Comparative studies on *ENOD40* in legumes and non-legumes

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Contents

Outline

Plants, unlike animals, continue to form organs after the completion of embryogenesis. This continuous formation of new organs allows plants to be flexible in a constantly changing environment. A unique example of adaptation to an environmental signal can be found among members of the family of *Leguminosae*. Legumes can enter a symbiosis with *Rhizobium* bacteria that leads to the formation of a complete new organ, the root nodule. Inside these nodules, the hosted bacteria fix atmospheric nitrogen into ammonia that can be used by the plant. Root nodule formation is considered to be acquired by legumes in the course of evolution (Gualtieri and Bisseling, 2000). Several studies show that evolution of new traits involves changes of transcription factors, signalling molecules and structural proteins (Carroll *et al*., 2001). Homologues of nodulin genes (genes that are highly induced during nodule formation) can be found in non-legumes. This indicates that at least some of the genes and maybe even processes necessary for nodule development must be functioning in non-legumes, suggesting that rhizobia have recruited genes involved in general plant development for nodule formation. The presence of nodulin homologues in non-legumes opens the possibility to compare the regulation and function of nodulins and nodulin homologues in legumes and non-legumes. From such studies one might learn how the regulation or function of these genes was adjusted to establish a symbiosis with *Rhizobium*.
We studied the regulation and function of the legume *ENOD40* gene and its tomato homologue *LeENOD40*. *ENOD40* homologues have been found in legumes as well as non-legumes such as rice (Kouchi *et al*. 1999), maize, citrus and tobacco (Van de Sande *et al*. 1996). In the legume *Medicago truncatula*, *ENOD40* was shown to be required for proper nodule development (Charon *et al.* 1999). Ectopic expression of soybean *ENOD40* in the non-legume tobacco leads to reduced apical dominance (Van de Sande *et al.* 1996). This indicates that *ENOD40* plays an important role in both nodule formation and non-symbiotic plant development.

In chapter 1 an introduction on root nodule development in legumes is described and the current knowledge on the role of *ENOD40* in this process is summarised.
In chapter 2 the isolation of the *LeENOD40* gene from tomato and its mapping position on the tomato genome is described. To enable detailed expression studies of *ENOD40*, *LeENOD40::GUS* is introduced in tomato. Expression of *LeENOD40::GUS* is analysed throughout the plant life cycle. *LeENOD40::GUS* expression strikingly co-localises with sites of increased ethylene production in plant development such as in the seed after germination and in flowers before the onset of and during flower senescence. Furthermore, the expression studies show that *LeENOD40* is negatively regulated during

initiation of lateral root formation, suggesting that the gene plays a role in lateral root formation.

In chapter 3 we compare regulation of *LeENOD40::GUS* and *GmENOD40::GUS* expression in a legume and a non-legume background. Our studies show that *LeENOD40::GUS* and *GmENOD40::GUS* expression are similarly regulated in non-legumes. We also show that *LeENOD40::GUS* is expressed in similar nodular tissues as the endogenous *ENOD40*.

In chapter 4 we investigate the effect of ectopic expression of *GmENOD40* (*35S::GmENOD40*) on tomato development in transgenic tomato plants. Preliminary studies show that ectopic expression of *GmENOD40* causes an increase in flower and leaf size in 2 transgenic tomato plants. The leaves and flowers of these plants contain cells larger than wild-type cells in the epidermis.

In chapter 5 we investigate whether we can assign a function to the peptide encoded by *ENOD40* by searching for protein binding partners for the peptide. For this we used the yeast Two-Hybrid system to screen a cDNA library of young pea nodules. This resulted in the isolation of Ps-p40, a pea homologue of the ribosomal protein p40. *In situ* hybridisation studies show that the expression sites of *p40* and *ENOD40* partly overlap in nodules. Further investigation is necessary to confirm or disprove the interaction between p40 and ENOD40.

In the concluding remarks of this thesis (chapter 6) we discuss a possible function of *ENOD40* and we discuss whether *ENOD40* represents a gene that is recruited during evolution for nodule formation.

CHAPTER 1

Introduction

Ingrid Vleghels, Tom Ruttink, Bert Compaan, Ton Bisseling and Henk Franssen

Adapted from: **Vleghels I, Ruttink T, Compaan B and Franssen H** (2001) Root nodule formation in legumes; a molecular chatbox. *Recent research developments in plant physiology* **2**: 187-199

Introduction

Plants of the family of *Leguminosae* can enter a symbiosis with soil bacteria in such a way that differentiated root cells start to dedifferentiate and form a completely new organ, the root nodule, which hosts the bacteria. This symbiosis is only established when plants are grown under nitrogen limiting conditions. Root nodule formation is beneficial for plants as the bacteria fix atmospheric nitrogen into ammonia, which can be utilised by the plant. The interaction leading to root nodule formation is in general restricted to plants of the family of *Leguminosae* (legumes) and bacteria belonging to the genera *Rhizobium, Bradyrhizobium, Sinorhizobium* and *Azorhizobium* (here collectively called rhizobia). To establish an infection, rhizobia attach to the root hairs and cause root hair curling. The bacteria become trapped in the curled root hair and can enter the root through a newly formed infection thread. Simultaneously, mitotic reactivation of cortical cells leads to the formation of the nodule primordium. The infection threads grow towards the primordium and upon arrival bacteria are released into the cytoplasm of the primordium cells and become surrounded by a plant-derived peribacteroid membrane. The nodule primordium develops into a nodule and the bacteria differentiate into their endosymbiotic form, the bacteroids. Studies aiming at the understanding of a process as complex as nodule formation, could certainly benefit from integrated multidisciplinary approaches which have been shown to be productive in studies on many facets of the plant life cycle of model plant *Arabidopsis thaliana*. In the last decade, *Medicago truncatula* (Barker *et al.,* 1990) and *Lotus japonicus* (Schauser *et al.*, 1998) have been chosen as model legumes. Gene mutagenesis -, gene tagging -, DNA sequence -, and proteomic programs have been initiated in these model legumes.

In several legumes, mutants impaired in symbiosis are described. These can roughly be grouped into three classes: class I, non-nodulators (nod^-); class II, hypernodulators (nod^{+++}) and class III, nodulators with impaired fixation (nod^+, fix^-). In this review we focus on the current knowledge of the interaction between legumes and rhizobia (for other reviews see: Albrecht *et al.*, 1999; Foucher and Kondorosi, 2000; Stougaard, 2000; Hirsch *et al.,* 2001).

Starting of the interaction

Nodule formation is the result of a two-way communication: Plants secrete host signals such as flavonoids into the rhizosphere, which can act as chemo attractants to rhizobia. Together with the rhizobial NodD transcriptional activator, flavonoids induce expression of the rhizobial *nodulation* (*nod*) genes (Goethals *et al.,* 1992). The *nod* genes encode proteins that are involved in the production of Nod factors (Figure 1).

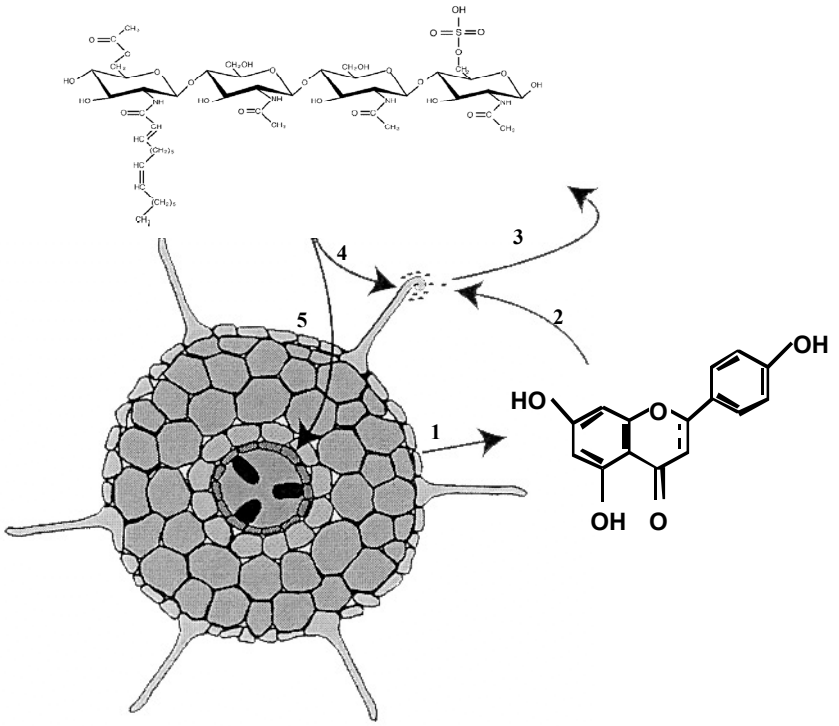

Figure 1: Schematic representation of the primary signals involved in the *Sinorhizobium meliloti* and *Medicago truncatula* interaction. Flavonoids secreted by the plant (1) attract *S. meliloti* (2) and induce the transcription of bacterial *nod* genes. The proteins encoded by the *nod* genes are involved in synthesis and transport of the Nod factor, a lipochito-oligosaccharide (3). The Nod factor provokes responses in the plant at spatially separated sites, the root hairs (4), the cortex and the pericycle (5).

These Nod factors play a major role in the host specificity of the symbiosis between rhizobia and legumes. Most rhizobia possess multiple copies of *nodD* and in some cases it has been shown that different NodD proteins are involved in the recognition of different plant signals, leading to an extension of the host range in these particular bacterial strains (Denarié *et al.,* 1996; Spaink, 1996).

In the early 1990s, Nod factors were characterised (Lerouge *et al.,* 1990) and to date the Nod factor structure of several rhizobial species has been elucidated. They all share a β-1,4-linked *N* acyl-D-glucosamine backbone of three to six units. The non-reducing terminal sugar moiety is substituted with a fatty acid, of which the structure is variable. Substitutions may be present at the reducing terminal sugar, including sulfuryl, fucosyl, mannosyl or arabinosyl groups and an acetyl group may be present at the non-reducing terminal sugar. Genetic and molecular analyses have shown that the bacterial *nodA*,

nodB and *nodC* genes, common to all rhizobia, are responsible for the synthesis of Nod factors (for reviews on genes involved in Nod factor production and on Nod factor structure, see Denarié *et al.*, 1996; Spaink, 1996). The different structural features of Nod factors find a basis in presence or absence of specific *nod* genes. Several studies have shown that the substitutions on the core backbone of the Nod factor confer host-specificity of the interaction between the bacterium and its host (Spaink *et al.*, 1991; Bloemberg *et al.*, 1994).

Purified Nod factors applied in the nano- to picomolar range are able to induce developmental processes that are needed to form the nodule (Lerouge *et al.*, 1990; Ardourel *et al.*, 1994). Responses to Nod factors are provoked at spatially separated sites; the epidermis, cortical cells and pericycle (Geurts and Bisseling, 2002). This observation raises the question whether the observed responses are directly provoked by Nod factors or by Nod factor induced diffusible signals. Ballistic targeting of the chito-oligosaccharide part of the Nod factor to inner cortical cells proved to be sufficient to initiate cell divisions (Schlaman *et al.*, 1997). These experiments suggest that the mitotic activity of Nod factors resides in the chito-oligosaccharide part, and that this part either is transported to the inner cortical cells to induce cell divisions or is sufficient for the production of a secondary signal that leads to cell division. The low concentration in which Nod factors can already provoke responses as well as the structural specificity of Nod factors are indicative for a receptor-mediated activity. Studies using various pharmacological drugs indicate that Nod factor signalling might be a G protein mediated transduction mechanism (Pingret *et al* 1998). However, until now there are no experimental data supporting the presence of this particular transduction system in plants (Meyerowitz, 1999).

At the epidermis

Nod factors induce several responses in the root epidermis of their host plant. The earliest responses to Nod factors are physiological responses in the root hairs. Immediately after Nod factor application, a decrease of the calcium concentration in the environment between the root hairs occurs, which could be due to an influx of calcium into the root hairs (Felle *et al.*, 1998). This is followed by depolarisation of the root hair plasma membrane associated with an efflux of chloride ions (Ehrhardt *et al.*, 1992; Felle *et al.*, 1995; Felle *et al.*, 1998). Several minutes after Nod factor application, spiking of cytoplasmic calcium levels occurs in the root hairs (Ehrhardt *et al.*, 1996). These Nod factor induced ion fluxes are followed by morphological changes in root hairs. Vetch root hairs that deform after Nod factor application have almost terminated growth (Heidstra *et al.*, 1994). When Nod factor is applied, the tips of these root hairs swell. Renewed root hair growth takes place in a different direction leading to root hair deformation (De Ruijter *et al.*, 1998). Furthermore, Nod factor application to vetch root

hairs was shown to change the configuration of the actin cytoskeleton (De Ruijter *et al.*, 1999).

Several studies on marker genes for Nod factor activity have been performed. These marker genes are expressed in hairs as early as two hours after Nod factor application. These marker genes have been shown to encode proline-rich proteins (*PsENOD5*, *PsENOD12*, *MtENOD11*, *MtENOD12*), a peroxidase (*Mtrip1*) and a Ca^{2+} binding protein (*LjCjp1*) (Horvath *et al.*, 1993; Cook *et al.*, 1995; Vijn *et al.*, 1995; Bauer *et al.*, 1996; Web *et al.*, 2000).

The described physiological changes, morphological changes and expression profile of marker genes imply that several processes are set in motion by Nod factors. Whether these processes are really important for nodule formation needs to be confirmed. Instrumental to this task may be the mutants that are impaired in root hair deformation. For example, pea *sym8, sym10* and *sym19* (Markwei and LaRue, 1992; Walker *et al.*, 2000) and alfalfa *MN1008* (Ehrhardt *et al.*, 1992; Ehrhardt *et al* 1996) show no morphological response to rhizobia. *Medicago truncatula dmi1*, *dmi2*, and *dmi3* are blocked in Nod factor induced polar outgrowth of the root hair (Catoira *et al.*, 2000) (Figure 2). For all these mutants, except for *dmi3*, the absence of Ca^{2+} oscillation has been shown, suggesting a relation between Ca^{2+} oscillation and root hair deformation (Ehrhardt *et al.*, 1996; Oldroyd *et al.*, 2001). The genes mutated in alfalfa *MN1008* and *M. truncatula dmi2* encode homologous receptor kinases (Stracke *et al.*, 2002; Endre *et al.*, 2002). How this receptor kinase could be involved in Nod factor perception or signal transduction has not been investigated yet.

Since the characterisation of Nod factors the hunt for a Nod factor receptor has been opened, but at present its structure is still illusive. Alfalfa *MN1008* and *M. truncatula dmi2* were shown to encode homologous receptor kinases. These genes could be candidates to encode the Nod factor receptor. However, the presence of LRR motifs in the encoded protein (NORK) indicates that a protein-protein interaction is involved in activation of the receptor. If NORK encodes the Nod factor receptor it would require Nod factors to bind with a protein before interaction with the LRR motif could occur (Stracke *et al.*, 2002; Endre *et al.*, 2002).

A biochemical approach to identify a Nod factor receptor has led to the identification of two binding sites, NFBS1 and NFBS2, in microsomal fractions from alfalfa roots and tissue cultured cells. The first site has low affinity for the *Sinorhizobium meliloti* specific Nod factor, in contrast to NFSB2 that binds this Nod factor with higher affinity. Both NFSB1 and NFSB2 however, can also bind to derivatives devoid of the sulfuryl substitution that appears to be required for *in vivo* activity of the *S. meliloti* Nod factors (Gressent *et al.*, 1999).

The chemical nature of Nod factors suggests that a putative Nod factor receptor contains a sugar recognising part. A role in Nod factor perception for lectins, well-known sugar

binding proteins, has been suggested (Kijne, 1992; Van Rhijn *et al.,* 1998; Etzler *et al.,* 1999; Diaz *et al.,* 2000). A purified lectin with apyrase activity from *Dolichos biflorus* was shown to bind Nod factors with high affinity (Etzler *et al.,* 1999). This lectin is present on the root hair surface and treatment of root hairs with antiserum against this lectin inhibits root hair deformation and nodule formation. From these experiments however, the role of lectins in Nod factor transduction remains unclear.

Derivatives of *Rhizobium leguminosarum* bv *viciae* 248 Nod factors, in which the acyl chain is provided with a fluorescent group, were used for FCM (Fluorescence Correlation Microscopy) studies on *Vicia sativa* root hair deformation. These Nod factors become rapidly immobilised in the root hair cell wall and upon deformation, Nod factors hardly migrate to the newly synthesised cell wall. Apparently, Nod factors are immobilised in the root hair cell wall (Goedhart *et al.,* 2000). This indicates that a Nod factor receptor may have a part residing in the cell wall. Studies with *Rhizobium* mutants showed that a difference in Nod factor structure induced a different type of response. Based on these studies a two-receptor model for Nod factor perception was proposed by Ardourel *et al* (1994). This model implies that there is an entry receptor, which is highly selective for Nod factor structure and a signalling receptor, which is less selective for Nod factor structure. The highly selective receptor is in control of the infection process and will only recognise Nod factors with the appropriate substitutions. The signalling receptor triggers root hair deformation and cortical cell division upon binding of Nod factor. The pea *SYM2* gene is involved in Nod factor induced infection thread formation and its activity depends on the structure of the Nod factor (Geurts *et al.,* 1997). In *sym2* plants, induction of primordium formation does not depend on the right Nod factor structure. Therefore, *SYM2* is an interesting candidate to encode the entry receptor. However, it is also likely that pathways leading to infection and primordium formation interact. Based on histological studies on the rearrangements of the microtubular cytoskeleton during early steps in the symbiosis, it has been proposed that infection thread growth and primordium formation should occur in close concert in order to be successful (Timmers *et al.,* 1999). In line with this is also the observation that the *L. japonicus Nin* gene, which encodes a protein with homology to transcription factors, is required for both infection thread formation and initiation of nodule primordia (Figure 2). Disruption of *Nin* by insertion of an *Ac* element causes excessive root hair deformation and curling (Schauser *et al.,* 1999).

The infection process

To establish a successful symbiosis, rhizobia have to penetrate the root and enter nodule primordium cells. In the infection threads, bacteria are imbedded in a matrix. The major plant glycoprotein (MGP) in pea infection threads is also released from the root tip. This soluble MGP can be made insoluble by peroxidases and diamineoxidase (Wisniewski *et*

al., 2000). Based on the assumption that such an insolubilisation of MGP occurs in the lumen of the infection thread, it has been suggested that bacteria become entrapped in the infection thread matrix. In this way, the rate of solidification might then be a means of the plant to regulate the entrance of compatible and incompatible bacteria.

Bacterial surface exo- or lipo-polysaccharides, like EPSI, EPSII and LPS, have been shown to be essential in infection, as mutants impaired in the production of these surface components are unable to establish a successful infection process. Intriguingly, EPS mutants can partially be rescued by low Mr-polysaccharide fractions of EPSI and EPSII (Gonzalez *et al.,* 1996). This suggests that these molecules may act as signalling molecules. For the *R. leguminosarum* bv *viciae*-pea interaction, it was shown that the bacterial NodO secreted protein positively contributes to the progression of infection (Sutton *et al.,* 1994). However, the NodO ortholog in other *Rhizobium* species has not been discovered yet. Thus, it remains to be solved whether other rhizobia also make use of secreted proteins that can aid the infection process. Concomitant with the infection process, differentiated cortical cells start to dedifferentiate and subsequently to divide, giving rise to the nodule primordium. Roughly, nodule primordia arise either in the outer cortical cell layers, like in soybean and common bean, or in the inner cortical cells as is the case in pea and *Medicago spp*. In plants where the primordium is formed in the inner cortex, the infection thread passes the outer cortex. Prior to infection thread penetration, cytological rearrangements occur in the cortex cells. The nuclei move to the centre of these cells and the microtubules and cytoplasm rearrange to form a radially oriented conical structure, which resembles a phragmoplast. The infection threads traverse the cortical cells through radially aligned cytoplasmic bridges, which are called pre-infection threads (Van Brussel *et al.,* 1992). In *Lotus japonicus*, the nodule primordium is formed in the middle root cortex and also there cytoplasmic bridges are formed to allow infection thread progression (Van Spronsen *et al.,* 2001).

In situ hybridisation experiments scoring for the expression of cell cycle phase specific genes demonstrated the expression of the S phase specific *H4* gene, but not of an M phase mitotic cyclin gene in cells forming the pre-infection thread (Yang *et al.,* 1994). This observation shows that cells that form the pre-infection thread re-enter the cell cycle, but become arrested in the G2 phase. Based on this, it was proposed that rhizobia apparently have been able to manipulate a general process such as cell division to penetrate plant roots. Purified Nod factors can induce pre-infection thread formation, but for the formation of a complete infection thread bacteria are required (Van Brussel *et al.,* 1992). The final destination of infection threads are cells of the primordium where, upon arrival, the threads ramify and infect cells at the base of the primordium. Abortion of infection threads seems to take place predominantly in the epidermal cell layer (Kijne, 1992). This could indicate that a strong selection barrier is laid down in the epidermis. On the other hand, since formation of pre-infection threads relies on the proper

regulation of genes controlling the cell cycle, it might be that mutations leading to defects in infection thread progression would also affect normal cell cycle progression and could therefore be lethal.

Primordium formation

Local cortical cell divisions can be induced by Nod factors but can also be provoked by exposure of the roots to auxin transport inhibitors or cytokinin (Cooper and Long, 1994; Hirsch *et al.*, 1989; Mathesius *et al.*, 1998). These observations indicate that a disturbance of the auxin/cytokinin balance could be a crucial early event in nodule formation. Nodulation impaired mutants have been identified in which the nodulation phenotype is accompanied with either ethylene hyper- or insensitivity. In the pea mutant *sym5* the number of infections is similar as in wild-type pea but a lower number of primordia are formed. This results in a lower number of nodules. The *sym5* phenotype can be rescued by treatment with either AVG or Ag^{+} inhibitors of ethylene formation and action, respectively. It was concluded that in the *sym5* mutant increased sensitivity to ethylene causes a block on primordium formation (Figure 2). As *sym5* plants show no other morphological differences, the importance of the *sym5* gene product is only clear in nodule development (Guinel and LaRue, 1991). In *Medicago truncatula* the *sickle* mutation induces insensitivity to ethylene and ACC (Penmetsa and Cook, 1997). The mutation also leads to a more than two-fold increase in the number of nodules when compared to wild-type plants. Based on the pea *sym5* phenotype it can be inferred that ethylene is a negative regulator of nodule primordium formation. Hence, it is more likely that the *sickle* mutation has an effect on cortical cell divisions leading to nodule development rather than on infection as was suggested (Figure 2). However, ethylene was also shown to inhibit the initiation of calcium spiking and regulate the degree of root hair deformation in response to Nod factors (Oldroyd *et al.*, 2001). It was suggested that ethylene acts at or before the point of calcium spiking in Nod factor signal transduction.

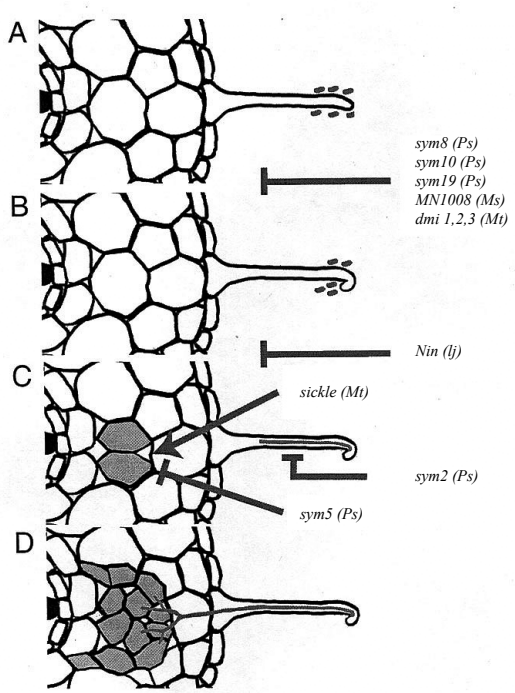

Figure 2: Schematic representation of the early steps in the *Rhizobium*-legume interaction and mutants impaired in particular steps. A: attachment of rhizobia to the root hairs B: induction of a curl into which the bacteria become entrapped. From here infection thread formation is initiated. C: Infection threads grow inwards via the hair into the cortex towards the cortical cells that have been mitotically reactivated. D: Penetration of infection threads into nodule primordium cells and release of bacteria into the cytoplasm. *Ms = Medicago sativa*, *Mt = Medicago truncatula*, *Ps = Pisum sativum*, *Lj = Lotus japonicus*, ⊥= blocking, ↓= stimulating. Names refer to mutants in different species affected in these particular steps.

Heidstra *et al.*, (1997) showed that ACC oxidase is expressed in the pericycle opposite phloem poles in pea roots. Strikingly, the cortical cells that are recruited for primordium formation are predominantly positioned opposite proto-xylem poles, facing the region in the pericycle devoid of ACC oxidase expression. Treatment of pea seedlings with *R. leguminosarum* bv *viciae* in the presence of AVG or Ag^{+} resulted in an increased number of nodules formed opposite phloem poles. From these observations it can be inferred that ethylene is negatively regulating nodule primordium formation opposite phloem poles whereby it provides positional information for the nodule primordium to form opposite protoxylem poles. Remarkably, the effect of ethylene as described has only been noticed

in legumes where nodule primordia are formed in the inner cortex. For instance in soybean, where the nodule primordium forms in the outer cortex, primordium formation and infection have been shown to be insensitive to ethylene (Schmidt *et al.,* 1999). Primordia that are initiated in the inner cortex give rise to nodule meristems that persist, while meristematic activity of outer cortex primordia derived meristems ceases early after establishing symbiosis. It was shown that ethylene has an influence on meristem life span in *Sesbania rostrata*, in which the primordia are formed in the outer cortex (Fernandez-Lopez, 1998). In addition to negative regulators of nodule formation, signals positively influencing primordium formation other than cytokinin have also been characterised, such as uridine (Smit *et al.,* 1995). However, the effect of uridine in legumes other than pea has not been reported.

