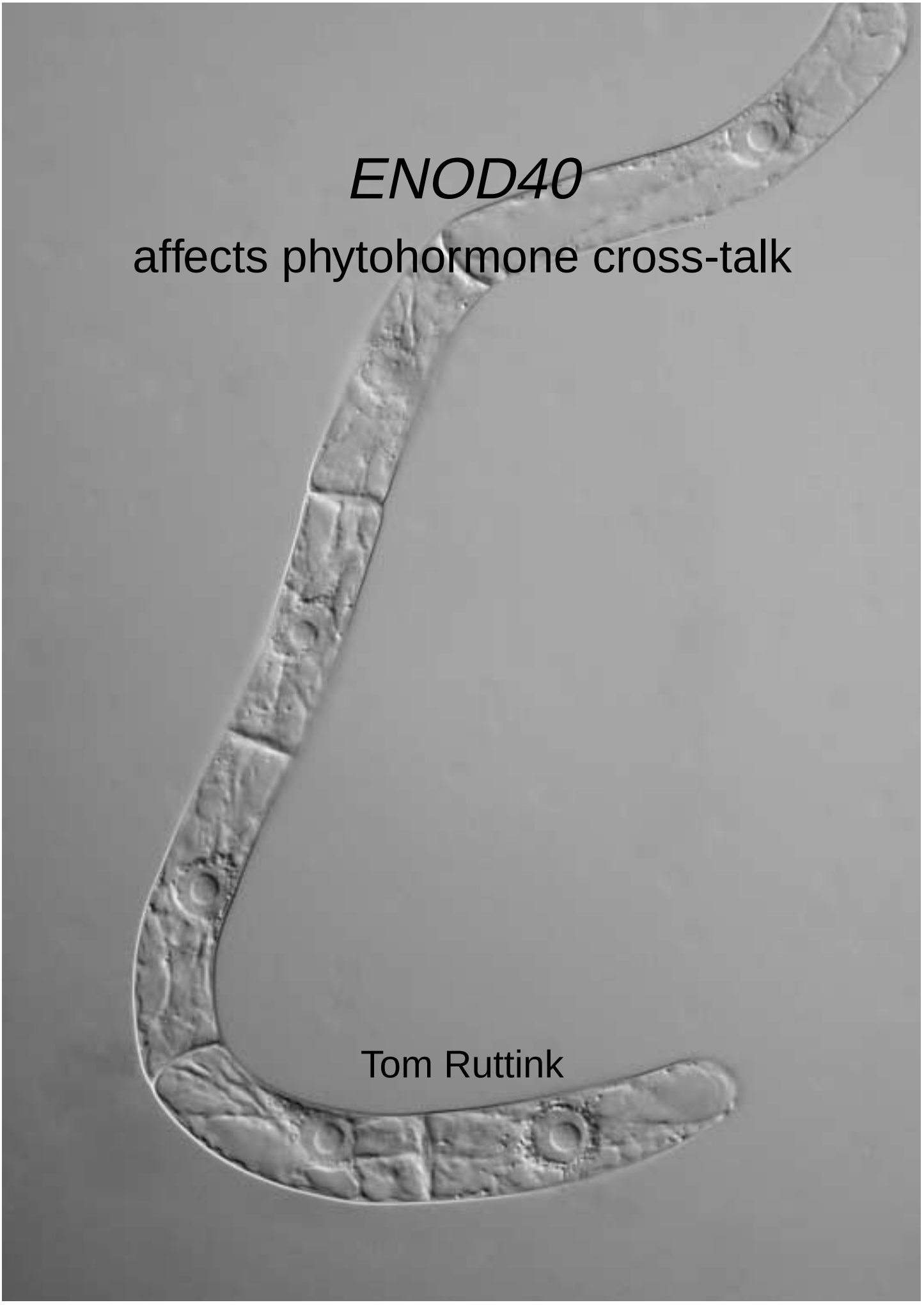

ENOD40

affects phytohormone cross-talk

Tom Ruttink

ENOD40 affects phytohormone cross-talk

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ENOD40 affects phytohormone cross-talk

Tom Ruttink

Proefschrift

Ter verkrijging van de graad van doctor

Op gezag van de rector magnificus

Van Wageningen Universiteit

Prof. dr. ir. L. Speelman

In het openbaar te verdedigen

Op vrijdag 5 december 2003

Des namiddags te vier uur in de Aula

ENOD40 affects phytohormone cross-talk

Ruttink, Tom

Thesis Wageningen University, The Netherlands
With references – with summary in Dutch

ISBN 90-5808-979-7

CONTENTS

Scope

SCOPE

During the interaction between legumes and rhizobia a completely new organ, the nodule, is formed on the roots of leguminous plants to host the bacteria. Upon infection with rhizobia, differentiated cortical cells are triggered to dedifferentiate, divide, and give rise to the nodule primordium. After the bacteria have reached and entered the primordium cells, the primordium develops into a nodule and the bacteria differentiate into their endosymbiotic form, the bacteroids. The developmental process is coordinated by an intricate network of signaling pathways. In addition to signaling molecules like Nod-factors released by the bacteria and plant factors like the phytohormones ethylene, auxin and cytokinin, a specific set of plant genes is induced during the early stages of nodulation (Mylona et al., 1995). These are known as the early nodulin (*ENOD*) genes, and some are thought to have a regulatory role during nodule organogenesis. One of the earliest activated *ENOD* genes is *ENOD40*. The spatio-temporal *ENOD40* expression pattern is closely associated with the nodule developmental program (Yang et al., 1993; Kouchi et al., 1993). Strikingly, *ENOD40* is also expressed in tissues not related to symbiosis indicating that the role of *ENOD40* is probably not restricted to nodulation (Crespi et al., 1994; Papadopoulou et al., 1996; Varkonyi Gasic and White, 2001). *ENOD40* genes are present in non-legumes, suggesting that *ENOD40* has a general role in plant development (van de Sande et al., 1996; Kouchi et al., 1999). However, the precise function of *ENOD40* genes is poorly understood. In this thesis, we explored new ways to elucidate the function of this gene.

In chapter 1, we describe what is known about *ENOD40* regulation, structure and function, based on *ENOD40* genes from plant species across the plant kingdom in search for common features of *ENOD40* action. By now, almost 40 different *ENOD40* homologs have been identified in species across the plant kingdom. Among these are monocots such as rice, sorghum, maize and ryegrass; but also dicots like pineapple, tomato, tobacco and citrus and several leguminous species, showing that *ENOD40* is a common gene. Mainly based on the *ENOD40* expression patterns several functions of *ENOD40* have been proposed. These, in combination with the phenotypes induced by

misexpression of *ENOD40*, have lent support to the hypothesis that *ENOD40* genes could be involved in regulation of plant growth and development. In addition, several studies have indicated that the function of *ENOD40* potentially overlaps with phytohormone action (Crespi et al., 1994; van de Sande et al., 1996; Charon et al., 1999). This led to our working hypothesis that *ENOD40* could act on the phytohormone status of a cell, thus participating in regulation of the (cellular) responses to phytohormones. Therefore, we decided to focus on the development of bioassays specifically designed to test the effect of *ENOD40* on responses to phytohormones. In this thesis we set up two new and complementary test systems in a dicot, but non-legume, plant background; a cellular system, using the tobacco BY-2 cell-suspension and a whole plant system, using *Arabidopsis*.

An important advantage of the BY-2 cell suspension is the observation that elongation growth and cell division frequency of cells are regulated by the balance between cytokinin and auxin in the culture medium (Hasezawa and Syono, 1983). In this thesis we have adapted their approach to turn the morphological response to phytohormones into a bioassay for the effect of *ENOD40*. In this bioassay, elongation growth rate and cell division frequency are morphological growth parameters that can be quantified in dose response curves for phytohormones, and it now forms the basis for an experimental system to test the effect of *ENOD40* on phytohormone signaling. In chapter 2, we describe the experiments that showed that *ENOD40* could indeed affect cellular responses to some phytohormones.

The comparison of *ENOD40* transcripts in chapter 1 revealed the presence of two short yet highly characteristic conserved regions that together make up only 10-20% of the length of transcripts. *ENOD40* transcripts are further characterized by the absence of a long open reading frame. Therefore, it is not obvious which *ENOD40* gene product has biological activity. A short ORF resides in one of the two conserved regions and the encoded oligopeptide may have biological activity. Strikingly, in only about 50% of *ENOD40* transcripts, an ORF is present in the second conserved nucleotide region and therefore this region may be active as RNA.

As we have developed a bioassay for *ENOD40* activity, it is possible to use this bioassay to determine which *ENOD40* gene products (oligopeptides encoded by the short ORF or RNA) have biological activity. Since we studied the effect of *ENOD40* by a reverse genetics approach, we used a set of constructs carrying mutations in the two conserved regions to test their biological activity in the bioassay. In chapter 3, we describe experiments that were performed to test whether translation of the small ORF located in the first conserved region occurs and is required for biological activity of *ENOD40*. In order to test conservation of gene function between plant species, we also tested biological activity of a distantly related *ENOD40* transcript in tobacco BY-2 cells.

To come to a detailed description of the molecular mechanism underlying the activity of *ENOD40*, it is required to identify the biologically active *ENOD40* gene product as well as direct interactors, but also factors acting further up- or down-stream in the pathway. As this is not possible in the cellular model system, we searched for effects of *ENOD40* in whole plants as this could raise the possibility to develop a suppressor screen for interactors of *ENOD40* in an un-biased genetic approach. We chose *Arabidopsis* as a genetic model system and generated stable transgenic lines overexpressing *NtENOD40*. Analysis of these lines is described in chapter 4.

In chapter 5 we present our concluding remarks in which we discuss the implications of the proposed interaction of *ENOD40* with phytohormone responses for the role of *ENOD40* on organ or plant level.

REFERENCES

Charon, C., Sousa, C., Crespi, M., and Kondorosi, A. (1999). Alteration of *ENOD40* expression modifies *Medicago truncatula* root nodule development induced by *Sinorhizobium meliloti*. Plant Cell **11,** 1953-1965.

Cohn, J., Day, R.B., and Stacey, G. (1998). Legume nodule organogenesis. Trends Plant Sci. **3,** 105-110.

Crespi, M.D., Jurkevitch, E., Poiret, M., d'Aubenton-Carafa, Y., Petrovics, G., Kondorosi, E., and 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.

Hasezawa, S., and Syono, K. (1983). Hormonal Control of Elongation of Tobacco Cells Derived from Protoplasts. Plant Cell. Physiol. **24,** 127-132.

Kouchi, H., and Hata, 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.

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

Mylona, P., Pawlowski, K., and Bisseling, T. (1995). Symbiotic Nitrogen Fixation. Plant Cell **7,** 869-885.

Papadopoulou, K., Roussis, A., and 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.

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

Varkonyi Gasic, E., and White, D.W.R. (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.

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

Chapter 1

Introduction

INTRODUCTION

The symbiotic interaction between legumes and rhizobia leads to the development of a new organ, the nodule, which is formed on the roots of leguminous plants to host the bacteria. A set of plant genes is induced during initial stages of nodulation. These genes are called the early nodulin (*ENOD*) genes. One of these *ENOD* genes is *ENOD40*, and its expression pattern is closely associated with the nodule developmental program (Yang et al., 1993; Kouchi et al., 1993). *ENOD40* is induced in pericycle cells within three hours after infection and is also expressed in the dividing cortical cells that give rise to the nodule primordium. In later stages of nodule development, *ENOD40* is expressed in vascular tissue and in the zone between the meristem and the cells of the central tissue of the mature nodule in which nitrogen fixation takes place (Compaan et al., 2001). Overexpression of *ENOD40* leads to acceleration of nodulation whereas silencing impairs nodule development and aberrant nodules are formed, indicating that *ENOD40* has a regulatory role in this process (Charon et al., 1999). The function of *ENOD40* is probably not restricted to nodule development because *ENOD40* expression is also found in tissues that are not related to symbiosis (Asad et al., 1994; Papadopoulou et al., 1996). *ENOD40* genes are also present in non-legumes and expression patterns are comparable between legume and non-legume plant species (Kouchi, Takane et al., 1999; Varkonyi Gasic and White, 2002). Moreover, overexpression affects non-legume plant development (van de Sande et al., 1996). Thus, it has been suggested that *ENOD40* may also have a regulatory role during different stages of plant development but its precise function is poorly understood.

For the vast majority of genes, the encoded polypeptide is the biologically active molecule. In this respect, *ENOD40* is unusual due to the lack of a long open reading frame. However, several short ORFs are present in *ENOD40* transcripts, so it is possible that oligopeptides are translated from *ENOD40* transcripts and that these are biologically active. Sequence comparison between *ENOD40* transcripts revealed two regions of high nucleotide sequence similarity named region I and region II. Some of the short ORFs reside in these regions and therefore are conserved among plant species. It

is likely that region I and II are important for the function of *ENOD40*, but it remains to be solved whether the transcripts are active as RNA or that a short conserved oligopeptide has biological activity. To better understand the molecular mechanism of *ENOD40* action and its role in plant development, two important questions remain to be solved and are the subject of this thesis; what is the function of *ENOD40* and which gene product has biological activity? In this introduction, we describe the current knowledge on *ENOD40* structure and function.

ENOD40 Homologs Are Present in Plant Species Across the Plant Kingdom

We start by comparing the sequence of *ENOD40* transcripts to study which nucleotide regions are likely to be important for biological activity. We wanted to include representative transcripts of different plant clades across the plant kingdom and therefore we searched for *ENOD40* genes in clades in which no *ENOD40* homologs have yet been described. All known *ENOD40* transcripts are relatively short, around 400-800 bases. Overall sequence homology is about 70% in closely related species but is down to at most 30% in distantly related plant species. The stretches of high sequence homology in region I and II span about 30-40 bp and 60-100 bp, respectively. The highest level of nucleotide sequence conservation is found in region II (Kouchi, Takane et al. 1999; Flemetakis, Kavroulakis et al. 2000; Compaan et al., 2001). Due to low homology between distantly related transcripts, it is difficult to identify novel members by cross-hybridization. Large-scale sequencing programs provide genomic and transcript (EST) sequence information from species across the plant kingdom (Figure 1.1). The short stretches of conserved nucleotide sequence in *ENOD40* transcripts are specific and long enough to be used for sequence homology searches. Therefore, nucleotide sequence databases are an easily accessible source of unidentified *ENOD40* homologs from distantly related plant species.

From 27 different plant species, in total 35 different genes homologous to *ENOD40* can be identified by means of standard BLAST searches in the Genbank sequence

database. Figure 1.1 shows the occurrence of *ENOD40* homologs in angiosperms. These *ENOD40* homologs were present in the Genbank sequence database, as of May 2003 and include *NtENOD40-2* and *LeENOD40*, which are not yet present in the sequence database, but have been cloned in our laboratory (Matvienko et al., 1996; Vleghels et al., 2003). In this way we identified novel *ENOD40* homologs from *Populus*, *Prunus*, *Solanum*, *Citrus*, and *Sorghum*. These sequences contain both conserved nucleotide regions. The *Antirrhinum majus*, *Lactuca sativa* and *Vigna radiata* transcripts only contain the region II sequence. Because these are shorter than all other *ENOD40* transcripts, these may be partial cDNA sequences. Most *ENOD40* sequences (including the novel sequences) that are available were identified as an entry in an EST database, showing that these sequences are transcribed. For all *ENOD40* genes for which this information is available, the cDNA sequence is identical to the corresponding genomic sequence, indicating that splicing does not occur.

The occurrence of *ENOD40* homologs in monocots and different clades within the core eudicots shows that *ENOD40* is an ancient gene that has been maintained in these plant clades during evolution. For most families for which a substantial number of sequences (>20.000) are available, *ENOD40* homologs can be identified. Because *ENOD40* is a commonly occurring gene, we anticipate that when similar numbers of sequences will become available for other families, many more *ENOD40* homologous sequences will appear. It must be noted though that for the families Caryophyllales, Malvales and Brassicales, 52.000, 60.000 and 1.124.000 sequences, respectively, are already available but none are homologous to *ENOD40*, even when *ENOD40* sequences from closely related plant families (e.g. Sapindales for the Malvales and Brassicales families) are used for the search. The reason for the absence of *ENOD40* homologs in these sets of sequences is as yet unclear. In most non-legume plant species *ENOD40* is not abundantly expressed, so it is possible that *ENOD40* is not represented in these data sets when sequences are mainly obtained from ESTs. The sequence database contains a complete genome (*Arabidopsis thaliana*) belonging to the Brassicales, suggesting that *ENOD40* is not present in this plant species. In Citrus species, which belong to the Sapindales family, *ENOD40* homologous sequences can

be found that contain both regions, indicating that at least the ancestor of the Brassicales family to which *Arabidopsis* belongs, still contained an *ENOD40* homologous sequence.

Several plant species contain multiple copies of *ENOD40* in their genomes (Figure 1.1). As this is found in most plant clades, monocots as well as dicots, this suggests that *ENOD40* belongs to a small gene family in most plant species. Thus, extensive and targeted searches based on the highly characteristic conserved regions are expected to reveal additional copies in plant species for which single *ENOD40* genes are already known. Some sequence divergence can be found between *ENOD40* transcripts within a plant species. Strikingly, in a number of cases, *ENOD40* transcripts in closely related species contain a higher percentage sequence similarity between plant species, than within plant species (Compaan et al., 2003; Varkonyi-Gasic and White, 2002), suggesting that *ENOD40* genes have been duplicated and diverged in an ancestor of these plant species.

➢ **Figure 1.1**

The numbers of nucleotide sequence entries were obtained from www.ncbi.nlm.gov/taxonomy/browser/wwwtax.cgi in May 2003. The angiosperm phylogenetic tree is redrawn after "an update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG II, Botanical journal of the Linnean Society, 2003, 141, 399-436". Plant species abbreviations and Genbank Accession Numbers: Am, *Antirrhinum majus*, snapdragon (AJ559999); Cu, *Citrus unshiu* (C95533); Cg, *Casuarina glauca* (AJ487686); Cs, *Citrus sinensis*, pineapple (BQ624698); Gm, *Glycine max* (1, X69154; 2, D13503; 3, AI431225); Hv, *Hordeum vulgare* (BQ765935); Ls, *Lactuca sativa*, lettuce (BQ854021); Le, *Lycopersicon esculentum*; Lj, *Lotus japonicus* (1, AF013594; 2, AJ271788); Lp, *Lolium perenne*, ryegrass (1, AF538350; 2, AF538351); Ms, *Medicago sativa*, alfalfa (L32806); *Medicago truncatula* (1, X80264; 2, X80262); Nt, *Nicotiana tabacum* (X98716); Ob, *Oryza brachyantha* (AB024055); Os, *Oryza sativa* (1, AB024054, (CA755970); 2, AU101849); Pa, *Prunus armeniaca* (CB822805); Ps, *Pisum sativum* (X81064); Pt, *Populus tremula* x *Populus tremaloides* (BU883953); Pv, *Phaseolus vulgaris* (X86441); Sb, *Sorghum bicolor* (BE362667); Sr, *Sesbania rostrata* (Y12714); St, *Solanum tuberosum* (AJ276864); Ta, *Triticum aestivum* (BJ278615); Tr, *Trifolium repens* (1, AF426838; 2, AF426839; 3, AF426840); Vr, *Vigna radiata* (AF061818); Vs, *Vicia sativa* (X83683); Zm, *Zea mays* (1, AI001271+W21740; 2, AI491369).

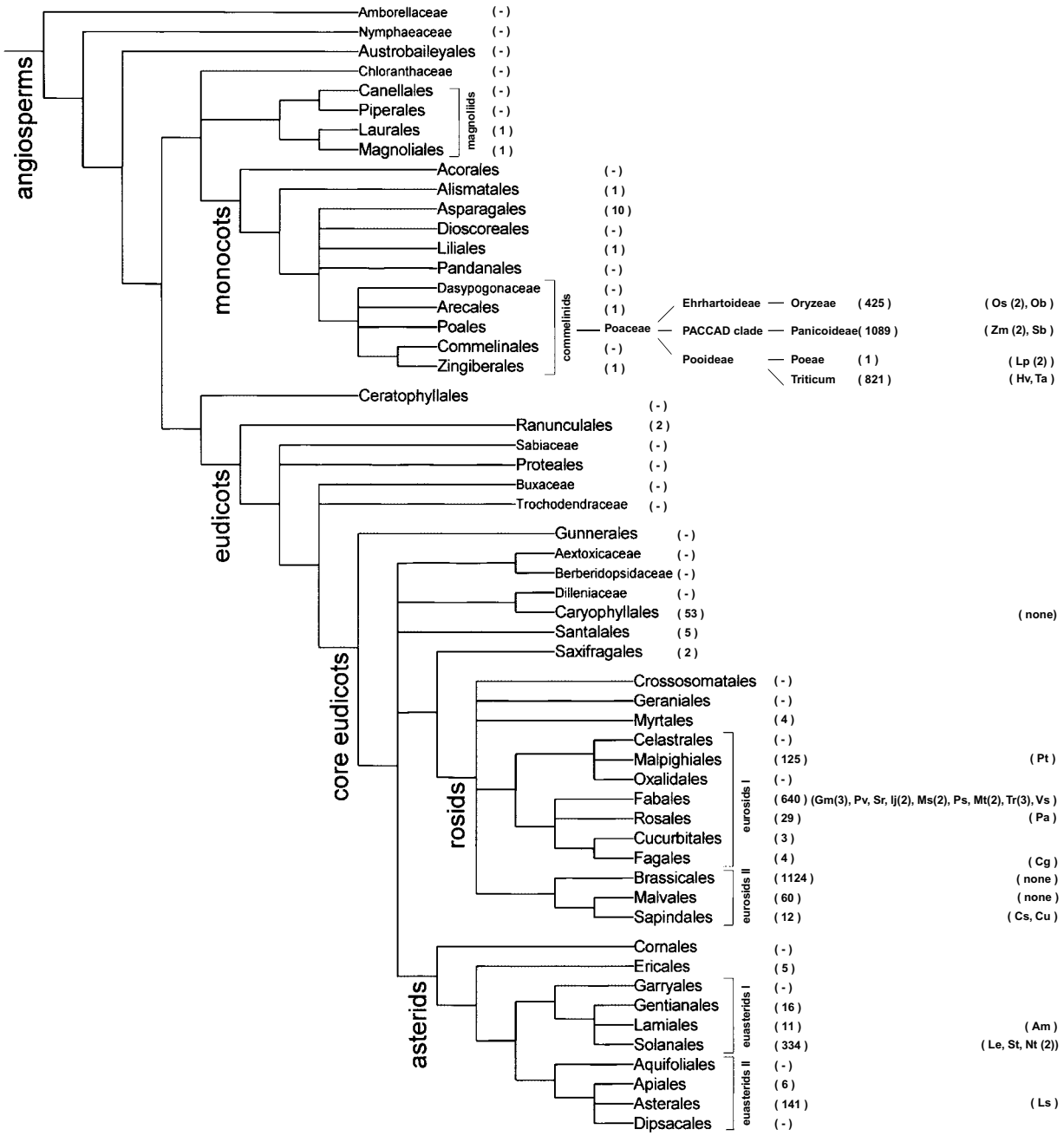


Figure 1.1. Occurrence of *ENOD40* Homologs in Angiosperms.

Phylogenetic tree with the total number of nucleotide sequence entries per plant family indicated between brackets (*10^3). (-) Indicates less than 500 nucleotide sequence entries. *ENOD40* homologs were obtained from the Genbank database by BLAST homology searches. Plant species containing *ENOD40* homologs are listed and the number of copies per plant species is indicated between brackets. This reveals that in most plant families for which a substantial number of sequences are available, *ENOD40* homologs can be identified. Striking exceptions are the Malvales and the Brassicales family. Two different Citrus species (Cs, Cu) belonging to the closely related Sapindales family do contain *ENOD40* homologs indicating that their common ancestor still contained an *ENOD40* homolog.

Common Features of *ENOD40* Transcripts

To understand the molecular mechanism of *ENOD40* action, it is required to identify the biologically active gene product. For most genes this is the encoded protein but for some genes it has been shown that they are biologically active at the RNA level. It has been suggested that the lack of a long conserved open reading frame (ORF) indicates that *ENOD40* RNA is the active product (Crespi et al., 1994). As *ENOD40* transcripts do contain many short ORFs (Figure 1.2) encoding putative oligopeptides, these might also have biological activity. The *ENOD40* sequences have been identified by the occurrence of the conserved regions I and II. In case the peptides are the biologically active gene products it seems probable that ORFs encoding these peptides are conserved. The only ORFs that are conserved must be located within region I or region II and these ORFs were analyzed in further detail.

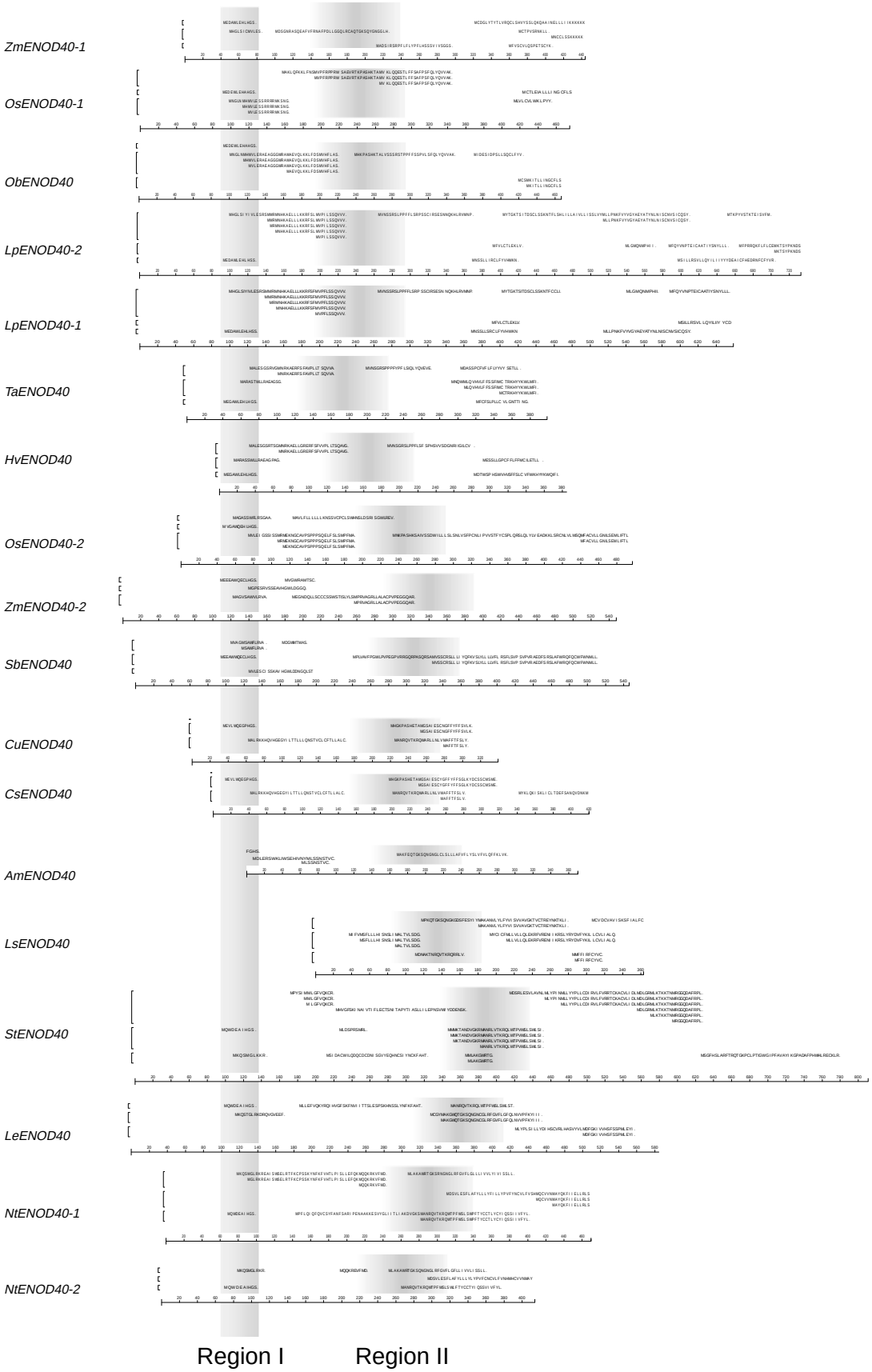
Region I

Nucleotide sequence alignment of region I and amino-acid alignment of ORF I are presented in Figure 1.4A. The length of ORF I varies slightly in different plant species, but is usually identical in closely related plant species. In species in which multiple *ENOD40* genes have been identified, the length and sequence of ORF I in different transcripts is not always identical (Figure 1.4A, Zm, Mt and Tr). Taken together, all requirements for a functional ORF are present in region I, even in distantly related species. These include: in all *ENOD40* genes (except *LjENOD40*-2) it contains the first start codon of the transcript, the ORF encodes a conserved peptide, conservation on amino-acid level but not necessarily nucleotide level between closely related species, comparable length of the ORF indicating a conserved stop. Because all requirements for a functional ORF are met within region I, it is highly likely that this region is translated and that the ORF I encoded peptide is biologically active.

We identified the [M-X_{1-4}-W-X_4-HGS*] sequence as the characteristic peptide motive encoded by region I. There are six exceptions with regard to the amino-acid sequence of

ORF I: *Lolium perenne*-1,2 [M-X_{1-4}-W-X_4-HSS*] ; *Prunus armeniaca* [M-X_5-W-X_5-HGT*] ; *Populus tremula x tremaloidus*, [M-X_{10}-QGP*] ; *Medicago truncatula*-2 [M-X_3-W-X_3-IYD*], whereas *Casuarina glauca ENOD40* does not encode the conserved peptide due to a frame shift and the lack of the conserved stop codon in region I (Santi et al., 2003). However, all these transcripts have significant homology to *ENOD40* as they contain a perfect region II motive.

Several approaches have been undertaken to study translation of ORF I. The *GmENOD40* ORF I is translated in an *in vitro* assay (Rohrig et al., 2002) and, translationally fused to GFP, is translated in tobacco protoplasts (van de Sande et al., 1996). The *NtENOD40* ORF I is translated in cowpea protoplasts (Compaan et al., 2001). Translation of *MtENOD40* ORF I was demonstrated in *in vitro* assays and in root epidermal and cortical cells of *Medicago truncatula* using GUS translational reporter fusions (Sousa et al., 2001). Van de Sande (1996) detected an antigenic determinant in extracts of soybean nodules with an antibody raised against a synthetic soybean ORF I peptide. Together, these data support that ORF I is translated *in vivo* but whether the peptide encoded by ORF I has biological activity remains to be elucidated.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ObENOD40
LpENOD40-2
LpENOD40-1
TaENOD40
HvENOD40
OsENOD40-2
ZmENOD40-2
SbENOD40
CuENOD40
CsENOD40
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LeENOD40
NtENOD40-1
NtENOD40-2
Region I
Region II

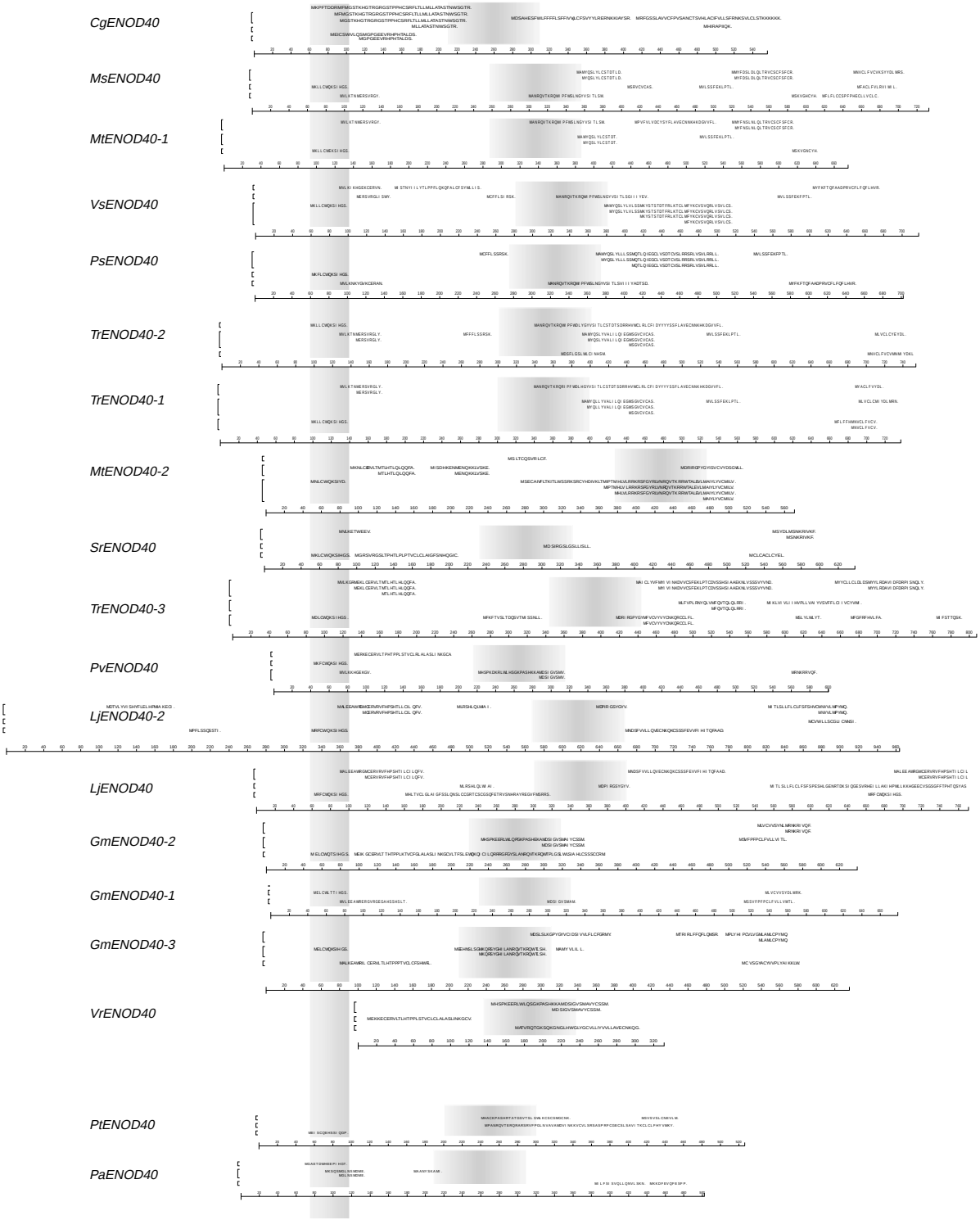
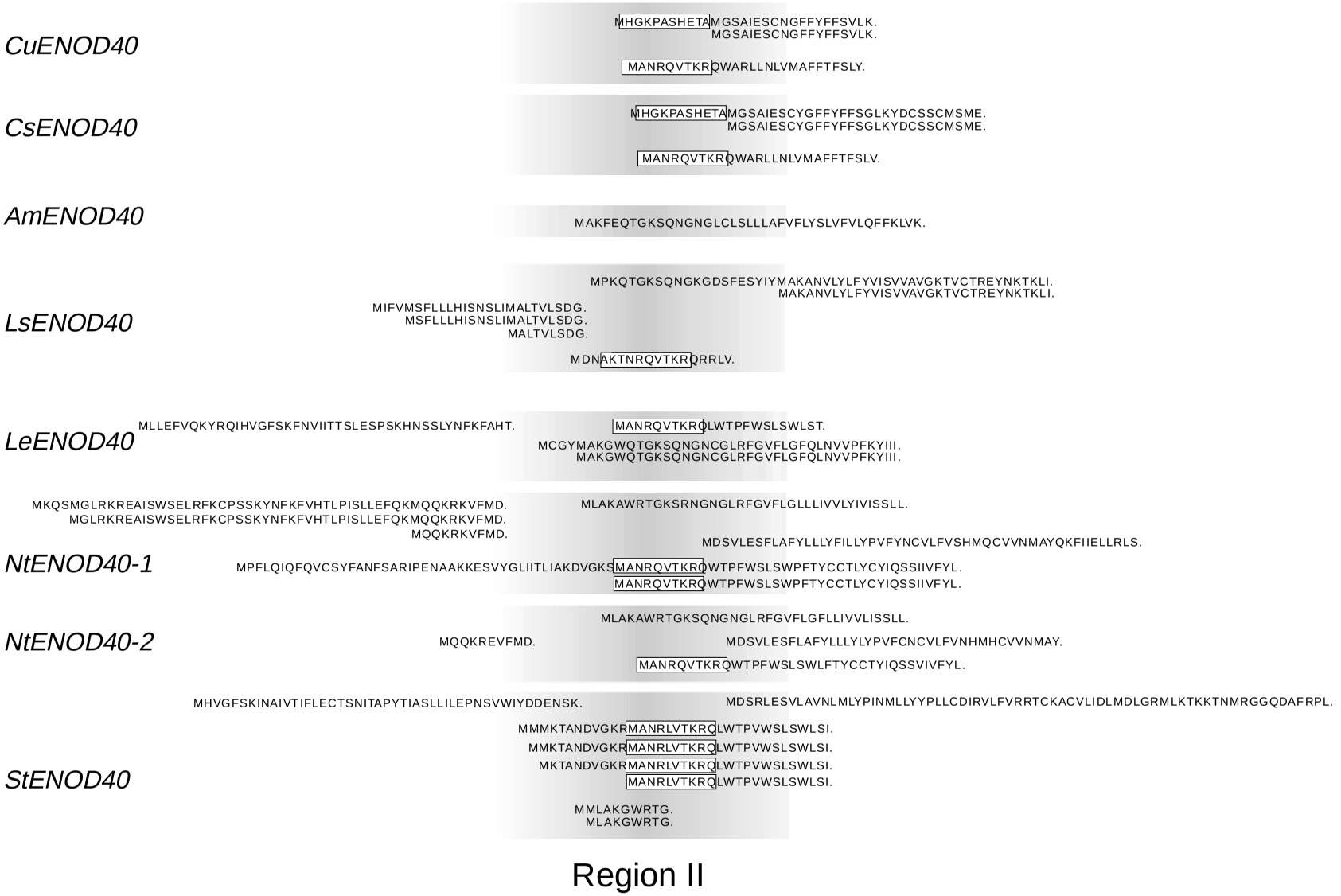


Figure 1.2.
Schematic overview of *ENOD40* transcripts across the plant kingdom reveals the absence of long ORFs. Conserved nucleotide sequences are indicated as shaded boxes. *ENOD40* transcripts are aligned at region I and transcript lengths are on scale. All ORFs with a length of 9 amino acids or longer are indicated.


