Analyses of nodule meristem persistence and *ENOD40* functioning in *Medicago truncatula* nodule formation

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CONTENTS

OUTLINE

Medicago truncatula nodules are initiated in inner root cortical cells by *Sinorhizobium meliloti* secreted Nod factors. To understand how Nod factor signaling pathway can trigger nodule formation, the role of *ENOD40,* as one the genes that is activated by this pathway, was studied. *ENOD40* is induced in pericycle cells before cortical cell division starts. Its expression is also observed in the nodule primordium and mature nodule, indicating that *ENOD40* plays a role at several steps of nodule development. However, the precise function as well as the biological activity residing in *ENOD40* is unclear. The effect of co-suppression of *MtENOD40* resulted in a reduction of nodule number. However, the recent discovery of a new *M. truncatula ENOD40* EST makes it essential to clarify whether these two genes act redundantly or not, before it is possible to assign the observed effect to the *ENOD40* gene discovered first. In chapter 2, the effect of down-regulation of the two different Medicago *ENOD40* genes by gene-specific RNA interference on nodule number and structure was studied.

ENOD40 is most likely functional, through products of the conserved regions box1 and 2. However, the nature of the biological activity of box2 is unclear. To clarify this we set up a bioassay to investigate whether box2 activity is peptide or RNA mediated. This will be described in Chapter 3.

Once a *M. truncatula* nodule is formed, growth is maintained by the nodule meristem (NM), which continuously adds new cells to the growing nodule. How nodule meristem cells maintain their activity is unknown. In roots, persistence has been shown to be dependent on the presence of stem cells of which the identity is determined and maintained by quiescent center cells. Since nodules

are root-borne organs, we investigated whether in nodules a similar mechanism maintains meristematic activity. We studied whether the NM contains cells in which quiescent center and stem cell markers of the root meristem are active. This is described in Chapter 4.

In Chapter 5, the data described in chapters 2, 3 and 4 will be discussed.

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Wan Xi, 万 曦 30, October, 2007

CHAPTER 1

General Introduction

Chapter 1: General Introduction

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In nitrogen-deficient soil, legume roots exude host-specific compounds, flavonoids, which induce the expression of *Rhizobium nod* genes (Redmond *et al.,* 1986). These genes encode proteins involved in the synthesis and secretion of highly host-specific bacterial signal molecules, so-called Nod factors (NFs; Lerouge *et al.,* 1990). Perception of NFs induces root hair deformation and curling, and infection threads formation. Further, de-differentiation of cortical cells and a subsequent recapitulation of cell division activity leading to the formation of a nodule primordium in the cortex are also induced by Nod factors (Roche *et al.,* 1991). When infection threads reach a nodule primordium cell, rhizobia are released and the primordium will develop into a nodule meristem. The nodule meristem adds cells to form the nodule.

Histology of Medicago truncatula nodules

Two types of legume nodules can be distinguished on the basis of the persistence of their meristem. Soybean, bean and lotus form determinate nodules that have a temporary nodule meristem. They originate from outer cortical cells and division activity of the meristem stops shortly after infection thread entrance in the primordium cells (Newcomb, 1981; Calvert *et al*., 1984). The increase in organ size is mediated through cell growth. *Medicago truncatula* nodules, like those of pea and clover, originate from inner cortical cells. These nodules have a persistent meristem and have an elongated shape.

Medicago nodules are built up of peripheral tissues and a central tissue. The former one includes root cortex, nodule endodermis and nodule parenchyma in which nodule vascular bundles are located. The central tissue consists of infected and non-infected cells. As a consequence of the presence of a persistent meristem, cells in the central tissue are of graded age and form from the distal to the proximal end distinct zones: the meristem region is composed of dividing cells (Zone I). Cells proximal to this zone compose the infection zone (Zone II). In cells of this zone the nuclear DNA undergoes multiple rounds of duplication without further division (Roudier *et al*., 2003). In the fixation zone (Zone III), nitrogen fixation takes place. When nodules get older, Zone IV, the senescence zone is present in which cells are degenerated (Vasse, *et al.*, 1990; van de Wiel, 1991).

The nodule meristem

Little is known of how the activity of the nodule meristem (NM) is maintained in contrast to the two known persistent meristems in plants; the root apical meristem (RM) and shoot apical meristem (SM), respectively. The RM contains a stem cell niche where a group of mitotic less active cells, so called quiescent center cells (QC), is surrounded by stem cells (Dolan *et al*., 1993; Stahl and Simon, 2005). This quiescent center acts as an organizer for the surrounding stem cells in keeping them undifferentiated (van den Berg *et al*., 1997). A maximium in the concentration of the phytohormone auxin, and transcription factors like PLETHORA1 and 2 (PLT1,2), SHORTROOT (SHR) and SCARECROW (SCR) are required for positioning and maintaining the stem cell niche in RM (Aida *et al*., 2004; Blilou *et al.,* 2005).

In SM, the organizing center (OC) is located proximal to the stem cells at the meristem tip. A homeobox gene, *WUS (WUSCHEL)* is specifically expressed in

OC cells and induces stem cell fate in the apical part in a non-cell autonomous manner (Mayer *et al*., 1998). The *CLAVATA3 (CLV3)* gene, encoding a small secreted protein, is only translated in stem cells (Fletcher *et al.,* 1999) and the peptide is signaling back to the OC where it is perceived by CLV1 and CLV2, which may form a heterodimeric receptor complex to transmit signals (Schoof *et al*., 2000). The feedback regulation between WUS and CLV3 is believed to create a balance between OC and stem cells (Schoof *et al.,* 2000).

Although a description of the NM in terms of stem cells and organizers is currently lacking, several studies are in favor of considering nodules as root-like organs (Mathesius *et al*., 2000; Bright *et al*., 2005). For instance, the requirement of the *LATD* gene in NM and lateral RM formation strongly indicates that both organs share developmental pathways (Bright *et al*., 2005). The Lotus mutant *har1-1* that shows a supra-normal number of nodules after inoculation has an increased number of lateral roots in the absence of *Rhizobium* (Krusell et al., 2002; Nishimura et al., 2002). This indicates that the number of meristems, and consequently the number of primordia, formed in the root is controlled by the plant and suggests that lateral root and nodule formation share developmental pathways.

Phytohormones have been shown to be involved in both lateral root and nodule formation (Vanneste *et al.,* 2005; Van Noorden *et al.,* 2006). Auxin induces lateral root formation (Casimiro *et al*., 2001). In contrast, cytokinin has a negative role in lateral root formation, but induces nodule formation, as addition of cytokinin to Medicago roots induces nodule formation (Cooper and Long, 1994; Lohar *et al*., 2004). Down-regulation of the cytokinin receptor *MtCRE1* expression enhanced numbers and growth of lateral roots while both infection and nodule inception were disturbed (Gonzalez *et al*., 2006). An ortholog of

MtCRE1 (*LHK1*) has recently been cloned from *L. japonicus* twice by screening for suppressors of *har1-1* (hypernodulation) phenotype (*hit1-1*; Murray *et al*., 2006). A continuous active form of LHK1 is causing spontaneous nodule formation (Tirichine *et al.*, 2006). Thus loss- and gain-of-function of LHK1 indicate that cytokinin signaling is the main drive for cortical cell division and nodule formation.

Nod factor signaling pathways is essential for Rhizobium infection and nodule formation

Recently, screening for legume mutants that lost NF-induced responses has led to the cloning of a number of genes that are critical for nodule formation. These genes have been placed in a sequential order of action during NF perception and transduction. NF are perceived by putative NF receptors NFP, LYK3, and LYK4 in *M. truncatula*, and NFR1 and NFR5 in *L. japonicus* and PsSYM10 in pea (Geurts *et al*., 2005; Limpens *et al.*, 2003; Madsen *et al.*, 2003; Radutoiu *et al.*, 2003; Amor *et al.*, 2003; Walker *et al.*, 2000). These receptor kinases contain two or three LysM extra-cellular domains. In *M. truncatula*, after NF perception a DMI (Doesn't Make Infection)-dependent pathway is activated. It contains components encoded by genes *DMI1*, *DMI2* and *DMI3* (Ane *et al*., 2004; Levy *et al.*, 2004; Catoira *et al*., 2003). *DMI1* encodes a putative cation channel. *DMI2* codes for a leucine-rich repeats receptor kinase, while DMI3 is a calcium-calmodulin-dependent protein kinase. DMI1 and DMI2 are essential for intracellular Ca^{2+} spiking while DMI3 functions downstream of Ca^{2+} spiking. Two putative transcription factors NSP1 (Nodulation Signaling Pathway) and NSP2 are active downstream of the DMIs and elicit NF-related gene expression (Smit *et al.,* 2005; Kaló *et al.,* 2005). Based on the nodulation phenotype observed in Lotus mutants *snf2* (Tirichine *et al.*, 2006) and *hit1-1*

(Murray et al., 2006), cytokine biosynthesis/accumulation/perception has been positioned downstream of DMI3 but upstream of NSP2. In addition to the DMI-dependent pathway another signal transduction cascade is activated that induces root hair deformation. Components of this pathway have not been identified.
Thus the number of identified NF-signaling genes essential for nodulation is small and therefore it is intriguing how such a small number of genes can redirect the fate of root cortical cells and trigger the nodule patterning upon rhizobial infection. One way to investigate this is to identify genes induced by the NF signaling pathway (e.g. Lohar *et al.,* 2006) and study their function in nodule formation. One of these NF signaling pathway-induced genes is *ENOD40*, which is also inducible by cytokinin (Fang and Hirsch, 1998).

ENOD40

Upon inoculation with *Rhizobium*, *ENOD40* is highly induced within a few hours in the root pericycle cells of *M. sativa* next to the protoxylem pole (Compaan *et al*., 2001; Fang and Hirsch, 1998). *ENOD40* expression is also observed in cells of the nodule primordium, and in mature nodules, in cells of the infection zone, as well as in pericycle cells of nodule vascular bundles (Yang *et al.*, 1993). This expression pattern suggests that *ENOD40* plays a role in the nodule initiation and subsequent nodule development. However, its precise function remains unknown. Over-expression of *ENOD40* in *M. truncatula* roots grown under nitrogen limitation induced extensive cortical cell divisions (Charon *et al.*, 1997). Upon *Rhizobium* inoculation (Charon *et al.*, 1999), kinetics of nodulation was accelerated, but the total number of nodules was not affected. Co-suppression of *ENOD40* resulted in a reduced number of nodules that further underwent early senescence (Charon *et al.*, 1999). RNA interference of *ENOD40-1* and 2 in transgenic *L. japonicus* resulted in an arrest

in nodule primordium formation, but rhizobial infection was not affected (Kumagai *et al.*, 2006). These studies show that *ENOD40* is required for normal nodule formation.

Comparison of *ENOD40* sequences across the plant kingdom reveals that the lengths of all *ENOD40* cDNA sequences are between 400 and 800 bases (Ruttink, 2003). At the nucleotide level there are two highly conserved regions, box1 and box2. Instead of containing a long open reading frame (ORF), several short ORFs are present within the *ENOD40* transcript. The ORF present in box1 can be translated into a 12-13 amino acid oligo-peptide (Compaan *et al*., 2001; Van de Sande *et al*. 1996; Rohrig *et al.*, 2002; Sousa *et al*. 2001), that share the consensus sequence –W-(X4)-HGS. In contrast, not all *ENOD40* genes have an ORF spanning box2 and in those cases there is an ORF the amino acid sequence of the peptide is less conserved compared to its strong conservation at nucleotide level. This indicates that box2 residing in *ENOD40* likely functions at the RNA level. However, Sousa *et al*. (2001) using a ballistic micro-targeting approach to introduce DNA of box1 and box2 into Medicago roots, proposed that both box1 and 2 could be translated into a peptide. Therefore, it remains unclear what the biological activity of *ENOD40* is, and what the nature of the activity is; peptide and/or RNA.

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CHAPTER 2

Medicago truncatula ENOD40-1 and *ENOD40-2* are Both Involved in Nodule Initiation and Bacteroid Development

Chapter 2: *Medicago truncatula ENOD40-1* and *ENOD40-2* are Both Involved in Nodule Initiation and Bacteroid Development

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ABSTRACT

The establishment of a nitrogen-fixing root nodule on legumes requires the induction of mitotic activity of cortical cells leading to the formation of the nodule primordium and the infection process by which the bacteria enter this primordium. Several genes are up-regulated during these processes, among them *ENOD40*. Here we show by using gene-specific knock-down of the two *Medicago truncatula ENOD40* genes, that both genes are involved in nodule initiation. Further, during nodule development, both genes are essential for bacteroid development.

INTRODUCTION

Root nodules are specialised organs on the roots of legumes in which soil-borne bacteria, collectively known as rhizobia, are hosted intracellularly and fix atmospheric nitrogen. The formation of these organs requires a complex communication between the bacteria and their host plants. Plant secreted flavonoids are inducers of bacterial genes that code for proteins involved in the production of so-called Nod factors. These molecules are lipo-chitooligosaccharides consisting of a skeleton of 4, 5 N-acetyl glucosamines, substituted with specific modifications (Spaink, 2000). Nod factors are recognised by plant receptors that activate a Nod factor signalling pathway.

This induces mitotic activity in already differentiated cortical cells. These dividing cortical cells form the nodule primordium. Meanwhile, bacteria enter the root hair through a tube-like structure, the so-called infection thread. These threads grow towards the primordium and upon arrival, bacteria are released from the threads. The bacteria become entrapped within plant-plasma membrane and form the so-called symbiosomes. After infection the nodule primordium differentiates into a nodule (Stougaard, 2001; Limpens and Bisseling, 2003). *Medicago truncatula* nodules have a persistent meristem at their apex and nodule cells are of graded age along the apical-basal axis. Therefore, based on both plant and rhizobial cell morphology (Vasse *et al.,* 1990; Patriarca *et al.,* 2004) and gene expression (Scheres *et al.,* 1990), indeterminate nodules can be divided into 4 distinct zones, while 5 stages of bacteria development can be distinguished (Vasse *et al.,* 1990; Patriarca *et al.,* 2004). At the distal end the meristem forms zone I. Cells of the meristem are small and rich in cytoplasm, while infection threads and bacteria are absent. Infection threads are entering cells at the distal end of zone II, the infection zone. Here, rhizobia are released from the infection threads and are surrounded by a plant membrane together forming the symbiosome (rhizobia in stage I of development). The symbiosomes divide and the short rod-like rhizobia start to elongate (stage II). Rhizobia from now on are named bacteroids (Bergersen, 1974). In cells in the proximal part of the infection zone, bacteroids stop elongating and have a long rod-shaped structure (stage III). In cells of the fixation zone, zone III, the infected cells are fully packed with elongated bacteroids and the vacuoles of the cells have almost completely disappeared. At the distal part of the fixation zone, stage IV bacteroids are morphologically more heterogeneous and capable of fixing nitrogen. In older nodules, at the base of the nodule a zone can be distinguished where bacteroids disintegrate (stage

V) and plant cells go into senescence. This zone is called the senescence zone (zone IV).

During nodulation several host genes are up-regulated, indicating that these genes are important for establishing a symbiosis between the plant and rhizobia. *ENOD40* is one of these genes (Kouchi and Hata, 1993; Yang *et al.,* 1993; Crespi *et al.,* 1994) and its expression level is increased at the onset of nodulation (Compaan *et al.,* 2001). *ENOD40* is first expressed in pericycle cells and later in nodule primordium cells. Later in symbiosis *ENOD40* is expressed in cells of the nodule where differentiation of host cells and rhizobia is initiated.

ENOD40 has an unusual structure, since it lacks a long open reading frame (ORF). However, several short ORFs are present (Sousa *et al.,* 2001) in *ENOD40* transcripts. Therefore, it is possible that these oligo peptides are translated and that a peptide represents the biological activity of *ENOD40*. Alternatively, due to the lack of a long ORF and the highly structured RNA (Girard *et al.,* 2003), it has been suggested that the *ENOD40* activity resides in the RNA (Crespi *et al.,* 1994; Sousa *et al.,* 2001; Girard *et al.,* 2003). At the nucleotide level *ENOD40* transcripts share two regions of high sequence similarity, named box1 and box2 (Kouchi *et al.,* 1999). Some of the short ORFs reside in these regions. In particular, a 10-13 amino acid oligo peptide encoded by the ORF in box1 is conserved among plant species (Compaan *et al.,* 2001; Varkonyi-Gasic and White, 2002) with the exception of the *Casuarina glutinosa* (Santi et al., 2003). The high degree of conservation of box1 and box2 sequences indicates that these regions are important for *ENOD40* function. However, it remains to be solved whether the *ENOD40* acitivity is peptide or RNA-mediated.

The spatial and temporal expression of *ENOD40* suggests that this gene could play an important role in nodule development. Recently, it was reported that knock-down of *Lotus japonicus ENOD40 (LjENOD40)* expression by RNA interference (RNAi) leads to a strong reduction in nodule number, but bacterial infection of root hairs was not infected (Kumugai *et al.,* 2006). Also in *Medicago truncatula ENOD40* might be important in nodule initiation as plants in which ENOD40 expression is down regulated due to co-suppression (Charon *et al.,* 1999) nodule number is markedly reduced. Although these studies indicate that *ENOD40* has an important role in nodule initiation, none of the forward genetic screens for legume mutants disturbed in nodule formation has resulted in an *ENOD40* mutant. This might be due to functionally redundancy, or alternatively, it indicates that *ENOD40* is not essential for nodule formation.

Several legumes, like Lotus (Flemenakis *et al.,* 2000) and *Trifolium repens* (Varkonyi-Gasic and White, 2002), have more than one copy of *ENOD40*. However, until now only one *ENOD40* gene has been identified in *Medicago truncatula.* Recently, an EST has been deposited in the *Medicago truncatula* database of which the nucleotide sequence shows homology to *MtENOD40*. The identification of a putative second *MtENOD40* gene, *MtENOD40-2*, in *Medicago truncatula* opens the possibility to test whether both genes are required for nodule initiation and development.

To this end, we applied gene-specific RNAi to knock-down the expression of both genes separately in *Medicago truncatula* roots.

