The role of CLE peptides during nodulation

Virginie Mortier

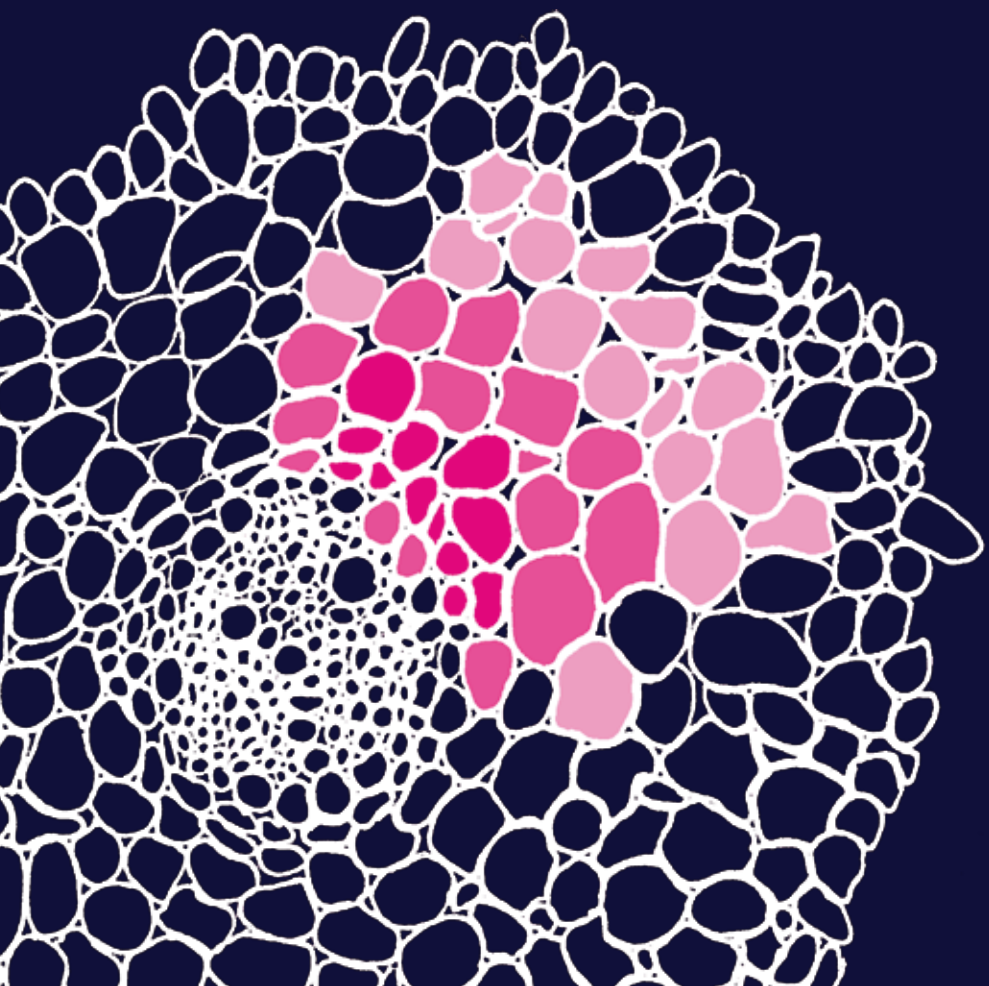

Figure cover: Drawing of a transversal root section showing the *MtCLE13* expression pattern during early nodule development. A high-to-low *MtCLE13* expression gradient, radiating from the inner towards the outer cortical cell layers is observed in a root cortical region defining the incipient nodule.

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Virginie Mortier

Promotor: Prof. Dr. Sofie Goormachtig
Co-promotor: Prof. Dr. Marcelle Holsters

Ghent University
Faculty of Sciences
Department of Plant Biotechnology and Genetics
VIB Department of Plant Systems Biology

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Examination committee

Prof. Dr. Ann Depicker (chair)
Faculty of Sciences, Department of Plant Biotechnology and Genetics, Ghent University

Prof. Dr. Sofie Goormachtig (promotor)
Faculty of Sciences, Department of Plant Biotechnology and Genetics, Ghent University

Prof. Dr. Marcelle Holsters (co-promotor)
Faculty of Sciences, Department of Plant Biotechnology and Genetics, Ghent University

Dr. Pascal Ratet
Institut des Sciences du Végétal, Centre National de la Recherche Scientifique, Gif sur Yvette, France

Dr. Florian Frugier
Institut des Sciences du Végétal, Centre National de la Recherche Scientifique, Gif sur Yvette, France

Prof. Dr. Tom Beeckman
Faculty of Sciences, Department of Plant Biotechnology and Genetics, Ghent University

Dr. Giel van Noorden
Faculty of Sciences, Department of Plant Biotechnology and Genetics, Ghent University