The gene *ENOD40* is thought to play an important role in nodule formation. Detailed expression studies in alfalfa showed *MsENOD40* is induced shortly after bacterial treatment of plants. Expression is observed 3 hours after inoculation with rhizobia in the pericycle facing the proto-xylem pole, while cortical cell divisions are observed 20 hours after inoculation (Compaan *et al.,* 2001). Ectopic expression of *ENOD40* leads to perturbation in plant growth in both legumes and the non-legume tobacco (Crespi *et al.,* 1994; Van de Sande *et al.*, 1996; Charon *et al.,* 1997). Application of *Sinorhizobium meliloti* to *M. truncatula* overexpressing *MtENOD40*, results in nodule formation in a region closer to the root tip. A similar effect is achieved when bacteria inoculated *Medicago* plants are grown in the presence of AVG (Charon *et al.,* 1999). In addition, ballistic targeting of *MtENOD40* DNA into alfalfa induces cortical cell divisions (Charon et al., 1997). Thus, in legume roots *ENOD40* may act as a signal to reactivate cortical cells and it may do so by suppressing the negative effect of ethylene.

A current working model for Nod factor induced nodule primordium formation is that Nod factors locally cause a block in polar auxin transport. This leads to an increase in cytokinin that together with positive regulators like uridine and *ENOD40* lead to reactivation of cell division. Positive regulators might be required to overcome the negative effect of ethylene on induction of cell division.

Late steps

Upon infection of particular primordium cells, bacteria are released from the infection threads and become internalised by a process reminiscent of endocytosis. In indeterminate nodules, it has been shown that rhizobia have a preference to infect those cells that have gone through several rounds of endoreduplication (Truchet, 1978). Recently, *ccs52* a gene involved in endoreduplication and ploidy-dependent cell enlargement has been characterised in *M. truncatula*. However, antisense silencing of this gene was not described to have an effect on nodule formation (Cebolla *et al.,* 1999). Thus, the importance of polyploid cells in nodule formation or functioning is not yet

clear. After release from the infection thread, the endosymbionts multiply and the intracellular bacteria differentiate into bacteroids, which then enlarge. At this stage, division of the microsymbiont and the surrounding membrane is synchronised in an unknown way. Cells at the proximal site of the persistent apical meristem stop dividing and differentiate into the various tissues that are present in the mature nodule: central tissue harbouring bacteroids adjacent to uninfected plant cells. The central tissue is surrounded by parenchyma cells in which the vascular bundles are embedded. The tissue organisation in the central region of the nodule has been studied in several ways in pea and *M. sativa* (Scheres *et al.*, 1990; Vasse *et al.*, 1990; Yang *et al.*, 1991). The results of these studies (Figure 3) suggest that a flow of signals between the two organisms guide the progression of the interaction. Bacterial *nod* genes are still expressed in the nodule region preceding the fixation zone, suggesting that Nod factors might still be produced and could act as a signalling molecule like in the early steps of the interaction (Schlaman *et al.*, 1991).

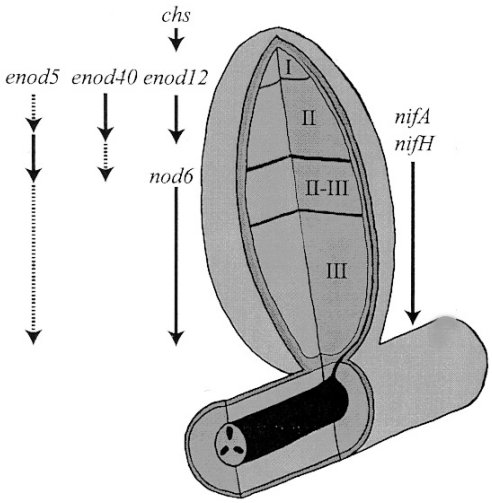

Figure 3: Schematic representation showing tissue organisation of the central region of the indeterminate pea nodule and alignment of regions where different plant (indicated at the left side of the nodule) and bacterial transcripts (indicated at the right side of the nodule) are located. The meristematic zone (zone I): expression of chalcone synthase (*chs*, involved in the production of flavonoids). The infection zone (zone II): expression of early nodulins *ENOD5*, *ENOD12* (proline rich proteins) and *ENOD40*. The interzone (zone II-III, nitrogen fixation starts) and the nitrogen fixation zone (zone III): expression of late nodulin *NOD6* and expression of the bacterial genes *nifA* and *nifH*, genes involved in the transcription of genes involved in nitrogen fixation. Closed arrows: maximal expression; dotted arrows decreased expression.

The presence of bacterial strains with functional *nod* genes, that are impaired in release and bacteroid development, strongly argue for the involvement of additional bacteria or plant derived signals in these late symbiotic steps. It will be a major challenge to characterise the nature of these signals. A glimpse of the complexity one may encounter, is best illustrated by the observation that silencing of a Krüppel-like zinc finger protein encoding gene, which is normally expressed in vascular tissue, leads to non-functional nodules due to hampered differentiation of the invasion- and fixation zone (Frugier *et al.*, 2000). This observation shows that in addition to the ongoing signalling between the bacteria and the plant, also intercellular signalling between plant cells are major events in establishing of the symbiosis.

Perspective

Nitrogen fixation in root nodules is an advantageous trait that crops growing on nitrogen poor soils can benefit from. Also non-legumes could benefit from nitrogen fixation. However, transferring the possibility of nitrogen fixation is a difficult task. It depends largely on how much of the processes that are needed for nodule development are functioning in non-legumes and how many additional functions are required (Parniske, 2000). Homologues of nodulin genes have been found in non-legumes. Moreover, identification of genes mutated in non-nodulating mutants such as alfalfa *MN1008*, *Medicago truncatula dmi2* and *Lotus japonicus Nin*, shows that homologues of these genes can be found in non-legumes (Stougaard, 2000; Stracke *et al.*, 2002; Endre *et al.*, 2002). The presence of these genes in non-legumes indicates that processes needed for nodule formation could be partly present in non-legumes. Application of Nod factors to rice plants transgenic for the *ENOD12* promoter shows that the *ENOD12* promoter can be induced in these plants (Reddy *et al.*, 1998). Therefore, a mechanism must be present in these plants to recognise Nod factors or signals reminiscent of Nod factors. It seems likely that rhizobia have recruited genes involved in general plant development for nodule formation. The identification of several non-nodulating mutants that are also impaired in the arbuscular mycorrhiza (AM) formation led to the hypothesis that nodule formation evolved from this, more widespread, symbiosis between plants and endomycorrhizal fungi (Gianinazzi-Pearson, 1996; Albrecht *et al.*, 1999; Gualtieri and Bisseling, 2000). Investigation of the function of non-legume homologues of genes involved in nodule formation might give more insight into their function in nodule formation. Such studies could also give insight into differences between species that can be a basis for symbiosis between legumes and rhizobia.

CHAPTER 2

Expression studies of *ENOD40* in tomato plant development suggest a role for *ENOD40* in lateral root development and ethylene associated processes

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Abstract

In legumes, *ENOD40* expression is dramatically increased upon interaction of plants with rhizobia. Therefore, it is suggested that this gene plays a key role in nodule development. The presence of *ENOD40* homologues in non-legumes suggest that this gene, apart from its involvement in root nodule development, functions in other developmental processes as well. However, little is known of the expression pattern of *ENOD40* during other stages of the plant life cycle. Expression studies of *ENOD40* in non-legume development may give an indication on a function of the gene. To investigate the *ENOD40* expression pattern during plant development, a fusion between the *ß-glucuronidase* (*GUS*) reporter gene and 150 bp of 5' UTR plus 3000 bp of 5' untranscribed tomato *ENOD40* sequence was constructed and introduced in *Lycopersicon esculentum*. Analysis of *GUS* expression in transgenic tomato plants suggests that *ENOD40* in tomato has a role in counteracting ethylene-provoked responses. The expression pattern of *ENOD40* in roots suggests that *ENOD40* plays a role in lateral root development.

Introduction

While animals develop all their organs during embryogenesis, for plants the continuous formation of new organs is an important feature as they are sessile organisms that unlike animals cannot move away from an unfavourable environment. The continuous formation of new organs after completion of embryogenesis allows plants to be flexible in a constantly changing environment. An example of adaptation to an environmental cue can be found among members of the family of *Leguminosae*. These plants develop unique organs on their roots, the so-called nodules. Nodules are the result of a symbiotic interaction between legume plants and *Rhizobium* bacteria. When nitrogen sources in the soil are low, this symbiosis gives the plants access to a pool of ammonia assimilated by the rhizobia. Although many plants could benefit from this symbiosis, the phenomenon is largely restricted to a distinct plant family, the legumes. This leads to the question what determines the competence for symbiosis in these plants. Homologues of genes expressed at elevated levels during nodule formation have been found in non-legumes (Matvienko *et al.,* 1996; Kouchi *et al.,* 1999; Reddy *et al.,* 1999; Becker *et al.,* 2001). The establishment of a symbiotic interaction could cause or require a different regulation of expression of these genes than normally during plant development. A gene responsible for the nod$^-$ phenotype of the alfalfa mutant MN-1008 has recently been isolated (Stracke *et al.,* 2002; Endre *et al.,* 2002). This gene is not unique for legumes but homologues are present in the non-legume *Arabidopsis thaliana*. Furthermore, mutants have been described that are disturbed in both nodule formation and plant development (Penmetsa and Cook, 1997, Wopereis *et al.,* 2000). These data indicate that legumes have recruited genes for nodule formation that are involved in other, more

ancient, plant processes. The presence of nodulin homologues in non-legumes opens the possibility to study the regulation and function of these genes in non-legumes. From such studies one might learn how in legumes these genes have been modified so that they could be recruited to establish a symbiosis. In addition, one might learn about the function of these genes in symbiosis as well. Preferably these studies should be performed in a well-studied model plant, which can be efficiently transformed and used for genetic analyses.

We chose *ENOD40* for these studies, a gene thought to play a key role in nodule development. Reduction of *ENOD40* expression during nodule formation by cosuppression leads to the formation of aberrant nodules in *Medicago truncatula*, showing that in these plants *ENOD40* is essential for proper nodule formation (Charon *et al.*, 1999). *ENOD40* can be found in legumes and in non-legumes like rice, maize, citrus and the *Solanaceae* tobacco and tomato. A detailed expression study is already available for *ENOD40* in rice (Kouchi *et al.*, 1999). However, this study gave no further insight into the function of *ENOD40*.

Here, we show detailed expression studies of *ENOD40* in tomato. We chose the non-legume tomato for these studies because it is a well-studied plant and genetic maps are available, which also allows us to search for mutations in *ENOD40*. Our studies suggest that *ENOD40* in tomato has a role in counteracting ethylene-provoked responses; a function that it could have in root nodule formation as well as in non-symbiotic processes.

Materials & methods

Plant material

Tomato plants (*Lycopersicon esculentum* cv Moneymaker) were grown in a growth chamber at 25°C and a 16 hour photoperiod (7W/m^2). For generation of offspring, plants were moved to a greenhouse (at 18 to 21°C) with additional light during the winter (16 hour photoperiod, 150 W lights).

Transgenic plants were grown aseptically in jars on 1/2 MS (Murashige and Skoog medium, Duchefa) containing 10 g sucrose (1/2 MS 10) and 15 mg/l hygromycin in a growth chamber (conditions described above). After two weeks plants resistant to hygromycin were transferred to soil (and moved to a greenhouse) or to medium without antibiotics for further treatments.

RFLP mapping

To identify RFLPs suitable for mapping of *LeENOD40*, the cDNA was hybridised to Southern blots containing *L. esculentum* and *L. pennellii* DNA digested with a number of different enzymes. The enzyme that gave an RFLP was used to digest DNA samples

of 79 plants of an F2 population of *L. esculentum* x *L. pennellii*. The computer package JoinMap (version 2.0, Stam and Van Ooijen, 1995) was used to translate the mapping data into a map position. The department of Plant Breeding, Wageningen University, kindly provided DNA samples and segregation data.

RT-PCR

RNA was isolated from different organs of tomato using the hot phenol method for RNA isolation (Pawlowski, 1994). After RNA isolation the samples were treated with DNaseI (Promega) to remove chromosomal DNA that could disturb the PCR reactions. Total cDNA was made from 2.5 µg of total RNA using an oligo dT primer in a volume of 20 µl (10 mM Tris-HCl pH 8.8, 50 mM KCl, 5 mM $MgCl_2$, 1 mM dNTPs, 1 µg oligo dT12-18 (Pharmacia), 20 U RNA guard (Pharmacia) and 200 U MMLV reverse transcriptase (Stratagene). The samples were incubated for 1 hour at 37°C. To inactivate the reverse transcriptase enzyme the samples were incubated at 95°C for 5 minutes. The RT samples were then diluted to 100 µl and 1 or 2 µl of the cDNA were used for PCR (10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 100 µM dNTPs, 50 ng primer and 1 U Taq polymerase (Boehringer Mannheim, USA) in a total volume of 50 µl). For isolation of the *LeENOD40* cDNA clone the primers Ka 5L: 5'-GGC (A/T)(C/A)(A/G)(C/A)A(A/T) C(C/A)ATCCATGGTTCTT-3' and oligo dT were used (60°C annealing temperature). For the RT-PCR reactions of the different tomato plant organs the primers Le40-3: 5'-GCAAGTTGGAGTGGAGG-3' and Le40-4: 5'-ACACACTGGCATGTAACC-3' were used (annealing temperature 58°C). For these reactions the following cycling program was used: denaturation 95°C 30 sec, annealing 30 sec (temperature dependent on primers), extension 70°C 30 sec. Amplified DNA samples were run on a 1% agarose gel, alkaline blotted to Nytran-N^+ membrane and hybridised to radiolabelled inserts of the corresponding cDNA clones.

5'-RACE

To determine the 5' end of the *LeENOD40* mRNA a 5'-RACE was performed on RNA of tomato flowers. For this, cDNA was constructed from 20 µg of total RNA using the primer Le40-4 (100 ng) (see RT-PCR) in 10 mM Tris-HCl pH 8.8, 50 mM KCl, 5 mM $MgCl_2$, 1 mM dNTPs, 20 U RNA guard (Pharmacia) and 20 U AMV reverse transcriptase (Stratagene). The samples were incubated for 10 minutes at room temperature, 30 minutes at 42°C, 60 minutes at 50°C and finally 10 minutes at 65°C. The sample volume was adjusted to 100 µl and purified using a PCR Purification Kit (Boehringer). The sample was eluted from the PCR purification column using 50 µl 1x TdT buffer. TdT tailing was performed in a total volume of 55 µl containing 1x TdT buffer, 25 µM dATP and 15 U TdT. This mixture was incubated for 30 minutes at 37°C and 5 minutes at 70°C to inactivate the TdT enzyme. 5µl of the tailed cDNA was used

for the first PCR for which the primers Le40-1: 5'-GGTTTGCCATCCTTTTGC-3' (100 ng) and T (200 ng) were used. A hot start PCR program was used: 5 min 94°C, 3 min 80°C (in this step the Taq polymerase was added); 94°C 15 sec, 55°C 30 sec, 72°C 40 sec, for 10 cycles; 94°C 15 sec, 55°C 30 sec, 72°C 40 sec with 20 sec extension, for 20 cycles; 5 min 72°C. A second PCR was performed with Le40-2 primer: 5'-GGACTCTCCAGAGATGTTG-3' and A primer (binds to linker on T primer). This product was hybridised to radiolabelled *LeENOD40* to confirm the presence of an *LeENOD40* insert and then cloned into the pGEM-T vector (Promega).

Cloning of the LeENOD40 promoter

A genomic library of *Lycopersicon esculentum* VFN8 in the EMBL3 vector (Clontech) was screened using radiolabelled *LeENOD40* as a probe. The library screen was performed according to the protocol provided by the company. This screen resulted in 51 positive clones. First, the λ clones were tested for the presence of *LeENOD40* by PCR with the primers Le40-3 and Le40-4. Out of the positive λ clones, 1 clone was analysed further. From this clone the *LeENOD40* promoter was amplified by PCR with primers F 5'-GAGACGTCGACAGTGGTAAGTTAGCCCATA-3' and R 5'-AAGCGGATCC CCATATCAATGGAATGAG-3' with *Bam*HI and *Sal*I sites at the ends. The resulting PCR product was cloned into pBluescript. Then cloned into pCambia 1381Z (Cambia, Australia) which contains the *GUS* gene.

Fluorescence in situ hybridisation

FISH was performed according to the protocols described by Zhong *et al.*, (1996). For hybridisation genomic clones λT15 and λTG441 were used. λT15 and λTG441 were PCR labelled with biotin-16-dUTP (Boehringer Mannheim, Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Clone TGR1 was labelled with digoxigenin-11-dUTP using the high-primed labelling kit (Boehringer Mannheim). Biotin labelled probes were detected wit Avidin-Texas Red and amplified with biotin-conjugated goat-anti-Avidin and Avidin Texas Red. Digoxigenin-labelled probes were detected with sheep-antidigoxigenin-fluorescin (FITC) and amplified with rabbit-anti-sheep-FITC.

Tomato transformation procedure

Tomato MM seeds were sterilised in 1% bleach for 8 minutes and successively washed with sterile water 5 times. The seeds were germinated on plates with MS medium and used for transformation just after the germination was complete. The two ends of the cotyledons were cut off and these cotyledon explants were incubated 24 hours on R3B plates. For transformation of the cotyledon explants, *Agrobacterium tumefaciens* AGL0 containing (transformed by electroporation) the *LeENOD40::GUS* construct was used.

An *Agrobacterium tumefaciens* preculture was prepared from a colony from a freshly plated stock and grown O/N in 2YT (10 g/l NaCl, 10 g/l yeast extract, 16 g/l trypton). This preculture was used to inoculate a second culture that was grown until OD 0.4-0.8 in 3 to 4 hours and was then used for cocultivation with the tomato explants. Explants were incubated in the *Agrobacterium* culture for 10 minutes. Explants were transferred very carefully one by one to a Whatman filter to drain off the excess culture. Finally the explants were transferred to R3B (1x MS, 30 g/l sucrose, 2 mg/l NAA, 1 mg/l BA) plates which were closed off with parafilm. This cocultivation was incubated in low light for two days. Following the incubation the explants were taken from the cocultivation plates and washed in 100 mg/ml timentin (1500 mg Ticarcillin/100 mg Potassium Clavulanate, Duchefa). The washed explants were drained on Whatman filter paper and transferred to selective medium (1x MS, 1 mg/l zeatin, 20 g/l sucrose, 100 mg/l timentin) containing 15 mg/l hygromycin. Normally, after two or three weeks transgenic calli would appear and form shoots. Shoots were transferred to rooting medium (1x MS, 0.5 mg/l IAA, 100mg/l timentin). When roots had formed, usually after 1 or 2 weeks, the plants were transferred to MS medium containing timentin (100 mg/l) and hygromycin (15 mg/l). After 1 or 2 weeks these plants were ready for transfer to soil. Primary transformants were selected on hygromycin and once the transgenic plants developed roots T0 plants were transferred to the greenhouse and selfed. T1 seeds were selected on hygromycin and analysed for presence of the promoter-*GUS* construct by Southern analysis.

GUS assays

GUS assays were performed according to the protocol of Kosugi *et al.,* (1990). Plant material was stained in GUS buffer (0.1 M Na_2PO_4 pH 7.2, containing 1mM X-Gluc dissolved in DMF and 0,1% TRITON X-100). The plants were put 10 minutes under vacuum and were incubated at 37°C for 4 hours or longer until the blue GUS stain was visible.

Results

Isolation of the LeENOD40 gene

To obtain a cDNA clone representing an *ENOD40* transcript from tomato, total RNA was isolated from tomato flowers and stems. This RNA was used to make cDNA that subsequently served as template to amplify *ENOD40* in a PCR using primers Ka-5L and oligo dT (see Materials and Methods). The 480 bp product was purified and cloned into pGEM-T (Promega). Two clones, one of flower and one of stem RNA origin, respectively, were sequenced. These cDNA sequences were identical and they were shown to be 64% homologous to tobacco *ENOD40* (Figure 1). A full size cDNA of the

mRNA was generated through 5'-RACE using primer Le40-1 and primer Le40-2 (see Materials and Methods). The product was purified and cloned into pGEM-T. Sequencing showed that the 5' end of the cDNA had been extended resulting in a cDNA clone of 600 bp. The sequence of the cDNA contains the two conserved regions, region 1 and region 2, that are characteristic for all *ENOD40* cDNAs known so far (Van de Sande *et al.*, 1996; Corich *et al.*, 1998; Kouchi *et al.*, 1999; Flemetakis *et al.*, 2000) and the peptide encoded within region 1 is identical to the *NtENOD40* peptide (Figure 1). We therefore concluded that this cDNA is tomato *ENOD40* (*LeENOD40*). To determine how many copies of *LeENOD40* are present in the tomato genome, a radiolabelled *LeENOD40* probe was hybridised to a blot containing tomato Moneymaker DNA digested with various restriction enzymes. The autoradiogram showed that there is one DNA fragment hybridising, suggesting that tomato has only one *LeENOD40* gene, which is consistent with the observation that the sequences of the different cDNA clones are identical.

An *LeENOD40* genomic clone was isolated by screening a tomato genomic library in λEMBL3. Three hundred thousand plaques were screened using radiolabelled *LeENOD40* as a probe. The screen resulted in 4 positive clones, 1 of which was analysed further. This λ phage was purified and the tomato genomic DNA was subcloned. The subclones were sequenced and the sequences were assembled leading to a contig of 15000 bp. Comparison of the genomic *LeENOD40* sequence (g*LeENOD40*) (Figure 2) and the cDNA sequence (c*LeENOD40*) revealed that the *ENOD40* gene in tomato does not contain introns as is the case for all other studied *ENOD40* genes (Roussis *et al.*, 1995; Fang and Hirsch, 1998; Flemetakis *et al.*, 2000).

```
1    A C C A C T A G C T T T G T C T C A A G A G C C T A T T G G A T A C A A T T A A        LeENOD40
1    G - - A C T A G C T T - G T C T C A A G A A C A C A C T - - - - - - A A C T A -        NtENOD40

41   A G A A A A T A A C A C T A T T C A G T T T C C C T T C T T G A A A C C T A T A        LeENOD40
31   - - - - - - - - - - - - - - - - T C A G T T T C A A A C T C T G A - - - - - - T A        NtENOD40

81   C A T T A T T A A G T G A G A G A A A A A A A A A  A T G C A G T G G G A T G A A        LeENOD40
50   G A T T A T T A A G T G A G A - - - - - - A A A A  A T G C A G T G G G A T G A A        NtENOD40
                                                      M   Q   W   D   E

121  G C A A T C C A C G G G T C T T A G  - A A A A G A T A G G C A A G T T G G A G T        LeENOD40
84   G C A A T C C A T G G G T C T T A G  G A A A A G A G A G G C A A - T T A G T - T        NtENOD40
     E   I   H   G   S

160  G G A G G A G T T T T A A A C T G C A G G G C A G T T A T G T T G C T C G A A T        LeENOD40
122  G G A G - - - - - - T G A A T T A - A G G A C T T T T A A A T - G C C C - - - T        NtENOD40

200  T T G T T C A A A A A T A T C G A C A A A T A C A T G T T G G A T T C T C C A A        LeENOD40
151  T C C T C C A A A T A C A A T T T C A A G T T T G T - T C A T A C T T T G C C A        NtENOD40

240  A T T C A A C G T G A T T A T A A C A A C A T C T C T G G A G A G T C C G A G C        LeENOD40
190  A T T T - - - - - - - C T C T G C T A G A A T - T C C A G A A A A T G C - A G C        NtENOD40

280  A A A C A T A A T T C C T C C C T A T A C A A T T T C A A G T T T G C T C A T A        LeENOD40
221  A A A A A A G G A A A G T G T T T A T G G A - - - T T G A - - - T A A T C A C A        NtENOD40

320  C T T A G C C A A A T T A T G T G T G G A T A T A T G G C A A A A G G  A T G G C        LeENOD40
255  C T - - - - - - - A A T A G C A A A - G G A T G T - T G G C A A A A G C  A T G G C        NtENOD40

360  A A A C C G G C A A G T C A C A A A A C G G C A A T T G T G G A C T C C G T T T        LeENOD40
287  G A A C C G G C A A G T C A C G A A A C G G C A A T - - - G G A C T C C G T T T        NtENOD40

400  T G G A G T C T T T  C T T G G C T T T C A A C T T A A T G T T G T A C C C T T T        LeENOD40
324  T G G A G T C T T T  C T T G G C C T T T T T A C T T A T T G T T G T A C T T - - -        NtENOD40

440  A A G T A T A T T A T T A T A T G A T A T C C A C T C T T G T G T A A G G T T A        LeENOD40
361  - - - T A T A T T G T T A T A T - - - - - C C A G T C T T C T A T A A - - T T G        NtENOD40

480  C A T G C A A G T G T G T A T G T T C T A A T G G A T T T T G G T A A G A T T G        LeENOD40
391  T G T T T T A - T T T G T A A G T C A C A - T G C A G T - - - G T G T A G T T -        NtENOD40

520  T A G T A C A T T C C T T C T C T A G T C C T A T G T T G G A A T A T A T A T A        LeENOD40
425  - A A T A T G G C T T A T C A A A A A T T - T A T A A T A G A G T - T G T - T A        NtENOD40

560  A C T T G A A T A T C A T G A G T A T                                              LeENOD40
461  A - - - G A T T G T C A T                                                          NtENOD40
```

Figure 1: Alignment of *LeENOD40* and *NtENOD40* cDNA sequences. *ENOD40* region 1 and region 2 are marked black. The peptide encoded by region 1 of *LeENOD40* and *NtENOD40* is also marked black.