RESULTS

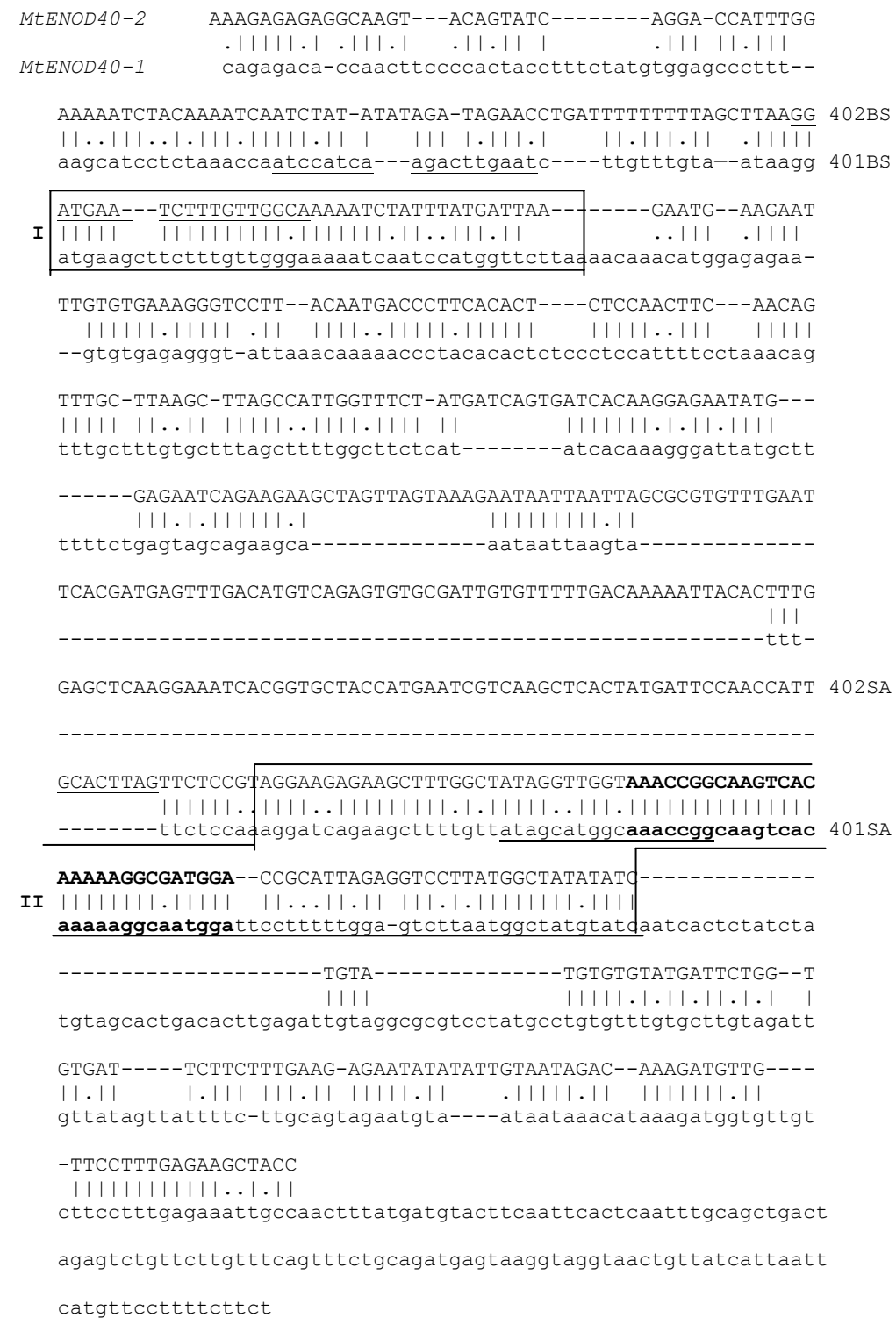
The *Medicago truncatula* genome contains two *ENOD40* genes

The *Medicago truncatula* EST BF519327 (Genbank X80262) has high homology to the 3'UTR of *MtENOD40*. To obtain genomic sequences

corresponding to the BF519327 encoding gene, a BAC library of *Medicago truncatula* (Nam *et al.,* 1999) was screened with BF519327. A 3.9 kb DNA fragment from a BAC clone was sequenced and this contained a new *ENOD40* gene, which has a region that is 100% identical to BF519327. Comparison of the nucleotide sequence of *MtENOD40-2* and *MtENOD40-1* (Fig.1A) shows that the two genes only share 50% homology. Furthermore, the *MtENOD40-2* gene contains the two conserved boxes (Fig.1A-B) present in all leguminous *ENOD40* genes identified to date of which one contains an open reading frame for a small peptide (Fig.1B). Whereas in all legumes the *ENOD40* peptide contains the motif C-W-(X3)-I-H-G-S, in the *MtENOD40-2* peptide the -I-H-G-S amino acid sequence is substituted by -I-Y-D (Fig.1B).

On a Southern blot containing *Eco*RI digested *Medicago truncatula* genomic DNA the *MtENOD40-2* probe hybridised to two fragments, which is expected as *MtENOD40-2* contains one *Eco*RI site. In contrast, *MtENOD40-1* probe hybridized to one fragment, as *MtENOD40-1* does not contain an *Eco*RI site (data not shown). Thus these data show that the *Medicago truncatula* genome contains two *ENOD40* genes.

A


MtENOD40-2
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MtENOD40-1
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AAAAATCTACAAAATCAATCTAT-ATATAGA-TAGAACCTGATTTTTTTTTAGCTTAAGG 402BS
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I
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--gtgtgagagggt-attaaacaaaaaccctacacactctccctccattttcctaaacag
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------GAGAATCAGAAGAAGCTAGTTAGTAAAGAATAATTAATTAGCGCGTGTTTGAAT
ttttctgagtagcagaagca--------------aataattaagta--------------
TCACGATGAGTTTGACATGTCAGAGTGTGCGATTGTGTTTTTGACAAAAATTACACTTTG
ttt-
GAGCTCAAGGAAATCACGGTGCTACCATGAATCGTCAAGCTCACTATGATTCCAACCATT 402SA
GCACTTAGTTCTCCGTAGGAAGAGAAGCTTTGGCTATAGGTTGGTAAACCGGCAAGTCAC
--------ttctccaaaggatcagaagcttttgttatagcatggcaaaccggcaagtcac 401SA
II
AAAAAGGCGATGGA--CCGCATTAGAGGTCCTTATGGCTATATATC--------------
aaaaaggcaatggattccttttttgga-gtcttaatggctatgtatcaatcactctatcta
----------TGTA----------TGTGTGTATGATTCTGG--T
tgtagcactgacacttgagattgtaggcgcgtcctatgcctgtgtttgtgcttgtagatt
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gttatagttattttc-ttgcagtagaatgta----ataataaacataaagatggtgttgt
-TTCCTTTGAGAAGCTACC
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agagtctgttcttgtttcagtttctgcagatgagtaaggtaggtaactgttatcattaatt
catgttccttttcttct

B

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

Fig. 1. Comparison of *MtENOD40* sequences. A. Nucleotide sequence alignment of *MtENOD40-1* (lower case) and *MtENOD40-2* (upper case). Box1 and box2 sequences are boxed. Within the box2, the nucleotide sequences conserved in all *ENOD40* sequences (Kouchi *et al.,* 1999) are in bold. Nucleotide sequences of primers for gene specific knock-down are underlined. Primer names are indicated at the right. B. Nucleotide sequence alignment of box1 and amino-acid sequence alignment of the ORF in box1 of legume *ENOD40* genes. Plant species abbreviations and Genbank Accession Numbers: *MsENOD40*, *Medicago sativa* (L32806); *MtENOD40*, *Medicago truncatula* (1, X80264; 2, X80262); *VsENOD40*, *Vicia sativa* (X83683); *PsENOD40*, Pisum sativum (X81064); *TrENOD40*, *Trifolium repens* (1, AF426838; 2, AF426839; 3, AF426840); *SrENOD40*, *Sesbania rostrata* (Y12714); *PvENOD40*, *Phaseolus vulgaris* ((X86441); *LjENOD40*, *Lotus japonicus* (1, AF013594; 2, AJ271788); *GmENOD40*, *Glycine max* (1, X69154; 2, D13503).

MtENOD40-2 is induced during nodulation

To determine whether MtENOD40-2 is expressed in nodule primordia and nodules, like *MtENOD40-1*, hairy roots containing a 1.8Kb DNA fragment upstream of the coding region of *MtENOD40-2* to drive the *GUS* reporter gene were analysed for GUS activity 2 and 21 days after inoculation with *S. meliloti*. In sections of roots collected 2 days post-inoculation, GUS activity was present in dividing cortical cells, indicating that *MtENOD40-2* is expressed in cells of

the nodule primordium (Fig. 2C). Whole-mount staining for GUS activity of nodules showed that GUS activity is detected near the apex of the nodule and in vascular bundles (Fig. 2D). To precisely localise the site of expression of *MtENOD40-2* in the nodule in situ hybridisation using 35S-UTP labelled anti-sense *MtENOD40-2* RNA was conducted (Fig. 2A-B). This showed that *MtENOD40-2* is expressed in cells of the infection zone (Fig. 2B, IZ). Thus, the *MtENOD40-2* expression pattern is similar to the *MtENOD40-1* expression pattern (Crespi et al., 1994). Furthermore, the *GUS* expression studies are consistent with the in situ hybridisation data, indicating that the 1.8kb DNA fragment used, contains the elements required for the regulation of *MtENOD40-2* expression. Based on the combination of expression data and the sequence homology between both genes it is likely that *MtENOD40-2* is functional in nodule initiation and development.

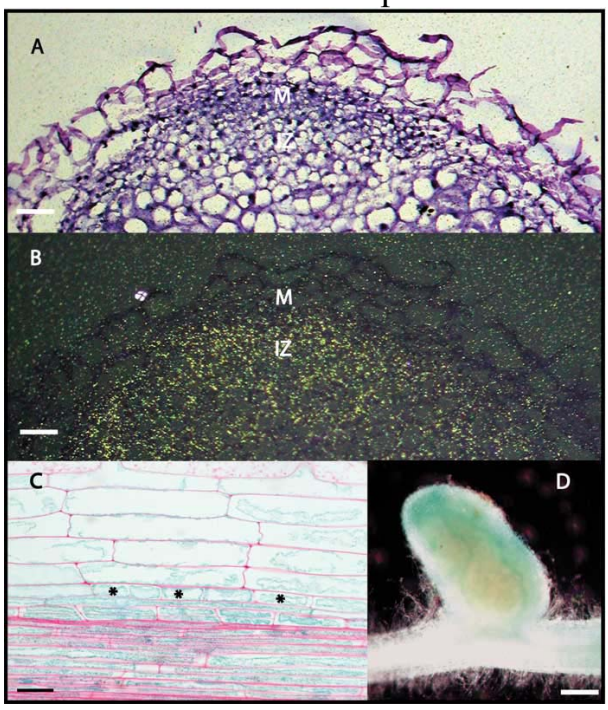

Fig.2 Expression of *MtENOD40-2* during nodulation. (colorful picture in appendix). A, B, *In situ* hybridization of a longitudinal section hybridized to 35S-UTP labelled anti-sense *MtENOD40-2* RNA. A, bright field micrograph; B, dark field micrograph, where signal appears as white grains. Signal is present in the infection zone (IZ) and absent from the meristem (M), bar=200µm. C, histochemical localization of GUS activity in semi-thin (7 µm) section of a *pMtENOD40-2: GUS* roots, 2 days after inoculation with *S.meliloti*. Dividing cortical cells are indicated by an asterisk (*), bar=25µm. D, whole mount detection of *pMtENOD40-2: GUS* activity in 21-days-old nodules, showing promoter activity in the apical part of the nodule and in vascular bundles.Bar=0.5mm.

Gene specific knock-down of *MtENOD40-1* and *MtENOD40-2*

To find out whether *MtENOD40-1* and *MtENOD40-2* are both required for nodule formation, the effect of reduction in expression of each gene individually on nodule formation has been investigated.

To reduce *MtENOD40-1* and *MtENOD40-2* gene expression we applied *A. rhizogenes* mediated RNAi in *Medicago truncatula* hairy roots. (Limpens *et al.,* 2004). Therefore, we designed one vector (pRRsil401) that is expected to lead to a reduction in expression of *MtENOD40-1*, and a second vector (pRRsil402) that is expected to lead to a reduction in *MtENOD40-2* expression. To knock-down transcription of both genes simultaneously a third vector (pRRsil4012) was used (Material and Methods).

Two week old transgenic roots were analysed for the levels of *MtENOD40-1* and *MtENOD40-2* transcripts by RT-PCR reaction using *MtENOD40-1* and *MtENOD40-2* specific primers (Fig. 3). The transcript level of *MtENOD40-1* was reduced about 25-fold in RNA isolated from *MtENOD40-1* RNAi roots as compared to that of control roots, that did not show red fluorescence (Fig. 3A, compare column a and d), while *MtENOD40-2* expression was not altered in *MtENOD40-1* RNAi roots (Fig3B, compare column a and d). In RNA isolated from *MtENOD40-2* silenced roots, the transcript level of *MtENOD40-2* is reduced 5 to 25-fold compared to transcript level in control roots (Fig3B, compare column b and d), while the level of *MtENOD40-1* in *MtENOD40-2* RNAi and control roots is similar (Fig. 3B, compare column a and d). Thus by using the pRRsil401 and pRRsil402, the expression of the two related genes *MtENOD40-1* and *MtENOD40-2* can be knocked down specifically. In roots containing RRsil4012 DNA the level of transcript of *MtENOD40-1* and *MtENOD40-2* is reduced more than 5-fold compared to the transcript levels of *MtENOD40-1* and *MtENOD40-2* in control roots (Fig. 3A, compare column c

and d; B, compare column c and d). This shows that application of pRRsil4012 leads to a reduction in expression of both *MtENOD40-1* and *MtENOD40-2*.

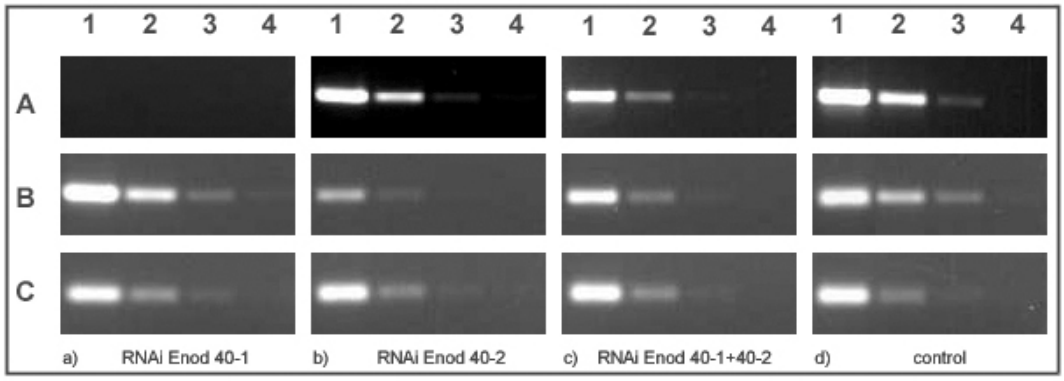

Fig. 3. RT-PCR analyses of *MtENOD40-1* and *MtENOD40-2* expression in knock-down roots using gene-specific primers. A, *MtENOD40-1* RNA level in *MtENOD40-1* RNAi (column a), *MtENOD40-2* RNAi (column b) and double RNAi (column c). Reduction of *MtENOD40-1* RNA level in *MtENOD40-1* RNAi and double RNAi, but not in *MtENOD40-2* RNAi (column b) roots, compared to control root (column d). B, *MtENOD40-2* RNA level in *MtENOD40-1* RNAi (column a), *MtENOD40-2* RNAi (column b) and the double RNAi (column c). *MtENOD40-2* RNA level is reduced in *MtENOD40-2* RNAi and double RNAi, but not in *MtENOD40-1* RNAi roots, compared to control root (column d). C, *Mtactin2* RNA levels. Amplification is shown in 0 (1), 5 (2), 25 (3) and 125-fold dilutions (4) of the cDNA mix at a fixed number of cycles; 30 cycles for *MtENOD40-1*, 30 cycles for *MtENOD40-2* and 22 cycles for *Mtactin.*

Reduced nodule number in *MtENOD40-1* and *MtENOD40-2* knock-downs

Nodules formed on roots in which the *MtENOD40-1* and *MtENOD40-2* genes are knocked down were determined three weeks after inoculation (Table 1). Whereas an average of 3.2 nodules/*MtENOD40-1* RNAi root was formed, 5.9 nodules /root are formed on control roots (46.4% reduction). On *MtENOD40-2* RNAi roots, the average number of nodules per root was 3.4, which corresponds to a reduction in nodule numbers of 38.5%. These data indicate that *MtENOD40-1* as well as *MtENOD40-2* is involved in nodule initiation. On roots, in which the expression of both *MtENOD40-1* and *MtENOD40-2* is

reduced, the average number of nodules per root is 1.5 (75% reduction). Thus knocking down of the expression of both genes has an additive effect suggesting that *MtENOD40* act in a dose dependent manner.

Table1: Effect of RNAi on number of nodules

	nodules/root	Reduction
MtENOD40-1 RNAi	3.2±0.3 (n=51)	46.4% (P<0.001)
Wild type	5.9±0.2 (n=61)	
MtENOD40-2 RNAi	3.4±0.2 (n=55)	38.5% (P<0.01)
Wild type	5.5±0.2 (n=62)	
MtENOD40-1 & -2 RNAi	1.5±0.2 (n=60)	75.0% (P<0.000001)
Wild type	6.0±0.3 (n=62)	

Reduced *MtENOD40-1/40-2* expression affects symbiosome development

To determine whether *MtENOD40-1* and *MtENOD40-2* are required for nodule development, we analysed nodules formed on *MtENOD40-1* and *MtENOD40-2* RNAi plants in more detail. Whereas 3 week-old nodules on control plants are rod-shaped (Fig. 4A), the nodules formed on *MtENOD40-1* or *MtENOD40-2* RNAi plants are spherical and small (Fig.4E). Longitudinal sections were prepared from control nodules and nodules of *MtENOD40-1* or *MtENOD40-2* RNAi roots to analyse their cytology.

Analyses of sections of *MtENOD40-1* RNAi nodules showed that in about half of the *MtENOD40-1* RNAi nodules (Fig 4F, J) the zonation of the central tissue can not be recognized (compare Fig. 4A-D and 4F, J). The majority of these nodules were senescent. Some cells in the proximal part are repopulated by rhizobia. These are rod-shaped and electron microscopy (EM) studies show that

they lack a plant-derived membrane and the ultrastructural differentiation features of bacteroids. Therefore, the bacteria colonize cells in a saprophytic manner (Timmers *et al.,* 2000; Fig. 4J-K). In about half of the *MtENOD40-2* RNAi and 40 % of the nodules from plants in which the expression of both genes was reduced growth disturbances are similar to those observed in the *MtENOD40-1* RNAi nodules.

Among the nodules studied we identified also nodules that were less disturbed in their development (Fig.4F-H). In cells of the infection zone of these nodules, a few bacteria are released from the infection threads and can be recognized as small rod-shape bacteroids. However, bacteroids remain short rod-shaped. At later stages of development, in the middle of the nodule lysed cells with irregular shape that lost turgor are present (Fig. 4F-G) as well as senescent cells (Fig. 4G-H). Thus light microscopy (LM) analyses showed that bacteroid development was impaired. To identify which step in bacteroid development is affected we studied bacteroids in these nodules by EM.

In *MtENOD40-1* RNAi nodules (Fig.5A-B), bacteria are released from infection threads and EM analyses revealed that each bacteroid was surrounded by symbiosome membrane as in wild type (Fig.5C). In contrast to wild type nodules, bacteroid development was arrested at stage II-III and never developed into bacteroids of stage IV (Fig.5). Further, in cells of the fixation zone bacteroids undergo premature senescence (Fig. 5D). This is characterized by the presence of electron dense cytoplasm, an enlarged peribacteroid space and an irregular shape. Furthermore, in cells of this zone fusion of symbiosomes leads to the formation of vacuole-like structures with lysis of bacteroids that are entrapped inside (Fig.5E). The latter is a typical feature for (premature) symbiosis termination (Vasse *et al.,* 1990; Vance and Johnson, 1983). The

bacteroid lysis was followed by mitochondria destruction, as cells contained swollen organelles with degraded matrix and a very low number of cristae (Fig. 5E). These observations show that senescence of both partners occurs concomitantly. Analyses of *MtENOD40-2* RNAi and nodules in which the expression of both *MtENOD40* genes is reduced, also showed that bacteroid development was blocked at stage II-III. These data indicate that reduction of *MtENOD40-1* and *MtENOD40-2* expression level in nodules interfered with bacteroid development. Strikingly, irrespective of which gene is knocked down, the percentage of the nodules with growth disturbances is similar (around 50%). This indicates that *MtENOD40-1* and *MtENOD40-2* genes are not acting redundantly.

