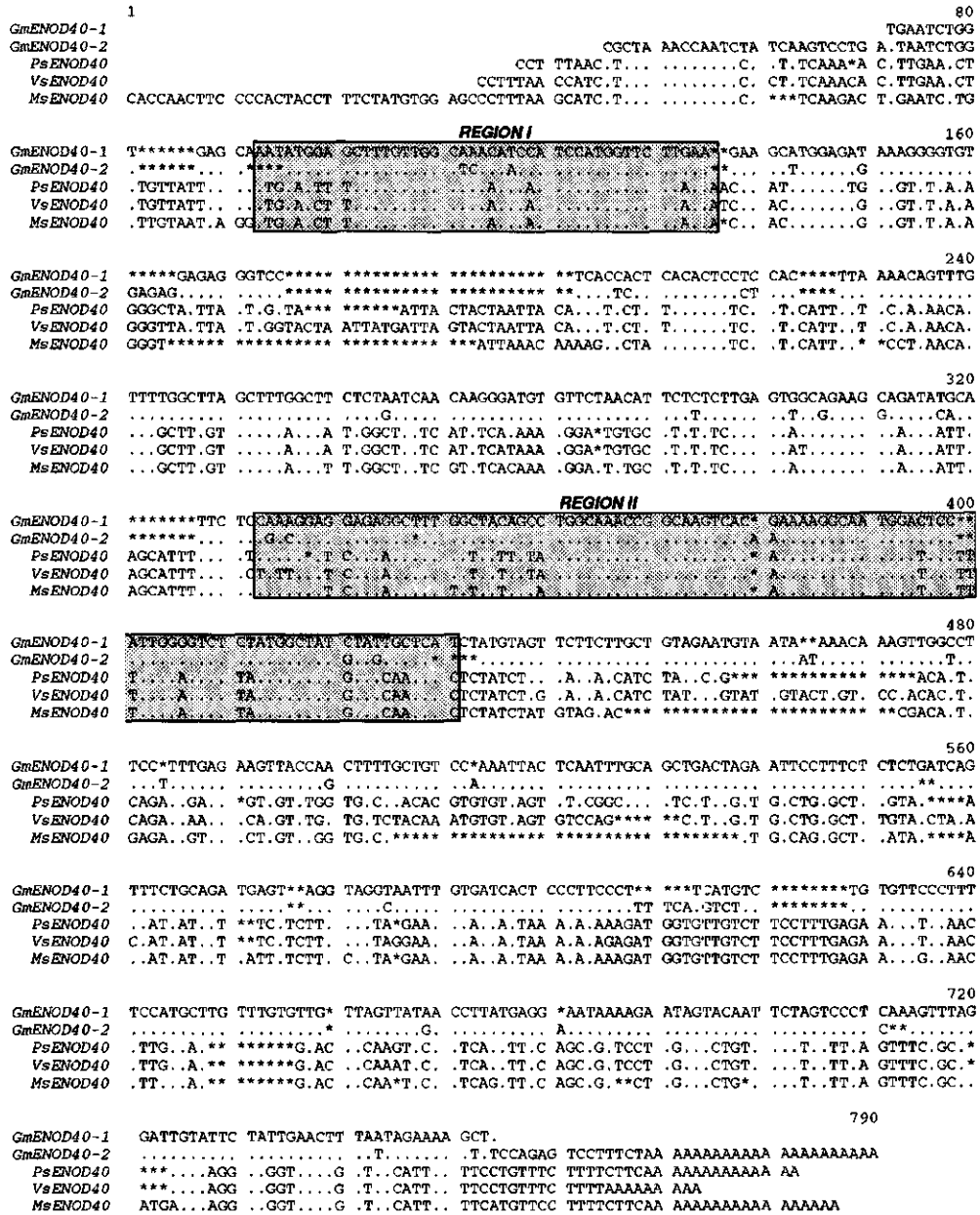
To identify the conserved regions of *ENOD40* we compared the *Vicia* ENOD40 cDNA clone with the pea, soybean and the recently cloned alfalfa (Crespi *et al.*, 1994) ENOD40 sequence (Figure 2.2A). Comparison on the nucleotide level of the complete cDNA clones showed that the *Vicia* and pea cDNA clone are 87% homologous to the alfalfa cDNA clone, and that the *Vicia,* pea and alfalfa clones are 55% homologous to the soybean cDNA clones. However, the comparison showed that the sequences contain two conserved regions, indicated in figure 2.2A as region I and region II. The homology of region I between *Vicia,* pea and alfalfa is 98%, whereas the homology of these sequences with soybean is 82%. The homology of region II is 91% between *Vicia,* pea and alfalfa and compared to soybean the sequences show a homology of 76%. Comparison of all potential ORFs, that means sequences starting with an AUG start codon, showed that all five different clones encode a highly conserved small polypeptide of 12 or 13 aa (Fig 2.2B), encoded by an ORF located in region I. The second conserved region does not encode a polypeptide. This could mean that *ENOD40* encodes a short polypeptide.

Matvienko *et al.* (1994) and Crespi *et al.* (1994) suggested that *ENOD40* might be active as a RNA transcript, because of the absence of significant ORFs and the tendency of ENOD40 RNA to form secondary structures. However, the methods used by Crespi *et al.* (1994) to show that *ENOD40* codes for a non-translatable RNA might not be applicable for messengers encoding small proteins. The Test Code program (GCG software package) was designed for detection of coding regions longer than 200 bp, so small polypeptides are overlooked and for a good comparison of the free energies of folding between coding and non-coding RNAs also RNAs encoding small polypeptides should have been used. Therefore these studies do not exclude that *ENOD40* encodes a small conserved protein. However, it should be noticed that this conserved peptide is unusual small.

Expression of early nodulin genes during nodule development.

The expression of *ENOD5*, *ENOD12* and *ENOD40* was studied by *in situ* hybridization at different stages of nodule development. Four days after spot inoculation a globular nodule primordium has been formed in the inner cortex of the root. The peripheral tissues have started to form, but the apical meristem has not yet been formed (Figure 2.3A). Some branches of the infection thread have penetrated the primordium. One day later (day 5) the nodule meristem is visible at the distal part of the young nodule and at the proximal part infected cells are present, which are larger than the cells at the distal part (Figure 2.3B). Twenty days after inoculation a mature nodule is formed (Figure 2.3C). Like pea, *V. sativa* forms indeterminate nodules, which have a persistent meristem that

A.



B.

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          1          13
GmENOD40-1  ME LCWQTSI HGS
GmENOD40-2  ME LCWLTTI HGS
  PsENOD40  MKFLCWQKSI HGS
  VsENOD40  MKLLCWQKSI HGS
  MsENOD40  MKLLCWQKSI HGS
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continuously differentiates in the different nodule tissues. As a result the central tissue of indeterminate nodules has been divided into zones representing successive stages of development. The following nomenclature for these zones from the apex to the root attachment point, are used in alfalfa and pea: the meristematic zone I, prefixation zone II, interzone II-III, fixation zone III and in older nodules a senescent zone IV is present. In both alfalfa and pea the sudden start of amyloplast accumulation in the uninfected cells marks the transition of prefixation zone II into interzone II-III (Franssen *et al.*, 1992; Vasse *et al.*, 1990). Such a sudden accumulation of amyloplasts in the uninfected cells was also found in *V. sativa* nodules and this will be considered as the transition of prefixation zone II into interzone II-III (Figure 2.4). Adjacent cross sections of vetch roots 4, 5 and 20 days after inoculation with *R. leguminosarum* bv. *viciae* were hybridized with ^{35}S-labelled anti-sense RNA transcribed from the three cloned early nodulin cDNAs.

At 4 days after spot inoculation both ENOD12 and ENOD40 mRNA are present in all cells of the centre of the nodule primordium, while ENOD40 mRNA is also present in the region of the root pericycle facing the nodule primordium (Figure 2.3G and J). Remarkably ENOD12 mRNA also occurs in the root cortical cells containing the infection thread (G, arrowhead). In contrast to the expression pattern of *ENOD12* and *ENOD40* in the primordium, *ENOD5* transcript is only found in a small cluster of cells of the primordium (Figure 2.3D). In this cluster infection threads are present (data not shown). At day 5, one day later, all three early nodulin genes are now expressed in the complete central tissue, but in the meristem none of these genes is active (Figure 2.3E, H and K).

The *in situ* expression patterns 4 and 5 days after inoculation are in agreement with the *in situ* hybridization studies in pea, which showed that *ENOD12* and *ENOD40* are already induced in nodule primordia cells before they become infected by *Rhizobium*, whereas ENOD5 mRNA is only present in infected cells (Scheres *et al.*, 1990a; Scheres *et al.*, 1990b; Yang *et al.*, 1993)

In 20 day old root nodules of *V. sativa* plants *VsENOD5* is expressed in the complete prefixation zone II, where it is only active in the infected cells. Maximal

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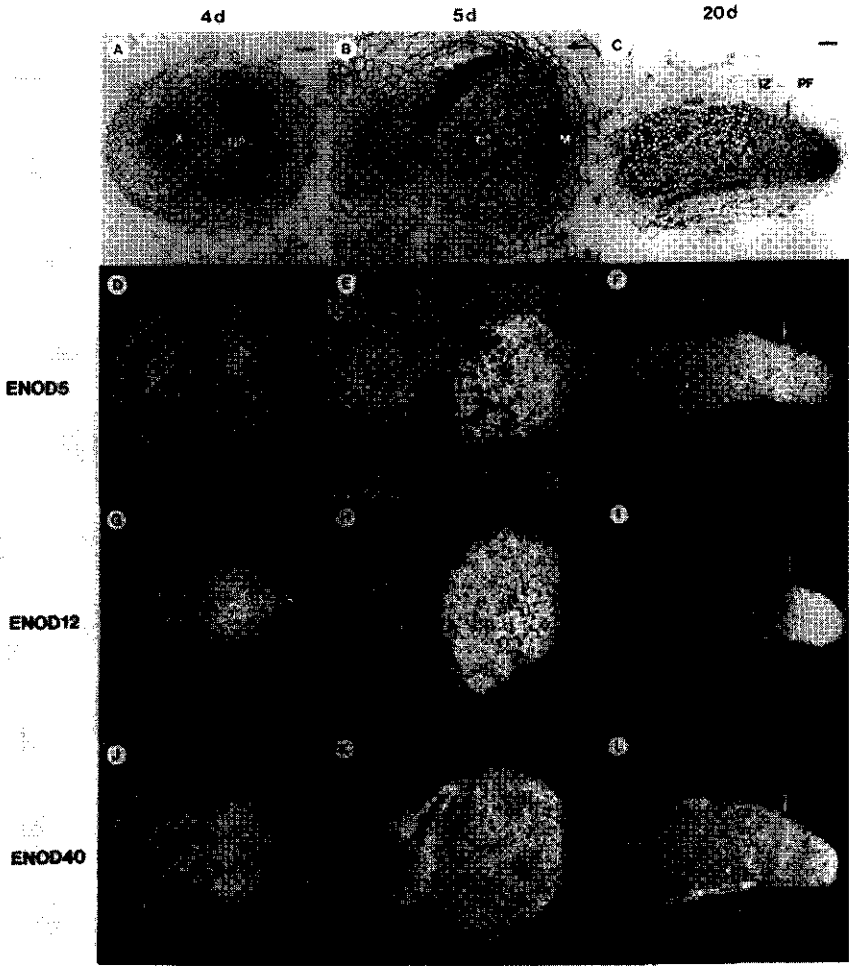
Figure 2.2: Alignment of nucleotide and amino acid sequences of *ENOD40*.

A: Alignment of nucleotide sequences of different ENOD40 sequences. *GmENOD40-1* and *GmENOD40-2* are isolated from soybean by Kouchi and Hata (1993) and Yang *et al.* (1993), respectively; *PsENOD40* has been isolated from pea by Matvienko *et al.* (1994); *MsENOD40* has been isolated from alfalfa by Crespi *et al.* (1994) and *VsENOD40* is isolated from *Vicia sativa* as described in this paper. The conserved regions I and II are boxed.

Dots represent identical nucleotides, stars represent deletions.

B: Alignment of the conserved polypeptide encoded by region I of all *ENOD40* genes.


4d
5d
20d
A
B
C
IZ
PF
ENOD5
ENOD12
ENOD40

accumulation of ENOD5 mRNA occurred in the proximal part of this zone (Figure 2.3F). The level of VsENOD5 RNA suddenly decreases to a lower level from one cell layer to another at the transition of prefixation zone II into interzone II-III and remains at this reduced level in the fixation zone III. The pattern of *VsENOD5* expression is identical to that of *PsENOD5* in pea nodules (Scheres *et al.*, 1990b), although the ENOD5 mRNA, in pea, drops to a lower level at the transition point.

VsENOD12 is in 20 day old nodules expressed in the complete prefixation zone II as well and its transcript is present at a similar level throughout the whole zone. In the distal part of prefixation zone II *VsENOD12* is probably expressed in all cells, whereas in the more proximal part *VsENOD12* expression is only found in the infected cells. *VsENOD12* expression decreases to an undetectable level at the transition of prefixation zone II into interzone II-III (Figure 2.3I). So this drop of *ENOD12* expression exactly coincides with the

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Figure 2.3: In situ localization of *VsENOD5, VsENOD12* and *VsENOD40* in 4, 5 and 20 day old *Vicia sativa* root nodules.

NP, nodule primordium; CT, central tissue; PE, pericycle, M, meristem; X, proto-xylem pole; ic, infected cells; uc, uninfected cells; PF, prefixation zone II; IZ, interzone II-III. A, B, C are bright field micrographs; D, E, F, G, H, I, J, K and L are dark field micrographs were signals are represented by white dots.

The sections were hybridized with ENOD5 (D, E, F), ENOD12 (G, H, I) and ENOD40 (J, K, L) ^{35}S-labelled antisense RNA.

A, D, G, J: Serial cross sections of a 4 day old root nodule showing the nodule primordium (NP) formed in the inner root cortex. The sections were hybridized with ENOD5 (D), ENOD12 (G) and ENOD40 (J) ^{35}S-labelled antisense RNA, showing the expression of *ENOD12* and *ENOD40* in all cells of the nodule primordium (NP), whereas ENOD5 mRNA is only detected in a small cluster of cells in the nodule primordium (NP). In addition ENOD40 mRNA is detected in the root pericycle (PE) near one of the xylem poles. Note that *ENOD12* is also expressed in the root cortical cells containing the infection thread (arrow). Bar = 80 µm.

B, E, H, K: Serial cross sections of a 5 day old root nodule showing that at the distal part of the nodule a meristem (ME) is visible and at the proximal part infected cells (IC) are present. The sections were hybridized with ENOD5 (E), ENOD12 (H) and ENOD40 (K) ^{35}S-labelled antisense RNA, showing that all three early nodulin genes are expressed in the complete central tissue (CT), but not in the meristem (ME). Bar = 80 µm.

C, F, I and L: Serial cross sections of a 20 day old fully developed nodule. The sections were hybridized with ENOD5 (F), ENOD12 (I) and ENOD40 (L) ^{35}S-labelled antisense RNA. Note that all three early nodulin genes are expressed in the whole prefixation zone and that their expression pattern is changing at the transition from prefixation zone II (PF) into interzone II-III (IZ)(arrow); the level of ENOD5 mRNA decreases, *ENOD12* expression drops to an undetectable level and the expression of ENOD40 drops in the infected cells and in the fixation zone III *ENOD40* expression is induced in the uninfected cells. ENOD40 mRNA is also detected in the pericycle (PE) of the nodule vascular bundle (arrowhead). Bar = 200 µm.

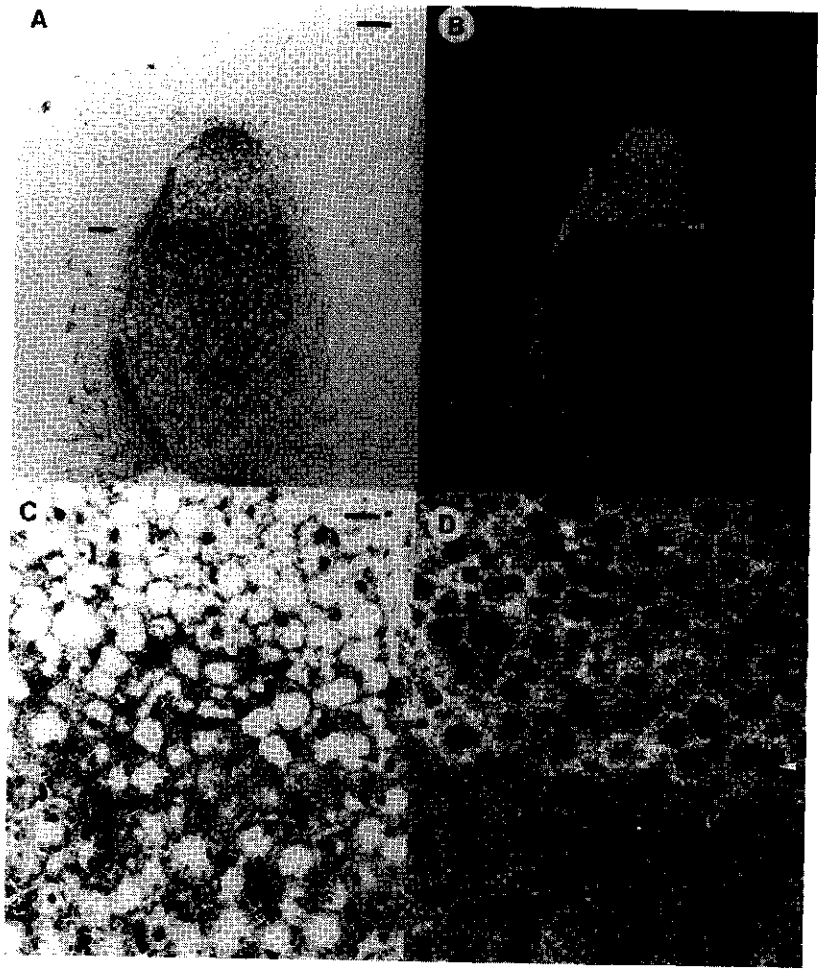

Figure 2.4: Amyloplast accumulation in 20 day old nodules.

A, B: Iodine stained cross-section of 20 day old nodule hybridized with ENOD40 ^{35}S-labelled antisense RNA, showing the accumulation of amyloplasts (AP) in the uninfected cells. The sudden accumulation of amyloplasts in the uninfected cells marks the transition of prefixation zone II (PF) into interzone II-III (IZ) (arrow). Bar = 200 µm.

C, D: Higher magnification of A and B. Bar = 40 µm.

A, C are bright field micrographs. B and D are dark field micrographs were signals are represented by white dots. Arrowheads indicate same cells in A, B, C and D.

decrease of the level of ENOD5 RNA. The expression pattern of *VsENOD12* in *V. sativa* nodules is strikingly different from *PsENOD12* expression in pea nodules, where *ENOD12* is only expressed in the most distal part of prefixation zone II (Scheres *et al.*, 1990a). In the distal part infection threads grow and infect new meristematic cells and for this reason it was proposed that *ENOD12* expression correlates with the infection process (Scheres *et al.*, 1990a). In the proximal part of the prefixation zone the bacteria, surrounded by a plant membrane, called the peribacteroid membrane, proliferate in the cytoplasm of the infected plant cells. This suggests that at least in vetch, the function of *ENOD12* is not strictly linked to the infection process and that *ENOD12* might have an additional function in the proliferation of the bacteria.

Like *VsENOD5* and *VsENOD12*, *VsENOD40* is expressed in the complete prefixation zone II. In the proximal part of this zone ENOD40 mRNA is present in the infected cells but not in the uninfected cells. At the transition of prefixation zone II into interzone II-III the level of *VsENOD40* expression drops markedly in the infected cells, and in fixation zone III expression of *VsENOD40* is now induced in the uninfected cells. In the proximal (older) part of the fixation zone the expression of *VsENOD40* is not detected anymore. In the pericycle of the nodule vascular bundle *VsENOD40* is expressed at a high level (Figure 2.3L). In both pea and vetch nodules *ENOD40* expression is maximal in the pericycle of the nodule vascular bundle and the gene is active in all infected cells of the prefixation zone. However, in the fixation zone of pea nodules *ENOD40* expression is found in both the infected and uninfected cells (Matvienko *et al.*, 1994), whereas in vetch *ENOD40* is only expressed in the uninfected cells in this zone.

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

Materials and Methods

Growth conditions for plants and bacteria

Vicia sativa spp. *nigra* seeds were sterilized and germinated for two days as described by Van Brussel *et al.* (1982). 3 day old seedlings were planted in gravel and grown as described by Moerman *et al.* (1987). *Rhizobium leguminosarum* bv *viciae* strain 248 was grown in liquid YEM medium (Bhuvaneswari *et al.*, 1980) at 28 °C. Inoculation with *R.l.* bv *viciae* 248 occurred when the seedlings were planted in gravel.