CuENOD40
MHGKPASHETAMGSAIESCNGFFYFFSVLK.
MGSAIESCNGFFYFFSVLK.
MANRQVTKRQWARLLNLVMAFFTFSLY.
CsENOD40
MHGKPASHETAMGSAIESCYGFFYFFSGLKYDCSSCMSME.
MGSAIESCYGFFYFFSGLKYDCSSCMSME.
MANRQVTKRQWARLLNLVMAFFTFSLV.
AmENOD40
MAKFEQTGKSQNGNGLCLSLLLAFVFLYSLVFVLQFFKLVK.
LsENOD40
MPKQTGKSQNGKGDSFESYIYMAKANVLYLFYVISVVAVGKTVCTREYNKTKLI.
MAKANVLYLFYVISVVAVGKTVCTREYNKTKLI.
MIFVMSFLLLHISNSLIMALTVLSDG.
MSFLLLHISNSLIMALTVLSDG.
MALTVLSDG.
MDNAKTNRQVTKRQRRLV.
LeENOD40
MLLEFVQKYRQIHVGFSKFNVIITTSLESPSKHNSSLYNFKFAHT.
MANRQVTKRQLWTPFWSLSWLST.
MCGYMAKGWQTGKSQNGNCGLRFGVFLGFQLNVVPFKYIII.
MAKGWQTGKSQNGNCGLRFGVFLGFQLNVVPFKYIII.
NtENOD40-1
MKQSMGLRKREAISWSELRFKCPSSKYNFKFVHTLPISLLEFQKMQQKRKVFMD.
MGLRKREAISWSELRFKCPSSKYNFKFVHTLPISLLEFQKMQQKRKVFMD.
MQQKRKVFMD.
MLAKAWRTGKSRNGNGLRFGVFLGLLLIVVLYIVISSLL.
MDSVLESFLAFYLLLYFILLYPVFYNCVLFVSHMQCVVNMAYQKFIIELLRLS.
MPFLQIQFQVCSYFANFSARIPENAAKKESVYGLIITLIAKDVGKSMANRQVTKRQWTPFWSLSWPFTYCCTLYCYIQSSIIVFYL.
MANRQVTKRQWTPFWSLSWPFTYCCTLYCYIQSSIIVFYL.
NtENOD40-2
MLAKAWRTGKSQNGNGLRFGVFLGFLLIVVLISSLL.
MQQKREVFMD.
MDSVLESFLAFYLLLYLYPVFCNCVLFVNHMHCVVNMAY.
MANRQVTKRQWTPFWSLSWLFTYCCTYIQSSVIVFYL.
StENOD40
MHVGFSKINAIVTIFLECTSNITAPYTIASLLILEPNSVWIYDDENSK.
MDSRLESVLAVNLMLYPINMLLYYPLLCDIRVLFVRRTCKACVLIDLMDLGRMLKTKKTNMRGGQDAFRPL.
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MMKTANDVGKRMANRLVTKRQLWTPVWSLSWLSI.
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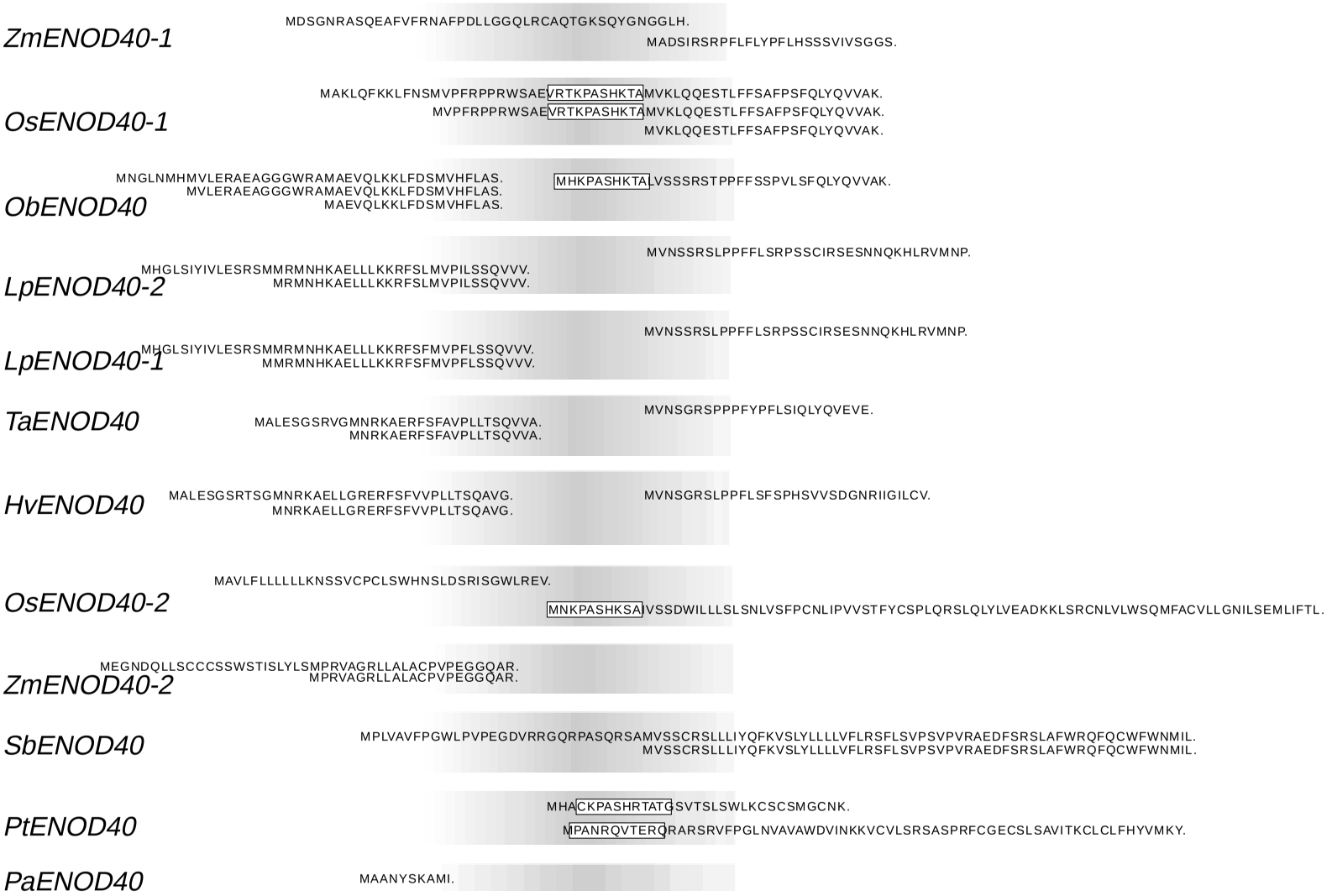
MMLAKGWRTG.
MLAKGWRTG.

Region II


ZmENOD40-1
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MADSIRSRPFLFLYPFLHSSSVIVSGGS.
OsENOD40-1
MAKLQFKKLFNSMVPFRPPRWSAEVRTKPASHKTAMVKLQQESTLFFSAFPSFQLYQVVAK.
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MVKLQQESTLFFSAFPSFQLYQVVAK.
ObENOD40
MNGLNMHMVLERAEAGGGWRAMAEVQLKKLFDSMVHFLAS.
MVLERAEAGGGWRAMAEVQLKKLFDSMVHFLAS.
MAEVQLKKLFDSMVHFLAS.
MHKPASHKTALVSSSRSTPPFFSSPVLSFQLYQVVAK.
LpENOD40-2
MVNSSRSLPPFFLSRPSSCIRSESNNQKHLRVMNP.
MHGLSIYIVLESRSMMRMNHKAELLLKKRFSLMVPILSSQVVV.
MRMNHKAELLLKKRFSLMVPILSSQVVV.
LpENOD40-1
MVNSSRSLPPFFLSRPSSCIRSESNNQKHLRVMNP.
MHGLSIYIVLESRSMMRMNHKAELLLKKRFSFMVPFLSSQVVV.
MMRMNHKAELLLKKRFSFMVPFLSSQVVV.
TaENOD40
MVNSGRSPPPFYPFLSIQLYQVEVE.
MALESGSRVGMNRKAERFSFAVPLLTSQVVA.
MNRKAERFSFAVPLLTSQVVA.
HvENOD40
MALESGSRTSGMNRKAELLGRERFSFVVPLLTSQAVG.
MNRKAELLGRERFSFVVPLLTSQAVG.
MVNSGRSLPPFLSFSPHSVVSDGNRIIGILCV.
OsENOD40-2
MAVLFLLLLLLKNSSVCPCLSWHNSLDSRISGWLREV.
MNKPASHKSAIVSSDWILLLSLSNLVSFPCNLIPVVSTFYCSPLQRSLQLYLVEADKKLSRCNLVLWSQMFACVLLGNILSEMLIFTL.
ZmENOD40-2
MEGNDQLLSCCCSSWSTISLYLSMPRVAGRLLALACPVPEGGQAR.
MPRVAGRLLALACPVPEGGQAR.
SbENOD40
MPLVAVFPGWLPVPEGDVRRGQRPASQRSAMVSSCRSLLLIYQFKVSLYLLLLVFLRSFLSVPSVPVRAEDFSRSLAFWRQFQCWFWNMIL.
MVSSCRSLLLIYQFKVSLYLLLLVFLRSFLSVPSVPVRAEDFSRSLAFWRQFQCWFWNMIL.
PtENOD40
MHACKPASHRTATGSVTSLSWLKCSCSMGCNK.
MPANRQVTERQRARSRVFPGLNVAVAWDVINKKVCVLSRSASPRFCGECSLSAVITKCLCLFHYVMKY.
PaENOD40
MAANYSKAMI.

Region II

Figure 1.3.
Schematic overview of open reading frames residing in region II of *ENOD40* genes across the plant kingdom. Conserved amino-acid motives are boxed.

A

```
ZmENOD40-1              ATGGAAGATGCA---TGGCTTGAGCATCTGCATGGTTCTTGA    MEDA-WLEHLHGS.
OsENOD40-1              ATGGAAGATGAA---TGGCTTGAACATGCACATGGTTCTTGA    MEDE-WLEHAHGS.
ObENOD40                ATGGAAGATGAA---TGGCTTGAACATGCACATGGTTCTTGA    MEDE-WLEHAHGS.
LpENOD40-2              ATGGAAGATGCA---TGGCTTGAGCATCTACATAGTTCTTGA    MEDA-WLEHLHSS.
LpENOD40-1              ATGGAAGATGCA---TGGCTTGAGCATCTACATAGTTCTTGA    MEDA-WLEHLHSS.
TaENOD40                ATGGAAGGCGCA---TGGCTCGAGCATCTACATGGCTCTTGA    MEGA-WLEHLHGS.
HvENOD40                ATGGAAGGCGCA---TGGCTCGAGCATCTTCATGGCTCTTGA    MEGA-WLEHLHGS.
OsENOD40-2              ATGGTGGGTGCA---TGGCAGGAGCATCTTCATGGTTCTTGA    MVGA-WQEHLHGS.
ZmENOD40-2              ATGGAGGAGGAAGCATGGCAGGAGTGTCTGCATGGGTCCTGA    MEEEAWQECLHGS.
SbENOD40                ATGGAGGAAGCATGGTGGCAGGAATGTCTGCATGGTTCTTGA    MEEAWWQECLHGS.

CuENOD40                ATGGAAGTGCTT---TGGCAAGAAGGCCCTCATGGCTCTTAG    MEVL-WQEGPHGS.
CsENOD40                ATGGAAGTGCTT---TGGCAAGAAGGCCCTCATGGCTCTTAG    MEVL-WQEGPHGS.

LeENOD40                ATG---------CAGTGGGATGAAGCAATCCACGGGTCTTAG    MQ---WDEAIHGS.
NtENOD40-1              ATG---------CAGTGGGATGAAGCAATCCATGGGTCTTAG    MQ---WDEAIHGS.
NtENOD40-2              ATG---------CAGTGGGATGAAGCAATCCATGGGTCTTAG    MQ---WDEAIHGS.
StENOD40                ATG---------CAGTGGGATGAAGCAATCCATGGGTCTTAA    MQ---WDEAIHGS.
AmENOD40                                    TTCCATGGATCTTGA                FHGS.

CgENOD40                ATGAAGCCTTTTACTGATGATAGAATGTT-CATGGGTTCTAC    MKPFTDDRMFMGS~
MsENOD40                ATGAAGCTTCTTTGTTGGCAAAAATCAATCCATGGTTCTTAA    MKLLCWQKSIHGS.
MtENOD40-1              ATGAAGCTTCTTTGTTGGGAAAAATCAATCCATGGTTCTTAA    MKLLCWEKSIHGS.
VsENOD40                ATGAAGCTTCTTTGTTGGCAAAAATCAATCCATGGTTCTTAA    MKLLCWQKSIHGS.
PsENOD40                ATGAAGTTTCTTTGTTGGCAAAAATCAATCCATGGTTCTTAA    MKFLCWQKSIHGS.
TrENOD40-2              ATGAAGCTTCTTTGTTGGCAAAAATCAATTCATGGTTCTTAA    MKLLCWQKSIHGS.
TrENOD40-1              ATGAAGCTTCTTTGTTGGCAAAAATCAATTCATGGTTCTTAA    MKLLCWEKSIHGS.
MtENOD40-2              ATGAAT---CTTTGTTGGCAAAAATCTATTTATGAT---TAA    MNL-CWQKSIYD-.
SrENOD40                ATGAAG---CTCTGTTGGCAAAAATCCATCCATGGTTCTTAA    MKL-CWQKSIHGS.
TrENOD40-3              ATGGAC---CTTTGTTGGCAAAAATCAATTCATGGTTCTTAA    MDL-CWQKSIHGS.
PvENOD40                ATGAAG---TTTTGTTGGCAAGCATCCATCCATGGTTCTTAA    MKF-CWQASIHGS.
LjENOD40-2              ATGAGA---TTTTGTTGGCAAAAATCCATCCATGGCTCTTGA    MRF-CWQKSIHGS.
LjENOD40-1              ATGAGA---TTTTGTTGGCAAAAATCCATCCATGGCTCTTGA    MRF-CWQKSIHGS.
GmENOD40-2              ATGGAG---CTTTGTTGGCAAACATCCATCCATGGTTCTTGA    MEL-CWQTSIHGS.
GmENOD40-1              ATGGAG---CTTTGTTGGCTCACAACCATCCATGGTTCTTGA    MEL-CWLTTIHGS.
GmENOD40-3              ATGGAG---CTTTGTTGGCAAAAATCCATCCATGGCTCTTAA    MEL-CWQKSIHGS.

                                                                      M-X1-4-W-X4-HGS.

PtENOD40          ATGGAAATCTCTTGTCAAGAACAC---TCATCCATCCAAGGGCCCTGA    MEISCQEH-SSIQGP.
PaENOD40          ATGGGTGCGGAAACAGATTGGCATGAAGAGCCAATCCATGGGACTTAA    MGAETDWHEEPIHGT.
```

Figure 1.4.
(A) Nucleotide sequence alignment of region I and amino-acid sequence alignment of ORF I of *ENOD40* genes shows that this region encodes a conserved peptide.

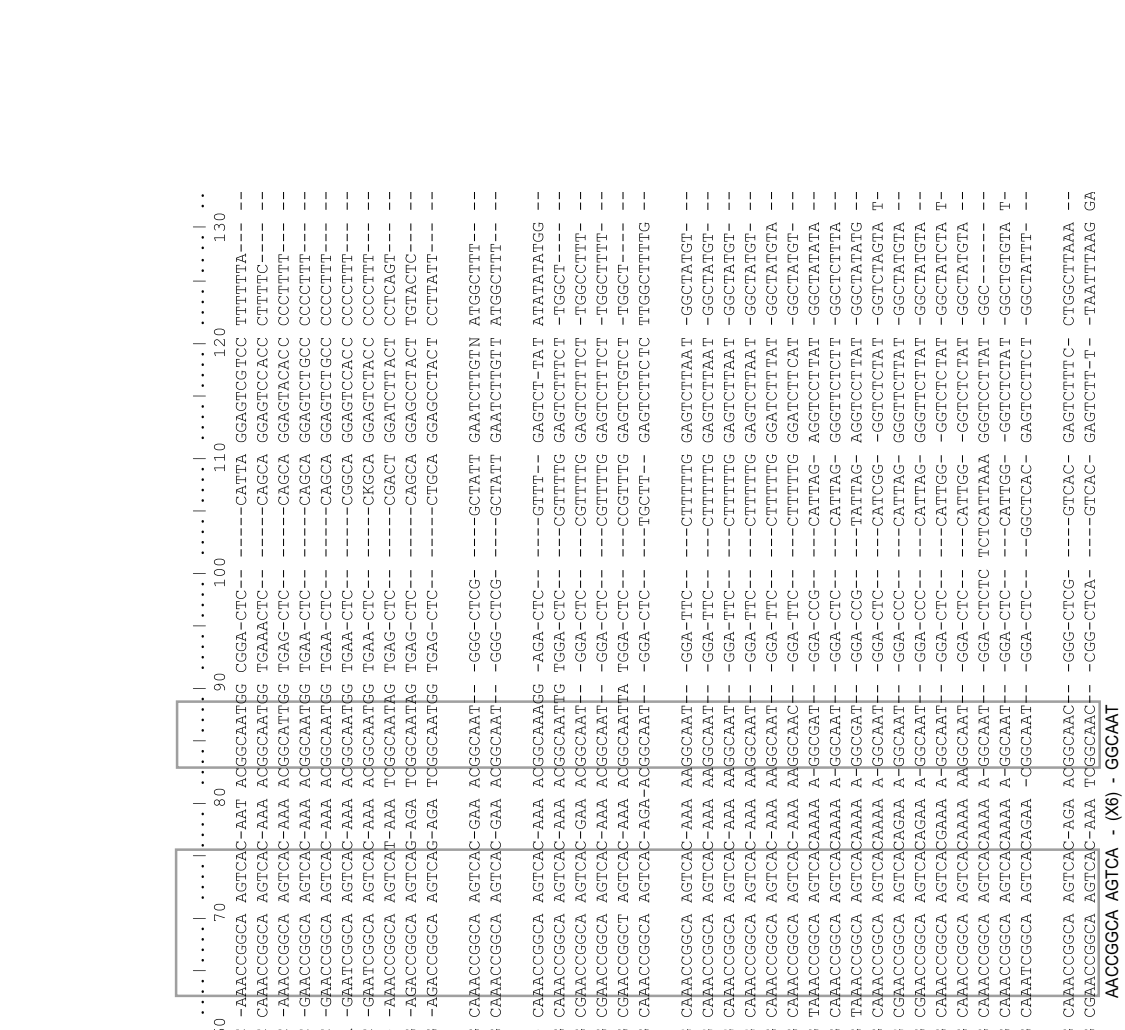


Figure 1.4.
(B) Nucleotide sequence alignment of region II of *ENOD40* genes.

Region II

The length of region II is not strictly defined as sequence similarity gradually decreases in domains more remote from the conserved core. We have used 50 bp up- and 30 bp down-stream of the most conserved core (25 bp) of region II for these comparisons (Figure 1.3, 1.4B). The AACCGGCAAGTCA-(X_6)-GGCAAT nucleotide motive represents the highest level of sequence conservation in region II. In region II no ORFs are found that are absolutely conserved (Figure 1.3). In about 50% of the *ENOD40* transcripts, an ORF starts in the middle of region II encoding the MANRQVTKRQ (or similar) peptide motive. In some plant species containing multiple *ENOD40* genes, an ORF encoding this peptide is not present in all transcripts (Tr, Gm, Lj). This ORF is not present in any of the monocot transcripts. In some *ENOD40* transcripts (25%), a start codon is located in a different reading frame, leading to an other peptide motive: M- - KPASHET (Figure 1.3, e.g. Ob, Pv, Os-2). Four transcripts contain both these ORFs. In about 33% of the *ENOD40* transcripts, there is no ORF present which spans the highly conserved center of region II (Figure 1.3, e.g. Sr, Lj, Lp, Ta, Gm-1). Finally, the nucleotide sequence alignment of region II presented in Figure 1.4B, shows that frame-shift inducing single as well as multiple nucleotide gaps are present which would disrupt a conserved amino-acid sequence within the ORF. The translation of ORF II peptides has been tested using reporter fusions. The *NtENOD40* ORF II is only efficiently translated in the absence of the preceding ORF I in cowpea protoplasts (Compaan et al., 2001). Likewise, translation of *MtENOD40* ORF II was demonstrated in *in vitro* assays (Sousa et al., 2001) and in root epidermal and cortical cells of *Medicago truncatula* using GUS translational reporter fusions but translation efficiency depends on the presence (and translational activity) of the preceding ORF I. If a peptide from ORF II is translated that would have biological activity, about 50% of the *ENOD40* transcripts encodes a similar region II peptide. Since these peptides are not absolutely conserved, but the nucleotide sequence is strongly conserved, this suggests that region II is active at the RNA level.

	Symbiotic tissues				Non-symbiotic tissues																	
	Nodule				Root			Vegetative tissues							Reproductive tissues					Inducers		
Plant species	Root pericycle facing nodule primordium	Dividing cortical cells/ nodule primordium	Nodule vascular tissue pericycle	Nodule central tissue	Root	Early stages of adventitious nodal roots	lateral root	Stem	Stem + root procambial cells	Meristem lateral shoots	Young leaf / stipule primordia	Mature leaf	Hypocotyl	Cotyledon	Flower	Fruit / green seed pods	Germinated seeds	Pollen	Embryonic tissues ovules and embryo	Cytokinine (-N)	NOD factor	Author (proposed function)
Glycine max (determinate) *Pisum sativum* (indeterminate)	++[1c] +[1c]	+[1c] +[1c]	++[1c] ***[I] ++[1c]	++[1c] *[I]	-[1a,b,c]		-[1c]	+[1c] phloem		-[1c]												Yang, 1993, ¥ (Transport)
Glycine max (determinate)	+[1c]	+[1a,b,c]	++[1c]	+[1c]	-[1a], +[1b]			+[1a,b,c] §	+[1c] stem			-[1a]										Kouchi, 1993 (morphogenesis / function of nodule vascular system, transport photosynthate)
Medicago sativa (indeterminate)	+[1c]	+[1c]	+[1c]	+[1c]	+[1a], -[1c]		+[1c] ‡	+[1a,c]	+[1c]	+[1c] §§	+[1c]	-[1a]			+[1a], -[1c]					+	+	Asad, 1994 ,¥ (meristematic tissues, mitosis or protein synthesis)
Pisum sativum (indeterminate)	+[1c]	+[1c]	++[1a,c]	++[1c] **[I]	+[1a]			+[1a]							+[1a]							Matvienko, 1994
Medicago sativa (indeterminate)		+[1c]	+[1a,b,c] ***[III]	++[1c]	-[1a], +[1b]			-[1a], +[1b]				-[1a]			-[1a], -[1b]						+	Crespi, 1994 †,¥ (function in differentiation process in relation to hormonal status of tissue)
pGm-2 in *Vicia sativa* (indeterminate)	+[2]		+[2] ***[II]	-[2] *[I]																		Roussis, 1995
Vicia sativa (indeterminate)	+[1c]	+[1c]																			+	Vijn, 1995[a] ,¥
Vicia sativa (indeterminate)	+[1c]	+[1c]	++[1c]	++[1c] **[II]																		Vijn, 1995[b]
Phaseolus vulgaris (determinate)	++[1c]	+[1c]	++[1c] ***[III]	+[1c] *[I]	+[1a]	+[1c] ‡‡	-[1c]	+[1a]					+[1a]									Papadopoulou, 1996 (hormonal status of plant differentiation process of vascular tissue, also in non-symbiotic organogenic processes)

Glycine max (determinate)	+[1c]	+[1c]	+[1c]		+[1b]		-[1c]														+[1c]	Minami, 1996, ¥
Medicago sativa (indeterminate)					+[1c]															+[1a]		v. Rhijn, 1997, ¥¥
Medicago sativa pMs-1 symbiotic	±[2]	+[2] not in cortex	+[2]	+[2] **III	-[2]		+[2] ‡												-[2] æ	+[2] ß	+[2] ß	Fang, 1998, ¥¥¥ (Induction of E40 (by Ck or NF) could serve as amplification mechanism, triggering localised hormone imbalance, leading to ccd).
pMs-2 Non- and symbiotic	+[2]	+[2]	++[2]	++[2] **III	+[2]		+[2] ‡‡‡[I]		+[2]		+[2] §§§I								-[2] æ	+[2] ß	+[2] ß	
Trifolium repens																				+		Mathesius, 1998
Sesbania rostrata		+[1c]	+[1c] ***IV	+[1c] *II		+[1c] ‡‡‡[I]	+[1c]		+[1c]		+[1c]											Corich, 1998 (establishment of zones and patterns of cells with different susceptibility to prevailing hormone concentrations,leading to different outcomes)
pGm-2 in *A. thaliana*					+[2]		+[2] ‡‡				+[2]		-[2]		+[2]						-[2]	Mirabella, 1999
Oryza sativa					-[1a]			+[1a,b] §§§§I	+[1b]			-[1a]										Kouchi, 1999 (function in differentiation of vascular bundles)
pOs in *G. max* (determinate)	+[2]		+[2]																			
L. japonicus 1/2			++[1c]		+[1b]			+[1b]			±[1b]		+[1b]	+[1b]	+[1b]	+[1b]			+[1c]		+[1b]	Flemetakis, 2000 (organogenesis)
Trifolium repens Tr-1/2			++[1c,2]	++[1c,2] **IV	+[1a,2]	+[1c] ‡‡‡[II]	++[1c]	+[1c,2] §§§II	+[1c,2] §§§§II			-[1a]			+[1c,2] ¶							Varkonyi-Gasic, 2002 (role at sites of intensive lateral transport of solutes)
Trifolium repens Tr-3			±[1c,2]	±[1c,2]	+[1a,2]	+[1c] ‡‡‡[II]	+[1c]	+[1c,2] §§§II	+[1c,2] §§§§II			-[1a]			+[1c,2] ¶							
pTr-1/2 in *N.Tabaccum*								+[2] §§§III	+[2] §§§§III													
pTr-3 in *N.Tabaccum*								+[2] §§§III														
Lycopersicon esculentum					+[1b,2]		+[2] ‡‡‡[II]	+[1b,2]			+[2]	+[1b]	+[1b,2]	+[1b,2]	+[1b,2] ¶¶	+[1b]	+[2]	+[2]		-[1b,2]		Vleghels, 2003 (counter-acting effects of ethylene)
Gm in *A. thal.*																				-[2]		
Le in *M. trunc.*			+[2]	+[2]																		

1. *ENOD40* RNA expression; 1a, RNA gel blot; 1b, RT-PCR; 1c, in situ hybridization.
2. *ENOD40* promoter driven GUS expression.
++ high levels of expression, + detectable levels of expression, - undetectable levels of expression.

Nodules:
*^{I} boundary layer and uninfected cells of central tissue, no expression in infected cells in determinate nodules.
*^{II} not in meristematic cells, in later stages: residual meristematic cells beginning to differentiate, invasion zone, uninfected cells of fixation zone (in stem-borne nodules).
**^{I} in infected cells of pre-fixation zone (in indeterminate nodules, these are differentiating tissues). In fixation zone low level of expression.
**^{II} in infected cells of pre-fixation zone. In fixation zone only in uninfected cells.
**^{III} localised in nodule meristem, including pre-fixation zone, and cells on the periphery of the central region.
**^{IV} in meristem, infection zone and vascular bundles.
***^{I} *ENOD40* expression in nodule vascular bundle when procambial cells differentiate into vascular tissue.
***^{II} in young not fully developed nodules: exclusively in root pericycle where provascular strands of the nodule will be initiated.
***^{III} vascular tissue: differentiating cells, not in meristematic or terminally differentiated cells (Crespi: in infected (++) and uninfected (+¥)) indeterminate nodules).
***^{IV} parenchymatous cells surrounding the developing connecting vascular bundles (in stem-borne nodules).
¥: *ENOD40* expression in empty nodules induced by mutant bacteria, or spontaneous nodules, or NOD factor induced nodule primordia.
¥¥: *ENOD40* expression upon Arbuscular Mycorrhizal symbiosis both in uninfected (epidermal and pericycle) and infected cortical (containing immature arbuscules) cells.
¥¥¥: pMs-1: no GUS in NPA induced pseudonodules, but normally expressed in *R.meliloti exo* mutant induced empty nodules.
pMs-2: in NPA induced pseudonodules: dividing cortical cells and pericycle, later stage: around central vascular bundle and peripheral area; normally expressed in *R.meliloti exo* mutant induced empty nodules.

Lateral roots :
‡ pre-emerging lateral root tips.
‡‡ pericycle cells giving rise to lateral root primordium, at later stages: in developing vascular cylinder.
‡‡‡^{I} parenchymatous cells surrounding central vascular bundle in adventitious root primordia (inducible upon inoculation, comparable to root pericycle facing nodule primordium).
‡‡‡^{II} Tr-1/2: dividing cells of primordium of adventitious root formed in the cortex of the first visible node, remains strong in zone that corresponds to vascular initials
Tr-3: developing root cap, meristematic cells, vascular initials of the nodal root.
‡‡‡^{I} in developing lateral root: central cells of lateral root primordia, after emergence: restricted to central vascular tissue at proximal end of lateral root, remained high at the root tip.
‡‡‡^{II} expression in lateral root primordium is low, flanked by regions of expression in vascular bundle of root, later stages also in connecting vascular tissue of main root and lateral root.

Stem:
§ adjacent to secundary phloem (procambial region).
§§ vegetative apical shoot meristems: at leaf primordia, particularly at the margins consisting of developing leaflets.
§§§I node, where leaf is attached
§§§II at developing lateral shoot; Tr-1/2: up-regulation confined to vascular tissue at base of lateral shoot, Tr-3 also in other tissues of axillary bud and more mature lateral shoot.
§§§III Tr-1/2: stem vasculature in the internode on the side where the petiole is attached, at the node at the point of petiole attachment; Tr-3: only at base of axillary shoot bud, not in stem vascular tissue.
§§§§I in rice: only in early developmental stages of stem vascular bundles that conjoin the emerging leaf.
In lateral vascular bundles exclusively in the xylem parenchyma cells surrounding the protoxylem.
§§§§II in stolon vascular bundles: in the phloem-cambium region of all vascular bundles and the parenchyma surrounding the xylem vessels in the leaf vascular traces.
Confined to nodes, rather than internodes, not detectable in petiole vascular bundles.
§§§§III parenchyma surrounding xylem vessels, the internal phloem, and the cells between xylem and internal phloem.

Flower:
¶ in pedicels that connect florets with the inflorescence axes, in pedicel vascular tissue after onset of senescence in lower floret whorls.
¶¶ in stigma coinciding with anthesis, in petals after pollination just preceding petal senescence.

æ absent during somatic embryogenesis: not in callus or somatic embryos.
ß in root cortex and epidermal cells in root elongation zone; treatment induced (inner) cortical cell divisions, leading to small nodule primordia expressing GUS.
† alteration of *ENOD40* expression by overexpression or antisense silencing affects regeneration of transgenic explants

Table 1.1 Overview of expression patterns of *ENOD40* genes in 12 different plant species shows that these are conserved across the plant kingdom.

Several Functions for *ENOD40* Genes Have Been Proposed

In general, gene function assignment can benefit from the availability of mutants in that particular gene. However, only a *Zea mays* line has been described in which a transposon has been inserted in region II of one of its *ENOD40* genes and this line has no clear growth aberrations. The lack of a phenotype could be due to either a partial loss of function or to functional redundancy of the two *ZmENOD40* genes (Compaan et al., 2003). In general, overexpression or silencing of *ENOD40* seems not to induce very severe growth aberrations, but nonetheless supports a role for *ENOD40* in nodulation and plant developmental processes possibly through interaction with phytohormone signaling pathways (Crespi et al., 1994; van de Sande et al., 1996; Charon et al., 1997; Charon et al., 1999). Assignment of the function of *ENOD40* has mainly been based on spatio-temporal expression patterns in symbiotic as well as non-symbiotic tissues. An overview of *ENOD40* expression patterns of 12 different plant species is presented in Table 1.1. Here, we will focus on common features of expression in different plant backgrounds and refer to the original reports for full details. Possible functions of *ENOD40* fall into three groups, namely transport, organogenesis and regulation of phytohormone status.