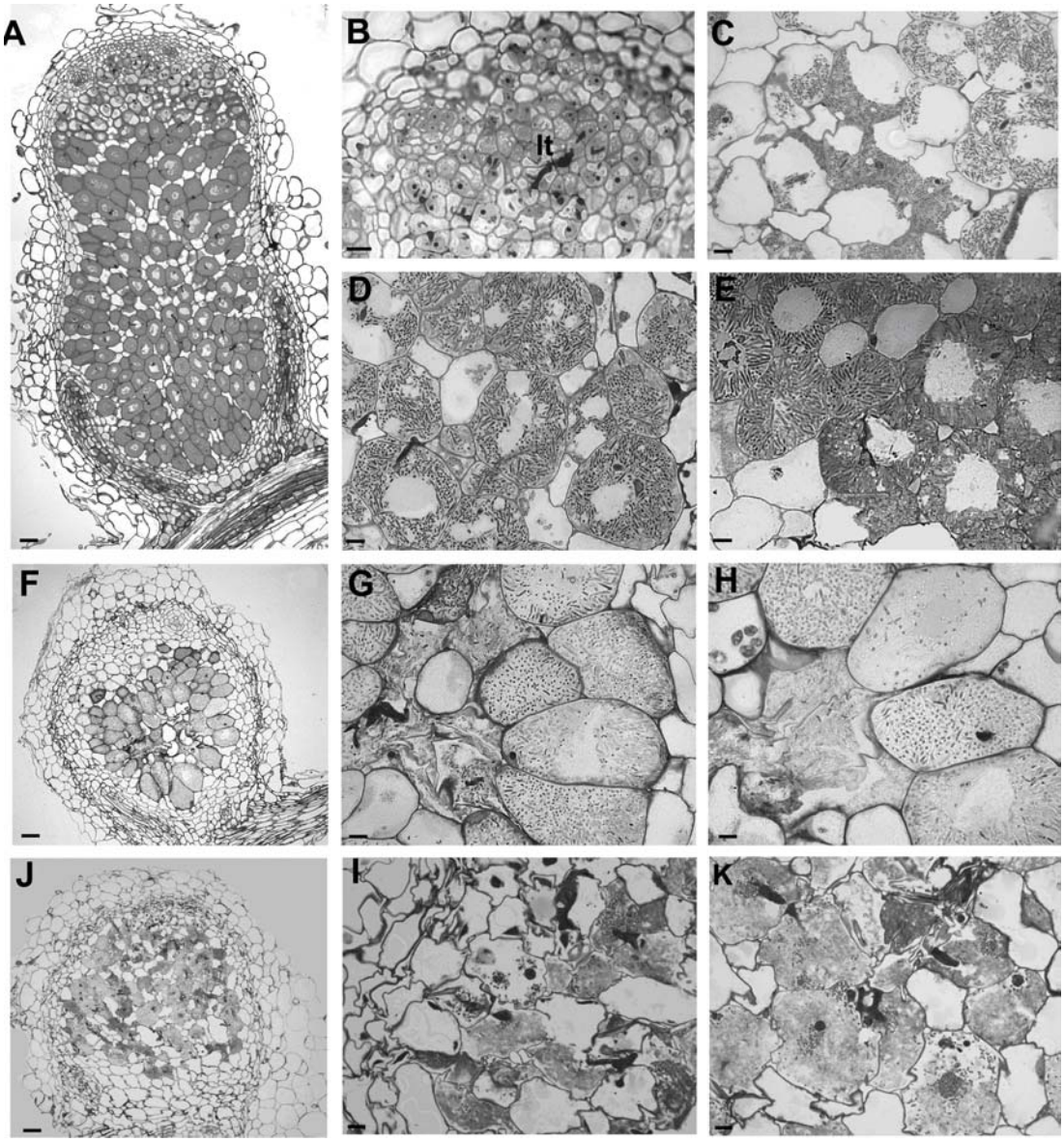

Fig. 4 Histology of 3-week-old RNAi nodules (F-K) compared to control nodules (A-E). Light microscopic of control nodule (A-E). A, median longitudinal section of a three-week-old nodule. B, magnification of meristem and distal part of infection zone of wild type nodule, where bacteria are released from infection threads (It). C, magnification of infection zone, where short rod-shaped bacteriods (arrow) are present at the distal part and (D) long rod-shaped bacteroids in the proximal part. E, magnification of fixation zone containing cells fully packed with elongated bacteroids. F, Light microscopic analyses of longitudinal section of aberrant *MtENOD40-1* RNAi nodule. Note that the zonation is not clear. G, Senescent nodule wherein cells in the middle of zone III lost turgor and collapsed. H, note the size of bacteroids compared to bacteroids in E. J, dead nodule recolonized by saprophytic rhizobia. I, magnification of collapsed cells being repopulated with bacteria. K, release of rhizobia from intracellular colonies. Bars= 20 μm.

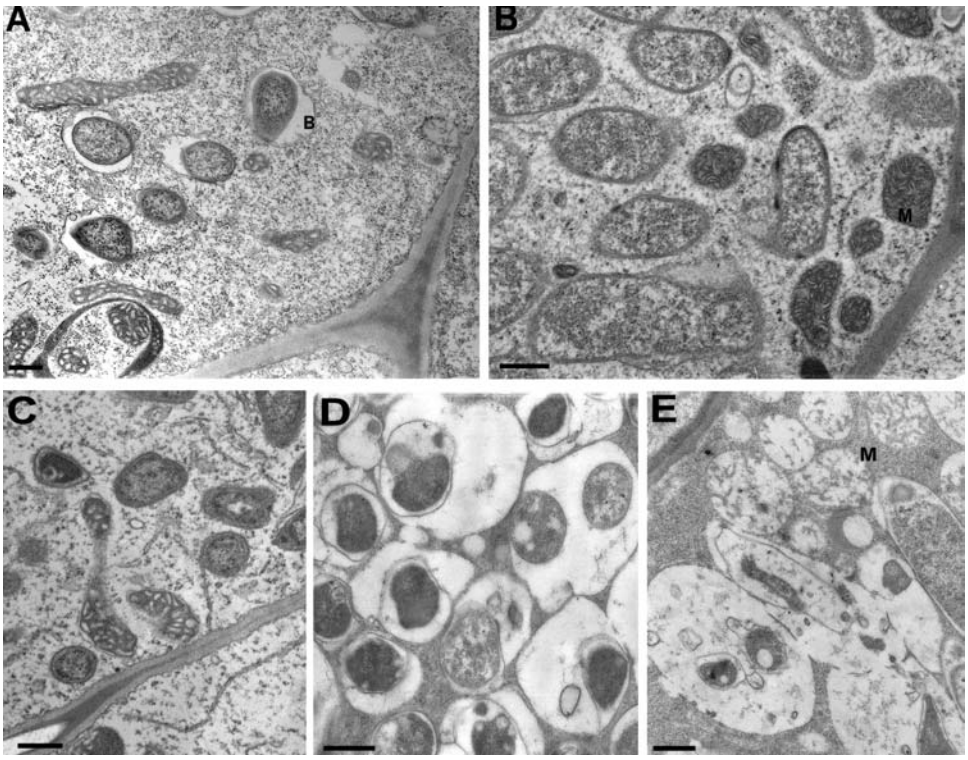

Fig. 5 Ultrastructure of control (A, B) and 3-week-old RNAi nodules (C, D, E).
A, wt nodules, young bacteroids in distal part of infection zone, B , wt nodules, proximal part of infection zone, C, RNAi nodules, infection zone, D, bacteroids with big peribacteroid space, fusing. E, lysis of bacteroids entrapped inside vacuole-like structure. Note swollen mitochondria of the host cell (B- bacteroids, M-mitochondria. Bars= 500 nm)

DISCUSSION

Here we described the identification of a second *MtENOD40* gene, *MtENOD40-2,* and the involvement of this gene in nodule formation.

MtENOD40-2 contains the two regions, box1 and box2, that are conserved among all *ENOD40* genes known so far. However, whereas all legume *ENOD40* genes contain an ORF encoding for a peptide with conserved amino acid motif –C-W-(X3)-I-H-G-S, the peptide encoded within the ORF of *MtENOD40-2* lacks the three carboxy-terminal amino acids –H-G-S. It is not

clear whether the activity of *ENOD40* is determined by the peptide encoded within box1 of *ENOD40*. Hence, the significance of the change in amino acid order of *MtENOD40-2* peptide with respect to activity of *MtENOD40-2* remains unknown.

ENOD40 gene expression has been shown to be highly induced during the interaction of the roots of legumes with *Rhizobium*. Here, we show that *MtENOD40-2* is expressed in the nodule primordium and in the infection zone of the nodule, like *MtENOD40-1*. Co-localization of different *ENOD40* genes within one species has also been shown in *Medicago sativa* (Fang and Hirsch, 1998), *Lotus japonicus* (Flemetakis *et al.,* 2000) and *Trifolium repens* (Varkonyi-Gasic and White, 2002). Although we have not compared the levels of *MtENOD40-1* and *MtENOD40-2* expression in nodules, the detection of 42 ESTs for *MtENOD40-1* among several cDNA libraries of *Medicago truncatula* nodules (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=medicago; http://medicago.toulouse.inra.fr/Mt/EST/), compared to 1 for *MtENOD40-2*, strongly indicates that *MtENOD40-1* is much higher expressed in nodules than *MtENOD40-2*.

The spatial and temporal expression pattern of *MtENOD40-1* and *MtENOD40-2* during nodule formation suggests a role of these genes in this process. In *Medicago truncatula* knock-down of *MtENOD40-1* expression led to a 50% reduction in the number of nodules (Charon *et al.,* 1999) and RNAi of *LjENOD40-1* in *Lotus japonicus* led to an even more drastic reduction in nodule numbers. Thus, these data all show that *ENOD40* is involved in nodule initiation. However, since in both experiments the introduced *ENOD40-1* DNA contained the conserved box2 sequences, the observed reduction in nodule number can not be assigned to the reduction of *ENOD40-1*, per se. Here we

show, by using a gene specific knock-down, that *MtENOD40-1* and *MtENOD40-2* are both involved in nodule initiation. The observation that a decrease in expression levels of either *MtENOD40-1* or *MtENOD40-2* reduces nodule initiation suggests that the two *MtENOD40* genes do not act redundantly in nodule initiation. Further, as a reduction of the expression of both *MtENOD40* genes leads to a higher reduction of nodule initiation, the effect on nodule initiation of the *MtENOD40* genes is synergistic. Therefore, we propose that the effect of *MtENOD40* on nodule initiation is dose dependent. This dose dependent effect of *ENOD40* genes on nodule initiation explains why no mutant in *ENOD40* came out of the genetic screens for mutants impaired in nodule initiation. In both *Lotus japonicus* and *Medicago truncatula* a reduction, but not a complete knock-down, in *ENOD40* expression leads to significant inhibition of nodule formation. Therefore, our results and the results in *Lotus japonicus* (Kumagai et al., 2006) strongly suggest that *ENOD40* genes are essential for nodule initiation.

Furthermore, we show that in all knock-downs tested the percentage (50%) of aberrant nodules formed is similar, and that all aberrant nodules show an impaired bacteroid developmental progression from stage II to III. This suggests that the *MtENOD40* genes do not act redundantly in bacteroid development and in contrast to their involvement in nodule initiation, neither act synergistically. This shows that both *MtENOD40* genes are essential for bacteroid development.

It is likely that in the aberrant nodules bacteroids are unable to fix nitrogen. In the nodules that do not show an impaired bacteroid development we expect that bacteroids are able to fix nitrogen. Our data are consistent with the observations in nodules formed on RNAi *LjENOD40-1* plants (Kumagai *et al.,*

2006). Some of these *Lotus japonicus* nodules are also white and small, suggesting an impaired nodule development.

Here we show that *MtENOD40-1* and *MtENOD40-2* are both required for bacteroid development. This observation offers therefore, an opportunity for unraveling the nature of the biological activity of *ENOD40*.

MATERIALS AND METHODS

Primers

p402Hind: GGAAGCTTATCCTTAAGCTAAAAAAAAATCAGG

p402Bam: GGGGATCCATTTCAGTTATAGGATGATTC

Mt42Xba: GGTCTAGACAGGACCATTTGGAAAAATC

Mt42Bam: GGGGATCCAGAATCATACACACATACAG

Mt401SA: GGACTAGTGGCGCGCCGGTTTGCCATGCTAT

Mt401BS: GGGGATCCATTTAAATCCATCAAGACTTGAATCT

Mt402BS: GGGATCCATTTAAATGGATGAATCTTTGTTGGCAA

Mt402SA: GGACTAGTGGCGCGCCTAAGTGCAATGGTTGG

401N1: GAGAAGTGTGAGAGGGTATTAAAC

402N2: CAGTTACCTACCTTACTCATCTG

402N1: GGATGAATCTTTGTTGGCAA

402N2: ACTTGCCGGTTTACCAACCT

MtACTIN2F: TGGCATCACTCAGTACCTTTCAACAG

MtACTIN2R: ACCCAAAGCATCAAATAATAAGTCAACC

Plasmids and vectors

For the construction of the RNAi vector sil401, a DNA fragment containing 330 bp of the 5'UTR including box1 sequences was amplified using primers Mt401SA and Mt401BS, while for the construction of vector sil402 a DNA fragment comprising the 5'UTR including box1 of *MtENOD40-2* was amplified

using primers Mt402BS and Mt402SA. Fragments were cloned into pGEM-T (Promega), yielding pGem401 and pGem402, respectively. Primers are provided with restriction enzyme recognition sites at their 5' end to facilitate subsequent cloning.

The amplified fragments were released from pGEM-T and cloned in two orientations by two sequential cloning steps in pBS-d35S-RNAi (Limpens, *et al.,* 2004), using *Asc*I and *Swa*I in the first cloning step and *BamH*I and *Spe*I in the second step, respectively. The inverted repeat preceded by the 35S promoter was released by digestion and subsequently cloned in the binary vector pRedroot (Limpens *et al.,* 2004) using *Kpn*I/*Pac*I restriction enzymes delivering the pRRsil401 and pRRsil402 vectors, respectively. To be able to knock-down the expression of *MtENOD40-1* and *MtENOD40-2* simultaneously, we fused the fragments the *Spe*I-*Nco*I/blunted of pGem401 and the *BamH*I-*Ecl*I fragment of pGem402. The obtained fragment was then ligated into pGem402 from which the insert was removed after digestion with *BamH*I and *Spe*I, yielding pGem4012. The insert of pGem4012 was then cloned in inverted orientation in used to generate pRRsil401 and pRRsil402 and introduced them into pBS-d35S-RNAi. The obtained inverted repeat was then cloned into pBS-d35S-RNAi as described above and subsequently in pRedroot yielding pRRsil4012. To obtain *MtENOD40-2* promoter sequence, a 1.8 kb DNA fragment upstream of the coding region of *MtENOD40-2* was obtained by PCR using primers p402*Hind* and p402*Bam* using BAC DNA as template. The sequences of the primers were designed based on available nucleotide sequences of the 3.9 kb DNA fragment from the BAC clone that hybridized to the *Medicago truncatula* EST BF519327. The obtained fragment was cloned into pCAMBIA1381Z digested with *Hind*III and *BamH*I.

Plant transformation

Vectors were transformed to *Agrobacterium rhizogenes* MSU 440, containing the helper plasmid pRiA4 (Sonti *et al.,* 1992), by means of electroporation.

A.rhizogenes mediated root transformation was performed according to Limpens *et al.,* (2004). Nitrogen starved plants were inoculated with *S.meliloti* SM2011. To prevent nitrogen deficiency, 15 days after inoculation with bacteria, plants were provided with Fahreus medium supplemented with 1.5 mM ammonium nitrate.

RNA isolation and Reverse Transcription mediated-PCR

At least 5 *Medicago truncatula* roots 2 weeks after transformation with *A. rhizogenes*, were selected by screening for red fluorescence and collected for RNA isolation using the RNeasy Plant Mini Kit (Qiagen). As control a similar number of roots that did not show red fluorescence were collected. Synthesis of complementary DNA and subsequent semi-quantitative reverse transcriptase PCR was performed as described (Ruttink *et al.,* 2006). Successful removal of genomic DNA has been checked prior to cDNA synthesis. The rest of the plants were inoculated with *S.meliloti* SM2011 (Limpens *et al.,* 2004).

Histochemical analyses, *in situ* hybridization and microscopy

Histochemical analyses of GUS activity was performed as described by Limpens *et al.,* (2005). Sections of 21-day old nodules were generated as decribed (Limpens *et al.,* 2005). 35S-UTP labelled RNA of *MtENOD40-2* was produced by T7 RNA polymerase on *MtENOD40-2* DNA cloned in plasmid pT7-5 (Scheres *et al.,* 1990). Hybridization and subsequent detection and analyses of signal were performed as described (Limpens *et al.,* 2005). Sections of nodules for light and electron microscopy were obtained as described (Limpens *et al.,* 2005).

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CHAPTER 3

Medicago truncatula ENOD40 box2 is involved in translational regulation of the box1 encoded peptide that is required for nodule development

Chapter 3: *Medicago truncatula ENOD40* box2 is involved in translational regulation of the box1 encoded peptide that is required for nodule development

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ABSTRACT

A characteristic feature of *ENOD40* genes is the absence of a long open reading frame and the presence of two conserved regions, box1 and box2, respectively, suggesting that these two region are important for *ENOD40* activity. It is very probable that box1 encodes a peptide. However, how box2 contributes to *ENOD40* activity is not clear. Here we show that over-expression of *MtENOD40*, box1 and box2 induces premature nodule senescence of *Medicago truncatula* nodules. Box1 activity is mediated by a 13 amino acid peptide encoded within box1, but box2 activity is not peptide mediated. We used transgenic *Medicago truncatula* lines containing a gene encoding RED FLUORESCENT PROTEIN and with or without box2 sequences in its 3'UTR. We showed that box2 is involved in the translational control of the RED FLUORESCENT PROTEIN. This suggests that box2 regulates the translation of the peptide coded by box1 in *MtENOD40*. In this way an interdependency of the two boxes in the regulation of *MtENOD40* activity can be explained.

INTRODUCTION

During the formation of root nodules in the symbiosis between legume plants and rhizobia, the expression of several genes, so-called nodulin genes (van Kammen, 1984), is markedly upregulated. One of these genes is *ENOD40*, a gene not restricted to legumes. A common feature of *ENOD40* genes is the absence of a long open reading frame (ORF). At the nucleotide level *ENOD40* genes share two regions of high conservation designated box1 and box2, respectively. Their conserved nature suggests that both regions are important for the biological activity of *ENOD40*. In all *ENOD40* genes known to date, box1 has a short ORF coding for a peptide of 10-13 amino acids with the exception of *Casuarina glutinosa ENOD40* (Santi et al., 2003). Several studies showed that the ORF in box1 of *ENOD40* could be translated into such a peptide (Van de Sande et al., 1996; Compaan et al., 2001; Rohrig et al; 2002; Sousa et al., 2001). In contrast, box2 contains an ORF in only a few *ENOD40* genes. Further, the encoded peptides are not highly homologous in these cases, although at the nucleotide level box2 sequences are strongly conserved. Nevertheless, it has been claimed that box2 activity is peptide mediated (Sousa et al., 2001). Since this seems in conflict with the lack of a conserved ORF, we decided to analyse the Medicago box2 activity, but using a different bioassay.