Preparing and screening of a Vicia sativa nodule cDNA library

Nodules were harvested 7, 9, 12 and 17 days after inoculation, immediately frozen in liquid nitrogen and stored at -70 °C. Total RNA from these root nodules was isolated as described by Govers *et al.* (1985). Equal amounts of these RNAs were mixed and poly(A)+ RNA was obtained by oligo-dT cellulose purification (Pharmacia, type 7). First strand cDNA synthesis (Krug and Berger, 1987) was performed by using an oligo(dT)-*Not*I primer (Promega). After second strand cDNA synthesis according to (Gubler, 1987), double stranded cDNA fragments larger than 400 bp were purified on a sepharose CL-4B column. These cDNA fragments were provided with *Eco*RI adaptors (Promega), digested with *Not* I and ligated into *Eco*RI-*Not*I digested λ-ZAPII arms (Stratagene). The DNA was packed by using a Gigapack Gold II kit (Stratagene), as described by the manufacturer. Starting from 5 µg poly(A)+ RNA, 500 ng of double stranded cDNA, larger than 400 bp, was obtained. From 75 ng of cDNA ligated to λ-ZAPII arms, $8x10^5$ independent recombinant phages were obtained. The library was amplified once and this amplified library was screened with ^{32}P-labelled inserts of pPsENOD5, pPsENOD12 and pPsENOD40. Screening and isolation of the clones was performed according to the Stratagene protocol.

DNA-sequencing

By *in vivo* excision (Stratagene), the inserts of the different early nodulin phage clones were obtained in pBluescript SK(-). Nucleotide sequences were determined by using the dideoxy chain termination method using the T7 Sequencing™ kit (Pharmacia). The nucleotide and deduced amino acid sequences of pPsENOD5, pPsENOD12 and pPsENOD40 will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers X83681, X83682 and X83683, respectively. In

this thesis the nucleotide sequence data are reported in appendices 2, 3 and 4.

Labelling of antisense/sense RNAs

For preparing antisense and sense RNAs 5' fragments of pVsENOD5, pVsENOD12 and pVsENOD40 were subcloned into pKS(+) vector (Stratagene). The 230 bp *Eco*RI-*Stu*I fragment of pVsENOD5 and the 190 bp *Eco*RI-*Rsa*I fragment of pVsENOD12 were subcloned in the *Eco*RI-*Sma*I digested pKS(+) vector. The 430 bp *Eco*RI-*Spe*I fragment of pVsENOD40 was subcloned into analogous digested pKS(+) vector. The plasmids were digested with *Eco*RI before antisense RNA was made with T7 polymerase. Before making sense RNA with T3 polymerase the plasmids containing the ENOD5 and ENOD12 fragments were digested with *Bam*HI, the plasmid containing the ENOD40 fragment was digested with *Spe*I. The probes were radioactively labelled with ^{35}S-UTP (1000-1500 Ci mmol^{-1}, Amersham) and degraded to about 150 nucleotides long fragments before hybridization (Van de Wiel *et al.*, 1990). Hybridization with ^{35}S-labelled sense RNAs did not show any hybridization.

In situ hybridization

For *in situ* hybridization nodules were harvested 20 days after inoculation and fixed in 4% paraformaldehyde supplemented with 0.25% glutaraldehyde in 10 mM sodium phosphate buffer for 3 hours (Van de Wiel *et al.*, 1990). To study the *in situ* expression of early nodulin genes at early stages of nodule development, seedlings were grown for 5 days on 2% B&D agar plates (Broughton and Dilworth, 1971), before *R. leg.* bv. *viciae* strain 248 was spot-inoculated on the roots (Yang *et al.*, 1994). Four and 5 days after spot-inoculation the inoculated part of the roots were harvested and fixed as described. Fixed material was dehydrated and embedded into paraffin by routine methods (Van de Wiel *et al.*, 1990; Yang *et al.*, 1991). Sections (7 mm thick) were hybridized with ^{35}S-UTP labelled antisense or sense RNA probes according to a procedure derived from Cox and Goldberg (1988) (Van de Wiel *et al.*, 1990). Sections exposed for 2 to 4 weeks were stained with toluidine blue and photographed with a Nikon microscope with dark field and epipolarization optics.

To detect starch accumulation the section of a 20 day old nodule hybridized with ENOD40 ^{35}S-UTP labelled antisense RNA was stained with a 0.2% I_2 - 2% KI solution for a few minutes.

CHAPTER 3

A 200 bp region of the pea *ENOD12* promoter is sufficient for nodule specific and Nod factor induced expression

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Submitted.

Abstract

ENOD12 is one of the first expressed nodulin genes, upon inoculation with *Rhizobium*. The *ENOD12* gene family in pea (*Pisum sativum*) has two members, named *PsENOD12A* and *PsENOD12B*. A cDNA clone representing *PsENOD12A* (Scheres *et al.*, 1990a) and a *PsENOD12B* genomic clone (Govers *et al.*, 1991) have been previously described. The isolation and characterization of a *PsENOD12A* genomic clone is presented in this paper. Futhermore it is shown by using a *Vicia hirsuta-Agrobacterium rhizogenes* transformation system that both genes have a similar expression pattern in transgenic *V. hirsuta* root nodules. 5' deletion analyses of chimaeric *PsENOD12-gusA*-int genes in transgenic root nodules of *V. hirsuta* were performed to identify the promoter regions involved in nodule specific gene expression. The results of these promoter analyses showed that the 200 basepairs immediately upstream of the transcription start are sufficient to direct nodule specific gene expression. The Nod factors (substituted oligomers of N-acetyl-D-glucosamine) of *Rhizobium leguminosarum* bv. *viciae* (NodRlv factors) are also able to induce the expression of the chimaeric *PsENOD12-gusA*-int gene in transgenic roots of V. hirsuta and the same 200 bp promoter region is sufficient for the induction by Nod factors.

Introduction

The interaction between rhizobia and leguminous plants results in the formation of new organs, the root nodules, in which the rhizobia are able to fix atmospheric nitrogen into ammonia. The formation of these root nodules involves different developmental steps, like root hair curling, the formation of infection threads and the induction of cell division in the root cortex (Hirsch, 1992). During these processes nodule specific plant genes, called nodulin genes (Van Kammen, 1984) are expressed.

The first event in the interaction between rhizobia and legumes is the activation of the bacterial nodulation (*nod*) genes by plant secreted flavonoids. The Nod proteins are involved in the synthesis of specific lipo-oligosaccharides called Nod factors. The Nod factors are secreted by the bacteria and are able to induce, even in the absence of the bacteria, the first steps of nodule formation (Fisher and Long, 1992; Lerouge *et al.*, 1990; Spaink *et al.*, 1991; Van Brussel *et al.*, 1992). The Nod factors produced by *R. leguminosarum* bv. *viciae* (NodRlv factors) consist of tetra- or pentamers of N-acetyl-D-glucosamine with an unsaturated C18 fatty acid chain and an O-acetyl-group at the non-reducing terminal sugar. The fatty acid chain can have either one or four unsaturated bonds (Spaink *et al.*, 1991). These Nod factors are able to induce in pea the expression of early nodulin genes (Horvath *et al.*, 1993). One of these genes is *ENOD12* (Scheres *et al.*,

1990a).

The *PsENOD12* gene family of pea has two members (Scheres *et al.*, 1990a), *PsENOD12A* and *PsENOD12B*. The pPsENOD12 cDNA clone representing the *PsENOD12A* gene, has been described by Scheres *et al.* (1990a) and the characterization of a *PsENOD12B* genomic clone has been reported by Govers *et al.* (1991). Both *PsENOD12* genes encode a polypeptide that contains a putative N-terminal signal peptide followed by a stretch of repeating proline rich pentapeptide units. The major difference between the two genes is the presence of two small deletions in the region of the *PsENOD12B* gene encoding the proline rich part of the protein. From *Medicago sativa* and *M. truncatula* early nodulin genes homologous to the *PsENOD12* genes have been isolated. The *MsENOD12* genes (Allison *et al.*, 1993) and the *MtENOD12* gene (Pichon *et al.*, 1992) show a homology of 70 to 80% at the amino acid level with their pea counterparts.

In situ hybridization experiments on infected pea (*Pisum sativum*) roots showed that *PsENOD12* expression occurs in root cortical cells containing the infection thread, in root cells preparing for infection thread penetration and in the nodule primordia (Scheres *et al.*, 1990a). In mature pea nodules, which have a persistent meristem at the apex, *PsENOD12* is expressed in a small region immediately adjacent to the apical meristem, the distal part of pre-fixation zone II (Franssen *et al.*, 1992; Scheres *et al.*, 1990a). In this part of zone II infection threads continuously infect new meristematic cells. A similar expression pattern for *MtENOD12* is seen in *in situ* experiments on mature root nodules of *M. truncatula* (Pichon *et al.*, 1992) and *M. sativa* (Bauer *et al.*, 1994). However, *in situ* hybridization experiments on infected *Vicia sativa* root nodules showed *VsENOD12* expression in whole prefixation zone II (Chapter 2).

NodRIv factors are able to induce the expression of *PsENOD12* in root hairs of pea plants within 12 hours after addition of the factors (Horvath *et al.*, 1993). The NodRIv factors are also able to induce cortical cell division in vetch (*Vicia sativa*) roots (Spaink *et al.*, 1991) and *ENOD12* is expressed in these nodule primordia (Vijn *et al.*, 1993). Journet *et al.* (1994) showed that in transgenic *M. varia* plants a chimaeric *MtENOD12-gusA* gene is induced in the root epidermis 2 hours after addition of the *R. meliloti* Nod factor (NodRm factor). Since *ENOD12* expression can be induced by the Nod factors within hours, it is interesting to study the mechanisms controlling ENOD12 expression. We have carried out a promoter deletion analysis of the *PsENOD12* genes in *V. hirsuta* and demonstrate that for *PsENOD12B* the 200 bp immediately upstream of the transcription start are sufficient to direct nodule specific gene expression in transgenic *V. hirsuta* root nodules. The same 200 bp region was also sufficient for NodRIv induced expression in transgenic *V. hirsuta* roots. For the *PsENOD12A* gene, isolated in this study, the 100 bp immediately upstream of the transcription start were able to direct nodule specific expression. Interestingly, a

comparison of the pea and *Medicago* ENOD12 promoter sequences showed a strong homology in these first 100 bp, while more upstream the sequences showed less homology. This observation, together with the results of our promoter deletion analysis, strongly suggests that this particular region is of general importance for *ENOD12* gene expression triggered by the presence of Nod factors.

Results

Isolation and characterization of the PsENOD12A genomic clone

Screening of an EMBL3 library of genomic pea DNA (variety Feltham First) (Govers *et al.*, 1991) with the cDNA clone pPsENOD12 resulted in one positive clone. Further analysis showed that the hybridizing sequence was located on a 2.8 Kb *Eco* RI-*Sal* I fragment. This fragment was sequenced and contained one open reading frame (ORF) coding for a polypeptide of 110 amino acids, which is 100% identical to the ENOD12 protein encoded by the previously described pPsENOD12 (Scheres *et al.*, 1990a), and is now defined *PsENOD12A* (Appendix 1, genbank accession number X81366). The coding sequence is flanked by 2187 bp upstream of the transcription initiation site and 235 bp downstream of the stop codon. Two basepair substitutions in the 3' untranslated region might be due to cultivar specificity, since the cDNA clone was isolated from the variety Sparkle and the genomic clone is isolated from the variety Feltham First.

Rhizobium induced expression of PsENOD12A and PsENOD12B promoter - gusA-int gene fusions in transgenic V. hirsuta root nodules

The expression pattern and expression level of *PsENOD12A* and *PsENOD12B* are very similar as was previously shown by Northern analysis (Govers *et al.*, 1991), indicating that the two genes are similarly regulated. To further analyse the expression pattern of both genes a 2.2 Kb fragment of the *PsENOD12A* promoter and a 2.0 Kb fragment of the *PsENOD12B* promoter were amplified by PCR and fused to a *ß-glucuronidase*-intron (*gusA*-int) gene to generate transcriptional fusions (See Materials & Methods). A translational fusion of *PsENOD12B* was made as well. These constructs were introduced into *V. hirsuta* using an *A. rhizogenes* transformation system (Quandt *et al.*, 1993), resulting in a non transformed shoot and a varying number of transformed hairy roots. The hairy roots were inoculated with *R. leguminosarum* bv. *viciae* strain VH5e and pink nodules were formed nine days after inoculation. GUS activity was histochemically detected in nodules

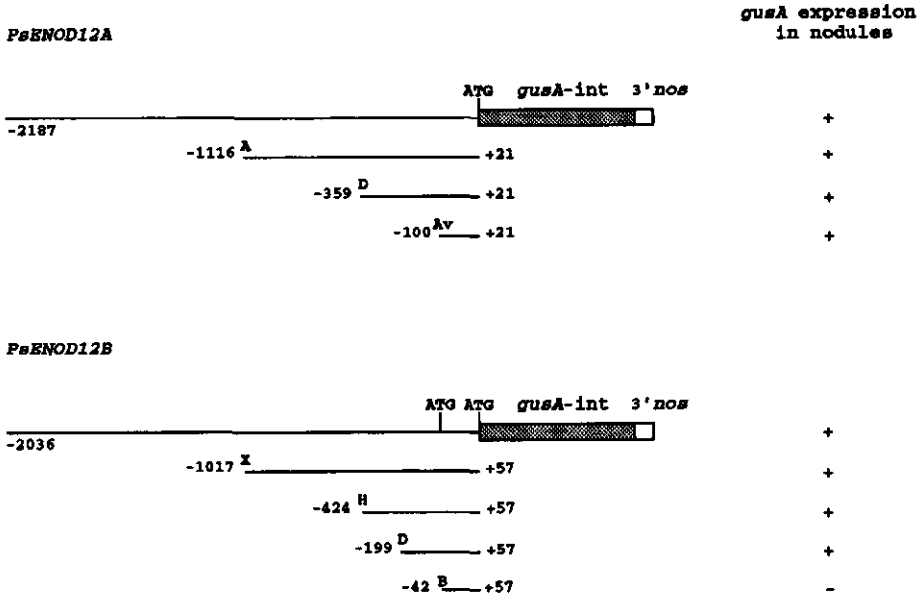


Figure 3.1: 5' deletion analysis of the *PsENOD12A* and *PsENOD12B* promoter. Of *PsENOD12B* only translational fusions were used. The restriction sites used for constructing the deletions are indicated by an abbreviation. A, *Acc* I: Av, *Ava* II; B, *Bam* HI; D, *Dra* I; H, *Hind* III; X, *Xba* I. Behind the constructs is shown if the construct was able to induce *PsENOD12-gusA*-int expression in transgenic nodules.

one to two weeks after inoculation with *Rhizobium*.

Roots transformed with either *PsENOD12A*-, *PsENOD12B*- or *PsENOD12B* (translational)-*gusA*-int fusions formed transgenic nodules in which GUS activity occurred only in the distal part of the nodules. A typical example of *gusA*-int expression in these transgenic nodules is shown in figure 3.2A. With all three fusion constructs GUS activity was clearly detectable in about 30% of the tested nodules. Control experiments using the CaMV 35S constitutive promoter fused to *gusA*-int showed GUS activity in 70-80% of the roots emerging from the wound site.

5' Deletion-analysis of the PsENOD12 promoters

Despite the similar expression pattern of *PsENOD12A* and *PsENOD12B*, the two promoters show significant differences in the DNA sequences. To delineate the regulatory elements conferring the highly specific expression of the two genes, a series of 5' deletion analyses were performed with both *PsENOD12A*- and *PsENOD12B-gusA*-int (Figure 3.1). For *PsENOD12B* only the translational fusion was used. These deletion constructs were

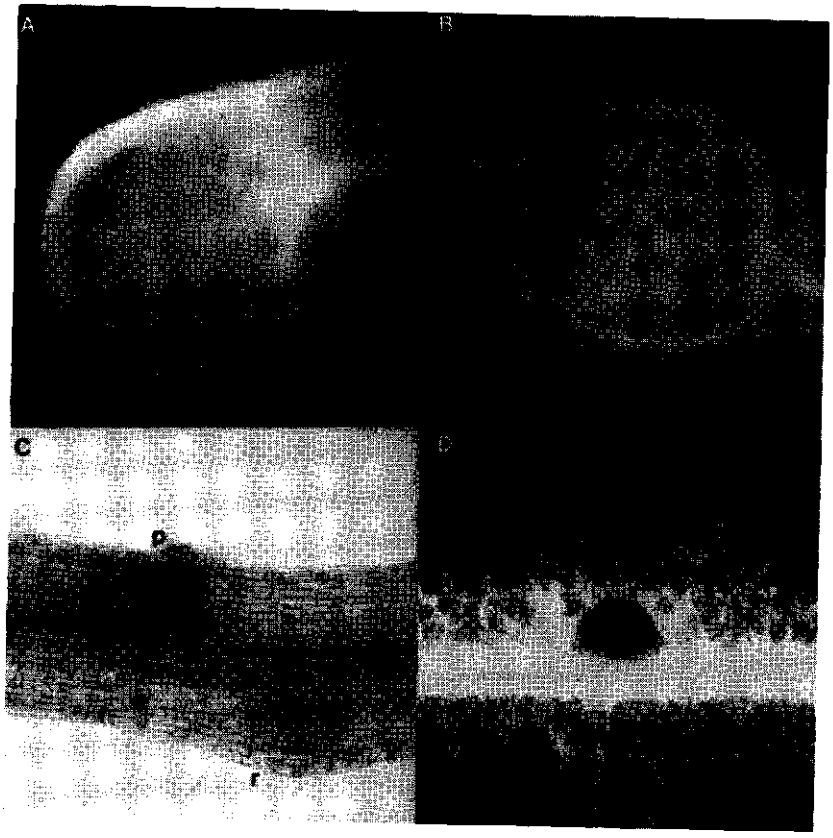


Figure 3.2: Histochemical localization of *PsENOD12-gusA*-int activity in transgenic *Vicia hirsuta* nodules.

A, B: Cross sections of two week old transgenic root nodules, harbouring the -2036 (A) or the -199 (B) *PsENOD12-gusA*-int chimaeric gene. The GUS assay was performed for 1 hr. The blue precipitate indicates GUS activity and is detected in the distal part of the nodule.

C, D: Transgenic *Vicia hirsuta* roots 4 (C) and 7 (D) days after NodRlv-V(Ac, C18:4) inoculation. The GUS assay was performed o/n. GUS activity is observed in the nodule primordia (p) (C, D), but not in the lateral root (r) (C).

transferred to *V. hirsuta*. Transgenic roots were inoculated with *R. leguminosarum* bv. *viciae* strain VH5e and 2 weeks after inoculation transgenic nodules were analysed histochemically for GUS activity. The -2187, -1116 and -359 constructs of *PsENOD12A* and the -2036, -1017, -424 and -199 deletion constructs of *PsENOD12B* had a similar nodule specific gene expression, as the GUS activity only occurred in the distal part of the transgenic nodules. The *gusA* expression of the -2036 and the -199 deletion constructs of *PsENOD12B-gusA*-int are shown in figure 3.2A and B respectively. In all cases *gusA* expression was detectable in about 30% of the transgenic nodules. The -100 deletion construct of *PsENOD12A* also showed nodule specific expression, however, *gusA* expression was only found in about 10% of the nodules tested. The -42 deletion construct of *PsENOD12B* was not active in transgenic nodules. These findings suggest the presence of a specific promoter regulatory element located between -199 and -42 in the PsENOD12B promoter and downstream of -100 in the PsENOD12A promoter.

Induction of PsENOD12A and PsENOD12B promoter - gusA-int gene fusions in nodule primordia induced by Nod factors

NodRlv factors containing a highly unsaturated acyl group (NodRlv-V(Ac, C18:4)) have the ability to induce cortical cell divisions in *V. sativa* roots and a special bioassay has been developed to study this mitosis inducing activity of the Nod factors (Spaink *et al.*, 1991). We applied this assay on transgenic *V. hirsuta* hairy roots, containing the chimaeric *PsENOD12B-gusA*-int gene. Addition of NodRlv-V(Ac, C18:4) at a final concentration of 10^{-7} M to the medium resulted in nodule primordia formation in only 1 or 2 hairy roots out of 10. On average 3 to 4 primordia per hairy root were observed, in contrast to a range of 4 to 14 in untransformed *V. sativa* roots. A typical example of a nodule primordium induced by NodRlv-V(Ac, C18:4) after 4 days is seen in figure 2C. At one side of the vascular bundle a nodule primordium (p) is formed, while on the other side a lateral root primordium is present (lr). GUS activity was determined 4 and 7 days after Nod factor addition and *ENOD12-gusA*-int expression was found in the nodule primordia induced by NodRlv-V(Ac, C18:4) (Figure 3.2C and D), but not in the lateral root primordium (Figure 3.2C). Transformed roots that were not treated with Nod factors did not show any GUS activity.

Since the -199 deletion construct of *PsENOD12B* is expressed in a nodule specific manner we also tested whether the expression of this construct could be induced by NodRlv factors. Transgenic *V. hirsuta* roots were treated with 10^{-7} M NodRlv(Ac, C18:4) as described above and GUS activity was determined 4 days later when nodule primordia were formed. *GusA*-int expression was detectable in the primordia (data not shown), indicating that the 199 bp upstream of the transcription start are sufficient for nodule specific

as well as for Nod factor induced *ENOD12* expression.

Discussion

By using a *V. hirsuta-A. rhizogenes* transformation system it was shown that both *PsENOD12A* and *PsENOD12B* are expressed in transgenic *V. hirsuta* root nodules and that their expression patterns are similar. This in contrast to the expression patterns found for *MsENOD12A* and *MsENOD12B* in *Medicago sativa*. In *M. sativa MsENOD12B* is expressed in roots and nodules, whereas *MsENOD12A* is only expressed in nodules (Bauer *et al.*, 1994). *In situ* hybridization experiments showed that in *V. sativa* root nodules *VsENOD12* was expressed in the whole pre-fixation zone II (Chapter 2). The chimaeric *PsENOD12-gusA*-int genes were expressed in a relatively broad region at the distal part of transgenic *V. hirsuta* root nodules. This region probably coincides with pre-fixation zone II. In pea nodules *PsENOD12* is only expressed in the distal part of pre-fixation zone II (Scheres *et al.*, 1990a). Since the pea *ENOD12* promoter was introduced into the transgenic *V. hirsuta* nodules these results indicate that also the host plant is involved in determining the exact expression pattern.