Transport

A function of *ENOD40* in transport processes has been proposed on the basis of high expression levels in pericycle or xylem parenchyma cells of vascular bundles of root, shoot and nodule. The expression in vascular tissue is found in all plant species analyzed to date and the highest level of expression is usually found in (nodule) vascular tissues. Yang, (1993), Papadopoulou, (1996), Kouchi, (1999) and Varkonyi-Gasic (2002) argue for a role of *ENOD40* in vascular bundle functioning and underline that the expression pattern suggests a role in lateral transport of solutes. Although *ENOD40* expression correlates with a function in transport or differentiation of vascular tissue, there are no experimental data available which could support an effect of *ENOD40* on vascular development or functioning.

Organogenesis

ENOD40 is often transiently expressed during early stages of organogenesis in various organs. In later stages, *ENOD40* is expressed at lower levels in vascular tissue of developing organs. *ENOD40* transcripts are present in developing lateral roots (Corich et al., 1998; Fang and Hirsch, 1998; Mirabella et al., 1999; Varkonyi-Gasic and White, 2002; Vleghels, 2003), embryonic tissues (Flemetakis et al., 2000) and during early stages of lateral shoot development (Asad et al., 1994) and nodal root development (Corich et al., 1998; Varkonyi-Gasic and White, 2002). Therefore, it has been proposed that it functions in early stages of organogenesis. This function is further supported by reverse genetic data showing that misregulation of *ENOD40* expression interferes with regulation of *Rhizobium* induced cortical cell division (Charon et al., 1997), nodule development (Charon et al., 1999), somatic embryogenesis during regeneration of transgenic calli (Crespi et al., 1994) and development of adventitious shoots in tobacco (van de Sande et al., 1996).

Hormone Status

Since phytohormones are signaling molecules in developmental processes like organogenesis, the third function that was proposed for *ENOD40* is a modifier of hormone status of cells. This function is mainly based on the correlation between the timing of *ENOD40* expression, the effects of misexpression of *ENOD40* and phytohormone action during organogenesis.

It was reported that transformation with anti-sense *ENOD40* constructs arrested callus growth of *Medicago sativa* explants, whereas calli overexpressing *MtENOD40* developed in teratomas. In proliferating explants, these phenotypes can be mimicked by alteration of the cytokinin/auxin ratio (Crespi et al., 1994). Transgenic tobacco plants overexpressing a *GmENOD40* transcript had an increased number of adventitious shoots at the base of the main shoot. This suggests reduced apical dominance, raising the possibility that the transgenic plants were changed in terms of auxin metabolism or perception (van de Sande et al., 1996). The expression in differentiating vascular tissue (Yang et al., 1993; Varkonyi-Gasic and White, 2002) could indicate that the function of

ENOD40 is linked to auxin action since vascular tissue patterning and development is regulated by auxin (Mattson et al., 2003). Constitutive *ENOD40* expression in stable transgenic *Medicago truncatula* lines induced dedifferentiation and divisions of cortical cells (Charon et al., 1997). This effect is observed in the absence of *Rhizobium*, but only under nitrogen limiting conditions. Transient expression in epidermal and outermost cortical cells after bombardment induces dedifferentiation and division of inner cortical cells and expression of *MsENOD12*, a molecular marker for cortical cell division (Charon et al., 1997). Both auxin and cytokinin accumulate in cortical cells opposite protoxylem poles upon inoculation, at the site of cortical cell divisions (Hirsch et al., 1997). Application of auxin transport inhibitors or cytokinin can trigger pseudo-nodule development (Cooper and Long, 1994; Hirsch et al., 1989). These observations suggest that perturbation of the auxin/cytokinin ratio is a developmental cue during nodule development. Thus, cell division could be triggered by an *ENOD40* induced modification of phytohormone status of the cortical cells.

Several phenotypes are observed in *ENOD40* overexpressing plants upon bacterial inoculation. Primary root growth is slightly enhanced and nodulation kinetics is accelerated, accompanied by extensive cortical cell divisions in the region close to the root tip. A considerable increase in the number of persistent infection threads reaching the inner cortex was observed in these plants at early time points (Charon et al., 1999). *Sym5* and *sickle* mutants are impaired in nodulation and this is accompanied by either ethylene hyper- or insensitivity. Their nodulation phenotypes showed that both infection thread formation and cortical cell division are under control of ethylene (Guinel and LaRue, 1991; Penmetsa and Cook, 1997). Therefore, it is possible that *ENOD40* action affects either ethylene responses during early stages of nodulation, or that it affects cytokinin /auxin status, as induction of cortical cell divisions is also under the control of auxin and cytokinin. The hypothesis that *ENOD40* function is linked to ethylene action would be in line with the finding that transient *ENOD40* expression coincides with a temporal burst of ethylene in germinating seeds and flowers of tomato (Vleghels, 2003). The proposed function of *ENOD40* to modify phytohormone status in the cells in which it is expressed, is consistent with the observation that *ENOD40* expression itself is under control of ethylene and cytokinin (Fang and Hirsch, 1998; Vleghels, 2003).

CONCLUSION

In this chapter we described the current knowledge on *ENOD40* structure and function to answer two questions; what is the function of *ENOD40* and which gene product has biological activity? In the case of *ENOD40,* identification of the gene product that has biological activity is not trivial. The study of the structure of *ENOD40* transcripts revealed that all *ENOD40* transcripts lack a long open reading frame that could encode a polypeptide with biological activity. However, two conserved regions, likely required for biological activity, are present in *ENOD40* transcripts. Of these, one contains a conserved ORF and the other only in 50% of the cases. This indicates that the biologically active gene products of *ENOD40* may either be a (oligo)peptide and/or RNA. It has been shown that the conserved peptides are translated *in vivo*, but no biological function has yet been assigned to them. On the other hand, the second conserved region also seems to contain biological activity, but only in some transcripts an ORF is present in this region and it could be active as RNA. Therefore, these options should be studied in further detail to better understand the molecular mechanism of *ENOD40* action.

ENOD40 expression is transiently induced during early developmental stages of several organs, suggesting that it has a regulatory role in development. Based on the timing of *ENOD40* expression and the effects of misexpression of *ENOD40*, it has been proposed that *ENOD40* is involved in early stages of organogenesis, and that it acts by modifying phytohormone status. Whether and how *ENOD40* modifies the hormone status of cells in early stages of organogenesis is difficult to evaluate in whole plants because the action of phytohormones leads to complex responses. Furthermore, the effects of *ENOD40* misexpression in various organs are diverse and these phenotypes do not give further insight in the function of *ENOD40*. Therefore, the function of *ENOD40* and its mode of action are still poorly understood. To study the function of *ENOD40* in further detail requires a less complex test system than intact plants. Using a cellular system would be very helpful to reduce the complexity of responses. Since the function of *ENOD40* is closely related to morphological changes in early stages of organogenesis,

the cellular test system should allow to study the effect of *ENOD40* on cell division and growth. In addition, because we want to study the effect of *ENOD40* on hormone status of cells, these cells should also have a marked morphological response to phytohormones.

Taking these requirements into consideration only one such cellular plant system is available, namely the tobacco BY-2 cell suspension which is generally recognized as the HeLa cell of plant biology. The BY-2 cell suspension has several important advantages for our studies. *ENOD40* genes have been isolated from tobacco, so we can use a tobacco *ENOD40* homolog for our studies. The cell suspension is transformable, allowing a reverse genetics approach. With a simple, yet well described morphology, the characterization of the parameters cell growth and cell division in this filamentous cell suspension is straightforward. Furthermore, a specific and strong effect of phytohormones on cell growth and cell division has already been described. By exogenously applying phytohormones or blockers of phytohormone action at closely defined conditions, it is possible to study effects of *ENOD40* on hormone action by monitoring the morphological response.

We decided to explore the possibility to set up a bioassay for *ENOD40* function in BY-2 cells using a transgenic approach. If *ENOD40* induces a phenotype in the BY-2 cells, it will be possible to test the function of the two conserved regions in *ENOD40* transcripts using a series of constructs carrying mutations in either of the two regions.

REFERENCES

Asad, S., Fang, Y., Wycoff, K.L., and Hirsch, A.M. (1994). Isolation and characterization of cDNA and genomic clones of *MsENOD40*; Transcripts are detected in meristematic cells of alfalfa. Protoplasma **183,** 10-23.

Charon, C., Johansson, C., Kondorosi, E., Kondorosi, A., and 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., and Kondorosi, A. (1999). Alteration of *ENOD40* expression modifies *Medicago truncatula* root nodule development induced by *SinoRhizobium meliloti*. Plant Cell **11,** 1953-1965.

Compaan, B., Ruttink, T., Albrecht, C., Meeley, R., Bisseling, T., and Franssen, H. (2003). Identification and characterization of a *Zea mays* line carrying a transposon tagged *ENOD40*. BBA **-** Gene Structure and Expression, **1629 (1-3)** 84-91.

Compaan, B., Yang, W.C., Bisseling, T., and 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.

Cooper, J.B., and Long, S.R. (1994). Morphogenetic Rescue of *Rhizobium* meliloti Nodulation Mutants by trans-Zeatin Secretion. Plant Cell **6,** 215-225.

Corich, V., Goormachtig, S., Lievens, S., VanMontagu, M., and Holsters, M. (1998). Patterns of *ENOD40* gene expression in stem-borne nodules of *S. rostrata*. Plant Mol. Biol. **37,** 67-76.

Crespi, M.D., Jurkevitch, E., Poiret, M., d'Aubenton-Carafa, Y., Petrovics, G., Kondorosi, E., and 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.

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

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

Guinel, F.C., and LaRue, T.A. (1991). Light microscopy study of nodule initiation in *Pisum sativum* L. cv. Sparkle and its low-nodulating mutant E2 (*sym5*). Plant Phys. **97,** 1206-1211.

Hirsch, A.M., Bhuvaneswari, T.V., Torrey, j.G., and Bisseling, T. (1989). Early nodulin genes are induced in alfalfa root ougrowths elicited by auxin transport inhibitors. Proc. Natl. Acad. Sci. USA **86,** 1244-1249.

Hirsch, A.M., Fang, Y., Asad, S., and Kapulnik, Y. (1997). The role of phytohormones in plant-microbe symbioses. Plant and Soil **194,** 171-184.

Kouchi, H., and Hata, 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.

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

Mathesius, U., Schlaman, H.R.M., Spaink, H.P., Sautter, C., Rolfe, B.G., and Djordjevic, M.A. (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.

Mattsson, J., Ckurshumova, W., and Berleth, T. (2003). Auxin signaling in *Arabidopsis* Leaf Vascular Development. Plant Physiol. **131,** 1327-1339.

Matvienko, M., Van de Sande, K., Yang, W.C., van Kammen, A., Bisseling, T., and 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., and Bisseling, T. (1996). *Nicotiana tabacum* SR1 contains two *ENOD40* homologs. In: Biology Of Plant Microbe Interactions, G. Stacey, B. Mullin, and P. M. Gresshoff, eds. Int. Soc. MPMI. St. Paul USA, 387-391**.**

Minami, E., Kouchi, H., Cohn, J.R., Ogawa, T., and Stacey, G. (1996). Expression of the early nodulin, *ENOD40* , in soybean roots in response to various lipo-chitin signal molecules. Plant J. **10,** 23-32.

Mirabella, R., Martirani, L., Lamberti, A., Iaccarino, M., and 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.

Papadopoulou, K., Roussis, A., and 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.

Penmetsa, R.V., and Cook, R.V. (1997). A legume ethylene-insensitive mutant hyperinfected by its Rhizobial symbiont. Science **275,** 527-530.

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

Roussis, A., Van, D.S.K., Papadopoulou, K., Drenth, J., Bisseling, T., Franssen, H., and Katinakis, P. (1995). Characterization of the soybean gene *GmENOD40*-2. J. Exp. Bot. **46**, 719-724.

Santi, C., von Groll, U., Ribeiro, A., Chiurazzi, M., Auguy, F., Bogusz, D., Franche, C., Pawlowski, K. (2003). Comparison of nodule induction in legume and actinorhizal symbiosis: the induction of actinorhizal nodules does not involve *ENOD40*. MPMI **16**, 808-816.

Sousa, C., Johansson, C., Charon, C., Manyani, H., Sautter, C., Kondorosi, A., and 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. Mol. Cell. Biol. **21,** 354-366.

van de Sande, K., Pawlowski, K., Czaja, I., Wieneke, U., Schell, J., Schmidt, J., Walden, R., Matvienko, M., Wellink, J., vanKammen, A., Franssen, H., and 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., Shaul, O., Atzmon, N., Wininger, S., Eshed, Y., Lum, M., Li, Y., To, V., Fujushige, N., Kapulnik, Y., and Hirsch, A.M. (1997). Expression of Early nodulin genes in alfalfa mycorrhizae indicates that signal transduction pathways used in forming arbuscular mycorrhizae and *Rhizobium*-induced nodules may be conserved. Proc. Natl. Acad. Sci. USA 94, 5467-5472.

Varkonyi Gasic, E., and White, D.W.R. (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.

Vijn, I., Martinez, A.F., Yang, W.C., Das, N.L., Van, B.A., Van, K.A., and Bisseling, T. (1995a). Early nodulin gene expression during Nod factor-induced processes in *Vicia sativa*. Plant J. **8,** 111-119.

Vijn, I., Yang, W.C., Pallisgard, N., Jensen, E.O., Van, K.A., and Bisseling, T. (1995b). *VsENOD5*, *VsENOD12* and *VsENOD40* expression during *Rhizobium*-induced nodule formation on *Vicia sativa* roots. Plant Mol. Biol. **28,** 1111-1119.

Vleghels, I. (2003). Comparative studies on *ENOD40* in legumes and non-legumes. Thesis, Wageningen University and Research Center Wageningen, the Netherlands**,** pp 99.

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

Chapter 2

ENOD40 and Hormonal Control of Cell Size in Tobacco Bright Yellow-2 Cells

ABSTRACT

We show that *ENOD40* overexpression leads to suppressed elongation growth of BY-2 cells, whereas cell division frequencies are not affected in 35S:*NtENOD40* BY-2 cell suspensions. As auxin, cytokinin and ethylene control elongation growth and cell division of tobacco BY-2 cell suspensions we used these growth parameters in a bioassay to investigate interaction between *ENOD40* and auxin, cytokinin and ethylene signaling pathways. Thus, we show that *ENOD40* affects cytokinin/auxin dependent control of cell size. Analysis of ethylene homeostasis shows that ethylene accumulation is accelerated in 35S:*NtENOD40* lines. Furthermore, *ENOD40* action can be counteracted by ethylene receptor blockers, suggesting that ethylene is a negative regulator of elongation growth in BY-2 cells and that acceleration of ethylene accumulation is a primary cause and not a consequence of suppressed elongation growth in 35S:*NtENOD40* cells. Our data show that overexpression of *NtENOD40* results in a marked different behavior of phytohormone regulated cellular processes and support the hypothesis that the regulatory function of *ENOD40* in organogenesis depends on cross-talk with ethylene and cytokinin/auxin signaling pathways. Implications of our findings for the role of *ENOD40* during nodule primordium formation are discussed.

INTRODUCTION

Homologs of *ENOD40* genes have been identified in plant species across the plant kingdom including monocots like rice, maize and sorghum, and dicots such as tomato, tobacco, citrus and several leguminous species. The highest expression levels of *ENOD40* have been found during legume nodule formation, and therefore its function has been studied in most detail during this process. However, the presence of *ENOD40* homologs in genomes of non-leguminous plant species, and the presence of transcripts in non-symbiotic tissues indicates that the function of *ENOD40* is not confined to nodule development in leguminous species. Low levels of *ENOD40* expression have been detected during lateral root formation, flower development and vascular tissue development in several different plant species, indicating that *ENOD40* expression is

associated with certain specific stages of organogenesis. This suggests that *ENOD40* has a general role in plant development.

During nodulation, *ENOD40* expression is induced within 3 hrs after inoculation with *Rhizobium* in pericycle cells positioned opposite the protoxylem poles, prior to the onset of cortical cell divisions which lead to formation of the nodule primordium (Compaan et al., 2001; Kouchi and Hata, 1993; Mylona et al., 1995; Yang et al., 1993). Misregulation of *ENOD40* by co-suppression reduces the number of nodules and nodule development is arrested which indicates that *ENOD40* has a regulatory role in these processes (Charon et al., 1999; Crespi et al., 1994). *ENOD40* overexpression on the other hand, induces cortical cell divisions and accelerates nodule development in *Medicago* (Charon et al., 1999). However, *ENOD40* expression alone is not sufficient for nodule primordium formation (Mathesius et al., 2000; Minami et al., 1996), and interaction with other plant factors is probably required for the initiation of nodule morphogenesis. Whereas Rhizobial Nod-factor signaling sets the process of nodulation in action, the process is further regulated by the phytohormones ethylene, cytokinin and auxin. *ENOD40* expression is induced by Nod-factors or cytokinin in legumes suggesting that these could be candidate interactors of *ENOD40*.

The role of phytohormones during early stages of nodule primordium development has been implicated by experiments showing that either an auxin transport inhibitor or cytokinin are able to induce cortical cell divisions and eventually nodule-like structures on roots of legumes as well as non-legumes (Cooper and Long, 1994; Hirsch et al., 1989). Additionally, both auxin and cytokinin accumulate in cortical cells opposite protoxylem poles upon inoculation, at the site of cortical cell divisions. These observations suggest that perturbation of auxin and/or cytokinin flow through the root acts as a developmental cue during nodule development. Inhibitors of ethylene biosynthesis have been reported to increase nodule formation and to modify positioning of cortical cell division in roots (Heidstra et al., 1997; Lee and LaRue, 1992; Peters and Crist-Estes, 1989). Further, a blocker of ethylene production, AVG, partially mimics the effect of *ENOD40* overexpression in *Medicago* (Charon et al., 1997). Taken together,

these observations suggest that during nodule development cross-talk between *ENOD40* and phytohormone signaling exists. Ectopic overexpression of *ENOD40* affected certain hormonal responses of somatic embryos of alfalfa under *in vitro* culture conditions (Charon et al., 1999; Crespi et al., 1994). Also, overexpression of *ENOD40* led to reduced apical dominance in tobacco suggesting that phytohormone signaling is affected by *ENOD40* in non-legumes (van de Sande et al., 1996).

To date, the function of *ENOD40* and its mode of action are poorly understood. Direct evidence of an interaction between *ENOD40* activity and phytohormone signaling pathways is lacking. Establishing whether the function of *ENOD40* involves interaction with phytohormone signaling pathways is an important step towards unraveling the role of *ENOD40* during developmental processes to which its expression is associated. We searched for a bioassay to test the effect of *ENOD40* on cellular processes and chose the tobacco BY-2 cell suspension as a model system. We found that overexpression of *ENOD40* suppresses elongation growth but that cell division frequency is not affected. An important advantage of the BY-2 cell suspension for our study is the observation that elongation growth and cell division frequency of cells are regulated by the balance between cytokinin and auxin in the culture medium (Hasezawa and Syono, 1983). In addition, we provide evidence that ethylene is a negative regulator of elongation growth in BY-2 cells. These observations raised the possibility to test whether the morphological changes induced by *ENOD40* are caused through interaction with phytohormone signaling.

RESULTS

Generation of Stably Transformed Cell Lines Carrying 35S:*NtENOD40*

To determine whether overexpression of *NtENOD40* affects morphology of BY-2 cells, we generated a set of six 35S:*NtENOD40* BY-2 cell lines by *Agrobacterium*-mediated transformation (Methods). These lines were named line Nt1 through Nt6. Each transgenic line was derived from a different callus, which means that they cannot be

siblings. PCR analysis on genomic DNA showed that all lines contain the construct (data not shown). Expression of the transgenes was detected by RNA gel blot analysis performed with total RNA isolated from these transgenic lines in three independent experiments, each time with similar results. One representative set of data is presented in Figure 2.1D. In the wild type line, *NtENOD40* mRNA could not be detected, indicating a very low expression level of the endogenous *NtENOD40* gene. In the six lines that carry the 35S:*NtENOD40* construct *NtENOD40* transcripts were expressed at varying levels but this level was, in all lines except line Nt6, much higher than in the wild type line. Therefore, we concluded that *NtENOD40* is overexpressed in all 35S:*NtENOD40* transgenic lines, except in line Nt6. Hybridization with the *HPTII* probe was performed as a positive control, and revealed that in all transgenic lines *HPTII* transcripts could be detected, conferring resistance to hygromycin.

Further, hybridization with the *NtENOD40* probe resulted in two bands on the RNA gel blot, indicating the presence of two *NtENOD40* RNAs with different lengths. To further characterize the nature of these two RNAs, 3' RACE-PCR was performed on *NtENOD40* transcripts of the transgenic lines (Methods). Analysis of nucleotide sequences of 11 cloned RACE-PCR fragments revealed that all sequences that were obtained are 100% identical to the transgene sequence and that read-through occurs through the NOS terminator that flanks the *NtENOD40* cDNA sequence in the construct resulting in transcripts of two different lengths (data not shown).

- **Figure 2.1**

The value for each repetition is the means of 100-150 individual cells per sample. Error bars indicate SD between independent experiments. An * marks transgenic cell lines with a significant reduction of elongation growth compared to the wild type ($p < 0.001$).
(A) Elongation growth rate (cell file length expressed in width units).
(B) Cell division frequency (number of cells per cell file).
(C) Cell size (Ratio of elongation growth rate over cell division frequency).
(D) The level of transgene expression is determined at the start of protoplast culture by RNA gel blot analysis. Hybridization with the *NtENOD40* probe and *HPTII* probe show expression of the transgene transcripts. Hybridization with the *Ubiquitine* (*UBI*) probe is performed in order to compare loading of the separate samples.

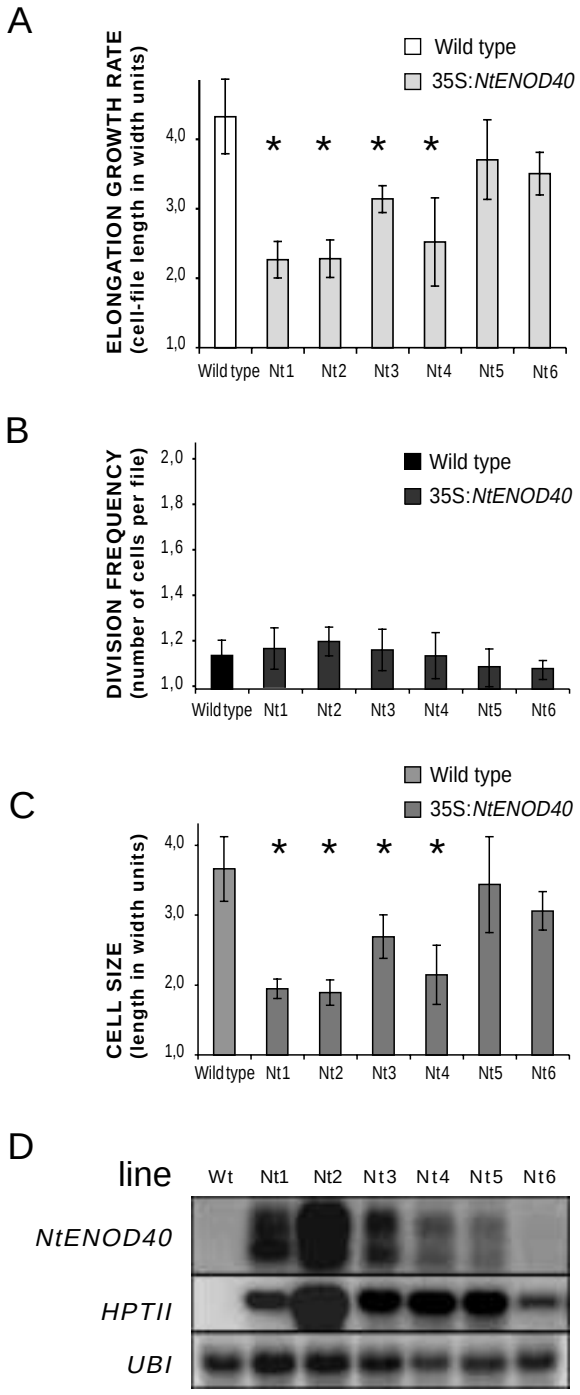


Figure 2.1. Elongation Growth Rate, Cell Division Frequency, Cell Size and Transgene Expression Levels of Cultured Cells of Wild Type and 35S:*NtENOD40* BY-2 Cell Lines.

Growth parameters of protoplast derived cells are determined after four days of culture in medium supplemented with 0.1 mg/l NAA and 1.0 mg/l BA. Data are average of 11 (Wild type), 8 (Nt1), 9 (Nt2) and 5 (Nt3-Nt6) independent repetitions.

NtENOD40 Suppresses Elongation Growth But Does Not Affect Cell Division Frequency of Cultured Protoplasts

It has been shown that a protoplast bioassay provides the most accurate data concerning elongation growth and cell division frequency in BY-2 cell suspensions. Therefore, we adapted this protoplast-based bioassay described by Hasezawa (1983), to quantitatively determine the contribution of cell division frequency and elongation growth to cell morphology. By preparing protoplasts from the cell suspension, a population of single cells with a similar size is obtained. This bioassay has two main advantages in comparison to direct use of suspension-cultured cells. Firstly, the cell division frequency can be directly scored by counting the number of cells in cell files that are formed from protoplasts. Under our growth conditions BY-2 cells normally form cell files. The number of cell files during culture remains similar to the number of protoplasts at the start of the experiment. This means that the vast majority of the cells remain attached to each other after division. Therefore, by starting from single cells, the average number of cells per cell file reflects the number of cell divisions that took place during the incubation time, and this parameter is from hereon called the ‘cell division frequency’. For example, cultured protoplasts remain single cells when no cell division takes place, whereas finding 2 cells per file means that one round of cell division has occurred during the incubation time.

The second advantage of protoplasts as starting material for our tests concerns quantification of elongation growth. The width of cells remains similar to the diameter of protoplasts during culture. This means that no radial expansion growth occurs and the length (expressed in width units) of the cell files is a parameter for elongation growth of cells during the incubation period. This parameter is from hereon called the ‘elongation growth rate’. The average length of individual cells, again expressed in width units, is from hereon called ‘cell size’. This parameter depends on the relative rates of elongation growth and cell division and therefore should not be used to indicate growth rates. Instead, in a population of simultaneously elongating and dividing cells, this parameter

(length per cell) reflects the ratio of the elongation growth rate (length per cell file) divided by the cell division frequency (number of cells per file).

To study the relation between cell size, elongation growth rate, cell division frequency and *NtENOD40* expression level, protoplasts were obtained from the wild type and 35S:*NtENOD40* cell lines (Methods), (Nagata et al., 1992). Cells were subsequently cultured for four days in protoplast culture medium (PCM) in the presence of 1.0 mg/l BA and 0.1 mg/l NAA (Methods). We determined elongation growth rate, cell division frequency and cell size, respectively, for each line in at least 5 independent experiments (Methods). Data are presented in Figure 2.1A-C. We found that four lines have a strongly reduced elongation growth rate as compared to the wild type (Nt1 2.27 ± 0.27; Nt2 2.31 ± 0.25; Nt3 3.09 ± 0.25 and Nt4 2.48 ± 0.65, respectively, versus 3.93 ± 0.52 for the wild type) (Figure 2.1A). The reduction of elongation growth rate is significant ($p<0.001$) for the lines Nt1, Nt2, Nt3 and Nt4. These data show that in the lines with the strongest phenotype (Nt1 and Nt2), elongation growth is reduced by about 60% in comparison to that of the wild type. The RNA gel blot analysis (Figure 2.1D) revealed that from the set of six transgenic lines, lines Nt1 and Nt2 express 35S:*NtENOD40* at the highest level. In addition, lines Nt5 and Nt6 have an elongation growth rate that is similar to wild type (3.70 ± 0.57 and 3.24 ± 0.37 respectively). In line Nt5 only a limited level of *NtENOD40* transcripts can be detected, and line Nt6 contains no detectable *NtENOD40* mRNA. So, a good correlation between *NtENOD40* expression level and reduction of elongation growth is found. Under the used hormonal conditions, the average number of cells per cell file in the wild type line is 1.15 ± 0.09. This means that about 15% of the cells has undergone one round of cell division during the culture period. The average number of cells per cell file in the transgenic lines is Nt1 1.17 ± 0.10; Nt2 1.20 ± 0.07; Nt3 1.17 ± 0.09; Nt4 1.14 ± 0.09; Nt5 1.09 ± 0.08 and Nt6 1.08 ± 0.03 (Figure 2.1B). This shows that division frequency is not affected in the transgenic lines, indicating that overexpression of *NtENOD40* does not alter cell division frequency.

The cell size reflects the ratio of elongation growth rate divided by cell division frequency and is depicted in Figure 2.1C. The average size of cells in the different lines is: Nt1 1.94 ± 0.16; Nt2 1.92 ± 0.17; Nt3 2.64 ± 0.18; Nt4 2.18 ± 0.50; Nt5 3,44 ± 0,68 and Nt6 3,06 ± 0,33; versus 3.42 ± 0.47 for the wild type). The reduction of cell size is significant ($p<0.001$) for the lines Nt1, Nt2, Nt3 and Nt4. These data reveal that overexpression of *NtENOD40* results in reduced cell size in cultured BY-2 cells. Representative photographs taken after four days of culture of wild type cells and cells of a transgenic line with a strong phenotype (line Nt1) are presented in Figure 2.4D,E. These pictures show the clear elongated appearance of wild type cells and reveal that cell size is smaller in line Nt1. Thus, the protoplast bioassay allows us to differentiate between effects of *ENOD40* on cell division and elongation growth. We show that cell size is reduced in *NtENOD40* overexpressing cell lines by a reduced elongation growth rate, rather than by increased cell division frequency under these conditions. Two lines (line Nt1 and Nt2) that have the highest level of *NtENOD40* expression and have a strong phenotype were selected for further analysis.

- **Figure 2.2**

(A) Wild type elongation growth rate, cell division frequency and cell size at increasing concentrations of BA, each in the presence of 0.1 mg/l NAA.
(B) Wild type elongation growth rate, cell division frequency and cell size at increasing concentrations of NAA, each in the presence of 1.0 mg/l BA.
(C), **(D)** Dose response curves measuring cell division frequency as a function of **(C)** BA concentration and **(D)** NAA concentration.
(E), **(F)** Dose response curves measuring elongation growth of cell files as a function of **(E)** BA concentration and **(F)** NAA concentration.
(G), **(H)** Dose response curves measuring cell size as a function of **(G)** BA concentration and **(H)** NAA concentration.

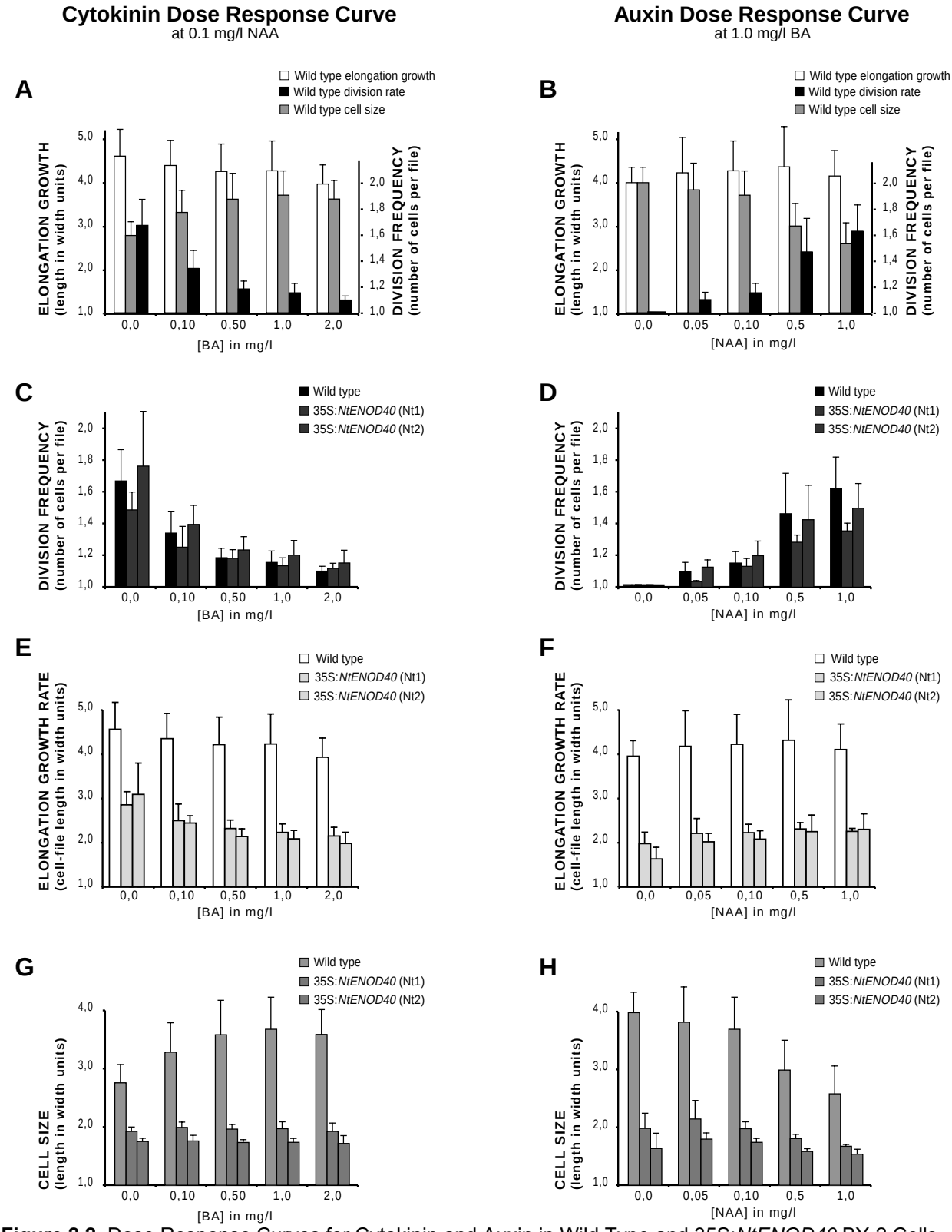


Figure 2.2. Dose Response Curves for Cytokinin and Auxin in Wild Type and 35S:*NtENOD40* BY-2 Cells.

Protoplasts are cultured for four days in medium supplemented with various concentrations of cytokinin or auxin. Data represent average (± SD) of 4 (Wild type), 3 (Nt1) and 4 (Nt2) independent experiments.