Bioassays to test *ENOD40* activity were developed in legumes, since *ENOD40* is best known from its high expression level during root nodule formation in legumes. Nodule formation on the roots of legumes is initiated by soil-borne bacteria collectively called rhizobia. Within a few hours *ENOD40* expression is elevated in pericycle cells opposite a protoxylem pole, where a nodule primordium will be formed in the cortex. In these primordium cells *ENOD40* expression is also induced. Upon infection by rhizobia the nodule primordium develops into a nodule. Medicago nodules have an apical meristem and its

tissues are of graded age, with the youngest cells near the meristem. Meristem cells are penetrated by infection threads, upon which the rhizobia are released by an endocytotic process by which they become surrounded by a host membrane. A *Rhizobium* bacterium surrounded by the host membrane is named a symbiosome. These symbiosomes divide and differentiate into nitrogen fixing "organelles". The zone adjacent to the meristem, where symbiosomes divide, is named the infection zone (zone II). In the fixation zone, (zone III), nitrogen fixation takes place (Vasse et al., 1990). In old nodules, a fourth zone, the so-called senescence zone, can be recognized at the proximal part where bacteroids degrade and host cells undergo lysis. Of all plant tissues in which *ENOD40* is expressed, the expression level is highest in cells in the proximal part of the infection zone (Crespi et al., 1994), where rhizobia differentiate into bacteroids (Vasse et al., 1990; Patriarca et al., 2004).

Although *ENOD40* expression during nodule formation and development is highest in cells of the infection zone, the effect of ectopic *ENOD40* expression on nodule development has not been used as an assay to address the nature of the biological activity residing in *ENOD40*. Rather a bioassay to test *ENOD40* activity was developed (Sousa et al., 2001) that was based on the role of *ENOD40* in the regulation of the division of cortical cells that will form the nodule primordium (Charon et al., 1997, 1999). It has been shown that expression of *P35S:MtENOD40* in transgenic Medicago can lead to induction of cell divisions in the roots, when grown under nitrogen limitation (Charon et al., 1997). Therefore, Sousa et al., (2001) monitors the induction of cell divisions in the Medicago root after ballistic targeting of *MtENOD40*-derived constructs (Sousa et al., 2001). Introduction of box1 or box2 induced cell division in a non-cell autonomous manner. Disruption of the translation start of

the ORF spanning box2, abolished the biological activity, suggesting that the box2 residing activity might be peptide mediated.

As *ENOD40* is expressed all dividing cortical cells, but not in any other cortical cell, it can not be excluded that the observed non-cell autonomous activity is not related to the symbiotic activity. Further, box2 in some *ENOD40* genes contain an ORF, and those cases the encoded peptides are not highly homologous.

Therefore, we decided to study box2 activity, developing an assay based on legume root nodule development, as it is known that *ENOD40* expression is highest in cells of the infection zone of the mature nodules. Nodules are easy to recognize on roots thus allowing the isolation of large numbers for analyses. Furthermore, the cytology of nodules is well documented (Vasse et al., 1990), therefore, allowing the identification of growth aberrations caused by manipulation of gene expression.

Here, we show that over-expression of *MtENOD40* induces a premature senescence of nodules. Further we show that over-expression of the entire *MtENOD40* gene as well as box2 and box1, separately, provoke a similar response. The box2 activity is not protein-mediated. In contrast, box1 activity is provoked by the peptide encoded by the ORF present in box1. Interestingly, the translation of the box1 peptide is controlled by the box2 sequence. Hereby we present a frame-work to explain the regulation of *MtENOD40* activity.

RESULTS

Over-expression of *MtENOD40* affects nodule development

To study the effect of over-expression on nodule development *P35S:MtENOD40* was introduced into Medicago (hairy) roots by *Agrobacterium rhizogenes*-based transformation. In this way, composite plants

containing transformed roots, that can be recognized by red fluorescence (see material & methods), and non-transformed roots are generated (Limpens et al., 2004). After 2 weeks, plants were inoculated with *Sinorhizobium meliloti*. In order to study whether expression of *P35S:MtENOD40* had an effect on nodule development transgenic and control nodules were harvested 21 days post inoculation (d.p.i.) and 7 μm thick sections were analysed by light microscopy. All control nodules (n=16; Table 2) are rod-shaped and show clearly three distinct zones from apex to base: a meristematic, an infection and a fixation zone, respectively (Figure 1A). Cells in the meristematic zone are small, cytoplasmic rich and devoid of infection threads and bacteria (Figure 1B). In cells in the distal part of the infection zone, rhizobia are released from the infection threads and become surrounded by a plant membrane. The released rhizobia are named bacteroids (Bergersen, 1974). Subsequently, these symbiosomes divide (Figure 1C). At the proximal part of the infection zone, bacteroids elongate (Patriarca et al., 2004; Figure 1D). In the fixation zone the infected cells are fully packed with elongated bacteroids and the vacuoles of the cells have almost completely disappeared (Figure 1P). In older nodules, a senescence zone is present at the basis of the nodule. The characteristics of cells in each zone are discernable at the light-microscopic (LM) level. For instance, hallmarks of “infected cells” in the senescence zone are their irregular shape and the presence of membrane-like material. Further, cells do no longer contain elongated bacteroids, but instead are eventually colonized by free-living bacteria (Timmers et al., 2000).

Since transgenic roots arose from independent transformation events, the phenotypes of transformed root nodules can vary. Half of the transgenic nodules (7 out of 15; Table 2) were more severely affected in their development than the other 8 nodules. In these 7 nodules (Figure 1E), infection threads are present in

the infection zone (Figure 1F) and symbiosomes are present in cells of the distal part of the infection zone, but the rhizobia remain short and rod-like (Figure 1G). Plant cells at the proximal part of the infection zone show the hallmarks of cells in the senescence zone of wild-type nodules, namely irregular shaped cells containing membrane-like material (Figure 1H). So senescence is induced in cells in the infection zone prematurely. In the remaining 8 nodules (table 2; Figure 1I) cells of the infection zone and the first 4 cell layers of the fixation zone are like in wild type nodules (Figures 1J and 1K). However, in subsequent layers, cells have the hallmarks of senescence cells (Figure 1L).

Thus, over-expression of *MtENOD40* leads to premature senescence. In the most severely affected nodules (phenotype I), senescence already is initiated in the infection zone while in the more mildly affected nodules (phenotype II), senescence becomes apparent in cells in the proximal part of the fixation zone.

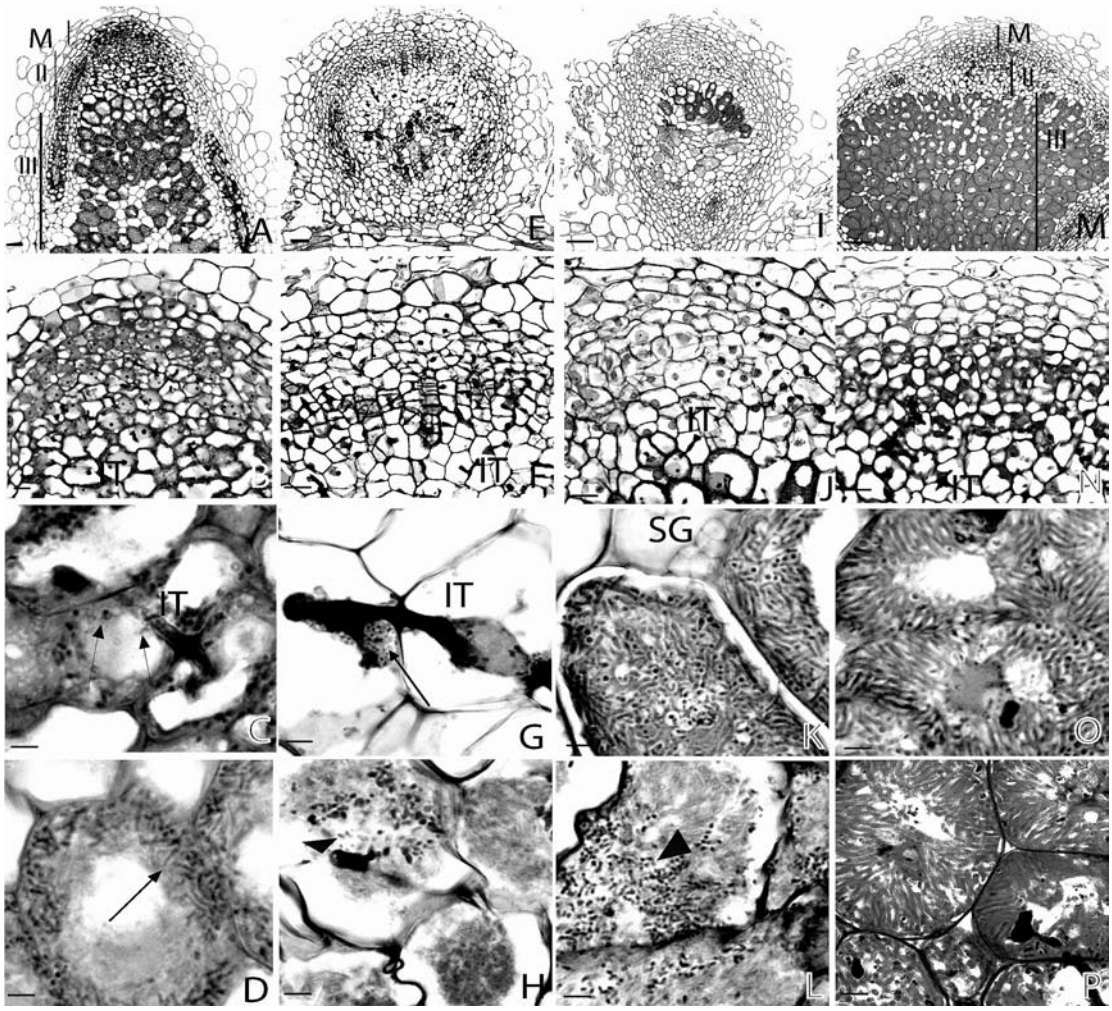

Fig.1 Histological analyses of wild-type (A-D, P), *P35S:MtENOD40* type I (E-H) and type II (I-L), and *P35S:MtENOD40 Mutbox1* Δ*box2* nodules (M-O).

A, wild-type nodules. B, magnification of meristem and the distal part of the infection zone (IT: infection threads). C, rhizobia are released from the infection threads (IT). D, magnification of the proximal part of the infection zone. P, magnification of the fixation zone. Arrows: released bacteria.

E, *P35S:MtENOD40* nodules type I. F, magnification of meristem and the distal part of the infection zone. G, intracellular bacteria released from ITs in the infection zone. H, cells are in saprophytic status. Arrow: released bacteria. Arrowhead: degraded bacteroids.

I, *P35S:MtENOD40* nodules type II. J, magnification of meristem and the distal part of the infection zone. K, magnification of the distal part of the fixation zone (SG: starch gradule). L, magnification of the proximal part of the fixation zone. Arrowhead: degraded bacteroids.

M, *P35S:MtENOD40 Mutbox1* Δ*box2* nodules. N, magnification of meristem and the distal part of the infection zone. O, magnification of the fixation zone. In A, E, I, M: Bars=1.5mm; In B, F, J, N: Bars=0.8mm; In C: Bar=25 μm; In D, G, H, K, L, O, P: Bars=15μm.

Over-expression of *MtENOD40* box2 or box1 is sufficient to affect nodule development

The premature senescence induced by over-expression of *MtENOD40* can now be used as an assay to analyse Medicago box2 activity. To do so, two constructs were made; one containing an *MtENOD40* gene devoid of box2 sequences (*P35S:MtENOD40*Δ*box2*; Table 1), and the other as a control, containing the *MtENOD40* gene lacking box1 sequences (*P35S:MtENOD40*Δ*box1*; Table 1). Both constructs were introduced into Medicago roots by *A.rhizogenes*-based transformation. After 2 weeks the composite plants were inoculated with *S. meliloti*. Nodules formed on transgenic roots and non-transgenic roots were analysed 21 d.p.i. The majority of the *P35S:MtENOD40*Δ*box1* nodules (9 out of 11; Table 2) displayed characteristics of the premature senescence induced in the infection zone (phenotype I). In the remaining nodules (2 out of 11; Table 2) senescence is apparent in cells in the proximal part of the fixation zone (phenotype II). In 29 out of 34 (Table 2) of the *P35S:MtENOD40*Δ*box2* nodules premature senescence is initiated in the infection zone (phenotype I). The remaining 5 nodules have a phenotype II appearance (Table 2).

Hence, over-expression of *MtENOD40*, *MtENOD40*Δ*box1* or *MtENOD40*Δ*box2* affects the development of Medicago root nodules in a similar fashion. This suggests that the box1 as well as box2 sequence are contributing to the biological activity residing in the *MtENOD40* gene. Strikingly, reduction of *MtENOD40* expression through RNA interference also induces premature nodule senescence (Wan et al., 2007).

Table1 Constitution of *MtENOD40* and *MtENOD40*-based genes introduced in *Medicago truncatula* through *A.rhizogenes*-mediated transformation.

DNA	sequence	Introduced point mutations
MtENOD40	1 – 670	
MtENOD40Δbox2	1 - 282; 345 – 670	
MtENOD40mutbox1Δbox2	1 - 282; 345 – 670	G60 – C; A61 – T
MtENOD40Δbox1	115 – 670	
MtENOD40Δbox1mutbox2	115 – 670	A293 – G; G295 – C
"box1"* Δbox2	"box1"; 115 - 282; 345 – 670	

* "box1": agatcttgtaataaggatgaaattgttgtgttgggagaagtctattcatggatcataaaacaaacatctaga

Introduced DNA	I#	II#	I+II#	WT#
-	-	-	-	10
MtENOD40	42	58	100	-
MtENOD40Δbox2	85	15	100	-
MtENOD40mutbox1Δbox2	-	10	10	90
MtENOD40Δbox1	82	18	100	-
MtENOD40Δbox1mutbox2	79	16	95	5
"box1"* Δbox2	55	22.5	77.5	22.5

Table 2 Percentage of nodules showing wild-type and phenotype I and II #
percentage of nodules displaying phenotypes of total number of analysed nodules
* "box1": agatcttgtaataaggatgaaattgttgtgttgggagaagtctattcatggatcataaaacaaacatctaga

MtENOD40 box2 does not encode a peptide

Since *MtENOD40* box2 is part of an ORF (Table 1), we tested whether translation of the putative peptide encoded by this ORF could cause the premature senescence phenotype. To this end we mutated the putative start codon (Table 1) yielding plasmid pRR-*P35S:MtENOD40□box1Mutbox2*. As control we studied the effect of mutation of the start codon in the box1 sequence (plasmid pRR- *P35S:MtENOD40Mutbox1Δbox2*; Table 1). Both plasmids were introduced into Medicago (hairy) roots. Transformed roots were inoculated with

S.meliloti and 21 d.p.i. transgenic nodules were analysed. Ninety-five percent (18 out of 19; Table 2) of *P35S:MtENOD40*Δ*box1Mutbox2* nodules showed premature senescence as described for phenotype I (79%) and phenotype II (16%), a percentage similar to that observed among *P35S:MtENOD40*Δ*box1* (82%) nodules. In contrast, the cytology of *P35S:MtENOD40Mutbox1*Δ*box2* (8 out of 9) and control nodules are indistinguishable (table 2; Figures 1M, 1N and 1O). These observations strongly suggest, that it is very unlikely that the effect of over-expression of *MtENOD40*Δ*box1* is mediated by the putative box2-encoded peptide, whereas the box1 sequence codes for a peptide that upon ectopic expression induces premature senescence.

However, if the RNA structure formed by box1 would be responsible for the induced phenotype, the mutation of the start codon might affect the RNA structure in such a way that the RNA would be no longer active. To exclude this possibility, a DNA fragment encoding the box1 peptide was constructed, but with an altered codon usage. In *MtENOD40*Δ*box2*, box1 is replaced by "box1" containing the altered codon usage yielding vector pRR-*P35S:"box1"* Δ*box2* (Table 1). This vector was introduced into Medicago (hairy) roots. Plants were inoculated with S.meliloti and after 3 weeks transgenic nodules were analysed. Twelve out of 22 analysed *P35S:"box1"* Δ*box2* nodules have a phenotype I of premature senescence, 5 out of 22 nodules have phenotype II, while 5 out of 22 nodules are like wild type (Table 2). Therefore, our data strongly suggests that the peptide encoded by box1, is involved in induction of premature senescence.

Taken together, the activity of *MtENOD40* is residing in box1 as well as box2, as both are sufficient to cause premature senescence upon over-expression. The activity of box1 is peptide-mediated, while the activity of box2 is RNA-structure mediated. This implies that box1 and box2, despite of their different

mode of action, can induce the same response. This could mean that box1 and box2 activate the same molecular mechanism or that there is an interdependence of the two boxes in the induction of premature senescence.

Box2 acts as a negative regulator of translation in *cis*

It has been described that in some mRNAs 3'UTR sequences can be involved in translational regulation of the mRNA, which is mediated by proteins binding specifically to the 3'UTR of such mRNAs (e.g. Kuersten et al., 2003). Therefore, we investigated whether box2 has an effect on translational efficiency of *MtENOD40*. Based on our observations so far, we hypothesize that over-expression of box2 in trans has a positive effect on the translation of box1 of the endogenous *MtENOD40* by titrating out the translational repressor that binds to the 3'UTR of the endogenous *MtENOD40* mRNA. As a consequence of this hypothesis, we therefore hypothesize that box2 can have a negative effect on translation of box1 when present in cis. To test the effect of box2 on translation efficiency, two constructs where made (Methods) in which the reporter-gene coding for the red fluorescence protein, dsRED, is under the control of the *MtENOD40* promoter and provided at its 3'UTR with (CAM40BC) or without box2 (CAM40BCΔbox2). If box2 has a negative effect on translation, it can be expected that in CAM40BC and CAM40BCΔbox2 plants with equal *dsRED* mRNA levels, the intensity of fluorescence is higher in CAM40BCΔbox2 than in CAM40BC plants. Thus determination of an effect of box2 on translation efficiency involves the quantification of red fluorescence in plants carrying either of the constructs. Each root obtained after hairy root transformation is the result of an independent transformation and by itself provides too little material for quantitative analyses. Therefore, we decided to generate lines into which the mentioned 2 constructs were integrated stably into the genome. Therefore, we introduced CAM40BC and CAM40BCΔbox2 into

the easy transformable Medicago accession R108 (Trinh et al., 1998). The two sets of transformed lines were subsequently called *cam40bc* and *cam40bc*Δ*box2*, respectively, and we selected lines with equal *dsRED* mRNA levels. Two lines per set, designated *cam40bc-1*, *cam40bc-6*, *cam40bc*Δ*box2-8* and *cam40bc*Δ*box2-20*, with equal *dsRED* mRNA level in nodules were selected for further studies (Methods). As *MtENOD40* activity has been observed in nodules, the intensity of dsRED fluorescence in 21 d.p.i. nodules on *cam40bc*Δ*box2* and *cam40bc* lines is quantified. Half of the nodule batches were used to confirm that *dsRED* mRNA levels are similar (data not shown). The rest of the nodule batches, were used to measure fluorescence intensities (Methods). Quantification of dsRED fluorescence shows that the amount of dsRED in nodules from 2 different cam40bc lines is similar (Figure 2), like in nodules from two *cam40bc*Δ*box2* lines (data not shown), when compared to each other. However, when compared to the fluorescence in *cam40bc* nodules the dsRED fluorescence in extracts from *cam40bc*Δ*box2* nodules is at least 4 times higher than in the *cam40bc* nodules (Figure 2). Thus dsRED fluorescence is higher in *cam40bc*Δ*box2* nodules than in *cam40bc* nodules, whereas the RNA levels of *dsRED* transcripts are similar. This shows that box2 has a negative effect on the translation efficiency of *dsRED* mRNA when box2 is in *cis*. Based on this observation, we postulate that this is the function of box2 in *ENOD40* mRNA.