The transformation of *V. hirsuta* with *A. rhizogenes* containing a 35S-*gusA*-int chimaeric gene showed that 70-80% of the newly formed roots were transformed. These results are consistent with transformation experiments performed by Quandt *et al.* (1993). When we transformed *Vicia* with chimaeric *PsENOD12-gusA*-int constructs only 30% of the tested nodules showed *gusA* expression. Since the transformation with either the 35S-*gusA*-int or the *PsENOD12-gusA*-int constructs were performed in parallel and the chimaeric genes were both cloned in pBIN19 vectors and transformed into the same *A. rhizogenes* strain, we assume that also in the case of the *PsENOD12-gusA*-int transformation 70-80% of the roots will be transgenic. That we, however, only in 30% of the nodules found detectable GUS activity might indicate that the PsENOD12 promoters are weaker than the 35S promoter. Another explanation for this low percentage of GUS activity might be that more upstream of the 2 Kb of the promoter, used for construction of the chimaeric *PsENOD12-gusA*-int genes, or more downstream of the *PsENOD12* gene, in the 3' region, enhancer sequences are located.

The deletion analyses of the PsENOD12 promoters showed that the +1,-199 bp region of *PsENOD12B* and the +1, -100 bp region of *PsENOD12A* are sufficient for *Rhizobium* induced expression. This 200 bp region is 89% conserved between *ENOD12A* and *ENOD12B* (Figure 3.3) and therefore it is likely that the expression of both genes is induced by the same trans-acting factors. Comparison with the *ENOD12* promoter sequences of the MsENOD12 and MtENOD12 promoters shows a strong homology just

```
MsENOD12A                      T* TTTAAA*A** ***CC----A C*CC*ATATG -175
MtENOD12                         TTTAAA*A** ***CC*CG*A C*CC*ATACG -173
PsENOD12A    CC*CTC*C A**TA*GCA* **TTCA*ATC ****T*GT** ***A****** -156
PsENOD12B    TTTAAAATAA TATAGAATTT AACCACTCAT ATTTAATATT TATGAGATGT -153
                 DraI
MsENOD12A    TTCTT**A*A A**TTCCACA C*CA*T*T*G AT*TT*CGT* CAGTGAT*A* -125
MtENOD12     TTCTT****A A**TTCGACA C*-A*T*T*G A*ATT*C*T* GAGTGAT*A* -124
PsENOD12A    ********** *****TTG** G********* ******T*GT **TA**T*AT -106
PsENOD12B    AAGAATGTTG CTACA---TT CAATAATATT TCTCAAAAAA TT--TTATTA -108

MsENOD12A    ***T*TT*TT TT**T*A*CT *-TA*****C *CCGA*TAAA *C***A**** - 76
MtENOD12     ***T*A*TTT AT*G**CT*T *GTA****** *CCTA*TAAA *C*G*C**** - 74
PsENOD12A    *A******** ********** *****-**** *--*A*TA** ********** - 59
PsENOD12B    TTAGTGGACC GATTATTGAG ATATATAATA A--ATTAGTT TTGATTGAGA - 60
                 AvaII
MsENOD12A    *A**G***** *C*******C CCTAAT*A*G ********** ***G****C* - 26
MtENOD12     *A**G***** *C*******C C*---**A** **C******* ***G****** - 27
PsENOD12A    ******-*** *C**C***C* C*---T***** ********** ****G***** - 13
PsENOD12B    CCTTCCACTT CTATTGAGGA TC---CTTAC TAGTATAAAA CCTTATTATT - 13
                            BamHI
MsENOD12A    ***T-A**CC CTATATGTTA TATGC*TT** **TT******T *C*****C** + 24
MtENOD12     ***T*A**CC CTATATGTTA TATGC**T** **TT******T *C******** + 24
PsENOD12A    ********-- ---------- --**T***** ********** *C******** + 24
PsENOD12B    CCCCATAT-- ---------- --GTGACAAA CACAAAAATC ATTACTTAAA + 24

MsENOD12A    *** + 27
MtENOD12     *** + 27
PsENOD12A    *** + 27
PsENOD12B    ATG + 27
```

Figure 3.3: Comparison of the 200 bp immediately upstream of the start codon of the promoters of *PsENOD12A* and *PsENOD12B* (Govers *et al.*, 1991), *MtENOD12* (Pichon *et al.*, 1993) and *MsENOD12A* (Allison *et al.*, 1993). The TATAA box and the restriction sites in the *PsENOD12B* promoter used for some of the deletion constructs are underlined. Identical nucleotides are replaced by an *, deletions by an -. Nucleotides of *PsENOD12A* and *PsENOD12B* are numbered relative to the putative transcription start (+1) as determined by Scheres *et al.* (1990a), nucleotides of *MsENOD12A* and *MtENOD12* are numbered relative to the A residue (+1) of the initiator ATG codon.

upstream of the start codon, around the TATAA box and just upstream of the TATAA box, while more upstream the homology declines. The conserved sequences AAAGAT and CTCTT found in the promoters of several late nodulin genes (Sandal *et al.*, 1987) are not present in the ENOD12 promoters. Thus apparently the regulatory mechanism of early nodulin genes is different from the mechanism controlling the expression of genes expressed later in nodule development.

All the deletion constructs of the *PsENOD12* promoters, except the -100 *PsENOD12A* deletion construct, showed histochemically detectable GUS activity in about 30% of the tested nodules. Thus the region of the *PsENOD12B* promoter between -199 and -2036 is not essential for induction of nodule specific *PsENOD12B* expression.

By using transgenic *V. hirsuta* roots, *PsENOD12-gusA*-int expression could be

detected in nodule primordia induced by NodRlv-V(Ac, C18:4) within 4 days after Nod factor addition. We were not able to detect GUS activity in the epidermis within a few hours after Nod factor addition as was observed in transgenic *M. varia* plants containing a chimaeric *MtENOD12-gusA* gene (Journet *et al.*, 1994). Nevertheless we expect that *ENOD12* is induced in the epidermis since we could detect, with PCR-based experiments, the expression of *PsENOD12* in root hairs of pea seedlings (Horvath *et al.*, 1993). Probably the expression of the chimaeric *PsENOD12-gusA*-int gene in the epidermis of vetch is to low to detect *gusA* expression histochemically. NodRlv induced nodule primordium formation in *V. hirsuta* hairy roots was less efficient than in the originally used wild type *V. sativa* roots. Only in a few hairy roots primordia were induced and also the number of primordia formed per root was lower. This might be due to the fact that the transgenic hairy roots are morphologically heterogeneous.

Since *ENOD12* expression can be induced within hours by Nod factors (Horvath *et al.*, 1993; Pichon *et al.*, 1993) a signal transduction pathway starting with Nod factor perception might control *ENOD12* expression. A final step in this pathway will be the activation or the synthesis of transcription factors controlling *ENOD12* expression. Most likely these factors interact with the promoter region that confers nodule specificity to *PsENOD12*.

Materials and Methods

Isolation and characterization of a genomic clone containing PsENOD12A

Screening of the genomic EMBL3 library (*P. sativum* variety Feltham First) with the cDNA clone pPsENOD12 as a probe provided a clone containing *PsENOD12A*. The EMBL3 library was a generous gift of Dr. A Shirsat and Dr. D. Boulter form Durham University, United Kingdom. To determine the nucleotide sequence, DNA fragments were subcloned in Bluescript vectors (Stratagene) and nested deletions were prepared by using Exonuclease III (Sambrook *et al.*, 1989). The dideoxy chain termination reaction (Sanger *et al.*, 1977) was carried out using single stranded templates. To obtain the sequence from both strands overlapping clones were used and in addition sequencing was performed with synthetic oligonucleotides.

Plant growth conditions

V. hirsuta seeds were obtained from John Chambers Ltd., London. Seeds were sterilized and germinated according to Quandt *et al.* (1993) with some modifications. Seeds were surface sterilized with 95-97% sulphuric acid for 30 min. After rinsing in tap water the seeds were sterilized for 10 min in 2% NaOCl and soaked o/n in 0.02% NaOCl in the dark, at room temperature. Then the seeds were washed 5x in sterile water and placed on 1% water-agar plates. The seeds were kept for 4 days at 4 °C before germination in the dark at 26 °C. Germinated seedlings were transferred to petri-dishes containing 2% B&D-agar (Broughton and Dilworth, 1971) and the seedlings were grown at 22 °C with a day/night cycle of 16/8 h.

Construction of PsENOD12 promoter-gusA-int fusions.

2.2 Kb of the *PsENOD12A* and 2.0 Kb of the *PsENOD12B* promoter were amplified by using the polymerase chain reaction (PCR) by which *Sal*I and *Sma*I restriction sites were added at either end of the fragment. The primers used in the PCR were:

PsENOD12A forward: 5'-GCG GCC GTC GAC GGG ATT AGA ATC TAG GTG-3' (-2176 - -2159)

reverse: 5'-GCG CGC CCC GGG AAG TAA/G TGA TTT TTG TG-3' (+21 - +5)

PsENOD12B forward: 5'-GCG GCC GTC GAC GTC AAC ATC ATG CC-3' (-2036 - -2023)

reverse: 5'-GCG CGC CCC GGG AAG TAA/G TGA TTT TTG TG-3' (+21 - +5)

In the forward primers the *Sal*I sites and in the reverse primers the *Sma*I sites are underlined. Thirty cycles of amplification were performed (92 °C, 2 min; 45 °C, 1.5 min; 72 °C, 5 min) under standard PCR conditions (Sambrook *et al.*, 1989). After digestion with *Sal*I and *Sma*I the PCR products were cloned as *Sal*I- *Sma*I fragments in the polylinker of PIV20 (Stougaard *et al.*, 1987). For the PsENOD12B promoter a translational fusion was made as well. The reverse PCR primer used for this construct is: 5'-CAG GCG CCC GGG CAA CAC TAG TGA GGA C-3'(+57 - +42). Before cloning this fragment in PIV20 the *Sal*I-*Sma*I fragment was cloned in frame with *ß-glucuronidase* (*gusA*) in pBI101.3 (Jefferson *et al.*, 1987). From this construct the *PsENOD12-gusA* fusion was cloned into the PIV20 vector (Ramlov *et al.*, 1993) as a *Sal*I-*Eco*RI fragment. The second intron (IV2) of the potato ST-LS1 gene (Eckes *et al.*, 1986) was introduced as a *Sna*BI-*Sfu*I fragment, derived from a 35S-*gusA*-int construct made by (Vancanneyt *et al.*, 1990), in *gusA* in PIV20. The *PsENOD12-gusA*-int chimaeric genes were cloned into pBIN19 (Bevan, 1984) as *Sal*I-*Eco*RI fragments.

The deletion constructs of the *PsENOD12A* and *PsENOD12B* promoters were made by using appropriate restriction sites (Figure 3.1). For *PsENOD12B* only the translational

fusion was used.

The DNA-constructs were introduced into *Agrobacterium rhizogenes* (ARqua1) (Quandt *et al.*, 1993) by electroporation (Mattanovich *et al.*, 1989).

Plant transformation

Hypocotyls of seedlings that had been grown for 3 days at 22 °C were wounded with a 26 G needle containing *A. rhizogenes* (ARqua1) harbouring a chimaeric gene construct. The wounded plants were grown for 24 h in the dark at 22 °C. Then the seedlings were grown with a day/night cycle of 16/8 h at 22 °C. After 2 weeks the main root was cut off from the plants containing hairy roots and the plants with the hairy roots were transferred to fresh B&D agar plates. Inoculation with *R. leguminosarum* bv. *viciae* strain VH5e of transformed roots was performed as described by Quandt *et al.* (1993). All deletion constructs were at least three times independently transformed to *V. hirsuta* and each time a minimum of 50 nodules was used for histochemical GUS analysis.

Transformation efficiency was checked by using *A. rhizogenes* ARqua2, which contained a 35S-*gusA*-int gene (Quandt *et al.*, 1993), as described in the Results.

Histochemical detection of GUS activity

Root nodules were hand-sectioned and incubated for 30 min to 16 h in 1 mM 5-bromo-4-chloro-3-indolyl-glucuronide (X-gluc) (Clonetech, USA), 0.1 mM potassium ferricyanide, 0.1 mM potassium ferrocyanide in 0.1 M sodium phosphate buffer pH 7.0 (Jefferson *et al.*, 1987) at 37 °C. The reaction was stopped by transferring the stained nodules to 70% ethanol. Photographs were taken with a Nikon Optiphot-2 microscope equipped with a Nikon FX-35DX camera (Fig. 3.2A, B and C) or a Nikon Diaphot microscope equipped with a Nikon FM-2 camera (Fig. 3.2D).

Induction of nodule primordia on transgenic V. hirsuta roots by NodRlv factors.

Composite plants containing hairy roots were transferred to amber glass vials (Spaink *et al.*, 1991) containing B&D liquid medium (Broughton and Dilworth, 1971). At the same time purified NodRlv-V(C18:4) (Spaink *et al.*, 1991) was added to the plant medium at a final concentration of 10^{-7} M. Four and seven days after Nod factor addition *PsENOD12-gusA*-int expression was assayed histochemically.

CHAPTER 4

Isolation of cDNA clones encoding proteins binding to the *PsENOD12* promoter

The work presented in this chapter was performed in a close collaboration with Henning Christiansen and Niels Pallisgård from the University of Aarhus, Denmark.

Introduction

ENOD12 is one of the early nodulin genes that can be induced by rhizobia secreted lipo-oligosaccharides (Nod factors). In pea (*Pisum sativum*) root hairs, *PsENOD12* expression is found 12 h after treatment with *Rhizobium leguminosarum* bv. *viciae* Nod factors (Horvath *et al.*, 1993), while in transgenic *Medicago varia* plants, carrying a chimaeric *MtENOD12-gusA* gene, *gusA* expression could already be detected in the epidermis 2 hours after addition of the *R. meliloti* Nod factor (Pichon *et al.*, 1993). Therefore it is of interest to study the mechanisms controlling *ENOD12* expression, since this may help in elucidating the pathway from Nod factor to the induction of *ENOD12* gene expression.

In general gene expression is controlled by the interaction of several DNA-binding proteins (*trans*-acting factors) with specific sequences (*cis*-acting elements) of the promoter of a gene. *Cis*-acting elements in plants have been identified by promoter deletion studies. By such studies *cis*-acting elements have been identified that; 1. repress gene expression, the so-called negative elements (NE), 2. stimulate gene expression, the positive elements (PE) and 3. are necessary for organ specific gene expression, the organ specific elements (OSE). These kinds of *cis*-acting elements have also been identified in the promoters of various nodulin genes, like the leghemoglobin genes of soybean (*lbc3*) (Ramlov *et al.*, 1993; She *et al.*, 1993a; She *et al.*, 1993b; Stougaard *et al.*, 1987) and *Sesbania rostrata* (*Srglb*) (de Bruijn *et al.*, 1989; Metz *et al.*, 1988; Szabados *et al.*, 1990), the soybean *N23* gene (Jørgensen *et al.*, 1988; Sandal *et al.*, 1987), a nodule enhanced *Phaseolus* glutamine synthetase gene (*gln-γ*) (Forde *et al.*, 1989) and the early nodulin *ENOD2* (Lauridsen *et al.*, 1993). The *ENOD12* promoter has not been studied in detail, but it was shown that the 200 bp immediately upstream of the transcription start are sufficient for nodule specific and Nod factor induced expression, and must therefore contain the OSE (Chapter 3). Positive or negative elements have not been identified so far.

Well-characterized *cis*-elements have successfully been used to identify DNA-binding proteins by gel mobility studies and DNase I footprinting assays (Katagiri and Chua, 1992). These approaches have also been used to identify DNA binding proteins interacting with the promoters of late nodulin genes (de Bruijn *et al.*, 1989; Forde *et al.*, 1990; Jacobsen *et al.*, 1990; Jensen *et al.*, 1988; Metz *et al.*, 1988). Two of these binding proteins, NAT1 and LAT1 have been characterized in more detail and were shown to be high-mobility group (HMG) proteins (Jacobsen *et al.*, 1990). HMG proteins have been isolated from many plant and animal species and are non-histone chromosomal proteins (see for example Mayes, 1982). By affecting the conformation of chromatin they might be involved in DNA replication and/or RNA transcription. Although proteins have been identified that form a complex with nodulin promoter regions, it is surprising that none of the binding proteins identified so far interacts with OSE of late nodulin promoters.

Cloning of a gene encoding a transcription factor can be performed by purification of the DNA-binding protein, followed by partial amino acid sequencing and screening of a cDNA library. This procedure is time consuming, and in some cases even impossible, for example when the transcription factor is present at a very low concentration, or occurs only in tissues that are hard to obtain in large quantities. An alternative approach to clone transcription factors involves the use of synthetic oligonucleotides, corresponding to specific *cis*-elements, in screening an expression library for clones producing DNA-binding proteins. When the cis-elements are less well defined, the expression library can be screened with several overlapping double stranded oligonucleotides covering a stretch of DNA up to 200 or 300 bp (see for example Nielsen *et al.*, 1992). The major advantage of the latter method is that it does not require a very detailed promoter analysis to identify the cis-elements of interest. Therefore we have used this method in an effort to clone genes encoding DNA-binding proteins that bind to the *PsENOD12* promoter. The promoter analysis has been described in Chapter 3 and showed that the 200 bp immediately upstream of the transcription start were sufficient for nodule specific expression. Therefore we screened the library with double stranded overlapping oligonucleotides covering these 200 basepairs.

In this chapter we describe the results of this screening and present a preliminary characterization of the isolated cDNA clones. This includes a sequence comparison with sequences in the NCBI database and *in situ* hybridization experiments showing the expression pattern of the cloned genes in young root nodules.

Results and Discussion

Isolation of cDNA clones encoding proteins that bind to the 200 bp fragment of the PsENOD12 promoter that is essential for nodule specific expression

To identify cDNAs encoding proteins that bind to the 200 bp fragment of the *PsENOD12* promoter, we screened a λgt11 cDNA expression library of poly(A)+ RNA from young *Vicia sativa* root nodules. Nine double-stranded overlapping oligonucleotides of about 50 nucleotides were designed to cover the complete 200 bp region, located immediately upstream of the transcription start of *PsENOD12* (Figure 4.1 and table 4.1). These oligonucleotides were individually concatenated and labelled with [a-^{32}P]-dATP. The λgt11 cDNA expression library used for this screening was not amplified and contained partial clones, since the first strand cDNA synthesis was performed by using random dN$_7$-*Not*I primers. This was deliberately done to avoid loss of certain clones during amplification and to prevent the presence of long 3' nontranslated regions in the inserts.

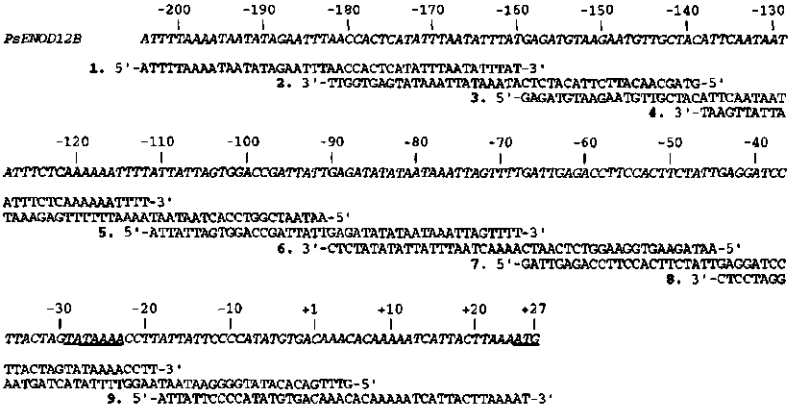


Figure 4.1: The sequence of the *PsENOD12* promoter region and the position of the nine oligonucleotides used to screen the cDNA expression library. Numbering of the nucleotides starts at +1 at the putative transcription start site as determined by Scheres *et al.* (1990a). The start codon and the TATA box are underlined.