Effect of *ENOD40* on Auxin and Cytokinin Responses

To test the effect of increased *NtENOD40* transcript levels on hormone responses, we made use of the effects of exogenously applied auxin and cytokinin on elongation growth and cell division of BY-2 cells. These have previously been determined by means of dose response curves for auxin and cytokinin (Hasezawa and Syono, 1983). Low concentrations of auxin stimulate BY-2 cell elongation, whereas high concentrations reduce the average cell length by promoting cell division. Increasing the concentration of cytokinin in the culture medium causes an opposite response since this reduces the cell division frequency and promotes elongated growth of cells (Hasezawa et al., 1988; Iwata, 1995; Tamura et al., 1999). In order to study the effect of overexpression of *ENOD40* on elongation growth and cell division in response to auxin and cytokinin, dose response curves were made for the wild type cell line and transgenic lines Nt1 and Nt2. For the cytokinin dose response curve, we determined elongation growth rate, cell division frequency and cell size as a function of cytokinin (BA) concentration at a fixed concentration of auxin (0.1 mg/l NAA). For the auxin dose response curve, we determined the same parameters as a function of auxin (NAA) concentration at a fixed concentration of cytokinin (1.0 mg/l BA). The dose response curves were made four times, in independent experiments, for the wild type line and line Nt2, and three times for line Nt1. The results from independent experiments were similar and we calculated the average value for each parameter (Figure 2.2A-H).

We first describe the response of wild type cells to exogenously applied cytokinin and auxin. The dose response curve for elongation growth rate as a function of cytokinin concentration in the presence of 0.1 mg/l NAA (Figure 2.2A) shows that elongation growth is almost constant although it is possible that an increasing cytokinin concentration has a slight negative effect on elongation growth. Elongation growth in the absence of cytokinin is similar to that in the presence of various concentrations of cytokinin. Therefore, exogenous application of cytokinin is not essential, nor does it markedly influence elongation growth in the presence of auxin. The auxin dose response curve for elongation growth (Figure 2.2B) shows that elongation growth is hardly affected by the concentration of auxin as in the absence of auxin it is similar to

that at the various concentrations of exogenous auxin. A low concentration of auxin only has a slight positive effect on elongation growth. This shows that elongation growth neither requires auxin, nor does auxin markedly affect it, when cytokinin is applied to the medium. Taken together, these data show that either cytokinin or auxin is sufficient to sustain the growth rates achieved under our culture conditions.

The dose response curve for cell division frequency as a function of cytokinin concentration (Figure 2.2A) shows that the cell division frequency is reduced at increasing concentrations of cytokinin in the presence of 0.1 mg/l NAA, and that the cell division frequency is maximal in the absence of exogenous cytokinin. The dose response curve for cell division frequency as a function of auxin concentration in the presence of 1.0 mg/l BA (Figure 2.2B) shows that the cell division frequency increases at increasing concentrations of auxin. In the absence of auxin, the cell division frequency is '1 cell per file' which means that cells have not divided during the culture period. These results show that exogenously applied auxin is essential for cell division in BY-2 cells. Taken together, these observations show that exogenously applied cytokinin has an inhibitory effect on cell division and that exogenous auxin has a promoting effect on cell division.

The size of individual cells in these filamentous cell suspensions is controlled by two processes, elongation growth and cell division, and the ratio of these two parameters reflects the individual cell size in our samples (file-length / (nr cells/file) = cell-length). Thus, the effect of external application of cytokinin and/or auxin on the average size of cells can be determined by calculating the ratio of elongation growth rate over cell division frequency in a dose response curve. The results are depicted in Figure 2.2A-B. These data show that cell size increases at increasing concentrations of cytokinin (Figure 2.2A) in the presence of 0.1 mg/l NAA. Further, the auxin dose response curve (Figure 2.2B) shows that the cell size decreases at increasing concentrations of auxin in the presence of 1.0 mg/l BA.

Taken together, the analysis of these three parameters reveals how the cell size can be regulated by phytohormones in wild type BY-2 cells. Cytokinin has an inhibiting effect on cell division frequency whereas auxin has a stimulating effect. By quantifying the division frequency in the two complementary dose response curves, we show that the effect of increasing the auxin concentration at a fixed cytokinin concentration is a mirror image to the effect of increasing the cytokinin concentration in the presence of a fixed auxin concentration. This means that the division frequency of BY-2 cells is dependent on the cytokinin to auxin balance, within the concentration range tested. Since elongation growth is almost constant in these dose response curves, these observations further infer that a high cytokinin to auxin ratio suppresses cell division by which individual cells become longer. The observation that cell division frequency can be affected by the cytokinin to auxin balance, without affecting elongation growth, infers that the two processes are not strictly coupled in wild type cells.

Cell Division and Elongation Growth Rates Are Strictly Coupled in 35S:*NtENOD40* Cells

To determine whether overexpression of *NtENOD40* affects the response of BY-2 cells to cytokinin and auxin, dose response curves for 35S:*NtENOD40* lines Nt1 and Nt2 are compared to those of the wild type line (Figure 2.2C-H). Figure 2.2C shows that the inhibitory effect of cytokinin on cell division is similar in wild type and 35S:*NtENOD40* lines. Further, the stimulating effect of auxin on cell division is also similar in wild type and 35S:*NtENOD40* lines, as revealed by the auxin dose response curve depicted in Figure 2.2D. The promoting effect of auxin on cell division at higher auxin concentrations is slightly less strong in the 35S:*NtENOD40* lines than in the wild type. However, at the highest auxin to cytokinin ratio (0.1 mg/l NAA and 0 mg/l BA, i.e. a condition that most strongly induces cell division in the wild type line), on average about 60% of the cells within the population has divided in the wild type line as well as in the 35S:*NtENOD40* lines. This means that under these conditions, wild type and 35S:*NtENOD40* cells can reach similar maximal division frequencies.

The effect of *ENOD40* on elongation growth at various cytokinin concentrations is shown in the cytokinin dose response curve in Figure 2.2E. In the absence of cytokinin, at 0.1 mg/l NAA, elongation growth is reduced in 35S:*NtENOD40* cells by about 40% in comparison to the wild type (Figure 2.2E). So, *ENOD40* can reduce elongation growth in the absence of exogenous application of cytokinin. Addition of cytokinin has only a slight negative effect on elongation growth of wild type cells since at most a 20% reduction of elongation growth is induced by 2 mg/l cytokinin in comparison with wild type elongation growth in the absence of cytokinin. In the presence of 2 mg/l cytokinin, elongation growth in 35S:*NtENOD40* cells is reduced by about 70% of wild type levels. Thus, the combined effect of *ENOD40* and cytokinin is slightly stronger than the sum of the separate effects of *ENOD40* and cytokinin. So, addition of cytokinin to 35S:*NtENOD40* cells attenuates elongation growth, suggesting that *ENOD40* and cytokinin act synergistically in inhibition of growth.

The auxin dose response curve (Figure 2.2F) shows that elongation growth of wild type cells is largely unaffected by application of NAA. Elongation growth of 35S:*NtENOD40* cells in the absence of auxin (at 1.0 mg/l BA) is reduced by about 75% of wild type levels whereas elongation growth is reduced by about 60% upon application of 1.0 mg/l NAA. This shows that the negative effect of *ENOD40* and cytokinin on elongation growth can be partially counter-acted by auxin in 35S:*NtENOD40* cells. So, whereas cytokinin reduces elongation growth of *ENOD40* overexpressing cells, this negative effect can be counter-acted by auxin. This shows that in 35S:*NtENOD40* lines, elongation growth is dependent on the ratio of exogenously applied cytokinin and auxin.

We showed that cell size is reduced in 35S:*NtENOD40* lines by reduction of elongation growth, rather than by increased division frequency (Figure 2.1). By using these dose response curves we can determine how *ENOD40* affects the control on cell size in relation to auxin and cytokinin responses by first discriminating between the effects on elongation growth and cell division frequency and subsequently calculating the ratio between both parameters at the various concentrations of cytokinin and auxin. Both the

cytokinin dose response curve (Figure 2.2G) and the auxin dose response curve (Figure 2.2H) show that this ratio remains constant for 35S:*NtENOD40* cells at all different cytokinin to auxin ratios tested. These data reveal that in 35S:*NtENOD40* lines Nt1 and Nt2, elongation growth and cell division frequency depend on the cytokinin to auxin ratio in a similar fashion. Since the size of individual cells is the net result of elongation growth and cell division frequency, these data show that the processes of elongation growth and cell division are strictly coupled in 35S:*NtENOD40* cells whereas in wild type cells they are not (Figure 2.2G-H). Thus, 35S:*NtENOD40* cell lines have a marked different behavior with respect to the control of cell size in response to phytohormones. In wild type, the balance between cytokinin and auxin regulates the cell division frequency and this regulation is not affected in 35S:*NtENOD40* lines. The observation that in 35S:*NtENOD40* lines the cell size remains constant at different division frequencies, suggests that *ENOD40* controls cell size by regulating elongation growth in accordance with the frequency at which cell division takes place. The observation that *ENOD40* and cytokinin may act synergistically in inhibition of elongation growth and that auxin can counter-act this effect provides evidence that elongation growth of 35S:*NtENOD40* cells is (at least partially) controlled by the auxin to cytokinin balance. However, the strict control of *ENOD40* on cell size is not influenced by exogenous application of either cytokinin or auxin or different combinations of both, suggesting that the effect of *ENOD40* may rely on other plant factors. One such plant factor may be ethylene.

ENOD40 And Ethylene Both Influence Elongation Growth

Genetic as well as physiological studies have shown that at least part of the diverse effects of ethylene on plant growth and development are caused by regulation of cell elongation growth rather than cell division frequency. Specific cellular phenotypes of ethylene perception mutants show that ethylene affects leaf expansion by suppressing cell enlargement rather then division (Kieber et al., 1993; Rodrigues-Pousada et al., 1993). For example, the *ctr1* mutation which leads to constitutive ethylene signaling, results in a dramatically reduced stature and unexpanded leaves in *ctr1* mutants; in

contrast, most ethylene insensitive mutants have a larger rosette than the wild type (Ecker, 1995), resulting from cell enlargement (Hua et al., 1995). Additionally, ethylene has been implicated to control the rate of elongation growth in cells in the elongation zone of the *Arabidopsis* root (Le et al., 2001). Ethylene can influence expression of genes encoding enzymes involved in cell wall loosening on one hand and microtubule organization on the other (Shibaoka, 1994). For example, a role for ethylene has been suggested in regulating the expression of cell wall peroxidases involved in the control of cell wall extensibility and cell growth (Ridge and Osborne, 1971).

Although direct evidence for a role of ethylene in the regulation of elongation growth in BY-2 cells is lacking, several observations do suggest such a role. A number of studies have used the BY-2 cell suspension to investigate the role of cell wall components and the cytoskeleton in elongation growth. These studies include cell wall associated ascorbate oxidase (Kato and Esaka, 2000), as well as peroxidases (Iwata, 1995) and expansins (Link and Cosgrove, 1998) which have been suggested to have a role in cell wall loosening and acid growth response. Furthermore, cortical microtubule orientation and stability as well as actin filament organization have been shown to be major determinants of directional expansion growth in BY-2 cells (Collings et al., 1998; Iwata, 1995; Kuss and Cyr, 1992). When either of these components is limited or disturbed by molecular techniques or chemical blockers, a reduction of elongation growth is observed, rather then severe morphological abnormalities of BY-2 cells. Since the expression of the genes encoding these structural components is under control of ethylene, this suggests that ethylene has a central role in regulation of elongation growth of BY-2 cells. Because the role of ethylene in regulating elongation growth would closely resemble the effect of overexpression of *ENOD40* in BY-2 cells, we hypothesize that ethylene homeostasis can be affected by overexpression of *ENOD40* and that this leads to altered regulation of elongation growth in our transgenic cell lines.

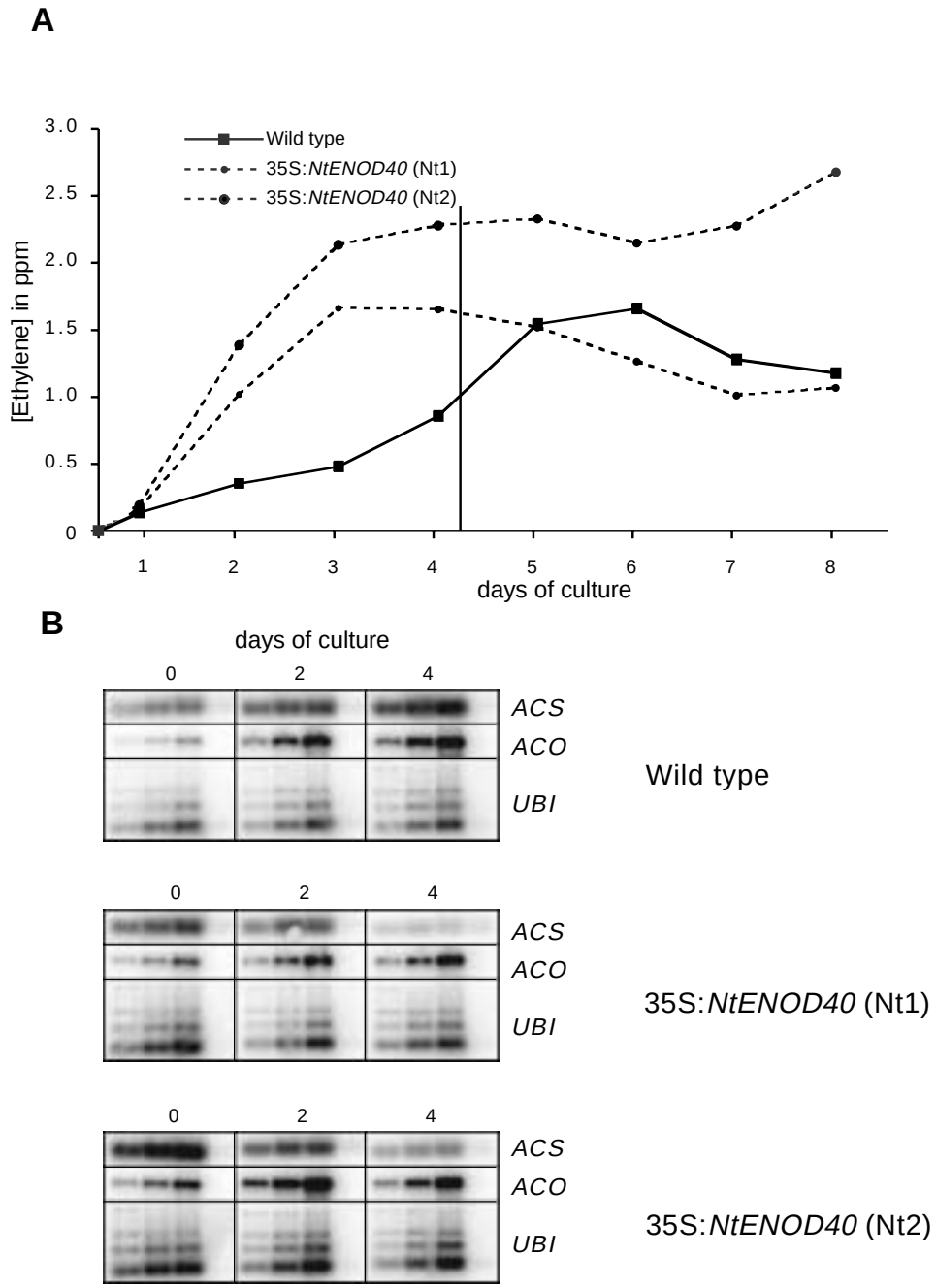


Figure 2.3. Temporal Ethylene Accumulation Profile and Transcript Profiles of Genes Required for Ethylene Biosynthesis in Wild Type and 35S:*NtENOD40* BY-2 Cells.

(A) Kinetics of ethylene accumulation in the headspace of protoplast derived cells, cultured in the presence of 0.1 mg/l NAA and 1.0 mg/l BA. The vertical bar at day 4 indicates the typical time point for quantifying growth parameters of cultured cells.
(B) RT-PCR analysis on ACC synthase (*ACS*) and ACC oxidase (*ACO*) transcript levels of Wild type and 35S:*NtENOD40* cells on day 0, 2 and 4. Amplification is shown for 3 consecutive PCR cycles; 16, 18 and 20 cycles for *UBI*; 28, 30 and 32 cycles for *ACS*; 22, 24 and 26 cycles for *ACO*, including a control on genomic DNA contamination (equivalent amount of RNA, without cDNA synthesis) in the fourth lane of each block.

ENOD40 Affects Ethylene Homeostasis

We tested whether ethylene production kinetics is affected by overexpression of *ENOD40*. As a first step we determined whether ethylene production is altered in transgenic cell lines Nt1 and Nt2, as compared to wild type lines. To this end, protoplasts are cultured in the presence of 1.0 mg/l BA and 0.1 mg/l NAA since under those conditions the strongest *ENOD40* phenotype is obtained (See Figure 2.1A, 2.2E-H). The headspace of protoplast cultures was sampled with 24 hrs intervals during 8 days and ethylene concentrations were determined by GC analysis (Methods). The experiment was performed five times, with similar results. In the wild type culture ethylene gradually accumulates during up to 6 days of culture to a maximal level of about 2 ppm (Figure 2.3A). In cultures of the 35S:*NtENOD40* lines, ethylene accumulates to similar maximal levels as in wild type cultures but transgenic lines already reached maximal levels at day 3. These results show that ethylene production is accelerated in 35S:*NtENOD40* lines while the final level that accumulates is not severely affected.

To determine whether early ethylene accumulation causes reduced elongation growth in 35S:*NtENOD40* lines, the effect of ethylene perception blockers on elongation growth was tested for the two transgenic lines, Nt1 and Nt2, and the wild type line. Thus, $AgNO_3$ or 1-MCP were applied during culture of BY-2 protoplasts in medium supplemented with 1.0 mg/l BA and 0.1 mg/l NAA. In three independent experiments, elongation growth rate, cell division frequency and cell size were scored after four days of culture. The results from independent experiments were similar and we calculated the average value for each parameter (Figure 2.4). The results show that elongation growth of wild type cells is similar in the absence and presence of a range of $AgNO_3$ concentrations (Figure 2.4A). Thus, a block of ethylene perception has no effect on elongation growth in wild type cells. In the absence of ethylene perception blockers, elongation growth of lines Nt1 and Nt2 is about 30% of wild type levels (Figure 2.4A), which is consistent with previous experiments (Figure 2.1A, 2.2E). By application of 10 µM $AgNO_3$ at the start of the protoplast culture, elongation growth increases to about 70% (Nt1) and 75% (Nt2) of wild type levels. Since elongation growth increases to about 60% of wild type levels by

application of 30 ppb 1-MCP to the headspace of transgenic cell cultures (data not shown), 1-MCP treatment is similar to, though slightly less effective than, $AgNO_3$ treatment in counter-acting the negative effect of *ENOD40* on elongation growth.

Further, at all concentrations of ethylene perception blockers tested, the division frequency of wild type as well as transgenic cells is similar to the division rate in the absence of ethylene perception blockers indicating that neither $AgNO_3$ (Figure 2.4B) nor 1-MCP (data not shown) severely affects the division frequency. Since ethylene perception blockers do not affect the division frequency, the increase in elongation growth leads to an increase in cell size in transgenic cell cultures (Figure 2.4C). This shows that a blocker of ethylene perception releases the strict control of cell size in 35S:*NtENOD40* lines. Representative photographs of a population of wild type cells (Figure 2.4D) and Nt1 cells show that the cell size of transgenic cell cultures is strongly reduced in the absence of $AgNO_3$ (Figure 2.4E). In the presence of 10 µM $AgNO_3$ cell size of Nt1 cells is similar to wild type (Figure 2.4F). Thus, ethylene perception blockers specifically counter-act the effect of overexpression of *ENOD40*. Since application of blockers of ethylene perception lead to increased elongation growth in 35S:*NtENOD40* cells, we show that elongation growth of 35S:*NtENOD40* cells is suppressed by ethylene. So, both the timing of ethylene accumulation as well as the studies with ethylene perception blockers strongly suggest that accelerated ethylene accumulation in *ENOD40* lines is a primary cause for reduced elongation growth of these cells.

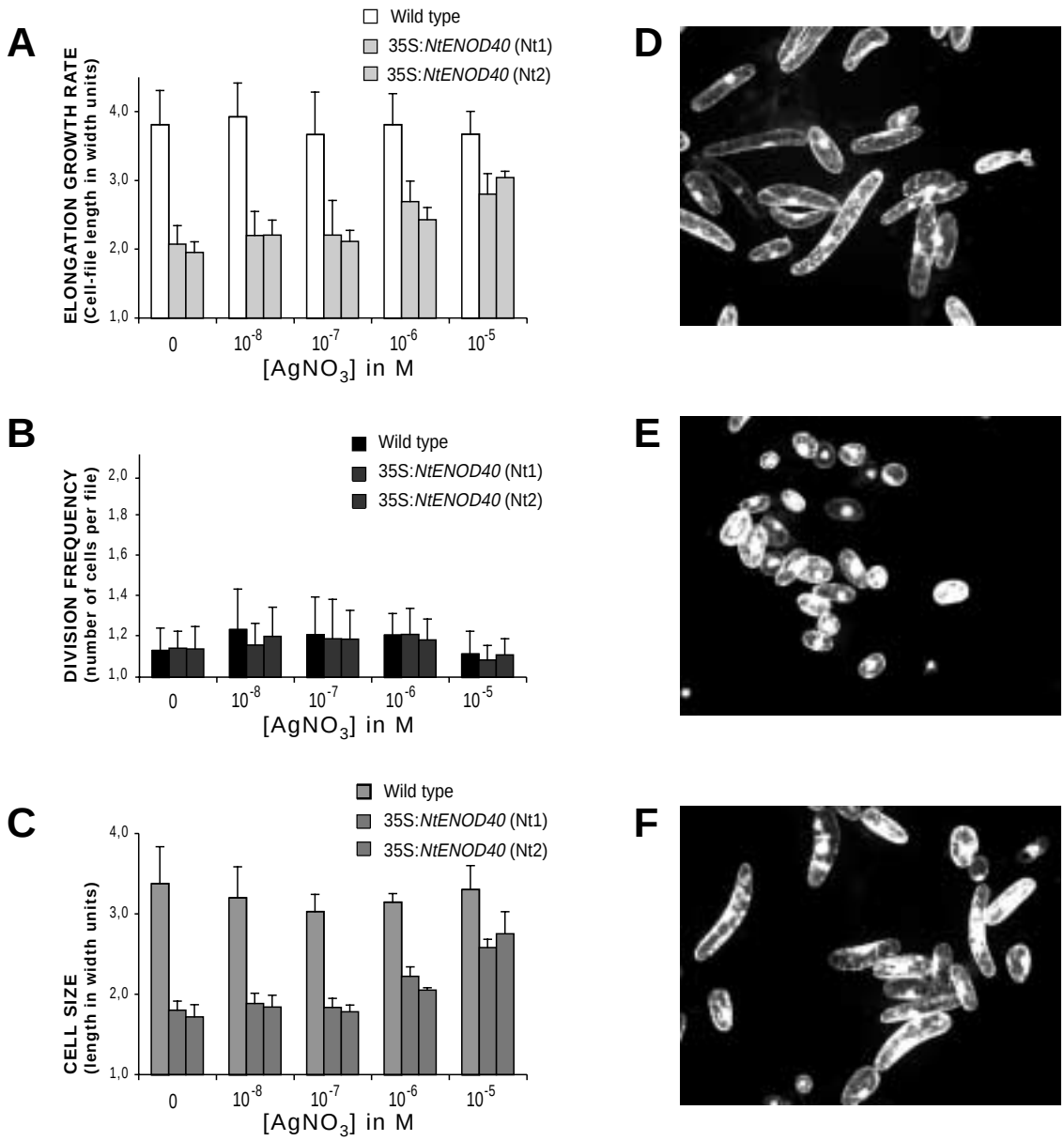


Figure 2.4. Recovery of Elongation Growth of 35S:*NtENOD40* Cells by $AgNO_3$ Treatment.

Cells are cultured in the presence of 0.1 mg/l NAA, 1.0 mg/l BA and various concentrations of $AgNO_3$. Pictures are taken with a fluorescence microscope after four days of culture. FDA staining facilitates selection of viable protoplasts for measurements and aids object recognition with the NIH-image software for quantification of growth parameters. Data are average (±SD) of three independent experiments.
Left panels:
(A) Elongation growth rate in wild type and 35S:*NtENOD40* cell lines.
(B) Cell division frequency in wild type and 35S:*NtENOD40* cell lines.
(C) Cell size in wild type and 35S:*NtENOD40* cell lines.
Right panels: Representative photographs of populations of:
(D) Wild type cells.
(E) 35S:*NtENOD40* (Nt1) cells.
(F) 35S:*NtENOD40* (Nt1) cells cultured in the presence of 10 µM $AgNO_3$.

Ethylene Accumulation Is Regulated by *ACS* But Not *ACO* Expression

We wanted to know how ethylene biosynthesis is accelerated in 35S:*NtENOD40* lines. Ethylene biosynthesis is known to be regulated at different levels, including control of gene expression and post-translational regulation of ACC Synthase (*ACS*) and ACC Oxidase (*ACO*) (Wang et al., 2002). Therefore, we tested whether regulation of expression of these genes encoding enzymes involved in ethylene biosynthesis is affected by *ENOD40* expression. In order to compare *ACS* and *ACO* expression levels and ethylene accumulation during culture of protoplasts, cells of each line (wild type, Nt1 and Nt2) were harvested at day 0, 2 and 4 for RNA extraction and RT-PCR based *ACS* and *ACO* transcript quantification. In many plant species, both the ACC synthase and the ACC oxidase family comprise a small number of genes. We designed primers to highly conserved sequences, such that most likely all *ACS* mRNAs that are expressed in BY-2 cells can be amplified in a single RT-PCR reaction. Since regions of high sequence conservation also occur in the ACC oxidase gene families of different plant species, the same approach was taken for the ACC oxidase gene family. Although the sequence in between these primers is slightly variable, the distance between the primers is very similar in different members. Therefore, the RT-PCR products that are amplified using these primers are detected as a single band on an RT-PCR blot after hybridization with the respective probes (Methods).

Figure 2.3A shows a time-course of ethylene accumulation for the wild type culture and for two 35S:*NtENOD40* lines, Nt1 and Nt2. In Figure 2.3B, corresponding *ACS* and *ACO* transcript accumulation profiles are shown. In 35S:*NtENOD40* cultures, ethylene accumulates to maximal levels as early as day 2-3, whereas in wild type cultures ethylene accumulation does not take place before day 5. The maximal level of ethylene as well as the time at which the maximum is reached is comparable to the former experiments. In wild type cultures, *ACS* transcripts gradually accumulate during the 4-day culture period, and maximal *ACS* expression levels are found at day 4. In contrast, in 35S:*NtENOD40* lines the maximal *ACS* transcript level is found at day 0, directly after protoplast isolation, and gradually decreases during the culture period (Figure 2.3B).

These results show that in 35S:*NtENOD40* lines *ACS* transcripts accumulate at an earlier time point and this is consistent with the accelerated ethylene production. In wild type as well as in 35S:*NtENOD40* cultures, *ACS* transcript accumulation precedes ethylene production. Further, the maximal level of *ACS* expression, as well as the maximal level of ethylene accumulation is similar in wild type and 35S:*NtENOD40* lines but the timing is different.

In wild type protoplast cultures, *ACO* transcripts are present directly after protoplast culture has started, and their level only slightly increases during the 4-day culture period. The *ACO* transcript accumulation profile in both 35S:*NtENOD40* lines is similar to that in wild type (Figure 2.3B). The temporal regulation of *ACO* transcript accumulation does not correlate with the timing of ethylene production in the different lines. Thus, these data indicate that *ACO* expression levels are probably not rate-limiting for ethylene production in these cell lines and regulation of *ACO* transcript levels does not contribute to regulation of ethylene production in wild type and 35S:*NtENOD40* cultures. Since a tight correlation between ethylene biosynthesis and *ACS*, but not ACO, transcript accumulation is found, regulation of ethylene biosynthesis can be largely attributed to transcriptional regulation of *ACS*.

It is known that different members of the *ACS* family are differentially expressed. Therefore, we wanted to know whether the expression of a specific *ACS* member is altered in 35S:*NtENOD40* cells. By using a 3' RACE-PCR based approach, we cloned partial 3' cDNA sequences of *ACS* genes that are expressed in elongating BY-2 cells (Methods). In total six different genes were identified. Based on their 3' UTR sequences, cDNAs could be pooled in three major groups in which members have more than 92% sequence similarity between clones within the group. One group includes sequences highly homologous to *NtACS*. The second group consists of sequences that are highly homologous to *NtACS2*. The third group is only represented by *NtACCS2*. Since we were not able to design specific primers for each of the different transcripts separately (except *ACCS2*), reverse primers were designed on the 3' UTR for the three ACC synthase cDNA groups. RT-PCR analysis indicated that the expression level of *ACCS2*

transcripts is much higher than that of any other *ACS* gene. Furthermore, the *ACCS2* expression profile closely reflects the total *ACS* transcript profile, suggesting that *ACCS2* transcripts predominantly represent ACC synthase expression. Furthermore, the increase of *ACCS2* expression occurs at an earlier time point in 35S:*NtENOD40* cultures than in wild type cultures, indicating that overexpression of *NtENOD40* affects *ACCS2* expression kinetics.

Next, we wanted to know whether altered regulation of *ACCS2* leads to reduced elongation growth in BY-2 protoplasts. Stably transformed BY-2 cell lines carrying the full size *ACCS2* cDNA driven by the CaMV 35S promoter were generated. Four independent lines were tested in the bioassay for elongation growth and cell division during culture in the presence of 1.0 mg/l BA and 0.1 mg/l NAA. These lines showed a reduction of elongation growth rate by at most about 50 % of wild type levels, whereas no severe effects on cell division frequency were observed (data not shown). Hence, their phenotype closely resembles the phenotype of cell lines that overexpress *ENOD40*. Further, this observation is consistent with the proposed hypothesis that ethylene is a negative regulator of elongation growth in BY-2 cells. Taken together, our data strongly suggest that altered regulation of elongation growth in 35S:*NtENOD40* lines is caused by accelerated expression of *ACCS2*.

DISCUSSION

In this study we showed that overexpression of *ENOD40* reduces BY-2 cell size by suppressing elongation growth, whereas the cell division frequency is unaffected. These observations offered the possibility to examine the relation between the *ENOD40* induced effect and the effect of cytokinin and auxin on elongation growth and cell division frequency in BY-2 cells. The protoplast bioassay developed by Hasezawa (1983) was originally used to determine experimental conditions that are optimal for uniform semi-synchronous elongation growth in single cell populations. In this report we have used the morphological response to phytohormones in a bioassay to study *ENOD40* activity. In this bioassay, elongation growth rate and cell division frequency are

morphological growth parameters that can be quantified in dose response curves for phytohormones, and now form the basis for an experimental system to test the effect of *ENOD40* on phytohormone signaling.

Auxin / cytokinin dose response curves showed that in wild type cells auxin induces cell division, while cytokinin lowers the number of cell divisions. The opposite effects of cytokinin and auxin explain why the division rate depends on the balance between these hormones in wild type BY-2 cells. Our observations are consistent with studies reported by Hasezawa (1983). When auxin is omitted from the culture medium, cell division ceases and cell differentiation sets in, associated with morphological changes in the golgi, an accumulation of starch, and an increase in cell length (Miyazawa et al., 1999; Winicur et al., 1998). BY-2 cells do not contain sufficient endogenous auxins to sustain their growth, since they are unable to divide in the absence of exogenous 2,4D (Ishida et al., 1993) or 1-NAA (Figure 2.2D). BY-2 cells are cytokinin autotroph. Specific cytokinins were shown to transiently accumulate at G_2/M and (although to lower levels) at G_1/S phase in synchronized BY-2 cells, indicating a regulatory role at those specific phases of cell cycle progression (Laureys et al., 1998; Laureys et al., 1999). It has been proposed that cytokinins modulate the activity of cell cycle regulatory components (Geelen and Inze, 2001). It is likely that down-regulation of cytokinin activity at specific phases of the cell cycle is equally important for progression as is transient accumulation. This could explain the observation that exogenous cytokinin is inhibiting for cell division activity as increasing concentrations of exogenous cytokinin may interfere with the control of endogenous cytokinin levels (Laureys et al., 1999). We found that *ENOD40* does not affect phytohormone control on cell division frequency, since in 35S:*NtENOD40* cells the cell division frequency at various cytokinin / auxin ratios is similar to that in wild type BY-2 cells.

ENOD40 Accelerates Ethylene Biosynthesis and Ethylene Acts As A Negative Regulator of Elongation Growth in BY-2 Cells

Three lines of evidence in our study indicate that suppressed elongation growth in 35S:*NtENOD40* cells is primarily caused by acceleration of ethylene accumulation. Firstly, we found a temporal correlation between ethylene accumulation and reduced elongation growth during culture of wild type cells and transgenic cells that overexpress *ENOD40*. Wild type cells begin to elongate 1-2 days after subculture and elongate most rapidly between day 3 and 7. Elongation then ceases and stops completely after day 9 (Hasezawa and Syono, 1983). In wild type cells, maximal ethylene accumulation is reached at day 5-6, whereas in 35S:*NtENOD40* lines similar maximal ethylene levels occur but this takes place as early as day 3. These data suggest that the duration (and therefore degree) of elongation growth can be controlled by the timing of ethylene accumulation, but without necessarily changing the maximal level of ethylene accumulation.

Secondly, recovery of elongation growth in 35S:*NtENOD40* lines can be achieved by application of $AgNO_3$ or 1-MCP, both blockers of ethylene action at the ethylene receptor. In the wild type cultures these treatments do not affect elongation growth. These data strongly suggest that accelerated ethylene production in 35S:*NtENOD40* cells is a primary cause and not a consequence of the reduced elongation growth and this shows that ethylene is a negative regulator of elongation growth. This is consistent with the absence of an effect of ethylene perception blockers on elongation growth of wild type protoplasts within 5 days of culture, since ethylene does not accumulate to high levels in wild type cultures until day 5-6. The observation that elongation growth of 35S:*NtENOD40* cells cannot be fully restored to wild type levels indicates that ethylene action may not be completely blocked and/or that ethylene accumulation is not the only signal that regulates elongation growth of these cells.

Thirdly, our data showed that the mRNA accumulation profile of a specific member (*NtACCS2*) of the *ACC synthase* family correlates with the respective ethylene

accumulation curves in wild type and in 35S:*NtENOD40* cells. Since *ACO* mRNA levels are unaffected in 35S:*NtENOD40* lines, it is likely that regulation of ethylene production can be mainly attributed to transcriptional regulation of *NtACCS2*. Elongation growth is reduced in transgenic 35S:*NtACCS2* lines, whereas the cell division frequency is not affected. This phenotype strongly resembles the morphological changes induced by overexpression of *ENOD40* and this confirms that ethylene accumulation negatively regulates elongation growth in BY-2 cells. Together, these data show that *ENOD40* provoked effects on elongation growth involve altered transcriptional regulation of *ACCS2*.