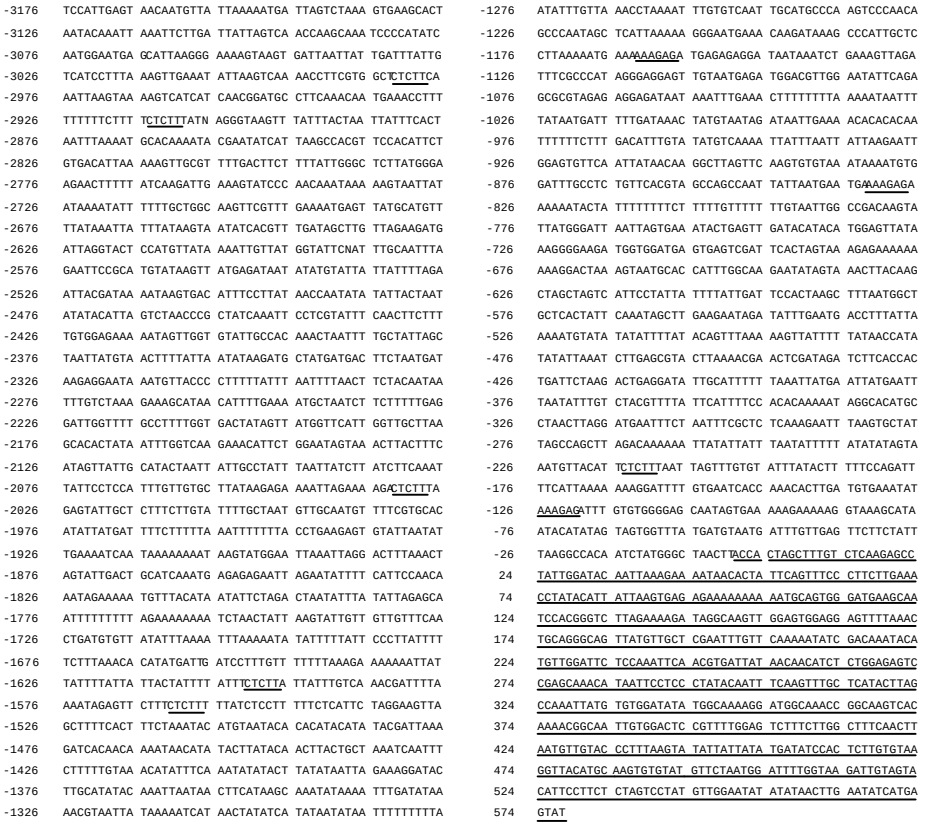


Figure 2: Nucleotide sequence of the genomic sequence of *LeENOD40*. The underlined sequence is the cDNA sequence of *LeENOD40*. Also underlined are the nodule specific motif CTCTT and an imperfect nodule specific motif AAAGAG.

RFLP linkage analysis and FISH

Tomato is a well-studied plant and many mutants have been described and their loci mapped. To determine whether *LeENOD40* maps in the vicinity of one of the described mutations, we set out to map *LeENOD40* on the tomato genome by RFLP linkage analysis. For RFLP mapping we used a mapping population derived from a cross between *L. esculentum* and *L. pennellii* (courtesy of Plant Research International, Wageningen). Digestion of genomic DNA of *L. esculentum* and *L. pennelli* with *Dra* V resulted in an RFLP for *LeENOD40*. This enzyme was used to digest the genomic DNA of all individual members of the mapping population and a Southern blot of this population was hybridised with a radiolabelled *LeENOD40* probe.

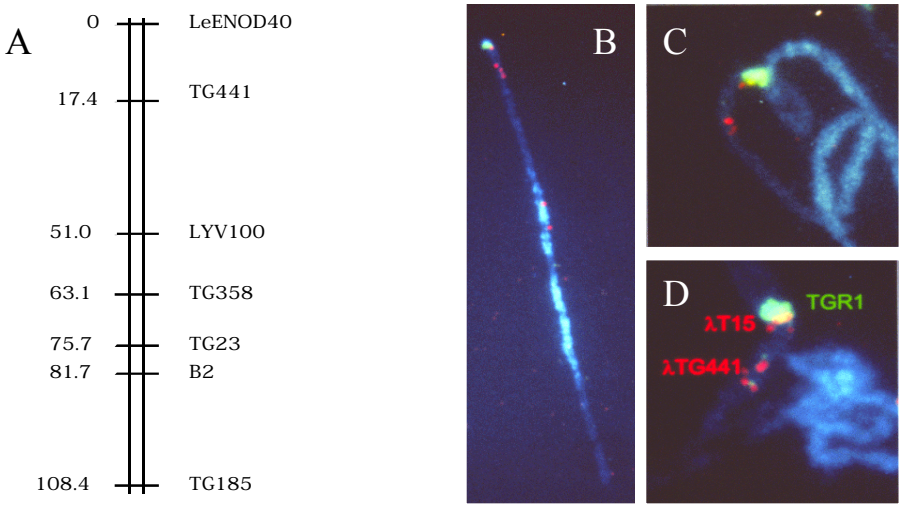


Figure 3: RFLP map of chromosome 5 of tomato and FISH on pachytene chromosomes. A: RFLP map of chromosome 5 of tomato showing the map position of *LeENOD40* 17.4 cM from the closest marker TG441. B-D: FISH (fluorescence *in situ* hybridisation) was performed on tomato pachytene chromosomes to determine the order and distance between *LeENOD40*, TG441 and the subtelomeric repeat TGR. B: Pachytene chromosomes hybridised with TGR1 (green) and λT15, which contains g*LeENOD40* (red) and λTG441 (red). λT15 hybridises so close to the TGR1 marker that the red and the green signal partly overlap giving a yellow fluorescence. C and D: Pachytene chromosomes hybridised with TGR1, λT15 and g*LeENOD40*. Enlargement of the region where the probes hybridise.

Segregation data of the mapping population were analysed using the computer program JoinMap (Stam and Van Ooijen, 1995). Linkage was observed between the marker TG441 and *LeENOD40* with a distance of 17.4 cM. This places the *LeENOD40* locus on the top of chromosome 5 (Figure 3). As TG441 was the only marker to which linkage could be shown we wanted to determine more precisely where *LeENOD40* maps on the top arm of chromosome 5. To this end, we used DNA of the marker TG441 (courtesy of Tanksley, CU) to isolate a genomic clone of this marker to be used for FISH (fluorescence *in situ* hybridisation) analysis. The λ-EMBL3 tomato genomic library was screened using radiolabelled TG441 DNA and a clone was isolated (λTG441). FISH was performed on pachytene chromosomes using as probes the two λ clones that contain the genomic regions of *LeENOD40* and TG441 (λT15, which contains *LeENOD40* and λTG441), respectively. Positions of λT15 and λTG441 were compared to that of the subtelomeric repeat TGR1 of tomato (Zhong *et al.*, 1998) to determine the distance of

both to the end of chromosome 5. Samples were first hybridised with λT15 (labelled red) and TGR1 (labelled green) and subsequently hybridised with λTG441 (labelled red). g*LeENOD40* is located in between the telomere and TG441. This confirmed the position of g*LeENOD40* and TG441 on the RFLP map of chromosome 5 with g*LeENOD40* located closest to the telomere (Figure 3). λT15, which contains *gLeENOD40*, is so close to the telomere marker TGR1 that the fluorescent colours green and red partly overlap and give a yellow fluorescence. TGR1 is at the border of the heterochromatic region of the chromosome end (Zhong *et al.*, 1998) and λT15 hybridises in the euchromatin (Figure 3). Since the condensation degree of DNA in heterochromatin (6.3 Mb/µm) is much higher than the condensation degree of DNA in euchromatin (0.6 Mb/µm) (Zhong, 1998), we cannot determine the exact distance of λT15 to TGR1 although the two markers are very close. No mutations have been mapped near the position of *LeENOD40* and therefore, the mapping of the *LeENOD40* locus does not give information on a putative function of the gene.

Expression studies of LeENOD40 in tomato

Northern analysis failed to detect *LeENOD40* mRNA, indicating that it is expressed at a low level. Therefore, expression of *LeENOD40* was studied by semi-quantitative RT-PCR using *LeENOD40* specific primers Le40-3 and Le40-4 on cDNA transcribed from total RNA isolated from different plant organs (see Materials and Methods). *LeENOD40* transcript was found in all organs tested at a similar level with the exception of roots and stems where the expression level is higher (Figure 4).

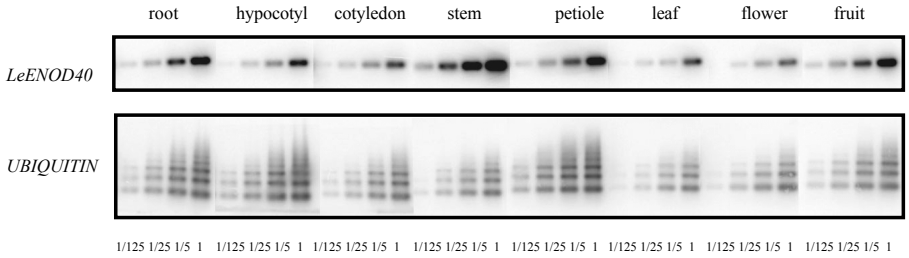
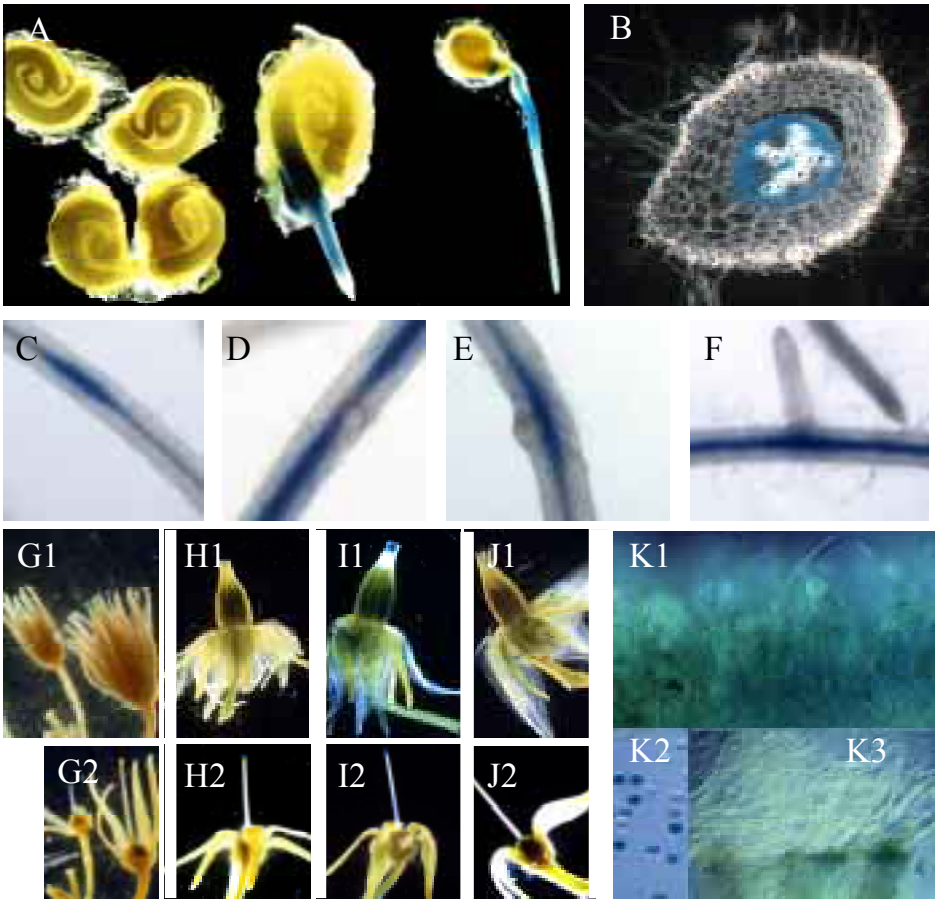


Figure 4: Expression level of *LeENOD40* compared to ubiquitin. RT-PCRs were performed on dilutions of 1, 1/5, 1/25 and 1/125 of the original cDNA samples, 25 cycles for *LeENOD40* and 20 cycles for ubiquitin.

Construction and analysis of transgenic tomato lines

In legumes, the expression of *ENOD40* has mainly been studied in roots. *ENOD40* expression can be markedly increased in the pericycle by inoculation with rhizobia or

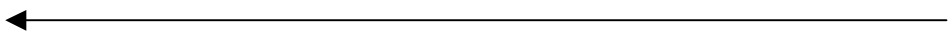
application of Nod factors, the triggers for switching on a programme leading to nodule formation. However, in the absence of symbiosis the level of *ENOD40* expression in legume roots is as low as observed for *LeENOD40* in tomato. To investigate whether we could find developmental processes in tomato where *ENOD40* might play a role, we decided to study the expression of *LeENOD40* using promoter-GUS studies in relation to tomato development. To this end, 3 kb upstream of the transcription start of *LeENOD40* (Figure 2) was fused to the *GUS* gene in pCambia1381Z (*LeENOD40::GUS*) and this construct was introduced into tomato. Transformation of tomato Moneymaker plants with *LeENOD40::GUS* resulted in 3 independent lines. Southern blot analysis showed that lines 20 and 30 contain a single insertion, whereas line 2 contained at least two insertions (data not shown). The segregation pattern (3:1) of the T2 population of lines 20 and 30 was consistent with the presence of a single T-DNA insertion site.



Analysis of promoter activity

GUS expression was studied in lines 20 and 30 in which the pattern of *GUS* expression is completely identical with only a slight difference in expression level. Immediately after germination, *LeENOD40::GUS* expression is visible just above the meristematic zone of the root and in the endosperm cap where the radicle has protruded the endosperm cap (Figure 5A). Two to 4 days after germination, *GUS* expression is observed in the vascular tissue of root, hypocotyl and cotyledons (data not shown). The expression pattern during early seedling development is very consistent and was found in all 20 seedlings studied. In 20 root systems of 4 week-old plants, we observed *LeENOD40::GUS* expression associated with the vascular bundles. The intensity of expression in the vascular bundle is variable (Figure 5C). Although in different parts of the root system *GUS* expression can be present or absent, 50-75% of the root shows *GUS* activity. Cross-sections of *LeENOD40::GUS* expressing roots show expression in the pericycle (Figure 5B) and sometimes patches of expression in the outer cortex and epidermis (data not shown).

Figure 5: LeENOD40::GUS and *GmENOD40::GUS* in tomato during different stages of development. Promoter induced *GUS* expression is visible as a blue stain. A: *LeENOD40::GUS* in imbibed seeds (72 hrs). Before germination (left) no *GUS* expression is observed. Germinating seeds (middle) show *GUS* expression in the protruding root starting just above the meristem, in the hypocotyl and in the endosperm cap. Germinated seedling (80 hrs imbibition, right); *GUS* expression in the endosperm cap, the boundary between hypocotyl and root and in the differentiation zone above the root tip. B: Cross section through the root of a two-week-old seedling. *GUS* expression is visible in the pericycle of the main root. C: Variable *LeENOD40::GUS* expression in the main root. D-F: *GUS* expression in the vascular tissue of the root during different stages of lateral root development. D: Decreased *GUS* expression in the vascular bundle at the site where a root primordium has formed. E: *GUS* expression at the site of a root primordium that is just emerging the main root. F: After emergence of the lateral root through the main root, *GUS* expression is variable in both the main root and in the lateral roots. G-J: Flowers during different stages of development. G: Two flowers before pollination, no *GUS* expression detectable in anthers (G1) or style (G2). H,I and J were flowers growing on 1 inflorescence and therefore represent three stages of development. H: Flower at anthesis; *GUS* expression is visible in petals, anthers (H1) and stigma (H2). I: Pollinated flower; strong *GUS* expression in petals, anthers (I1) and stigma (I2). J: Pollinated flower, very likely after fertilisation and in the process of senescence. *GUS* expression is visible in petals, anthers (J1) and stigma (J2). K1: Germinating pollen on style. K2: Pollen before germination. K3: Squashed sample of style with pollen tubes. *GUS* expression is only visible in the stigma and not in the pollen tubes.



After comparison of *LeENOD40::GUS* expression patterns of different root systems, we could not find a correlation between the *GUS* expression pattern and the developmental stage of the root. However, there is a rather strict correlation between lateral root formation and *LeENOD40::GUS* expression. In an early stage of lateral root development the lateral root primordium is flanked by regions of the vascular bundle where *LeENOD40::GUS* is expressed, whereas in the region where the primordium is located *ENOD40* expression is low (Figure 5E). When the lateral root is protruding the main root, *GUS* expression in the vascular bundle of the main root is spanning the region where the lateral root protrudes (Figure 5F). This regulation of *LeENOD40::GUS* expression was observed for all lateral root primordia present in the studied roots. Mature lateral roots show a variable expression pattern as was observed for the main root (Figure 5F).

LeENOD40::*GUS* is also expressed in a specific manner during flower development. For these studies, we used 5 complete inflorescences with flowers representing different developmental stages before and after anthesis. *LeENOD40* driven *GUS* expression is hardly visible before anthesis in any flower organ (Figure 5G1, G2) At this stage of development GUS stain was sometimes (2 out of 5 times) observed in the stigma. At anthesis, *LeENOD40::GUS* expression is found in the anther cone and in the stigma (Figure 5H1, H2). After anthesis *GUS* expression in the anthers increases and can still be found in the stigma after pollination of the flower. After anthesis but before petal senescence a strong staining is also found in the petals (Figure 5I1, I2). At later stages after anthesis where it is very likely that fertilisation has taken place and overall flower senescence has started, *GUS* expression is reduced in the anthers and petals but remains visible in the stigma (Figure 5J1, J2). *GUS* expression is also present in mature pollen (Figure 5K2). Strikingly, not all the pollen have detectable *GUS* expression, even when the plant is homozygous for *LeENOD40::GUS*. *GUS* expression is not detectable in germinated pollen, pollen tubes or ovules (data not shown) and remains restricted to the stigma after pollen germination (Figure 5K).

Discussion

We present detailed studies on the expression pattern of *LeENOD40* during tomato development. These studies were done on tomato transformed with *LeENOD40::GUS*. This study allows determination of the expression of *ENOD40* in the entire plant during its life cycle. Expression of *LeENOD40::GUS* in tomato is found in flowers and germinated seeds. Furthermore, it is active in vascular tissues of stems, young leaves (data not shown) and roots. This is consistent with *in situ* expression studies as well as promoter-*GUS* studies in legumes that have shown that expression of *ENOD40* is usually associated with vascular tissue (Yang *et al.*,1993; Matvienko *et al.*, 1994; Fang and Hirsch, 1998; Varkonyi-Gasic and White, 2002). The expression pattern of

LeENOD40::GUS in tomato root systems is variable and we could not correlate *ENOD40* expression with a specific developmental stage. However, we did find a correlation between lateral root formation and *LeENOD40::GUS* expression in main root vascular tissue. We suggest that *LeENOD40::GUS* expression marks the sites in the root where lateral roots can form. This hypothesis is based on the observation that lateral root primordia in the main root are flanked by high *LeENOD40::GUS* expression, which could reflect that *LeENOD40::GUS* is actively repressed at that site. When the lateral root primordium grows out of the root the expression increases to a level comparable to or higher than the basic level in the vascular bundle. Expression of *ENOD40* in emerging lateral roots has been described in legumes (Papadopoulou *et al.,* 1996; Fang and Hirsch, 1998; Varkonyi-Gasic and White, 2002). In these plants *ENOD40* expression is observed in emerging lateral roots as is observed for *LeENOD40* in tomato. During the tomato life cycle *LeENOD40* is expressed after germination in the seed and in flowers before the onset of and during flower senescence. Like in nodule development, in these processes the phytohormone ethylene plays an important role. In endospermic tobacco seeds radicle protrusion is accompanied by a burst of ethylene production (Khalil, 1992). Ethylene induces production of β-glucanases, which are important for weakening of the endosperm cap before penetration of the root. In tobacco, induction of class I β-1,3-glucanase occurs exclusively in the micropylar region of the endosperm where the radicle will penetrate (Leubner-Metzger *et al.,* 1998). In tomato this is exactly the position where *LeENOD40::GUS* expression is found. While ethylene induction of β-glucanases takes place before germination, *LeENOD40::GUS* expression occurs after germination.

During flower senescence, *LeENOD40* could act in a similar way as during germination. In many flowers, among which tomato flowers, the initial response to pollination is an increase in ethylene production by the stigma. This is followed by an increased ethylene production from ovaries and petals. Pollination-induced ethylene production by different floral organs is necessary for regulation of pollination associated events such as ovary growth (reviewed in: Larsen *et al.,* 1993; Woltering *et al.,* 1994). *LeENOD40::GUS* expression in the stigma coincides with anthesis although a light staining is sometimes observed before anthesis. Therefore, it is likely that *LeENOD40::GUS* expression marks the time of pollination of the stigma. *LeENOD40::GUS* expression also occurs in the petals after pollination. *LeENOD40::GUS* expression precedes petal senescence but appears to coincide with ethylene production by petals, after pollination has occurred. *ENOD40* is expressed in white clover flowers in the pedicels that connect florets with the inflorescence axes. This expression was observed in the pedicel vascular tissue after the onset of senescence in the lower floret whorls (Varkonyi-Gasic and White, 2002). Although the location of expression is different, in both cases *ENOD40* expression is

observed after the onset of senescence. From our observations we conclude that *LeENOD40* is expressed in tomato flowers after an increase of ethylene production as occurs in germination. This suggests that *ENOD40* expression might be under the control of ethylene. However, this is not likely because *LeENOD40* expression is not associated with all ethylene-related processes. For example, during tomato fruit ripening, no clear change in *LeENOD40::GUS* expression level is observed. We therefore conclude that expression of *LeENOD40* coincides with specific ethylene related processes in plant development. These processes have in common that ethylene activity is first induced and then reduced unlike a process as fruit ripening where ethylene induces its own production. Based on our observations, we suggest that *LeENOD40* could have a role in reducing ethylene production or in reducing local effects of ethylene production. Ethylene is known to have an inhibitory effect on nodule formation. Nodulation mutants impaired in nodule formation and with increased nodule formation have been found that can be related to an increased or decreased sensitivity to ethylene respectively (Guinel and LaRue, 1991; Penmetsa and Cook, 1997; Heidstra *et al.*, 1997). These studies demonstrate that ethylene acts as an inhibiting factor for primordium formation. In pea roots ACC oxidase is expressed in the region opposite phloem poles (Heidstra *et al.*, 1997), while *ENOD40* is expressed in the region opposite the protoxylem poles, which is also the region where nodules form. This suggests that ethylene provides positional information for nodule primordia to form opposite protoxylem poles. Overexpression of *MtENOD40* in *Medicago* roots leads to a transient acceleration of nodule formation and this effect can be partially mimicked by addition of AVG, an inhibitor of ethylene formation, suggesting a relation between ethylene and *ENOD40* activity (Charon *et al.*, 1999). Whether *ENOD40* is recruited in legumes to limit ethylene production or to reduce ethylene effects in nodule primordia is an interesting hypothesis to test.

CHAPTER 3

Legume and non-legume *ENOD40* promoters function identically in legumes and non-legumes

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Abstract

The presence of homologues of genes involved in nodule development (nodulins) in non-legumes indicates that legumes have recruited genes for nodule formation involved in other, more ancient, plant processes. The presence of nodulin homologues in non-legumes opens the possibility to study the regulation and function of these genes in non-legumes. We investigated whether *ENOD40* is regulated in the same manner during tomato plant development and nodule development. We studied the effect of cytokinin, ethylene and rhizobia on *LeENOD40::GUS* expression in transgenic tomato plants. Similar studies were repeated in *GmENOD40::GUS Arabidopsis* roots. Our studies show that during evolution essential elements for temporal and spatial expression of *ENOD40* have not been changed in legume and non-legume *ENOD40* promoters.

Introduction

The ability to establish a symbiosis with rhizobia leading to nodule formation is in general restricted to one plant family, the legumes. During this process several nodule specific genes, called nodulin genes are expressed at markedly elevated levels. Homologues of nodulin genes have been found in non-legumes (Kouchi *et al*. 1999; Gualtieri and Bisseling, 2000; Stougaard, 2000; Spaink, 2002). *Rhizobium* induced symbiosis is unique for legumes while AM formation, which is the result of an endosymbiotic interaction of plant roots and fungi of the order of *Glomales*, occurs in most higher plants. It has been suggested that nodule formation evolved from arbuscular endomycorrhiza (AM) formation as identical genes are involved in both interactions (Gianinazzi-Pearson, 1996; Albrecht *et al.,* 1999; Staehelin *et al*., 2000; Endre *et al.,* 2002). Following this reasoning, the wide occurrence of AM could explain in part why homologues of nodulins could be found in non-legumes. On the other hand it has become clear that processes that contribute to nodule development have been recruited from processes that are common to most or even all higher plants. Several observations described in Gualtieri and Bisseling (2000) are in line with the view that rhizobia have recruited genes from general developmental processes to establish a symbiotic interaction. This implies that expression of a set of genes involved in plant development might have been changed and their function is now related to nodule formation. To study the relation between nodulins that are expressed during nodulation and their non-symbiotic homologues, we compared regulation of expression of a nodulin gene and its non-legume homologue during non-legume plant development as well as during nodule development. We chose to study the early nodulin *ENOD40* homologue from tomato during tomato plant development. *ENOD40* is one of the early induced genes during nodule development (Yang *et al.,* 1993). *ENOD40* homologues are found in non-legumes like rice (Kouchi *et al.,* 1999), maize, citrus and the *Solanaceae* tobacco (Matvienko *et al.,* 1996) and tomato (this thesis, chapter 2). Strikingly, no *ENOD40*

homologue has been found in the model plant *Arabidopsis thaliana*, but the soybean *ENOD40* promoter has been shown to be active in *Arabidopsis* (Mirabella *et al.,* 1999) with an expression pattern comparable to *LeENOD40* in tomato (this thesis, chapter 2). *ENOD40* was shown to be essential for proper nodule formation in *Medicago truncatula* (Charon *et al.,* 1999), since the reduction of *ENOD40* expression by cosuppression during nodule formation leads to the formation of aberrant nodules. Efforts to generate *Medicago sativa* plants overexpressing *ENOD40* in the sense or antisense orientation led to the formation of teratomas and callus growth arrest, respectively (Crespi *et al.*, 1994). Furthermore, overexpression of soybean *ENOD40* in tobacco plants led to suppression of apical dominance (Van de Sande *et al.,* 1996). These experiments showed that *ENOD40* might not only be important for nodule formation but also for plant development.

To investigate whether *ENOD40* is regulated in the same manner during tomato plant development as during nodule development, we studied *LeENOD40* expression in previously described *LeENOD40::GUS* plants (this thesis, chapter 2) and *GmENOD40* expression in *Arabidopsis thaliana*. We studied the effect on *LeENOD40* expression in tomato roots of the phytohormones ethylene and cytokinin for which it has been shown that they affect the legume *ENOD40* expression level. Our studies suggest that cis-regulatory elements of the tomato *ENOD40* promoter are sufficient for symbiosis specific expression during nodule formation on legumes. Furthermore, the legume *GmENOD40* promoter behaves like a non-legume *ENOD40* promoter in roots of a non-legume.