Nr.	Sequence[a]	Position[b]
1.	5'-ATTTTAAAATAATATAGAATTTAACCACTCATATTTAATATTTAT-3' 3'-TATCTTAAATTGGTGAGTATAAATTATAAATAtaaaattttatta-5'	-204 - -160
2.	5'-aagaatgttgctacAACCACTCATATTTAATATTTATGAGATGT-3' 3'-TTGGTGAGTATAAATTATAAATACTCTACATTCTTACAACGATG-5'	-182 - -139
3.	5'-GAGATGTAAGAATGTTGCTACATTCAATAATATTTCTCAAAAAATTTT-3' 3'-TTACAACGATGTAAGTTATTATAAAGAGTTTTTTAAAActctacattc-5'	-159 - -112
4.	5'-ggaccgattattATTCAATAATATTTCTCAAAAAATTTTATTATTAGT-3' 3'-TAAGTTATTATAAAGAGTTTTTTAAAATAATAATCACCTGGCTAATAA-5'	-138 - -91
5.	5'-ATTATTAGTGGACCGATTATTGAGATATATAATAAATTAGTTTT-3' 3'-CTAATAACTCTATATATTATTTAATCAAAAtaataatcacctgg-5'	-111 - -68
6.	5'-ttccacttctattGAGATATATAATAAATTAGTTTTGATTGAGACC-3' 3'-CTCTATATATTATTTAATCAAAACTAACTCTGGAAGGTGAAGATAA-5'	- 90 - -45
7.	5'-GATTGAGACCTTCCACTTCTATTGAGGATCCTTACTAGTATAAAACCTT-3' 3'-TGAAGATAACTCCTAGGAATGATCATATTTTGGAActaactctggaagg-5'	-67 - -19
8.	5'-atatgtgacaaacGAGGATCCTTACTAGTATAAAACCTTATTATTCCCC-3' 3'-CTCCTAGGAATGATCATATTTTGGAATAATAAGGGGTATACACAGTTTG-5'	-44 - +5
9.	5'-ATTATTCCCCATATGTGACAAACACAAAAATCATTACTTAAAAT-3' 3'-CACTGTTTGTGTTTTTAGTAATGAATTTTAtaataaggggtata-5'	-18 - +26

Table 4.1. Sequence of the 9 double stranded oligonucleotides used to screen the cDNA expression library. **a.** The sequence present in the *PsENOD12* promoter is printed in capital letters, the extra sequence added to the oligonucleotides to create sticky ends for ligation is printed in small letters. **b.** The position relative to the transcription start of *PsENOD12* (see figure 4.1) is given.

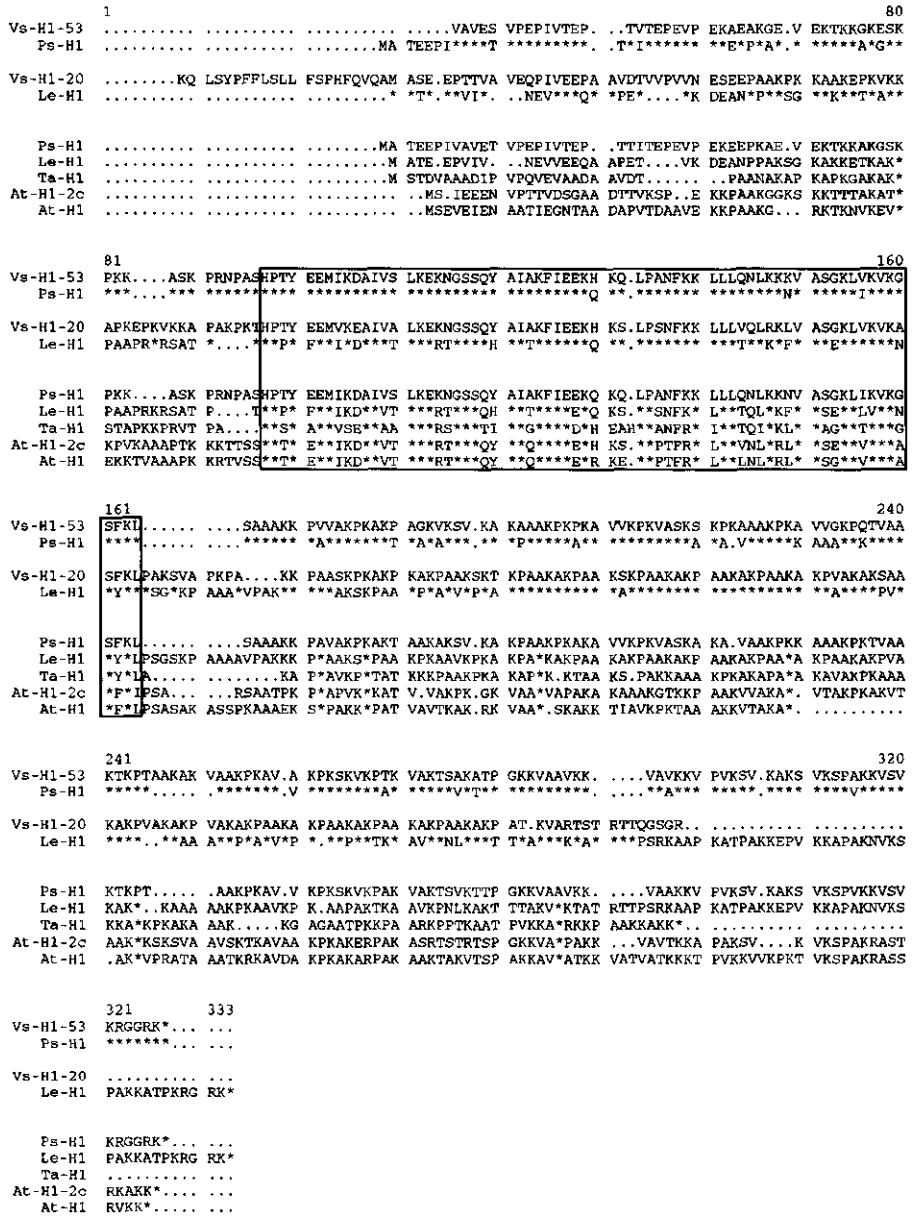
After plating of the library two replica nitrocellulose filters were prepared. To induce expression of the fusion protein, the nitrocellulose filters were submerged in 10 mM IPTG before applying onto the plates. A mixture of the nine labelled oligonucleotides was used for the screening. For the screening we used the method described by Vinson *et al.* (1988). Screening ±700.000 recombinant phages yielded 5 clones, called λVs14, λVs20, λVs28, λVs34 and λVs53. The cDNA inserts of the clones λVs14 and λVs28 cross-hybridized. Since the insert of λVs14 (2.8 Kb) was longer than the insert of λVs28 (1.7 Kb), we initially only studied λVs14. The cDNA inserts of the phages were subcloned in pGEMEX-2 or pBluescript and sequenced. The inserts of pVs20, pVs34 and pVs53 were ± 900 bp long and were sequenced completely, whereas of pVs14 only 1 Kb of the 5' end was sequenced. The reading frame used for translation in the λgt11 clones was taken for deduction of the amino acid sequence.

Characterization of pVs20 and pVs53

The cDNA insert of pVs20 is 851 nucleotides long. This insert encodes a polypeptide of 283 amino acids. The open reading frame encoding this polypeptide extends along the whole cDNA insert and does not contain a stop-codon. The encoded polypeptide is rich in lysines (23%), alanines (22%) and prolines (12%) and its sequence shows 64% identity to the histone H1 gene from tomato (Jayawardene and Riggs, 1994) and 56% identity to the pea histone H1 gene (Gantt and Key, 1987). This comparison also showed that the cDNA insert encodes 21 aa upstream of the first encoded methionine. These 21 aa do not show homology to histone H1 polypeptides (Figure 4.2A), suggesting that the nucleotide sequence encoding these 21 aa is normally not translated and is part of the 5' leader sequence and that the first methionine is the first aa of the encoded polypeptide.

pVs53 contains a cDNA insert of 917 nucleotides and contains a poly(A) tail at the 3' end. This is remarkable since for construction of the expression library a random dN7-*Not*I primer was used for first strand cDNA synthesis to avoid long 3' nontranslated regions in the inserts. The encoded polypeptide that is in frame with the *β-galactosidase* is 231 amino acids long. The stopcodon is located 204 nucleotides upstream from the start of the poly(A) stretch. The encoded polypeptide is lysine (29%), alanine (15%) and proline (9%) rich and its sequence is 88% identical to pea histone H1 and 55% identical to tomato histone H1. The Vs53 polypeptide showed 61% identity to the polypeptide of Vs20 (Figure 4.2A).

The lysine-rich histone H1 genes are considered to have three structural domains, a central folded globular region of about 80 residues in length, which is flanked on both sides by random-coil sequences rich in lysine, alanine and proline (Hartman *et al.*, 1977). Comparison of different plant histone H1 polypeptides showed that the central globular


A
B
A
B
A
B
A
B

region is highly conserved (60-75% identity) in the different genes and that the flanking sequences have less identical amino acids (47-62%) (Figure 4.2B). If the polypeptides encoded by pVs20 and pVs53 are divided in these three regions, the central globular region is more conserved than the N- and C-terminal ends (Figure 4.2A). For example, the central globular regions of the Vs20 polypeptide and tomato histone H1 show 75% identity, whereas the N-terminal regions are only 45% identical and the C-terminal region 67%. Since the C-terminal sequence of the histone H1 polypeptides is very rich in alanines and lysines, the sequence similarity in this region might be more a reflection of the amino acid composition rather than a sequence conservation.

With *in situ* hybridization experiments the expression pattern of *Vs20* and *Vs53* was studied in 5 day old *Vicia sativa* root nodules (Figure 4.5A, D, G). Both genes are mainly expressed in the meristem, but not in all cells of the meristem. Furthermore these genes are active in a few cells of the central tissue (Figure 4.5D and G, arrowheads). This expression pattern is similar to that found for histone H4 in pea nodules (Yang *et al.*, 1994). Expression of histone H4 is cell cycle dependent and only occurs in the S-phase (Yang *et al.*, 1994). Since only part of the meristematic cells are in the S-phase of the mitotic cell cycle, only in about 20% of the nodule meristem cells histone H4 mRNA is found. The expression pattern of *Vs20* and *Vs53* indicates that also the expression of these genes is cell cycle dependent, like that of the histone H1 genes. In the central tissue endoreplication takes place (Truchet, 1978). Hence the expression of *Vs20* and *Vs53* in cells of the central tissue might mark the occurrence of endoreplication.

Figure 4.2: A. Comparison of the amino acid sequence of the pVs53 encoded polypeptide (Vs-H1-53) with the amino acid sequence of histone H1 from pea (Ps-H1) and the pVs20 encoded polypeptide (Vs-H1-20) with histone H1 from tomato (Le-H1). Identical amino acids are replaced by a *; dots represent deletions.

B. Amino acid sequence comparison of histone H1 genes isolated from different plant species. In both A and B the conserved central globular domain is boxed. Conserved amino acids are replaced by a *. Dots represent deletions. Le-H1, *Lycopersicon esculentum* histone H1 (Jayawardene and Riggs, 1994) (EMBL accession number U03391); Ta-H1, *Triticum aestivum* histone H1 (Yang *et al.*, 1991a) (EMBL accession number X59872); Ps-H1, *Pisum sativum* histone H1 (Gantt and Key, 1987) (EMBL accession number X05636). At-H1 en At-H1-2c, *Arabidopsis thaliana* histone H1's (Gantt and Lenvik, 1991) (EMBL accession number At-H1: X62458, At-H1-2c: X62459).

This comparison shows that the central globular region (about 70 residues in length) is highly conserved (60-75% identity), whereas the flanking sequences show a much lower identity (47-62%). Also for the polypeptides encoded by pVs53 and pVs20 the central globular region is more conserved than the N- and C-terminal ends. For the Vs53 polypeptide the central region is 96% identical to the pea central region, whereas the N-terminal ends are 50% identical and the C-terminal ends are 71% identical. For the Vs20 polypeptide these numbers are 76%, 45% and 65% identity, respectively.

Vs14

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  1  GPALSTRNKL RSTEFEGCKH PGMSSNVRLE ENDGTASGLE ITTLAPLREA EKGMPFESAK
 61  HIENLTTPPI VKRGRPKGSK NKKKTLADQE HIGHGGDIIK LIGMESSEAA VSVGDQELVV
121  QPLVKVRFRM LNPKMGRPKG SKNKKKNVDG EAETGYIKEG KKRGRPKGSG NKQKETGNEK
181  IAKGLVSESS NVHKIERRGR PKGSAKKQKE NASRLDAEIE REKNTHVYGI LSTTMPHKHI
241  HEESILLLED HVNKKDDADF VLECSKESGI EKIAKGLVSE SDNVHKTHDV EVGDIFYEKE
301  VKETIDHRLE PSDMLGDCET KKEPRN
```

Vs34

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  1  AESHSNLLTH HLKRLKSDGL LQTIKNSYII PRSAPPTQPP STTPTASLSQ PSKPRGRPRK
 61  SVTPASASAP PQPLPVVVAN NDNDHSPLQN AEPVWAALGL SDEPLDVQPA ATPGEGSKRR
121  PGRPPGSKNV PKNSTAPSPS PSQNPTPIEG QVPPTPASRG RGRPPGSKAK LKKKPGRPPK
181  AKIDTPTPAA SAAAASDGTK RRPGRPPKNQ QQNPTPIPFA TTATETPQVV VPEVAVPVTA
241  VDGGTVLTPR SRGRPRKSPA VAAVPVSVVA GGGRGRGRSI PAERRSLPLP VGLVWPHA
```

Figure 4.3: Amino acid sequence of the polypeptides encoded by the inserts of pVs14 and pVs34. The heptapeptide repeats K/RRGRPKG in the Vs14 polypeptide and the octapeptide repeats R/KNP/RGRPP/RK/G in the Vs34 polypeptide, showing homology to AT-hooks found in HMG proteins, are underlined.

	GRP motifs								amino acids
	K	P	R	**G**	**R**	**P**	R	K	56 - 63
	R	R	P	**G**	**R**	**P**	P	G	122 - 129
Vs34	R	G	R	**G**	**R**	**P**	P	G	162 - 169
	K	K	P	**G**	**R**	**P**	P	K	176 - 183
	R	R	P	**G**	**R**	**P**	P	K	204 - 211
	R	S	R	**G**	**R**	**P**	R	K	253 - 260
		K	R	**G**	**R**	**P**	K	G	72 - 78
		K	M	**G**	**R**	**P**	K	G	134 - 140
Vs14		K	R	**G**	**R**	**P**	K	G	162 - 168
		R	R	**G**	**R**	**P**	K	G	197 - 203
	R	P	R	**G**	**R**	**P**	K	K	HMG-I consensus
	R	P/G	R	**G**	**R**	**P**	P/K	K	soybean embryo HMG consensus

Figure 4.4: Comparison of the GRP motifs or AT-hooks of the Vs14 and Vs34 polypeptide with the mammalian HMG I consensus (Reeves and Nissen, 1990) and the soybean embryo HMG consensus (Laux *et al.*, 1991). The conserved GRP core sequence is printed bold.

K, lysine; R, arginine; P, proline; G, glycine; S, serine.

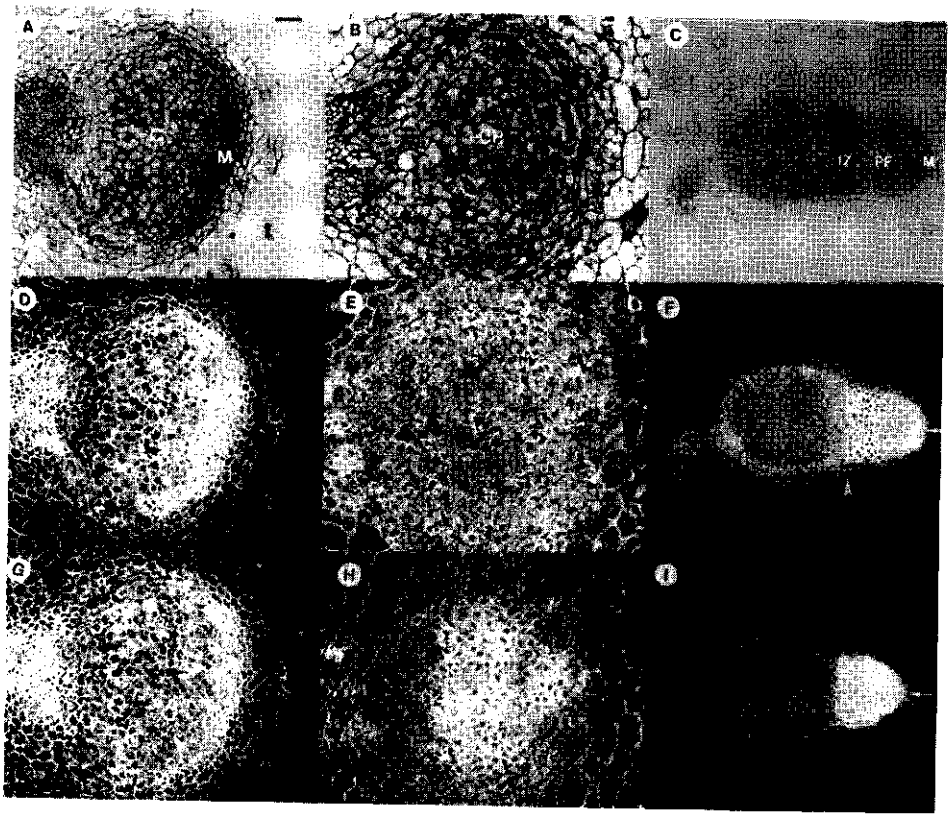
The results obtained from the sequence comparison showed that *Vs20* and *Vs53* are both encoding histone H1 polypeptides. The expression pattern of *Vs20* and *Vs53* in young root nodules confirmed this conclusion. That the clones represent different histone H1 genes is not surprising since plants have many H1 subtypes (reviewed by Spiker, 1988). It is very unlikely that histone H1 is involved in regulation of *ENOD12* expression. Therefore these clones were not further investigated in our study on *ENOD12* regulation.

Characterization of pVs34

The insert of pVs34 is 894 nucleotides long and can be read as one long open reading frame coding for a polypeptide of 298 aa. Since neither a start nor a stop codon are present, the encoded open reading frame will most likely be larger and continue further upstream and downstream. No homology was found with sequences in the NCBI database (22 Sept. 1994). However, the encoded polypeptide contains six amino acid repeats of the octapeptide R/KNP/RGRPP/RK/G. This sequence is very similar to the "AT-hook" motif, which has been found in mammalian HMG (High Mobility Group) I and HMG Y proteins (Reeves and Nissen, 1990) and in a soybean HMG protein (Laux *et al.*, 1991) (Figure 4.3 and 4.4). These AT-hooks, also called GRP motifs, have been shown to bind to AT-rich regions in the minor groove of double stranded DNA and alter the conformation and thermal stability of these regions (Lehn *et al.*, 1988; Reeves *et al.*, 1990). Besides the GRP motifs there is no sequence homology between the Vs34 polypeptide and HMG proteins. HMG proteins have a molecular weight of less than 30 kD and contain 25% of both acidic and basic residues and have a proline content of at least 7% (Johns, 1982). The partial polypeptide encoded by pVs34 of 298 amino acids contains 5% acidic residues and 16% basic residues while the proline content of 18% is very high. This composition is so much different from that of HMG proteins that we concluded that the polypeptide encoded by pVs34 does not belong to that class of proteins.

Recently a few DNA binding proteins have been identified in plants that indeed contain AT-hooks. Some of these proteins are homologous to HMG I or HMG Y proteins, like the DNA-binding protein PF1 isolated from rice and oat and that contains four GRP motifs (Nieto-Sotelo *et al.*, 1994a; Nieto-Sotelo *et al.*, 1994b). Tjaden and Coruzzi (1994) isolated a DNA binding protein containing seven AT-hooks from tobacco, called ATBP-1. Apart from the GRP motifs this polypeptide had no sequence homology with HMG proteins and it contained a glutamine-rich N-terminal end. This finding shows that AT-hooks also occur in DNA-binding proteins, which are not HMG proteins.

So far we were not able to detect expression of the *Vs34* gene with *in situ*


A
M
B
CP
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E
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hybridization experiments on 5 day old *Vicia sativa* root nodules.

On the basis of the sequence of pVs34, which showed that the encoded polypeptide has characteristics also found in other DNA binding proteins, this clone is of interest for further study.

Characterization of pVs14

pVs14 contains an insert of 2.8 Kb. Only 978 bp of the 5' end have been sequenced until now. This fragment contains one long open reading frame coding for a polypeptide of 326 aa. Since there is neither a stop codon nor a start codon present, the encoded open reading frame in this gene will most likely be larger and continue further downstream and more upstream. No homology was found with sequences in the NCBI database (22 Sept. 1994). The polypeptide encoded by pVs14 contains four GRP motifs or AT hooks (Figure 4.3). Just like the protein encoded by the cDNA insert of pVs34, the Vs14 protein does not fit the criteria for HMG proteins as it has, apart from the GRP motifs, no further sequence homology to known AT-hook containing HMG proteins. Recently part of the insert of pVs28, which cross-hybridized with pVs14, has been sequenced starting from the 3' end and it was found that the last 700 bp of the insert of pVs28 overlap with the first 700 bp of pVs14 (Henning Christiansen, pers. comm). Since the insert of pVs28 is 1.7 Kb long it contains, compared with the insert of pVs14, 1 Kb more upstream sequence and might

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Figure 4.5: *In situ* localization of Vs20, Vs53, Vs14 and ENOD12 mRNA in young root nodules of *V. sativa*. The pictures show cross sections of 4 (B, E, H), 5 (A, D, G) and 20 (C, F, I) day old *V. sativa* root nodules. A, B and C are bright field micrographs; D, E, F, G, H and I are dark field micrographs were signals are represented by white dots. C, central tissue; M, meristem; CP, central part of nodule primordium; PF, prefixation zone II; IZ, interzone II-III.

A, D, G: Cross sections of 5 day old root nodules. The sections were hybridized with *Vs20* (A, D) and *Vs53* (G) ^{35}S-labelled antisense RNA, showing the expression of *Vs20* and *Vs53* in the meristem of the nodule (M) and in some cells of the central tissue (arrowheads).