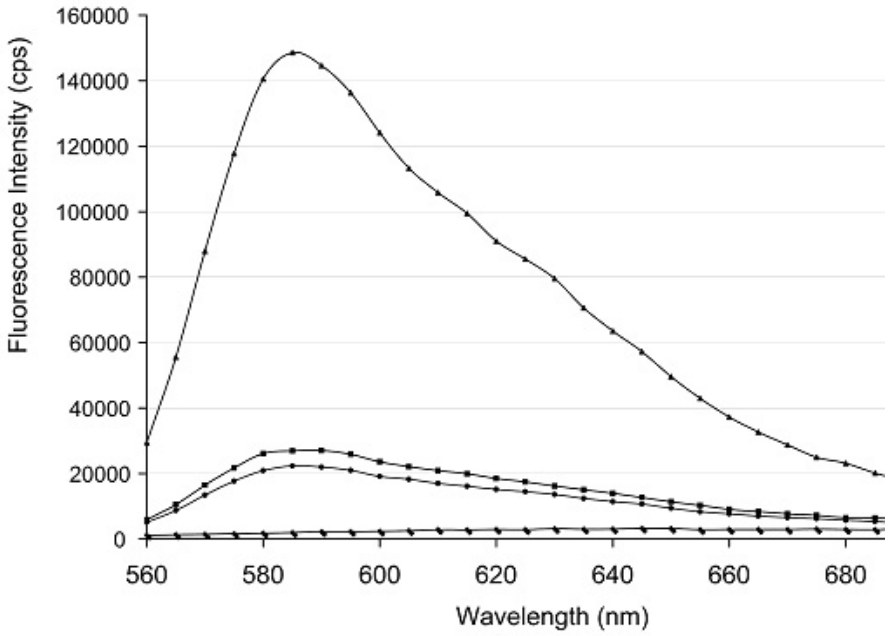

Fig. 2. *DsRED* expression in *cam40bc*, *cam40bc*Δ*box2* and wild-type nodules.

Expression of *dsRED* (counts per second), is determined by measurement of fluorescence present in protein extracts of *cam40bc-1*(■), *cam 40bc-6* (◆), *cam40bc*Δ*box2* (▲), and wild-type nodules (◥). Samples were excitated at 550 nm and the emission spectrum between 565 to 650 nm was analysed. In all samples a dsRED peak is observed around 583 nm.

DISCUSSION

Here we show that box2 of *MtENOD40* mRNA acts as a translational regulator of the peptide encoded by box1. Therefore, we propose that box1 represents the biological activity of *MtENOD40*. Further, the described activity of ectopically expressed box2 is indirect by affecting translational efficiency of endogenous *MtENOD40* mRNA. We hypothesize that over-expression of box2 sequences leads to out-titration of box2-specific RNA-binding proteins in the cell, which then releases the box2-controled translation of the endogenous expressed *MtENOD40*. This hypothesis provides a mechanistic explanation for the

biological activity of box2 sequences when this sequence is introduced in plants (this study; Sousa et al., 2001).

Whereas there are a few examples of translational regulation in plants described (e.g. Danon et al., 1991; Yohn et al., 1998; Fedoroff, 2002), in animals, numerous mRNAs have been reported to be translationally regulated (Kuersten et al., 2003; Leatherman et al., 2003). In most cases this is mediated by binding of protein complexes to specific sequences in their 3'UTR affecting the formation of the closed loop translation initiation complex or a post-initiation step (de Moor et al., 2004). The best described case is the translation repression of Drosophila *Caudal* mRNA, which codes for a transcription factor necessary for posterior segmentation, by the homeo-domain containing protein Bicoid. In the Drosophila embryo the Bicoid protein forms a morphogenic gradient in anterior to posterior direction. This gradient represses translation of the uniformly distributed caudal mRNA, thereby creating a gradient of Caudal protein in posterior to anterior direction (Dubnau and Struhl, 1996; Rivera-Pomar et al., 1996). Bicoid contains an eIF4E-binding motif, through which it, after binding to the 3'UTR of *Caudal* mRNA interacts with eIF4E. As a result of this protein-protein interaction, eIF4G, which is required for initiation of translation, is excluded from the cap-binding initiation complex (Niessing et al., 2002). Thus the Bicoid-mediated exclusion of eIF4G from the cap-initiation complex can explain how Bicoid is repressing translation of *Caudal* mRNA. In analogy with the mechanism of translation repression of *Caudal* mRNA by Bicoid, we expect that translation of *MtENOD40* is regulated by the presence of box2-specific RNA-binding proteins and that over-expression of box2 mRNA leads to out-titration of a box2-binding protein which then leads to an increased translation efficiency of the endogenous *MtENOD40*. To test this hypothesis, it will be important to identify a protein that specifically binds to box2 sequences

to regulate translation of *ENOD40* mRNA. An *MtENOD40* RNA binding protein has been identified (Campalans et al., 2003), but it is currently not known whether the binding requires the present of box2 sequences.

An intriguing question that arises from our data is why *ENOD40* is under translational control? Translation regulation of *Caudal* mRNA by Bicoid, leads to a gradient. However, whether the ENOD40 peptide forms a gradient is not known. Alternatively, the translation regulation of *ENOD40* mRNA could be a mean to produce ENOD40 peptide rapidly. To discriminate between these two options, the identification of regulators of ENOD40 activity, the targets and the function of *ENOD40* is required. Thus, currently the purpose of translation regulation of *ENOD40* remains obscure.

Box2 has also been shown to induce cortical cell divisions after ballistic targeting of box2 DNA in the Medicago root (Sousa et al., 2001). This is in line with our observations. However, the activity was abolished after mutation of the start of translation codon for the ORF in box2, suggesting that the box2 activity is protein-mediated. Since *MtENOD40* is not expressed in cells targeted by box2 DNA, the observed activity of box2 is these cortical cells can not mediated by the translation regulation of *MtENOD40*. Further, although it can not be completely ruled out that the box2-encoded peptide may be formed in the cortex and there display a cell division induction activity, a major argument opposes the presence of a peptide activity residing in box2; not in all *ENOD40* genes box2 contains an ORF and the encoded peptides are not highly homologous in these cases.

In conclusion, *ENOD40* harbours coding capacity for a peptide in the range from 10-13 amino acids, while the largest part of the transcript is a 3'UTR (> 90%), which contains sequences involved in translation regulation of the

mRNA. In analogy with known models in Drosophila, we speculate that the translation of *ENOD40* mRNA is regulated by the binding of a protein to box2 specific sequences. Mis-expression in cells of the infection zone leading to a lack (Wan et al., 2007) or an access of peptide (this study), induces premature nodule senescence.

MATERIAL and METHODS

Primers

5KPNMON	GGGGTACCGGCCAGTGAATTGCGG
3PACMON	TTTTAATTAACCATGATTACGCCAAGCTGC
Mt40F-xba	GCTCTAGACCCTTTAAGCATCCTCTA
Mt40R-bam	CGGGATCCCACAAACAAACAAGCATAC
Mt40box2F-xba	TTTTCTAGAGTGTGAGAGGGTATTAAAC
Mt40r-hind	GGAAGCTTCTGATCCTTTGGAGAAAA
Mt40f-hind	GGAAGCTTATGGCTATGTATCAATCAC
Mt40b1mutf	GTTTGTAATAAGGATCTAGCTTCTTTGTTGGG
Mt40b1mutr	CCCAACAAAGAAGCTAGATCCTTATTACAAAC
Mt40b2mutf	TTGTTATAGCGTCGCAAACCGGCA
Mt40b2mutr	TGCCGGTTTGCGACGCTATAACAA
Box1A1	GGGAGATCTTGTAATAAGGATGA
Box1A2	AATTGTTGTGTTGGGAGAA
Box1A3	GTCTATTCATGGATCATAAA
Box1A4	ACAAACATCTAGAGGGG
Box1A5	ACAACAATTTCATCCTTATTACAAGATCTCCC
Box1A6	CATGAATAGACTTCTCCCAAC
Box1A7	CCCCTCTAGATGTTTGTTTTATGAT
Mtprom1	GGAATTCGTAAATTGTCAGTC

StartkpnI	GGGGTACCTTATTACAAACAAGATTCAAGTC
Red1kpnI	GGGGTACCACAATGGCGCGCTCCTC
Red2xhoI	CCCTCGAGTACAGGAACAGGTGGTG
Mt564xhoI	CCCTCGAGGTGAGAGGGTATTAAACAAAAAC
Mt564xbaI	GCTCTAGAGGGCATTGGAAAAGTTGAGC

Plasmid construction

To generate binary vectors containing the entire *MtENOD40* gene or parts of it, first the 35S promoter, the multiple cloning site and the Nos terminator region of pMon999 (Van de Sande et al., 1996) was amplified by PCR using primers 5KPNMON and 3PACMON. This fragment was cloned into pGem-T (Promega, Madison, WI), generating pGemTKPMON. The *MtENOD40* (Genbank X80262) gene was amplified on genomic DNA using primers Mt40F-xba and Mt40R-bam, yielding a fragment of 670 bp (Table 1). The obtained fragment was introduced after digestion with *Xba*I and *BamH*I into pGemTKPMON digested with the same enzymes by ligation to generate pGemTKPMON40. To generate an ENOD40 gene devoid of the first 115 bp containing the putative peptide encoding part box1, pGemTKPMON40 was used as template in a PCR using and Mt40box2F-xba and Mt40R-bam as primers. The obtained DNA fragment was cloned into pGemTKPMON after digestion of both insert and plasmid with *Xba*I and *BamH*I, to generate plasmid pGemTKPMONMtENOD40ΔBOX1 (Table 1).

To remove the conserved stretch of nucleotides representing box2 present in all *ENOD40* genes known so far, pGemTKPMON40 was used as a template for two different PCR reactions; one including primers 5KPNMON and Mt40r-hind and the second including primers 3PACMON and Mt40f-hind. The obtained DNA fragments were digested with *Hind*III and *Kpn*I and *Hind*III and *Pac*I,

respectively and subsequently ligated into pGemTKPMON digested with *Kpn*I and *Pac*I, to generate pGemTKPMONMtENOD40ΔBOX2 (Table 1). This latter plasmid was used as a template in a PCR including primers Mt40box2F-xba and Mt40R-bam to obtain the 3'UTR devoid of box2. The obtained fragment after digestion with *Xba*I and *BamH*I was ligated in pGemTKPMON digested with *Xba*I and *BamH*I to generate plasmid pGemTKPMONΔBOX2. To mutate the putative start codons for translation giving rise to peptides derived from box1 or box2 regions plasmids pGemTKPMONMtENOD40ΔBOX2 and pGemTKPMONMtENOD40ΔBOX1 were used as template following the protocol included in Quick-a-change kit (Stratagene Inc, La Jolla, CA). To change the start codon in box1 primers Mt40b1mutf and Mt40b1mutr were used while for changing start codon in box2 primers Mt40b2mutf and Mt40b2mutr were used.

For construction of plasmids in which the nucleotide sequence of box1 is changed but the codon information is preserved, Primers Box1A1-7 were dissolved in TE (10mM Tris/HCL pH 7; 1mM EDTA) to 0.5nmol/µL. One microliter of each primer solution was added to 62 µl TE. Of this mix 2 µl was used in a total volume of 50 µl including 20U of T4 DNA kinase and 1mM ATP. After incubation for 10 minutes at 37^{o} C 50 µl TE + 150 mM NaCL was added. After boiling for 5 minutes, the mix was allowed to return to room temperature within 2 hours. Subsequently 2 µl of the annealed primers were added to 18 µl of ligation mix including 0.5 U of ligase. After ligation for 3hrs at 25^{o} C, 5 µl of the ligation mixture was used to amplify the generated DNA fragment in a total volume of 50 µl including primers Box1A1 and Box1A7 and 1U of tag polymerase (94^{o}C 15s, 48^{o}C 15 s, 72^{o}C 20s, 25 cycli). Subsequently, the PCR mix was purified and the DNA was digested with *Bgl*II and *Xba*I. Three microliter of the digestion mixture was used for ligation into plasmid

pGemTKPMONΔBOX2 digested with BglII and XbaI to obtain plasmid pGemTKPMON"BOX1"ΔBOX2. The nucleotide sequence of both the "BOX1" part in the plasmid was determined by sequencing and confirmed to be identical to the nucleotide sequences of the primers involved.

To generate binary vectors containing the appropriate inserts for plant transformation inserts from the respective pGemKPMON plasmids were released after digestion with *Kpn*I and *Pac*I and ligated into pRedRoot (pRR) digested with the same enzymes (Table 1).

Plant transformation

The various pRR plasmids were introduced into *A.rhizogenes* MSU440 and used to generate hairy roots on Medicago A17 (Limpens et al., 2004). Since pRR contains *dsRED* reporter gene that encodes for the red fluorescence protein, application of this plasmid offers the possibility to select transformed roots based on red fluorescence using a stereo fluorescence microscope equipped with dsRED specific filters. In this way transgenic, chimaeric and non-transgenic roots can be distinguished.

After two weeks the roots were inoculated with *S.meliloti* SM2011 constitutively expressing GREEN FLUORESCENT PROTEIN (GFP). Three weeks later roots were analysed for the presence of nodules. Of each transformation event nodules were isolated and embedded in Technovit 7100 (Limpens et al., 2004). Thin sections were made and after staining with 1% toluidine blue, subjected to microscopic analyses.

Generation of *R108* lines with *dsRED* expression under the control of the *MtENOD40* promoter

The promoter of *MtENOD40* including the start codon of the box1 peptide was isolated by PCR amplification on genomic DNA with primers mtprom1 and startkpnI designed on nucleotide sequences of BAC clone CR954187 and cloned in pCAMBIA1300 (Cambia, Australia) using *EcoR*I and *Kpn*I generating pCAM-PROM1.

The dsRED coding sequence was isolated by PCR amplification on with primers red1kpnI and red2xhoI and cloned in pGem-T, yielding pGem-RED. The intergenic fragment (IF) from the stop codon of the box 1 peptide to the stop codon of the hypothetical protein down stream of the *MtENOD40* gene on the genome was isolated by PCR amplification on genomic DNA with primers mt564xhoI and mt564xbaI designed on nucleotide sequences of BAC and cloned in pGem-T yielding pGem-IF. The RED fragment was released from pGem-RED by digestion with *Sph*I and *Xho*I and cloned in the *Sph*I/*Xho*I sites of pGem-IF, generating pGem-RED-IF. The RED-IF fragment was released from this vector by digestion with *Kpn*I and *Xba*I and cloned in the *Kpn*I/*Xba*I sites of pCAM-PROM1 generating pCAM40BC.

To generate pCAM40BC∆box2 regions of *MtENOD40* flanking box2 were amplified using primers mt564xhoI and Mt40r-hind for the upstream region and primers Mt40f-hind and mt564xbaI for the downstream region of box2. After digestion of obtained PCR fragments with *Hind*III, fragments were ligated into in pGem-T yielding pGem-IF∆box2. The RED fragment was released from pGem-RED by digestion with *Sph*I and *Xho*I and cloned in the S*ph*I/X*ho*I sites of pGem-IF∆box2, generating pGem-RED-IF∆box2. The RED-IF∆box2 fragment was released from this vector by digestion with *Kpn*I and *Xba*I and cloned in the *Kpn*I/*Xba*I sites of pCAM-PROM1 generating pCAM40BC∆box2.

The pCAM40BC and pCAM40BCΔbox2 plasmids were transformed into A.tumefaciens GV3101 containing helper plasmid C53C1 by means of electroporation.

Medicago truncatula R108-1(c3) plants were transformed using regeneration-transformation (Trinh et al., 1998). In total, 8 CAM40BC and 10 CAM40BCΔbox2 T_0 plants were generated. To obtain homozygous lines that contain a single T-DNA insertion, from each set 20 T_1 plants were analysed for the presence of a single T-DNA insertion in the genome by Southern analysis (data not shown). Offspring for which in the T2 the dsRED fluorescence did not segregated as expected for homozygous lines, were selected.

Expression studies

To determine levels of transcripts, RNA isolation, RT-PCR analyses were performed as described (Ruttink et al., 2006).

To determine red fluorescence intensities, proteins were extracted in a FPS buffer (120 mM KCL, 50 mM Tris 8.0, 10% glycerol and 1 tablet per 10 ml protease inhibitor (Boehringer). Total protein concentrations of these extracts were determined using the Biorad reagents (Biorad, The Netherlands) and all the samples were diluted to a final concentration of 0.5µg/µl. From these diluted samples, the fluorescence intensities were measured in a SPEX FluoroLog-3 spectrofluorometer. The samples were excitated at 550 nm and the emission spectrum between 565 to 650 nm was analysed. The height of the obtained emission peak at 583 nm was determined for each sample. The amount of auto-fluorescence as measured in the protein extract from the control plants was subtracted from this value.

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CHAPTER 4

The Medicago Root Nodule Meristem Has Two Peripheral Stem Cell Domains that Resemble the Root Meristem Stem Cell Domain

Chapter 4: The Medicago root nodule meristem has two peripheral stem cell domains that resemble the root meristem stem cell domain

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SUMMARY

Root nodules are organs that host nitrogen-fixing bacteria. The organization of the *Medicago truncatula* nodule meristem is studied using quiescent center and stem cell specific promoters of *Arabidopsis thaliana*. Their behavior in transgenic Medicago roots is similar to that in Arabidopsis. Nodule meristem cells, in which the Arabidopsis markers are activated, are positioned in a ring located at the periphery of the nodule. In this ring two different types of domains can be recognized on the basis of differences in promoter activities. One type of domain abuts on pro-vascular tissue, and the other on non-pro-vascular peripheral tissue. Our data indicate that the nodule meristem cells expressing the Arabidopsis markers form two different stem cells domains in the nodule meristem. Further, we propose that cells showing quiescent center specific promoter activity act as organizers and share properties with quiescent center cells in the root.

INTRODUCTION

The interaction between legumes and soil-borne bacteria collectively known as rhizobia, leads to the formation of a new organ, the root nodule (Stougaard, 2001; Limpens and Bisseling, 2003). Two types of nodules can be distinguished. Determinate nodules have a transient meristem that disappears at an early stage after rhizobia have entered cells, after which they grow by cell enlargement (Dart, 1977). Indeterminate nodules have a persistent meristem at the apex that remains active throughout the life span of the organ. *Medicago truncatula* (Medicago) nodules have such a nodule meristem (NM). From this NM all nodule tissues are derived; the central tissue, consisting of infected and non-infected cells, and the peripheral tissues including the cortex, endodermis and the nodule parenchyma. The latter contains several vascular bundles.