Materials & methods

Plant material

Tomato plants (*Lycopersicon esculentum* Moneymaker) and *Arabidopsis* were grown in a growth chamber at 25°C and a 16 hour photoperiod (7W/m^2). For the analysis of full-grown plants and for generation of offspring plants were moved to a greenhouse (at 18 to 21°C) with additional light during the winter (16 hour photoperiod, 150 W lights).

Transgenic *LeENOD40::GUS* tomato plants (this thesis, chapter 2) were grown aseptically in jars on 1/2 MS (Murashige and Skoog medium, Duchefa) containing 10 g/l sucrose (1/2 MS 10) and 15 mg/l hygromycin in a growth chamber (conditions described above). After two weeks plants resistant to hygromycin were transferred to soil (and moved to a greenhouse) or to medium without antibiotics for further treatments.

Transgenic *Arabidopsis* plants (Enod40-1 and Enod40-2; Mirabella *et al.*, 1999) were grown aseptically on 1 MS (Duchefa) containing 10 g/l sucrose.

Root treatments

T2 tomato plants were grown on 1/2 MS (with 10 g/l sucrose) selection medium containing hygromycin (15 mg/l) or kanamycin (50 mg/l). After two weeks when the selection was clear, antibiotic resistant plants were transferred to 20 ml brown flasks containing 10 ml of Fåhreus medium (Fåhreus, 1957) without NO_3^-. Each flask contained 3 plants held together by cotton wool in the top of the flask. The plants were incubated on Fåhreus medium for 10 days. After these 10 days the medium was refreshed and hormones were added.

Arabidopsis seeds of line Enod40-1 and Enod40-2 were germinated on medium containing 1 µM or 10 µM BA, respectively. After 3, 5 or 7 days the plants were stained for *GUS* expression.

GUS assays

GUS assays were performed according to the protocol of Kosugi *et al.,* (1990). Plant material was stained in GUS buffer (0.1 M Na_2PO_4 pH 7.2, containing 1mM X-Gluc dissolved in DMF and 0,1% TRITON X-100). The plants were put 10 minutes under vacuum and were incubated at 37°C for 4 hours or longer until the blue GUS stain was visible.

RT-PCR

RNA was isolated from different organs of tomato using the hot phenol method for RNA isolation (Pawlowski, 1994). After RNA isolation the samples were treated with DnaseI (Promega) to remove all chromosomal DNA that could disturb the PCR reactions. Total cDNA was made from 2.5 µg of total RNA using an oligo dT primer in a volume of 20 µl (10 mM Tris-HCl pH 8.8, 50 mM KCl, 5 mM $MgCl_2$, 1 mM dNTPs, 1 µg oligo dT12-18 (Pharmacia), 20 U RNA guard (Pharmacia) and 200 U MMLV reverse transcriptase (Stratagene). The samples were incubated for 1 hour at 37°C. To inactivate the reverse transcriptase enzyme the samples were incubated at 95°C for 5 minutes. The RT samples were then diluted to 100 µl and 1 or 2 µl of the cDNA were used for PCR (10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 100 µM dNTPs, 50 ng primer and 1 U Taq polymerase (Boehringer Mannheim, USA) in a total volume of 50 µl). Primers Le40-3: 5'-GCAAGTTGGAGTGGAGG-3' and Le40-4: 5'-ACACACTGGCATGTAACC-3' were used (annealing temperature 58°C) for the PCR reactions on tomato roots. For these reactions the following cycling program was used: denaturation 95°C 30 sec, annealing 30 sec (temperature dependent on primers), extension 70°C 30 sec. Amplified DNA samples were run on a 1% agarose gel, alkaline blotted to Nytran-N^+ membrane and hybridised to radiolabelled inserts of the corresponding cDNA clones.

Hairy root transformation of Medicago truncatula

Hairy root transformation of *Medicago truncatula* was performed according to Limpens *et al.* (in preparation).

Results

The effect of cytokinin on LeENOD40 *promoter activity*

We investigated the effect on *LeENOD40::GUS* expression in tomato by cytokinin. This hormone is known to induce *ENOD40* expression in legumes grown under limited nitrogen conditions (Fang and Hirsch, 1998; Mathesius *et al.,* 2000). For these experiments we used *LeENOD40::GUS* tomato lines 2, 20 and 30. These lines show *LeENOD40::GUS* expression in root vascular tissue. *LeENOD40::GUS* expression generally occurs in young as well as old parts of the root, although the expression can be off in some areas without a clear relation to the developmental stage of such a region (this thesis, chapter 2). Lines 20 and 30 have the same expression level of *LeENOD40::GUS*, but in line 2 *LeENOD40::GUS* expression level is markedly lower.

Plants were grown for two weeks and then starved for NO_3^- for 10 days, since *ENOD40* expression in legumes, grown at nitrogen limiting conditions, is induced by cytokinin. At day 0, the medium was replaced by medium supplemented with the factors to be tested and plants were grown for 2 days. In control experiments we replaced the growth medium at day 0 by medium without the supplemented factors. Plants were scored negative for *LeENOD40* activity when *GUS* activity would be detected in less than 20% of a tested root system. In control plants 75% or more of the root system has *GUS* activity.

Four experiments were performed in which we tested the effect on *LeENOD40* expression by 1 µM cytokinin (BAP). For each treatment, we tested the roots of 30 *LeENOD40::GUS*-2, 30 *LeENOD40::GUS*-20 and 30 *LeENOD40::GUS*-30 plants. A considerable decrease of *LeENOD40::GUS* expression in the roots of lines 20 and 30 was observed in all of the tested plants (Table 1, Figure 1A, B). *LeENOD40::GUS* expression in line 2 was hardly detectable after growth on Fåhreus medium. The expression level in this line was too low to determine the effect of treatments with cytokinin.

Table 1: LeENOD40::GUS expression after treatment with BAP, ACC and AVG.

	Control	BAP	ACC	AVG*[2]
Line 2*[1]	-	-	+	ND
Line 20	+	-	+	-
Line 30	+	-	+	-

In 4 experiments a total of 21 plants per line, per treatment were used. *[1] For line 2, 18 plants were tested in 3 experiments. *[2] For the AVG treatment 18 plants were tested in 3 experiments. Four different treatments were performed on the lines in which plants were incubated for 48 hours. The results were comparable for all plants per treatment. Control: medium without tested factors. BAP: control medium with1 µM BAP. ACC: control medium with 100 µM ACC. AVG: control medium with 50 µM AVG. -: Less than 20% of the root system shows a clear *GUS* staining. +: Over 75% of the roots show a clear *GUS* staining with intermediate intensity. ND: Experiment not performed.

The effect of cytokinin on GmENOD40 promoter activity in Arabidopsis thaliana

The observation that cytokinin reduces *LeENOD40::GUS* expression in tomato roots whereas it stimulates *ENOD40* expression in legume roots prompted us to investigate the response to cytokinin of a legume *ENOD40* promoter in another non-legume. To this end, we investigated how expression of *GmENOD40::GUS* in *Arabidopsis* is regulated by cytokinin. We studied *Arabidopsis* lines Enod40-1 and Enod40-2 (Mirabella *et al.*, 1999). 15 plants of line Enod40-1 and 15 plants of Enod40-2 were treated for 7 days with 1 µM BA (cytokinin). The comparison with control plants showed that the promoter activity was markedly reduced by cytokinin in all of the tested plants (Figure 1D,E). In conclusion, cytokinin decreases *ENOD40* promoter activity in *Arabidopsis* as well as tomato. This is the case for the legume *GmENOD40* promoter as well as for the non-legume *LeENOD40* promoter.

The effect of ethylene and AVG on LeENOD40 promoter activity

Ethylene is known to inhibit nodule formation and in line with this, application of AVG or Ag^+, blockers of ethylene production and perception, respectively, has a positive effect on the number of nodules that are formed (Peters and Crist-Estes, 1989; Lee and LaRue 1992a; Lee and LaRue 1992b). We tested the effect of exogenous application of ACC (precursor of ethylene) and AVG on *LeENOD40::GUS* expression in tomato roots. The effect of AVG was tested on the tomato *LeENOD40::GUS* lines 20 and 30. In these experiments 21 plants per line were used for each growth condition. After application of 50 µM AVG we observed a decrease of *LeENOD40::GUS* activity in the roots of lines 20 and 30 (Table 1, Figure 1A, C). *LeENOD40::GUS* expression is decreased in all of the 21 plants incubated with AVG. We applied the ethylene precursor ACC (100 µM) to

tomato lines 2, 20 and 30. Only in line 2 we observed an increase of *GUS* expression by ACC application in 6 out of 18 plants. This increase in *GUS* expression was not observed in lines 20 and 30 even when shorter GUS staining periods were used (Table 1). It is possible that the endogenous ethylene concentration is already at a level leading to a maximal expression level. In conclusion, application of AVG to *LeENOD40::GUS* tomato roots leads to a decrease in *ENOD40* promoter activity in these roots. Furthermore, application of ACC to *LeENOD40::GUS* tomato roots leading to higher ethylene concentration may also cause an increase in *ENOD40* promoter activity.

LeENOD40 is similarly regulated as LeENOD40::GUS in transgenic tomato roots

To test whether the effect of cytokinin and ethylene on expression of *LeENOD40::GUS* in the transgenic line reflects that of the endogenous *LeENOD40*, we investigated expression of *LeENOD40* in roots by RT-PCR. Line 20 plants were treated with BAP and AVG, respectively, which reduced *LeENOD40::GUS* expression, and ACC, which could increase *LeENOD40::GUS* expression. Three plants per treatment as well as three control plants were pooled and RNA was isolated from the roots. This RNA was subjected to RT-PCR using *LeENOD40* mRNA specific primers. The application of BAP caused a decrease of *LeENOD40* mRNA level by approximately 50% (Figure 1F). This result is similar to that obtained by the *LeENOD40::GUS* analysis where application of BAP causes a decreased *LeENOD40::GUS* expression level.

The application of AVG to tomato roots led to a marked decrease of the *LeENOD40* expression level to about 15% of the control level (Figure 1F). Therefore, these RT-PCR data confirm the result of AVG application to *LeENOD40::GUS* plants, which causes a decreased *GUS* expression. Application of ACC slightly increased the expression level of *LeENOD40* in these plants (Figure 1F). However, such a small difference would not have been observed in the *GUS* expression studies. These RT-PCR expression studies on *LeENOD40* in tomato confirm the results obtained by *LeENOD40::GUS* expression analysis in tomato. Therefore, we conclude that cytokinin and AVG have a negative effect on *LeENOD40* expression and that ethylene might have a positive effect on *LeENOD40* expression in tomato.

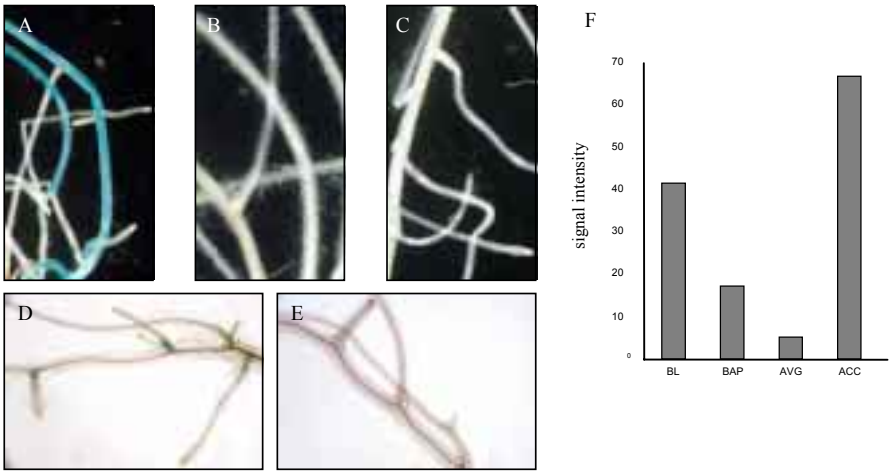

Figure 1: Regulation of *ENOD40* expression in tomato and *Arabidopsis*. A-C: Comparison of *LeENOD40* promoter induced *GUS* expression after treatment of *LeENOD40::GUS* line 20 roots with cytokinin and AVG. A: *LeENOD40::GUS* control stained for *GUS* expression. B: *LeENOD40::GUS* treated with 1 µM BAP for 48 hours, stained for *GUS* expression. C: *LeENOD40::GUS* sample treated with 50 µM AVG for 2 days, stained for *GUS* expression. D-E: Expression of *GmENOD40*::*GUS* expression is regulated by cytokinin in roots of *Arabidopsis thaliana*. D: Untreated *Arabidopsis GmENOD40::GUS* roots. E: *Arabidopsis GmENOD40::GUS* roots treated with 1 µM BA for 7 days. F: Expression level of *LeENOD40* compared to *Ubiquitin* standard. RT-PCRs were performed on dilutions of 1, 1/5, 1/25 and 1/125 of original cDNA samples of *LeENOD40::GUS* line 20. 30 cycles were performed for the PCR on *LeENOD40* and 25 cycles for the PCR on *Ubiquitin*. The intensity of the hybridised fragments was measured using Imagequant (Molecular Dynamics) and the *LeENOD40* expression levels were standardised using the ubiquitin profile. BL = control medium without added factors.

LeENOD40::GUS *expression in nodules on* Medicago truncatula *hairy roots*

From the studies described above, we conclude that the effect on *LeENOD40* expression by exogenously applied cytokinin in tomato is different from that of cytokinin on *ENOD40* expression during legume nodule formation. This also seems to be the case for the effect of ethylene on *ENOD40* expression in tomato and legumes. Therefore, it was surprising that *GmENOD40* in *Arabidopsis* is regulated in a similar manner as *LeENOD40* in tomato, which suggests that legume and non-legume promoters in non-legumes are regulated in a similar way. However, the non-legume *LeENOD40* promoter could lack domains present in legume *ENOD40* promoters responsible for the *ENOD40* expression pattern in legumes.

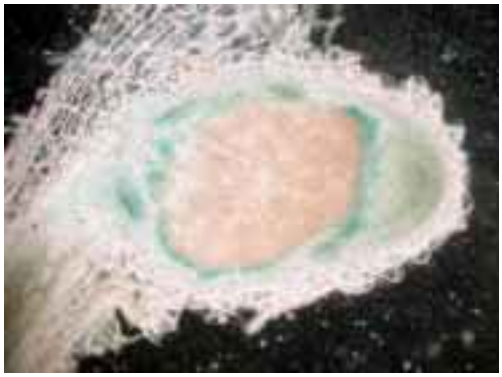

Figure 2: LeENOD40::GUS expression in nodules grown on *Medicago truncatula* hairy roots. Roots were infected with *Sinorhizobium meliloti* Rm2011, the symbiont of *Medicago truncatula* and harvested after three weeks. For these observations, 25 µm agarose sections were made from agarose blocks. *LeENOD40::GUS* is expressed in in the vascular bundle and in the boundary layer of the nodule.

To test the latter possibility we decided to compare the expression behaviour of the *LeENOD40* promoter in root nodules formed on *Medicago truncatula* hairy roots to the expression behaviour of the *MtENOD40* promoter in root nodules formed on *Medicago truncatula*. For the transformations we infected the roots of 15 plants. These roots, transgenic for *LeENOD40::GUS*, were tested for *GUS* expression after inoculation with *Sinorhizobium meliloti* Rm2011, a symbiont of *Medicago truncatula*. *LeENOD40*::*GUS* expression is found in nodules, harvested three weeks after infection with rhizobia. In 25 µm thick nodule sections, we observed *LeENOD40::GUS* expression in the vascular bundles and central tissue of the nodule (Figure 2). This is in agreement with *ENOD40* expression patterns in nodules of various legumes (Hatta and Kouchi, 1993, Crespi *et al*., 1994; Roussis *et al*., 1995; Papadopoulou *et al*., 1996; Fang and Hirsch, 1998). We conclude that in a legume background the non-legume *LeENOD40* promoter and legume *ENOD40* promoters are regulated in a similar manner.

Discussion

In this study we compared regulation of *ENOD40* expression in a legume and non-legume background to investigate whether *ENOD40* is a gene that was recruited for nodule formation from a non-symbiotic plant developmental process.

We showed that *GmENOD40* in *Arabidopsis* and *LeENOD40* in tomato are regulated similarly by cytokinin, suggesting that regulation of legume and non-legume promoters in non-legumes is comparable. Furthermore, we showed that *LeENOD40::GUS* is expressed in nodules in the same tissues as *MtENOD40::GUS* and so, the *LeENOD40* promoter is active during the *Rhizobium*-legume symbiosis and expressed in similar nodular tissues as the endogenous *ENOD40*. From this we conclude that all sequences required for a symbiotic response are present in the *LeENOD40* promoter. This result is similar to the result of Kouchi *et al*., (1999) who showed that in nodules grown on soybean transgenic hairy roots the rice *ENOD40* promoter is active in a similar pattern

as the soybean *ENOD40* promoter. Therefore, the *ENOD40* promoter activity of legumes and non-legumes is similar and furthermore this similarity extends from monocots to dicots. This suggests that the *cis*-regulatory sequences required for nodule specific expression are present in the non-legume promoters. This hypothesis is consistent with the observation that the legume *GmENOD40* and non-legume *LeENOD40* promoters are expressed in non-legumes in the same tissues, and regulated by cytokinin in a similar manner. This shows that during evolution essential elements for temporal and spatial expression of *ENOD40* have not changed in legume and non-legume *ENOD40* promoters. Since these sequences are conserved, it is probable that these regulatory elements are involved in both non-symbiotic and symbiotic regulation of *ENOD40* expression. This implies that most likely *ENOD40* is not a key gene in nodule development that has been recruited during evolution for this specific process. Rather, legumes have found another way to provoke the unusual high levels of *ENOD40* mRNA during nodule formation. This could have been achieved by influencing *ENOD40* mRNA stability or by interference with the regulation of *ENOD40* transcription. We are in favour of the latter as it is more likely that the unique increase in *ENOD40* mRNA is caused by increased transcription than by increased mRNA stability. A candidate for such a gene involved in this regulation must be active within 3 hours after inoculation with rhizobia as this is the time frame in which elevated levels of *ENOD40* expression are apparent. There may be specific transcription factors involved in symbiotic and non-symbiotic expression of *ENOD40* or modification of a transcription factor may occur after the plant senses the presence of rhizobia. Isolation and characterisation of this transcription factor may discriminate between these two possibilities.

The observation that cis-regulatory elements are conserved in the promoter of *ENOD40* in non-legumes and legumes suggests that the function of *ENOD40* might be conserved as well. The elevated expression of *ENOD40* during nodule development might reflect the need for this function. We showed that addition of ACC leads to a slight increase in *LeENOD40* expression in tomato roots, while addition of AVG leads to a decrease of *LeENOD40* expression. These results indicate that ethylene is involved in the regulation of *ENOD40* expression. This is supported by the observation that *LeENOD40* expression is induced during processes where ethylene is known to play a role such as germination and flower senescence. It was suggested that *ENOD40* expression is involved in counteracting ethylene-provoked responses (this thesis, chapter 2).

Several studies show that ethylene has a negative effect on primordium formation (Guinel and LaRue, 1991; Lee and LaRue, 1992b; Penmetsa and Cook, 1997) and infection during nodule formation (Penmetsa and Cook, 1997; Guinel and Sloetjes, 2000; Oldroyd *et al*., 2001). Furthermore, ethylene produced at the phloem poles provides positional information for nodule primordia to form opposite protoxylem poles

(Heidstra *et al.*, 1997). During nodule formation *ENOD40* is expressed in the pericycle facing the protoxylem pole. Expression of *ENOD40* in this region might be required to establish of a zone where the negative effect that ethylene has on primordium formation is counteracted.
In this view, the function of *ENOD40* might be similar in tomato non-symbiotic development and during nodule development.

CHAPTER 4

Towards unravelling *ENOD40* function by overexpression studies of *GmENOD40* in tomato

Ingrid Vleghels, Jan Hontelez, Ton Bisseling and Henk Franssen

Abstract

The gene *ENOD40* is considered to play an important role during nodule and plant development. All *ENOD40* genes share the conserved regions 1 and 2. Region 1 encodes a small peptide of 10-13 amino acids depending on the plant species. Region 2 does not contain a conserved ORF. To investigate *ENOD40* function in a non-legume we generated tomato plants overexpressing *ENOD40*. For this, we introduced the heterologous soybean *ENOD40* gene in tomato (*35S::GmENOD40*). To be able to investigate the role of the ENOD40 peptide in the establishment of a phenotype we also introduced *35S::GmENOD40* in tomato in which we changed the ATG start codon into AAG to inhibit production of the ENOD40 peptide. The two sets of transgenic tomato lines have been produced. Although phenotypic effects are observed in the *GmENOD40* overexpression lines, the transgenic lines are still under investigation to determine whether these phenotypic effects are due to *GmENOD40* overexpression.

Introduction

Nodule formation is the result of the symbiotic interaction between legume plants and rhizobia (*Azorhizobium*, *Bradyrhizobium*, *Rhizobium* and *Sinorhizobium*). Inside these nodules, rhizobia fix atmospheric nitrogen into ammonia. Nodule formation has been the subject of many studies and several genes have been identified that are expressed at a markedly higher level during this process. *ENOD40* is one of the early induced genes during nodule development. Soon after the plant root surface is colonised by rhizobia, expression of *ENOD40* is induced in the pericycle opposite the position where the nodule primordium will be formed (Compaan *et al.*, 2001). Furthermore, *ENOD40* is expressed in the nodule primordium and in the infection zone and vascular bundles of mature nodules (Yang *et al.*, 1993; Matvienko *et al.*, 1994). The timing and patterning of expression indicates that *ENOD40* could play an important role in the formation of nodules. This is further underlined by studies using a reverse genetics approach. Overexpression of *ENOD40* in *Medicago truncatula* leads to an increased number of nodule primordia and more dividing cortical cells near the root tip (Charon *et al.*, 1997). Reduction of *ENOD40* expression during nodule formation by co-suppression leads to the formation of aberrant nodules (Charon *et al.*, 1999), suggesting that *ENOD40* is essential for proper nodule formation. Homologues of *ENOD40* are also found in non-legumes like rice, maize, citrus and the *Solanaceae* tobacco and tomato. These plants cannot form nodules and therefore, the function of *ENOD40* in these non-legumes must be related to non-symbiotic plant development. Overexpression of *GmENOD40* in tobacco leads to the formation of extra shoots (Van de Sande *et al.*, 1996). Comparison of nucleotide sequences of *ENOD40* homologues in legumes and non-legumes reveals that they all share the conserved regions 1 and 2 (Van de Sande *et al.*, 1996; Corich *et al.*, 1998; Kouchi *et al.*, 1999; Flemetakis *et al.*, 2000). Region 1 of all these *ENOD40*

homologues encodes a remarkably small peptide of 10-13 amino acids. Region 2 is about 55 nucleotides long and is located at the 3' end of the mRNA. However, it does not contain a conserved ORF. To be able to investigate *ENOD40* function in non-symbiotic development we studied the effect of *ENOD40* overexpression in a non-legume. We introduced the heterologous *GmENOD40* in tomato to circumvent problems with transgenic lines overexpressing the endogenous gene, as has been reported in *Medicago sativa* (Crespi *et al.,* 1994). Furthermore, *35S::GmENOD40* has been successfully introduced in tobacco. In these plants *35S::GmENOD40* caused a reduction of apical dominance (Van de Sande *et al.,* 1996). We chose the non-legume tomato for these studies because it is a well-studied plant, for which detailed genetic maps, cDNA and genomic libraries are available and a sequence programme is running. To investigate the function of the *ENOD40* peptide we also introduced *35S::GmENOD40* in tomato in which we changed the ATG start codon into AAG to inhibit production of the ENOD40 peptide.

Materials & methods

Plant material

Tomato plants (*Lycopersicon esculentum* Moneymaker) were grown in the greenhouse at 25 °C and a 16 hour photoperiod ($7W/m^2$). For generation of offspring, plants were moved to a greenhouse (at 18 to 21°C) with additional light during the winter (16 hour photoperiod, 150 W lights).

Transgenic plants were grown aseptically in jars on 1/2 MS (Murashige and Skoog medium, Duchefa) containing 10 g sucrose and 15 mg/l hygromycin in a growth chamber (conditions described above). After two weeks plants resistant to hygromycin were transferred to soil (and moved to a greenhouse) or to medium without antibiotics for further treatments.

Cloning of constructs

The *GmENOD40* sequence was taken from a pMON999 vector (Monsanto) containing the full size *GmENOD40* sequence fused to a *35S* promoter (Van de Sande *et al.,* 1996). *35S::GmENOD40* was isolated form this construct as a *Hind* III and *Bam* HI fragment and ligated into pCAMBIA1390. For construction of 35S::*GmENOD40* AAG two PCR's on pCAMBIA 1390-*35S::GmENOD40* were performed. The first PCR was performed with 35S-F 5'-GGATTGATGTGATATCTCC-3' as a forward primer and GmNcoR: 5'-AACCATGGATGGTTGTGAGCCAACAAAGCTCCTTG-3' as a reverse primer (at 56° annealing temperature). The second PCR was performed with GmNcoF: 5'-CAACCATCCATGGTTCTTG-3' as a forward primer and NOSR 5'-CCAAATATT TGAACGATCGGGG-3' as a reverse primer. Subsequently, the first product was

digested with *Xba* I and *Nco* I and the second product was digested with *Nco* I and *Bam* HI. The two *GmENOD40* fragments were ligated into pCAMBIA1300 (Cambia, Australia) in a three point ligation. The replacement of the ATG start codon of the *GmENOD40* peptide sequence by AAG was confirmed by sequencing.

RT-PCR

RNA was isolated from leaves of tomato using the hot phenol method for RNA isolation (Pawlowski, 1994). After RNA isolation the samples were treated with DnaseI (Promega) to remove chromosomal DNA that could disturb the PCR reactions. Total cDNA was made from 2.5 µg of total RNA using an oligo dT primer in a volume of 20 µl (10 mM Tris-HCl pH 8.8, 50 mM KCl, 5 mM $MgCl_2$, 1 mM dNTPs, 1 µg oligo dT12-18 (Pharmacia), 20 U RNA guard (Pharmacia) and 10 U AMV reverse transcriptase (Stratagene). The samples were incubated for 10 minutes at room temperature, 30 minutes at 42°C, 60 minutes at 50°C and 10 minutes at 65°C. To inactivate the reverse transcriptase enzyme the samples were incubated at 95°C for 5 minutes. The RT samples were then diluted to 100 µl and 1 or 2 µl of the cDNA were used for PCR (10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 100 µM dNTPs, 50 ng primer and 1 U Taq polymerase (Boehringer) in a total volume of 50 µl). For the RT-PCR reactions of the different tomato plant organs the primers Le40-3: 5'-GCAAGTTGGAGTGGAGG-3' and Le40-4: 5'-ACACACTGGCATGTAACC-3' were used.