B, E, H: Cross sections of 4 day old root nodules. The sections were hybridized with *Vs14* (B, E) and *ENOD12* (H) ^{35}S-labelled antisense RNA, showing that *Vs14* is expressed in all cells of the primordium. *ENOD12* expression is only observed in the central part of the primordium (H).

C, F, I: Cross sections of 20 day old root nodules. The sections were hybridized with *Vs14* (C, F) and *ENOD12* (I) ^{35}S-labelled antisense RNA, showing that *Vs14* is expressed in the meristem, the prefixation zone II and the nodule surrounding tissue (F). *ENOD12* expression is only observed in the prefixation zone (I) and not in the meristem, in contrast to *Vs14* (arrow; F and I). Note that *Vs14* expression decreases at the transition from prefixation zone II into interzone II-III (F, arrowhead).

contain the start codon. So two clones representing different parts of the same gene are isolated, demonstrating that during the screening specific clones have been selected.

With *in situ* hybridization experiments the expression of *Vs14* was studied in 4 and 20 day old *V. sativa* root nodules. In a 4 day old root nodule primordium the peripheral tissues have started to form, but the apical meristem has not yet been formed (Figure 4.5B), whereas in a 20 day old mature root nodule the central tissue has been differentiated into successive nodule tissues (Figure 4.5C). In a 4 day old *V. sativa* nodule *Vs14* is expressed in all cells of the young nodule (Figure 4.5E), whereas *ENOD12* is only expressed in the central part of the young nodule (Figure 4.5H). In 20 day old root nodules *Vs14* is expressed in the nodule meristem, the prefixation zone and the surrounding tissues (Figure 4.5F). The expression of *Vs14* decreases at the transition from prefixation zone II into interzone II-III (Figure 4.5F, arrowhead). *ENOD12* is in 20 day old nodules only expressed in prefixation zone II (Figure 4.5I). Thus the expression of *Vs14* overlaps with *ENOD12* expression, but is extended to several other tissues. This suggests that if the Vs14 polypeptide is involved in controlling gene regulation, it will probably regulate the expression of several genes.

On the basis of the sequence, which has characteristics also found in other DNA-binding proteins and on the expression pattern, pVs14 and pVs28 are of interest to further investigate a possible role in regulating *ENOD12* expression.

Concluding remarks

Although the results presented here concern partial cDNA clones and need to be completed, some preliminary conclusions can be drawn. Two of the 5 isolated clones showed a strong sequence homology to histone H1 genes. The other 3 clones did not show sequence homology to known proteins but they contain repeated AT-hooks, which have been shown to be involved in DNA-binding. Therefore we can conclude that the screening of the expression library successfully resulted in the isolation of cDNA clones encoding DNA-binding proteins.

pVs34 and pVs14/pVs28 encode polypeptides containing AT-hooks, but the encoded proteins are not homologous to HMG proteins, which also contain these AT-hooks. A characteristic of he AT-hooks is that they bind AT-rich DNA (Reeves and Nissen, 1990). Since the 200 bp fragment of the *PsENOD12* promoter used for the screening is AT-rich it was not surprising that the isolated clones encode polypeptides containing AT-hooks. Most likely the Vs34 and Vs14/Vs28 polypeptides will not only, if at all, have a role in the regulation of the *ENOD12* gene but might be involved in the regulation of other genes as well. This is also suggested by the *in situ* hybridization expression pattern of *Vs14*.

Vs14 expression was observed in the meristem, the prefixation zone and in the surrounding tissues of a 20 day old nodule, whereas *ENOD12* expression was only observed in the prefixation zone. Comparison of the expression pattern of *Vs14* with the expression pattern of several proline-rich nodulin genes, like *ENOD2, ENOD12* and the recently cloned *mtprp4,* shows that *Vs14* is expressed in all the tissues of the nodule where one of these genes is expressed (Van de Wiel *et al.*, 1990; Wilson *et al.*, 1994). This suggests that *Vs14* might play a role in controlling the expression of proline-rich nodulin genes. At this moment we do not know whether *Vs14* is a nodule specific gene or that it is also expressed in other plant tissues, since this has not yet been tested.

For the polypeptide encoded by pVs14 two independent clones were isolated, pVs14 and pVs28. At this moment *Vs14/Vs28* are studied in more detail. With DNA binding assays, foot prints and RNAse protection assays it will be studied whether the encoded DNA-binding polypeptide is somehow involved in the regulation of *ENOD12* expression. Also effort will be put in the isolation of a full-size clone.

Finally, when it will have been shown that the isolated clones interact specifically with the *PsENOD12* promoter their involvement in *ENOD12* expression has to be tested in a functional assay. An assay that can be used for this purpose is for example micro injection of the protein in transgenic *Vicia hirsuta* root cells, carrying a reporter gene linked to the *ENOD12* promoter. Also overexpression or antisense experiments might give clues about the function of the cloned genes.

Materials & Methods

Growth conditions for plants and bacteria

Vicia sativa spp. *nigra* seeds were sterilized and germinated for two days as described by Van Brussel *et al.* (1982). Seedlings were planted in gravel and grown as described by Moerman *et al.* (1987). *Rhizobium leguminosarum* bv *viciae* strain 248 was grown in liquid YEM medium (Bhuvaneswari *et al.*, 1980) at 28 °C. Inoculation with *R.l.* bv. *viciae* 248 occurred at the day that the seedlings were planted in gravel.

Preparing and screening of a Vicia sativa nodule cDNA expression library

Nodules were harvested 7, 9, 12 and 17 days after inoculation, immediately frozen in liquid nitrogen and stored at -70 °C. Total RNA from these root nodules was isolated as described by Govers *et al.* (1985). Equal amounts of these RNAs were mixed and

poly(A)$^+$ RNA was obtained by using an oligo-dT cellulose column (Pharmacia, Type 7).

First strand cDNA synthesis (Krug and Berger, 1987) was performed by using dN$_7$-*Not*I primers. After second strand cDNA synthesis according to Gubler (1987), double stranded cDNA fragments larger than 400 bp were purified on a sepharose CL-4B column. These cDNA fragments were provided with *Eco*RI adaptors (Promega), digested with *Not*I and ligated into *Eco*RI-*Not*I digested λgt11*Sfi*I-*Not*I arms (Promega). The DNA was packed by using a Gigapack Gold II kit (Stratagene), as described by the manufacturer. Starting from 5 µg poly(A)+ RNA , 500 ng of double stranded cDNA, larger than 400 bp, was obtained. From 75 ng of cDNA ligated to λgt11*Sfi*I-*Not*I arms, 4x10^5 independent recombinant phages were obtained.

Probes for screening the library were obtained by annealing the phosphorylated oligonucleotides in pairs (see table 4.1), followed by concatenation by ligation. The probes were labelled with [a-^{32}P]-dATP by filling in the ends (Sambrook *et al.*, 1989). The probes were used without further purification.

For screening the library 3 x 10^4 pfu were plated on 500 cm^2 plates. Two replica nitrocellulose filters were prepared; the first was incubated overnight, the second for 4 h the following day. To induce expression of the fusion protein, the nitrocellulose filters were submerged in 10 mM IPTG before applying onto the plates. The expression library was screened according to Vinson *et al.* (1988): After removal from culture plates, the nitrocellulose filters were air-dried for 15 min at room temperature. The rest of the procedure was carried out at 4 °C. Denaturation/renaturation of the fusion proteins was performed by a guanidine hydrochloride concentration series, ranging from 6 M to 0.01 M guanidine hydrochloride in 1x SW buffer (25 mM Hepes pH 7.9, 3 mM $MgCl_2$, 40 mM KCl, 0.01 mM $ZnSO_4$, 1 mM DTT). After blocking in 5% nonfat dry milk in 1x SW buffer, the filters are exposed to the probes for 2 hr in 1x SW buffer supplemented with 0.25% dry milk. After exposure to the DNA probes the filters were washed over a 15 min period for 5 times with 1x SW buffer plus 0.25% dry milk. The filters were briefly blotted dry on 3 MM paper and exposed to Kodak X-Omat AR film at -70 °C.

DNA hybridization of isolated λgt11*Sfi*I-*Not*I phages was performed by transferring plaques to Hybond-N+ membranes (Amersham), as described by the manufacturer. The DNA fragments used as probes were purified by agarose gel electrophoresis and labelled with [a-^{32}P]-dATP, using a random primer labelling kit (Boehringer).

DNA-sequencing

After purification of the phages λVs14, λVs20, λVs34 and λVs53, the cDNA inserts were amplified by PCR, by using the λgt11 primers 5'-ACACCAGACCAACTGGTAATGGT

AGCGACC-3' and 5'-TTCCATATGGGGATTGGTGGCGACGACTCC-3', and after digestion with *Sfi*I and *Not*I subcloned in pGEMEX-2 (Promega). These constructs were called pVs20, pVs34 and pVs53, respectively. Only the insert of λVs14 was blunt ended with Klenow after PCR and subcloned in pUC18. Since the insert of λVs14 was ± 2.8 Kb, 1 Kb of the 5' end was subcloned from pUC18 as a *Bam*HI-*Eco*RI fragment in Bluescript pKS (Stratagene). This construct was called pVs14. The sequence of the different clones was determined with an automatic sequencing apparatus of Applied Biosystems, Inc. by using a Sequenase Terminator kit (Applied Biosystems, no. 401459).

In situ hybridization

For *in situ* hybridization nodules were harvested 5 days after inoculation and fixed in 4% paraformaldehyde supplemented with 0.25% glutaraldehyde in 10 mM sodium phosphate buffer for 3 h (Van de Wiel *et al.*, 1990). Fixed material was dehydrated and embedded into paraffin by routine methods (Van de Wiel *et al.*, 1990; Yang *et al.*, 1991). Sections (7 mm thick) were hybridized with [^{35}S]-UTP labelled antisense or sense RNA probes according to a procedure derived from Cox and Goldberg (1988) (Van de Wiel *et al.*, 1990). Sections exposed for 2 to 4 weeks were stained with toluidine blue and photographed with a Nikon microscope with dark field and epipolarization optics.

Labelling of antisense/sense RNAs

For preparing antisense and sense RNAs the cDNA inserts were subcloned from pGEMEX-2 into Bluescript pKS(+) vector (Stratagene). For *Vs14* the 1 Kb *Bam*HI-*Eco*RI subclone in pKS was used. The insert of pVs34 was subcloned in pKS as an *Hinc*II-*Hin*DIII fragment, pVs20 as an *Eco*RI-*Not*I fragment and pVs53 was subcloned as a *Sfi*I(filled in)-*Not*I fragment into pKS, predigested with *Sma*I and *Not*I. The plasmids containing the Vs20 and Vs53 cDNA inserts were digested with *Eco*RI and the plasmid containing the Vs34 cDNA insert was digested with *Hinc*II before antisense RNA was made with T7 polymerase. The plasmid containing the Vs14 cDNA insert was digested with *Bam*HI and antisense RNA was made with T3 polymerase. Sense RNA of pVs14 was made with T7 polymerase after digestion with *Eco*RI. The probes were radioactively labelled with [^{35}S]-UTP (1000-1500 Ci mmol^{-1}, Amersham) and degraded to about 150 nucleotides long fragments before hybridization (Van de Wiel *et al.*, 1990).

CHAPTER 5

Early nodulin gene expression during Nod factor induced processes in *Vicia sativa*

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Abstract

Rhizobium leguminosarum bv. *viciae* secreted Nod factors are able to induce root hair deformation, the formation of nodule primordia and the expression of early nodulin genes in *Vicia sativa* (vetch). To obtain more insight in the mode of action of Nod factors we followed the expression of early nodulin genes during Nod factor induced root hair deformation and nodule primordium formation. The results of these studies suggested that the expression of *VsENOD5* and *VsENOD12* is not required for root hair deformation. In the Nod factor induced primordia both *VsENOD12* and *VsENOD40* are expressed in a similar spatially controlled manner as found in *Rhizobium* induced nodule primordia. In contrast, *VsENOD5* expression has never been observed in Nod factor induced primordia, showing that the induction of *VsENOD5* and *VsENOD12* expression is not coupled. *VsENOD5* expression is induced in the root epidermis by Nod factors and in *Rhizobium* induced nodule primordia only in cells infected by the bacteria, suggesting that the Nod factor does not reach the inner cortical cells.

Introduction

Rhizobium leguminosarum bv. *viciae* has the ability to nodulate plants of the genera *Pisum*, *Lens*, *Lathyrus* and *Vicia*. During early stages of this *Rhizobium*-legume interaction, *R. leguminosarum* bv. *viciae* secretes at least five different specific lipo-oligosaccharides, the so-called Nod factors. These Nod factors, named NodRlv factors, are either tetra- or pentamers of N-acetyl-D-glucosamine with an unsaturated C18 fatty acid chain at the C-2 position of the non-reducing terminal sugar. The acyl chain has either one or four unsaturated bonds and the non-reducing terminal sugar can also be substituted with an O-acetyl group at the C-6 position (Spaink *et al.*, 1991).

Studies with purified Nod factors have shown that these compounds are able to induce some of the early steps of nodulation. The factors induce, at concentrations as low as 10^{-10} - 10^{-12} M, root hair deformation, pre-infection thread formation, cortical cell division and in some legume species even genuine nodules (Spaink *et al.*, 1991; Stokkermans and Peters, 1994; Truchet *et al.*, 1991; Van Brussel *et al.*, 1992). Furthermore Nod factors can elicit the expression of some early nodulin genes, like *ENOD5* and *ENOD12* (Horvath *et al.*, 1993; Journet *et al.*, 1994; Pichon *et al.*, 1993), *Chalcone synthase* (*CHS*) (Lawson et al., 1994) and a recently isolated peroxidase gene, *rip1*, from *Medicago truncatula* (Cook *et al.*, 1995).

The biological activity of Nod factors secreted by *R. leguminosarum* bv. *viciae* (NodRlv factors), has been studied on *Vicia sativa* (vetch) (Heidstra *et al.*, 1994; Spaink *et*

al., 1991), and *Pisum sativum* (pea) (Horvath *et al.*, 1993). The recently isolated homologues of the early nodulin genes *ENOD5*, *ENOD12* and *ENOD40* from *V. sativa* (Chapter 2) enabled us to study the correlation between nodulin gene expression and Nod factor induced morphological changes, which might provide new insights in the mode of action of Nod factors.

Root hair deformation occurs in *V. sativa* in a spatially and temporally controlled way (Heidstra *et al.*, 1994). Within 3 hours after Nod factor addition, root hair deformation is clearly visible in a narrow zone of the root, called the susceptible zone. This susceptible zone is about 2 mm long and contains the root hairs that have almost reached their mature size, but does not include young elongating root hairs or old mature root hairs. One hour after the addition of Nod factor, swelling of the root hair tips is observed and after 2 h polar growth is initiated, resulting in clearly detectable deformation at 3 h (Heidstra *et al.*, 1994). Root hair deformation has been used as a semi-quantitative assay to study the ability of Nod factors to deform root hairs (Heidstra *et al.*, 1994). The availability of a reliable root hair deformation assay made it possible to determine by using cycloheximide (CHX) and actinomycin D (AcD) whether protein and RNA synthesis are required for root hair deformation. By comparing the timing of *VsENOD5* and *VsENOD12* expression and root hair deformation and by using protein and RNA synthesis inhibitors, we have analysed whether these early nodulin genes are required for root hair deformation.

Besides root hair deformation, NodRlv factors are able to induce cortical cell division in *Vicia* roots. The primordia induced by either Nod factors or *Rhizobium* resemble each other, but Nod factor induced primordia do not develop into nodules (Spaink *et al.*, 1991). In *Rhizobium* induced nodule primordia *ENOD5* is only induced in the infected cells, whereas *ENOD12* and *ENOD40* are induced in all primordium cells (Scheres *et al.*, 1990a; Scheres *et al.*, 1990b; Yang *et al.*, 1993). In the presented paper we studied whether these early nodulin genes are activated in Nod factor induced primordia. Preliminary experiments on *ENOD12* and *ENOD40* expression in Nod factor induced primordia were reported earlier (Vijn *et al.*, 1993).

Results

Root hair deformation requires de novo RNA and protein synthesis

V. sativa roots were incubated in plant growth medium (pgm) supplemented with 10^{-10} M NodRlv-V(Ac, C18:4). By replacing the pgm medium by medium containing 10^{-10} M NodRlv-V(Ac, C18:4) and 100 µM cycloheximide (CHX) at 0, 1/2, 1 or 1 1/2 h after the incubation with only Nod factor, the effect of CHX on root hair deformation was studied.

Table 5.1: Effect of cycloheximide (CHX) and actinomycin D (AcD) on root hair deformation. At 0 h NodRlv-IV(C18:4, Ac) 10^{-10} M was applied to Fåhraeus slides containing 6 *Vicia* plants. AcD (20 μM) and CHX (100 μM) were added simultaneously or 1/2, 3/4, 1 or 1 1/2 h after Nod factor addition. Root hairs in the susceptible zone were examined 3 h after Nod factor addition. Each experiment was performed at least 2 times using 2 Fåhraeus slides.

Drug	Time of drug addition	root hair deformation after 3 h
CHX	0 h	<5%
	1/2 h	<5%
	1 h	<18%*
	1 1/2 h	<25%*
AcD	0 h	<5%
	1/2 h	<20%*
	3/4 h	>70%
	1h	>80%
	1 1/2 h	>80%
	No drug added	>80%

* These root hairs have swollen tips, but new tip growth has not been initiated.

Root hairs in the susceptible zone were examined 3 h after Nod factor addition. When CHX was added at 0 h or 1/2 h later, less than 5% of the root hairs deformed (table 5.1). When CHX was applied 1 or 1 1/2 h after starting the incubation with Nod factor, less than 25% of the root hairs were deformed after 3 h (table 5.1) or even 7 h (data not shown), showing that deformation became arrested. These data showed that not only the initiation but also the continuation of root hair deformation required protein synthesis.

The effect of actinomycin D (AcD) on root hair deformation was studied by treating *V. sativa* roots with 10^{-10} M NodRlv-V(Ac, C18:4) to which after different time periods (0, 1/2, 3/4, 1 and 1 1/2 h) AcD was added to a final concentration of 20 μM. Root hairs were examined 3 h after Nod factor addition (table 5.1). When AcD was added simultaneously with Nod factor (0 h) root hair deformation was observed in less than 5% of the root hairs, demonstrating that transcription of genes is essential to induce root hair deformation. Addition of AcD at 1/2 h resulted in deformation in less than 20% of the root hairs (table 5.1), even at 7 h after Nod factor addition (data not shown). Addition of AcD at 3/4 h or later resulted in deformation of more than 70-80% of the root hairs, just as on the roots not treated with AcD (table 5.1). So, in contrast to CHX, AcD was only able to block root hair deformation when it was added within the first 3/4 h after Nod factor addition, showing that

the transcription of genes within this time period was essential for root hair deformation to occur.

Expression of VsENOD5 and VsENOD12 during V. sativa root hair deformation

The early nodulin genes *ENOD5* and *ENOD12* are rapidly induced in the root epidermis by Nod factors (Horvath *et al.*, 1993; Journet *et al.*, 1994; Pichon *et al.*, 1993) and we wondered whether the expression of these genes could be essential for root hair deformation. Therefore we investigated the expression of *VsENOD5* and *VsENOD12* using a RT-PCR based assay as described by (Horvath *et al.*, 1993).

V. sativa plants were treated with 10^{-10} M NodRlv-V(Ac, C18:4) and the susceptible zone of 60 roots was collected 0, 3 and 8 h after addition of the Nod factor. Total RNA was isolated and *VsENOD5* and *VsENOD12* RNA was amplified by RT-PCR. As an internal standard ubiquitin mRNA was used (Horvath *et al.*, 1993) and as a positive control we used total RNA from 2 week old *V. sativa* nodules. The results are shown in figure 5.1A.

The expression of the ubiquitin genes is not affected by the addition of Nod factors and is therefore a useful internal standard (Figure 5.1A, panel ubiquitin). Amplification of *VsENOD12* RNA resulted in one band of 250 bp (table 5.2), which was not present when the reverse transcriptase step was omitted (Figure 5.1A, panel *ENOD12*, lane -rt). *VsENOD12* expression was not detected at 0 and 3 h after Nod factor addition, but was only detected after 8 h and in two week old nodules (Figure 5.1A, lane 0, 3, 8 and 2w).

Amplification of *VsENOD5* RNA resulted in two DNA fragments of 348 and 433 bp (Figure 5.1A, panel *ENOD5*). The 348 bp fragment was conform the expectation as the primers used for *VsENOD5* RNA amplification should produce a fragment of that size (Table 5.2). The 348 bp fragment was not found at 0 and 3 h after Nod factor addition (Figure 5.1A, lane 0 and 3), whereas at 8 h this fragment was clearly detectable (lane 8) as well as in two week old nodules (Figure 5.1A, lane 2w). The 433 bp fragment was not expected and was further analysed by sequencing. The 433 fragment appeared to have the *VsENOD5* sequence, but it contained an extra stretch of 85 bp that might reflect the presence of an intron, as also the pea *ENOD5* gene contains an intron (Horvath *et al.*, 1993). Therefore the 433 bp long fragment could be due to the presence of remaining genomic DNA, although in all experiments DNase-treated samples were used.