Coupling of Processes Implies Coupling of Regulating Pathways

The quantitative analysis of the effects of cytokinin, auxin and ethylene on cell division and cell elongation growth indicate that at least two phytohormone dependent signaling pathways exist that together control morphology in BY-2 cells. We found that ethylene acts as a negative regulator of elongation growth, but does not affect the cell division frequency under these conditions. In contrast, the cytokinin to auxin ratio controls cell division frequency, independently of elongation growth in the concentration range tested. Since in wild type cells these processes can be influenced independently of each other under changing cytokinin/auxin regimes, this indicates that the processes of elongation growth and cell division are not strictly coupled in wild type BY-2 cells. In contrast, elongation growth and cell division are not independent in the presence of *ENOD40* since our assays showed that a strict coupling of the elongation growth rate to the cell division frequency occurs in 35S:*NtENOD40* cells. Our results suggest that *ENOD40* and cytokinin act synergistically in suppressing elongation growth and that the negative effect of *ENOD40* on elongation growth is counter-acted by auxin (Figure 2.2). Since the cytokinin/auxin dependent pathway primarily regulates cell division frequency and the ethylene dependent pathway regulates elongation growth, we hypothesize that coupling of the two processes relies on cross-talk between ethylene and cytokinin/auxin signaling pathways and that this provides a mechanism to regulate cell size. The

observations that the reduction of elongation growth in *ENOD40* cells is, at least in part, dependent on the cytokinin to auxin ratio and that the reduction of elongation growth is mediated by accelerated ethylene accumulation in 35S:*NtENOD40* lines support this hypothesis.

Coordinated control of cell division and cell growth determine cell shape and patterning during plant growth and development. Although little is known about the mechanism that connects these processes during plant development, our studies revealed that *ENOD40* and phytohormones are likely involved in this mechanism. Cytokinin and auxin together control cell division and cell growth rates in the root meristem (Beemster and Baskin, 2000). Additionally, it has been postulated that ethylene levels act as a local 'fine-tuner' of cell size by controlling the rate of elongation growth in cells in the elongation zone of the *Arabidopsis* root (Le et al., 2001). These BY-2 protoplast assays provide a novel experimental system to validate the function of candidate components that affect regulation of cell growth and division.

Our studies suggest that transcriptional regulation of a specific *ACS* is part of the molecular mechanism that underlies cross-talk between phytohormone signaling pathways. Many reports have described that ACC Synthase and/or ACC Oxidase regulate ethylene production in response to diverse environmental or developmental stimuli (Johnson and Ecker, 1998). *ACS* and *ACO* both are multigene families comprising a small number of genes. Their expression is known to co-localize with ethylene production, and mRNA expression levels correlate with ethylene production levels as the same inducers that stimulate ethylene production induce *ACS* and/or *ACO* expression. Each of the members is induced by a small specific array of inducers including auxin and cytokinin (Johnson and Ecker, 1998; Van Der Straeten and Van Montagu, 1991; Wang et al., 2002). For example, it was shown that other hormones do not induce the auxin inducible *Vigna radiata ACS6* promoter, but that they greatly modified the response to auxin (Yoon et al., 1999). Taken together, these observations suggested that cross-talk between phytohormone and other signaling pathways takes place at the level of transcriptional regulation of different *ACS* (or in some cases *ACO*)

family members. Our data showing that regulation of one *ACS* gene is specifically affected in 35S:*NtENOD40* lines is consistent with this model. However, *ENOD40* is not simply a trigger of *ACS* expression, as indicated by the *ACS* transcript accumulation curves. Instead, we propose that altered regulation of *ACS* expression is part of the mechanism that leads to phytohormone cross-talk. It will be interesting to find out if and how *ENOD40* can attenuate promoter activity of *ACCS2*, and which other inducers are able to regulate transcriptional activity of this specific promoter. Such studies may provide clues on how *ENOD40* is integrated with developmental, hormonal or environmental cues in order to affect ethylene biosynthesis.

Role of *ENOD40* in Nodulation

Our data in BY-2 cells provide experimental evidence that *ENOD40* interferes with cytokinin, auxin and ethylene signaling pathways and suggests that *ENOD40* couples phytohormone dependent processes through an unknown mechanism. Thus, our studies in a cellular system are in support of the hypothesis that *ENOD40* acts in several developmental processes in order to fine-tune phytohormone signaling (Crespi et al., 1994; van de Sande et al., 1996). In nodulation, a regulatory role of *ENOD40* has been suggested based on its highly regulated expression pattern (Compaan et al., 2001; Yang et al., 1993), and on the nodulation phenotypes observed in plants with perturbed *ENOD40* expression (Charon et al., 1999). Based on our BY-2 studies we propose that during nodulation *ENOD40* changes the coupling between cytokinin, auxin and ethylene signaling pathways. During early stages of nodulation, *ENOD40* expression is induced by Nod factors in pericycle cells opposite the protoxylem poles as early as 3 hrs after inoculation. Subsequently, expression is found in inner cortical cells during nodule primordium initiation and development. So, if the function of *ENOD40* is to couple phytohormone dependent processes, what are the processes that have to be coupled in the cells that express *ENOD40*? Prior to answering this question, we need to address what is the function of these phytohormones in the cells that express *ENOD40*.

The *ACO* expression pattern suggests that ethylene is predominantly produced opposite phloem poles, whereas cortical cell divisions are specifically induced opposite protoxylem poles (Heidstra, Yang et al. 1997). The correlation between the *ACO* expression pattern and the location of cortical cell division suggests that ethylene is a negative regulator of cortical cell division, and that localized ethylene production provides positional information for the location of the nodule primordium. The causal role of ethylene distribution in the positioning of cortical cell divisions is supported by biochemical studies using blockers of ethylene biosynthesis or perception which showed that when ethylene production or perception were suppressed nodules were also formed opposite phloem poles (Heidstra, Yang et al. 1997). Genetic evidence is provided by the ethylene insensitive mutant *sickle* in which primordia also form opposite phloem poles (Penmetsa and Cook, 1997).

In both wild type and 35S:*NtENOD40* lines, ethylene is produced at a concentration that does not affect the cell division frequency under the conditions that we used, as shown by the absence of an effect of $AgNO_3$ application on the cell division frequency, but instead is involved in the control of elongation growth of cells. The negative effect of ethylene on root cortical cell division is different, from the role of ethylene in the control on elongation growth that we observed in BY-2 cells and is likely provoked at higher concentrations. The *ACO* expression pattern suggests that *ENOD40* expressing cells in the inner cortical cell layers, opposite protoxylem poles have low ethylene production. Based on our observations in BY-2 cells, we propose that in these cells, ethylene could be involved in the regulation of elongation growth.

It was shown that exogenous application of cytokinin could induce nodule formation (Cooper and Long, 1994). Upon inoculation with *Rhizobium*, or Nod factor treatment, auxin transport is inhibited and auxin accumulates transiently in the inner cortical cells that are located opposite protoxylem poles as shown using auxin reporter lines. Treatment of roots with auxin transport blockers can induce cell division and pseudo-nodule formation in the inner cortex (Hirsch et al., 1989). Taken together, these findings suggested that it is unlikely that either the cytokinin or the auxin level alone determines

the conditions for nodule initiation, but that the ratio of cytokinin to auxin levels are important for nodule initiation. So, a combination of positively acting factors like uridine (Smit et al., 1995) and the auxin to cytokinin ratio and negatively acting signals like ethylene, control induction of cortical cell divisions. Each of these factors has a specific distribution in the root and this leads to induction of cortical cell divisions at the right place. It seems plausible that coordinated control of cell division and cell growth is important during formation of the nodule primordium and that phytohormones play an important role in these stages of the developmental program. In line with the observation that *ENOD40* couples the phytohormone dependent relative rates of elongation growth and cell division in BY-2 cells, we propose that *ENOD40* couples phytohormone signaling pathways in order to closely coordinate growth in the dividing cells of the nodule primordium. *ENOD40* expression by itself is not sufficient to induce nodule primordium formation indicating that *ENOD40* is not a key-regulator of the participating processes. However, *ENOD40* function may be required to mediate cross-talk between phytohormone signaling pathways during development.

METHODS

Construction of Binary Vectors p35S:*NtENOD40* and p35S:*ACCS2*

Nicotiana tabacum contains two *ENOD40* homologs that are 96% identical at the nucleotide level (Matvienko et al., 1996). The Cauliflower Mosaic Virus 35S promoter was used for ectopic expression of *NtENOD40*-1. To this end, the 35S promoter from pMON999 (Monsanto) was transferred to pCambia 1390 (Cambia, Australia) yielding p35S:Tnos. A 470 bp PCR fragment corresponding to the *NtENOD40*-1 cDNA sequence was then cloned in p35S:Tnos using primers
5'-GCTCTAGACTAGCTTGTCTCAAGAAC-3' and
5'-CGGGATCCATGACAATCTTAACAACTCTAT-3'.
The full size *ACCS2* cDNA sequence (1568 bp) was cloned from cDNA that was prepared from total RNA obtained from elongating BY-2 cells and was transferred to p35S:Tnos using primers
5'- GCTCTAGAGGCACGAGGAGAAGATG-3', and
5'- CGGGATCCGTGGTTAAGACTTGATTATTC-3'.
The resulting constructs were introduced in *Agrobacterium tumefaciens* strain C58C1.

Liquid BY-2 Cultures and BY-2 Transformation

Nicotiana tabacum BY-2 cell suspensions were sub-cultured weekly by 40 times dilution in fresh medium (Nagata et al., 1981). BY-2 transformation was performed using a modification of the procedure reported by Gu and Verma (1997). Five ml of a 3 day-old BY-2 cell suspension was co-cultivated for 2 days at 25ºC in the dark with 60 µl of log-phase *Agrobacterium*, harboring the binary vectors. Cells were then washed three times with fresh medium containing 200 µg/l ticarciline/clavuline and plated on agar solidified selection plates containing the normal culture medium supplemented with 0.8 % Daishin Agar, 200 µg/l ticarciline/clavuline and 40 µg/l Hygromycin B. Individual transgenic calli that appeared after 3-4 weeks, were first cultured on fresh selection plates for one more week, and were subsequently transferred to liquid selection medium to initiate suspension cultures from independent transgenic calli. Transgenic lines were continuously maintained in selection medium.

Protoplast Isolation

Protoplasts were obtained from 6 day-old suspension cultures using 1% Cellulase-YC and 0.1% Pectolyase Y23 in 0.4 M D-mannitol, pH 5.5 (Nagata et al., 1981). Cells were incubated in the enzyme solution for 3 hours at RT, filtered through 63 µm nylon mesh, washed two times with 0.4 Osm KCl, purified over a one-step (18% w/v) sucrose gradient and subsequently washed three times with Protoplast Culture Medium (PCM) containing 4.3 g/l MS salts (without vitamins) supplemented with 1 mg/l Thiamine-HCl, 100 mg/l Myo-inositol, 10 g/l sucrose, 255 mg/l KH_2PO_4 and 0.4 M D-mannitol at pH 5.7. Elongation growth inducing PCM contained 0.1 mg/l 1-Naphthalene-acetic acid (1-NAA) and 1.0 mg/l Benzyl-adenine (BA). For the dose response curves, protoplasts were cultured in PCM supplemented with various concentration of NAA and/or BA as indicated in the text and/or graphs. Protoplasts were cultured in 3 ml liquid medium at a density of approximately 10^5/ml in small sealed petridishes at 25ºC in the dark (Kuss and Cyr, 1992).

Protoplast Assay Growth Parameter Measurements

Cell size measurements were performed on random photographs of the protoplast derived cells after four days of culture. Viable protoplasts were selected for measurements after FDA (fluorescein-diacetate) staining. Fluorescent images were captured using a cooled CCD camera

mounted on a Leica DMR microscope with a 20x objective. The digital fluorescent images facilitated computer based morphometric measurements using the NIH-IMAGE program (http:/rsb.info.nih.gov/nih-image) in which objects can be contoured by applying the invert/threshold option. Cell-file length, cell width, and number of cells/file were scored for 100-150 cells per sample. The mean cell-file length as well as the mean division frequency was then calculated for each protoplast sample. Subsequently, the average elongation growth rate and cell division frequency for each line was determined as the average over values from independent repetitions at different dates. Also, the standard deviation between measurements at different dates is determined for each line. Significance is tested using a two-tailed students T test.

RNA Gel Blot Analysis

Total RNA was isolated using the TRIzol method (GibcoBRL). 16 µg of total RNA was subjected to electrophoresis on a 1% agarose gel in 0.01 M NaH_2PO_4 (pH 7.0) using the glyoxal/DMSO method. RNA was subsequently transferred to a genescreen membrane in 20xSSC. RNA gel blots were hybridized with radiolabeled PCR fragments of the respective transcripts in formamide hybridization buffer O/N at 42ºC. Autoradiograms were obtained using a Molecular Dynamics Phosphorimager (Sunnyvale, CA).

Reverse Transcriptase-Mediated PCR

Total RNA was isolated using the TRIzol method (GibcoBRL). After DNaseI (Promega) treatment to remove chromosomal DNA that could disturb the PCR reactions, cDNA is synthesized from 2.5 µg of total RNA 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 oligod$T_{(12)}$V anchor primer, 20 U RNA guard (Pharmacia) and 200 U MuMLV reverse transcriptase (Stratagene). The samples were incubated for 1 hr at 37ºC. To inactivate the reverse transcriptase enzyme the samples were incubated at 95ºC for 5 minutes. The RT samples were then diluted to 100 µl and 1 or 2 µl of the cDNA were used for PCR analysis (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.
RACE-PCR on *ACS* transcripts was as follows: cDNA is synthesized from RNA isolated from the wild type BY-2 cell suspension, using the RACE-T anchor primer 5'-CATCTAGAGGATCGAATTC-$T_{(16)}$-3'. The first PCR cycles were: 94°C for 5 min; 3 cycles of 94°C for 1 min, 54°C for 1 min, 72°C for 1 min then 28 cycles of 94°C for 1 min, 50°C for 1 min

and 72°C for 1 min and a final extension at 72°C for 5 min, run with primers: RACE-A: 5'-CATCTAGAGGATCGAATTC-3' and 5'- GTTGTTCTTTTCATTGTTC-3'. The second PCR is run with primers RACE-A and *ACS*-3'race: 5'-GGTTGGTTTAGAGTTTGTTT-3', 94°C for 5 min; 30 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1 min and then a final extension at 72°C for 5 min. After the second PCR, the total RACE-PCR product mixture was purified using a PCR purification kit (Boehringer) and was cloned in pGEM-T (Promega). From a total set of 28 cloned cDNAs, 18 representative clones were sequenced in both directions.
RACE-PCR on *NtENOD40* transcripts of RNA isolated from the transgenic lines was identical to RACE-PCR on *ACS*, except primers and PCR cycles. First run; RACE-A primer, reverse primer: 5'-CGGGATCCTAGTTGGAGTGAATTAAGGA-3', second run; RACE-A primer, reverse primer: 5'-AAGCTTTTGGAGTCTTTCTTGGCCTTT-3'. Both PCR cycles were as follows: 94°C for 5 min; 30 cycles of 94°C for 20 sec, 50°C for 20 sec, 72°C for 30 sec and a final extension at 72°C for 5 min.

Specific primers are designed for reverse transcriptase mediated (RT) PCR based transcript quantification for each of the analyzed genes. Specificities of the primer sets were verified by sequencing the RT-PCR products. The number of PCR cycles was adapted to the linear range of the PCR amplification reaction for each gene, corresponding to the relative expression levels. All samples were normalized on *ubiquitine* levels. Primers that were used for RT-PCR are: *UBI*-f: 5'-ATGCAGAT(C/T)TTTGTGAAGAC-3'; *UBI*-r: 5'-ACCACCACG(G/A)AGACGGAG-3', General *ACS* primers: *ACS*-f: 5'-GATTTAATACAAGAATGGG-3' / *ACS*-r: 5'-GAACAATGAAAAGAACAAC-3'; *NtACO*f: 5'-GGGCTTCTTTGAGTTGGTG-3' / *NtACO*r: 5'-CTCCGCTGCCTCTTTCTC-3'. Primer combinations for specific *ACS* members: *ACS*-3'race / *ACCS*2-r : 5'-AAAGAAAAAGAAACATTACAAG-3'; *ACS*-3'race / *ACS 2*-r: 5'-TCCCATTTTGATACACTTTAC-3'; *ACS*-3'race / *ACS1*-r: 5'-TTCTTTTCCTTTATCTTCTTC-3'. Amplified DNA fragments were run on a 1% agarose gel, alkaline blotted to Hybond-N^+ membrane (Amersham Pharmacia) and hybridized to radiolabelled PCR fragments of the corresponding cDNA clones. Autoradiograms were obtained by using a Molecular Dynamics Phosphorimager (Sunnyvale, CA).

Ethylene Measurements

For each line, protoplasts were divided over six petridishes at the start of the experiment and were cultured in parallel. Each petridish was sampled every 24 hrs during 8 days in 5 independent experiments. A gas sample of 1 ml from a total of 30 ml headspace volume was

used for analysis on a gas chromatograph. In order not to severely alter accumulating ethylene levels, gas samples were taken with a syringe through a rubber gasket in the lid of the petridish without opening the sealed petridishes. Ethylene concentration was determined by standard GC-analysis on a gas chromatograph equipped with an alumina column and a flame ionisation detector (Gilissen and Hoekstra, 1984). Ethylene accumulation at each time point was determined as the average ethylene concentration in the headspace of these six cultures.
1 mg of 1-MCP was freshly dissolved in 10 ml of water in a sealed 600 ml bottle with a rubber gasket to give a stock concentration of 1000 ppb. At the start of the culture period, 1 ml volumes of serial dilutions of 1-MCP were transferred to the headspace of sealed petridishes containing the protoplasts by injection through a rubber gasket in the lid of the petridish, to give the appropriate final concentration of 1-MCP in the headspace. $AgNO_3$ was freshly dissolved in water to a stock concentration of 1 mM. To give the appropriate final concentrations of $AgNO_3$, 30 μl of a serial dilution was transferred to the culture medium containing the protoplasts just before sealing the petridishes at the start of the culture period.

Accession Numbers

The accession numbers are *NtENOD40-1* (X98716); *NtACS1* (X65982); *NtACS2* (AJ005002) and *NtACCS2* (X98492).

ACKNOWLEDGMENTS

We thank Danny Geelen (University of Gent, Belgium) for kindly supplying the BY-2 cell suspension. We gratefully acknowledge Gerard van der Krogt (Dutch Cancer Institute) for his valuable contribution to the BY-2 transformations and the excellent technical assistance of Ciska Braam and Maelle Lorvellec. We are thankful to Mark Hink and Jan-Willem Borst (Wageningen University Microscopy Center) for use of the microscope facilities. T.R. is supported by the Netherlands Organization for Scientific Research (NWO 805.49.004).

REFERENCES

Asad, S., Fang, Y., Wycoff, K.L., and Hirsch, A.M. (1994). Isolation and characterization of cDNA and genomic clones of *MsENOD40*; Transcripts are detected in meristematic cells of alfalfa. Protoplasma **183,** 10-23.

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

Beemster, G.T., and Baskin, T.I. (2000). Stunted plant 1 mediates effects of cytokinin, but not of auxin, on cell division and expansion in the root of *Arabidopsis*. Plant Physiol. **124,** 1718-1727.

Charon, C., Johansson, C., Kondorosi, E., Kondorosi, A., and 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., and Kondorosi, A. (1999). Alteration of *ENOD40* expression modifies *Medicago truncatula* root nodule development induced by *SinoRhizobium meliloti*. Plant Cell **11,** 1953-1965.

Chen, J.-G., Shimomura, S., Sitbon, F., Sandberg, G., and Jones, A.M. (2001). The role of auxin-binding protein 1 in the expansion of tobacco leaf cells. Plant J. **28,** 607-617.

Collings, D.A., Asada, T., Allen, N.S., and Shibaoka, H. (1998). Plasma membrane-associated actin in Bright Yellow-2 tobacco cells. Evidence for interaction with microtubules. Plant Physiol. **118,** 917-928.

Compaan, B., Yang, W.C., Bisseling, T., and 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.

Cooper, J.B., and Long, S.R. (1994). Morphogenetic Rescue of *Rhizobium* meliloti Nodulation Mutants by trans-Zeatin Secretion. Plant Cell **6,** 215-225.

Crespi, M.D., Jurkevitch, E., Poiret, M., d'Aubenton-Carafa, Y., Petrovics, G., Kondorosi, E., and 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.

Ecker, J.R. (1995). The ethylene signal transduction pathway in plants. Science **268,** 667-675.

Geelen, D.N.V., and Inze, D. (2001). A Bright Future for the Bright Yellow-2 Cell Culture. Plant Physiol. **127,** 1375-1379.

Gilissen, L.J.W., and Hoekstra, F.A. (1984). Pollination-induced corolla wilting in Petunia hybrida: rapid transfer through the style of a wilting-inducing substance. Plant Physiol. **75,** 496-498.

Gu, X., and Verma, D.P.S. (1997). Dynamics of phragmoplastin in living cells during cell plate formation and uncoupling of cell elongation from the plane of cell division. Plant Cell **9,** 157-69

Hasezawa, S., Hogetsu, T., and Syono, K. (1988). Rearrangement of cortical microtubules in elongating cells derived from tobacco protoplasts - A time-coarse observation by immunofluorescence microscopy. Plant Physiol. **133,** 46-51.

Hasezawa, S., and Syono, K. (1983). Hormonal Control of Elongation of Tobacco Cells Derived from Protoplasts. Plant Cell Physiol. **24 (1),** 127-132.

Heidstra, R., Yang, W.C., Yalcin, Y., Peck, S., Emons, A.M., van Kammen, A., and Bisseling, T. (1997). Ethylene provides positional 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.

Herbert, R.J., Vilhar, B., Evett, C., Orchard, C.B., Rogers, H.J., Davies, M.S., and Francis, D. (2001). Ethylene induces cell death at particular stages of the cell cycle in the tobacco TBY-2 cell line. J. Exp. Bot. **52,** 1615-1623.

Hirsch, A.M., Bhuvaneswari, T.V., Torrey, j.G., and Bisseling, T. (1989). Early nodulin genes are induced in alfalfa root ougrowths elicited by auxin transport inhibitors. Proc. Natl. Acad. Sci. USA **86,** 1244-1249.

Hua, J., Chang, C., Sun, Q., and Meyerowitz, E.M. (1995). Ethylene insensitivity conferred by *Arabidopsis* ERS gene. Science **269,** 1712-1714.

Ishida, S., Takahashi, Y., and Nagata, T. (1993). Isolation of cDNA of an auxin-regulated gene encoding a G protein beta subunit-like protein from tobacco BY-2 cells. Proc. Natl. Acad. Sci. USA **90,** 11152-11156.

Iwata, K. (1995). Effects of reduction of auxin and destruction of microtubules on cell wall proteins and cell morphology of the BY-2 line of tobacco cells. J. Plant Res. **108,** 469-476.

Johnson, P.R., and Ecker, J.R. (1998). The ethylene gas signal transduction pathway: a molecular perspective. Annu. Rev. Genet. **32,** 227-254.

Kato, N., and Esaka, M. (2000). Expansion of transgenic tobacco protoplasts expressing pumpkin ascorbate oxidase is more rapid than that of wild-type protoplasts. Planta **210,** 1018-1022.

Kieber, J.J., Rothenberg, M., Roman, G., Feldmann, K.A., and Ecker, J.R. (1993). CTR1, a negative regulator of the ethylene response pathway in *Arabidopsis*, encodes a member of the raf family of protein kinases. Cell **72,** 427-441.

Kouchi, H., and Hata, 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.

Kuss, W.C.L., and Cyr, R.J. (1992). Tobacco protoplasts differentiate into elongate cells without new microtubule depolymerization. Protoplasma **168,** 64-72.

Laureys, F., Dewitte, W., Witters, E., Van Montagu, M., Inze, D., and Van Onckelen, H. (1998). Zeatin is indispensable for the G_2-M transition in tobacco BY-2 cells. FEBS Lett. **426,** 29-32.

Laureys, F., Smets, R., Lenjou, M., Van Bockstaele, D., Inze, D., and Van Onckelen, H. (1999). A low content in zeatin type cytokinins is not restrictive for the occurrence of G_1/S transition in tobacco BY-2 cells. FEBS Lett. **460,** 123-128.

Le, J., Vandenbussche, F., Van Der Straeten, D., and Verbelen, J. (2001). In the early response of *Arabidopsis* roots to ethylene, cell elongation is up- and down-regulated and uncoupled from differentiation. Plant Physiol. **125,** 519-22.

Lee, K.H., and LaRue, T.A. (1992). Exogenous ethylene inhibits nodulation of *Pisum sativum* L. cv Sparkle. Plant Physiol. **100,** 1759-1763.

Link, B.M., and Cosgrove, D.J. (1998). Acid-growth response and alpha-expansins in suspension cultures of Bright Yellow-2 tobacco. Plant Physiol. **118,** 907-916.

Mathesius, U., Charon, C., Rolfe, B.G., Kondorosi, A., and 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. MPMI **13,** 617-628.

Mattsson, J., Ckurshumova, W., and Berleth, T. (2003). Auxin signaling in *Arabidopsis* Leaf Vascular Development. Plant Physiol. **131,** 1327-1339.

Matvienko, M., van de Sande, K., Pawlowski, K., van Kammen, A., Franssen, H., and Bisseling, T. (1996). *Nicotiana tabacum* SR1 contains two *ENOD40* homologs. In: Biology Of Plant Microbe Interactions, G. Stacey, B. Mullin, and P. M. Gresshoff, eds. Int. Soc. MPMI. St. Paul USA, 387-391.

Minami, E., Kouchi, H., Cohn, J.R., Ogawa, T., and Stacey, G. (1996). Expression of the early nodulin, *ENOD40*, in soybean roots in response to various lipo-chitin signal molecules. Plant J. **10,** 23-32.

Miyazawa, Y., Sakai, A., Miyagishima, S.-y., Takano, H., Kawano, S., and Kuroiwa, T. (1999). Auxin and Cytokinin Have Opposite Effects on Amyloplast Development and the

Expression of Starch Synthesis Genes in Cultured Bright Yellow-2 Tobacco Cells. Plant Physiol. **121,** 461-469.

Mylona, P., Pawlowski, K., and Bisseling, T. (1995). Symbiotic Nitrogen Fixation. Plant Cell **7,** 869-885.

Nagata, T., Nemoto, Y., and Hasezawa, S. (1992). Tobacco BY-2 cell line as the He-la cell in the cell biology of higher plants. Int. Rev. Cytol. **132,** 1-30.

Nagata, T., Okada, K., Tabeke, I., and Matsui, C. (1981). Delivery of tobacco mosaic virus RNA into plant protoplasts mediated by reverse-phase evaporation vesicles (liposomes). Mol. Gen. Genet. **184,** 161-165.

Penmetsa, R.V., and Cook, R.V. (1997). A legume ethylene-insensitive mutant hyperinfected by its Rhizobial symbiont. Science **275,** 527-530.

Peters, N.K., and Crist-Estes, D.K. (1989). Nodule formation is stimulated by the ethylene inhibitor aminoethoxyvinylglycine. Plant Physiol. **91,** 690-693.

Redig, P., Shaul, O., Inze, D., van Montagu, M., and van Onckelen, H. (1996). Levels of endogenous cytokinins, indole-3-acetic acid and abscisic acid during the cell cycle of synchronized tobacco BY-2 cells. FEBS Lett. **391,** 175-180.

Ridge, I., and Osborne, D.J. (1971). Role of peroxidase when hydroxyproline-rich protein in plant cell walls is increased by ethylene. Nat. New. Biol. **229,** 205-208.

Rodrigues-Pousada, R.A., De Rycke, R., Dedonder, A., Van Caeneghem, W., Engler, G., Van Montagu, M., and Van Der Straeten, D. (1993). The *Arabidopsis* 1-Aminocyclopropane-1-Carboxylate Synthase Gene 1 Is Expressed during Early Development. Plant Cell **5,** 897-911.

Tamura, K., Liu, H., and Takahashi, H. (1999). Auxin induction of cell cycle regulated activity of tobacco telomerase. J. Biol. Chem. **274,** 20997-21002.

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., and Bisseling, T. (1996). Modification of phytohormone response by a peptide encoded by *ENOD40* of legumes and a nonlegume. Science **273,** 370-373.

Van Der Straeten, D., and Van Montagu, M. (1991). The molecular basis of ethylene biosynthesis, mode of action, and effects in higher plants. Subcell. Biochem. **17,** 279-326.

Shibaoka, H. (1994). Plant hormone-induced changes in the orientation of corical microtubules: Alterations in the cross-linking between microtubules and the plasma membrane. Annu. Rev. Plant Physiol. Plant Mol. Biol. **45,** 527-544.

Smit, G., Koster, C.C., Schripsema, J., Spaink, H.P., van Brussel, A.A., Kijne, J.W. (1995). Uridine, a cell division factor in pea roots. Plant Mol. Biol. **29,** 869-873.

Wang, K.L., Li, H., and Ecker, J.R. (2002). Ethylene biosynthesis and signaling networks. Plant Cell **14,** 131-151.

Winicur, Z.M., Zhang, G.F., and Staehelin, L.A. (1998). Auxin Deprivation Induces Synchronous Golgi Differentiation in Suspension-Cultured Tobacco BY-2 Cells. Plant Physiol. **117,** 501-513.

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

Yoon, I.S., Park, D.H., Mori, H., Imaseki, H., and Kang, B.G. (1999). Characterization of an auxin-inducible 1-aminocyclopropane-1- carboxylate synthase gene, *VR-ACS6*, of mungbean (*Vigna radiata* (L.) Wilczek) and hormonal interactions on the promoter activity in transgenic tobacco. Plant Cell. Physiol. **40,** 431-438.

Chapter 3

The Two Conserved Regions of *ENOD40* Transcripts Have Biological Activity and Are Connected In A Complex Manner

INTRODUCTION

A remarkable feature of *ENOD40* genes is the absence of a long open reading frame, but instead several small ORFs are in general present in *ENOD40* transcripts. In chapter 1 we described that two regions are highly conserved in all *ENOD40* transcripts, which argues for biological importance of these regions. From the short ORFs that are present in different *ENOD40* transcripts, only the ORF within the first conserved region (ORF I) is conserved and encodes an oligopeptide of 10-13 amino-acids. In region II, an ORF can be found that contains the MANRQVTKRQ peptide motive and we refer to this ORF as ORF II. However, in only about 50% of the *ENOD40* transcripts this ORF is present. Further, it is striking that this region is more conserved at nucleotide level than at amino-acid level (chapter 1). Despite that in some *ENOD40* sequences an ORF II exists, it seems more likely that region II is active at the RNA level. As a consequence, it is still under debate which *ENOD40* gene product - protein or RNA - has biological activity. If *ENOD40* activity is of proteinaceous nature, the peptide encoded by the conserved ORF I is the best candidate. So, is this small ORF translated *in vivo*?

For several *ENOD40* transcripts it has been shown that ORF I is translated in *in vitro* translation assays (Rohrig et al., 2002) and also *in vivo* by making use of reporter fusions (Charon et al., 1997; Compaan et al., 2001). Van de Sande (1996) detected an antigenic determinant in extracts of 15-day old soybean nodules using an antibody raised against a synthetic *GmENOD40* peptide which suggests that the putative peptide encoded by ORF I indeed is produced *in planta*. Additionally, some other ORFs in *Medicago truncatula* and soybean *ENOD40* transcripts could be translated. Because the peptides that are derived from these ORFs are not conserved, it is unlikely that these are required for biological activity of *ENOD40* genes. Thus, how many ORFs in *ENOD40* transcripts are translated *in planta*, and whether they have activity *in vivo* remains to be elucidated. It has been suggested that the ORF I derived oligopeptides are highly instable in plant tissues since degradation of corresponding synthetic peptides in plant extracts occurs in less than 1 minute (Sousa et al., 2001). Thus, using synthetic

peptides may not allow an analysis of their activity and a bioassay system to test activity of *ENOD40* constructs would provide a useful alternative.

A bioassay system has already been described to study which *ENOD40* gene product has activity (Charon et al., 1997; Sousa et al., 2001). This system is based on ballistic targeting of *ENOD40* (or -derivative) constructs in root epidermal and outermost cortical cells of *Medicago* roots. Transient expression of *ENOD40* leads to induction of inner cortical cell division. Since cell divisions are induced at a spatially separated site from the cells that are targeted with the construct, the effect of *ENOD40* is provoked in a non-cell autonomous manner, suggesting that the *ENOD40* product is transported or that a secondary signal is required (Charon et al., 1997). Mutating the translation start or stop of ORF I in the complete transcript reduced activity, whereas replacing the nucleotide sequence of this ORF with an altered nucleotide sequence in a construct retaining the same amino-acids did not affect activity of the transcript. This confirms that the translation product rather than the primary nucleotide sequence is important for biological activity. Likewise, a point mutation of the start codon of ORF II of *MtENOD40* abolishing its translation reduced the activity of *MtENOD40*, suggesting that translation products of both regions are biologically active. This is striking, since this ORF in region II is not absolutely conserved among plant species (chapter 1).

These experiments further showed that transient expression of truncated constructs encompassing either region I with the small ORF I or a 3' sequence carrying region II evoked a response similar to that evoked by the complete transcript in *Medicago sativa* roots. Mutating the start codon of ORF I in a partial transcript spanning only region I, abolished the cell division inducing activity. This suggests that translation of this short ORF is required for activity in the absence of the downstream region. Similarly, mutating the start codon of ORF II in a construct spanning only the 3' region reduced activity of this transcript. Taken together, these data suggest that in the absence of the other region, either of the two regions is sufficient for biological activity of *ENOD40* and that the encoded peptides of ORF I and II are important for biological function. However, mutation of the start codon of ORF I in *MtENOD40* resulted in significantly reduced

activity in the complete transcript, although the activity of the 3' region was expected to be retained in this derivative. Thus, interaction between the two regions may be complex since this indicates that the activity of a complete *ENOD40* transcript is primarily caused by the peptide residing in region I and that in the presence of an ineffective ORF I, region II is no longer able to induce an effect, although region II by itself it is functional.

We have developed a novel bioassay based on the effect of overexpression of *ENOD40* in BY-2 cells (chapter 2). The effect of *ENOD40* on cell elongation growth and cell division has been tested in dose response curves in which different cell division frequencies can be induced by changing the hormone concentrations in the medium. These analyses showed that the strict coupling between these two processes in 35S:*NtENOD40* lines was primarily provoked by the negative effect of *ENOD40* on cell elongation growth. To study the effect of *ENOD40*-derived transcripts on this strict coupling would require the performance of these dose response curves for several individual lines for different constructs. As this would be too laborious and the effect of *ENOD40* is primarily caused by reduction of elongation growth, we decided to test the effect of *ENOD40*-derived transcripts at a specific concentration of cytokinin and auxin, which induces strong elongation growth. Since the negative effect of *ENOD40* on BY-2 cell elongation growth is enhanced by cytokinin and can be counter-acted by auxin, the most pronounced effect of *ENOD40* on elongation growth is obtained under culture conditions with a high cytokinin to auxin ratio. Under these conditions, overexpression of *ENOD40* also alters the timing of ethylene biosynthesis. Activities of *ENOD40* gene products could be scored by analyses of either of these effects. However, the temporal ethylene production curve has a broad maximum. Therefore, characterization of the ethylene accumulation profile is not very suitable for quantification, whereas quantifying elongation growth provides the least laborious bioassay to study *ENOD40* activity.