Transformation procedure

The transformation procedure was performed as described in chapter 3.

Southern analysis

Genomic DNA was isolated according to Kozik *et al.* (1996). 10 µg of genomic DNA were digested with *Eco* RI and *Eco* RV and separated on a 0.8% agarose gel. Gels were blotted onto a Nitran Plus membrane (Amersham). Blotting, hybridisation and washing were performed according to the manufacturer's recommendations. A 700 bp *GmENOD40* fragment was labelled with ^{32}P and used as a probe.

Cell size measurements

Silicon rubber impressions were made of the adaxial leaf surface using Xantopren (Bayer Ltd, Leverkusen, Germany). When hardened, impressions were removed from the leaf, a positive was produced with nail-varnish. This positive was subsequently studied with a light microscope.

Results

Construction and analysis of transgenic tomato lines

To investigate the role of *ENOD40* in tomato development we studied the effect of ectopic expression of *GmENOD40* in tomato. We also wanted to investigate the importance of the *ENOD40* peptide for *ENOD40* functioning. To this end, we generated two sets of transgenic plants. For the first set, we introduced *GmENOD40* under the control of the *CaMV 35S* promoter (*35S::GmENOD40* ATG) to obtain overexpression in tomato. For the second set, we generated plants in which we introduced *GmENOD40* with an AAG codon instead of the ATG start codon in the region 1 ORF of *GmENOD40* (*35S::GmENOD40* AAG). Primary transformants were analysed for the presence of the introduced transgenes by analysis of genomic DNA of the individual transformants digested with either *Eco* RI or *Eco* RV (Figure 1). The digested DNA was separated by agarose gel electrophoresis and subsequently blotted. The blot was hybridised to a ^{32}P labelled *GmENOD40* fragment. *GmENOD40* does not hybridise to the endogenous *LeENOD40* gene because their overall homology is too low. Therefore, only the introduced transgene can be visualised with the *GmENOD40* probe. Digestion with *Eco* RI results in an internal fragment of 450 bp (figure 1) of the introduced *35S::GmENOD40* construct, whereas there is no *Eco* RV site in the *GmENOD40* gene, by which an indication about the number of insertions can be obtained (Figure 1A). Eight independent transformants containing *35S::GmENOD40* ATG and 10 independent transformants containing *35S::GmENOD40* AAG were obtained (Figure 1). The analysis showed that the 8 *GmENOD40* ATG transformants, as well as the 10 *GmENOD40* AAG transformants, have 1 or 2 inserts. *GmENOD40* ATG 205.2 and *GmENOD40* AAG 305.24 are the 2 lines that were analysed in more detail and contain 1 and 2 inserts respectively.

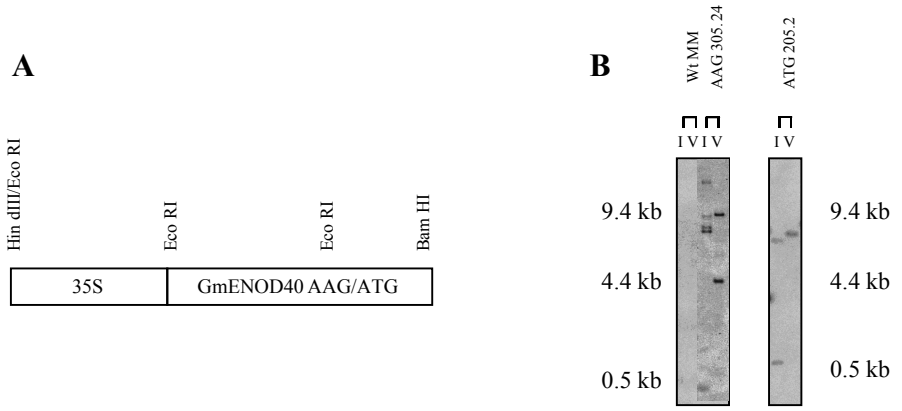

Figure 1: Southern analysis of transgenic tomato lines. Tomato plants were transformed with pCAMBIA 1390-*35S::GmENOD40* ATG and -*35S::GmENOD40* AAG. A: Schematic representation of *35S::GmENOD40* in pCAMBIA1390. B: Southern blots of gel with DNA of the studied transgenic lines *35S::GmENOD40* ATG 205.2 and *35S::GmENOD40* AAG 305.24. The lanes contain 5 µg of genomic DNA digested with *Eco*RI (I) and *Eco*RV (V) respectively. As a probe the entire *GmENOD40* coding sequence labelled with ^{32}P dATP was used.

Investigation of T1 plants

GmENOD40 *ATG and* GmENOD40 *AAG induce larger flowers and leaves*

All *35S::GmENOD40* T0 plants produced viable offspring. When possible, three T1 plants of all 18 transgenic tomato plants were analysed to investigate the effect of expression of *GmENOD40* ATG and *GmENOD40* AAG on tomato plant development. As controls tomato MM (wild-type) plants and *LeENOD40::GUS* plants were used (*LeENOD40::GUS* and *35S::GmENOD40* are constructed in pCambia 1381Z and pCambia 1390). *LeENOD40::GUS* plants develop similar to wild-type plants. We only observed differences in the development of 1 out of the 8 transgenic ATG lines (line ATG 205.2) and 1 out of the 10 transgenic AAG lines (AAG 305.24) when compared to wild-type plants.

When growing T1 plants of ATG 205.2 seeds, only one seedling out of 15 germinated seeds developed into a full-grown plant. The other seedlings stopped growing after the seedling stage. Consequently, from the T1 of this line only 1 plant was studied. Close inspection of this plant revealed that its leaves as well as its flowers were larger than wild-type leaves and flowers. Furthermore, ATG 205.2 formed a low number of flowers,

fruits and seeds, which were not viable. Therefore, the next generation of ATG 205.2 could not be studied.
From the T1 of AAG 305.24 6 out of 15 germinated seedlings did not develop into a full-grown plant. The leaves and flowers of these AAG 305.24 plants were larger than wild-type leaves and flowers (figure 2). The size of AAG 305.24 flowers was comparable to the size of ATG 205.2 flowers. Close inspection of AAG 305.24 and ATG 205.2 T1 flowers revealed that only sepals, petals and stamen were enlarged but not the carpel organs (Figure 2). AAG 305.24 plants formed a similar number of flowers and fruits as wild-type plants. However, a low number of seeds were produced, but these were viable. We conclude from these observations that the T1 of ATG 205.2 and AAG 305.24 plants display similar growth defects.

Expression studies

As we found only effects of *GmENOD40* overexpression on tomato development in *35S::GmENOD40* ATG 205.2 and *35S::GmENOD40* AAG 305.24 we decided to further investigate these plants in more detail. We investigated the level of expression of the introduced *GmENOD40* in young leaves of T1 plants of ATG 205.2 and AAG 305.24 and compared the expression level to endogenous *LeENOD40* expression and *LeENOD40* expression in wild-type plants (Figure 3). RNA was isolated from young leaves of the *GmENOD40* lines and of wild-type tomato. A semi-quantitative PCR was performed with specific primers for *GmENOD40* and *LeENOD40* on cDNA produced from the isolated RNA. The PCR products were analysed by Southern blotting with ^{32}P labelled *LeENOD40* and *GmENOD40* probes. This showed that endogenous *LeENOD40* expression in the transgenic and in wild-type tomato plants is similar (Figure 3). We conclude that in these 2 transgenic lines the endogenous expression level of *LeENOD40* is not affected by the expression of the introduced *35S::GmENOD40*. Furthermore, the RT-PCR showed that *GmENOD40* DNA is already at a maximum level after 20 PCR cycles while the level of *LeENOD40* DNA is at a maximum level at 25 cycles (Figure 3). From this we conclude that the expression level of *GmENOD40* in the transgenic plants is higher than the expression level of the endogenous *LeENOD40* gene.

Large cells in GmENOD40 *AAG 305.24 and ATG 205.2 flowers and leaves*

In *GmENOD40* ATG 205.2 and AAG 305.24 we found that leaves and flowers were larger than wild-type leaves and flowers. Larger organs can be the result of more cells per organ, larger cells or a combination of both. To determine what causes the increased size of flower organs, we made prints of mature leaves (second node) and flower petal surfaces of three month-old T1 tomato plants (Jansen *et al*., 2000). These prints can be examined to determine the number and the size of cells in the epidermis. Four prints were made from AAG 305.24 and ATG 205.2 plants. An example of a leaf print is

presented in Figure 2 and here it is visible that stomata and epidermal cells in line AAG 305.24 are markedly larger than cells in wild-type plants (Figure 2).

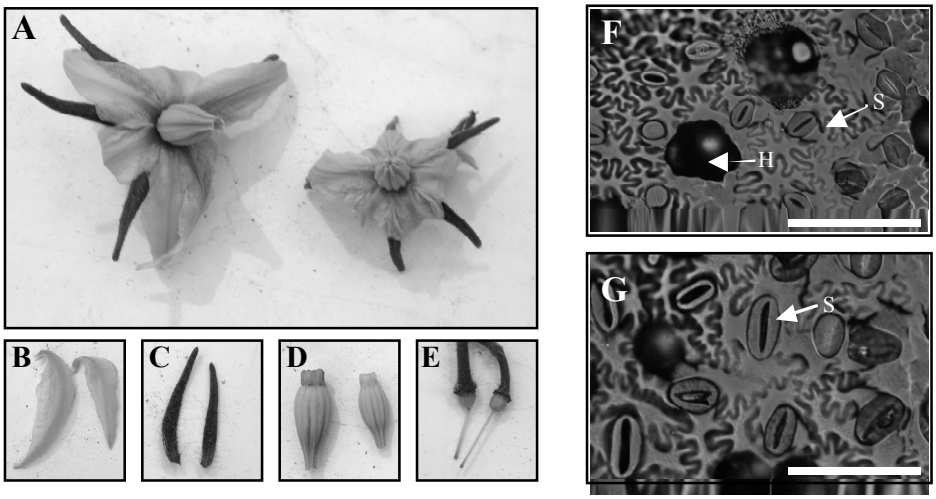

Figure 2: Comparison of flower size of transgenic tomato containing 35S::*GmENOD40* AAG (line 305.24) to wt MM flower size. A-E: Flower from line AAG 305.24 (left) compared to a wt flower (right). A: Complete flowers; B: Petals; C: Sepals; D: Stamen; E: Carpel. F: Epidermal cells of adaxial side of MM leaf. G: Epidermal cells of adaxial side of line *GmENOD40* AAG 305.24 leaf. H = opening in sample left by hair; S = stomata. Bars are 50 µm.

Investigation of T2 plants

Since ATG 205.2 T1 seeds were not viable we could only investigate the T2 generation of AAG 305.24 plants. We studied the development of 25 T2 plants of line AAG 305.24. The 25 studied plants were selected for the presence of the *GmENOD40* insert on medium with hygromycin. Germinated seedlings had in common that their cotyledons were larger and had an abnormal shape compared to wild-type cotyledons. In 3 to 4 week-old AAG 305.24 T2 plants, we observed a difference with the control plants in the development of the first two leaves. At this time in development, the 25 control tomato plants had formed their first two leaves and these leaves are similar in size. In contrast, two out of 25 AAG 305.24 plants that were studied did not form any leaves. Twelve out of the 25 plants stopped growing after the first leaf was formed. In the remaining plants, we observed that the first two leaves were not equal in size in contrast to control tomato plants. In all full-grown AAG 305.24 T2 plants we observed the same defects in leaf and flower size as in the T1 of this line described above.

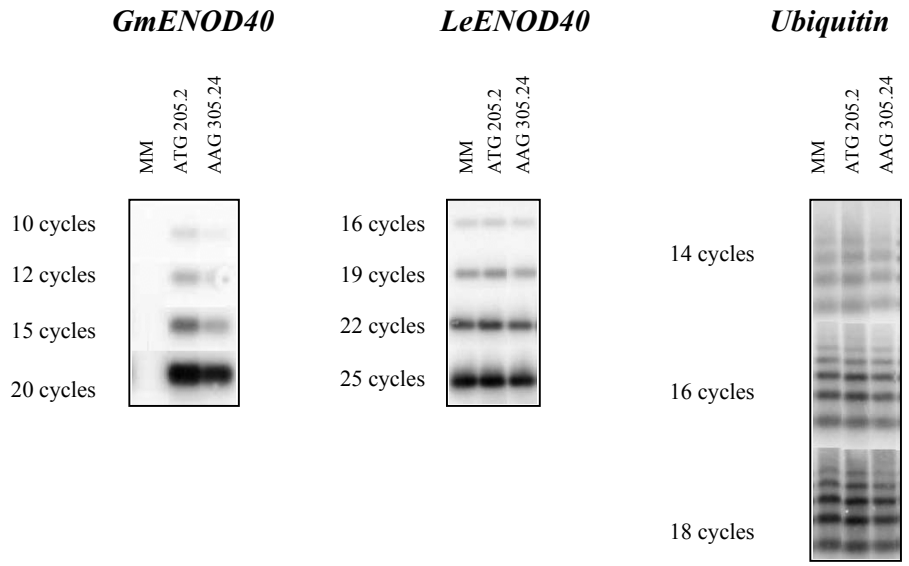

Figure 3: Expression analysis of *GmENOD40* and *LeENOD40* in T1 tomato plants transgenic for *35S::GmENOD40* ATG and *35S::GmENOD40* AAG. All genes were amplified by RT-PCR using total RNA isolated from leaf tissue of the different plants. The number of cycles is shown to the left of the panels. *Ubiquitin* RNA was amplified as a control.

Is the AAG 305.24 phenotype linked to the presence of the GmENOD40 construct?

To investigate whether these developmental defects are caused by *GmENOD40* overexpression we germinated *GmENOD40* AAG 305.24 seeds without selection on hygromycin of which 8 developed into a complete plant. All these 8 plants displayed the described growth defects. These plants were checked for presence of the *GmENOD40* gene by PCR using specific primers for *GmENOD40*. The PCR products were analysed by DNA gel electrophoresis and the analysis showed that all analysed plants contained the *GmENOD40* construct. Unfortunately, the seed set of the two remaining T1 plants was too low to determine whether the observed defects are strictly coupled to the presence of the *GmENOD40* construct. Therefore, we decided to cross the investigated T2 *GmENOD40* AAG 305.24 plants with wild-type tomato plants to investigate linkage of the presence of the *GmENOD40* gene and the observed phenotype in these plants.

Discussion

Here, we describe our ongoing studies on the effect of ectopic expression of *GmENOD40* on tomato development. We observed that. out of 18 transgenic *35S::GmENOD40* ATG 205.2 and AAG 305.24 T1 plants, 2 display larger flower

organs and leaves than wild-type plants. The leaves and flowers of these plants contain cells larger than wild-type cells in the epidermis.

The occurrence of similar growth defects in two out of the 18 produced plants transgenic for *GmENOD40* ATG and AAG indicates that the defects are caused by overexpression of *GmENOD40*. Unfortunately, the low seed set and the low viability of *GmENOD40* ATG 205.2 did not allow investigation of more generations of this plant. Therefore, we could only study the phenotype of *GmENOD40* AAG 305.24 T2 plants. Also *GmENOD40* AAG 305.24 plants produced few viable seeds. As the studied plants all contained the *GmENOD40* construct, these plants will be crossed with wild-type plants to get a population of heterozygous in which linkage of the phenotype and presence of the *GmENOD40* construct can be studied.

Although we did find similar defects in *GmENOD40* ATG 205.2 and *GmENOD40* AAG 305.24 plants, the number of plants that display developmental defects as a result of *GmENOD40* overexpression is too low to draw conclusions on a function of the ENOD40 peptide.

CHAPTER 5

Identification of proteins interacting with the ENOD40 peptide using a Two-Hybrid system approach

Ingrid Vleghels, Xi Wan, Ton Bisseling and Henk Franssen

Abstract

The gene *ENOD40* encodes a small peptide of 10-13 amino acids depending on the plant species. We investigated the function of the ENOD40 peptide by searching for protein binding partners. For this, we screened a Two-Hybrid library of young pea nodules using the pea ENOD40 peptide as a bait. We found that the peptide interacts with a protein with high homology to *p40*, a gene that codes for a ribosome associated protein. *In situ* expression studies of the *pea p40* and *ENOD40* showed that their expression patterns partly overlap in the infection zone of the nodule (zone II). More experiments are required to confirm the interaction between the pea ENOD40 and p40 proteins.

Introduction

Peptides are commonly used in animals as signalling molecules and have been the subject of many studies. Based on these studies, a stereotype peptide signalling system can be inferred consisting of the following components: a signalling peptide that is produced as part of a larger (pre)proprotein, a specific protease involved in the release of the signalling peptide and a receptor that recognises the mature peptide and transduces the signal, usually through a protein phosphorylation cascade. In plants, components of peptide signalling systems have been identified, either through genetic or biochemical approaches (reviewed in: Franssen and Bisseling, 2001; Ryan and Pearce, 2001; Bisseling, 1999). In contrast to the large number of putative receptors (McCarthy and Chory, 2000) only a few peptide-signalling molecules have been identified. The peptide systemin was discovered in a bioassay aiming to identify the molecule responsible for the systemic induction of genes encoding proteinase inhibitors in tomato leaves exposed to herbivorous insects (McGurl *et al.,* 1992). Biochemical studies in other *Solanaceae* have identified systemin homologues (Constabel *et al.,* 1998). Phytosulfokines, sulphated peptides were discovered as the components that can induce proliferation of asparagus mesophyl cells (Matsubayashi and Sakagami, 1996). The peptide CLAVATA3, which is the ligand of the CLAVATA1 receptor kinase, was discovered by a genetic search for genes required for SAM (shoot apical meristem) formation (Fletcher *et al.,* 1999). The peptide SP11, belonging to a large family of small cystein-rich proteins (SCR), was identified as the ligand for the SRK receptor involved in self-incompatibility in *Brassicaceae* (Takayama *et al.*, 2001). The peptide RALF , which was isolated from tobacco, was found to induce rapid alkalinisation of tobacco suspension culture cells (Pearce *et al.*, 2001).

All these peptides are synthesised as part of a longer (pre)protein, as is the case for peptide signalling molecules in other eukaryotes. An exception to this rule is the ENOD40 peptide. *ENOD40* encodes as a primary translation product an unusually small peptide of 10-13 amino acids. *ENOD40* was discovered as a gene involved in legume nodule formation, a process induced by *Rhizobium* bacteria (Yang *et al.,* 1993).

Transient expression studies in protoplasts and alfalfa roots, involving translational fusions of ENOD40 to GFP or GUS respectively, showed that the start codon of the *ENOD40* peptide is recognised by the translation machinery and the peptide ORF is translated *in vivo* (Van de Sande *et al.,* 1996; Compaan *et al.,* 2001; Sousa *et al.,* 2001). Furthermore, an antibody raised against a synthetic GmENOD40 confirmed the presence of an antigenic determinant in soybean nodule extracts, most likely being the GmENOD40 peptide (Van de Sande *et al.,* 1996). These data suggest that it is likely that the GmENOD40 peptide is produced *in vivo*.

All identified *ENOD40* cDNAs consist of two highly homologous regions, named region 1, which contains the peptide encoding ORF, and region 2 (Van de Sande *et al.,* 1996; Corich *et al.,* 1998; Kouchi *et al.,* 1999; Flemetakis *et al.,* 2000). Bombardment of gold particles into alfalfa roots coated with plasmids containing either the *MtENOD40* sequence region 1 or region 2, was found to induce cell divisions. This suggests that region 1 and region 2 can function separately and that both are important for *ENOD40* functioning. Furthermore, cell divisions were induced in cells that were not hit by the particles, indicating that *ENOD40* might be operating in a non-cell autonomous way (Charon *et al.,* 1997). As a first step to determine the function of region 1, we decided to study the function of the peptide encoded by region 1. The function of known plant signalling peptides has been clarified by either biochemical or by genetic approaches. A genetic approach requires the presence of a collection of mutants that can be screened for defects in expression of *ENOD40* or production of the ENOD40 peptide. The large mutant collection of *Arabidopsis thaliana* is unfortunately of no help since sequences similar to *ENOD40* are not present in the *Arabidopsis* genome.

Our approach to investigate the function of ENOD40 is based on the rational that a way to position a protein in a signal transduction pathway is to search for its interacting protein partners. By identifying partners of a protein with an unknown function, a putative function can often be assigned (Tucker *et al.,* 2001). Binding studies involving a chemically produced ENOD40 peptide tagged with biotin failed to identify an ENOD40 binding protein (H. Franssen, unpublished data). Therefore, we chose another approach to find an ENOD40 binding protein. We explored the yeast based Two-Hybrid system, which can be used to screen an expression library for proteins interacting with a protein of interest. In animal as well as in plant research the Two-Hybrid system has proven to be very useful for finding protein partners (Xenarios and Eisenberg, 2001; Pelletier and Sidhu, 2001). Although there are no published reports on peptides as small as the *ENOD40* peptide being used successfully as a bait in the Two-Hybrid system, according to Zhu and Khan (1997) it is possible to use small peptides as baits in the Two-Hybrid system. To investigate the signalling cascade of the *ENOD40* peptide during nodule development a Two-Hybrid library of young pea nodules was constructed and screened. For this purpose we chose the Lex-A Two-Hybrid system (Gyuris *et al.,* 1993), which is

a Lex-A-based version of the yeast Two-Hybrid system originally developed by Fields and Song (1989).

Materials & methods

Plant material and bacterial strains

Pisum sativum cv Rondo NOD3 plants were inoculated with *Rhizobium leguminosarum* bv *viciae* 248. *R. l.* bv *viciae* was grown in YEM medium (Jossey *et al.*, 1979).

Saccharomyces cerevisiae EGY48, the yeast strain used for the Two-Hybrid screen was grown in YPD (20 g/l peptone, 10 g/l yeast extract, and 20 g/l glucose). When selection was needed EGY48 was grown in selective medium (1.7 g/l yeast nitrogen base without amino acids and without ammonium sulphate, 5 g/l NH4SO4, 20 g/l glucose, 100 ml/l 10 x dropout mix) or in galactose/raffinose selective medium (1.7 g/l nitrogen base w/o amino acids, 5 g/l NH4SO4, 20 g/l galactose, 10 g/l raffinose, 100 ml/l dropout mix) (dropout mix: 300 mg/l L-isoleucine, 1500 mg/l L-valine, 200 mg/l L-adenine hemisulphate salt, 200 mg/l L-arginine-HCl, 200 mg/l L-histidine-HCl monohydrate, 1000 mg/l L-leucine, 300 mg/l L-lysine-HCl, 200 mg/l L-Methionine, 500 mg/l L-phenylalanine, 2000 mg/l L-threonine, 200 mg/l L-tryptophan, 300 mg/l L-tyrosine, 200 mg/l L-uracil. The media were made selective for the plasmids transfected to EGY48 by leaving out amino acids from the dropout mix that were produced by the enzymes encoded by the transfected plasmids.

cDNA library construction

P. sativum plants were grown for three days before they were inoculated with rhizobia. Roots with young nodules were harvested and stored in liquid nitrogen 6 days after inoculation. Total RNA was isolated according to the protocol of Pawlowski et al., (1994). RNA samples were tested for quality by RT-PCR studies on ubiquitin profile and occurrence of the mRNAs of ENOD40, ENOD12 and ENOD5 (data not shown). From these experiments we concluded that the RNA was of sufficient good quality to proceed with cDNA library construction. Poly A^+ RNA was purified from total RNA with Dynabeads according to the protocol provided by the company. RNA from young P. sativum nodules (6 days after inoculation) was transcribed into cDNA using an XhoI-oligo d(T) primer. An EcoRI adapter (5'-AATTCGGCACGAG-3') was ligated onto the 5' end of the cDNA. This allowed EcoRI-XhoI cloning of the cDNA's into pJG4-5. The DupLEX-ATM Yeast Two-Hybrid System was made in the B42 activation domain of the vector pJG4-5. The cDNA was cloned unidirectionally between the EcoRI and XhoI sites of pJG4-5 (OriGene Technologies, Inc). This resulted in a Two-Hybrid library of 6.1 106 independent clones.

Identification of proteins interacting with the ENOD40 peptide

Construction of bait plasmid

PsENOD40 fragment was isolated from a pGBT9-*PsENOD40* fusion plasmid (Vleghels, unpublished results) by *Eco*RI/*Bam*HI digestion and fused to the DNA-binding domain of the pEG202 vector, which was also digested with *Eco*RI and *Bam*HI (OriGene Technologies, Inc.). The fusion between the Lex-A DNA binding domain and the *ENOD40* peptide sequence was confirmed by sequencing. To test the production of the *PsENOD40* bait/LexA DNA binding protein fusion, a double transformation with the plasmid pJK101 into EGY48 was performed. pJK101 contains the *LacZ* reporter driven by a complete transcriptional activator. The introduction of pEG202-*PsENOD40* reduced the β-galactosidase activity indicating that the LexA-*PsENOD40* fusion protein is expressed and transferred to the nucleus where it competes with the transcriptional activator produced by pJK101. The pEG202-*PsENOD40* bait was tested with empty vector and several control proteins provided by the system and did not show any activation of the reporter genes. There were no indications of aspecific interactions of *PsENOD40* with unrelated proteins and pEG202-*PsENOD40* was used for screening the pea nodule Two-Hybrid library.

Two-Hybrid screen

EGY48 pretransformed with the plasmids pSH18-34 (containing the *LacZ* gene under control of 8 *LexA* operons) and pEG202-*PsENOD40* was transformed with the cDNA library in pJG4-5 and this resulted in 3.8 10^6 primary transformants growing on selective glucose medium. These primary transformants were collected by scraping off colonies from the plates. After collection, approximately 3.8 10^7 cfu were plated on gal/raff medium and analysed for activation of the *LEU2* and *LacZ* reporters by an interaction between bait and library proteins now induced by the galactose in the medium. Positive clones were grouped by digestion of colony PCR products with *Sau*3A.