These results show that *VsENOD5* and *VsENOD12* transcripts are first present several hours after root hairs have deformed. This strongly suggests that *VsENOD5* and *VsENOD12* are not essential for root hair deformation

To confirm that *VsENOD5* and *VsENOD12* are not involved in root hair deformation transcription was blocked by adding AcD (20 µM) 1 h after Nod factor addition. In this case

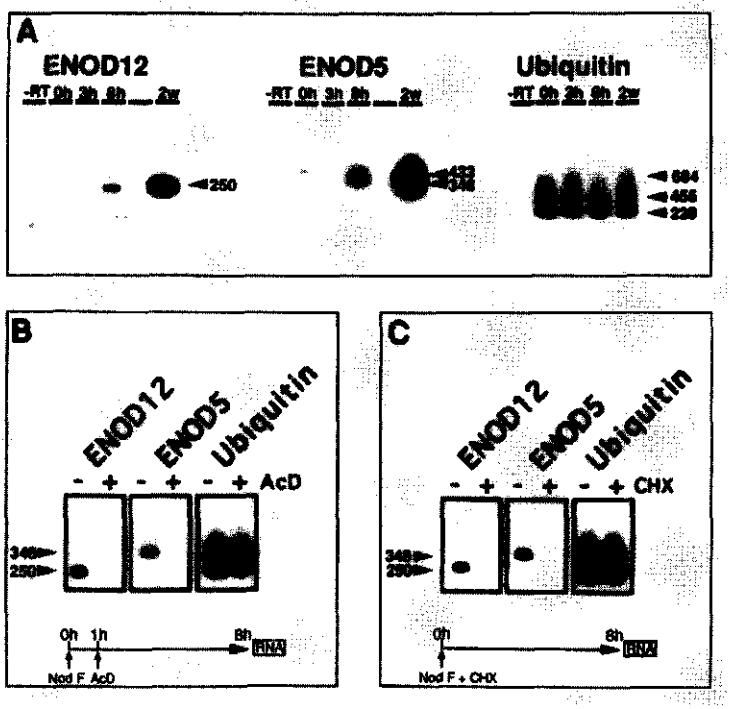


Figure 5.1: Timing of *VsENOD5* and *VsENOD12* expression during NodRlv induced root hair deformation and the effect of actinomycin D (AcD) and cycloheximide (CHX) on their expression.

A: *V. sativa* plants were treated with 10^{-10} M NodRlv-V(Ac, C18:4) at 0 h and the susceptible zone was collected 0, 3 and 8 h after Nod factor addition. Total RNA was isolated and *VsENOD5* and *VsENOD12* mRNA was amplified by RT-PCR. As an internal control ubiquitin RNA was amplified and as a positive control total RNA from 2 week old nodules (2w) was used. In the first lane of each panel the reverse transcriptase was omitted during the cDNA synthesis step on RNA isolated after 8 h (-rt). *VsENOD5* amplification resulted in two DNA fragments of 433 bp (genomic DNA) and 348 bp (mRNA). The *VsENOD12* fragment is 250 bp long. The ubiquitin fragments are 228, 456 and 684 bp long.

B: *V. sativa* plants were treated with 10^{-10} M NodRlv-V(Ac, C18:4) at 0 h and (as indicated in the figure) 1 h later AcD (20 μM) was added (+) or not (-). At 8 h the susceptible zone was collected and total RNA was isolated. *VsENOD5*, *VsENOD12* and ubiquitin mRNA was amplified by RT-PCR.

C: *V. sativa* plants were treated at 0 h with 10^{-10} M NodRlv-V(Ac, C18:4) in the presence (+) or absence (-) of CHX (100μM). The susceptible zone of the root was collected 8 h later and after total RNA isolation *VsENOD5*, *VsENOD12* and ubiquitin RNA was amplified by RT-PCR.

root hair deformation was not affected and took place within 3 h (table 1). On the other hand, *VsENOD5* and *VsENOD12* mRNA were not present 8 h after Nod factor treatment (Figure 5.1B, + lanes), showing that their expression is not induced within the first hour after Nod factor treatment. Therefore we conclude that *VsENOD5* and *VsENOD12* do not play a role in root hair deformation.

To exclude the possibility that the induction of *VsENOD5* and *VsENOD12*, although they are induced at a relatively late time point after Nod factor addition, does not require the synthesis of new proteins we applied at 0 h 10^{-10} M NodRlv-V(Ac, C18:4) and 100μM CHX to *V. sativa* roots and collected the susceptible zone of the roots 8 h later. After total RNA extraction *VsENOD5* and *VsENOD12* mRNA's were amplified by RT-PCR. As is shown in Figure 5.1C (+ lanes) neither *VsENOD5* nor *VsENOD12* mRNA's were present. This strongly suggests that *de novo* protein synthesis will be required for the activation of both *VsENOD5* and *VsENOD12*.

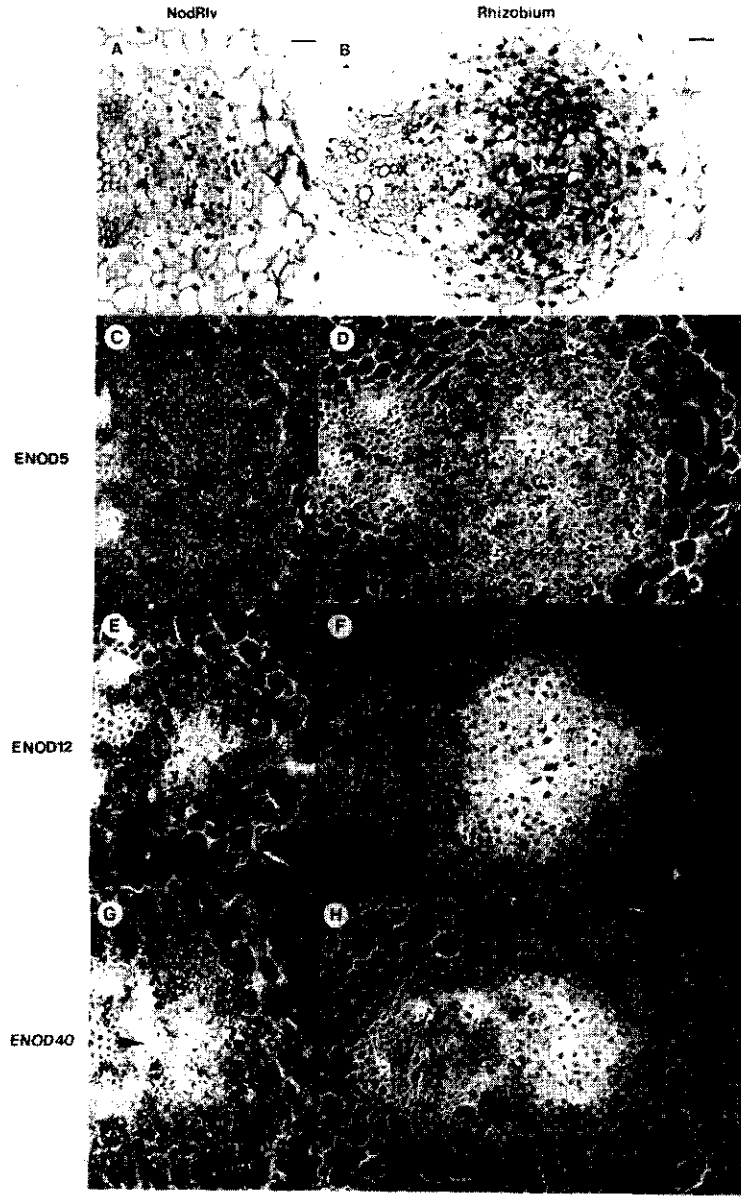
Expression of early nodulin genes in Nod factor induced nodule primordia

During the *Rhizobium*-legume interaction the expression of *VsENOD5* and *VsENOD12* is induced in the root epidermis as well as in nodule primordia, formed in the inner cortex. In these nodule primordia *VsENOD5* is only activated in cells infected by *Rhizobium* (Figure 5.2D, arrow), whereas *VsENOD12* expression is triggered in all primordium cells (Figure 5.2F). Therefore we were interested how the expression pattern of *VsENOD5* and *VsENOD12* would be in Nod factor induced nodule primordia.

If *V. sativa* roots are treated with 10^{-9} M NodRlv-V(Ac, C18:4) about 2 primordia per root are formed, predominantly opposite proto-xylem poles (Figure 5.2A). Serial sections of such primordia were hybridized with ^{35}S-labelled *VsENOD5* and *VsENOD12* antisense RNA. As is shown in Figure 5.2E *VsENOD12* is expressed, whereas *VsENOD5* mRNA (Figure 5.2C) is not present in these primordia.

When a 100 fold higher concentration (10^{-7} M) of NodRlv-V(Ac, C18:4) was applied to *V. sativa* roots the number of primordia formed per root increased to about 10. *In situ* hybridization showed that also in these primordia *VsENOD12* is active but *VsENOD5* is not (data not shown). Thus even a 100 fold higher concentration of Nod factor can not elicit *VsENOD5* expression.

ENOD40 is induced by *Rhizobium* in nodule primordium cells as well as in the region of the root pericycle, opposite the proto-xylem pole (Figure 5.2H). To determine whether purified Nod factors elicit *ENOD40* in a similar spatial manner we hybridized sections of Nod factor (10^{-9} M) induced primordia with ^{35}S-labelled *ENOD40* antisense RNA. As is shown


NodRlv
Rhizobium
A
B
C
D
ENOD5
E
F
ENOD12
G
H
ENOD40

in figure 5.2G *ENOD40* expression is detected in the mitotically activated cortical cells and in the pericycle opposite the primordium (arrowhead), just as in the *Rhizobium* induced primordia (Figure 5.2H).

The early nodulin gene *ENOD2* is first activated during *Rhizobium* induced nodule development when the primordium differentiates into different nodule tissues and it is only expressed in the nodule parenchyma (Van de Wiel *et al.*, 1990). To determine whether Nod factor induced nodule primordia also start to differentiate into nodule tissues, sections of 10 different primordia induced by Nod factor concentrations varying from 10^{-7} to 10^{-9} M were hybridized with a ^{35}S-labelled antisense *ENOD2* probe. As a control sections of *Rhizobium* formed nodules (20 days) were included on the slides and hybridized simultaneously. In none of the nodule primordia induced by Nod factors *ENOD2* expression occurred, whereas in the nodules used as a control the nodule parenchyma was very intensively labelled (Data not shown).

Discussion

Within three hours after addition of Nod factor to *Vicia sativa* roots, root hair deformation takes place in a narrow zone, the so-called susceptible zone, of the root. We have demonstrated that root hair deformation can be inhibited by AcD and CHX, suggesting that the deformation process depends on plant DNA specified RNA and protein synthesis. The inhibiting effect of AcD decreases rapidly and 3/4 h after adding Nod factor, root hair deformation has become insensitive to the inhibitor. The process remains, however, sensitive to the protein synthesis inhibitor CHX during the whole period of 3 h

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Figure 5.2. *In situ* localization of *VsENOD5*, *VsENOD12* and *VsENOD40* in NodRlv induced nodule primordia and in 4 day old *Rhizobium* induced root nodules of *Vicia sativa*.

N, nodule primordium; P, pericycle; X, proto-xylem pole. A and B are bright field micrographs; C, D, E, F, G and H are dark field micrographs were signals are represented by white dots. The sections were hybridized with *VsENOD5* (C, D), *VsENOD12* (E, F) and *VsENOD40* (G, H) ^{35}S-labelled antisense RNA.

A, C, E and G show serial cross sections of a NodRlv-V(Ac, C18:4) induced primordium. B, D, F and H show serial cross sections of a 4 day old *Rhizobium* induced root nodule.

Note that the NodRlv induced primordium (A) is formed opposite a proto-xylem pole (X) just as the *Rhizobium* induced nodule (B). In the *Rhizobium* induced 4 day old nodule *VsENOD12* is expressed in all primordium cells (F), whereas *VsENOD5* is only expressed in the infected cells (D, arrow). In the NodRlv factor induced primordium *VsENOD12* is expressed (E), but *VsENOD5* is not expressed (C). *VsENOD40* is in the NodRlv induced primordium expressed in the primordium cells and in the pericycle opposite the proto-xylem pole (arrowhead) (G), just as in the *Rhizobium* induced young nodule (H).

required for root hair deformation. The observation that DNA depended RNA and protein synthesis are prerequisites for root hair deformation suggests that some essential plant encoded proteins of which the synthesis is induced by the Nod factor, are involved.

VsENOD5 and *VsENOD12* are the first expressed early nodulin genes after addingNod factors. The expression of both genes was first detectable at 8 h after addition of Nod factor which is ample time after root hair deformation. When transcription was blocked by adding actinomycin D 1 h after Nod factor, root hair deformation normally occurred but *VsENOD5* and *VsENOD12* were not expressed. These observations show that both *VsENOD5* and *VsENOD12* are not involved in root hair deformation. The plant proteins responsible for root hair deformation remain to be determined.

The induction of *VsENOD5* and *VsENOD12* expression could be blocked by cycloheximide, indicating that the activation of the expression of these genes requires *de novo* synthesis of plant proteins. This further demonstrates that *VsENOD5* and *VsENOD12* are not among the first genes that are induced by Nod factors. In future studies on the mode of action of Nod factors effort should be put in the identification of plant genes that are induced within the first hour after Nod factor addition.

The expression of both *VsENOD5* and *VsENOD12* in *Vicia* roots is activated 8 h after Nod factor addition in the region where root hair deformation has occurred. By using RT-PCR for the detection of the specific mRNA's, we have not been able to localize exactly the expression of these genes in the root at that early stage. It was not possible to study whether *VsENOD5* and *VsENOD12* are activated in *V. sativa* root hairs because it is not feasible to isolate root hairs from plants grown in liquid medium. However, in pea root hairs the expression of these genes was induced after a similar incubation period (Horvath *et al.*, 1993), whereas in *Medicago ENOD12* is already induced in the epidermis within 2-3 hours (Journet *et al.*, 1994). At later stages *VsENOD12* is expressed in nodule primordia, but cortical cell division resulting in nodule primordia was not induced in the roots of the vetch plants grown in Fåhraeus slides under our experimental conditions. Therefore it seems plausible to assume that also in *V. sativa* roots both *VsENOD5* and *VsENOD12* expression is first induced in the root epidermis.

In Nod factor induced primordia *VsENOD12* expression is induced, whereas *VsENOD5* is not activated, even not when a 100 fold excess of Nod factor was used for induction of nodule primordium formation. So there is a striking difference with the responses in the epidermis, where both genes are activated by purified Nod factors.

In *Rhizobium* induced primordia there is a difference in the spatial distribution of cells expressing *VsENOD5* and *VsENOD12*. *VsENOD12* is expressed in all cells, whereas *VsENOD5* is only induced in cells infected by the bacteria. In the infection threads the bacterial *nod* genes are still expressed (Schlaman *et al.*, 1991), suggesting that the Nod factors are still made. These observations show that *VsENOD5* can only be induced by

Nod factors in cells which are in direct contact with the Nod factor, whereas *VsENOD12* is induced in epidermal cells that are directly in contact with Nod factors as well as in nodule primordia. This strongly suggests that the signal transduction pathway resulting in *VsENOD5* activation is different from the pathway controlling *VsENOD12* induction. The lack of *VsENOD5* expression in Nod factor induced primordia suggests that exogenously applied Nod factors do not reach the inner cortical cells. This implies that mitotic reactivation of cortical cells as well as *VsENOD12* and *VsENOD40* expression are induced by a diffusible secondary signal, generated by Nod factors.

Nod factor induced cortical cell division and early nodulin gene expression is induced in a spatially controlled way, despite the fact that the *V. sativa* roots were bathed in plant growth medium containing Nod factor. Cortical cell division only occurred opposite a proto-xylem pole and *VsENOD40* expression was only found in that part of the pericycle facing the nodule primordium just as in *Rhizobium* induced nodule primordia, showing that only certain pericycle and inner cortical cells are susceptible to Nod factors. Since primordia are preferentially formed opposite proto-xylem poles it is likely that a diffusible substance released at the xylem poles controls the susceptibility of cortical cells to Nod factors. The recently characterized stele factor (Kijne, pers. comm.), a plant compound released by the proto-xylem poles, is capable of inducing cell division in the inner cortex of pea root explants (Libbenga and Bogers, 1974). Whether this stele factor is involved in controlling the induced cell division and early nodulin gene expression in the primordia is not known. Also components involved in controlling *VsENOD40* expression in a specific region of the pericycle remains to be elucidated.

The Nod factor induced primordia in *V. sativa* roots never develop into genuine nodules. The *in situ* hybridization experiments showed that *ENOD2* expression was not induced in the primordia, showing that the development of the primordia is blocked before differentiation into nodule tissues starts. This suggests that for differentiation of the *V. sativa* primordia an additional signal is needed, which might be generated when infection by the bacteria takes place. This in contrast to other legumes where complete (empty) nodules are induced by Nod factors (Stokkermans and Peters, 1994; Truchet *et al.*, 1991).

Materials and Methods

Growth conditions for plants and bacteria

Vicia sativa spp. *nigra* seeds were sterilized and germinated for two days as described by Van Brussel *et al.* (1982). 3 day old seedlings were planted in gravel and grown as described by Moerman *et al.* (1987). *Rhizobium leguminosarum* bv *viciae* strain

248 was grown in liquid YEM medium (Bhuvaneswari *et al.*, 1980) at 28 °C. Inoculation with *R.l.* bv *viciae* 248 occurred when the seedlings were planted in gravel.

Root hair deformation assay

Vicia sativa spp. *nigra* seeds were sterilized and germinated for two days as described by Van Brussel *et al.* (1982). Seedlings were transferred to modified Fåhraeus slides (Bhuvaneswari and Solheim, 1985) and treated as described by Heidstra *et al.* (1994). Each slide contained 6 plants. Before applying NodRlv factors the roots of the plants were examined microscopically and slides containing roots with deformed root hairs were discarded. The plant growth medium (pgm) (Fåhraeus, 1957) was replaced by pgm containing NodRlv factor (NodRlv-V(Ac, C18:4)) at a final concentration of 10^{-10} M and as a negative control in some slides the medium was replaced by pgm without NodRlv factor. Deformation was examined 3 h after addition of NodRlv factor. At least two Fåhraeus slides were used for each incubation and deformation was scored blindly by two persons.

A similar assay was done to check the effect of specific inhibitors. In these experiments the pgm containing 10^{-10} M NodRlv-V(Ac, C18:4) was replaced by pgm containing 10^{-10} M NodRlv-V(Ac, C18:4) with 20 µM actinomycin D (AcD) or 100 µM cycloheximide (CHX) at 0, 1/2, 3/4, 1 and 1 1/2 h after the start of the incubation with Nod factor. Root hair deformation was scored as described above. The concentration of CHX and AcD needed was determined by applying different concentrations of each inhibitor to *V. sativa* roots and investigation of the effect on the growth of the root tip after 48 hours. The growth of root tips was completely inhibited at a concentration of 100 µM or higher for CHX and 20 µM or higher for AcD. At 100 µM CHX and 20 µM AcD cytoplasmic streaming in the root hairs was not affected, showing that root hairs are still alive. These concentrations were therefore used in the experiments.

PCR assays to detect VsENOD5 and VsENOD12 mRNA

The susceptible zone of 60 *Vicia sativa* spp. *nigra* plants, treated with 10^{-10} M NodRlv factors for 0, 3 or 8 h, was collected in liquid nitrogen. Total RNA was isolated (Pawlowski *et al.*, 1994) and quantified using spectrophotometry and agarose gel electrophoresis. Only RNA preparations having both A_{260}/A_{280} and $A_{260}/A_{240} \geq 2$ were used for further studies. Normal yield was 15-30 µg of total RNA. Total RNA from two week old nodules was used as a positive control.

The *VsENOD12* and *VsENOD5* mRNA levels were determined by the following

PCR assay as described by Horvath *et al.* (1993): 1µg of total RNA and 200 ng of 3'-primer 1 (*VsENOD12*) or 3 (*VsENOD5*) (table 2) were incubated in 10 µl annealing buffer (250 mM KCl, 10 mM Tris-HCl pH 8.3, 1 mM EDTA) for 3 min at 85°C and subsequently for 30 min at 38°C (*VsENOD12*) and 52°C (*VsENOD5*). After gradual cooling to 42°C, 15 µl of reverse transcription buffer (24 mM Tris-HCl pH 8.3, 16 mM MgCl , 8 mM DTT, 0.4 mM dNTPs) including 2.0 U AMV reverse transcriptase (Stratagene) was added and cDNA was synthesized at 42°C for 1 h. Then 75 µl amplification buffer (33.3 mM KCl, 7.2 mM Tris-HCl pH 8.3, gelatin 0.01 w/v) supplemented with 1 µl of 25 mM dNTPs, 1,5 U AmpliTaq DNA Polymerase (Perkin Elmer, Cetus) and 200 ng 5'-primer 2 (*VsENOD12*) or 4 (*VsENOD5*) (table 2) was added to the cDNA samples. The *VsENOD12* cDNA was amplified during 23 cycles (94°C, 1 min; 38°C, 1 min; 72°C, 3 min), while amplification of *VsENOD5* cDNA was carried out during 25 cycles (94°C, 1 min; 52°C, 1 min; 72°C, 3 min). 5 µl of the amplified DNA samples were run on a 2% agarose gel, and after blotting to Hybond-N+ (Amersham) hybridized to ^{32}P-labelled inserts of pVsENOD12 or pVsENOD5 (Sambrook *et al.*, 1989).