The best studied meristems are the shoot (SM) and the root meristem (RM; e.g. Nakajima and Benfey, 2002; Stahl and Simon 2005; Scheres, 2007). Knowledge on the organization of these meristems has increased over the last years especially by studies on Arabidopsis. Both RM and SM contain a group of stem cells, which by division are able to self-renew as well as to create progenitor cells that can differentiate. Stem cell identity is maintained by a so-called organizer. In the central region of the SM, cells that form the Organizing Center (OC) (Mayer *et al.*, 1998) are overlaid by the domain of stem cells. In the Arabidopsis root the organizer is formed by 4 quiescent center (QC) cells (van den Berg *et al.,* 1997) and these cells are surrounded by stem cells. Thus, both SM and RM contain stem cells and cells that function as organizer to preserve stem cell identity, strikingly involving related regulators (Sarker *et al.,* 2007).

In contrast, knowledge on the organization of the NM is scanty. NM is assumed to be composed of a few cell layers of dividing cells that add cells to form the central and peripheral tissues. To form the central tissue, cells switch from mitosis to endo-reduplication (Truchet, 1978; Cebolla *et al.,* 1999; Vinardell *et al.,* 2003). In Medicago, the gene encoding for the mitotic inhibitor *MtCCS52A*, which activity is required for endo-reduplication, is expressed in cells proximal of the meristem, forming the infection zone. Thus growth of the central tissue of the nodule is accomplished through ploidy-dependent cell enlargement (Cebolla *et al.,* 1999). In contrast, *MtCCS52A* is not expressed in cells of the peripheral tissue, showing that cells leaving NM to form peripheral tissue do not undergo endo-reduplication. As all tissues are derived from NM, it is likely that cells in the NM are stem cells for these tissues. However, it is not clear whether the NM also contains a region that functions as organizer to maintain stem cell identity.

As it has been suggested that the nodule is a root-derived organ (Hirsch and LaRue, 1997; Mathesius *et al.,* 2000; de Billy *et al.,* 2001; Roudier *et al*., 2003; Bright *et al.,* 2005), we investigated whether promoters specifically active in RM QC and stem cells, like *AtQC25*, *AtQC46*, *AtQC184* (Sabatini *et al.,* 1999; 2003), *SCARECROW (SCR)* (Di Laurenzio *et al.,* 1996), *PLETHORA1/2* (*PLT1*, *PLT2*) (Aida *et al.,* 2004; Blilou *et al.*, 2005) and *WOX5 (WUSCHEL*-related homeobox gene 5; Sarkar *et al.,* 2007) are active in specific regions of the NM.

Our data show that RM QC and stem cell markers are expressed in cells positioned in a ring located at the most distal region of the peripheral tissue of the nodule.

RESULTS

AtWOX5, *AtQC25*, *AtPLT2*, *DR5*, and *AtSCR* promoter activities mark QC and stem cells in the Medicago root meristem (RM)

To test whether the Arabidopsis QC markers are active in the Medicago root QC, we first identified this QC using histological characteristics. In the Arabidopsis RM, cell files converge to the QC. Therefore, we analysed median longitudinal sections of primary Medicago roots (Fig.1A&B). Each tissue in the Medicago RM consists of vertically continuous cell files that converge to a small group of cells (Fig.1A&B). This group of cells is organized into two tiers (Fig.1B). Furthermore, these cells have no clonal linkage with their neighboring cells. Therefore, these cells most likely form the QC.

To investigate whether QC cell-type specific Arabidopsis promoters are active in the Medicago QC cells, we transformed Medicago roots using *Agrobacterium rhizogenes* with the following constructs; *AtWOX5::GUS*, *AtQC25::GUS*, *AtPLT2::GUS*, *DR5::GUS*, and *AtSCR::GUS* (Fig. 1C-H).

The Arabidopsis QC specific promoters *AtWOX5* (Fig.1C&D) and *AtQC25* driving *GUS* (data not shown) expression are active in the two tiers of cells that we identified as the Medicago QC. This shows that the transcriptional regulation of these 2 QC specific promoters is conserved between Arabidopsis and Medicago.

Whole mount GUS staining of *AtPLT2::GUS* Medicago roots shows that GUS activity occurs in the QC region (Fig.1E). However, compared to whole mount GUS activity staining pattern in *AtWOX5::GUS* roots (Fig.1C) the *AtPLT2::GUS* pattern in this region is broader (Fig.1E). This suggests that also

in Medicago the *AtPLT2* promoter is active in the QC as well as in surrounding stem cells.

In Arabidopsis roots, the auxin marker *DR5* is expressed at the highest level in the QC (Fig.1F). As shown in a longitudinal section (Fig.1G) of a Medicago root expressing *DR5::GUS*, the maximum GUS activity is highest in the two tiers of QC cells. This suggests that also in the Medicago RM, like in the Arabidopsis RM, an auxin concentration maximum is present in QC cells.

In Arabidopsis, *AtSCR* is expressed in the root endodermis and the QC. In median longitudinal sections of Medicago roots expressing *AtSCR::GUS*, the *AtSCR* promoter is shown to be active in the endodermal cell layer as well as in the QC (Fig.1H).

Taken together, our data show that QC and stem cell expressed Arabidopsis promoters are active in the QC and stem cells in the Medicago root. This shows that the Arabidopsis promoters are reliable markers for identification of root-like QC and stem cells in Medicago nodules.

Root QC and stem cell markers are expressed in the Medicago nodule meristem (NM)

To assess whether the NM contains root QC-like cells, we studied whether the QC promoters *AtQC25::GUS*, *AtWOX5::GUS* and stem cell promoter *AtPLT2::GUS* are active in this meristem. In *AtWOX5::GUS* nodules the cells that display the highest GUS activity form a ring at the apex of the nodule (Fig.2A). Within this ring, a few clusters of cells have a markedly higher GUS activity (Fig.2A, arrow). *AtQC25::GUS* (data not shown) has a similar expression pattern. Thus our studies show that root QC promoter activity locates to specific cells of the NM that we will name nodule QC (NQC) cells. Also *AtPLT2::GUS* (Fig. 2B) expressing cells form a ring. However, it is not

clear whether *AtPLT2::GUS* is active in a broader region of cells than *AtWOX5::GUS*, like is the case in the RM.

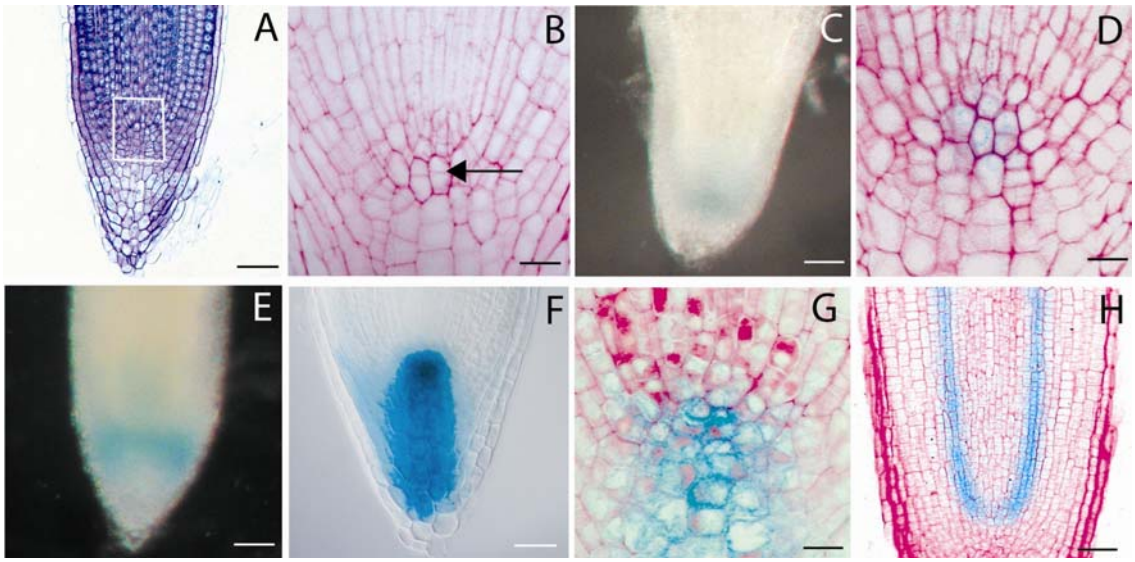

Fig.1 Cellular organization and expression patterns of *AtWOX5::GUS*, *AtPLT2::GUS* and *DR5::GUS* in the *Medicago* RAM. (colorful picture in appendix). (A) Medium longitudinal section of Medicago truncatula root apical meristem. Cell files converge to a central point (white rectangular). (B) Cellular organization of the Medicago pro-meristem showing the presence of 4 QC cells (arrow). (C) Whole mount *AtWOX5::GUS* root. (D) A median longitudinal section of *AtWOX5::GUS* root shows that *AtWOX5* promoter is active in a cluster of cells located in the presumptive QC cells of the RAM. (E) Whole mount *AtPLT2::GUS* roots shows that GUS activity is detectable in a similar, yet broader, region comparing to the *AtWOX5::GUS* and *AtQC25::GUS* expression. (F) Whole mount *DR5::GUS* root. (G) Maximum *DR5::GUS* activity is restricted to two tiers of cells and reduced towards the mature columella cap cells. (H) The *AtSCR* promoter is active in the endodermal cell layer of the *Medicago* root. Weak blue staining extends through cells in the RAM at the position of the QC. Bars=250µm (A, C, E); =100µm (B, D, G); =150µm (F); =120 µm (H).

Therefore, to localize the NQC and the *AtPLT2::GUS* expressing stem cells within the NM, series of longitudinal and cross-sections of *AtWOX5::GUS, AtQC25::GUS and AtPLT2::GUS* nodules were analyzed. In longitudinal *AtWOX5::GUS* sections, GUS expressing cells are confined within a 1-2-cell-high layer (Fig.2C) and are located in the periphery of the NM that is

continuous to the peripheral tissues. Cross-sections show that the *AtWOX5::GUS* expressing cells form a contiguous ring (Fig.2D), which is 1-2 cells broad. Further, the most intensive GUS staining is apparent in clusters of 2-4 cells that are continuous to nodule pro-vascular bundles (Fig. 2C). A similar expression pattern was observed in sections of nodules expressing *AtQC25::GUS* (data not shown). *AtPLT2::GUS* expressing cells are forming a cluster of 4-6 cells, including cells with a size bigger than *AtWOX5::GUS* expressing cells, and are located more to the outside of the nodule (data not shown). This suggests that, like in the RM, also in the NM *AtPLT2::GUS* expression occurs in a broader region of cells compared to *AtWOX5::GUS* expression and that nodule stem cells are positioned in the region of the NQC. However, our data do not allow determining whether the *AtPLT2::GUS* expressing stem cells surround the NQC, like is the case in the RM.

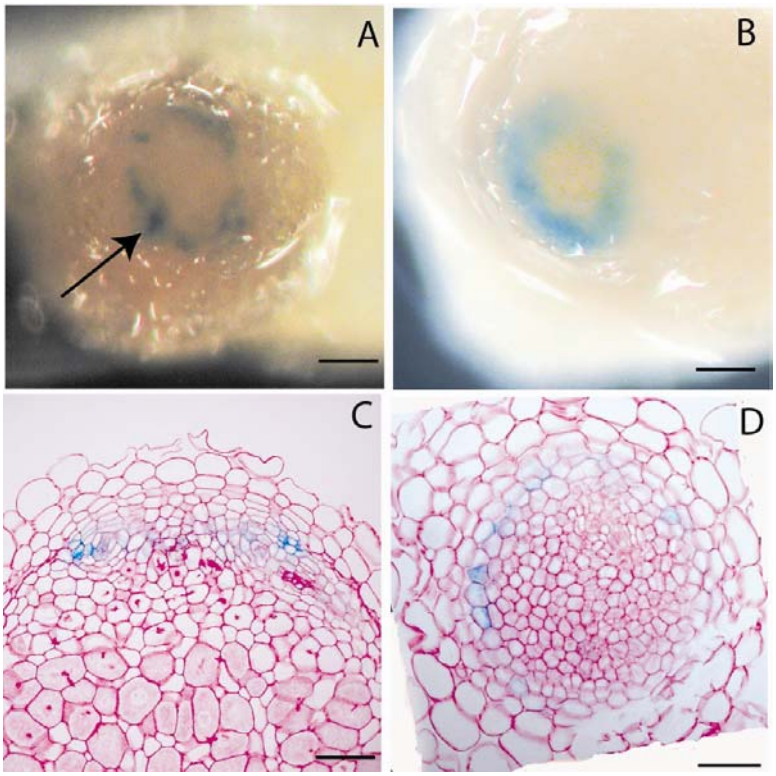

Fig.2 GUS expression patterns in *AtWOX5::GUS* and *AtPLT2::GUS* Medicago nodules. (colorful picture in appendix).

Cells showing *AtWOX5* promoter activity (A) and *AtPLT2* activity (B) are located in a ring. (C) A median longitudinal section of nodule shows that *AtWOX5::GUS* expressing cells are confined within 1-2-cell high and 2-4-cell wide. (D) A cross-section shows that cells form a ring, which is 1-2 cells broad. Bar=2.5mm (A); =1.5mm (B); =0.8mm (C, D).

Thus our studies show that NQC cells form a ring and this ring of cells is positioned in the periphery of the NM. The difference in expression level of the tested QC and stem cell markers in the NQC suggests that the NQC consists of different domains. One type of domain abuts on pro-vascular tissue, while the other on the remaining of the peripheral tissues. SCR is required for QC functioning in the Arabidopsis root (Sabatini *et al.,* 2003; Blilou *et al.,* 2005), and is a marker for the endodermis (Di Laurenzio *et al.,* 1996; Helariutta *et al*., 2000; Nakajima *et al.,* 2001). The expression of *AtSCR* is regulated by SHR which is expressed in the vascular tissue (Helariutta *et al*., 2000; Nakajima *et al.,* 2001). Based on this SHR dependency for SCR expression, it is expected that in the NQC SCR promoter activity is restricted to the domain in the NQC that abuts on pro-vascular tissue. Therefore, analyses of the *AtSCR* promoter activity in nodules could underline the presence of different domains in the NQC. The expression of *AtSCR* promoter is detected in nodule vascular bundle tissue and the highest expression occurs in the pro-vascular tissue that abuts on the NM (Fig.3A&B). Cross-sections show that *AtSCR::GUS* is expressed in the endodermis of nodule vascular bundles (Fig.3C&D). Longitudinal sections showed that the *AtSCR* promoter activity is highest in the NQC domain that abuts on a nodule pro-vascular bundle (date not shown). Even after prolonged GUS staining, expression of *AtSCR::GUS* was not detectable in the NQC domains of the peripheral tissues. So in contrast to *AtWOX5::GUS, AtPLT2::GUS* and *AtQC25::GUS, AtSCR::GUS* expression remains restricted to NQC cells that abut on nodule vascular bundles, thereby underlining that the NQC consists of different domains.

It has been shown that auxin is involved in QC and stem cell positioning in the RM and that an auxin concentration maximum co-localizes with the RM QC (Sabatini *et al.,* 1999; Blilou *et al.,* 2005). The level of *DR5* expression is

correlated with the auxin concentration. Therefore, the position of an auxin concentration maximum in nodules was determined by studying *DR5::GUS* expression in nodules (Fig.3E-H). After 2 hours of staining for DR5 activity, *DR5* expression occurs in regions around the QC that abut on nodule vascular bundles (Fig.3E&F), while only after prolonged incubation (16h) *DR5* promoter activity becomes detectable in cells of the NM including the ring of NQC cells (Fig.3G&H). Thus these data further underline the presence of two types of NQC domains in the ring of NQC cells (Fig.4); "peripheral tissue QCs" (PT-NQCs) in which the *AtPLT2*, *AtQC25*, *AtWOX5* promoters are active and "vascular bundle QCs" (VB-NQCs) in which in addition to the same promoters also the *AtSCR* promoter is active and that have a maximum in auxin concentration compared to PT-NQCs.

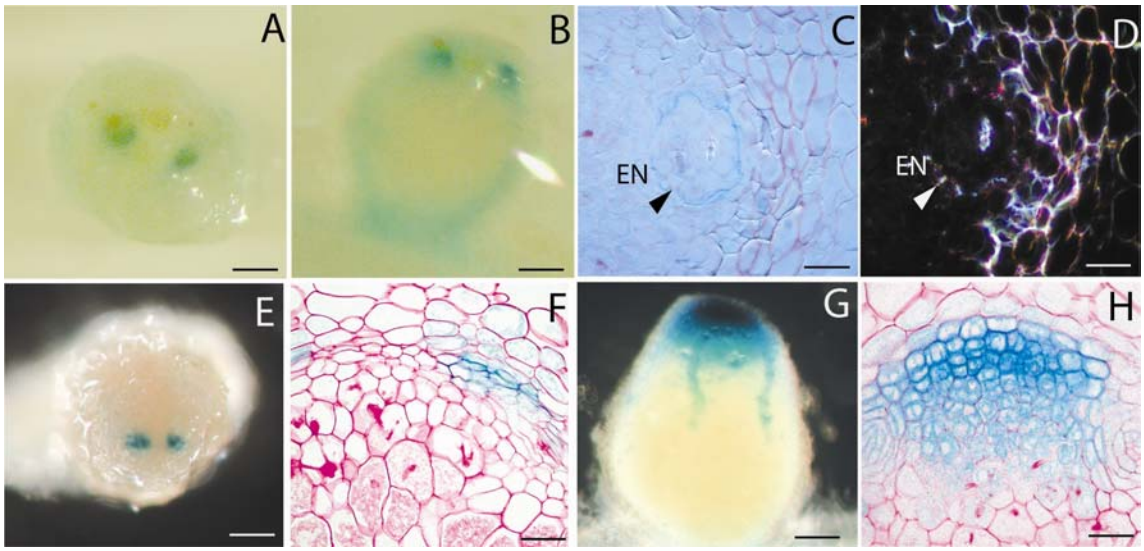

Fig.3 GUS expression patterns in *AtSCR::GUS* and *DR5::GUS* nodules. (colorful picture in cover). (A-D) GUS staining of a *AtSCR::GUS* nodule. (A) top view; (B) side view; (C) light micrograph of a crossc-section of such a nodule shows *AtSCR* promoter activity in the endodermis of the nodule vascular bundle (arrow: endodermis (EN)). (D) dark micrograph of cross section of a nodule in (C). (arrow: Casparian strips). (E-H) GUS staining of a DR5*::GUS* nodule. (E-F) Two clusters of blue cells appeared at the apical part of *DR5::GUS* nodule after 2 hours GUS staining. (E) top view; (F) A median longitudinal section reveals that cells at these two locations are present within 2-3 cell layers

and abut to pro-vascular bundles of the nodule. (G-H) After prolonged GUS staining, nodule meristem (NM) cells including the ring of NQC cells also display *DR5::GUS* expression. Bar in =3.5mm (A, B); =3mm (E,G); = 500µm (F) ; =0.8mm (H).