Positive clones were selected and their plasmids were isolated by the plasmid preparation protocol provided by the manufacturer. To isolate the library plasmid *E. coli* KC8 cells (OriGene, Inc.) were transfected with the product of the plasmid preparation. Cells were selected on M9 minimal medium without tryptophan containing 50 mg/l ampicillin (12.8 g/l Na_2HPO_4, 3 g/l KH_2PO_4, 0.5 g/l NaCl, 1.0 g/l NH_4Cl, 100 ml/l 10x dropout mix, 20 g/l glucose, 1mM thiamine-HCl). Each library plasmid was analysed by retransformation to EGY48 (pSH34-12). Retransformation of the library plasmids to EGY 48 was needed to test for autoactivation of the reporter genes by the inserts alone in the absence of the bait. Bait and prey plasmids were retransformed to reconfirm the interaction *PsENOD40* and the preys.

Sequencing

Library plasmids that showed no autoactivation were transfected to *E. coli* DH5α for plasmid preparation for sequence determination. For sequencing of inserts the primers BCO1 and BCO2 were used (BCO1: 5'-CCAGCCTCTTGCTGAGTGGAGATG-3'; BCO2: 5'-GACAAGCCGACAACCTTGATTGGAG-3').

In vitro binding studies

In vitro binding studies were performed as described in Jarillo *et al.,* (2001). DNA templates were cloned into the pT7-6 expression vector (Scheres *et al.*, 1990). Templates were transcribed using the TnT Quick coupled transcription/translation system (Promega). DNA fragments containing the sequence for an YFP-PsENOD40 fusion and YFP respectively, were isolated from pMON999-YFP and pMON999-YFP-PsENOD40 plasmid (Monsanto, H. Franssen, unpublished results; G. van der Krogt, unpublished results) with the enzymes *Xho*I and *Bam*HI, transferred to the plasmid RT105 and recloned into plasmid pT7-6 (Scheres *et al.*, 1990) using the enzymes *Sal*I and *Bam*HI. *PsP40* was amplified by PCR using the primers F 5'-CCCCTCG-AGCTACCTCCGCTGC-3' and R 5'-GGGGATCCGTAAACAGTATTTATACC-3'. These primers generated an *Xho*I and a *Bam*HI site respectively, which enabled a *Xho*I/*Bam*HI cloning into pT7-6. All plasmids were linearised with *Sca* I before transcription/translation. Translation was performed in the presence of ^{35}S-methionine (Amersham). For immuno-precipitation a GFP antibody (Clontech) was used.

Results

cDNA library construction

Pisum sativum cv Rondo NOD3 plants inoculated with *Rhizobium leguminosarum* bv *viciae* 248 were used for construction of the Two-Hybrid cDNA library. The supernodulator NOD3 was chosen for making this library because this mutant makes more nodules on its root system. *P. sativum* plants were grown for 3 days and then inoculated with *Rhizobium*. Roots with young nodules were harvested 6 days after inoculation with *Rhizobium*. Total RNA was isolated and transcribed into cDNA. This cDNA was used to make a Two-Hybrid library of 6.1 10^6 independent clones in the B42 (*Lex-A*) activation domain of the vector pJG4-5. The Two-Hybrid library was transformed to *E. coli* and DNA isolated from these cells was used for transformation of yeast.

Psp40 interacts with PsENOD40-peptide in the yeast Two-Hybrid system

In two separate experiments a plasmid DNA equivalent of 3.8 106 clones in total were transformed to yeast. Subsequently, these clones were amplified and 3.8 107 colonies were tested for their ability to activate the reporter genes *LEU* and *LacZ*. Positive clones are expected to appear more than once as the library is amplified before screening for Leu^+ colonies. The two screens resulted in 204 $Leu^+/LacZ^+$ clones. To determine which clones were identical, inserts of these clones were amplified by PCR and digested with *Sau*3A. Digestion patterns of the amplified inserts were compared. Sorting of identical clones showed that the majority of the clones were collected once or twice from screen 1 or screen 2, but never from both screens (data not shown). The number of clones appearing only once from the screen (162 clones) was too large to investigate. We investigated some of the 17 clones that appeared two times in the screen. However, based on sequence analysis, most of these clones were in the antisense direction or were fused to the Lex-A DNA binding domain in the wrong frame, we considered these clones to be false positives. Two other clones that appeared twice in the screen had a homology to the described false positives Therefore, we decided to discard clones that only appeared as positives once or twice from the screen and focussed on clones appearing more than twice within the screen. This was only 1 clone, clone B2, which was collected from the two-hybrid library 8 times. These 8 clones had completely identical *Sau*3A digestion patterns. Two of these clones were sequenced and their sequences turned out to be completely identical. The protein in frame with the *Lex-A* activation domain has very high homology to *p40*, a gene that codes for a ribosome associated protein. *p40* is found in plants as well as in animals (Ardini *et al.*, 1998).

Specificity of the interaction

The *p40* sequence is fused to the *Lex-A* activation domain 57 bases upstream of the ATG translation codon. This results in 19 extra amino acids between the polypeptide encoded by B2 and the Lex-A activation domain. *p40* from pea will from here on be referred to as *Psp40* and the B2 clone as pJG4-5-*Psp40*. The interaction between PsENOD40 and Psp40 was confirmed by isolation of the separate plasmids from yeast EGY48 and subsequent retransformation to EGY48. This retransformation leads to the same activation of the reporter genes as was initially found after the screen, thus confirming the interaction between the two proteins. As an extra control *Psp40* from pJG4-5-*Psp40* was cloned in frame with the *Lex-A* DNA binding domain of pEG202 (pEG202-*Psp40*) and *PsENOD40* was cloned in frame with the activation domain of pJG4-5 (pJG4-5-*PsENOD40*) (Table 1). In this case pEG202-*Psp40* was able to induce *LacZ* expression in the absence of pJG4-5-*PsENOD40*. Apparently, the pEG202-*Psp40* fusion of Psp40 and the Lex-A DNA binding domain, localised to the reporter gene DNA, can activate transcription in the absence of the Lex-A activation domain. Therefore, this experiment

provided no further support for the reliability of the interaction between PsENOD40 and Psp40. Since *ENOD40* and *p40* are present in the genome of many plants, we postulated that ENOD40 and p40 of other species, for instance tomato, would interact with each other. To this end *LeENOD40* and *Lep40* were cloned in pEG202 and pJG4-5, respectively, and co-transformed into EGY48. Although these cells could grow, no induction of *LacZ* expression was observed (Table 1), indicating that LeENOD40 and Lep40 do not interact in yeast.

Table 1: Two-Hybrid analysis of the interaction of ENOD40 and Psp40.

pEG202 BD	*pJG4-5AD*	*Gal/LacZ+*	*GAL/-Leu*	*Glu/LacZ+*	*Glu/-Leu*
PsENOD40	Psp40	++	+++	-	-
Psp40	PsENOD40	x	x	x	x
LeENOD40	Lep40	-	+-	-	-
Lep40	LeENOD40	x	x	x	x
PsENOD40	Lep40	+-	-	+-	+-
LeENOD40	Psp40	+-	-	+-	+-

Double transformations to yeast EGY40 were performed to test for interactions in the Two-Hybrid system. Transformants were grown on induction medium containing galactose (Gal), or control medium containing glucose (Glu). Both media were supplemented with X-GAL, which is a substrate of Lac Z and forms a blue product, or depleted from leucine (-Leu), which selects for production of leucine by the transformants. In the pEG202-BD (Binding Domain) column are the clones in the pEG202 vector indicated. In the pJG4-5-AD (Activation Domain) column are the clones in the pJG4-5 vector indicated. Blue staining caused by activity of the *Lac Z* gene or growth on medium without leucine was indicated by – (no growth/staining), +- (very little growth/staining), + to +++ (good to very good growth/staining). x: Experiment was not performed because of autoactivation of the reporter genes. Only combinations that were both Gal/Leu^+ and Gal/*Lac* Z^+ were scored as positives. Combinations that scored equally in the Glu/Leu^+ and Glu/*Lac* Z^+ were considered false positives.

Identification of proteins interacting with the ENOD40 peptide

The lack of an interaction between LeENOD40 and Lep40 in the Two-Hybrid system could be caused by a modification of one of the two proteins. To test if only *LeENOD40* or *Lep40* has lost this ability to bind we tested both with the corresponding pea binding partner. Cross testing p40 from pea and tomato and ENOD40 from pea and tomato in the Two-Hybrid system neither gave induction of the *LacZ* reporter gene (Table 1). From this we concluded that the interaction between Psp40 and PsENOD40 seems to be specific for legumes.

PsENOD40 and Psp40 are expressed in nodules

In situ hybridisation studies were performed on serial sections of nodules harvested 18 days after inoculation of pea roots with *Rhizobium leguminosarum* bv *viciae* 248. Hybridisation of antisense *PsENOD40* RNA showed that *PsENOD40* is expressed in the pericycle of the nodule vascular bundle (data not shown) and in the infection zone but is absent in the meristem. This location in agreement with published data on *ENOD40* expression (Yang *et al.,* 1993; Matvienko *et al.,* 1994). Hybridisation of antisense *Psp40* RNA showed that *Psp40* is expressed in the nodule meristem and in the infection zone of the nodule (Figure 1). We observed no expression of *Psp40* in the pericycle of the nodule vascular bundle. These observations show that expression of *PsENOD40* and *Psp40* is partly overlapping.

In vitro binding studies

To obtain independent evidence for the interaction between the PsENOD40 peptide and Psp40 we used an *in vitro* transcription/translation system. A tagged *ENOD40* sequence was used to study the interaction with p40. An antibody against this tag was used to precipitate ENOD40 as well as the complex of the two proteins. We used YFP to tag ENOD40, by a fusion to the N-terminal end of the peptide. We do not know whether this fusion is functional or allows proper binding of ENOD40 to its protein partner. However, the *PsENOD40* sequence was fused to the *Lex-A* DNA binding domain in the same manner and this fusion was able to interact with p40. Therefore, we assume that the YFP-ENOD40 fusion protein has maintained its ability to interact with p40. The *PsENOD40* peptide sequence fused to the carboxy-terminal end of YFP was combined with the *Psp40* sequence in the TnT *in vitro* transcription/translation system (Promega) to synthesise polypeptides for binding studies. Translation was performed in the presence of ^{35}S labelled methionine to allow detection of the proteins. The polypeptides of the separate translation mixes were incubated for 30 minutes to allow binding. The mixture was then incubated with GFP antibodies to test if PsENOD40 and Psp40 could be immuno-precipitated as a complex.

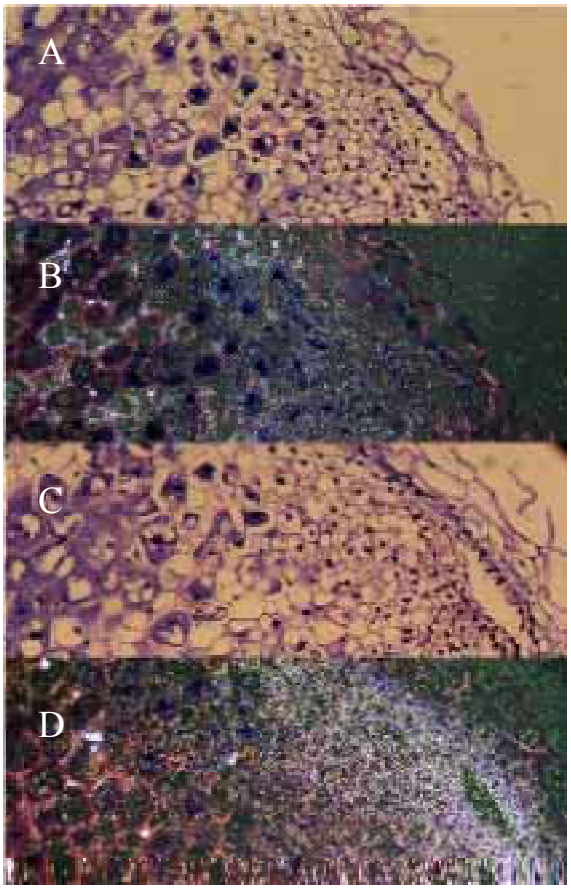

Figure 1: In situ localisation of *PsENOD40* and *Psp40* mRNA in full-grown pea nodules.
A and C are brightfield micrographs in which the signal is represented by black dots.
B and D are darkfield micrographs in which the signal is represented by white dots. Sections are hybridised with ^{35}S labelled RNA.
A and B: Cross-section through a pea root containing a nodule harvested 18 days after inoculation hybridised with *PsENOD40* RNA. Expression of *PsENOD40* can be observed in the infection zone.
C and D: Cross-section through a pea root containing a nodule harvested 18 days after inoculation hybridised with *Psp40* RNA. Expression of *Psp40* can be observed in the meristem and in the infection zone of the nodule.

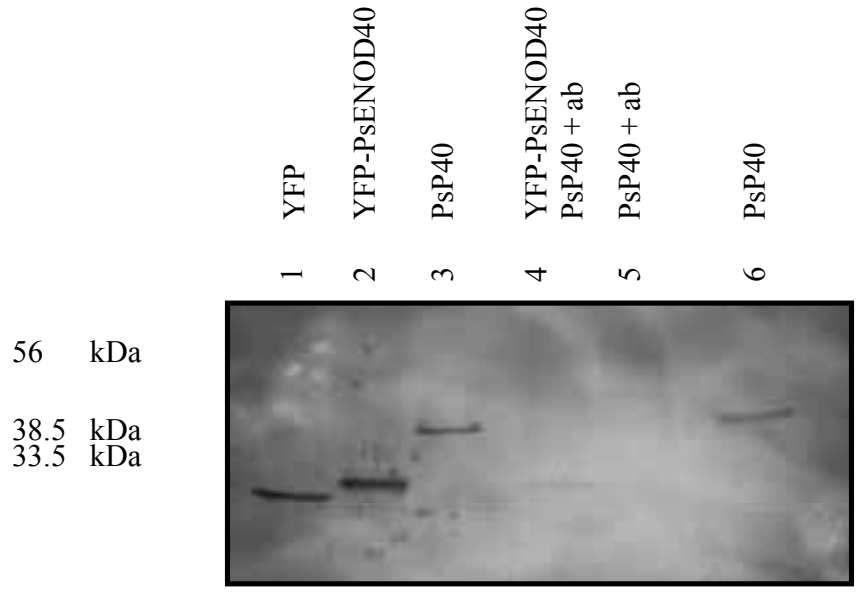

Figure 2: In vitro binding of PsENOD40 and Psp40. To test the interaction between Psp40 and PsENOD40, *in vitro* transcription/translation was used to generate peptides of Psp40 and YFP-PsENOD40. For translation of the polypeptides ^{35}S labelled methionine was used to enable detection of the proteins on an autoradiogram. The SDS-PAGE gel shows YFP (lane 1), YFP-ENOD40 fusion (lane 2), Psp40 (lane 3 and 6), the precipitated YFP-ENOD40 fusion product using GFP antibody (lane 4) and no precipitation product in the negative control Psp40 precipitated with GFP antibody (lane 5).

Figure 2 shows an autoradiogram of the ^{35}S labelled proteins, obtained in the *in vitro* translation mix that were separated by SDS-PAGE. The difference in MW of YFP in lane 1 and YFP-PsENOD40 in lane 2 shows that the YFP-ENOD40 fusion is successfully produced. Lanes 3 and 6 contain PsP40. Lane 4 shows the immuno-precipitation with GFP antibody of the mixture of PsENOD40-YFP and Psp40. Only a single protein is precipitated, which has the size of PsENOD40-YFP indicating that no Psp40 had formed a complex with PsENOD40-YFP. Although no Psp40 is detected, it should be kept in mind that only a small fraction of PsENOD40-YFP is immuno-precipitated. Therefore, this experiment does not exclude that a part of the ENOD40-YFP proteins could have formed a complex with PsP40 and that immuno-precipitated Psp40 is below the detection level in this experiment.

Discussion

The Two-Hybrid system was used to identify proteins interacting with the peptide encoded by *PsENOD40*. Identification of proteins interacting with ENOD40 could be a way to position ENOD40 in a signal transduction pathway and give insight into the function of ENOD40. Screening of a pea nodule Two-Hybrid cDNA library resulted in the identification of one protein Psp40 that interacted with the *PsENOD40* encoded peptide in the Two-Hybrid system.

In animals p40 is a protein that acquired a dual function in evolution (37LRP/p40). 37LRP/p40 can function as the laminin receptor protein LRP but also as a ribosome associated protein, found associated with 40S ribosomal units and essential for protein synthesis (Ardini *et al.,* 1998). The presence of p40 has now been reported in plants as well. Expression studies of *p40* in *Arabidopsis thaliana* showed that the gene is expressed in young dividing tissues (Scheer *et al.,* 1997). The protein is present in the cytoplasm as a ribosome associated protein (Garcia-Hernandez *et al.,* 1994), a receptor function for p40 has not been shown in plants. As p40 is important for protein synthesis, it is not surprising that expression is found in actively dividing cells. The formation of a nodule requires the mitotic reactivation of cortical cells. Therefore, it is probable that Psp40 is important for the formation of a nodule primordium. The function of ENOD40 in relation to Psp40 could be to regulate the efficiency of protein synthesis during nodule formation. Although binding of Psp40 to PsENOD40 could be shown in the Two-Hybrid system, whether this binding has biological relevance is not yet clear. A first prerequisite for the interaction of Psp40 and PsENOD40 is that both proteins must be present in the same cells. Like *PsENOD40, Psp40* is expressed during pea nodule development. *Psp40* expression is found in the nodule meristem (zone I) and the nodule infection zone (zone II). *PsENOD40* expression also occurs in zone II but not in zone I. Further, *PsENOD40* expression is also found in the nodule vascular bundle, whereas Psp40 is not expressed in the nodule vascular bundle. Therefore, Psp40 and PsENOD40 could only interact in zone II cells. *Psp40* expression in nodules does not exclude an interaction between p40 and ENOD40. Moreover, the specific nodule tissue (zone II) in which both are expressed suggests that the interaction of Psp40 and PsENOD40 might have a specific function for the infection zone. Recently, it has been postulated that the GmENOD40 peptide binds sucrose synthase (Röhrig *et al.,* 2002). When testing the PsENOD40 peptide in the Two-Hybrid system we have not identified sucrose synthase as a binding partner. Localisation studies of sucrose synthase in nodules (Komina *et al*., 2002) show that the protein is located in the fixation zone of the nodule, where *ENOD40* expression does not occur. This makes it unlikely that ENOD40 and sucrose synthase interact *in vivo*.

A pull-down experiment involving an *in vitro* transcription/translation system with plasmids containing *Psp40* and a *YFP-PsENOD40* fusion failed to immuno-precipitate Psp40 with YFP antibody. However, one must keep in mind that the amount of immuno-

precipitated Psp40/YFP-ENOD40 complex might have been below the detection level of the experiment. So far, the ENOD40 peptide has not been purified from plant tissues. Therefore, it is not known whether the peptide is post-translationally modified. In case the ENOD40 peptide needs modifications for proper functioning, the failure to immuno-precipitate a Psp40/YFP-ENOD40 complex could be explained by the absence of post-translational modifications to the peptide in the transcription/translation assay. These modifications are more likely to occur in yeast cells of the *in vivo* Two-Hybrid system. Furthermore, the physiological conditions of the yeast cells in the Two-Hybrid system could have been more optimal for binding than those in the *in vitro* transcription/translation experiment.

We further investigated the interaction between ENOD40 and p40 by testing whether this interaction can take place between the tomato homologues of ENOD40 and p40. For this, Lep40 and LeENOD40 were tested in the Two-Hybrid system. No interaction between the Lep40 and LeENOD40 could be shown and therefore, this experiment showed that the interaction between ENOD40 and p40 is not occurring in all plant species. We have not studied *Psp40* expression in other organs than nodules. However, expression studies in *Arabidopsis* show that *Atp40* has a high expression in young roots and is also expressed in the vascular tissue of stems and leaves (Scheer *et al.*, 1997). Therefore, the interaction between *p40* and *ENOD40* might be specific for nodule formation but it can not be excluded that this interaction also occurs in other organs.

In conclusion, the expression of *PsENOD40* and *Psp40* during nodule development is a prerequisite for a possible interaction but is not sufficient to prove that the interaction between p40 and ENOD40 occurs *in vivo*. To prove the interaction between Psp40 and ENOD40, more experiments need to be performed. As the Psp40 originates from a nodule primordium cDNA library it is most likely that at least there the interaction can take place. Therefore, the most promising approach would be to test an interaction between Psp40 and ENOD40 in *in vivo* experiments in nodule primordia.

CHAPTER 6

Concluding remarks

Ingrid Vleghels, Ton Bisseling and Henk Franssen

Rhizobium induced nodule formation is generally restricted to a distinct plant family, the legumes and is accompanied by the expression of nodulin genes. The presence of nodulin (nodule specific genes) homologues in non-legumes (Kouchi *et al*. 1999; Gualtieri and Bisseling, 2000; Stougaard, 2000; Spaink, 2002) indicates that rhizobia have recruited genes involved in general plant development for nodule formation. This is in line with empirical evidence that underlines that changes in the regulation of genes affecting morphology are implicated more frequently in the evolution of diversity than new genes (Carroll *et al*., 2001). We suggested that the early nodulin *ENOD40* could be such a gene recruited for nodule development as *ENOD40* homologues are found in non-legumes. In case *ENOD40* would be a recruited gene, we would expect that the regulation of *ENOD40* in legumes and non-legumes is different, while the function of *ENOD40* in legumes and non-legumes most likely is conserved.

In this thesis, we set out to investigate the regulation and function of *ENOD40*. We chose the non-legume tomato for these studies because it is a well-studied plant and genetic maps are available. We studied expression of the tomato *ENOD40* promoter in tomato and the legume *Medicago truncatula* and compared it to expression of the soybean *ENOD40* promoter in *Arabidopsis* and the *Medicago ENOD40* promoter in *Medicago*.

The *ENOD40* mRNA has an unusual structure consisting of two highly homologous regions, named region 1, which contains the peptide encoding ORF, and region 2, which does not contain a conserved ORF (Van de Sande *et al.,* 1996; Corich *et al.,* 1998; Kouchi *et al.,* 1999; Flemetakis *et al.,* 2000). We studied the function of the ENOD40 peptide in nodule development and we studied the role of *ENOD40* in tomato plant development.

In chapter 2 we describe the isolation of the *ENOD40* gene from tomato (*LeENOD40*). A promoter *LeENOD40::GUS* was introduced in tomato to study *ENOD40* expression during tomato development. We showed that *LeENOD40* is expressed throughout plant development in different organs, generally associated with vascular tissue. The expression pattern of *LeENOD40* in tomato is comparable to the expression pattern of *GmENOD40::GUS* in *Arabidopsis* described by Mirabella *et al*., (1999) and the expression pattern of *MsENOD40::GUS* in alfalfa (Fang and Hirsch, 1998). These experiments have shown that legume and non-legume *ENOD40* promoters in non-legume background are expressed in a similar manner.

In chapter 3 we studied regulation of *LeENOD40::GUS* in tomato and *GmENOD40::GUS* in *Arabidopsis*. We showed that application of cytokinin leads to a reduction of *ENOD40* promoter driven *GUS* expression in tomato as well as *Arabidopsis* roots. This shows that *GmENOD40* driven *GUS* expression in *Arabidopsis* is regulated by cytokinin in a similar manner as *LeENOD40* driven *GUS* expression in tomato indicating that regulation of legume and non-legume promoters in non-legumes is comparable. Furthermore, the *LeENOD40::GUS* expression pattern in nodules on *Medicago truncatula* hairy roots is similar to the endogenous *ENOD40* expression pattern in nodules of various legumes (Yang, *et al.*, 1993; Matvienko *et al*., 1994; Roussis *et al*., 1995; Papadopoulou *et al*., 1996; Fang and Hirsch, 1998). This shows that the *LeENOD40* promoter is activated in a similar way as the endogenous *ENOD40* promoters during the *Rhizobium*-legume symbiosis. This result is similar to the result of Kouchi *et al.,* (1999) who showed that in nodules grown on soybean transgenic hairy roots the rice *ENOD40* promoter is active in a similar pattern as the soybean *ENOD40* promoter. Together with the observation that the legume *GmENOD40* and non-legume *LeENOD40* promoters are expressed in non-legumes in the same tissues, and are regulated by cytokinin in a similar manner, this indicates that legume and non-legume *ENOD40* promoter activity is regulated in the same manner. We conclude that the regulation of *ENOD40* promoter activity is conserved between legumes and non-legumes and even between monocots and dicots.

ENOD40 expression during legume nodule formation is elevated to a level not observed in any other developmental process. This indicates that *ENOD40* is regulated in nodule development in a unique manner, although the promoter regulatory elements appear to be conserved. We conclude that the different regulation of *ENOD40* in nodule development was not achieved by changed regulatory elements in the *ENOD40* promoter sequence since also the *LeENOD40* promoter in a legume is subject to this unique regulation. Consequently, legumes must have adjusted either a factor influencing *ENOD40* mRNA stability or a step before *ENOD40* induction to allow the unusually high induction of *ENOD40* expression that precedes nodule primordium formation. We suggest that the second possibility is the most probable as it is more likely that the unique increase in *ENOD40* mRNA is caused by increased transcription than by increased mRNA stability. A candidate could be a gene encoding a transcription factor influencing *ENOD40* expression directly. We suggest that there could be different transcription factors or modified transcription factors involved in symbiotic and non-symbiotic expression of *ENOD40*.

Our data strongly suggests that *ENOD40* is not recruited for nodule formation, but that a regulator of *ENOD40* expression might be. This could imply that only a few critical regulator genes are recruited for nodule formation and these control the expression of several genes, among them *ENOD40*.