Ubiquitin (*ubi*) mRNA was used as an internal control (Horvath *et al.*, 1993). The *ubi* gene consists of five multiple units (Watts and Moore, 1989). The oligonucleotides 5 (3') and 6 (5') (table 2) hybridize with the ends of each single unit. After 25 cycles of amplification (94°C, 1 min; 50°C, 1 min; 72°C, 1.5 min) 3 of these 5 bands are clearly visible.

Table 2. Oligonucleotides used in the PCR assays. Nucleotides printed in bold are mismatches between pea (Scheres *et al.*, 1990a; Scheres *et al.*, 1990b) and *Vicia sativa* (Chapter 2) cDNA clones.

Oligonucleotides	Positions
1 5'-CATAAGATGGTTTTGTCACG-3'	Compl. to nt 272-253 of the *VsENOD12* cDNA
2 5'-CTTGTCCTCACTAGTGTTGT-3'	homologous to nucleotide 22-41 of *VsEnod12* cDNA
3 5'-AATTAAGAACATCAACATAG-3'	Compl. to 348-367 of *VsENOD5* cDNA
4 5'-CTTCTATTTTCCTACTCAGA-3'	homologous to nt 20-39 of *VsENOD5* cDNA
5 5'-ACCACCACG^{G}/$_{A}$AGACGGAG-3'	Compl. to the 3' end of a repeating ubiquitin unit
6 5'-ATGCAGAT^{C}/$_{T}$TTTGTGAAGAC-3'	Compl. to the 5' end of a repeating ubiquitin unit

Labelling of antisense/sense RNAs

For preparing antisense and sense RNAs 5' fragments of pVsENOD5, pVsENOD12 and pVsENOD40 were subcloned into pKS(+) vector (Stratagene). Of pVsENOD5 the 230 bp *Eco*RI-*Stu*I fragment and of pVsENOD12 the 190 bp *Eco*RI-*Rsa*I fragment was

subcloned in the *Eco*RI-*Sma*I digested pKS(+) vector. Of pVsENOD40 the 430 bp *Eco*RI-*Spe*I fragment was subcloned into analogous digested pKS(+) vector. The plasmids were digested with *Eco*RI before antisense RNA was made with T7 polymerase. Before making sense RNA with T3 polymerase the plasmids containing the *VsENOD5* and *VsENOD12* fragments were digested with *Bam*HI, the plasmid containing the *VsENOD40* fragment was digested with *Spe*I. The probes were radioactively labelled with [^{35}S]-UTP (1000-1500 Ci mmol^{-1}, Amersham) and degraded to about 150 nucleotides long fragments before hybridization (Van de Wiel *et al.*, 1990). Hybridization with ^{35}S-labelled sense RNAs did not show any hybridization (data not shown).

In situ hybridization

To study the *in situ* expression of early nodulin genes at early stages of nodule development, seedlings were grown on 2% B&D agar plates (Broughton and Dilworth, 1971) for 5 days, before *R. leguminosarum* bv. *viciae* strain 248 was spot-inoculated on the roots (Yang *et al.*, 1994). Four days after spot-inoculation the inoculated part of the roots was harvested and fixed in 4% paraformaldehyde supplemented with 0.25% glutaraldehyde in 10 mM sodium phosphate buffer for 3 h (Van de Wiel *et al.*, 1990). Fixed material was dehydrated and embedded into paraffin by routine methods (Van de Wiel *et al.*, 1990; Yang *et al.*, 1991). Sections (7 µm thick) were hybridized with [^{35}S]-UTP labelled antisense or sense RNA probes according to a procedure derived from Cox and Goldberg (1988) (Van de Wiel *et al.*, 1990). Sections exposed for 2 to 4 weeks were stained with toluidine blue and photographed with a Nikon microscope with dark field and epipolarization optics.

Plant growth conditions for the induction of Nod factor induced primordia

Vicia sativa spp. *nigra* seeds were sterilized and germinated for two days as described by (Van Brussel *et al.*, 1982). Seedlings were placed in amber glass containers of 30 ml filled with plant growth medium (pgm) (Fåhraeus *et al.*, 1957) containing the NodRlv factor (NodRlv-V(Ac, C18:4) at a final concentration of 10^{-9} M (Spaink *et al.*, 1991). Six days after transferring the seedlings to the glass containers, the roots were harvested and fixed. *In situ* hybridization was performed as described above.

CHAPTER 6

General discussion

Introduction

Nod factors play a pivotal role in the induction of early steps of nodule formation. The aim of the research described in this thesis was to gain more insight in the mechanisms by which the Nod factors induce these steps. Such an investigation requires a host plant in which several approaches can be combined in an efficient way. We have chosen *Vicia sativa* for our studies and in this chapter we will discuss first how suitable *V. sativa* is as host plant for studying the mode of action of Nod factors and then, in which ways the studies described in this thesis have contributed to elucidate the mechanism by which Nod factors elicit a diversity of plant responses.

Vicia as host plant to study the mode of action of Nod factors.

In chapter 1 of this thesis we have discussed why *Vicia sativa* could be a suitable host plant to study the mode of action of Nod factors secreted by *Rhizobium leguminosarum* bv. *viciae* (NodRlv factors). Main advantages are the size of the plant and the availability of assays in which the biological activity of the NodRlv factors, like root hair deformation and nodule primordium formation, can easily be tested.

At the time the work described in this thesis was started two essential tools for studying Nod factor activity on the molecular level were missing in *V. sativa*. Early nodulin genes from *V. sativa* were not yet cloned and there was no transformation and regeneration protocol for *V. sativa* available. We have isolated homologues of early nodulin genes from *V. sativa* (Chapter 2). This has enabled us to study the expression of these genes during Nod factor induced processes (Chapter 5). Meanwhile, a transformation system was developed for *V. hirsuta* and we have used this system for studies on the mechanisms controlling early nodulin gene expression (Chapter 3 and 4). So, many conditions for studying the mode of action of the Nod factors at the molecular level in *Vicia sativa* are now fulfilled.

However, for the identification of plant genes involved in root nodule formation *V. sativa* is not very suitable since the genetics of *V. sativa* is hardly developed and, for example, genes essential for normal nodulation, so-called *Sym* genes, have not been identified in *V. sativa*. On the other hand the genetics of pea has been studied extensively and various pea mutants disturbed in the interactions with *Rhizobium* are available and characterized. In pea at least 20 different *Sym* genes have been identified (Kneen *et al.*, 1994). The high degree of conservation of early nodulin genes of *V. sativa* and pea (Chapter 2) suggests that genetic information can easily be exchanged between both plant species and it seems plausible that, when in the future a *Sym* gene has been cloned

from pea, it will be possible to isolate the corresponding gene from *V. sativa* using the pea gene as a probe. Pursuing the properties of such *Sym* genes might conveniently be studied in *Vicia* using the transformation system for *Vicia hirsuta*.

The use of transgenic plants in studies on root nodule formation

There is a general demand for a good transformation system for legumes to study root nodule formation. The last few years new *Agrobacterium tumefaciens* mediated or biolistic transformation protocols have been described for pea, soybean and alfalfa, but for the time being, the methods for transformation are not very efficient and regeneration of transgenic plants takes a long time (for review see Christou *et al.*, 1993). One exception might be the *A. tumefaciens* mediated transformation of *Lotus japonicus* (Handberg and Stougaard, 1992), which goes efficiently, and by which transgenic plants can be obtained within 5 months. *L. japonicus* is a diploid self-fertilizer with a regeneration time of approximately 3-4 months that makes it a suitable plant for both classical and molecular genetic studies. Therefore *L. japonicus* has been proposed as a model plant to analyse the symbiosis between rhizobia and leguminous plants forming determinate nodules. However, the mutant collection of *Rhizobium loti* is not extensive and the Nod factors secreted by *R. loti* have not yet been identified.

An alternative transformation system uses the induction of transgenic hairy roots on the stem of legumes, such as *Lotus corniculatus* and *Vicia hirsuta*, with *A. rhizogenes* (Hansen *et al.*, 1989; Quandt *et al.*, 1993). This system has successfully been used for promoter studies of several nodulin genes, like, for example, leghemoglobin, *ENOD2* and *ENOD12* (Lauridsen *et al.*, 1993; Stougaard *et al.*, 1987; Vijn *et al.*, 1995) (see Chapter 3). The advantage of the *A. rhizogenes* transformation system is that it is very fast, since within 4-6 weeks transgenic root nodules are formed. A disadvantage is that the phytohormone balance in the transgenic root is changed, due to the introduction of *A. rhizogenes* phytohormone encoding genes. This changed phytohormone balance might in some cases interfere with *Rhizobium* or Nod factor induced processes. For example, root nodules are not induced on hairy roots of pea after inoculation with *Rhizobium* (Christiansen, pers. comm.) and in transgenic *V. hirsuta* roots, Nod factor induced nodule primordium formation is less efficient than in untransformed *V. sativa* roots (Chapter 3). Furthermore, root hair deformation can not be studied with transgenic *V. hirsuta* roots, since the root hairs on the transgenic hairy roots do not show root hair deformation in a temporally and spatially controlled way as observed for untransformed *V. sativa* root hairs (René Geurts, pers. comm.). Therefore *A. rhizogenes* induced transgenic hairy roots appear to be not very suitable for studies on Nod factor induced processes.

A. tumefaciens transformed *Medicago varia* plants have been used to study Nod factor induced expression of *ENOD12* (Journet *et al.*, 1994), suggesting that an *A. tumefaciens* mediated transformation might be useful for this type of studies. Although gene expression in the transgenic *M. varia* plants could not be quantified, due to the varying levels of expression in the plants from the S1 seeds, it was possible to perform a structure-function analysis of different Nod factors. At the moment *M. truncatula*, a diploid self-fertile plant, is used for transformation, which will make quantification of gene expression possible in the future (Journet *et al.*, 1994).

Studies on early nodulin gene expression to elucidate the mode of action of Nod factors

For the further elucidation of the mechanism by which the Nod factors elicit a diversity of plant responses, like root hair deformation and the induction of nodule primordia, questions whether the target of the Nod factor is only located at the outside of the root, or whether the Nod factor or secondary signal molecules derived from the Nod factor, also reach the inner cortical cells should be answered. Studies on the localization of the Nod factor are, however, not easily to perform. For localization the Nod factors have to be labelled. Radioactive-labelled Nod factors can be prepared *in vivo* by adding ^{14}[C]acetate to a culture of a Nod factor overproducing *Rhizobium* strain or, alternatively, by reduction of the double bonds in the acyl chain of the Nod factor with $^{3}H_2$ (Roche *et al.*, 1991; Spaink *et al.*, 1991), without complete loss of biological activity. However, the obtained specific activity of factors labelled in these manners is too low to be used for localization of Nod factors inside the root. To obtain labelled Nod factors with a higher specific activity, extra labelled groups can be added to the Nod factors, but this usually results in a decrease of biological activity (Heidstra, pers. comm.). Another problem in localization studies is that Nod factors are small molecules that are difficult to cross-link and that they are very soluble in organic solvents, used in embedding plant material for preparing sections for microscopic examination. Therefore we tried to get information about how, and where in the root, Nod factors are active by studying the mechanisms controlling the induction of *PsENOD12* expression and by investigating early nodulin gene expression during different Nod factor responses.

ENOD12 is one of the first early nodulin genes that are induced by Nod factors (Horvath *et al.*, 1993; Journet *et al.*, 1994). As a step towards the cloning of transcription factors controlling *ENOD12* expression we identified the region of the *PsENOD12* promoter necessary for nodule specific expression, using *A. rhizogenes* mediated transformation of *V. hirsuta*. For the promoter analysis this transformation system appeared to be very useful and we found that a region of 200 bp immediately upstream of the transcription start of

ENOD12 is essential for nodule specific and Nod factor induced expression (Chapter 3). Within these 200 bp the relevant *cis-acting* elements will be located.

As our final interest was to identify transcription factors involved in controlling *ENOD12* expression we screened an expression library with double stranded, overlapping oligonucleotides, covering the 200 bp region of the *PsENOD12* promoter, to isolate DNA-binding proteins. This screening resulted in the isolation of 5 clones, containing cDNA inserts encoding DNA-binding polypeptides (Chapter 4). Although the results described in Chapter 4 concern partial cDNA clones and need to be completed, they show that the screening of the library was successful in picking up DNA-binding proteins. The results of *in situ* hybridization experiments showed that the expression pattern of one of the isolated clones overlaps with *ENOD12* expression. Further research is required to show that the cloned polypeptides are involved in controlling *ENOD12* expression.

Although the exploratory experiments in the promoter region controlling *ENOD12* expression as described in this thesis went on rather successful, we may ask ourselves whether such studies will provide more insight in the signal transduction pathway that is elicited by Nod factor and results in specific gene expression. The results of the experiments described in chapter 5 showed that *ENOD12* expression is first induced in root tissue 8 h after addition of Nod factor and that within that time period other proteins need to be synthesized before induction of *ENOD12* can take place. This suggests that the signal transduction pathway starting with the Nod factor does not directly result in *ENOD12* expression, but is most likely more complicated.

The expression of early nodulin genes during Nod factor induced nodule primordium formation was studied with *in situ* hybridization. These experiments showed that in Nod factor induced primordia *ENOD12* and *ENOD40* are expressed, while *ENOD5* is not expressed. This raised the question why *ENOD5* is not expressed in Nod factor induced nodule primordia, while during root hair deformation exogenously applied Nod factors trigger the expression of both *ENOD5* and *ENOD12*. In *Rhizobium* induced nodule primordia *ENOD5* is only expressed in cells containing the infection thread (Chapter 2). Since in the infection threads the bacterial *nod* genes are expressed (Schlaman *et al.*, 1991) it is likely that Nod factors are then still produced. This suggests that *ENOD5* is only induced by Nod factors in cells that are directly in contact with the Nod factor, whereas *ENOD12* is induced in epidermal cells that are directly in contact with Nod factors as well as in primordia (Chapter 5). However, also the expression of *ENOD5* is first detectable in root tissue 8 h after the addition of Nod factors and the synthesis of new proteins is required before induction of *ENOD5* can take place (Chapter 5), showing that the Nod factor does not directly induce *ENOD5* expression. The observation that *ENOD5* is not expressed in Nod factor induced primordia suggests that exogenously applied Nod factors are not transported towards the primordia. This implies that *ENOD40* and *ENOD12* expression in Nod factor

induced primordia is most likely not induced by the Nod factor itself but by a diffusible secondary signal, generated by the Nod factors. Furthermore it suggests that the signal transduction pathway from Nod factor to *ENOD5* expression is not the same pathway as from Nod factor to *ENOD12* expression.

For more detailed studies on the mode of action of Nod factors, the identification of genes that are induced within 30 min after Nod factor addition, the time period in which the genes necessary for root hair deformation are transcribed (Chapter 5), seems now essential. This will require methods which enable the isolation of genes that are only expressed in the limited number of cell types affected by Nod factors. The subtractive hybridization-polymerase chain reaction-based technique described by Wang and Brown (1991) and the differential RNA display technique (Liang and Pardee, 1992) have recently been developed for the isolation of genes expressed in cells under specific conditions of development. These techniques might provide the tools to achieve the identification and isolation of the genes induced shortly after addition of Nod factors to the roots.

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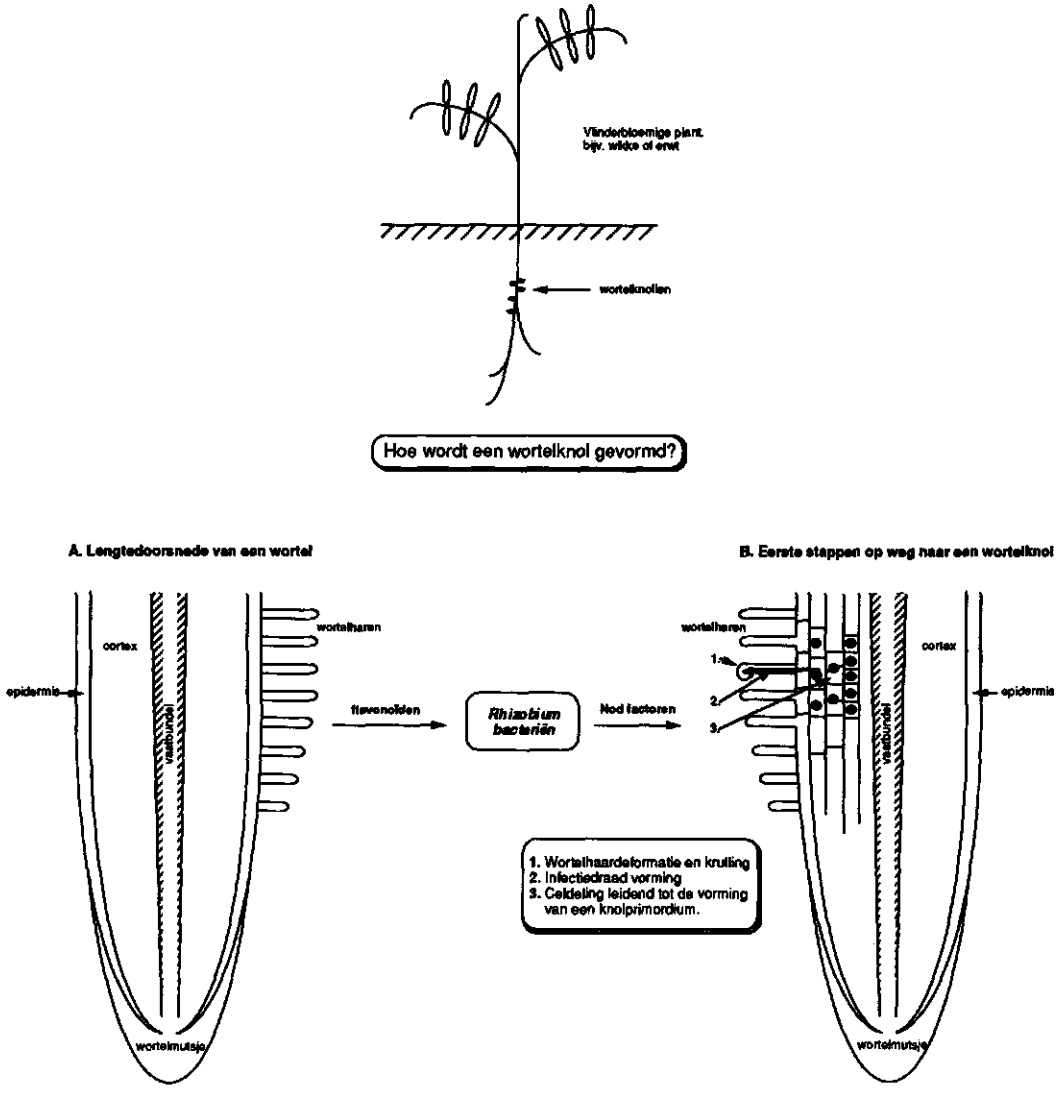
Samenvatting



Figuur 1. Schematische weergave van de eerste gebeurtenissen tijdens de wortelknolvorming. Flavonoïden zijn door de plant uitgescheiden signaalstoffen (A) die de bacteriën aanzetten tot het maken van signaalstoffen, Nod factoren genoemd. Deze Nod factoren induceren in de plant de eerste stappen van de worteknolvorming (B).

Vlinderbloemige planten, zoals erwt, wikke, klaver en soja, kunnen in symbiose met bacteriën van de geslachten *Rhizobium, Bradyrhizobium* of *Azorhizobium* stikstof uit de lucht binden en de zo gebonden stikstof gebruiken voor hun groei en ontwikkeling. Tijdens deze symbiose vormt de plant op de wortels speciale organen, wortelknollen, waarin de juiste omstandigheden zijn gecreëerd voor de bacterie om vrije stikstof uit de lucht om te zetten in ammonia.

Net als bij de ontwikkeling van andere planteorganen, zoals bloemen en stengels, doorloopt de vorming van een wortelknol een aantal ontwikkelingsstadia (voor een schematische weergave zie figuur 1). Het begint met de specifieke herkenning van plant en bacterie. De plant scheidt signaalstoffen uit, flavonoïden, die door de bacterie herkend worden en die er voor zorgen dat de bacterie zogenoemde Nod factoren gaat produceren en uitscheiden. Deze Nod factoren zijn moleculen die bestaan uit enkelvoudige ketens van 4 of 5 suikerresiduen met een vetzuurketen aan de niet-reducerende eindstandige suiker (lipo-oligosacchariden). Deze lipo-oligosacchariden worden Nod factoren genoemd omdat ze in staat zijn de eerste ontwikkelingsstadia van de knolvorming (Engels: nodule formation) in de wortels van de vlinderbloemige plant te induceren.

Hoe wordt een wortelknol gevormd? (zie ook figuur 1). Het eerst zichtbaar is deformatie en krulling van de wortelharen. In de gekrulde wortelharen wordt een infectiedraad gevormd, waardoor de bacteriën de plant kunnen binnendringen. Tegelijkertijd worden in de binnenste cortex van de wortel cellen aangezet tot deling en die delende cellen vormen het knolprimordium. De infectiedraden groeien van de wortelharen naar dit knolprimordium en daar aangekomen worden de cellen geïnfecteerd met de *Rhizobium* bacteriën. Het primordium groeit intussen uit en er ontstaat een volwassen wortelknol, waarin stikstoffixatie plaats vindt.