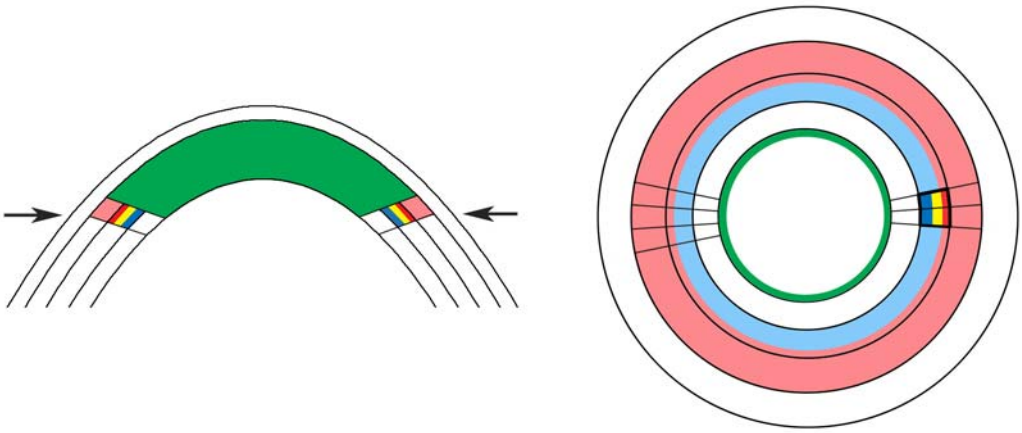

Fig.4 Schematic representation of the two domains present in the ring of NQC cells. (color picture in appendix). (A) In a longitudinal section the localization of the VB-NQC is visualized by the multicolored region at the periphery of the NM (arrow). (B) In a cross-section, the PT-NQC (visualized by red and blue) and VB-NQC (multi-colored) domains are shown.
Red=*AtPLT2*; blue = *AtWOX5*, *AtQC25* and *DR5*; yellow=*AtSCR* promoter activity; green = central part of NM. Intensity correlates to promoter activity. The location of *AtPLT2::GUS* activity in cells more towards the central part of the NM is hypothetical.

DISCUSSION

Here we show that the Medicago NM contains regions that resemble root stem cell domains using marker gene expression activity. These data strongly support that the developmental program of nodule tissue shares key properties with the root developmental program and therefore provide a molecular basis for the hypothesis that the nodule is a root-like organ (Hirsch and LaRue, 1997; Mathesius *et al.*, 2000; de Billy *et al.*, 2001; Roudier *et al.*, 2003; Bright *et al.* 2005). However, in the VB-NQC more RM QC markers are active than in the PT-NQC, and in addition, the highest auxin response appears to be associated with stem cell positioning in nodule vascular bundle tissue (Fig.4). These

observations indicate that the stem cell niche at the distal end of nodule vascular bundles is closer related to the root stem cell niche than the stem cell niche for the peripheral tissue (PT-NQC). Therefore, we suggest that nodule vascular bundle development has more properties in common with the root developmental program than the developmental program for the peripheral tissue.

Our data do not allow precise location of *AtPLT2::GUS* expressing cells with respect to the NQC. Therefore, it remains unclear whether stem cells surround the NQC like is the case in the QC of the root. Co-localization of *AtWOX5* and *AtPLT2* promoter activity should clarify this. However, since both promoters have a low activity and the use of fluorescent probes is hampered by the high auto-fluorescence of nodule tissue, this is not a trivial experiment. *In situ* hybridization with the Medicago orthologous of *AtPLT2* (Imin *et al.,* 2007) and *AtWOX5* could be an alternative approach. Hence, it remains unclear whether cells in the central part of the NM, that adds cells to the central tissue, are devoid of expression of the RM stem cell marker. If *AtPLT2* promoter activity is present in NM cells that add cells to central tissue, also this developmental program shares properties with root development. However, if *AtPLT2* promoter activity is absent from NM cells that add to the central tissue, it would imply that the nodule central tissue is derived from a third stem cell domain, which lacks a root identity. The phenotype of the mutant *cochleata* (Ferguson *et al*., 2005) in pea, in which at a late stage in nodule development lateral roots are formed from nodule vascular bundles while the central domain stops growing, is suggesting that the developmental program for central tissue and nodule vascular bundles can be uncoupled.

Whether the NQC functions as a RM QC awaits functional confirmation. One characteristic feature of RM QC cells is their low mitotic activity (Dolan *et al.,*

1993). Therefore, it is expected that the mitotic activity of the NQC cells is low, which should then be reflected by the absence of promoter activity of cell cycle-regulated genes. The second characteristic of the RM QC is the stem cell maintenance function. In Arabidopsis this has been shown by application of laser ablation of QC cells (van den Berg *et al.,* 1997). Such an approach in nodules will be difficult for technical reasons. Instead functional confirmation of the NQC may be achieved through analyses of nodules in Medicago mutants affected in orthologs of the Arabidopsis QC-expressed genes with a function in stem cell maintenance.

In conclusion, our data support that the nodule and root developmental programs share key properties. However, for the formation of the central tissue, the relation with root development is not as obvious as for the peripheral tissues including the nodule vascular bundle. Therefore, in addition to proving that the NQC has organizer activity, like the root QC has, identification of identity markers for cells in the central part of the NM, is another challenge.

MATERIALS AND METHODS

Primers

SCRPeco	gggaattccggaacacgtcgtccgtgtctc
SCRPbam	ggggatccgtaagaaaagggttaaatccaaaatcg
QC25sac	cccgagctcgtggatcccccatttttgt
QC25xba	cctctagaaacaatgtaacatcaatgcg

Plasmid construction

To isolate the *AtSCR* promoter, a 2500bp DNA fragment upstream from the ATG start codon of *SCR*, was amplified from Arabidopsis genomic DNA using primers SCRPeco and SCRPbam. The obtained fragment was cloned into the

*EcoR*I and *BamH*I sites of pCambia1381Z in front of the β-glucuronidase (GUS) coding sequence or of the gene encoding for the red fluorescent protein of ds (DsRed). The *AtPLT2* promoter was released after digestion with *Sal*I, *BamH*I and *EcoR*I, yielding a *Sal*I-*EcoR*I fragment of 5900bp and a *EcoR*I-*BamH*I fragment of 2800bp. These two fragments were ligated into a *BamH*I-*Sal*I digested pBinPlus (van Engelen et al., 1995) to drive the GUS gene. The *AtWOX5* promoter fragment was released after *Sal*I and *BamH*I digestion from pGEM-T-EASY (Promega, Madison, USA) and subsequently ligated into *Sal*I-*BamH*I linearized pCambia1381Z. A plasmid containing the The QC25 promoter was provided with restriction recognition sites *Xba*I and *Sac*I at the end by PCR using primers QC25sac and QC25xba. The fragment was cloned into a *Sac*I and *Xba*I linearized pCambia1391Z. DR5::GUS (kindly provided by Tom Guilfoyle) and cloned in pBinPlus (Van Engelen *et al.,* 1995).

Hairy root transformation

Binary vectors containing *AtWOX5::GUS,* AtQC25::GUS*, AtPLT2::GUS, DR5::GUS* and *AtSCR::GUS* were introduced into *M. truncatula* A17 through *A. rhizogenes*-mediated transformation as described (Limpens *et al*., 2004). Three weeks later, *S. meliloti* 2011 was added to induce nodules. For each purpose, at least 50 individual roots and nodules were under examination.

Histochemical GUS staining

Plant tissues containing promoter-GUS fusion, were incubated in 0.1 M NaH_2PO_4-Na_2HPO_4 (pH7) buffer including 3% sucrose, 0.05mM EDTA, 0.5mg/ml X-gluc, 2.5mM potassium ferrocyanide and potassium ferricyanide (to improve the specificity of the GUS expression localization), 100μg/ml chloramphenicol was added to inhibit bacterial growth. Incubation (at 37ºC) time varied from several hours to days depending on tissues containing different

promoter-GUS fusion. GUS stained tissues were fixed and embedded as described (Limpens et al., 2003). Semi-thin sections (7 μm) were analyzed after counter-staining with 0.5% ruthenium Red (Sigma, Germany).

Histological analysis and Microscopy

Root tips and nodules were fixed in 5% glutaraldehyde in 0.1M phosphate buffer (pH7.2) for 1-2 hours under vacuum, then washed with 0.1M phosphate buffer 15min (*4) and H_2O 15 min, dehydrated for 10min in 10%, 30%, 50%, 70%, 90% and 100% EtOH, respectively, and embedded in Technovit 7100 (Heraeus Kulzer, Germany). Sections were made at 4 μm using a microtome (Reichert-Jung, Leica, Holland), stained either by 1% toluidine blue (Sigma, Germany) or 0.5% ruthenium Red (Sigma, Germany), mounted in Canada balsam (MERCK, Holland), and analyzed with a Nikon Optiphot Microscopy (Nikon, Japan).

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CHAPTER 5

Conclusion Remarks

Chapter 5: Concluding Remarks

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In recent years genes crucial for Nod factor signaling (NFS) to establish the *Rhizobium*-legume symbiosis have been isolated from several legumes. From this it is clear now that the NFS pathway includes a limited number of genes. Most likely, these genes are used to regulate existing gene modules, among others, those for induction of cell divisions in the cortex of the root (e.g. Geurts *et al*., 2005). To further understanding how *Rhizobium* is using these existing modules to infect the plant and to form a nodule primordium, we focused on the characterization of the biological activity of *ENOD40* one of the genes that is induced during the primary cortical cell divisions (Compaan *et al.,* 2001). Further we examined the cellular organization of the nodule meristem that is formed from the nodule primordium.

Root nodule formation shares properties with root formation.

It is a longstanding discussion whether the nodule is as a root-borne organ, a modified lateral root or a unique organ (Hirsch and LaRue, 1997). The Medicago nodule has a persistent meristem, like a root meristem. In the latter, the persistence is maintained by a group of stem cells that surround a QC, which functions to maintain stem cell identity (Scheres, 2007). However, it is not clear whether the same concept underlies the persistence of the nodule meristem. With the advent of markers for the *Arabidopsis thaliana* root QC and stem cells, it came within reach to test whether the persistence of the nodule meristem is maintained in a similar fashion as in the root. Thereby we could test the hypothesis whether a nodule is related to a lateral root. Therefore, we

investigated whether we could identify cells in the nodule meristem in which the Arabidopsis QC and stem cell markers are active. Our data show that root QC and stem cell markers are active in particular cells in the nodule, and we named these cells NQC cells. The NQC cells form a ring at the proximal part of the meristem. Within this ring there are two different populations of cells that can be distinguished from each other by the level of activity of the markers; the group of cells, having the highest marker activity, is abutting the nodule vascular bundles. These NQC cells are characterized by a maximum in auxin concentration and in these cells the AtSCR promoter is activated. Most likely these NQC cells are organizers for stem cells for all the tissues in the nodule vascular bundle. The remaining cells in the ring of NQC markers activity cells are organizers for all other nodule tissue. Thus our observation suggests a root stem cell device forms the basis for the nodule.

The observation that the NQC cells form a ring suggests that the stem cells for the nodule tissues are contacting the NQC cells and divide into all directions to give rise to nodule tissues. A remarkably implication of our observation is that meristem cells that add cells to the central tissue, which forms the most nodule specific tissue, do not express any of the tested root stem cell markers. Thus these meristem cells represent a group of stem cells with a unique identity for which the identity genes remain to be uncovered.

Whether the NQC cells indeed are involved in the maintenance of the stem cell identity needs to be confirmed. Unfortunately the Medicago nodule is difficult to asses for technologies as have successfully been applied in Arabidopsis, like laser ablation and meristem cell labeling. Therefore, this support should come from mutant analyses of plants in which the orthologs of the Arabidopsis QC genes are mutated.

In conclusion, our data show that *Rhizobium* is able to exploit part of the root developmental program for the formation of a nodule, which is supported by the recent identification of mutants like HAR1 (Krusell *et al.,* 2002; Nishimura *et al.,* 2002) in Lotus and SUNN (Penmetsa and Cook, 1997) and LATD (Bright *et al.,* 2005) in Medicago and COCH (Ferguson *et al*., 2005) in pea. It will be interesting now to identify molecules commonly used in root and nodule development, and those that are involved in nodule formation only. Several experiments have implicated cytokinin as the key role phytohormone for induction of cortical cell division leading to nodule primordium formation (Cooper and Long, 1994). RNAi of the cytokine receptor MtCRE1 abolished nodule formation (Gonzalez et al., 2006). Strikingly, this gene is expressed in the nodule meristem (Lohar *et al*., 2006), whereas in roots expression is restricted to (pro)-vascular tissue (Lohar *et al.,* 2006). This observation underlines that the region of the nodule meristem adding cells to the central tissue is distinct from the region that adds cells to peripheral and nodule vascular tissue. It also shows that cytokinin plays a role throughout the entire life cycle of the nodule.

MtENOD40 genes are involved in nodule initiation and bacteroid development

Previously it has been shown that a reduction in *MtENOD40* expression as a result of co-suppression, during *Rhizobium* interaction leads to a reduction in nodule number on Medicago roots, suggesting that this gene is involved, but not crucial for nodule initiation (Charon *et al*., 1999). However, a recent discovery of the existence of a second copy of the *MtENOD40* gene in the Medicago genome with an overall homology of 30 % to *MtENOD40*, prompted us to investigate whether both genes are redundantly acting during nodule initiation. Using RNAi, we showed that reduction of expression of either of the genes affected the number of nodules formed by 20-50%. Reduction of the expression of both genes

led to an almost complete abolishing of nodule formation, suggesting that both genes are involved in a dose-dependent manner in nodule formation. This observation is in agreement with studies conducted in Lotus, where the most conserved region of *LjENOD40* was used in an RNAi to reduce expression of both genes simultaneously (Kumagai *et al.,* 2006).

In nodules that were still formed after RNAi, reduction of expression of either of the *MtENOD40* genes has been shown to induce premature senescence of nodules. Detailed analyses of sections of nodules formed on roots in which the expression of *MtENOD40-1* or *MtENOD40-2* was reduced showed that bacteroids do not reach the stage of nitrogen fixating bacteroids in these nodules. Hence, also at later stage in nodule formation both genes are required.

MtENOD40-1 **encodes a peptide, the translation of which is regulated by its 3'UTR**

Whereas the vast majority of eukaryotic mRNAs contain information for proteins, *ENOD40* mRNAs lack the presence of long ORFs. Instead, *ENOD40* mRNAs contain several small ORFs (Ruttink, 2003). At the nucleotide level *ENOD40* genes share two regions of high conservation designated box1 and box2, respectively, suggesting that these regions are important for the biological activity of *ENOD40*. With the exception of *Casuarina glutinosa ENOD40* (Santi et al., 2003) in all *ENOD40* genes known to date, box1 has a short ORF coding for peptides of 10-13 amino acids, that is active in plants (Van de Sande *et al*., 1996; Compaan *et al.,* 2001; Rohrig *et al;* 2001; Sousa *et al.,* 2001). The peptides share the W-(X4)-H-G-S motif. In contrast, the protein encoding capacity for the box2 region is not so obvious, since some *ENOD40* genes lack an ORF spanning box2 sequences and in the *ENOD40* genes in which an ORF is present the encoded peptides are not highly homologous, whereas at the

nucleotide level box2 sequences are strongly conserved. Furthermore, it has been shown that *ENOD40* transcripts are highly structured RNAs (Girard *et al*., 2003) and therefore, it was suggested that *ENOD40* does not encode for a peptide but acts as RNA.

Based on observation in a bioassay that monitors the capability of *ENOD40* to induce cell divisions in the Medicago root after ballistic targeting of *MtENOD40*-derived constructs, it has been shown that in addition to box1 encoded peptide, box2 activity is peptide mediated too (Sousa *et al.,* 2001). Since this seems in conflict with the lack of a conserved ORF, we decided to analyse the Medicago box2 activity, but using a different bioassay.

As *MtENOD40-1* is highest expressed in the proximal part of the infection zone we investigated the effect of ectopic expression of *MtENOD40-1* on nodule development. Ectopic expression of *MtENOD40-1* induced premature senescence of nodules. This observation has then been used to determine whether the peptide encoding box1 and/or the region 2 could induce the same effect. In this way we provided evidence for the peptide encoded by box1 as the activity, whereas the activity residing within box2 is not peptide-mediated. In addition we showed that box2 has a role in translational regulation of the box1 ORF. This translational regulation might be protein-mediated as at least one protein, MtRBP1, has been identified that binds to *MtENOD40-1* mRNA (Campalans *et al.,* 2004). We have hypothesized that over-expression of box2 sequences will titrate-out this RNA binding protein, thereby releasing the translational control of endogenous *ENOD40* mRNA, leading to a higher peptide production.

The identification of the biological active component within *MtENOD40-1* as well as the presence of a regulatory sequence within the mRNA, has several implications for the function of *MtENOD40* and we will discuss them below.

It remains difficult to predict what the function of this peptide could be. Peptides usually have a signaling function, and they are synthesized as part of pre-proproteins that are processed in the secretory pathway (e.g. Lodish *et al*., 2003). Since *ENOD40* peptides lack an amino-terminal signal sequences that will target it to the secretory pathway, it is most probably that this peptide acts intra-cellular. It is tempting to speculate that the translational regulation of the *ENOD40* mRNA is a means to control peptide production tightly. The observation that over-expression and reduction in expression of *MtENOD40-1* affects the same step in bacteroid development suggests that a delicate level of peptide is required during this step and underlines the need for a tight regulation of peptide production.

Knowledge of the mode of action of peptides that act intra-cellular is lacking and therefore, studies on the mode of action of ENOD40 may be of help in identifying the working mechanism of intracellular peptides. As a starting point, now an assay is available, it is possible to provide ENOD40 with a tag and monitor whether the peptide remains active and if so, localize the tagged peptide and use the tag as a bait to fish for partners.

The allocation of the biological activity of *MtENOD40-1* to the peptide that contains the motif W-(X4)-H-G-S raises the question whether the peptide in *MtENOD40-2* that lacks this motif, has biological activity. Therefore, it will be essential to study the effect of ectopic expression of box1 of *MtENOD40-2* on the induction of premature senescence. The outcome will be either showing that the *MtENOD40-2* peptide has the same activity as the *MtENOD40-1* peptide, or

not. In the former case, the importance of the conservation of the motif within *ENOD40* peptides is questioned. In the latter case, it remains to be elucidated whether the peptide of box1 of *MtENOD40-2* has an activity at all, and if so what the activity is. The outcome will also have implications for explaining the effect of knock-down of *MtENOD40-2*. If *MtENOD40-2* activity is mediated by the peptide and this peptide has the same activity as *MtENOD40-1*, reduction in transcript levels of *MtENOD40-2* leads to a similar effect as the reduction in transcript level of *MtENOD40-1*. However, if the *MtENOD40-2* encoded peptide has not the similar effect as the *MtENOD40-1* peptide, it is likely that the reduction of *MtENOD40-2* transcripts leads to an increase in the concentration of the protein through which the translation of box1 is controlled. The surplus of protein might bind on the *MtENOD40-1* transcripts leading to a reduction in peptide coded by box1 of *MtENOD40-1*.

The impact of the identification of a short peptide as being an active molecule in a eukaryote goes beyond the research on nodule formation. Until now, no criteria have been provided that sets a minimum for the length of proteins encoded by RNAs. Our data are in line with observations in Drosophila (Galindo *et al.,* 2007) showing that even peptides as short as 13 amino acids can have a biological activity *in vivo*. Recently, a whole scala of non-protein coding (small) RNAs with an activity in the cell have been discovered (Washietl *et al.,* 2005). With the notion that *ENOD40* contains an ORF for a peptide it will be challenging to reinvestigate the potential of non-coding RNA for the presence of small ORFs.