In search for a possible function of *ENOD40*, we showed (chapter 3) that application of AVG leads to a reduction of *ENOD40* expression, while an increase of ethylene concentration by addition of ACC leads to an increased *ENOD40* expression in tomato roots. This suggests that *ENOD40* expression is upregulated by ethylene. During the tomato life cycle *LeENOD40* expression is associated with certain processes such as seed germination and flower senescence, where ethylene concentration is first increased and then decreased (Khalil, 1992; Woltering *et al.*, 1994). The timing of *LeENOD40::GUS* expression just after the onset of ethylene production suggests that *LeENOD40* could have a role in reducing ethylene production or in reducing local effects of ethylene production, thereby limiting negative effects of ethylene. As ethylene is a negative regulator of nodule development, *ENOD40* could have a similar role in nodule development. Several nodulation-impaired mutants have been identified of which the nodulation phenotype is accompanied with either ethylene hyper- or insensitivity. For instance, in the pea mutant *sym5* infection takes place with a similar frequency as in wild-type pea but cortical cell division rarely occurs, resulting in a lower number of primordia. For *sym5* it was shown that nodule primordium formation is hypersensitive to ethylene (Guinel and LaRue, 1991). In the *Medicago truncatula* mutant *sickle* hyper-infection leads to an increase in the number of nodules. *Sickle* seedlings were shown to be insensitive to ethylene and ACC and application of ethylene did not inhibit nodulation in these plants (Penmetsa and Cook, 1997). Heidstra *et al.*, (1997) have shown in pea roots that ethylene also provides positional information for nodules to form opposite protoxylem poles. In *sickle* plants primordia also form opposite protoxylem poles and the number of primordia is higher in *sickle* compared to wild-type plants (Penmetsa and Cook, 1997; Geurts and Bisseling, 2002). From the phenotypes of *sym5* and *sickle* it can be inferred that ethylene controls nodule primordium formation as well as infection. The mutant R50 in which infection threads have lost their directional growth towards the nodule primordium, can be rescued by treating roots with ethylene inhibitors (Guinel and Sloetjes, 2000). This finding supports that ethylene is an important factor in the regulation of infection. Ethylene can block nodule development as early as at the process of calcium spiking, which is one of the first known reactions to the application of Nod factors (Oldroyd *et al.*, 2001). As a direct effect of Nod factors on ethylene production or action is not known, one may expect that Nod factors induce a local signal to repress the negative effect of ethylene. *ENOD40* is expressed within 3 hours after inoculation with *Sinorhizobium meliloti* 1021 in the pericycle opposite the

site where the nodule primordium forms (Compaan *et al.,* 2001). This is markedly before the first cortical cell divisions become apparent and strikingly in a region complementary to the site where in pea ACC oxidase expression was observed (Heidstra *et al.,* 1997; Compaan *et al.,* 2001). Moreover, ballistic targeting of *MtENOD40* DNA into alfalfa induces cortical cell divisions and *GUS* expression (Charon *et al.,* 1997). Ectopic expression of *ENOD40* in *Medicago truncatula* leads to an increased number of nodule primordia accompanied by enhanced cell proliferation in inoculated roots near the root tip. Root elongation measurements after ACC application show that these transgenic plants are still sensitive to ethylene. However, the increased nodule number of *M. truncatula* plants over expressing *ENOD40* can be mimicked by AVG application to wild-type *M. truncatula* plants, indicating that ethylene signalling in these plants is somehow affected (Charon *et al.*, 1999).

Thus, *ENOD40* might be a candidate through which Nod factors manipulate the ethylene homeostasis in cortical cells and facilitate cortical cell division by counteracting the negative effect of ethylene.

We observed that (chapter 2) lateral root primordia in the main root are flanked by high *LeENOD40::GUS* expression, while at the site of the lateral root primordia expression is absent. This could reflect that *LeENOD40::GUS* is actively repressed at the site where lateral root primordia are initiated. When the lateral root grows out of the main root the expression in the vascular bundle is increased to a level comparable to the basic level in the vascular bundle. We suggested that *LeENOD40::GUS* expression marks the sites in the root where lateral roots can form and that local repression of *ENOD40* is needed for the lateral root primordium to form. A transient decrease of *ENOD40* expression during lateral root primordium formation has now also been found in *Medicago truncatula* (B. Compaan, personal communication). Expression of *ENOD40* in emerging lateral roots has been described in legumes (Papadopoulou *et al.,* 1996; Fang and Hirsch, 1998; Varkonyi-Gasic and White, 2002) and confirms the expression of *LeENOD40::GUS* in emerging lateral roots in tomato. Whether during lateral root formation expression of *ENOD40* plays a role in limiting ethylene effects remains to be studied as the involvement of ethylene in lateral root formation is unknown.

All identified *ENOD40* cDNAs consist of two highly homologous regions, named region 1 and region 2. Only region 1 contains a conserved ORF (Van de Sande *et al.,* 1996; Corich *et al.,* 1998; Kouchi *et al.,* 1999; Flemetakis *et al.,* 2000). Transient expression studies showed that the start codon of the *ENOD40* peptide is recognised by the translation machinery and the peptide ORF is translated *in vivo* (Van de Sande *et al.,* 1996; Compaan *et al.,* 2001; Sousa *et al.,* 2001). Furthermore, an antibody raised against a synthetic GmENOD40 peptide confirmed the presence of an antigenic determinant in soybean nodule extracts, most likely being the GmENOD40 peptide (Van de Sande *et al.,* 1996). However, a function of the ENOD40 peptide is not known yet. We set out to

investigate the function of the *GmENOD40* peptide in tomato plant development by producing two sets of transgenic tomatoes: Tomato plants in which a complete *GmENOD40* sequence was introduced and tomato plants in which *GmENOD40* was introduced from which no region 1 peptide can be produced (chapter 4). These tomato plants are still being investigated. Furthermore, to obtain insight in the function of the ENOD40 peptide we used the Two-Hybrid system to identify putative interacting proteins. As the ENOD40 peptide was detected in nodule extracts (Van de Sande *et al*., 1996), we chose to search for interacting proteins from nodules. For this, we used the PsENOD40 peptide as bait in the Two-Hybrid system (chapter 5). Psp40, a protein with homology to ribosomal associated proteins, was shown to interact with PsENOD40 in the Two-Hybrid system. A first prerequisite for the interaction of Psp40 and PsENOD40 is that both proteins must be present in the same cells. Like *PsENOD40*, *Psp40* is expressed during pea nodule development. *Psp40* expression is found in the nodule meristem (zone I) and the infection zone (zone II). *PsENOD40* expression also occurs in zone II but not in zone I. Furthermore, *PsENOD40* expression is also found in the nodule vascular bundle, whereas Psp40 is not expressed in the nodule vascular bundle. Therefore, Psp40 and PsENOD40 could only interact in zone II cells, which could imply that the interaction of Psp40 and PsENOD40 might have a specific function in zone II. However, it cannot be excluded that the ENOD40 peptide interacts with other proteins in cells of the vascular bundle and the pericycle. It is a major challenge now to set up a system that allows the monitoring of the biological function of the ENOD40 peptide, so that subsequently the validity of the observed protein-protein interaction can be tested.

***ENOD40* in nodule and plant development**

We have shown that *ENOD40* expression occurs in several tissues of a non-legume. Our observations show that *ENOD40* expression does not coincide with a specific developmental stage. Rather it indicates that ENOD40 is involved in a process that different developmental stages have in common. Our studies on *LeENOD40* in the non-legume tomato indicate that *LeENOD40* plays a role in ethylene regulated processes and suggests that *LeENOD40* might be needed to counteract the effects of ethylene, a role that *ENOD40* could also have in nodule development.

We have shown it to be unlikely that *ENOD40* is a gene that was recruited from general plant development processes for nodule formation, as legume and non-legume *ENOD40* promoters are interchangeable and their regulatory elements do not seem to have changed during evolution. We suggest that the cause for the differential regulation of *ENOD40* by cytokinin and ethylene in legumes and non-legumes must be in the steps before induction of *ENOD40* expression. Furthermore, we suggest that specific transcription factors could be responsible for non-legume *ENOD40* expression and symbiotic *ENOD40* expression in legumes. Our findings suggest that although nodule

formation has many steps in common with general plant developmental processes, specific key regulators may be responsible for regulating these general processes in such a way that a completely new organ, the nodule, is formed.

REFERENCES

Albrecht C, Geurts R, Bisseling T (1999) Legume nodulation and mycorrhizae formation; two extremes in host specificity meet. *EMBO J* **18**: 281-288.

Ardini E, Pesole G, Tagliabue E, Magnifico A, Castronovo V, Sobel ME, Colnaghi MI, Ménard (1998) The 67-kDa Laminin Receptor originated from a ribosomal protein that acquired a dual function during evolution. *Mol Biol Evol* **15**: 1017-1025.

Ardourel M, Demont N, Debelle F, Maillet F, de Billy F, Prome J-C, Denarié J, Truchet G. (1994) *Rhizobium meliloti* lipoolichosaccharide nodulation factors: different structural requirements for bacterial entry into target root hair cells and induction of plant symbiotic developmental responses. *Plant Cell* **6**; 1357-1374.

Asad S, Fang Y, Wycoff KL, Hirsch AM (1994) Isolation and characterization of cDNA and genomic clones of *MsENOD40*; transcripts are detected in meristematic cells of alfalfa. *Protoplasma* **183**: 10-23.

Barker DJ, Bianchi S, Blondon F, Dattée Y, Duc G, Essad S, Flament P, Gallusci P, Génier G, Guy P, Muel X, Tourneur J, Dénarié J and Huguet T (1990) *Medicago truncatula*, a model plant for studying the molecular genetics of the *Rhizobium*-legume symbiosis. *Plant Mol Biol Rep* ***8***: 40-49.

Bauer P, Ratet P, Crespi M, Schultze M, Kondorosi A (1996) Nod factors and cytokinin induce similar cortical cell divisions, amyloplast deposition and *MsENOD12A* expression patterns in alfalfa roots. *Plant J* **10**: 91-105.

Becker JD, Moreira LM, Kapp D, Frosch SC, Puhler A, Perlic AM (2001) The nodulin *vfENOD18* is an ATP-binding protein in infected cells of *Vicia faba* L. nodules. *Plant Mol Biol* **47**: 749-759.

Bisseling T (1999) The role of plant peptides in intercellular signalling. *Curr Opin Plant Biol* **2**: 365-368.

Bloemberg GV, Thomas-Oates JE, Lugtenberg BJJ, Spaink HP (1994) Nodulation protein NodL of *Rhizobium leguminosarum* O-acetylates lipo-oligosaccharides, chitin fragments and N-acetylglucosamine in vitro. *Mol. Microbiol* **11**: 793-804.

Carroll SB, Grenier JK, Weatherbee SD (2001) From DNA to diversity. Blackwell Science Inc. Oxford UK.

Catoira R, Galera C, de Billy F, Penmetsa V, Journet E-P, Maillet F, Rosenberg C, Cook D, Gough C, Dénarié J. (2000) Four genes of *Medicago truncatula* controlling components of a nod factor transduction pathway. *Plant Cell* **12**: 1647-1665.

Cebolla A, Vinardell JM, Kiss E, Olah B, Roudier F, Kondorosi A, Kondorosi E (1999) The mitotic inhibitor *ccs52* is required for endoreduplication and ploidy-dependent cell enlargement in plants. *EMBO J* **18**: 4476-4484.

Charon C, Johansson C, Kondorosi E, Kondorosi A, Crespi M (1997) *enod40* induces dedifferentiation and division of root cortical cells in legumes. *Proc Natl Acad Sci USA* **94**: 8901-8906.

Charon C, Sousa C, Crespi M, Kondorosi A (1999) Alteration of *enod40* expression modifies *Medicago truncatula* root nodule development induced by *Sinorhizobium meliloti*. *The Plant Cell* **11**: 1953-1965.

Compaan B, Yang WC, Bisseling T, Franssen H (2001) *ENOD40* expression in the pericycle precedes cortical cell division in *Rhizobium*-legume interaction and the highly conserved internal region of the gene does not encode a peptide. *Plant and Soil* **230**: 1-8.

Constabel CP, Yip L, Ryan CA (1998) Prosystemin from potato, black nightshade, and bell pepper: primary structure and biological activity of predicted systemin polypeptides. *Plant Mol Biol* **36**: 55-62.

Cook D, Dreyer D, Bonnet D, Howell M, Nony E, Vandenbosch KA (1995) Transient induction of a peroxidase gene in *Medicago truncatula* precedes infection by *Rhizobium meliloti*. *Plant Cell* **7**: 43-55.

Cooper JB and Long SR (1994) Morphogenetic rescue of *Rhizobium meliloti* nodulation mutant by trans-zeatin secretion. *Plant Cell* **6**: 215-225.

Corich G, Goormachtig S, Lievens S, Van Montagu M, Holsters M (1998) Patterns of *ENOD40* gene expression in stem-borne nodules of *Sesbania rostrata*. *Plant Mol Biol* **37**: 67-76.

Crespi MD, Jurkevitch E, Poiret M, d'Aubenton-Carafa Y, Petrovics G, Kondorosi E, Kondorosi A (1994) *ENOD40*, a gene expressed during nodule organogenesis, codes for a non-translatable RNA involved in plant growth. *EMBO J* **13**: 5099-5112.

De Ruijter NCA, Rook MB, Bisseling T, Emons AMC (1998) Lipochito-oligosaccharides re-initiate root hair tip growth in *Vicia sativa* with high calcium and spectrin-like antigen at the tip. *Plant J* **13**: 341-350.

De Ruijter NCA, Bisseling T, Emons AMC (1999) Nod factors induce an increase in sub-apical fine bundles of actin filaments in *Vicia sativa* root hairs within minutes. *Mol Plant Microbe Interact* **12**: 829-832.

Dénarié J, Debelle F, Prome JC (1996) *Rhizobium* lipo-chitooligosaccharide nodulation factors: signaling molecules mediating recognition and morphogenesis. *Annu Rev Biochem* **65**: 503-535.

Diaz CL, Spaink HP, Kijne JW (2000) Heterologous rhizobial lipochitin oligosaccharides and chitin oligomers induce cortical cell divisions in red clover roots, transformed with the pea lectin gene. *Mol Plant Microbe Interact* **13**: 268-276.

Downie JA and Walker SA (1999) Plant responses to nodulation factors. *Curr Opin Plant Biol* **2**: 483-489.

Ehrhardt DW, Atkinson EM, Long SR (1992) Depolarization of alfalfa root hair membrane potential by *Rhizobium meliloti* Nod factors. *Science* **256**: 998-1000.

Erhardt DW, Wais R, Long SR (1996) Calcium spiking in plant root hairs responding to *Rhizobium* nodulation signals. *Cell* **85**: 673-681.

Endre G, Kereszt A, Kevel Z, Milhacea S, Kaló P, Kiss GB (2002) A receptor kinase gene regulating symbiotic nodule development. *Nature* **417**: 962-966.

Etzler ME, Kalsi G, Ewing NN, Roberts NJ, Day RB, Murphy JB (1999) A nod factor binding lectin with apyrase activity from legume roots. *Proc Natl Acad Sci USA* **96**: 5856-5861.

Fåhreus G (1957) The infection of clover root hairs by nodule bacteria studied by a simple glass technique. *J General Microbiol* **16**: 374-381.

Fang Y and Hirsch AM (1998) Studying early nodulin gene *ENOD40* expression and induction by nodulation factor and cytokinin in transgenic alfalfa. *Plant Physiol* **116**: 53-68.

Felle HH, Kondorosi E, Kondorosi A, Schultze M (1998) The role of ion fluxes in Nod factor signalling in *Medicago sativa*. *Plant J* **13**: 455-463.

Fernandez-Lopez M, Goormachtig S, Mengsheng G, D'Haeze W, Van Montagu M, Holsters M (1998) Ethylene-mediated phenotypic plasticity in root nodule development on *Sesbania rostrata*. *Proc Natl Acad Sci USA* **95**: 12724-12728.

Fields S and Song O (1989) A novel genetic system to detect protein-protein interactions. *Nature* **340**: 245-246.

Flemetakis E, Kavroulakis N, Quaedvlieg NE, Spaink HP, Dimou M, Roussis A, Katinakis P (2000) *Lotus japonicus* contains two distinct *ENOD40* genes that are expressed in symbiotic, nonsymbiotic, and embryonic tissues. *Mol Plant Microbe Interact* **13**: 987-994.

Fletcher JC, Brand U, Running MP, Simon R, Meyerowitz EM (1999) Signaling of cell fate decisions by CLAVATA3 in Arabidopsis shoot meristems. *Science* **283**: 617-619.

Franssen HJ and Bisseling T (2001) Peptide signaling in plants. *Proc Natl Acad Sci USA* **98**: 12855-12856.

Frugier F, Poirier S, Satiat-Jeunemaitre B, Kondorosi A, Crespi M (2000) A Krüppel-like zinc finger protein is involved in nitrogen-fixing root nodule organogenesis. *Genes and Development* **14**: 475-482.

Garcia-Hernandez M, Davies E, Staswick PE (1994) *Arabidopsis* p40 homologue. A novel acidic protein associated with the 40 S subunit of ribosomes. *J Biol Chem* **269**: 20744-20749.

Geurts R, Heidstra R, Hadri AE, Downie JA, Franssen H, van Kammen A, Bisseling (1997) Sym2 of *Pisum sativum* is involved in a Nod factor perception mechanism that controls the infection process in the epidermis. *Plant Physiol* **115**: 351-359.

Geurts R and Bisseling T (2002) *Rhizobium* Nod factor perception and signalling. *Plant Cell* **Supplement 2002**: S239-S249.

Gianinazzi-Pearson V (1996) Plant cell responses to arbuscular mycorrhizal fungi: getting to the roots of symbiosis. *Plant Cell* **8**: 1871-1883.

Goethals K, Mergaert P, Gao M, Geelen D, Van Montagu M, Holsters M (1992) Identification of a new inducible nodulation gene in Azorhizobium caulinodans. *Mol Plant Microb Int* **5**: 405-411.

Goedhart J, Hink MA, Visser AJWG, Bisseling T, Gadella TWJ (2000) In vivo fluorescence correlation microscopy (FCM) reveals accumulation and immobilization of Nod factors in root hair cell walls. *Plant J* **21**: 109-119.

Gonzalez JE, Reuhs BL, Walker GC (1996) Low molecular weight EPS II of *Rhizobium meliloti* allows nodule invasion in *Medicago sativa*. *Proc Natl Acad Sci USA* **93**: 8636-8641.

Gressent F, Drouillard S, Mantegazza N, Samain E, Geremia RA, Canut H, Niebel A, Driguez H, Ranjeva R, Cullimore J, Bono J-J (1999) Ligand specificity of a high-affinity binding site for lipo-chitooligosaccharidic Nod factors in *Medicago* cell suspension cultures. *Proc Natl Acad Sci USA* **96**:4704-4709.

Gualtieri G, Bisseling T (2000) The evolution of nodulation. *Plant Mol Biol* **42**: 181-194.

Guinel FC and LaRue TA (1991) Light microscopy study of nodule initiation in *Pisum sativum* L. cv Sparkle and in its low-nodulating mutant E2 (*sym* 5) *Plant Physiol* **97**: 1206-1211.

Guinel FC and Sloetjes LL (2000) Ethylene is involved in the nodulation phenotype of Pisum sativum (sym 16), a pleiotropic mutant that nodulates poorly and has pale green leaves. *J Exp Bot* **51**: 885-894.

Gyuris J, Golemis E, Chertkov H, Brent R (1993) Cdi1 a human G1 and S phase protein phosphatase that associates with Cdk2. *Cell* **75**: 791-803

Kouchi H and Hatta S (1993) Isolation and characterization of novel nodulin cDNAs representing genes expressed at early stages of soybean nodule development. *Mol Gen Genet* **238**: 106-119.

Heidstra R, Yang WC, Yalcin Y, Peck S, Emons AM, Van Kammen A, and Bisseling T (1997) Ethylene provides postitional information on cortical cell division but is not involved in Nod factor-induced root hair tip growth in *Rhizobium*-legume interaction. *Development* **124**: 1781-1787.

Hemerly A, Engler Jde A, Bergounioux C. Van Montagu M, Engler G, Inzé D, Ferreira P (1995) Dominant negative mutants of the Cdc2 kinase uncouple cell division from iterative plant development. *EMBO J* **14**: 3925-3936.

Hirsch AM, Bhuvaneswari TV, Torrey JG, Bisseling T (1989) Early nodulin genes are induced in alfalfa root outgrowths elicited by auxin transport inhibitors. *Proc Natl Acad Sci USA* **86**: 1244-1248.

Hirsch AM, Lum MR, Downie JA (2001) What makes the rhizobia-legume symbiosis so special? *Plant Physiol* 127: 1484-1492.

Horvath B, Heidstra R, Lados M, Moerman M, Spaink HP, Promé JC, Van Kammen A, Bisseling T (1993) Lipo-oligosaccharidesof Rhizobium induce infection-related early nodulin gene expression in pea root hairs. *Plant J* **4**: 727-733.

Hutchison CE and Kieber JJ (2002) Cytokinin signaling in Arabidopsis. *Plant Cell* Supplement 2002: S47-S59.

Jansen AK, van den Noort RE (2000) Ultraviolet-B radiation induces complex alterations in stomatal behaviour. *Physiol Plant* **110**: 189-194.

Jossey DP, Beynon JL, Johnston AWB, Beringer JE (1979) Strain identification in *Rhizobium* using intrinsic antibiotic resistance. *J Appl Bacteriol* **46**: 343-350.

Khalil MK (1992) Nature of growth regulators effects on Nicotiana tabacum seed germination. *Angew Bot* **66**: 106–108.

Kijne JW (1992) The Rhizobium infection process. In: *Biological Nitrogen Fixation*, eds Stacey G, Evans HJ, Burris RH. Chapman and Hall NY, 349-398.

Komina O, Zhou Y, Gautam S, Chollet R (2002) In vivo and in vitro phosphorylation of membrane and soluble forms of soybean nodule sucrose synthase. *Plant Physiol* **129**: 1664-1673.

Kosugi S, Ohashi Y, Nakajima K, Arai Y (1990) An improved assay for beta-glucuronidase in transformed cells: Methanol almost completely suppresses a putative endogenous beta-glucuronidase activity. *Plant Science* **70**: 133-140.

Kouchi H, Takane K, So RB, Ladha JK, Reddy PM (1999) Rice *ENOD40*: isolation and expression analysis in rice and transgenic soybean root nodules. *Plant J* **18**: 121-129.

Kozik A (1996) Fine mapping of the SYM2 locus of pea linkage group I, *thesis*, Agricultural University, Wageningen, The Netherlands, 111p.

Larsen PB, Woltering EJ, Woodson WR (1993) Ethylene and interorgan signalling in flowers following pollination. In: Schultz JC, Raskin I (eds) *Plant signals in interactions with other organisms*, pp. 171–181. American Society for Plant Physiology, Rockville, MD.

Lee KH and LaRue TA (1992a) Pleiotropic effects of *sym17*. *Plant Physiol* **100**: 1326-1333.

Lee KH and LaRue TA (1992b) Exogenous ethylene inhibits nodulation of *Pisum sativum* L. cv Sparkle. *Plant Physiol* **100**: 1789-1763.

Lerouge P, Roche P, Faucher C, Maillet F, Truchet G, Promé J-C, Dénarié J (1990) Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acytlated glucosamine oligosaccharide signal. *Nature* **344**:781-784.

Leubner-Metzger G, Petruzzelli L, Waldvogel R, Vögeli-Lange R, Meins F Jr (1998) Ethylene-responsive element binding protein (EREBP) expression and the transcriptional regulation of class I β-1,3-glucanase during tobacco seed germination. *Plant Mol Biol* **38**: 785-795.

Lorteau MA, Ferguson BJ, Guinel FC (2001) Effects of cytokinin on ethylene production and nodulation in pea (*Pisum sativum*) cv. Sparkle. *Physiol Plant* **112**: (421-428).

Markwei CM and Larue TA (1992) Phenotypic characterization of *sym8* and *sym9*, two genes conditioning non-nodulation in *Pisum sativum* 'Sparkle'. *Can J Microbiol* 38: 548-554.

Mathesius U, Schlaman HRM, Spaink HP, Sautter C, Rolfe BG, Djordjevic MA (1998) Auxin transport inhibition precedes root nodule formation in white clover roots and is regulated by flavonoids and derivatives of chitin oligosaccharides. *Plant J* **14**: 23-34.

Mathesius U, Charon C, Rolfe BG, Kondorosi A, Crespi M (2000) Temporal and spatial order of events during the induction of cortical cell divisions in white clover by *Rhizobium leguminosarum* bv. *trifolii* inoculation or localized cytokinin addition. *Mol Plant Microbe Interact* **13**: 617-628.

Matsubayashi Y, Sakagami Y (1996) Phytosulfokine, sulfated peptides that induce the proliferation of single mesophyll cells of *Asparagus officinalis* L. *Proc Natl Acad Sci USA* **93**: 7623-7627.

Matvienko, van de Sande K, Yang WC, van Kammen A, Bisseling T, Franssen H (1994) Comparison of soybean and pea *ENOD40* cDNA clones representing genes expressed during both early and late stages of nodule development. *Plant Mol Biol* **26**: 487-493.

Matvienko M, van de Sande K, Pawlowski K, van Kammen A, Franssen H, Bisseling T (1996) *Nicotiana tabacum* SR1 contains two *ENOD40* homologs. In: *Biology of Plant-microbe interactions*, Eds: Stacey G, Mullin B and Gresshoff PM, International Society for Molecular Plant-Microbe Interactions. St Paul pp. 387-391.

McCarty DR and Chory J (2000) Conservation and innovation in plant sigaling pathways. *Cell* **103**: 201-209.

McGurl B, Pearce G, Orozco-Cardenas M, Ryan CA (1992) Structure, expression and antisense inhibition of the systemin precursor gene. *Science* **255**: 1570-1573.

Meyerowitz E (1999) Plants, animals and the logic of development.*Trends Cell Biol* **9**: 65-68.

Mirabella R, Martirani L, Lamberti A, Iaccarino M, Chiurazzi M (1999) The soybean *ENOD40*(2) promoter is active in *Arabidopsis thaliana* and is temporally and spatially regulated. *Plant Mol Biol* **39**: 177-181.

Oldroyd GE, Engstrom EM, Long SR (2001) Ethylene inhibits the Nod factor signal transcudtion pathway of *Medicago truncatula*. *Plant Cell* **13**: 1835-1849.

Parniske M (2000) Intracellular accomodation of microbes by plants: a common developmental program for symbiosis and disease? *Curr Opin in Plant Biol* **3**: 320-328.

Papadopoulou K, Roussis A, Katinakis P (1996) *Phaseolus ENOD40* is involved in symbiotic and non-symbiotic organogenetic processes: expression during nodule and lateral root development. *Plant Mol Biol* **30**: 403-417.

Pawlowski K, Kunze R, De Vries S, Bisseling T (1994) Isolation of total, poly(A) and polysomal RNA from plant tissues. In: *Plant Mol Biol Manual*, (Gelvin SB and Schilperoort RA eds) Kluwer Academic Publishers, Dordrecht, The Netherlands, 1-13.