Tijdens de knolvorming worden zowel in de plant als in de bacterie specifieke eiwitten aangemaakt. In de plant worden deze eiwitten nodulines genoemd. De stukken DNA (genen) die voor nodulines coderen zijn de noduline genen. De noduline genen die tijdens de ontwikkeling van de wortelknol tot expressie komen, voordat stikstoffixatie plaatsvindt, worden vroege noduline genen genoemd (*ENOD* genen). De vroege noduline genen komen op verschillende tijdstippen van de wortelknolontwikkeling en in verschillende cellen en weefsels tot expressie en kunnen gebruikt worden als moleculaire merkers van de opeenvolgende ontwikkelingsstadia.

Uit eerdere studies was gebleken dat naast wortelhaar deformatie en de vorming van knolprimordia, ook de expressie van enkele vroege noduline genen geïnduceerd kan worden door de Nod factoren. Daardoor werd het mogelijk om de expressie van deze noduline genen te gebruiken als moleculaire merkers van Nod factor geïnduceerde processen. Door deze processen op moleculair niveau te bestuderen is het mogelijk ook een antwoord te vinden op de vraag hoe de Nod factoren deze processen induceren.

Experimenten om deze vraag te beantwoorden en meer inzicht te krijgen in die processen, lopen als een rode draad door dit proefschrift.

Om de wortelknolvorming te kunnen bestuderen is het nodig om over een plant-bacterie systeem te beschikken waarin de eerste ontwikkelingsstadia zichtbaar gemaakt kunnen worden. Omdat wij werken met *Rhizobium leguminosarum* bv. *vicia* wordt de keuze van gastheer planten beperkt tot de genera *Pisum*, *Lens*, *Lathyrus* en *Vicia*. In Hoofdstuk 1 van dit proefschrift staat beschreven waarom wij voor *Vicia sativa* (voederwikke) gekozen hebben. De belangrijkste voordelen van voederwikke zijn dat het een relatief kleine plant is, zodat het kweken weinig ruimte inneemt, en dat Nod factor geïnduceerde processen, zoals wortelhaardeformatie en de vorming van knolprimordia, gemakkelijk met een microscoop waargenomen kunnen worden.

Omdat Nod factor geïnduceerde wortelhaardeformatie en vorming van knolprimordia goed bestudeerd kunnen worden in *V. sativa* en wij verband willen proberen te leggen tussen de expressie van vroege noduline genen en deze processen (zoals later beschreven wordt in Hoofdstuk 5) zijn cDNA klonen geïsoleerd van een aantal vroege noduline genen (*ENOD5*, *ENOD12* en *ENOD40*) van *V. sativa* en met behulp van de *in situ* hybridisatie techniek is hun expressie tijdens de wortelknolvorming bestudeerd. De resultaten van deze experimenten staan beschreven in hoofdstuk 2.

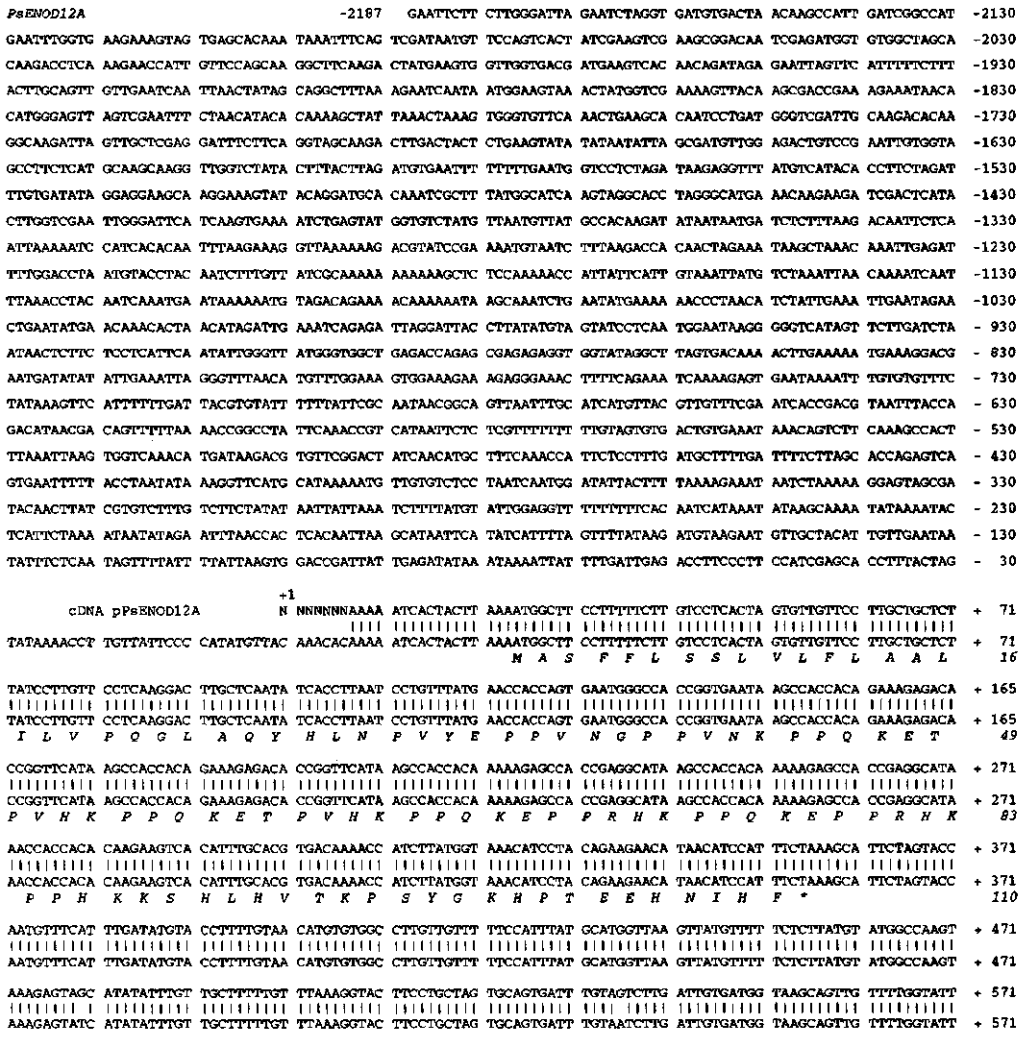
De expressie van één van deze vroege noduline genen, *ENOD12*, wordt door de Nod factoren geïnduceerd binnen enkele uren. Hierdoor leek het mogelijk dat de afgelegde weg van Nod factoren naar genexpressie relatief kort zou zijn. Omdat opheldering van de mechanismen die de genexpressie van *ENOD12* reguleren inzicht zouden kunnen geven over hoe Nod factoren genexpressie induceren werd besloten om na te gaan hoe *ENOD12* expressie gereguleerd wordt. In het algemeen wordt genexpressie gereguleerd door de interactie van verschillende DNA-bindende eiwitten ("*trans*-acting factors") met specifieke sequenties ("*cis*-acting elements") in de promoter van een gen. Met behulp van een promoter analyse kunnen de belangrijke sequenties in de promoter opgespoord worden. In hoofdstuk 3 staan de resultaten beschreven van de promoter analyse van *ENOD12*. Uit deze analyse bleek dat 200 baseparen voldoende zijn om knolspecifieke expressie te krijgen. Deze 200 baseparen liggen onmiddelijk voor de transcriptiestart van het gen. De volgende stap was bindende eiwitten, "trans-acting factors", te isoleren. Daartoe werd een cDNA expressie-bank gescreened met een mengsel van 40 tot 50 baseparen lange, dubbelstrengs oligonucleotiden die samen het gehele 200 baseparen fragment bestreken. Op die manier werden uit de expressie-bank 5 cDNA's geïsoleerd die coderen voor DNA-bindende eiwitten (Hoofdstuk 4). Twee van deze cDNA's coderen voor histone H1 eiwitten. De andere 3 cDNA's coderen polypeptiden die geen homologie vertonen met reeds bekende eiwitten, maar deze polypeptiden bevatten repeats, AT-hooks, waarvan bekend is dat ze betrokken zijn bij het binden van DNA. Verder kruishybridiseren 2 van

deze 3 cDNA's en coderen delen van hetzelfde polypeptide. Het expressie patroon van de geïsoleerde cDNA's is bestudeerd met behulp van de *in situ* hybridisatie techniek. Hieruit bleek dat de expressie van de kruishybridiserende cDNA's overlapt met de expressie van *ENOD12*. In de toekomst zal verder onderzocht worden of het eiwit dat gecodeerd wordt door deze cDNA's ook werkelijk betrokken is bij de regulatie van de expressie van het vroege noduline gen *ENOD12*.

In Hoofdstuk 5 zijn studies naar Nod factor geïnduceerde processen gecombineerd met studies naar de expressie van vroege noduline genen. Daarvoor is de expressie van *ENOD5* en *ENOD12* bestudeerd tijdens wortelhaardeformatie en verder is met behulp van de *in situ* hybridisatie techniek gekeken welke vroege noduline genen tot expressie komen in de door Nod factoren geïnduceerde knolprimordia. Een belangrijke conclusie uit de proeven die in dit hoofdstuk worden beschreven is dat *ENOD12* expressie slechts door de Nod factoren geïnduceerd kan worden nadat eerst andere eiwitten gemaakt zijn. De eerdere veronderstelling dat *ENOD12* één van de eerste genen zou zijn die rechtstreeks aangeschakeld worden door de Nod factoren is hiermee vervallen. Verder bleek uit de *in situ* hybridisatie experimenten dat *ENOD5* niet tot expressie komt in Nod factor geïnduceerde primordia, terwijl Nod factoren *ENOD5* expressie wel aanschakelen in de epidermis. In door *Rhizobium* geïnduceerde wortelknollen komt *ENOD5* alleen tot expressie in cellen die een infectiedraad bevatten, waarin zich bacteriën bevinden die nog Nod factoren produceren. Dit wijst er op dat de expressie van *ENOD5* alleen aangeschakeld kan worden in cellen die direct contact hebben met de Nod factor en maakt het onwaarschijnlijk dat de Nod factor zelf getransporteerd wordt in de plant van de wortelharen naar het zojuist gevormde knolprimordium. Toch wordt ook de expressie van het *ENOD5* gen niet direct door de Nod factor aangeschakeld, want ook voor de inductie van *ENOD5* expressie moeten eerst andere eitwitten gemaakt worden, zoals ook in hoofdstuk 5 wordt beschreven. Omdat *ENOD12* en *ENOD40* wel tot expressie komen in Nod factor geïnduceerde primordia wordt de expressie van deze genen waarschijnlijk aangeschakeld door een vervolgsignaal en niet door de Nod factor zelf. Deze resultaten wijzen er tevens op dat de signaaltransductie van Nod factor naar *ENOD5* expressie niet hetzelfde is als van Nod factor naar *ENOD12* expressie.

Tenslotte wordt in Hoofdstuk 6 bediscussieerd in welke mate *V. sativa* en het onderzoek, beschreven in dit proefschrift, hebben bijgedragen aan het vinden van antwoorden op de vraag hoe Nod factoren wortelknolvorming induceren.

Appendix 1: Nucleotide sequence of *PsENOD12A*.The genbank accession number is X81366



Appendix 2: Nucleotide sequence of the cDNA clone pVsENOD5. The genbank accession number is X83681.

The deduced amino acid sequence is printed in italics.

```
  1  GATCATTTTC TCAATGTGGC TTCTATTTTC CTTCTCAGAA TCCACAGAAT
  1   I  I  F   S  M  W  L   L  F  S   F  S  E   S  T  E  Y

 51  ATATTGCTGG AGACTCTGAA AGTTCATGGA AGGTTAACTT TCCATCACGA
 18    I  A  G   D  S  E   S  S  W  K   V  N  F   P  S  R

101  GAAGCACTCA TCGATTGGGC CACTAGACAC CAATTCACAT ACAGTGATAC
 34  E  A  L  I   D  W  A   T  R  H   Q  F  T  Y   S  D  T

151  TGTTGTCAAT GAGGATGAGG ATCATGACTG TAATACAAAG ATTCATTCCA
 51   V  V  N   E  D  E  D   H  D  C   N  T  K   I  H  S  K

201  AGCTAGGTGA TATGGTTGTT ACAAAGAGGC CTCTAGTTCT CCCACCTTTG
 68    L  G  D   M  V  V   T  K  R  P   L  V  L   P  P  L

251  ATTACCTTGC CACTCTCACC TTCGCCGGCA CCAGCACCAA ATACGTCCGG
 84  I  T  L  P   L  S  P   S  P  A   P  A  P  N   T  S  G

301  AGCTGCTGCG GGCTGTGGAT TCATGGCGTT TTTGGAGGTT TCAGTGGCTA
101   A  A  A   G  C  G  F   M  A  F   L  E  V   S  V  A  M

351  TGTTGATGTT CTTAATTTGG CTATAGTTGT TTGTTTCTTT AATAAGCTGA
118    L  M  F   L  I  W   L  *

401  ATGACTACAT GCATGAACAT GCAACAGATA TGTGTGTGCG GTGAAGGTCA

451  ATTACATCCT TATTTTGTTT TCATGTTTCC TTAATTTTTA TGGATAATAA

501  AGGCAGTTGT TAGTTTTTTT TGTTGATCAA AAAAAAAAA
```

Appendix 3: Nucleotide sequence of the cDNA clone pVsENOD12. The genbank accession number is X83682.

The deduced amino acid sequence is printed in italics.

```
  1  ACTTAAAATG GCTTCCTTTC TCTTGTCCAC ACTAGTTTTC TTCCTTGCTG
  1        M   A  S  F  L   L  S  T   L  V  F   F  L  A  A

 51  CTCTTATCCT TGTTCCTCAA GGACTTGCTC AATATCACCT TAATCCTGTT
 16    L  I  L   V  P  Q   G  L  A  Q   Y  H  L   N  P  V

101  TATGAGGCAC CAGTGAATGG GCCACCGGTG AATAAGCCAC CACAGAAAGA
 32  Y  E  A  P   V  N  G   P  P  V   N  K  P  P   Q  K  E

151  GACACCGGTG CAAAAACCAC CACAAAAAGA GCCACCGGTA CATAAATCGC
 49   T  P  V   Q  K  P  P   Q  K  E   P  P  V   H  K  S  P

201  CACGAAATGA GCCACCTAGG CATAAGCCAC CACACAAGAA ATCACATTTG
 66    R  N  E   P  P  R   H  K  P  P   H  K  K   S  H  L

251  CACGTGACAA AACGATCTTA TGGTAAACAT GCTACAGAAG AACATAGCAT
 82  H  V  T  K   R  S  Y   G  K  H   A  T  E  E   H  S  I

301  CCATTTCTAA AGCATTCTAG TATCAATGTT TTATTTTATA TGTACTTTCT
 99   H  F  *

351  GTAACATGTG TGGCCAAGTT GTTTTTTCTG TTATGTATGG TCTCAAGTTG

401  TGTTTTTTTC CCCTTATATG TATGTATGGC CAAGTTAAGA GGTATTTCCT

451  GTTCCTGCTA ATGCTATGAT TTGTAATCTT CTAGTTCTAT CTGGACACAG

501  TATTGTGTTG GGGTATAACT GTTTCAGTAA ATTTTCAATT AAAAAAAG
```

Appendix 4: Nucleotide sequence of the cDNA clone pVsENOD40. The genbank accession number is X83683.

The amino acid sequence of the small polypeptide is printed in italics.

```
  1  CCTTTAACCA TCCTCTAAAC CAATCCACTA TCAAACACTT TGAATCTTTG

 51  TTATTAGCAA TGAAGCTTCT TTGTTGGCAA AAATCAATCC ATGGTTCTTA
  1           M   K  L  L   C  W  Q   K  S  I  H   G  S  *

101  AAATCAAACA TGGAGAGAAG TGTGAGAGGG TTAATTAGTT GGTACTAATT

151  ATGATTAGTA CTAATTACAT CATCCTTTAC ACTCTCCCTC CATTTTTACA

201  AAAACAGTTT GCTTTGTGCT TTAGCTATTG GCTTCTCATA TCATAAAGGG

251  ATGTGCTTTT TTCTGAGTAT CAGAAGCAAA TAATTAAGCA TTTTTCTCTA

301  TTGGATCAGA AGCTTTGGTT ATAGTATGGC AAACCGGCAA GTCACAAAAA

351  GGCAATGGAT TCCTTTTTGG AGTCTTAATG GCTATGTATC AATCACTCTA

401  TCTGGTATTA TCATCTATGA AGTATAGTAC TAGTACCGAC ACCTTCAGAT

451  TAAAGACATG TCTGATGTTC TACAAATGTG TTAGTGTCCA GCGTCTGGTG

501  TCTGTGCTTT GTAGCTAAAC TATTATAGTT CTTCTTGTAG TAgggATGTA

551  ATAATAAACA TAGAGATGGT GTTGTCTTCC TTTGAGAAGT TTCCAACTTT

601  GTGATGTACT TCAAATTCAC TCAATTTGCA GCTGATCCTA GAGTCTGTTT

651  CTTGTTTCAG TTTCTGCATG TAAGGTAGGT AACTGTTATC ATTAATTCCT

701  GTTTCTTTTA AAAAAAAA
```

Curriculum vitae

Op 10 december 1964 ben ik in Hengelo (Ov) geboren. In 1983 behaalde ik het eindexamen Gymnasium ß (Lyceum de Grundel, Hengelo) en begon in datzelfde jaar met de studie Moleculaire Wetenschappen aan de Landbouwuniversiteit te Wageningen. De ingenieursstudie omvatte de hoofdvakken Moleculaire Biologie (prof. dr A. van Kammen), Immunologie (prof. dr W. van Muiswinkel) en Erfelijkheidsleer (prof. dr C. Heijting). De afstudeeropdracht voor de vakgroep Erfelijkheidsleer werd uitgevoerd in het Department of Toxicology, Sandoz Ltd. te Basel, Zwitserland (dr T.S.B. Zwanenburg). De stageperiode voor de vakgroep Moleculaire Biologie werd doorgebracht in het Department of Molecular Biology van het Massachusetts General Hospital/Harvard Medical School te Boston, USA (prof. F. Ausubel). In maart 1990 behaalde ik het ingenieursdiploma. Van juli 1990 tot juli 1994 was ik als onderzoeker in opleiding (OIO) in dienst bij de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO). Het onderzoek, beschreven in dit proefschrift, werd uitgevoerd onder leiding van dr T. Bisseling en prof. dr A. van Kammen bij de vakgroep Moleculaire Biologie van de Landbouwuniversiteit te Wageningen. Sinds 1 december 1994 ben ik als wetenschappelijk projectmedewerker (post-doc) in dienst bij NWO op een project gefinancierd door de Stichting Technische Wetenschappen (STW) en werkzaam bij de projectgroep Moleculaire Genetica van de vakgroep Moleculaire Celbiologie van de Universiteit Utrecht.

Nawoord

Deze laatste bladzijde van mijn proefschrift wil ik gebruiken om de mensen van de vakgroep Moleculaire Biologie van de Landbouwuniversiteit en het Laboratory of Gene Expression van de Universiteit van Aarhus te bedanken voor hun bijdrage aan het werk beschreven dit boekje, "mijn" proefschrift.

First I would like to thank *Henning Christiansen, Erik Østergaard Jensen, Niels Pallisgård, Peter Lauridsen* and *Kjeld Marcker* for the stimulating collaboration and their hospitality during my stays in Aarhus. Especially *Henning*, with whom I have spent many hours in the coldroom screening the expression library, I would like thank for keeping in with me, even at those crazy hours. Particularly your taste for good food and wine made working with you very pleasant.

Ab van Kammen, mijn promotor. *Ab*, vooral jouw "helicopter-view" en kritische correcties zijn van groot belang geweest bij het schrijven van de artikelen en dit proefschrift.

Ton Bisseling, mijn co-promotor. Jouw aanstekelijk en nooit aflatende enthousiasme voor de wetenschap heeft ervoor gezorgd dat ik de afgelopen jaren met veel plezier in jouw groep heb gewerkt. Zowel op wetenschappelijk als op organisatorisch gebied heb ik erg veel van je geleerd.

Janneke Drenth voor de grote hoeveelheid transformaties. Zonder jouw doorzettingsvermogen en liefde voor de planten had dit boekje er heel anders uitgezien. Helaas werd Nederland te klein voor je. Het ga je goed in Brisbane, Australië.

Wei-Cai Yang for the excellent *in situ* hybridizations. How boring would my thesis have been without these pictures.

Henk Franssen voor het fungeren als moleculaire vraagbaak en voor al de goede adviezen.

Renze Heidstra voor het voorkomen dat ik naar een grote rumoerige werkkamer moest verhuizen en voor het "Nod factor geïnduceerde primordia experiment" tijdens mijn verblijf in Denemarken. Het mooiste primordium is tenlotte uit dit experiment afkomstig.

De studenten *Simon Vroemen, Johan Spee, Karin van de Sande, Wilfred Vermerris* en *Lucinda das Neves* voor hun bijdrage tijdens hun afstudeervak.

Alle andere Knollen voor het creëren van de goede werksfeer op het lab.

Tony van Kampen voor het sequencen van de cDNA's beschreven in hoofdstuk 4. *Tony*, jij was (en bent nog steeds) een hele aanwinst voor de vakgroep, niet alleen omdat wij niets van computers wisten en jij heel veel en je ons bovendien al het sequentiewerk uit handen nam, maar vooral vanwege je humor waarmee je de lunches opfleurde.

Alle collega (ex-)aio's/oio's voor de mentale steun en de gezellige weekends.

Alle andere MolBiollers voor hun wetenschappelijke, technische, administratieve en/of mentale steun.