By preparing protoplasts from the BY-2 cell suspension, a population of single cells with a similar size is obtained. Since the vast majority of the cells remain attached to each other after division, the average number of cells per cell file reflects the number of cell divisions that took place during the incubation time, and this parameter is from hereon

called the ‘cell division frequency’. On the other hand, the width of cells remains similar to the diameter of protoplasts during culture. This means that no radial expansion growth occurs and the length (expressed in width units) of the cell files is a parameter for elongation growth of cells during the incubation period. This parameter is from hereon called the ‘elongation growth rate’. To determine whether the peptide encoded by ORF I and/or the nucleotide sequence of region II, or the peptide encoded by ORF II have biological activity, we constructed a series of *NtENOD40*-derived transcripts. In order to assay biological activity of these transcripts, we studied their effects in the BY-2 protoplast bioassay.

RESULTS

Six independent transgenic BY-2 cell lines carrying construct 35S:*NtENOD40* (Figure 3.1) were generated by *Agrobacterium*-mediated transformation and were named Nt1 to Nt6. Expression of the transgenes was detected by RNA gel blot analysis performed with total RNA isolated from these transgenic lines. In the wild type line, *NtENOD40* mRNA could not be detected, indicating a very low expression level of the endogenous *NtENOD40* gene. This analysis further showed that *NtENOD40* is overexpressed in all 35S:*NtENOD40* transgenic lines, except in line Nt6. In all transgenic lines *HPTII* transcripts can be detected (Figure 3.2A).

The protoplast bioassay data for these lines were obtained within the same set of experiments as for the lines described in this chapter, but have also been described in chapter 2. In short, in four out of six lines elongation growth was reduced by about 40-60% in comparison to that of the wild type, whereas the cell division frequency was unaffected. Elongation growth is most strongly reduced in cell lines containing a high expression level of 35S:*NtENOD40*, whereas elongation growth is not affected in lines containing low or undetectable levels of expression. So, a good correlation between 35S:*NtENOD40* expression levels and reduction of elongation growth was found. To exclude that introduction of vector sequences could provoke a similar response, the

control vector 35S:Tnos (Figure 3.1) was introduced in BY-2 cells. Three independent lines (named EV1, EV2 and EV3) were obtained. RNA gel blot analysis showed that 35S:*HPTII* was expressed to a high level in these transgenic lines (Figure 3.2B). A reduction of elongation growth of 15-25% in comparison to the wild type is observed in these lines. Although the negative effect on elongation growth of one of the lines (EV3) is statistically significant ($p<0.05$), the effect is not very strong. The division frequency of these lines is similar to that of the wild type (Table 3.1). Thus, neither elongation growth nor cell division frequency was severely affected in transgenic lines carrying a control vector.

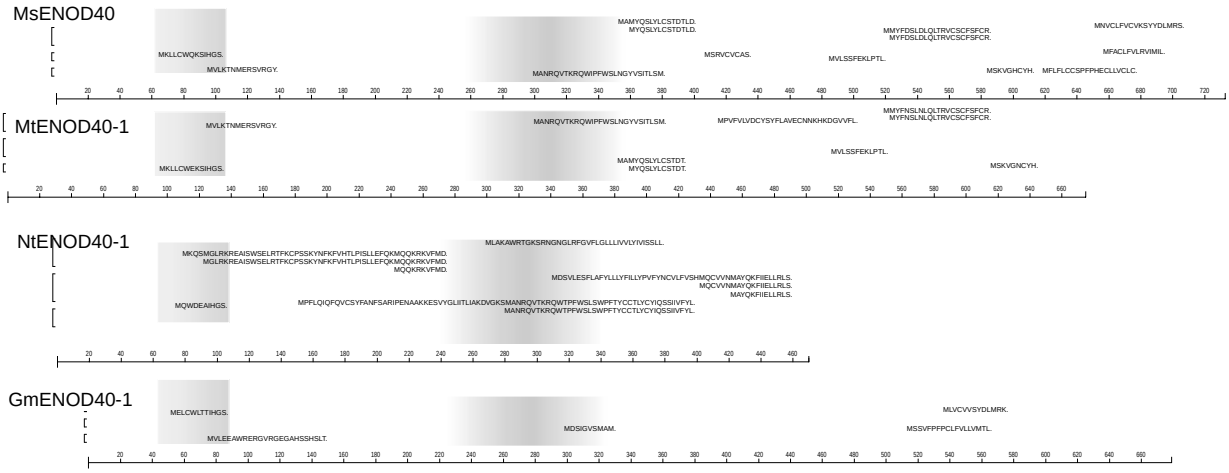
ORF I Is Translated But the Encoded Oligopeptide Is Not Essential for a Reduction of Elongation Growth

Two approaches were taken in order to test whether ORF I is functional and necessary for the reduction of elongation growth. We first determined whether ORF I is translated upon expression in stable transgenic BY-2 cells by using an *NtENOD40*-GFP reporter fusion. To this end, the GFP coding sequence without its own ATG was cloned in frame in the middle of ORF I, replacing the last six amino-acids. A schematic representation is given in Figure 3.1. Figure 3.3 shows the GFP fluorescence that was detected in a stable transgenic BY-2 cell line carrying the 35S:*NtENOD40*-GFP construct showing that ORF I is indeed translated. These data confirmed the results obtained by Compaan (2001).

Secondly, we tested whether the peptide has biological activity. We approached this by testing whether translation of ORF I is required for inducing a response in the protoplast assay. To this end, the ATG start codon of ORF I of *NtENOD40* was exchanged by AAG. Five independent lines carrying construct 35S:*NtENOD40*-AAG (Figure 3.1) were obtained. These lines were named 2, 6, 7, 8, and 49. RNA gel blot analysis showed that the lines express 35S:*NtENOD40*-AAG, but at different levels, and that *HPTII* transcripts were detected in all transgenic lines (Figure 3.2C). Cells from these lines were tested for

their ability to elongate and divide in the protoplast bioassay. The results showed that in these lines elongation growth is reduced to various degrees. In the lines with the strongest phenotype (2 and 6), elongation growth was reduced by about 50% of the wild type elongation growth. The elongation growth in 35S:*NtENOD40* lines is reduced by 40-60%. Thus, the degree of the elongation growth reduction induced by this transcript is comparable to that induced by the intact transcript. Thus, these results show that translation of ORF I is not required for the reduction of elongation growth. The average cell division frequency in wild type cells is 1.15. This means that on average about 15% of the cells in the population has undergone one round of cell division during the culture period. Strikingly, in some of the lines (2 and 6) only about 5-6 ± 4% of the cells divided during the culture time in comparison to 15 ± 9% in the wild type line, suggesting that cell division frequency is reduced in some of the 35S:*NtENOD40*-AAG lines whereas cell division frequency was not reduced in 35S:*NtENOD40* lines. This suggests that when the function of one region is tested by mutation in a construct containing both regions the observed reduction of cell division frequency is a gain of function since this effects was not observed using the intact construct. Since an additional effect is observed when translation of ORF I is disrupted, this indicates that the ORF I encoded peptide does have a biological function. These observations also indicate that the activity of the two regions are interdependent and that regulation of the activities of these two regions is complex.

A



B

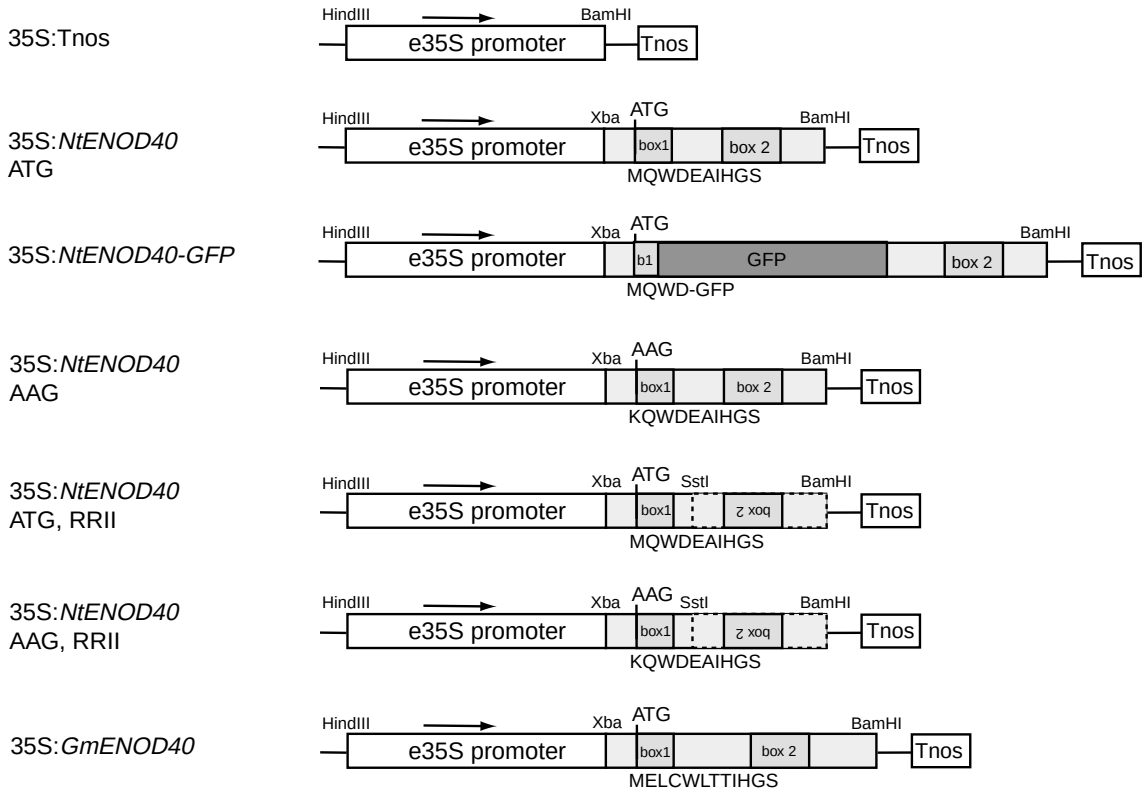


Figure 3.1.

(A) Comparison of ORFs in *MtENOD40*-1*, MsENOD40*, *NtENOD40*-1 and *GmENOD40*-1 transcripts shows the absence of a conserved ORF in region II of the *GmENOD40* -1 transcript.
(B) Constructs used for overexpression of *ENOD40* (-derived) transcripts in BY-2 cells.

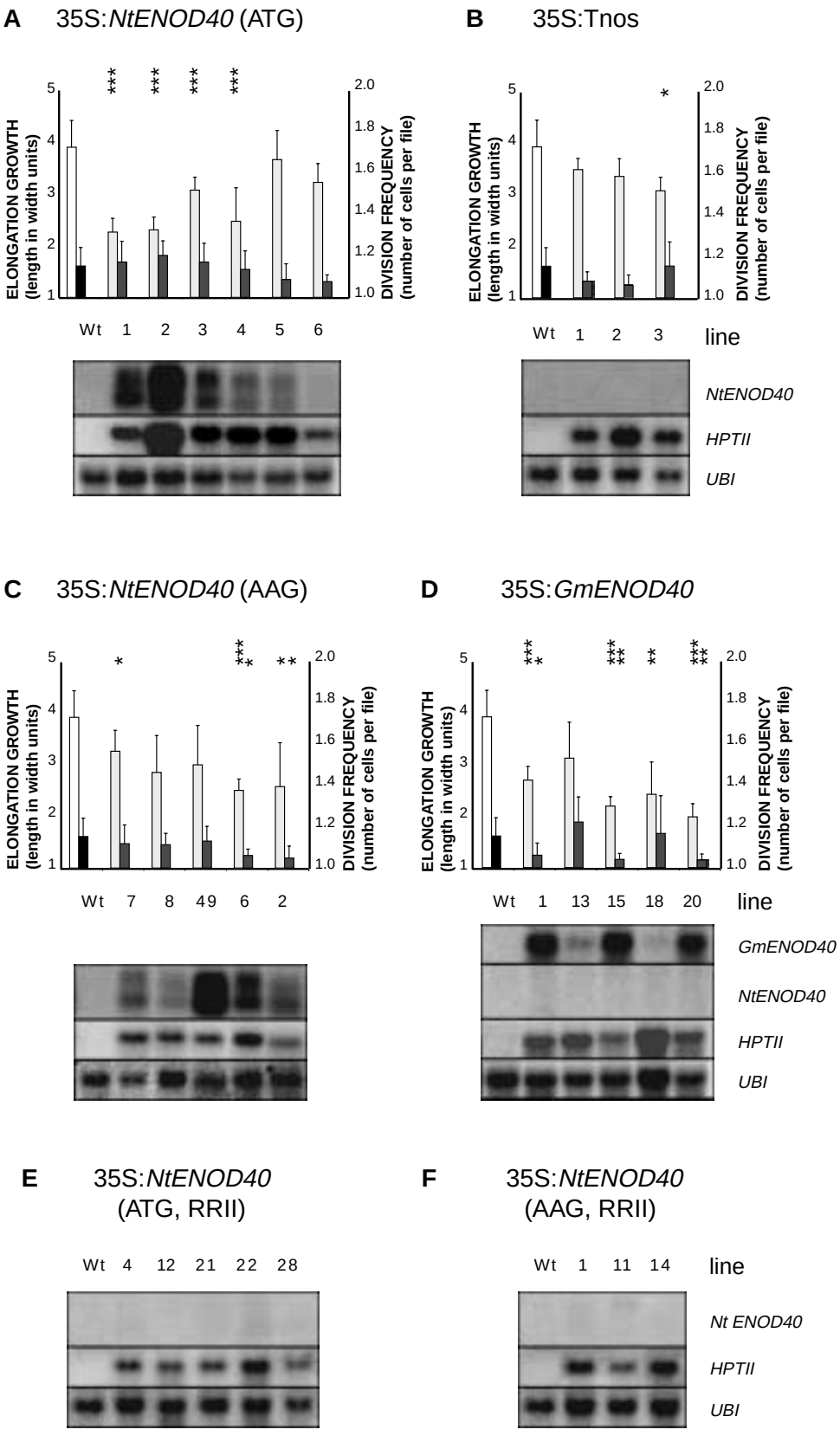


Figure 3.2. Elongation Growth Rate (white bars) and Cell Division Frequency (black bars) of Cultured Cells of Wild Type (Wt) and Transgenic BY-2 Lines.

Figure 3.2. Elongation Growth Rate (white bars) and Cell Division Frequency (black bars) of Cultured Cells of Wild Type (Wt) and Transgenic BY-2 Lines.

(A) Six independent transgenic cell lines carrying 35S:*NtENOD40* (ATG region I).
(B) Three independent transgenic cell lines carrying the 35S:Tnos control vector.
(C) Five independent transgenic cell lines carrying 35S:*NtENOD40* (AAG region I).
(D) Five independent transgenic cell lines carrying 35S:*GmENOD40*.
(E) Five independent transgenic cell lines carrying 35S:*NtENOD40* (ATG region I, RRII).
(F) Three independent transgenic cell lines carrying 35S:*NtENOD40* (AAG region I, RRII).
Data are average ± SD of at least three independent experiments. * Indicates significance in reduction of elongation growth rate or division frequency compared to wild type (* = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$). The level of transgene expression (*NtENOD40*, *GmENOD40* and *HPTII*) is determined at the start of protoplast culture by RNA gel blot analysis. All lines express the *HPTII* transcript, which confers resistance against hygromycin. *Ubiquitine* (*UBI*) hybridization is included to compare loading of separate samples on the RNA gel blot. The RNA gel blot analysis was repeated three times in independent experiments with similar results, one representative set of data is shown.

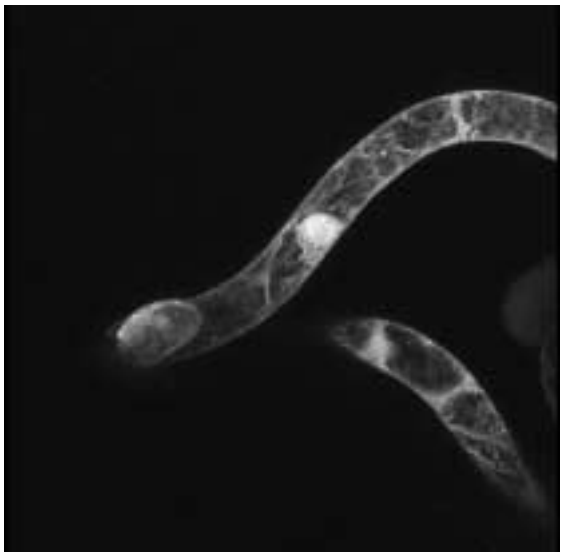


Figure 3.3. GFP Fluorescence of a 35S:*NtENOD40*-GFP Transgenic Line.

Confocal image showing fluorescence of a GFP reporter translationally fused to the ORF of region I indicating that this ORF is actively translated upon expression in stably transformed BY-2 cell suspensions.

Construct	Line	Elongation Growth Rate(E_{gr})	% Increase File-length	Cell Division Frequency	n
Wild type	Wt	3.93 ± 0.52	100 %	1.15 ± 0.09	11
p35S:Tnos	Ev1	3.49 ± 0.22	84 %	1.08 ± 0.05	3
	Ev2	3.36 ± 0.35	80 %	1.06 ± 0.05	3
	Ev3	3.18 ± 0.26	75 %	1.15 ± 0.12	3
p35S:*NtENOD40*	Nt1	2.27 ± 0.27	43 %	1.17 ± 0.10	8
ATG region I	Nt2	2.31 ± 0.25	44 %	1.20 ± 0.07	9
	Nt3	3.09 ± 0.25	70 %	1.17 ± 0.09	5
	Nt4	2.48 ± 0.65	50 %	1.14 ± 0.09	5
	Nt5	3.69 ± 0.57	91 %	1.08 ± 0.08	5
	Nt6	3.24 ± 0.37	75 %	1.08 ± 0.03	5
p35S:*GmENOD40*	Gm1	2.70 ± 0.27	58 %	1.06 ± 0.06	4
	Gm13	3.13 ± 0.70	72 %	1.22 ± 0.12	4
	Gm15	2.20 ± 0.18	40 %	1.04 ± 0.03	4
	Gm18	2.42 ± 0.63	48 %	1.17 ± 0.13	4
	Gm20	1.98 ± 0.26	33 %	1.04 ± 0.03	4
p35S:*NtENOD40*	2	2.58 ± 0.85	53 %	1.05 ± 0.06	4
AAG region I	6	2.51 ± 0.22	51 %	1.06 ± 0.03	4
	7	3.27 ± 0.41	77 %	1.12 ± 0.09	4
	8	2.86 ± 0.72	64 %	1.11 ± 0.05	3
	49	3.00 ± 0.76	66 %	1.13 ± 0.07	3

Table 3.1. Elongation Growth Rate, % Increase File-length and Cell Division Frequency of wild type and transgenic lines carrying constructs for overexpression of *ENOD40*–derived transcripts. Data are means ± SD of (n) number of repetitions in independent experiments. % Increase File-length from start of protoplast culture is calculated as $(E_{gr(line)} - 1)/(E_{gr(Wt)} - 1) \times 100\%$.

Next, we decided to test whether the ORF I encoded peptide has biological activity, but in the absence of the region II sequence. In order not to affect the length of the transcripts, we did not delete the region II sequence from the transcripts but instead, reversed the downstream region of the transcript (Reversed Region II). Like in the previous experiment, we also substituted the ATG codon by AAG in a second construct, thus disrupting the translation start of ORF I. For each construct several independent transgenic lines were generated, and these are named 35S:*NtENOD40* ATG, RRII (lines 4, 12, 21, 22 and 28) and 35S:*NtENOD40* AAG, RRII (lines 1, 11 and 14). The expression level of *NtENOD40* RRII and *HPTII* transcripts was determined by RNA gel blot analysis (Figure 3.2E-F). In none of the transgenic lines *NtENOD40* RRII transcripts

could be detected whereas *HPTII* transcripts were detected in all transgenic lines. Since we did not obtain any lines in which the *NtENOD40* RRII transcripts were present at a detectable level, we were not able to study the effect of these constructs.

Overexpression of *GmENOD40-1* Reduces Elongation Growth of BY-2 cells

The activity of an *NtENOD40* transcript with a disrupted translation start in ORF I suggests that translation of ORF I is not required to reduce elongation growth and therefore that the downstream region II contains activity. The *NtENOD40* transcript contains an ORF II and so we can not exclude that the encoded peptide has biological activity. To test this, we used a heterologous transcript that contains the highly conserved region II but a corresponding ORF II is not present in this transcript. For this purpose, we chose the *GmENOD40*-1 transcript. Five independent lines carrying construct 35S:*GmENOD40*-1 (Figure 3.1) were obtained and were named Gm1, Gm13, Gm15, Gm18 and Gm20. The levels of *GmENOD40* and *HPTII* transcripts were analyzed by RNA gel blot analysis (Figure 3.2D). This showed that these lines express 35S:*GmENOD40* but at different levels and *HPTII* is expressed at a high level in all transgenic lines. The effects of overexpression of *GmENOD40* on elongation growth rate and cell division frequency were tested for each individual line (Figure 3.2D, Table 3.1). Lines Gm1, Gm15 and Gm20 contained the highest level of 35S:*GmENOD40* expression, and each line had a strong reduction (35-60%) of elongation growth as compared to that of the wild type. These lines also showed a lower cell division frequency as compared to that of the wild type (around 4-6 ± 4% of the cells divided compared to 15 ± 9% in the wild type). Thus, elongation growth was reduced to a similar degree as that observed in the 35S:*NtENOD40* and 35S:*NtENOD40*-AAG lines, showing that this heterologous transcript has comparable activity in the protoplast elongation growth response. In addition, in the lines in which *GmENOD40* transcripts are expressed at a high level (Gm1, Gm15 and Gm20), cell division frequency is reduced in comparison to the wild type line. This difference is statistically significant ($p < 0.05$, $p < 0.01$, $p < 0.01$ respectively) and is similar to that observed in some of the 35S:*NtENOD40*-AAG lines (Table 3.1).

DISCUSSION

We studied which *ENOD40* gene product is biologically active, in order to reveal a molecular mechanism of *ENOD40* action. Here, we have used BY-2 cells overexpressing *ENOD40* derived constructs to study whether the peptide encoded by ORF I is biologically active. We demonstrated that ORF I is translated *in vivo* by using a GFP reporter gene fusion, and this supports the notion that this peptide can be required for biological activity of *ENOD40*. Subsequently, we determined whether translation of ORF I is required for biological activity of the *ENOD40* transcript by disrupting the translation start of ORF I. Strikingly, transgenic lines overexpressing the *NtENOD40* transcript with a disrupted translation start in ORF I show a reduction of elongation growth comparable to that in cell lines overexpressing the intact *NtENOD40* transcript. This indicates that the ORF I peptide is not required for the reduction of elongation growth and suggests that this activity may reside in the nucleotide sequence of region I or in region II. Our data are in disagreement with some of the results reported by Sousa (2001). In their assay, activity of a *MtENOD40* as well as a *MsENOD40* transcript was lost upon disruption of the translation start of ORF I, indicating that region II is not active in the presence of a disrupted ORF I and that the ORF I peptide is required for biological activity. Our results show that in *NtENOD40,* which contains ORF I and II, region II still contains biological activity when translation of ORF I is disrupted.

Strikingly, cell division frequency was reduced in some of the 35S:*NtENOD40*-AAG lines, whereas this effect was not induced by the intact *NtENOD40* transcript, indicating that the peptide encoded by ORF I does have some biological activity. To clarify this effect, we have attempted to test biological activity of the ORF I encoded peptide in the absence of the region II sequence by reversing the downstream region. Unfortunately, we could not detect expression of the *NtENOD40*-RRII transcript by RNA gel blot analysis in any of the lines carrying these constructs. It is unlikely that this is due to the integration-site in the genome (so-called position-effect), since this generally affects flanking genes in a similar way and we were able to detect substantial levels of *HPTII* transcripts in all these lines. Since high expression of both transgenes (*HPTII* and

ENOD40) is found in the majority of the other lines carrying 35S:*NtENOD40* or 35S:*GmENOD40* constructs, we assume that the absence of detectable levels of *NtENOD40* RRII transcripts is the result of either post-transcriptional silencing or severely reduced transcript stability in these lines.

The reduction of elongation growth in 35S:*GmENOD40* lines is comparable to that observed in 35S:*NtENOD40* lines, indicating that reduction of elongation growth can also be induced by overexpression of a heterologous *ENOD40* transcript. Both sequences contain the two conserved regions of *ENOD40* genes but have a low overall homology (~ 25%). Since these transcripts have a similar effect their activity likely resides in shared sequences. Therefore, these results indicate that the biologically active sequence is contained in either region I or region II, or a combination of both and suggests that the function of *ENOD40* genes is conserved among plant species. The results using the *NtENOD40*-AAG transcript suggest that translation of ORF I is probably not required for reduction of elongation growth and that this activity resides in region II. Thus, since region II, but not ORF II, is present in the *GmENOD40* transcript, it is unlikely that the peptide encoded by ORF II has this biological activity and it would be more likely that region II is active at the RNA level. Strikingly, 35S:*GmENOD40* lines most closely resemble 35S:*NtENOD40*-AAG lines, since both cell division and cell elongation growth are reduced in some lines expressing these constructs, whereas only elongation growth is affected by the intact *NtENOD40* construct.

The data using the 35S:*NtENOD40*-AAG construct showed that, when the function of one region is tested by mutagenesis in a construct containing both regions, this induced additional effects in comparison to the effect of the intact construct, indicating that the activity of the two regions is interdependent. Sousa (2002) tested the function of the two regions separately using truncated constructs carrying only one of the two regions. These data showed that the biological activity of either of the two regions is similar to the complete construct, which suggested that each region has biological activity on its own. In a second set of experiments, mutating the ATG of ORF I, or mutating the ATG of ORF II in the complete construct, reduced the biological activity of these constructs. This

shows that in both cases the effect of either region depends on the function of the other region, even though deletions spanning either region I or region II are active. Thus, our data as well as Sousa's data lead to a similar conclusion; testing the function of one region by mutagenesis in constructs containing both regions showed that the two regions influence each other, inferring that the functions of the two regions are somehow coupled.

There are two main reasons why it will be difficult to use these bioassays to further study the interaction between the two regions. The data using the 35S:*NtENOD40*-AAG construct indicate that the activity of the two regions is interdependent and may be regulated by a complex mechanism. The semi-quantitative nature of these bioassays is probably not sufficient to study the complexity of the combined effects of the two regions.

METHODS

Construction of Binary Vectors

Control Vector. The Cauliflower Mosaic Virus p35S promoter was used for ectopic expression of the different *ENOD40* sequences. To this end, the CaMV p35S promoter from pMON999 (Monsanto) was HindIII:BamHI transferred to pCambia1390 (Cambia, Australia) yielding p35S:Tnos. All cDNA sequences that are used for expression in these studies were cloned between the XbaI and BamHI restriction sites in the new MCS. The p35S:Tnos vector carries the hygromycin selection marker under control of a second CaMV 35S promoter in reverse orientation to the cloned inserts. The p35S:Tnos construct is used as the control vector for transformation.

p35S:NtENOD40. The endogenous full size *NtENOD40-1* cDNA sequence (Genbank accession number X98716, 470 bp) was cloned in p35S:Tnos after adding XbaI and BamHI linkers to the *NtENOD40* sequence by means of a PCR reaction using primers *NtENOD40*-1: 5'-GCTCTAGACTAGCTTGTCTCAAGAAC-3' and *NtENOD40*-8: 5'-CGGGATCCATGACAATCTTAACAACTCTAT-3'.

p35S:NtENOD40-GFP. The *NtENOD40*:ORFI:GFP fusion construct was obtained from Compaan (2001) and was XbaI:BamHI transferred to p35S:Tnos.

p35S:NtENOD40-AAG. The ATG codon that marks the start of ORF I in *NtENOD40* was mutagenized using a PCR based cloning method. To do so, we first amplified an *NtENOD40* DNA fragment by PCR using primers *NtENOD40*-1, and *NtENOD40*-AAG: 5'-CATGCCATGGATTGCTTCATCCCACTGCTTTTT-3' which has a single base mismatch to introduce an A at the T70 position. This fragment contains bases 1-90 and is digested with XbaI and NcoI. It was fused in a three-point ligation to an NcoI:BamHI PCR fragment containing bases 91-470 of *NtENOD40-1* and the XbaI:BamHI digested pBSK cloning vector. The construct was XbaI:BamHI transferred to p35S:Tnos after sequencing.

p35S:GmENOD40-1. A *Glycine max ENOD40*-1 cDNA clone (Genbank accession number X69154.1, 656 bp) was available as pKP1 in pMEX. Using primers *GmENOD40*-1 f: 5'-GCTCTAGACTAAACCAATCTATCAAGTCC-3' and *GmENOD40*-1 r: 5'-CGGGATCCAAAGGACTCTGGAAACTTTTC-3', we introduced XbaI:BamHI linkers by PCR amplification of the fragment, which was first cloned in pBSK and then transferred to p35S:Tnos.

p35S:*NtENOD40* ATG, RR II, **p35S:*NtENOD0* AAG, RR II**. Constructs containing a region II sequence in the reverse orientation were generated using the *NtENOD40*-ATG as well as the *NtENOD40*-AAG constructs as template. We generated fragments containing the first 117 bases with either an ATG or AAG codon marking the start of ORF I, with primers *NtENOD40*-1 and nt1asregionIISstI: 5'-GGGGCGAGCTCATTGCCTCTCTTTTCCTAA-3'. Both fragments were fused to the second fragment containing bases 117-470, encompassing the conserved region II and further 3' sequence of *NtENOD40*, which were generated with primers nt2asregionIIBamHI:5'-CGGGATCCTAGTTGGAGTGAATTAAGGA-3' and nt3asregionIISstI: 5'-GGGGCGAGCTCATGACAATCTTAACAACTCTAT-3'. The orientation of the second fragment is thereby reversed. The construct was XbaI:BamHI transferred to p35S:Tnos after sequencing. All binary vectors were sequenced in both directions before *Agrobacterium tumefaciens* transformation.

BY-2 Cell Suspension Culture, Protoplast Isolation and BY-2 Transformation

Liquid BY-2 culture and BY-2 transformation were as described in chapter 2. Transgenic lines were obtained from individual calli, which means that they can not be siblings. Transgenicity of each individual line was determined by PCR analysis of genomic DNA and by RNA gel blot analyses. The lines are continuously maintained in selection medium. Protoplast isolation and

growth parameter measurements in the Protoplast Bioassay were performed as described previously (Chapter 2). Significance was tested using a two-tailed students T-test.

RNA Gel Blot Analysis

Total RNA was isolated using the TRIzol method (GibcoBRL) according to the manufacturers protocol. 16 µg of total RNA was subjected to electrophoresis on a 1% agarose gel in 0.01 M NaH_2PO_4 (pH 7.0) using the glyoxal/DMSO method (Sambrook et al, 1989). RNA was subsequently transferred to a genescreen (Dupont) membrane in 20xSSC. RNA gel blots were hybridized with radiolabeled PCR fragments of the respective transcripts in formamide hybridization buffer O/N at 42ºC. Autoradiograms were obtained using a Molecular Dynamics Storm 840 Phosphorimager.

Confocal Microscopy

Confocal microscopy was performed on a Zeiss LSM 510 confocal microscope using standard GFP filter settings.

REFERENCES

Charon, C., Johansson, C., Kondorosi, E., Kondorosi, A., and Crespi, M. (1997). *ENOD40* induces dedifferentiation and division of root cortical cells in legumes. Proc. Natl. Acad. Sci. USA **94,** 8901-8906.

Compaan, B., Yang, W.C., Bisseling, T., and 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.

Fütterer, J., and Hohn, T. (1996). Translation in plants - rules and exceptions. Plant Mol. Biol. **32,** 159-189.

Lindsey, K., Casson, S., and Chilley, P. (2002). Peptides: new signalling molecules in plants. TRENDS in Plant Sci. **7,** 78-83.

Minami, E., Kouchi, H., Cohn, J.R., Ogawa, T., and Stacey, G. (1996). Expression of the early nodulin, *ENOD40*, in soybean roots in response to various lipo-chitin signal molecules. Plant J. **10,** 23-32.

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

Sambrook, J., Fritsch, E. F., Maniatis, T. (1989). Molecular Cloning a Laboratory Manual. Cold Spring Harbor Laboratory Press, USA.

Sousa, C., Johansson, C., Charon, C., Manyani, H., Sautter, C., Kondorosi, A., and 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. Mol. Cell. Biol. **21,** 354-366.

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., and Bisseling, T. (1996). Modification of phytohormone response by a peptide encoded by *ENOD40* of legumes and a nonlegume. Science **273**, 370-373.

Chapter 4

Arabidopsis thaliana:

Useful for Functional Analysis of *ENOD40*?

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Submitted to: Proceedings of Molecular Plant Microbe Interactions, 2003

ABSTRACT

In search of a molecular mechanism of *ENOD40* action, we aimed to identify interactors of *ENOD40* through a genetic approach. In order to facilitate a genetic screen, we searched for a phenotype in whole plants and chose *Arabidopsis thaliana* as a model system. In this report, we describe the generation and analysis of *Arabidopsis thaliana* transgenic lines that overexpress the tobacco homolog of *ENOD40*. Several independent transgenic lines were generated and were analyzed for growth aberrations. Interaction of *ENOD40* with cytokinin and ethylene signaling pathways has previously been suggested and we also tested the response to exogenous application of ACC and BA in our transgenic lines.

INTRODUCTION

To unravel the molecular mechanism underlying the function and activity of *ENOD40*, it is required to identify either direct interactors, or factors acting up- or down-stream of *ENOD40*. Studies in BY-2 cell suspensions (chapter 2) support the notion that the function of *ENOD40* depends on interaction with phytohormone signaling. Yet, it is difficult in this system to resolve the mechanism through which this interaction is established. As cell suspensions allow molecular biological, biochemical or pharmacological approaches, these can be used to test known candidate components of a signaling pathway, but this system does not provide the possibility to screen genetically for unknown interactors. Therefore, we search for phenotypes that are induced by overexpression of *ENOD40* in whole plants since this provides the possibility to develop a genetic screen through which unknown interactors of *ENOD40* can be identified in an un-biased manner. The effect of overexpression of *ENOD40* has been reported in *Medicago sativa*, *Medicago truncatula* and in tobacco. In the latter overexpression of *ENOD40* leads to reduced apical dominance (van de Sande et al., 1996). In *Medicago sativa* no transgenic plants could be generated (Crespi et al., 1994) and in *Medicago truncatula* a subtle effect on the timing of nodule formation is observed

(Charon et al., 1999). Although these data show that overexpression of *ENOD40* leads to impaired plant development, we decided not to use these plants for further studies, because tobacco is not suitable for genetic screens, and the phenotypes observed in the other two plant species are not suitable for suppressor screens.

The promoter of soybean *ENOD40* is active at similar sites in *Arabidopsis* and legumes (Mirabella et al., 1999). Moreover, the promoter is responsive to AVG and cytokinin in *Arabidopsis* similar to legumes, indicating that factors involved in the regulation of expression of *ENOD40* are conserved among legumes and *Arabidopsis* (Vleghels, 2003). Therefore, we chose to constitutively express *ENOD40* in *Arabidopsis* as it represents a plant model species with the established advantages of the fully available genomic sequence information. Due to its relatively small genome size and short generation time, genetic screens are relatively easy to perform in *Arabidopsis* as compared to other plant species. In addition, many *Arabidopsis* mutants (among these, mutants in hormonal production or signaling) are available that may become important in further studies on *ENOD40*. Relatively weak phenotypic changes or for example cell-type specific changes which require many steps of practical handling to visualize may well be very informative on the function of *ENOD40* in whole plants, but can not be used in a large screen. Therefore, these were not included in this study. Instead, we use assays that test the response to the phytohormones ethylene and cytokinin to analyze whether these responses are affected by *ENOD40* in *Arabidopsis*, since we found that in tobacco cell suspensions *ENOD40* affects elongation growth in response to cytokinin and that alteration of ethylene biosynthesis is involved (chapter 2).