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SUMMARY

Medicago root nodules are formed as a result of the interaction of the plant with the soil-borne bacterium *Sinorhizobium meliloti.* Several plant genes are induced during nodule formation and *MtENOD40* is one of the earliest genes activated. The precise function as well as the molecule harboring the biological activity of *ENOD40*, however, remains unknown. In this thesis, we conducted experiments aiming at filling this gap in knowledge concerning *ENOD40*. As two copies of *ENOD40* are present in the genome of Medicago, we used gene-specific knock-down of the two genes to determine whether both genes are involved in nodule formation. This is described in chapter 2. We showed that the number of nodules per root, in case expression of both genes was reduced, was lower than the number of nodules per root in case either of the two genes was reduced. This showed that both genes are involved in initiation of nodule formation and the two genes work in an additive manner in nodule initiation. Furthermore, we showed that reduced expression of either *MtENOD40* gene induced premature nodule senescence and that both genes are essential for the development of the bacterium into the nitrogen-fixing bacteroid.

Whereas the vast majority of eukaryotic mRNAs code for proteins, a common feature of *ENOD40* genes is the absence of a long open reading frame (ORF). Instead, *ENOD40*s share at the nucleotide level two highly conserved regions, box1 and box2, of which box1, in almost all *ENOD40* genes, contains an ORF for a peptide of 10-13 amino acids. In chapter 3, we showed that over-expression of *MtENOD40*, as well as box1 or box2 only, induced premature nodule senescence. We showed that the box1 activity was mediated by the 13 amino acid peptide encoded within box1, while the box2 activity is not peptide mediated. Using transgenic Medicago lines containing the marker gene coding

for RED FLUORESCENT PROTEIN (RFP) with or without box2 sequences in its 3'UTR, we showed that the translation of the mRNA with box2 yielded less RFP than the mRNA lacking box2. This showed that box2 is involved in the regulation of translation of RFP and suggests that box2 functions in a similar way in the regulation of the translation of the peptide encoded by box1 in *MtENOD40*. Thus our data as described in chapters 2 and 3 propose a role of the peptide in nodule initiation and at a later stage in nodule development, most likely in avoiding senescence. In this latter process the concentration of the peptide is critical as over-expression and reduction in expression of *MtENOD40* induces premature nodule senescence.

Medicago nodules have a persistent meristem, like roots. In the root meristem the persistence of the meristematic activity is maintained by a group of stem cells that surround the so-called quiescent center cells (QC). These maintain stem cell identity in the surrounding cells and are mitotically inactive themselves. However, it is not known whether a similar mechanism controls the persistence of a nodule meristem. As nodules are root-borne organs, we studied whether promoters of QC and stem cell-specific genes of Arabidopsis were activated in the nodule meristem of Medicago. Our data, as described in chapter 4, showed that three out of the five tested markers for QC and stem cell-specific genes are activated in cells that form a ring at the periphery of the nodule meristem. The activity of the other two markers was restricted to cells that are part of this ring of cells, but only were active in cells abutting on nodule vascular bundles. These data suggest that the nodule meristem contains two different stem cell domains and that the cells in which QC markers are activated may act as organizers and share properties with the QC of the root. As the cells expressing the tested stem cell marker are at the periphery of the nodule meristem, we propose that these cells form the stem cells for nodule peripheral

and vascular tissues. Strikingly, none of the tested promoters was activated in cells in the central part of the nodule meristem. This part of the meristem adds cells to the central tissue of the nodule. However, it remains to be determined whether or not the mitotic activity of cells in the nodule meristem is maintained by the QC cells identified at the periphery of the meristem.

SAMENVATTING

Medicago planten, behorend tot de familie van vlinderbloemigen, kunnen een interactie aangaan met *Sinorhizobium meliloti* bacteriën, die in de grond leven. Dit leidt tot de vorming van een compleet nieuw orgaan, de wortelknol, dat als huis fungeert voor de bacterie. Tijdens de vorming van de wortelknol wordt de activiteit van een groot aantal genen verhoogd. Een van deze genen is *MtENOD40*. De activiteit van dit gen wordt al heel snel na de ontmoeting tussen plant en bacterie verhoogd. De precieze functie van dit gen bij de wortelknolvorming is echter nog niet bekend. Eveneens is nog niet bekend of *ENOD40* actief is als eiwit of als RNA. In dit proefschrift staan experimenten beschreven die tot doel hadden antwoord te krijgen op deze vragen.

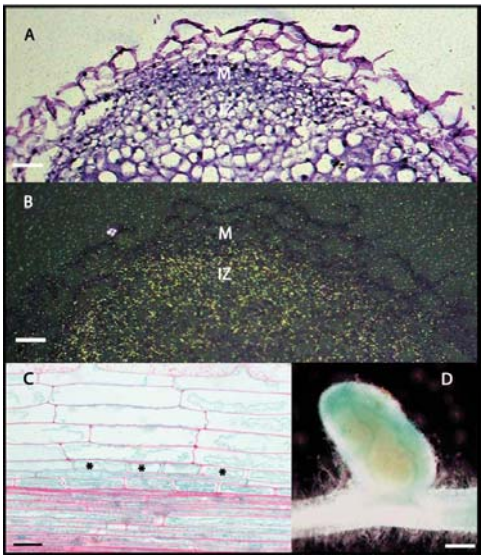
In het genoom van Medicago komen twee kopieën voor van *ENOD40*. We hebben gekozen voor een benadering waarbij we de afzonderlijke genen hebben uitgeschakeld om te bepalen of beide genen betrokken zijn bij wortelknolvorming. In hoofdstuk 2 laten we zien dat het aantal knollen per wortel lager is indien de expressie van beide *ENOD40* genen verlaagd is, vergeleken met het aantal knollen per wortel indien de expressie van slechts een *ENOD40* gen verlaagd is. Dit toont aan dat beide genen betrokken zijn bij de initiatie van knolvorming en dat beide genen elkaar hierin versterken in hun werking. Verder is uit onze experimenten naar voren gekomen dat een verlaging van de activiteit van beide *ENOD40* genen als ook van elk van de genen afzonderlijk leidt tot een vervroeging van de veroudering van knollen (senescence). Deze studies suggereren dat beide genen essentieel zijn voor de ontwikkeling van de bacterie in de knol.

De meeste eukaryotische mRNAs bevatten informatie voor een eiwit. Echter, *ENOD40* genen missen lange open leesramen (ORF). Als *ENOD40* genen

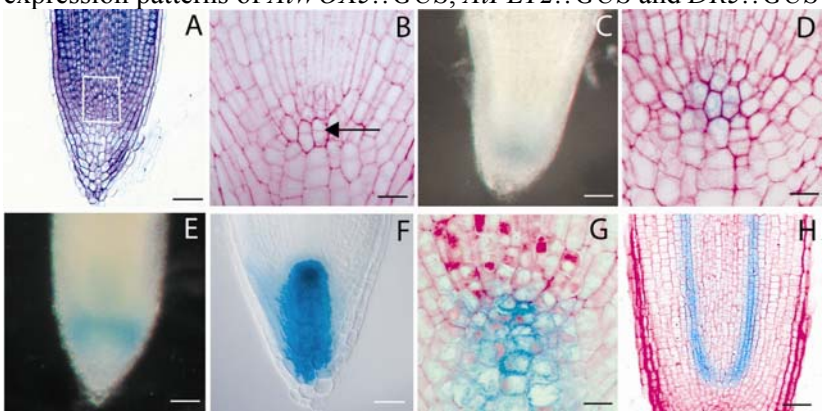
onderling vergeleken worden, dan valt op dat op het nivo van de nucleotiden er twee geconserveerde regio's zijn, genaamd box 1 en box 2. In de meeste *ENOD40* genen bevindt zich binnen box 1 een ORF voor een peptide van 10-13 aminozuren. In hoofdstuk 3 beschrijven we experimenten waarin we laten zien dat verhoogde expressie van *MtENOD40*, en van box 1 en box2 afzonderlijk, allen leiden tot een vervroegde intrede van senescence van de knol. We hebben laten zien dat het peptide gecodeerd binnen box 1 hiervoor verantwoordelijk is. Echter, de activiteit binnen box 2 is geen peptide. Om te begrijpen hoe box 2 toch eenzelfde effect heeft als het peptide van box 1 hebben we de volgende proef uitgevoerd; er zijn twee type transgene lijnen gemaakt. Een lijn bevat het marker gen coderend voor het RED FLUORESCENT PROTEIN (RFP) onder controle van de *MtENOD40* promoter en voorzien van box 2 sequenties in het 3'UTR, terwijl de andere lijn hetzelfde DNA bevat, maar nu zijn de box 2 sequenties niet toegevoegd aan het 3'UTR. We hebben laten zien dat de translatie van het mRNA waarin zich box 2 sequenties bevinden minder RFP opleverde dan de translatie van het mRNA waarin zich geen box 2 sequenties bevonden. Dit geeft aan dat box 2 betrokken is bij de regulatie van de translatie van RFP en dat suggereert dat box 2 op dezelfde wijze functioneert in de regulatie van de translatie van het peptide gecodeerd binnen box 1. Samenvattend, op basis van de data zoals beschreven in de hoofdstukken 2 en 3, poneren we dat het peptide een rol speelt tijdens initiatie van knolvorming en bij knolontwikkeling. In dit laatste proces heeft het peptide mogelijk een rol in het onderdrukken van de vervroegde intrede van senescence. De concentratie van het peptide is hierbij van cruciaal belang aangezien zowel verhoging als verlaging van MtENOD40 activiteit leidt tot de vervroegde intrede van senescence van de knol.

Medicago knollen bezitten net als wortels een persistent meristeem. De persistentie van het wortelmeristeem wordt gewaarborgd door de aanwezigheid van een groep stam cellen. Deze stamcellen omsluiten een groep cellen die het zogenaamde “quiescent center”(QC) genoemd worden. QC-cellen zorgen ervoor dat de stamcellen hun identiteit behouden, maar terwijl stamcellen delen, doen QC-cellen dat niet. Het is niet bekend of een vergelijkbaar mechanisme gebruikt wordt om de persistentie van het knolmeristeem te waarborgen. Om hierover inzicht te krijgen en omdat knollen ontstaan op wortels, hebben we de activiteit van promoters van QC- en stam-cel specifieke genen van Arabidopsis in het knolmeristeem van Medicago bestudeerd. Deze experimenten zijn beschreven in hoofdstuk 4. Het bleek dat 3 van de 5 geteste promoters actief zijn in cellen gelegen aan de rand van het meristeem. Deze cellen vormen een ring, die de rest van het meristeem omsluit. De overige 2 promoters waren actief in cellen die weliswaar een onderdeel vormen van de hierboven beschreven ring, maar deze cellen lagen in het verlengde van de vaatbundels van de knol. Deze data suggereren dat zich in het knolmeristeem twee verschillende stam-cel domeinen bevinden en dat de cellen waarin de QC-specifieke promoters actief zijn als organizers kunnen functioneren en dus eigenschappen delen met de QC in de wortel. Aangezien de cellen waarin de geteste promoters actief zijn, zich aan de rand van het meristeem bevinden poneren we dat deze cellen de stamcellen zijn die cellen leveren voor het perifere weefsel en de vaatbundel van de knol. Opvallend is dat geen enkele van de geteste promoters actief is in cellen in het centrum van het knol meristeem. Dit deel van het meristeem levert cellen die het centrale weefsel van de knol vormen, en dus het meest knol-specifieke weefsel vormen.

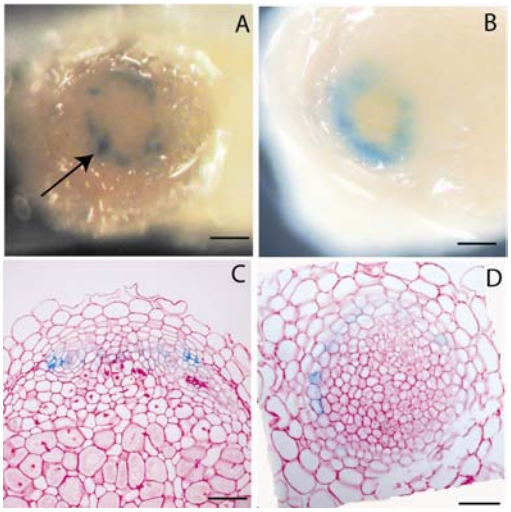
Chapter 2 Fig. 2 Expression of *MtENOD40-2* during nodulation.



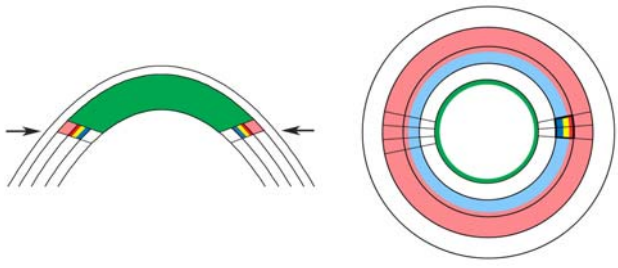
Chapter 4 Fig.1 Cellular organization and expression patterns of *AtWOX5::GUS*, *AtPLT2::GUS* and *DR5::GUS* in the *Medicago* RAM.



Chapter 4 Fig.2 GUS expression patterns in *AtWOX5::GUS* and *AtPLT2::GUS* Medicago nodules.



Chapter 4 Fig.4 Schematic representation of the two domains present in the ring of NQC cells.



Curriculum Vitae

Xi Wan was born on 4, January, 1969 in Chengdu, Sichuan province, China. She enjoyed her childhood in Beijing, Chengdu and Nanxi (a beautiful small town located at the upstream of Yangzi River). She was educated in the primary and high schools in Guiyang, Guizhou province. In 1991, she obtained a BSc. degree on Microbiological Pharmaceutics after 4 years study in Shengyang Pharmaceutical University. After graduation, she worked in the State Key Laboratory of Environmental Geochemistry, Chinese Academy of Sciences until 1996. Granted by EAWAG in 1995, she worked in Swiss Federal Institute of Environment and Technology (EAWAG), Switzerland. From 1996 she started to work in Chinese Collection of Agricultural Microorganisms, Institute of Soil and Fertilizer, Chinese Academy of Agricultural Science. In 1997, she followed a joint MSc. program on limnology between Institute of Limnology, Austrian Academy of Sciences, Austria and Institute Hydraulic Engineering (IHE), The Netherlands, supported by UNESCO. Five months later, she moved to Wageningen University to participate in the MSc.-biotechnology program. In 1999, she received a MSc. degree under the supervision of Prof. Dr. Ton Bisseling and Dr. Henk Franssen. After graduation, she went back to Chinese Collection of Agricultural Microorganisms. In 2001, she came again to Wageningen University for research and initiated the PhD program guided by Prof. Dr. Ton Bisseling and Dr. Henk Franssen. From August 2006, she has been working as a post-doc in the group of Dr. Colin Logie, Nijmegen Center of Molecular Life Science.

Publications and submissions

X. Wan, J. Hontelez, A. Lillo, C. Guarnerio, D. van de Peut, E. Fedorova, T. Bisseling, H. Franssen (2007) *Medicago truncatula ENOD40-1* and *ENOD40-2* are Both Involved in Nodule Initiation and Bacteroid Development. Journal of Experimental Botany, Vol. 58(8): 2033–2041.

X. Wan, I. Vleghels, T. Bisseling, H. Franssen (2001). Towards identification of proteins interactive with early nodulin ENOD40. *Journal of Agricultural Biotechnology*, 9(3): 293-296 (in Chinese with English abstract).

X. Wan, B. Compaan, J. Hontelez, A. Lillo, C. Guarnerio, T. Bisseling, H. Franssen. *Medicago truncatula ENOD40* box2 is involved in translational regulation of the box1 encoded peptide that is required for nodule development, submitted.

X. Wan, R. Korthouwer, A. Adams, R. Heidstra, B. Scheres, T. Bisseling and H. Franssen. The Medicago root nodule meristem has two peripheral stem cell domains that resemble the root meristem stem cell domain, submitted.

Education Statement of the Graduate School

Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: **Xi Wan**
Date: **03 December 2007**
Group: **Molecular Biology, Wageningen University and Research Center**

1) Start-up phase	*date*
► **First presentation of your project**	
Comparative study of the cellular organization of root and nodule meristems in legumes	Feb 2002
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
► **MSc courses**	
Advanced statistics MAT-20304	Nov-Dec 2002
► **Laboratory use of isotopes**	
Safe handling of Radio-active Materials and Sources, level 5B.	Jun 16-18, 2005
Subtotal Start-up Phase	*9.0 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
PhD students day 2002, Wageningen University	Jan 24, 2002
PhD students day 2003, Utrecht University	Mar 27, 2003
PhD students day 2004, Vrije Universiteit Amsterdam	Jun 03, 2004
PhD students day 2005, Radboud University Nijmegen	Jun 02, 2005
► **EPS theme symposia**	
Theme 1 symposium 'Developmental Biology of Plants', Leiden University	Feb 02, 2002
Theme 1 symposium 'Developmental Biology of Plants', Leiden University	Feb 06, 2003
Theme 1 symposium 'Developmental Biology of Plants', Wageningen University	Feb 17, 2004
Theme 1 symposium 'Developmental Biology of Plants', Wageningen University	Apr 26, 2005
Theme 4 symposium 'Genome Plasticity', Wageningen University	Dec 09, 2005
Theme 1 symposium 'Developmental Biology of Plants', Leiden University	May 16, 2006
► **NWO Lunteren days and other National Platforms**	
NWO-ALW Lunteren meeting	Apr 15-16, 2002

NWO-ALW Lunteren meeting	Apr 07-08, 2003
NWO-ALW Lunteren meeting	Apr 05-06, 2004
NWO-ALW Lunteren meeting	Apr 04-05, 2005
► **Seminars (series), workshops and symposia**	
Seminar Series " Frontier in Plant Development" (6 times)	2002-2006
Flying Seminars (8 times)	2002-2006
Symposium on Systems Biology in Honor of Prof. Dr. Pierre de Wit	Nov 11, 2004
► **Seminar plus**	
Flying seminar plus by Prof. Dr. Philip Benfey	Oct 24, 2005
► **International symposia and congresses**	
Dutch-Chinese Biotechnology Forum, Wageningen, NL	Oct 12, 2003
► **Presentations**	
Theme 1 symposium 'Developmental Biology of Plants', Leiden (oral)	May 16, 2006
EMBO Plant Development, Potugal (oral)	Mar 22-Apr 07, 2004
► **IAB interview**	Mar 28, 2003
► **Excursions**	
Subtotal Scientific Exposure	*9.6 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
Mathematics and Biology, the Netherlands Scientific Organization (NWO/IOP)	Dec 17-19, 2001
The analysis of natural variation within crop and model plants, Wageningen University	Apr 22-25, 2003
Bio-information Technology-1, Wageningen University	May 12-21, 2003
Functional genomics, Utrecht University	Aug 25-28, 2003
EMBO Plant Development, Oeiras, Portugal	Mar 22-Apr 07, 2004
System Biology, Wageningen University	Dec 11-14, 2006
► **Journal club**	
Literature Discussion, Once a week	2001-2006
► **Individual research training**	
Subtotal In-Depth Studies	*12.6 credits**

4) Personal development	*date*
► **Skill training courses**	
Dutch I - Beginner (CENTA)	Apr-Jun 2005
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*1.5 credits**

TOTAL NUMBER OF CREDIT POINTS*	**32.7**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the

Educational Committee of EPS which comprises of a minimum total of 30 credits

** A credit represents a normative study load of 28 hours of study*