Pearce G, Moura DS, Stratmann J, Ryan CA (2001) RALF, a 5-kDa ubiquitous polypeptide in plants, arrests root growth and development. *Proc Natl Acad Sci USA* **98**: 12843-12847.

Pelletier J, Sidhu S (2001) Mapping protein-protein interactions with cominatorial biology methods. *Curr Opin Biotechnol* **12**: 340-347.

Penmetsa RV, Cook RC, (1997) A legume ethylene-insensitive mutant hyperinfected by its rhizobial symbiont. *Science* **275**: 527-530.

Peters NK and Crist-Estes DK (1989) Nodule formation is stimulated by the ethylene inhibitor aminoethoxyvinylglycine. *Plant Physiol* **91**: 690-693.

Pingret J-L, Journet E-P, Barker DG (1998) Rhizobium Nod factor signaling: evidence for a G protein-mediated transduction mechanism. *Plant Cell* **10**:659-671.

Ramanna MS, Prakken R (1967) Structure of and homology between pachytene and somatic metaphase chromosomes of the tomato. *Genome* **36**: 404-417.

Reddy PM, Ladha JK, Ramos MC, Maillet F, Hernandez RJ, Torrizo LB, Oliva NP, Datta SK, Datta K (1998) Rhizobial lipochitooligosaccharide nodulation factors activate expression of legume early nodulin gene *ENOD12* in rice. *The Plant J* **14**: 693-702.

Reddy PM, Aggarwal RK, Ramos MC, Ladha JK, Brar DS, Kouchi H (1999) Widespread occurrence of the homologues of the early nodulin (*ENOD*) genes in *Oryza* species and related grasses. *Biochem Biophys Res commun* **258**: 148-154.

Röhrig H, Schmidt J, Miklashevichs E, Schell J, John M (2002) Soybean *ENOD40* encodes two peptides that bind to sucrose synthase. *Proc Natl Acad Sci USA* **99**: 1915-1920.

Roussis A, van de Sande K, Papadopoulou K, Drenth J, Bisseling T, Franssen H, Katinakis P (1995) Characterization of the soybean gene *GmENOD40-2*. *J Exp Bot* **46**: 719-724.

Ryan CA, Pearce G (2001) Polypeptide hormones. *Plant Physiol* **125**: 65-68.

Schauser L, Handberg K, Sandal N, Stiller J, Thykjaer T, Pajuelo E, Nielsen A, Stougaard J (1998) Symbiotic mutants deficient in nodule establishment identified after T-DNA transformation of *Lotus japonicus*. *Mol Gen Genet* **259**: 414-423.

Schauser L, Roussis A, Stiller J, Stougaard J (1999) A plant regulator controlling development of symbiotic root nodules. *Nature* **402**: 191-195.

Scheer I, Ludevid MD, Regad F, Lescure B, Pont-Lezica RF (1997) Expression of a gene encoding a ribosomal p40 protein and identification of an active promoter site. *Plant Mol Biol* **35**: 905-913.

Scheres B, van Engelen F, van der Knaap E, van de Wiel C, van Kammen A, Bisseling T (1990) Sequential induction of nodulin gene expression in the developing pea nodule. *Plant Cell* **2**: 687-700.

Scheres B, van Engelen F, van der Knaap E, van de Wiel C, van Kammen A, Bisseling T (1990) Sequential induction of nodullin gene expression in the developing pea nodule. *The Plant Cell* **2**: 687-700.

Schlaman HRM, Horvath B, Vijgenboom E, Okker RJH, Lugtenberg BJJ (1991) Suppression of nodulation gene expression in bacteroids of *Rhizobium leguminosarum* biovar *viciae*. *J Bacteriol* **173**: 4277-4287.

Schlaman HRM, Gisel AA, Quadvlieg N, Bloemberg G, Lugtenberg BJJ, Kijne J, Potrykus I, Spaink HP, Sautter C (1997) Chitin oligosaccharides can induce cortical cell division in roots of Vicia sativa when delivered by ballistic micro-targeting. *Development* **124**: 4887-4895.

Schmidt JS, Harper JE, Hoffman TK, Bent AF (1999) Regulation of soybean nodulation independent of ethylene signalling. *Plant Physiol* **119**: 951-959.

Schultze M, Kondorosi A (1998) Regulation of symbiotic root nodule development. *Annu Rev Genet* **32**: 33-57.

Smit G, de Koster CC, Schripsema J, Spaink HP, van Brussel AAN, Kijne JW (1995) Uridine, a cell division factor in pea roots. *Plant Mol Biol* **29**: 869-873.

Sousa C, Johansson C, Charon C, Manyani H, Sautter C, Kondorosi A, Crespi M (2001) Translational and structural requirements of the early nodulin gene *enod40*, a short-open reading frame-containing RNA, for elicitation of a cell-specific growth response in the alfalfa root cortex. *Moll Cell Biol* **21**: 354-366.

Spaink HP, Sheeley DM, Van Brussel AAN, Glushka J, York WS, Tak T, Geiger O, Kennedy EP, Reinhold VN and Lugtenberg BJJ (1991) A novel highly unsaturated fatty acid moiety of lipo-oligosaccharide signals determines host specificity of Rhizobium. *Nature* **354**: 125-130.

Spaink HP (1996) Regulation of plant morphogenesis by lipo-chitin oligosaccharides. *Crit Rev Plant Sci* **15**: 559-582.

Spaink HP (2002) A receptor in symbiotic dialogue. *Nature* **417**: 910

Staehelin C, Charon C, Boller T, Crespi M, Kondorosi A (2000) Medicago truncatula plants overexpressing the early nodulin gene enod40 exhibit accelerated mycorrhizal colonization and enhanced formation of arbuscules. *Proc Natl Acad Sci USA* **98**: 15366-15371.

Stam P, Van Ooijen JW (1995) JoinMap (tm) version 2.0: Software for the calculation of genetic linkage maps. CPRO-DLO Wageningen.

Stougaard J (2000) Regulators and regulation of legume root nodule development. *Plant Physiol* **124**:531-540.

Stougaard J (2001) Genetics and genomics of root symbiosis. *Curr Opin Plant Biol* **4**: 328-335.

Stracke S, Kistner C, Yoshida S, Mulder L, Sato S, Kaneko T, Tabata S, Sandal N, Stougaard S, Szczyglowski K, Parniske M (2002) A plant receptor-like kinase required for both bacterial and fungal symbiosis. *Nature* **417**: 959-962.

Sutton JM, Lea AJA, Downie JA (1994) The nodulation-signalling protein *nodO* from *Rhizobium leguminosarum* biovar *viciae* forms ion channels in membranes. *Proc Natl Acad Sci USA* **91**: 9990-9994.

Takayama S, Shimosata H, Shiba H, Funato M, Che FS, Watanabe M, Iwano M, Isogai A (2001) Direct ligand-receptor complex interaction controls *Brassica* self-incompatibility. *Nature* **413**: 534-538.

Timmers AC, Auriac MC, Truchet G (1999) Refined analysis of early symbiotic steps of the Rhizobium-Medicago interaction in relationship with microtubular cytoskeleton rearrangements. *Development* **126**: 3617-28.

Truchet G (1978) Sur l'tat diploide des cellules du meristeme des nodules radiculaire de Lgumineuses. *Ann Sci Nat Bot Paris* **19**:3-38.

Tucker CL, Gera JF, Uetz P (2001) Towards an understanding of complex protein networks. *Trends Cell Biol* **11**: 102-106.

Van Brussel AAN, Bakhuizen R, van Spronsen PC, Spaink HP, Tak T, Lugtenberg BJJ, Kijne JW (1992) Induction of pre-infection thread structures in the leguminous host plant by mitogenic lipo-oligosaccharides of *Rhizobium*. *Science* **257**: 70-72.

Van de Sande K, Pawlowski K, Czaja I, Wieneke U, Schell J, Schmidt J, Walden R, Matvienko M, Wellink J, van Kammen A, Franssen H, Bisseling T (1996) Modification of phytohormone response by a peptide encoded by *ENOD40* of legumes and a nonlegume. *Science* **273**: 370–373.

Van Rhijn P, Fang Y, Galili S, Atzmon N, Wininger S, Eshed Y, Lum M, Li Y, To VV, Fujishige N, Kapulnik Y, Hirsch AM (1997) Expresion of early nodulin genes in alfalfa mycorrhizae indictes that signal transduction pathways used in forming arbuscular mycorrhizae and *Rhizobium*-induced nodules may be conserved. *Proc Natl Acad Sci USA* **94**: 5467-5472.

Van Rhijn P, Goldberg RB, Hirsch AM (1998) Lotus corniculatus nodulation specificity is changed by the presence of a soybean lectin gene. *Plant Cell* **10**: 1233-1250.

Van Spronsen PC, Gronlund M, Bras CP, Spaink HP, Kijne JW (2001) Cell biological changes of outer cortical root cells in early determinate nodulation. *Mol Plant Microbe Interact* **14**: 839-847.

Varkonyi-Gasic E, White DWR (2002) The white clover enod40 gene family. Expression patterns of two types of genes indicate a role in vascular function. *Plant physiol* **129**: 1107-1118.

Vasse J, de Billy F, Camut S, Truchet G (1990) Correlation between ultrastructural differentiation of bacteroids and nitrogen fixation in alfalfa nodules. *J. Bacteriol*. **172**: 4295-4306.

Vijn I, Yang WC, Pallisgard N, Ostergaard Jensen E, van Kammen A, Bisseling T (1995) *VsENOD5*, *VsENOD12* and *VsENOD40* expression during *Rhizobium*-induced nodule formation on *Vicia sativa* roots. *Plant Mol Biol* **28**: 1111-1119.

Walker SA, Viprey V, Downie JA (2000) Dissection of nodulation using pea mutants defective for calcium spiking induced by nod factors and chitin oligomers. *Proc Natl Acad Sci USA* **97**: 13413-13418.

Webb KJ, Skot L, Nicholson MN, Jorgensen B, Mizen S (2000) *Mesorhizobium loti* increases root-specific expression of a calcium-binding protein homologue identified by promoter tagging in *Lotus japonicus*. *Mol Plant Microbe Interact* **13**: 606-616.

Wisniewski JP, Rathbun EA, Knox JP, Brewin NJ (2000) Involvement of diamine oxidase and peroxidase in insolubilization of the extracellular matrix: implications for pea nodule initiation by *Rhizobium leguminosarum*. *Mol Plant Microbe Interact* **13**: 413-420.

Woltering EJ, ten Have A, Larsen PB, Woodson WR (1994) Ethylene biosynthetic genes and interorgan signalling during flower senescence. In*: Molecular and Cellular Aspects of Plant Reproduction*, R.J. Scott and A.D. Stead (Eds.), Cambridge University Press, Cambridge, UK, pp. 285–307.

Wopereis J, Pajuelo E, Jiang Q, Gresshoff PM, De Bruin FJ, Stougaard J, Szczyglowski K, (2000) Short root mutant of *Lotus japonicus* with a dramatically altered symbiotic phenotype. *Plant J* **23**: 97-114.

Xenarios I, Eisenberg D (2001) Protein interaction databases. *Curr Opin Biotechnol* **12**: 334-339.

Yang WC, Horvath B, Hontelez J, van Kammen A, Bisseling T (1991) *In situ* localization of *Rhizobium* mRNAs in pea root nodules: *nifA* and *nifH* localization. *Mol Plant Microbe Interact* **7**: 276-281.

Yang WC, Katinakis P, Hendriks P, Smolders A, de Vries F, Spee J, van Kammen A, Bisseling T, Franssen H (1993) Characterization of *GmENOD40*, a gene showing novel patterns of cell-specific expression during soybean nodule development. *Plant J* **3**: 573-585.

Yang WC, de Blank C, Meskiene I, Hirt H, Bakker J, van Kammen A, Franssen H, Bisseling T (1994) *Rhizobium* nod factors reactivate the cell cycle during infection and nodule primordium formation, but the cycle is only completed in primordium formation. *Plant Cell* **6**: 1414-1426.

Zhong XB, Fransz PF, Wennekes-van Eden J, Zabel P, van Kammen A, de Jong JH (1996) High resolution mapping by fluorescence *in situ* hybridisation to pachytene chromosomes and extended DNA fibres. *Plant Mol Biol Rep* **14**: 232-242

Zhong XB, Fransz PF, Wennekes-van Eden J, Ramanna MS, van Kammen A, Zabel P, de Jong JH (1998) FISH studies reveal the molecular and chromosomal organisation of individual telomere domains in tomato. *Plant J* **13**(4): 507-517.

Zhu JW, Kahn R (1997) Analysis of a peptide hormone-receptor interaction in the yeast two-hybrid system. *Proc Natl Acad Sci USA* **94**: 13063-13068.

SAMENVATTING

Vlinderbloemige planten kunnen, als er weinig stikstof in de grond aanwezig is, een symbiose aangaan met *Rhizobium* bacteriën. Rhizobia induceren in de wortel van de gastheerplant de vorming van een nieuw orgaan, de wortelknol. De bacteriën worden gehuisvest in de wortelknol waar zij moleculaire stikstof reduceren tot ammonium. De gastheerplant gebruikt het geproduceerde ammonium als stikstofbron. Op haar beurt voorziet de gastheerplant de bacteriën van suikers.

Voor de vorming van de wortelknol in de wortel worden cellen in de wortel geherprogrammeerd. Binnen in de wortel vormt zich het knolprimordium. Tegelijkertijd ontwikkelt zich vanaf de wortelharen een infectiedraad richting het knolprimordium waarlangs de bacteriën het knolprimordium bereiken. Na infectie met de bacteriën ontwikkelt het knolprimordium zich tot een volwassen knol.

De symbiose met rhizobia beperkt zich grotendeels tot vlinderbloemige planten, ook al zou deze symbiose, die leidt tot stikstoffixerende wortelknolletjes, nuttig kunnen zijn voor allerlei plantensoorten. Dit leidt tot de vraag wat in vlinderbloemigen de mogelijkheid van een symbiose met rhizobia bepaalt. Genen waarvan de expressie tijdens knolvorming wordt geïnduceerd worden nodulinegenen genoemd. Homologen van deze nodulinegenen komen ook voor in niet-vlinderbloemige planten. Dit wijst erop dat nodulinegenen zijn gerekruteerd voor knolvorming. Twee vragen dienen zich dan aan: hoe zou deze rekrutering hebben kunnen plaatsvinden en waarom? Een antwoord op de laatste vraag zou kunnen zijn dat de functie van het betreffende gen ook vereist is in de ontwikkeling en het functioneren van de wortelknol. Knolvorming kan zich hebben ontwikkeld tijdens de evolutie door aanpassing van de expressie van een bepaalde set genen. Het kan hier gaan om een beperkte specifieke set van sleutelregulatoren of om een grote set van genen waarvan de regulatie specifiek voor knolvorming is aangepast. Om inzicht te krijgen in hoe en waarom nodulinegenen zijn gerekruteerd voor knolvorming hebben we gekozen om de regulatie van het nodulinegen *ENOD40* in vlinderbloemigen en niet-vlinderbloemigen te bestuderen.

Het gen *ENOD40* is gekozen voor deze studies omdat *ENOD40* een belangrijke rol tijdens knolontwikkeling wordt toebedacht. Dit komt onder andere doordat de expressie van *ENOD40* tijdens knolvorming wordt geïnduceerd op de plaats waar later het knolprimordium zich zal vormen. Verder heeft men laten zien dat in planten waar de expressie van *ENOD40* uitgeschakeld wordt er alleen misvormde wortelknollen ontstaan. *ENOD40* homologen zijn ook gevonden in niet-vlinderbloemigen, zelfs in monocotylen. Studies in verschillende planten waarin *ENOD40* tot overexpressie is

gebracht laten zien dat dit leidt tot defecten in de plantontwikkeling. *ENOD40* heeft dus een belangrijke rol in knolontwikkeling en plantontwikkeling en zou een gen kunnen zijn dat om zijn functie tijdens de evolutie is gerekruteerd voor knolvorming. In dit geval zou de regulatie van *ENOD40* tijdens knolontwikkeling en plantontwikkeling verschillend moeten zijn, maar de functie van *ENOD40* in vlinderbloemigen en in niet-vlinderbloemigen zou gelijk moeten zijn. Om de vraag te kunnen beantwoorden of *ENOD40* is gerekruteerd voor knolvorming is in dit proefschrift de regulatie en functie van het nodulinegen *ENOD40* in vlinderbloemige en niet-vlinderbloemige planten bestudeerd.

Om *ENOD40* in de ontwikkeling van (niet-vlinderbloemige) tomatenplanten te kunnen bestuderen is de homoloog van *ENOD40* uit tomaat geïsoleerd (*LeENOD40*). Gedetailleerde expressiestudies met een *LeENOD40* promoter *GUS* fusie (*LeENOD40::GUS*) laten zien dat *ENOD40* in de hele tomatenplant tot expressie komt, voornamelijk in het vaatweefsel. Het expressiepatroon van *LeENOD40::GUS* in tomaat is vergelijkbaar met de expressie van de vlinderbloemige promoter *GUS* fusie *GmENOD40::GUS* in de niet-vlinderbloemige modelplant *Arabidopsis*. In deze planten worden beide promotoren negatief gereguleerd door het phytohormoon cytokinine. Hieruit blijkt dat vlinderbloemige en niet-vlinderbloemige promotoren in een niet-vlinderbloemige achtergrond zich hetzelfde gedragen. Ook de expressie van *LeENOD40::GUS* is in wortelknollen op transgene (vlinderbloemige) *Medicago* wortels vergelijkbaar met de expressie van een vlinderbloemige *ENOD40* in knollen. Dit laat zien dat de *LeENOD40* promoter ook functioneel is in wortelknollen en dat vlinderbloemige en niet-vlinderbloemige promotoren uitwisselbaar zijn. Hetzelfde is aangetoond met een rijst *ENOD40* promoter *GUS* fusie in knollen op transgene soja wortels. Blijkbaar zijn er in de evolutie geen functionele promoterelementen in *ENOD40* promoters veranderd en is het functioneren van deze promoters *in planta* gelijk in monocotylen en dicotylen.

Ook al kunnen we laten zien dat vlinderbloemige en niet-vlinderbloemige promotoren uitwisselbaar zijn, tijdens knolvorming wordt *ENOD40* expressie geïnduceerd tot een niveau dat in geen enkel ander proces is geobserveerd voor dit gen. Blijkbaar gaat er toch een speciale regulatie van *ENOD40* expressie aan knolvorming vooraf. De hierboven beschreven experimenten laten zien dat de oorzaak hiervan niet in de promoter van *ENOD40* ligt. Waarschijnlijk is een andere factor van invloed op *ENOD40* expressie. Deze factor kan van invloed zijn op de mRNA stabiliteit of op de transcriptie van het *ENOD40* mRNA. De tweede optie is het meest waarschijnlijk omdat het meer voor de hand ligt dat een plotselinge verhoging van *ENOD40* expressie wordt veroorzaakt door een verhoogde transcriptie dan door een stabilisering van het mRNA.

Specifieke transcriptiefactoren zouden tijdens knolontwikkeling en tijdens plantontwikkeling verantwoordelijk kunnen zijn voor initiatie van *ENOD40* expressie.

We concluderen dat *ENOD40* zeer waarschijnlijk geen sleutelregulator van knolvorming is. Een knolspecifieke transcriptiefactor zou wel een sleutelregulator kunnen zijn en zou *ENOD40* expressie en misschien ook andere nodulinegenen kunnen reguleren tijdens knolvorming.
Nu de regulatie tussen *ENOD40* in vlinderbloemigen en niet-vlinderbloemigen is vergeleken en is gebleken dat *ENOD40* promotoren uit vlinderbloemigen en niet-vlinderbloemige uitwisselbaar zijn, blijft nog de vraag of de functie van *ENOD40* gelijk is in vlinderbloemigen en niet-vlinderbloemigen. In transgene tomatenlijnen is het effect van ethyleen en AVG (een remmer van ethyleenproductie) op *LeENOD40* expressie in wortels bestudeerd. AVG veroorzaakt een reductie van *ENOD40* expressie in tomatenwortels, terwijl ACC (een precursor van ethyleen) leidt tot een inductie van *ENOD40* expressie in tomatenwortels. Verder is de *LeENOD40::GUS* expressie geassocieerd met bloemveroudering en kieming, processen waarbij de ethyleen concentratie eerst stijgt en daarna afneemt. Het moment van *ENOD40* expressie tijdens deze processen direct na een toename van ethyleen productie kan erop wijzen dat *LeENOD40* een rol speelt in het voorkomen van negatieve effecten van ethyleen. Omdat ethyleen een negatief effect heeft op knolvorming zou *ENOD40* eenzelfde rol kunnen spelen tijdens knolvorming.
Medicago planten waarin *ENOD40* tot overexpressie is gebracht laten een versnelde knolvorming zien. Dit effect kan ook gedeeltelijk worden bereikt door *Medicago* wortels te behandelen met AVG. Dit laat zien dat in deze transgene *Medicago* planten een verandering is opgetreden in de ethyleen huishouding of de ethyleen signaal transductie. Uit het bovenstaande kan worden afgeleid dat *ENOD40* expressie ook in vlinderbloemigen gevoelig is voor ethyleen en hieruit leiden wij de hypothese af dat *ENOD40* nodig is om de celdelingen van het knolprimordium te faciliteren door de negatieve effecten van ethyleen op celdeling te voorkomen of teniet te doen.
De expressie van *ENOD40* tijdens zijwortelvorming kan ook een aanwijzing zijn voor de functie van *ENOD40*. In transgene *LeENOD40::GUS* tomatenplanten flankeert *LeENOD40::GUS* expressie in de vaatbundel van de hoofdwortel de zijwortelprimordia. Op de plaats van de zijwortelprimordia is een lokale afwezigheid van *LeENOD40::GUS* expressie. Na uitgroei van het zijwortelprimordium bereikt de *LeENOD40::GUS* expressie weer zijn basisniveau in de vaatbundel van wortel en zijwortel. De plaatselijke reductie van *ENOD40* expressie in de wortel zou erop kunnen wijzen dat een hoge *ENOD40* expressie op de plaats waar een zijwortelprimordium zich vormt remmend kan werken op de ontwikkeling van het zijwortelprimordium. Of *ENOD40* ook in dit proces negatieve effecten van ethyleen voorkomt is niet duidelijk omdat de rol van ethyleen tijdens zijwortelvorming niet bekend is.
Alle *ENOD40* genen coderen voor een klein peptide van 10-13 aminozuren afhankelijk van de plantensoort. Door het bestuderen van *ENOD40* expressie in tomaat en

Arabidopsis hebben we een aanwijzing gekregen over de functie van *ENOD40*. Deze studies kunnen geen aanwijzingen geven of de functie van *ENOD40* in het ENOD40 peptide ligt of in het *ENOD40* RNA. Omdat het ENOD40 peptide zo klein is, is het zeer moeilijk om het peptide volgens conventionele methodes te bestuderen. Dit is de reden dat het nog steeds niet helemaal duidelijk is in hoeverre het *ENOD40* gen een combinatie is van een RNA en peptide functie of dat de functie van het gen volledig in het peptide ligt. Om de functie van het ENOD40 peptide te bestuderen is het Two-Hybrid systeem gebruikt om ENOD40 bindende eiwitten te isoleren. We hebben aangetoond dat PsENOD40 in het Two-Hybrid systeem bindt met het Ps-p40 eiwit, een eiwit met homologie met ribosoom geassocieerde eiwitten. *In situ* expressie studies hebben aangetoond dat *Ps-p40* tot expressie komt in het knolmeristeem (zone I) en in de infectiezone van de knol (zone II). Dit overlapt gedeeltelijk met *ENOD40* expressie. *ENOD40* komt tot expressie in de infectiezone maar niet in het knolmeristeem. Interactie tussen ENOD40 en Ps-p40 zou kunnen voorkomen in de infectiezone van de knol. Een specifieke plaats van interactie zou erop kunnen wijzen dat het ENOD40 peptide een speciale functie heeft in het knolmeristeem.

Concluderend, onze experimenten aan *ENOD40* laten zien dat *ENOD40* een rol speelt bij processen waar het phytohormoon ethyleen ook een rol speelt. De functie van *ENOD40* zou van belang zou kunnen zijn voor het voorkomen van negatieve effecten van ethyleen. Verder hebben we laten zien dat het niet waarschijnlijk is dat *ENOD40* een sleutelregulator van knolvorming is maar dat het meer waarschijnlijk is dat een transcriptiefactor die de expressie van *ENOD40* reguleert een van de sleutelregulatoren van knolvorming is. Verschillende specifieke transcriptiefactoren zouden *ENOD40* expressie tijdens knolvorming en tijdens niet-vlinderbloemige plantontwikkeling kunnen reguleren. Deze specifieke regulatoren zouden tijdens de evolutie kunnen zijn gerekruteerd of aangepast om algemene processen, normaal betrokken bij plantontwikkeling, zo te sturen dat knolontwikkeling mogelijk werd.

Nawoord

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Curriculum vitae

Ingrid Jeannine Elisabeth Vleghels werd geboren op 27 september 1971 te Woerden. In 1989 haalde zij het VWO diploma aan het Veenlanden College te Mijdrecht. Haar studie HLO Biologie aan de Hogeschool van Amsterdam werd afgesloten met een stage Genetica (Dr. B. Overduin en Dr. J. Hille) aan de Vrije Universiteit te Amsterdam. Na afronding van haar HLO studie in 1993 begon zij aan een kopstudie Biologie aan de Wageningen Universiteit. In september 1996 behaalde zij het ingenieursdiploma met de afstudeervakken Plantenfysiologie aan de University of Guelph, Canada (Dr. Peter Toorop, Dr. Derek Bewley en Dr. Henk Hilhorst) en Moleculaire Biologie aan de Wageningen Universiteit (Dr. Henk Franssen en Prof. dr. Ton Bisseling). Vanaf september 1996 was zij als assistant in opleiding (AIO) verbonden aan de Wageningen Universiteit. Het in dit proefschrift beschreven onderzoek is uitgevoerd onder leiding van Dr. H. Franssen en Prof. dr. T. Bisseling bij de leerstoelgroep Moleculaire Biologie. Sinds augustus 2002 is zij werkzaam als beleidsmedewerker bij het gebied Aard- en Levenswetenschappen (ALW) van de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO) in Den Haag.