RESULTS

Arabidopsis ENOD40 Homologs Can Not be Identified

Preferentially, we would like to overexpress an *Arabidopsis* homolog of *ENOD40* in *Arabidopsis*. As an *Arabidopsis ENOD40* homolog has not been described, we first searched the Genbank sequence database for the presence of *ENOD40* homologous sequences. Sequence comparison between *ENOD40* genes, reveals two regions of high sequence similarity named region I and region II (Compaan et al., 2001; Flemetakis et al., 2000; Kouchi et al., 1999). The M-X_{1-4}-W-X_4-HGS* peptide motive present in the open reading frame of region I and the AACCGGCAAGTCA-(X_6)-GGCAAT nucleotide sequence motive in region II are characteristic to *ENOD40* sequences known to date. We examined the Genbank sequence databases for the presence of homologous sequences through blastn alignment searches (Figure 4.1). Several sequences with homology to region II of *ENOD40* can be identified in the *Arabidopsis* genome. However, these do not span the entire conserved sequence of region II. In addition, the small conserved ORFs that are present upstream of region II in all known *ENOD40* genes can not be recognized in *Arabidopsis*. Thus, in the *Arabidopsis* genome both motives do not occur within a relatively short (<1kb) sequence range and in the correct orientation. These results can not simply be explained by a partial gene deletion or rearrangement. Therefore, we conclude that the *Arabidopsis* genome does not contain an *ENOD40* homolog. In Citrus species, which are part of the Sapindales family, *ENOD40* homologous sequences can be found that contain both regions, indicating that the ancestor of the Brassicales family to which *Arabidopsis* belongs, still contained an *ENOD40* homologous sequence. These results do not exclude that a component able to recognize the secondary structure of *ENOD40* gene products is present in *Arabidopsis*. Studies in tobacco BY-2 cells showed that an *ENOD40* sequence from a leguminous species and tobacco, respectively, contain similar biological activity, indicating that *ENOD40* cDNA sequences are active in a heterologous plant background (chapter 3). Thus, we chose to use the *NtENOD40* cDNA sequence for constitutive overexpression in *Arabidopsis*.

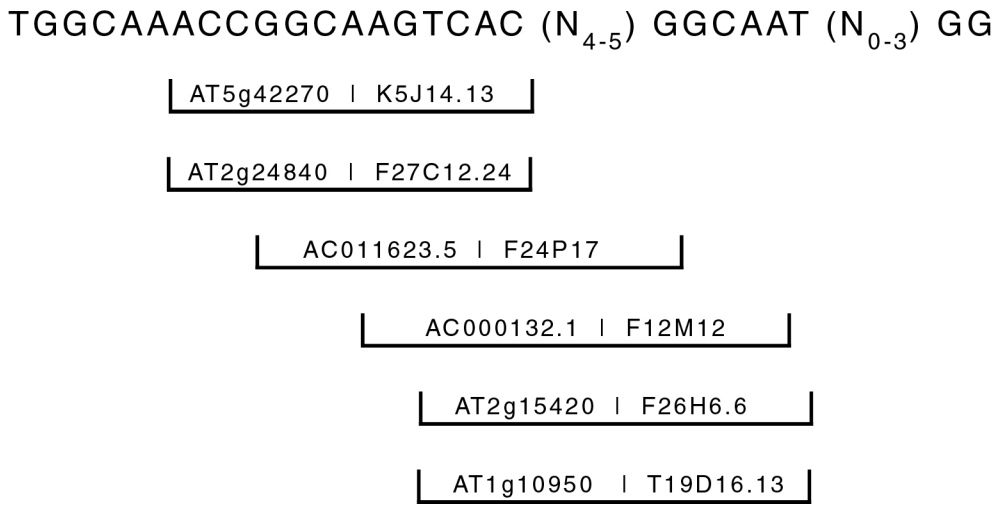

Figure 4.1. Short stretches of *ENOD40* Sequence Homology in *Arabidopsis thaliana* genome database.

BLASTn searches with the highly conserved nucleotide sequence of region II of *ENOD40* reveal the presence of sequences with homology to this region in the *Arabidopsis thaliana* genome. However, these sequences do not span the entire conserved sequence of region II and short ORFs with similarity to region I of *ENOD40* genes can not be identified upstream of these sequences. This indicates that an *ENOD40* homolog is not present in the *Arabidopsis thaliana* genome. Genbank accession numbers and corresponding BAC clones are indicated.

Generation of Transgenic Lines

Since the effect of overexpression of *ENOD40* is not yet known in *Arabidopsis*, we aim to analyze plant morphology in different developmental stages in all organs that can later be useful in a genetic screen. For this purpose it is important that *ENOD40* is expressed in all tissues under study. Thus, we chose the CaMV 35S promoter to constitutively drive the expression of the tobacco full-size *NtENOD40*-1 cDNA sequence. As a first step, we generated transgenic plants by *Agrobacterium*-mediated transformation and out of 32 primary transformants three independent homozygous transgenic lines were selected and were named line 1, 2 and 3 (Figure 4.2). Genomic DNA gel blot analysis showed that line 1 probably contains 2 copies; lines 2 and 3 one copy. Segregation analyses of T1, T2 and T3 generations (3:1) showed that for all three lines, the transgenes segregate as single insertion loci. From the selected lines RNA was isolated and inspected for the expression of the transgene by RNA gel blot analyses (Figure 4.2). All lines showed a high level of expression of the introduced gene.

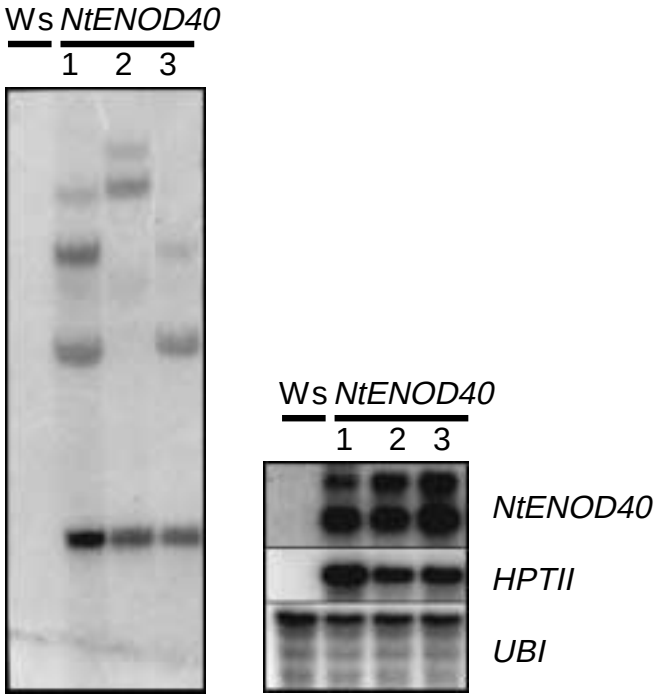

Figure 4.2. Genomic DNA and RNA Gel Blot Analysis of Wild type (Ws) and Three Independent Transgenic Lines Carrying the 35S:*NtENOD40* Construct.

Genomic DNA was HindIII:BamHI digested, electroforesed and blotted. The probe hybridizes to the HindIII:BamHI 35S:*NtENOD40* fragment in all lines, and both one upstream and one downstream fragment for each unique insertion site. Line 1 probably contains two copies; line 2 and 3 one copy. Segregation analysis of T1, T2 and T3 generations (3:1), revealed that for all three lines, transgenes segregate as single insertion loci. RNA gel blot analysis reveals high levels of expression for the *NtENOD40* transgene and the *HPTII* transgene conferring resistance against hygromycin. Hybridization with the *Ubiquitin* (*UBI*) probe confirms equal loading of RNA on the gel.

Overexpression of *NtENOD40* Does Not Lead to Growth Aberrations

To find out whether overexpression of *NtENOD40* interferes with *Arabidopsis* growth, we inspected plants of the T3 and T4 generation for abnormalities in growth. To this end T3 and T4 plants from homozygous lines were germinated on agar-solidified $^1/_2$MS medium containing 1% sucrose. Ten-day old plantlets were transferred to soil and grown in the greenhouse. Plants were screened for germination time, rosette size; number, size and

shape of leaves, flowering time, number and length of inflorescence shoots or number of seeds. No parameters could be identified that were affected in our 35S:*NtENOD40* lines.

Phytohormone Responses Are Not Affected in 35S:*NtENOD40* Lines

As ethylene and cytokinin responses are affected by overexpression of *ENOD40* in BY-2 cells (chapter 2), we set out to test whether *ENOD40* affects the response of *Arabidopsis* to the phytohormones cytokinin and ethylene. To this end we tested the effect of exogenous application of phytohormones on growth and development in *Arabidopsis* in a dose response curve for each hormone. Wild type (Ws) and 35S:*NtENOD40* plants were grown in the dark in the presence of a range of ACC concentrations and plants were photographed after 5 days (Figure 4.3). Ethylene or ACC (a direct precursor of ethylene) treatment induces the triple response in dark-grown seedlings (Guzman and Ecker, 1990). Hypocotyl length of wild type and 35S:*NtENOD40* plants were measured and the length was plotted as a function of the ACC concentration (Figure 4.4). In the absence of ACC hypocotyl length is similar in wild type and 35S:*NtENOD40* plants of all three independent transgenic lines, indicating that overexpression of *ENOD40* does not affect hypocotyl growth. Further, hypocotyl length of 35S:*NtENOD40* lines and wild type reduced similarly in response to ACC treatment. Comparison of the other hallmarks of the triple response by eye, including reduction of root growth and hypocotyl length, thickening of the hypocotyl, and enhanced apical hook curvature, indicates that wild-type plants and transgenic plants respond similarly to ACC. These data indicate that these responses to ethylene are not affected by overexpression of *NtENOD40* in *Arabidopsis*.

A linear relation exists between the ethylene concentration and the length of the root epidermal cell in which a new root hair just emerges, the length of the root elongation zone and the length of the primary root (Le et al., 2001). Further, ethylene regulates root hair development, by determining the number and position of root hairs (Tanimoto et al., 1995). To find out whether overexpression of *NtENOD40* affects these processes, the length of the elongation zone, defined by the distance between the root tip and the first

bulging root hair, is analyzed in 5-d old plants grown in the light. Neither the length of the elongation zone, nor the number, distance between nor length of the root hairs was affected in our transgenic lines (data not shown). Since ethylene signaling controls these processes, these data indicate that ethylene signaling is not severely affected by overexpression of *ENOD40* under these conditions. Taken together with the normal triple response to ACC, we have no indication that responses to ethylene are affected in these 35S:*NtENOD40* lines.

There are two ways to test the effect of exogenous application of cytokinin; BA treatment while plants are growing in the light or in the dark respectively. BA treatment in the light results in reduced root growth, while hypocotyl length is not affected (Cary et al., 1995). BA treatment in the dark induces a response similar to the triple response in *Arabidopsis*. This response can be counter-acted by application of ethylene receptor blockers, showing that this response is (at least partially) ethylene dependent. Both responses were tested in the three different 35S:*NtENOD40* lines. To this end, wild type and transgenic plants were grown on MS plates supplemented with different concentrations of BA. After 5 days of growth plants were subjected to visual inspection. Photographs of representative plants show that plants overexpressing *NtENOD40* respond similarly to the added BA as wild type plants, both in light (Figure 4.5) and dark (Figure 4.7) grown plants. Hypocotyl length of dark grown wild type plants and plants overexpressing *ENOD40* were measured and plotted as a function of BA concentration (Figure 4.6). This shows that hypocotyl length of plants of 35S:*NtENOD40* lines was similar to wild type plants at all tested BA concentrations. We did not measure the reduction in root length (dark and light grown seedlings) in 35S:*NtENOD40* plants and wild type plants as visible inspection indicated that differences would be marginal. Also, the other responses to BA treatment in the dark, thickening of the hypocotyl and enhanced apical hook curvature, do not seem to be affected in 35S:*NtENOD40* lines. Thus, *ENOD40* does not affect the response to cytokinin in our transgenic lines.

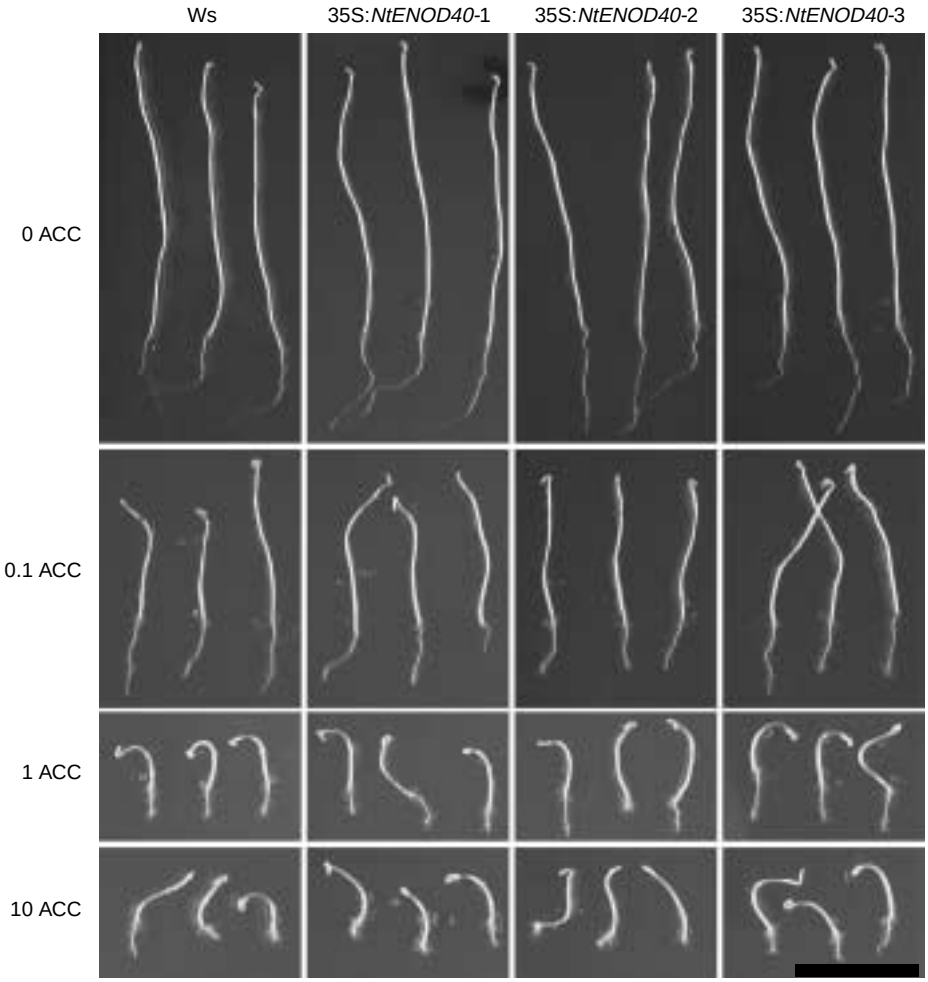

Figure 4.3. Application of an ACC concentration range (0-10^{-5}M) induces the triple response in dark grown wild type and 35S:*NtENOD40 Arabidopsis* seedlings. The reduction of root and hypocotyl growth, enhanced hypocotyl thickening and enhanced apical hook curvature is similar in wild type and *NtENOD40* expressing lines. Bar equals 1 cm.

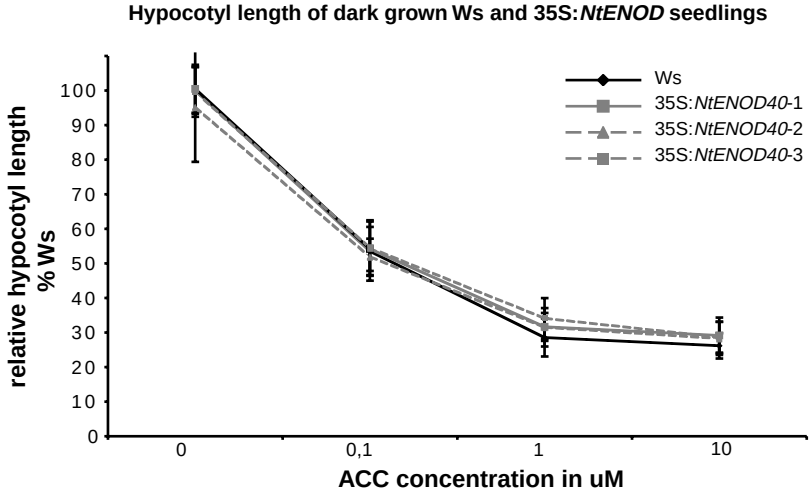

Figure 4.4. Quantification of hypocotyl length in a dose response curve shows that the response to ACC is not affected by overexpression of *NtENOD40*.

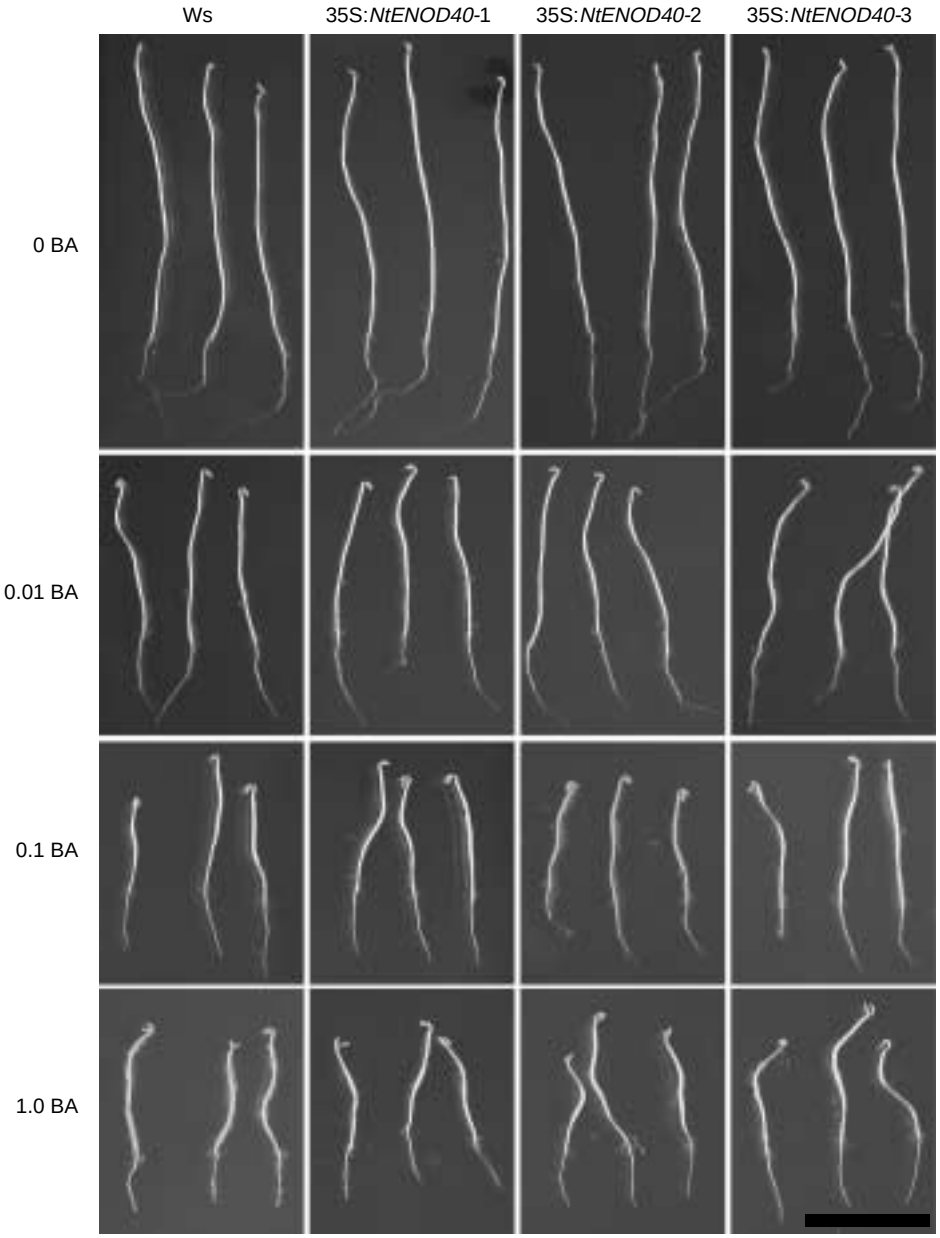

Figure 4.5. Application of a BA concentration range (0-10^{-6}M) induces a response similar to the triple response in dark grown wild type and 35S:*NtENOD40 Arabidopsis* seedlings. The response to BA is not affected by overexpression of *NtENOD40*. Bar equals 1 cm.

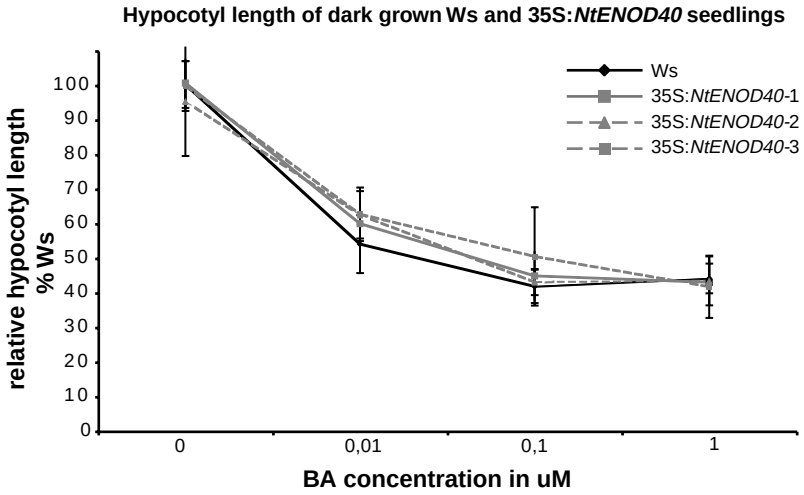

Figure 4.6. Quantification of hypocotyl length in a dose response curve shows that the response to BA is not affected by overexpression of *NtENOD40.*

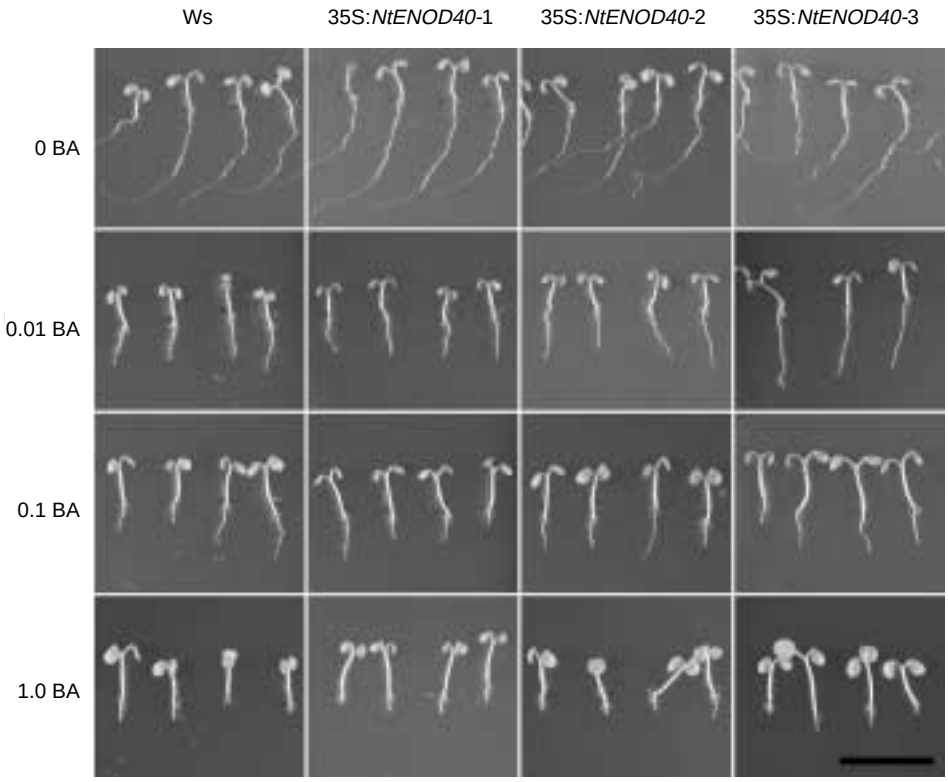

Figure 4.7. Photographs of representative plants show that BA treatment in the light reduces root growth of wild type and 35S:*NtENOD40* seedlings to a similar extent. Bar equals 1 cm.

CONCLUSIONS

Here we describe the generation of transgenic *Arabidopsis* plants that express *NtENOD40* under the control of the CaMV 35S promoter. We anticipated that these plants could facilitate the performance of genetic screens to identify interactors of *ENOD40* in *Arabidopsis*, once these plants display a clear, easily recognizable growth aberration. However, all analyzed parameters failed to reveal an effect of *ENOD40* on *Arabidopsis* growth and development. Neither did *ENOD40* affect the response to the phytohormones cytokinin and ethylene in *Arabidopsis*, whereas this is the case in tobacco BY-2 cells overexpressing *NtENOD40*. The lack of a clear phenotype as a result of overexpression of *NtENOD40* suggests that this gene does not have a critical function in the *Arabidopsis* life cycle. The finding that database searches do not reveal the presence of an *ENOD40* homolog in the genome of *Arabidopsis*, while the presence of *ENOD40* homolog in a close relative such as citrus is detectable in this way, suggests that this gene is not present in the genome and therefore the gene products of the *NtENOD40* gene might not be recognized. It does not seem to be feasible to unravel the molecular mechanism underlying the function of *ENOD40* by a genetic approach using *Arabidopsis*, due to the absence of a clear phenotype as a result of overexpression of *ENOD40*. With the development of plant model systems containing *ENOD40*, like *M.truncatula*, *L. japonicus*, rice and tomato, a forward genetic approach might come within reach soon.

METHODS

Generation of Transgenic Lines

The full-size *NtENOD40*-1 cDNA (Genbank accession number X98716) was cloned in pCambia1390 (Cambia, Australia) behind the pMON999 (Monsanto) derived CaMV 35S promoter as described previously (chapter 3). Transgenic *Arabidopsis* Ws lines were generated using the dipped plant method, essentially as described by Weigel and Glazebrook (2002) using *Agrobacterium tumefaciens* strain AGLO. Primary transgenic seeds were selected on

hygromycin selection medium and propagated on soil. Plants were grown in the greenhouse with 16h photoperiod. The T1 segregation ratio was used to identify lines carrying single insertions of the transgene (3:1). For each generated line, 10 hygromycin resistant plants were transferred to soil for propagation. The T2 seeds were again selected on hygromycin, and the segregation ratio was used to identify homozygous lines and were again propagated in the greenhouse. 10-d old homozygous T3 plants were used for genomic DNA and RNA gel blot analyses.

Genomic DNA and RNA Gel Blot Analysis

Genomic DNA was isolated according to standard protocols. 10 µg of genomic DNA was HindIII:BamHI digested, electroforesed on a 0.8 % agarose gel and blotted onto a Hybond-N^+ membrane (Amersham Pharmacia). Blotting, hybridization and washing were performed according to the manufacturer's recommendations. RNA was isolated using the TRIzol method (GibcoBRL). 22 µg of total RNA was run on a 1% agarose gel in 0.01 M NaH_2PO_4 (pH 7.0) using the glyoxal/DMSO method (Sambrook et al., 1989). RNA was subsequently transferred to a genescreen membrane (Dupont) in 20xSSC. Probes were hybridized in formamide hybridization buffer O/N at 42ºC. Blots were washed till 1xSSC at 60ºC. Autoradiograms were obtained by exposure to a phosphorscreen and scanned using a Storm 840 Phosphor-imager (Molecular Dynamics).

Phytohormone Response Assays

Seeds are surface sterilized, and sown (20 on a line, 60 plants per plate) on agar-solidified MS medium with 0,5 % sucrose. Seeds are kept at 4ºC for four days to enhance synchronous germination. Then, plates are transferred to 25ºC, either in the light (BA treatment) or in the dark (ACC and BA treatment) for five days. Plants are photographed, and mean hypocotyl length is determined for at least 20 plants per transgenic line per treatment using NIH image software according to Weigel and Glazebrook (2002). For each transgenic line, progeny of three different homozygous T3 plants are tested and compared to three different seed batches of the control Ws line. Relative hypocotyl length is calculated as percentage of the mean hypocotyl length in the Ws control treatment.

ACKNOWLEDGMENTS

This work was supported by a research grant from the Netherlands Organization of Scientific Research (NWO 805.49.004). We gratefully acknowledge Bert Essenstam for technical assistance.

REFERENCES

Cary, A.J., Liu, W., and Howell, S.H. (1995). Cytokinin action is coupled to ethylene in its effects on the inhibition of root and hypocotyl elongation in *Arabidopsis thaliana* seedlings. Plant Physiol. **107,** 1075-1082.

Charon, C., Sousa, C., Crespi, M., and Kondorosi, A. (1999). Alteration of *ENOD40* expression modifies *Medicago truncatula* root nodule development induced by *SinoRhizobium meliloti*. Plant Cell **11,** 1953-1965.

Compaan, B., Yang, W.C., Bisseling, T., and 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.

Crespi, M.D., Jurkevitch, E., Poiret, M., d'Aubenton-Carafa, Y., Petrovics, G., Kondorosi, E., and 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.

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

Guzman, P., and Ecker, J.R. (1990). Exploiting the triple response of *Arabidopsis* to identify ethylene-related mutants. Plant Cell **2,** 513-523.

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

Le, J., Vandenbussche, F., Van Der Straeten, D., and Verbelen, J. (2001). In the early response of *Arabidopsis* roots to ethylene, cell elongation is up- and down-regulated and uncoupled from differentiation. Plant Physiol. **125,** 519-522.

Mirabella, R., Martirani, L., Lamberti, A., Iaccarino, M., and 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.

Sambrook, J., Fritsch, E. F., Maniatis, T. (1989). Molecular Cloning a Laboratory Manual. Cold Spring Harbor Laboratory Press, USA.

Tanimoto, M., Roberts, K., and Dolan, L. (1995). Ethylene is a positive regulator of root hair development in *Arabidopsis thaliana*. Plant J. **8,** 943-948.

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., and Bisseling, T. (1996). Modification of phytohormone response by a peptide encoded by *ENOD40* of legumes and a nonlegume. Science **273,** 370-373.

Vleghels, I. (2003). Comparative studies on *ENOD40* in legumes and non-legumes. Thesis, Wageningen University and Research Center Wageningen, the Netherlands**,** pp 99.

Weigel, D., and Glazebrook, J. (2002). *Arabidopsis*, a laboratory manual. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, New York.

Chapter 5

Concluding Remarks

***ENOD40* affects hormone responses in BY-2 cells.**

The *Rhizobium*-legume interaction results in the formation of a completely new organ on the roots of leguminous plants to host the bacteria. A complex network involving signal molecules like Nod-factors, the phytohormones ethylene, auxin and cytokinin and some plant genes, coordinates this developmental process (Mylona et al., 1995). One of the plant genes that are highly induced during early stages of nodulation is *ENOD40* (Yang et al., 1993; Kouchi et al., 1993). Upon overexpression of *ENOD40*, nodule formation is accelerated, whereas silencing of *ENOD40* arrested nodulation at different stages of nodule development (Charon et al., 1999), indicating that the control on developmental progression is disturbed and that *ENOD40* has a regulatory role during development. In addition, *ENOD40* is also present in non-legumes (Figure 1.1). The expression patterns in different plant species show that *ENOD40* is transiently expressed in various developmental programs as we have summarized in chapter 1. Several putative functions have been suggested, one of which is that *ENOD40* modifies the phytohormone status of cells. To date, the function of the *ENOD40* genes and their mode of action are poorly understood. In this thesis, we have explored the possibility to set up bioassays to study *ENOD40* function in further detail.

We studied the effect of *ENOD40* on phytohormone signaling by developing a bioassay based on the morphological response of suspension cultured tobacco BY-2 cells to the phytohormones auxin, cytokinin and ethylene. We found that *ENOD40* overexpression leads to suppressed elongation growth of BY-2 cells, whereas cell division frequencies are not affected in 35S:*NtENOD40* BY-2 cell suspensions. In BY-2 cells, the cell division frequency is controlled by the cytokinin to auxin ratio (Hasezawa and Syono, 1983). By testing our 35S:*NtENOD40* lines at different cytokinin/auxin ratios in dose response curves, we showed that this control on cell division is not affected by *ENOD40*. Elongation growth is independent of the cytokinin to auxin ratio in wild type cells. In contrast, in the presence of *ENOD40*, elongation growth is reduced in both a cytokinin and an auxin dependent manner. That elongation growth in 35S:*NtENOD40* lines is not reduced to a similar degree under various cytokinin/auxin ratios shows that *ENOD40* is

not simply a blocker of elongation growth in BY-2 cells. Instead, cytokinin and *ENOD40* act synergistically whereas the effect of *ENOD40* is counter-acted by auxin. This shows that in the cell elongation growth response, but not in the cell division response, cross-talk between *ENOD40* and both cytokinin and auxin occurs. Close examination of the relative rates of both processes revealed that *ENOD40* expression causes the elongation growth rate to become rather strictly coupled to the cell division frequency. Furthermore, analysis of ethylene homeostasis showed that the cytokinin/auxin dependent effect of *ENOD40* on elongation growth is mediated by ethylene.

Ethylene biosynthesis is accelerated in 35S:*NtENOD40* lines, which is correlated with accelerated accumulation of *ACS* transcripts. The observation that constitutive expression of *ENOD40* does not lead to constitutive ethylene production indicates that *ENOD40* is not a direct inducer of ethylene biosynthesis. The observation that the *ENOD40* induced response can be counter-acted by ethylene receptor blockers leads to several important conclusions. (i) It suggests that ethylene is a negative regulator of elongation growth in BY-2 cells. This effect of ethylene had not been described in BY-2 cells, but is in line with the effect of ethylene on several plant processes, like in the control of elongation growth of cells in the elongation zone of the *Arabidopsis* root (Le et al., 2001). It is also consistent with the reduced size of an ethylene overproducing mutant (*eto*) or the constitutive ethylene response mutation (*ctr1*). Further, most ethylene insensitive mutants have larger leaves than the wild type (Ecker, 1995), resulting from cell enlargement (Hua et al., 1995). (ii) It shows that acceleration of ethylene accumulation is a primary cause and not a consequence of suppressed elongation growth of 35S:*NtENOD40* cells. Since *ENOD40* is probably not an inducer of ethylene biosynthesis per sé, it seems more likely that altered regulation of ethylene biosynthesis is subject to, or part of, the mechanism that couples elongation growth rates to cell division frequency. (iii) This indicates that ethylene perception is important for the coupling between these processes, since application of Ag^{+} leads to a loss of the strict coupling of cell division and cell elongation. In conclusion, our data show that overexpression of *NtENOD40* results in coupling between phytohormone regulated cellular processes. The observation that -specifically in the presence of *ENOD40* -, the cytokinin and auxin dependent elongation growth response is mediated by ethylene

signaling, supports the hypothesis that *ENOD40* affects phytohormone signaling (Crespi et al., 1994; van de Sande et al., 1996; Charon et al., 1999). These data indicate that the function of *ENOD40* is to facilitate cross-talk between ethylene and cytokinin/auxin signaling pathways.

Cross-talk Between *ENOD40* and Phytohormones

It is generally accepted that phytohormones act as key-regulators of developmental processes such as cell division, cell growth and cell differentiation and that coordinated control of these processes determines cell shape, size and patterning in developing organs. It has become clear that there is a significant amount of cross-talk between the phytohormone signaling pathways and that this could, at least in part, account for the observed diversity of cellular responses during development (Lindsey et al., 2002). In genetic screens for components of phytohormone signaling pathways, often mutations affecting responses to multiple hormones are isolated and these are beginning to give insight in the molecular mechanism that underlies phytohormone cross-talk (Swarup et al., 2002). Signaling of one hormone can be affected by a second hormone at any of the following steps: hormone level, perception, inhibition or stimulation of signal transduction processes, regulation of transcription, post-translational modification or interaction at the response level (Coenen and Lomax, 1997).

We showed that cytokinin and auxin act antagonistically in the regulation of cell division in wild type BY-2 cells. Several studies have shown that auxin and cytokinin regulate tobacco pith cell proliferation by synergistically regulating the expression of the cell cycle regulatory components *cdc2* and *cycD3.* Additionally, cytokinin regulates *cdc2* catalytic activity (John et al., 1993). A similar mechanism has been suggested to control pericycle cell division leading to lateral root formation, although here cytokinin and auxin act antagonistically, indicating that the interactions between auxin and cytokinin in this mechanism differ in a tissue or species-specific manner (Coenen and Lomax, 1997). Thus, it is likely that auxin/cytokinin cross-talk underlies regulation of BY-2 cell division. Since *ENOD40* does not affect the cell division frequency in BY-2 cells, it is not likely

that *ENOD40* is part of this mechanism. Instead, the observation that altered regulation of *ACS* transcription, and consequently altered ethylene biosynthesis kinetics, mediates the auxin/cytokinin dependent elongation growth response in tobacco BY-2 35S:*NtENOD40* cells, suggests that in the presence of *ENOD40*, cross-talk between auxin and ethylene but also cytokinin and ethylene takes place. This mechanism is consistent with that proposed for cytokinin/ethylene and auxin/ethylene cross-talk in several plant developmental processes (Swarup et al., 2002). Auxin and ethylene have been described to interact at the level of ethylene biosynthesis since auxin up-regulates the expression of *ACC synthase* in *Arabidopsis* (Abel et al., 1995), tomato (Abel and Theologis, 1996) and lupine (Beckman et al., 2000). Cytokinin treated dark-grown *Arabidopsis* seedlings display the triple response whereas this effect is not observed when plants are grown in the presence of ethylene-synthesis or -perception blockers, indicating that cytokinin and ethylene pathways interact at the level of ethylene biosynthesis. Both transcriptional and post-translational regulation seem to be involved (Cary et al., 1995; Vogel et al., 1998). Our data show that, like in intact plants, in BY-2 cell suspensions similar mechanisms leading to phytohormone cross-talk operate and control cell morphology. A remarkable result is that cytokinin/ethylene and/or auxin/ethylene cross-talk -resulting in altered regulation of the elongation growth response- requires *ENOD40*, suggesting that *ENOD40* is part of this pathway(s). How and at what level *ENOD40* acts in the pathway(s) remains to be clarified.

Which gene product is important for the function of *ENOD40*?

Further insight in the molecular mechanism of the mode of action of *ENOD40* requires the identification of the biologically active gene product. In the case of *ENOD40* this is not trivial since a long open reading frame that could encode a biologically active peptide is lacking in *ENOD40* transcripts. Instead, as described in chapter 1, all *ENOD40* genes contain two short conserved regions. Strikingly, in region I a conserved small ORF resides encoding a remarkably small peptide of 10-13 amino-acids, whereas in region II in only about 50% of the transcripts a conserved ORF can be found,

suggesting that this region may be active as RNA. We have used our protoplast bioassay to study the activity of the two regions (chapter 3). We showed that the ORF in region I is translated in BY-2 cells, as revealed by a GFP reporter fusion. These observations are consistent with previous reports (Compaan et al., 2001; Sousa et al., 2001; van de Sande et al., 1996) and support the hypothesis that the peptide encoded by ORF I has biological activity. However, translation of this oligopeptide was not required for the *ENOD40* induced elongation growth phenotype in the protoplast bioassay (chapter 3), indicating that biological activity may reside in the RNA sequence of region I and/or II. Strikingly, disruption of the translation start of ORF I led to a reduction of the cell division frequency that was not observed in lines expressing the intact construct. The observation that a disruption of translation of ORF I induces an additional phenotype in comparison to the effect of the intact construct, indicates that the encoded peptide has biological activity but also that the function of both conserved RNA regions may be interconnected. Since the semi-quantitative nature of our assay system is not sufficient to unravel the complex effects of both regions, the role of the two conserved regions and the nature of the *ENOD40* encoded biologically active component remain to be elucidated.

Sousa (2001) described a bioassay for *ENOD40* activity by scoring for cortical cell division induced upon ballistic targeting of *ENOD40*-derived constructs in *Medicago* roots. In this bioassay, both the peptide encoded by region I, as well as a peptide encoded in region II have biological activity, and lead to the same response. The effect of the *MtENOD40* region II in alfalfa roots depends on the presence, correct size and sequence of the region I encoded peptide, even though deletions spanning either region I or region II are active, indicating a complex level of regulation acting on the *MtENOD40* RNA (Sousa et al., 2001). Although the responses induced by the two regions differ in the two different assays, both our data and the data described by Sousa (2001) suggest that the function of the two conserved regions may be connected in a complex manner.

Rohrig (2002) showed that two partially overlapping ORFs located in region I were translated from a *GmENOD40-1* transcript *in vitro* and that two corresponding synthetic

peptides bind sucrose synthase *in vitro*. The enhanced expression of sucrose synthase during nodule development indicates that sucrose synthase may have an important function in phloem unloading of sucrose, which is translocated from shoots to nodules. Binding of the two *ENOD40* peptides may either regulate enzyme activity or may direct this enzyme to specific intracellular sites. The role of binding between the two *ENOD40* peptides to sucrose synthase for the function of *ENOD40* in phytohormone cross-talk as we have observed in BY-2 cells is unclear. Two structural requirements in *ENOD40* transcripts, namely the presence and conservation of the second short ORF partially overlapping with ORF I and the presence of a cysteine in the middle of the ORF I encoded peptide, which is required for binding, are only present in legume *ENOD40* transcripts. This suggests that the interaction between *ENOD40* peptides and sucrose synthase can only occur in legumes. The expression patterns of *ENOD40* and sucrose synthase overlap at least partially in mature soybean nodules (Yang et al, 1993; Komina et al., 2002). So, it is possible that *ENOD40* encoded peptides and sucrose synthase interact during nodulation.

In a two-hybrid screen, Vleghels (2003) identified a laminin-like protein (p40) with unknown function, as an interactor of *ENOD40*. *In situ* hybridization showed that the expression patterns of p40 and *ENOD40* partially overlap in pea nodules, yet in an other region as where *ENOD40* and sucrose synthase overlap. As a first step to show that these peptides are part of the molecular mechanism of *ENOD40* action, binding of *ENOD40* peptides either with sucrose synthase or with p40 should be confirmed *in planta*.

We have explored the possibility to use *Arabidopsis thaliana* as a genetic model system to identify *ENOD40* interacting components (chapter 4). Sequence homology searches did not reveal an *ENOD40* homolog in the *Arabidopsis* genome. Since we showed that *ENOD40* transcripts are biologically active in a heterologous plant background (chapter 3), we proceeded by using the tobacco *ENOD40* (*NtENOD40*) homolog to generate transgenic plants overexpressing *NtENOD40*. Unfortunately, in neither of the transgenic lines that were obtained a phenotype was observed, indicating that *NtENOD40* does not affect *Arabidopsis* growth and development. This can be explained in several ways,

most likely *Arabidopsis* does not contain a gene with significant homology to *ENOD40*, the gene products of the *NtENOD40* transgene are not recognized and therefore are not functional in the *Arabidopsis* background. Thus, it does not seem to be feasible to unravel the molecular mechanism underlying the function of *ENOD40* by a genetic approach using *Arabidopsis*, due to the absence of a clear phenotype as a result of overexpression of *NtENOD40*.

Is *ENOD40* a mediator of phytohormone cross-talk in other processes and/or other plant species?

A major challenge now is to confirm that interaction between *ENOD40* and phytohormone signaling pathways provides the basis for the biological function of *ENOD40* in organogenesis. An experimental system in intact plants that may well be exploited to study the function of *ENOD40* in further detail would be nodule formation. *ENOD40* is expressed in the nodule primordium where cell division and cell growth take place and in Zone II which, in indeterminate nodules, is the zone between the meristematic cells and the differentiated cells of the central tissue of the mature nodule. Especially in these tissues where different cellular processes occur, it is tempting to assume that the coupling between cellular processes is important for development and that *ENOD40* is required for the cross-talk between regulating pathways. To determine whether the coupling of these processes occurs via cross-talk between phytohormone pathways, one first needs to visualize what the cellular effects of the phytohormones auxin, cytokinin and ethylene in these tissues are. Subsequently, the effect of misexpression of *ENOD40* on (the coupling of) these processes should be tested. For this purpose, the investigation of the spatial and temporal correlation between phytohormone distribution and cellular responses in developing nodules in a plant background in which phytohormone distribution, synthesis or perception and/or *ENOD40* expression is disturbed seems necessary, yet requires a complex experimental set-up.
Whether the *ENOD40* induced responses in BY-2 cells provide insight in *ENOD40* function in other plant species, depends on whether its function is conserved across the

plant kingdom. The function of *ENOD40* can be thought of at two levels. *ENOD40* function may be thought of in terms of a molecular mechanism, or alternatively in terms of a physiological or morphological response. We would like to note that the level of sequence homology between transcripts and the observation that a *ENOD40* transcript is biologically active in a heterologous plant background, suggests that *ENOD40* gene function is at a molecular level similar in different plant species. However, this can only be confirmed by knowledge of the molecular mechanism of *ENOD40* action. On the other hand, if *ENOD40* mediates cross-talk between phytohormone pathways, the induced cellular responses may either be similar or opposing in different tissues or plant species depending on additional up- or downstream signaling events, even though the molecular mechanisms are the same.

In conclusion, the observation that *ENOD40* affects phytohormone responses in BY-2 cells is consistent with the spatio-temporal expression patterns, which correlates with developmental stages involving the activity of phytohormones. We propose that coupling between cell division, cell growth and cell differentiation may be achieved by transient and local expression of plant factors like *ENOD40* that are involved in phytohormone cross-talk pathways. The coupling of hormone responses may be crucial to many developmental programs, and this may explain the complexity of *ENOD40* expression patterns and the variety of tissues with which *ENOD40* expression is associated in different plant species.

REFERENCES

Abel, S., Nguyen, M.D., Chow, W., and Theologis, A. (1995). *ACS4*, a primary indoleacetic acid-responsive gene encoding 1-aminocyclopropane-1-carboxylate synthase in *Arabidopsis thaliana*. J. Biol. Chem. **270,** 19093-19099.

Abel, S., and Theologis, A. (1996). Early genes and auxin action. Plant Physiol. **111,** 9-17.

Beckman, E.P., Saibo, N.J.M., Di Cataldo, A., Regalado, A.P., Ricardo, C.P., and Rodrigues-Pousada, C. (2000). Differential expression of four genes encoding 1-aminocyclopropane-1-carboxylate synthase in *Lupinus albus* during germination and in response to indole-3-acetic acid and wounding. Planta **211**.

Cary, A.J., Liu, W., and Howell, S.H. (1995). Cytokinin action is coupled to ethylene in its effects on the inhibition of root and hypocotyl elongation in *Arabidopsis thaliana* seedlings. Plant Physiol. **107,** 1075-1082.

Charon, C., Sousa, C., Crespi, M., and Kondorosi, A. (1999). Alteration of *ENOD40* expression modifies *Medicago truncatula* root nodule development induced by *Sinorhizobium meliloti*. Plant Cell **11,** 1953-1965.

Compaan, B., Yang, W.C., Bisseling, T., and 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.

Coenen, C., and Lomax, T.L. (1997). Auxin-cytokinin interactions in higher plants: old problems and new tools. Trends Plant Sci. **2,** 351-356.

Crespi, M.D., Jurkevitch, E., Poiret, M., d'Aubenton-Carafa, Y., Petrovics, G., Kondorosi, E., and 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.

Ecker, J.R. (1995). The ethylene signal transduction pathway in plants. Science **268,** 667-675.

Hasezawa, S., and Syono, K. (1983). Hormonal Control of Elongation of Tobacco Cells Derived from Protoplasts. Plant Cell. Physiol. **24,** 127-132.

Hua, J., Chang, C., Sun, Q., and Meyerowitz, E.M. (1995). Ethylene insensitivity conferred by *Arabidopsis* ERS gene. Science **269,** 1712-1714.

John, P.C.L., Zhang, K., Dong, C., Diederich, L., and Wightman, F. (1993). P34-cdc2 related proteins in control of cell cycle progression, the switch between division and differentiation in tissue development and stimulation of division by auxin and cytokinin. Aust. J. Plant Physiol. **20,** 503-526.

Komina, O., Zhou, Y., Sareth, G., and Chollet, R. (2002). In vivo and in vitro phosphorylation of membrane and soluble forms of soybean nodule sucrose synthase. Plant Physiol. **129,** 1644-1673.

Kouchi, H., and Hata, 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.

Le, J., Vandenbussche, F., Van Der Straeten, D., and Verbelen, J. (2001). In the early response of *Arabidopsis* roots to ethylene, cell elongation is up- and down-regulated and uncoupled from differentiation. Plant Physiol. **125,** 519-522.

Lindsey, K., Casson, S., and Chilley, P. (2002). Peptides: new signalling molecules in plants. Trends Plant Sci. **7,** 78-83.

Mirabella, R., Martirani, L., Lamberti, A., Iaccarino, M., and 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.

Mylona, P., Pawlowski, K., and Bisseling, T. (1995). Symbiotic Nitrogen Fixation. Plant Cell **7,** 869-885.

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

Sousa, C., Johansson, C., Charon, C., Manyani, H., Sautter, C., Kondorosi, A., and 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. Mol. Cell. Biol. **21,** 354-366.

Swarup, R., Parry, G., Graham, N., Allen, T., and Bennett, M. (2002). Auxin cross-talk: integration of signaling pathways to control plant development. Plant Mol. Biol. **49,** 411-426.

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., and Bisseling, T. (1996). Modification of phytohormone response by a peptide encoded by *ENOD40* of legumes and a nonlegume. Science **273,** 370-373.

Vleghels. (2003). Comparative studies on *ENOD40* in legumes and non-legumes. Thesis, Wageningen University and Research Center Wageningen, the Netherlands**,** pp 99.

Vogel, J.P., Woeste, K.E., Theologis, A., Kieber, J.J. (1998) Recessive and dominant mutations in the ethylene biosynthetic gene *ACS5* of *Arabidopsis* confer cytokinin insensitivity and ethylene overproduction, respectively. Proc. Natl. Acad. Sci. USA **95,** 4766-4771.

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

SAMENVATTING

De interactie tussen *Rhizobium* bacterien en vlinderbloemige planten leidt tot de ontwikkeling van een nieuw orgaan, de wortelknol, op de wortels van leguminose planten. Een set van signaal moleculen zoals Nod-factoren en de planten hormonen ethyleen, auxine en cytokinine alsmede plant genen die mogelijk coderen voor een groeifactor, coördineren gezamenlijk dit ontwikkelingsproces. Een van de genen die sterk geïnduceerd wordt tijdens de vroege stadia van het proces is het *ENOD40* gen. Als gevolg van overexpressie van *ENOD40* wordt het nodulatie proces versneld, terwijl het uitschakelen van *ENOD40* door middel van co-suppressie tot knolletjes leidt die verstoord zijn in hun ontwikkeling (Charon et al., 1999). Deze observaties duiden erop dat *ENOD40* een regulerende rol zou kunnen hebben bij dit proces. Het *ENOD40* gen is niet alleen aanwezig in vlinderbloemigen maar komt ook voor in de genomen van niet-vlinderbloemigen. In hoofdstuk 1 hebben we een overzicht gegeven van het voorkomen van *ENOD40* genen in het plantenrijk en van de opbouw van de transcripten. Opvallend is dat geen van de *ENOD40* genen een lang open leesraam bevat dat zou kunnen coderen voor een eiwit. Wel zijn er twee sterk geconserveerde regio's aanwezig. Regio I bestaat uit een kort open leesraam dat codeert voor een opmerkelijk klein peptide van slechts 10-13 aminozuren terwijl er in regio II in slechts 50% van de transcripten een geconserveerd leesraam aanwezig is. Dit suggereert dat de tweede regio actief zou kunnen zijn op het RNA niveau. De sterke mate van conservering duidt erop dat de twee regio's belangrijk zouden kunnen zijn voor de biologische activiteit van *ENOD40*, maar een functie van de twee regio's of het (potentieële) peptide is nog niet bekend. De *ENOD40* expressie patronen in verschillende plantensoorten laten zien dat *ENOD40* transient tot expressie komt in verscheidene ontwikkelingsprocessen. Er zijn een aantal mogelijke functies voor het *ENOD40* gen gepostuleerd in deze processen, een ervan is dat het *ENOD40* gen de respons op fytohormonen, of de gevoeligheid of de concentraties van fytohormonen in cellen zou kunnen beïnvloeden. In deze thesis beschrijven we de bio-assays die we hebben ontwikkeld om de functie van het *ENOD40* gen in meer detail te kunnen bestuderen.

In hoofdstuk 2 hebben we het effect van *ENOD40* op fytohormoon signalering bestudeerd door een bioassay te ontwikkelen die gebaseerd is op de morfologische respons van BY-2 tabakscelcultures op de fytohormonen auxine, cytokinine en ethyleen. We hebben gevonden dat in transgene 35S:*NtENOD40* bevattende lijnen een verhoogde expressie van *ENOD40* de elongatiegroei van deze cellen onderdrukt, terwijl de delingsfrequentie niet beïnvloed wordt. Deze delingsfrequentie staat onder controle van auxine en cytokinine en wel via hun concentratie ratio. Door de delingsfrequentie van de transgene 35S:*NtENOD40* bevattende lijnen te bepalen bij varierende auxine tot cytokinine ratio's konden we aantonen dat de controle op de delingsfrequentie niet beïnvloed wordt door de verhoogde expressie van *ENOD40*. Elongatiegroei is onafhankelijk van de ratio tussen cytokinine en auxine in wild type cellen waar *ENOD40* niet of nauwelijks tot expressie komt. In tegenstelling tot de wild type cellen, is in cellen die *ENOD40* tot overexpressie brengen de elongatiegroei gereduceerd in zowel een cytokinine- als een auxine-afhankelijke manier. Dat de elongatiegroei niet onder alle omstandigheden in dezelfde mate gereduceerd is, laat zien dat *ENOD40* niet simpelweg een blokker is van elongatiegroei. Deze proeven tonen verder aan dat *ENOD40* en cytokinine synergistisch werken en dat het effect van *ENOD40* kan worden tegengewerkt door auxine. Deze experimenten suggereren dan ook dat bij de controle op elongatiegroei, maar niet bij de controle op de delingsfrequentie, er cross-talk plaats vindt tussen *ENOD40* en zowel auxine als cytokinine signalering. Analyse van de relatieve snelheden van de elongatiegroei en de delingsfrequentie laten zien dat in de transgene 35S:*NtENOD40* lijnen, de elongatiegroei sterk gekoppeld wordt aan de delingsfrequentie met als resultaat dat de grootte van cellen constant wordt. Analyse van de ethyleen homeostase laat zien dat het cytokinine- en auxine-afhankelijke effect op de elongatiegroei gemediëerd wordt door ethyleen. Ethyleen biosynthese vindt versneld plaats in de transgene *ENOD40* lijnen en is gecorreleerd met een versnelde accumulatie van ACC synthase transcripten, die coderen voor een enzym betrokken bij ethyleen productie. De observatie dat constitutieve expressie van *ENOD40* niet leidt tot constitutieve ethyleen productie suggereert dat *ENOD40* niet een directe inducer is van ethyleen biosynthese. Daarnaast leidt de observatie dat het effect van *ENOD40* kan worden tegengewerkt door toevoeging van een blokker van de ethyleen receptor tot een

aantal belangrijke conclusies. In de eerste plaats suggereert het dat ethyleen een negatieve regulator is van elongatiegroei in BY-2 cellen. Dit effect van ethyleen was nog niet beschreven in BY-2 cellen maar is vergelijkbaar met het effect van ethyleen in een aantal andere processen, zoals in de controle op elongatiegroei van cellen in de elongatiezone van *Arabidopsis* wortels. Ten tweede laten deze experimenten zien dat een versnelde accumulatie van ethyleen een primaire oorzaak en niet een gevolg is van de gereduceerde elongatiegroei van de tabakscellen. Omdat *ENOD40* waarschijnlijk geen directe inducer van ethyleen biosynthese is, is het waarschijnlijker dat de veranderde regulatie van ethyleen biosynthese onderhevig is aan, of onderdeel is van, het mechanisme dat de snelheid van elongatiegroei aan de delingsfrequentie koppelt. Omdat de toevoeging van de ethyleen perceptie blokker er toe leidt dat de koppeling tussen deze twee processen verloren gaat, suggereren deze observaties dat perceptie van ethyleen belangrijk is voor de koppeling tussen elongatiegroei en delingsfrequentie. Concluderend kunnen we stellen dat overexpressie van *ENOD40* resulteert in de koppeling tussen fytohormoon gereguleerde processen. De observatie dat de door *ENOD40* geïnduceerde cytokinine- en auxine-afhankelijke elongatiegroei respons gemediëerd wordt door ethyleen, ondersteunt de hypothese dat *ENOD40* de respons van cellen op fytohormonen kan beïnvloeden. Onze data wijzen er daarom op dat *ENOD40* de cross-talk tussen ethyleen en auxine/cytokinine signaal transductie routes faciliteert.

Om meer gedetailleerd inzicht te krijgen in het mechanisme van de *ENOD40* activiteit, is het nodig de biologisch actieve moleculen te identificeren. In het geval van *ENOD40* is dit verre van triviaal omdat een lang open leesraam dat voor een biologische actief eiwit zou kunnen coderen niet aanwezig is in de *ENOD40* transcripten.
In hoofdstuk 3 hebben we de functie van de twee geconserveerde regio's in the *ENOD40* transcripten onderzocht door de activiteit van verschillende transcripten te toetsen in de bio-assay die is beschreven in hoofdstuk 2. Experimenten waarbij we het *ENOD40* peptide aan de *GFP* reporter fuseren, tonen aan dat het korte leesraam van regio I daadwerkelijk vertaald wordt. Deze data komen overeen met eerdere experimenten (Compaan et al., 2001; Sousa et al., 2001; Van de Sande et al., 1996) en

ze ondersteunen de hypothese dat het kleine peptide biologische activiteit zou kunnen hebben. Echter, translatie van dit peptide is niet nodig voor de reductie van elongatiegroei in de protoplasten-assay, en dit suggereert dat de biologische activiteit in de nucleotide sequentie van regio I en/of II zou kunnen liggen. Een opvallend resultaat is dat een verstoring van de translatie van regio I leidt tot een afname van de delingsactiviteit, terwijl dit niet werd gevonden in de lijnen die het intacte construct tot overexpressie brengen. De observatie dat verstoring van translatie van ORF I een additioneel fenotype induceert ten opzichte van het effect van het intacte transcript, duidt erop dat het peptide toch activiteit heeft maar ook dat de functie van de twee regio's met elkaar in verband staat. Omdat de semi-kwantitatieve aard van deze bio-assays waarschijnlijk niet voldoende is om de complexe effecten van de twee regio's te ontrafelen, zijn andere experimentele systemen nodig om de rol van de twee geconserveerde regio's en de aard van de biologisch active component die door *ENOD40* gecodeerd wordt, op te helderen.

In hoofdstuk 4 hebben we onderzocht of model plant *Arabidopsis thaliana* te gebruiken is om de met *ENOD40* interacterende componenten te identificeren omdat *Arabidopsis* grote voordelen biedt met betrekking tot het uitvoeren van genetische screens. Hoewel uit het overzicht van het voorkomen van *ENOD40* genen in het plantenrijk (hoofdstuk 1) blijkt dat *ENOD40* een algemeen voorkomend gen is, hebben sequentie homologie analyses geen *ENOD40* homoloog in het *Arabidopsis* genoom opgeleverd. Omdat we in hoofdstuk 3 hebben laten zien dat *ENOD40* homologen ook in een heterologe plant achtergrond actief kunnen zijn, hebben we een tabaks homoloog van *ENOD40* (*NtENOD40*) gebruikt om transgene lijnen te genereren die *NtENOD40* tot overexpressie brengen. Om een genetische screen uit te kunnen voeren is het allereerst nodig een fenotype ten gevolge van *ENOD40* misexpressie in *Arabidopsis* te identificeren. We hebben de transgene lijnen op morfologische afwijkingen getoetst onder normale groei condities, alsook na behandeling met fytohormonen. Echter, in geen van de transgene lijnen die gegenereerd zijn, werd een fenotype geobserveerd, hetgeen suggereert dat *NtENOD40* geen effect heeft op *Arabidopsis* groei en ontwikkeling. De meest waarschijnlijke verklaring is dat, omdat *Arabidopsis* geen gen

met sterke homologie met *NtENOD40* heeft, de gen producten van het *NtENOD40* transgen niet herkend worden en daarom niet functioneel zijn in de *Arabidopsis* achtergrond. Helaas zullen daarom de transgene *Arabidopsis* lijnen die we gegenereerd hebben waarschijnlijk niet gebruikt kunnen worden om het werkingsmechanisme van *ENOD40* op te helderen en zullen andere model planten, zoals bijvoorbeeld *Medicago* die het *ENOD40* gen wel in zijn genoom heeft, gebruikt moeten worden voor een dergelijke screen.

De belangrijkste resultaten van deze thesis hebben we verkregen uit de bio-assays gebaseerd op een cellulair test systeem en deze duiden erop dat het koppelen van fytohormoon signaal transductie routes de functie van *ENOD40* is. Hoewel de fytohormonen cytokinine, auxine en ethyleen zeer vergelijkbare effecten hebben in het cellulaire systeem als in hele planten, is hiermee nog niet de functie van *ENOD40* in intacte planten bewezen. De grote uitdaging die nu volgt is aantonen of de interactie tussen *ENOD40* en fytohormoon signaal transductie routes ook daadwerkelijk de basis vormt voor de functie van *ENOD40* genen in hele planten. Een proces dat hiertoe zou kunnen worden aangewend is uiteraard de knolvorming in vlinderbloemige planten. Verschillende fytohormonen spelen een belangrijke regulerende rol in cellulaire processen tijdens de knolontwikkeling. *ENOD40* komt tot expressie in het knolprimordium waar celdeling en celgroei plaatsvinden en in Zone II welke, in knollen van het ‘indeterminate’ type, de zone is tussen cellen van de meristematische zone en de gedifferentieerde cellen van het centrale weefsel. Juist in deze weefsels waar verschillende cellulaire processen plaatsvinden is het aannemelijk dat de koppeling tussen cellulaire processen belangrijk is voor de voortgang van de ontwikkeling van het orgaan, en dat *ENOD40* benodigd is voor de communicatie tussen de regulerende factoren.

Een Woord van Dank

Als ik de ingredienten die mijn leven de laatste jaren zo mooi hebben gemaakt elk apart zou moeten noemen zou het een lange lijst worden die ieder geen eer meer aan zou doen. Ik zou moeten beginnen met de mij zeer aan het hart liggende collega's van de vakgroep Molbi, waar de grens tussen collega's en vrienden vervaagt, ik zou de polo, de koekjes, de cola en mijn trouwe radiootje willen noemen en natuurlijk mijn familie die me altijd door dik en dun gesteund heeft en kunnen eindigen met een hele schare vrienden die ik in Wageningen heb mogen leren kennen. Maar net zo goed zou ik kunnen eindigen met het bedanken van de leden van de fotoclub die mij opnieuw hebben leren kijken of meneer Nikon voor het maken van geweldige camera's.

Dat ik het promotie onderzoek dat in dit boekje beschreven is heb kunnen uitvoeren, heb ik in ieder geval te danken aan de enorme inzet van een aantal mensen die ik hier wel speciaal wil noemen. Henk, veel heb ik aan jou te danken, je enthousiasme en liefde voor het vak zijn waardevol en aanstekelijk. Ik ben er trots op bij jou te zijn gepromoveerd. Ton, het was kort maar krachtig, helderheid en snelheid; ik heb ongelofelijk veel van je geleerd en hoop het nog vaker mee te mogen maken. Bert en Ingrid, vrienden voor het leven, samen met jullie en Jan en Xi had ik het niet beter kunnen treffen in de "*ENOD40*" groep. "Mijn studenten" Silvia, Ciska en Maelle, het was geweldig om jullie te zien groeien en echt goed te worden in wat je doet. Gerard, jij hebt de BY-2 transformaties die de kern van dit onderzoek vormen in ons lab opgezet, fantastisch werk! Bert en Tinka, jullie hebben me ontzettend veel werk uit handen genomen bij het verzorgen en oogsten van de arabidopsis planten, bedankt! Verder iedereen met wie ik op Molbi heb samengewerkt: ik hoop dat we elkaar nog vaak en op onverwachte plekken tegen mogen komen en dat er meer dan alleen werk is om over te praten. Elf jaar Wageningen waren fantastisch maar lang genoeg. Ik mag mezelf gelukkig prijzen nooit alleen te zijn geweest, met zoveel vrienden om me heen heb ik me geen moment verveeld. Te veel herinneringen om op te noemen, genoeg verhalen voor heel wat nostalgische avondjes, maar ik leef liever vandaag dan gisteren dus laten we er meteen nog een flinke stapel nieuwe verhalen bij gaan maken. Laat het gezegd zijn.

Bedankt, het was een mooie tijd. tommie.

A word of gratitude

If I would have to list all ingredients that have made my life so valuable during the recent years, I would end up with a list so long it would not do justice to each of them. I'd have to start with the dear colleagues of the Molbi department where the difference between colleagues and friends has disappeared, I'd have to mention the polo, the cookies, the cola and my faithful radio, of coarse I'm grateful to my dearly beloved family who have always supported me in an amazing way and I could end with a whole bunch of friends with whom I've had the chance to meet. But just as well I could end by thanking the members of the photography club who have taught me to look at the world in new and different ways or Nikon for producing wonderful cameras, or…

This thesis is all about the research that I have performed in the last six years and it would never have been possible without the extraordinary input of a number of people. Henk, I owe a lot to you, your enthusiasm and love for the work are very valuable and encouraging. I'm proud to have had you as a mentor. Ton, clarity, speed and a number of eye-openers, I've learned an incredibly from our discussions and hope to do it more often. Bert and Ingrid, friends for life, together with Jan-the-pun-Ho and Xi, I could not have been more lucky than to work with you in the '*ENOD40*' group. As for my students Silvia, Ciska and Maelle; it was great to see you grow and become really good at what you do. Gerard, you introduced the BY-2 cell transformation in our lab which turned out to be so crucial, thank you for helping me generate the core of my research. Bert and Tinka, by taking care of the arabidopsis plants you took a load of work off my hands, thanks! And to everybody with whom I've worked at the Molbi department: I hope that we meet again and again at unexpected places and that we have more to discuss than just work.

Eleven years of Wageningen were fantastic but long enough. Too many memories to remember, enough stories to fill a lifetime of nostalgic evenings. But as I'd rather live today than yesterday, lets just start now with living new adventures. Let it be said. Those who know, know.

Thanks, 'had a great time. tommie.

Curriculum Vitae

Tom Ruttink werd geboren op 15 januari 1974 te Utrecht. In september 1992 haalde hij het VWO diploma aan het Montessori Lyceum Herman Jordan te Zeist. Tussen 1992 en 1997 studeerde hij Moleculaire Wetenschappen aan de Wageningen Universiteit. In september 1997 behaalde hij het ingenieursdiploma met afstudeervakken Plantenfysiologie (onder begeleiding van Dr. W. van Leeuwen en Dr. A.R. van der Krol), waarvoor de C.T. de Wit scriptieprijs werd uitgereikt, Moleculaire Fysica (onder begeleiding van Dr. L. Meulenkamp en Dr. H. van As) en Moleculaire Plantenfysiologie aan de University of Guelph, Canada (onder begeleiding van Prof. J. Strommer). Vanaf februari 1998 was hij Onderzoeker in Opleiding (OIO) 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 en is gefinancïerd door de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO 805 49 004). Per december 2003 is hij werkzaam als Post-doc aan het Vlaanderen Instituut voor Biotechnologie (VIB) in de onderzoeksgroepen van Prof. Dr. D. Inzé en Prof. Dr. W. Boerjan.

List of publications

van der Weerd, L., Ruttink T., van Dusschoten, D., Vergeldt, FJ., de Jager, PA., and van As, H. (1998). Plant growth studies using low field NMR. *In*: Spatially resolved magnetic resonance. P. Blumler, B. Blumich, R. Botto, and E. Fukushima, editors. Wiley-VCH, Weinheim. 473 - 479.

van Leeuwen, W., Hagendoorn, MJM., Ruttink, T., van Poecke, R., van der Plas, LH., and van der Krol, AR. (2000). The Use of the Luciferase Reporter System for in Planta Gene Expression Studies. Plant Mol. Biol. Reporter **18** (2),143

van Leeuwen W., Ruttink T., Borst-Vrenssen AW., van der Plas, LH., van der Krol, A.R. (2001). Characterization of position-induced spatial and temporal regulation of transgene promoter activity in plants. J. Exp. Bot. **52** (358) 949-959.

van der Weerd, L., Claessens, MM., Ruttink, T., Vergeldt, FJ., Schaafsma, TJ., Van As, H. (2001). Quantitative NMR microscopy of osmotic stress responses in maize and pearl millet. J. Exp. Bot. **52** (365) 2333-2343.

Vleghels, I., Ruttink, T., Compaan, B., and Franssen, H. (2001) Root nodule formation in legumes; a molecular chat-box. *In*: Recent Research Developments in Plant Physiology, Vol. 2 187-199.

Compaan, B., Ruttink, T., Albrecht, C., Meeley, R., Bisseling, T., Franssen, H. (2003). Identification of a *Zea mays* line carrying a transposon tagged *ENOD40*. BBA **-** Gene Structure and Expression, **1629** (1-3) 84-91.

The research described in this thesis was carried out at the Laboratory of Molecular Biology, Wageningen University, The Netherlands. This work was supported by a research grant from the Netherlands Organization for Scientific Research (NWO 805.49.004).