Prof. Dr. Ir. Monica Höfte
Faculty of Bioscience Engineering, Department of Crop Protection, Ghent University

Prof. Dr. Dirk Inzé
Faculty of Sciences, Department of Plant Biotechnology and Genetics, Ghent University

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Liefs,
Virginie*

Table of Contents

Abbreviations

AA	amino acid
ABA	abscissic acid
ACC	1-aminocyclopropane-1-carboxylic acid
ACR4	Arabidopsis crinkly4
Ag	agamous
AHK	Arabidopsis histidine kinase
AHP	Arabidopsis histidine phosphotransfer proteins
ANOVA	analysis of variation
AOC	allene oxide cyclase
AON	autoregulation of nodulation
ARR	Arabidopsis response regulator
At	*Arabidopsis thaliana*
AUT	autoregulation of nodulation
AUX	auxin-resistant
AVG	1-aminoethoxyvinylglycine
BAC	bacterial artificial chromosome
BAM	barely any meristem
BAP	benzyl amino purine
bit	branching infection threads
BL	brassinolide
BLAST	basic local alignment search tool
bp	base pair
BR	brassinosteroid
C	carbon
CaMV	cauliflower mosaic virus
CCaMK	calcium calmodulin dependent kinase
cDNA	complementary DNA
CDS	coding sequence
CHS	naringenin-chalcone synthase
CKX	cytokinin oxydase/dehydrogenase

CLE	CLV3/ESR
CLV	clavata
co-IP	co-immunoprecipitation
CRE	cytokinin response
CRF	cytokinin response factor
CRN	coryne
cv	cultivar
cyc	cyclin
cZ	cis-zeatin
DAG	days after germination
DFCI	Dana-Farber Cancer Institute
DMI	doesn't make infection
DNA	deoxyribonucleic acid
dpi	days post inoculation
DVL1	devil1
EFR	EF-TU receptor
EIN	ethylene insensitive
ENOD	early nodulation
ERF	ethylene response factor
ERN	ethylene-responsive binding domain factor required for nodulation
ESR	embryo-surrounding region
EST	expressed sequence tag
Fe	iron
FLS	flagellin sensitive
GA	gibberellic acid/gibberellin
gDNA	genomic DNA
GFP	green fluorescent protein
GLMM	generalized linear mixed model
Gm	*Glycine max*
GUS	beta glucuronidase
HAR	hypernodulation abberant root formation
HCL	hair curling locus
HIT	hyperinfected
HMM	hidden Markov model
HY5	hypocotyl5
IAA	indol-3-acetic acid
IDA	inflorescence deficient in abscission
iP	isopentenyl adenine

IPD3	interacting partner of DMI3
IPT	isopentenyl transferase
ISV	Institut de Sciences du Végétal
IT	infection thread
JA	jasmonic acid
KAPP	kinase associated protein phosphatase
kb	kilobase
kDa	kilodalton
KLV	klavier
Km	kanamycin
LATD	lateral root defective
LHK	Lotus histidine kinase
Lj	*Lotus japonicus*
LjCLE-RS	LjCLE-root signal
ln	logaritmus naturalis
LNOD	late nodulation
LOG	lonely guy
LRR	leucine rich repeat
LSS	like sunn supernodulator
LUC	luciferase
LYK	LysM receptor-like kinase
LysM	lysin motif
MAPK	mitogen activated protein kinase
Mbp	mega base pairs
MGI	Medicago gene index
mRFP	monomeric red fluorescent protein
Ms	*Medicago sativa*
Mt	*Medicago truncatula*
MTGI	Medicago truncatula gene index
N	nitrogen
NARK	nodule autoregulation receptor kinase
NAS	nicotianamine synthase
NCR	nodule specific cysteine rich
NF	nod factor
NFP	nod factor perception
NFR	nod factor receptor
NI	uninoculated
NIC	nitrate induced *CLE* gene

NIN	nodule inception
NIP	numerous infection threads, polyphenolics
NLS	nuclear localization signal
nM	nanomolar
NOD	nodulation
NORK	nodulation receptor kinase
NPA	1-naphthylphtalamic acid
NRT	nitrate transporter
NTS	nitrate tolerant symbiotic
OC	organizing centre
ODD	2-oxoglutarate-dependent dioxygenase
ORF	open reading frame
PAM	point accepted mutation
PAMP	pathogen associated molecular pattern
PAT	polar auxin transport
PCR	polymerase chain reaction
PIN	PIN-formed
PLL	POL-like
POL	poltergeist
PRR	pattern recognition receptor
Ps	*Pisum sativum*
PSK	phytosulfokine
PXL	PXY-like
PXY	phloem intercalated with xylem
QC	quiescent centre
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RALF	rapid alkalinisation factor
RAM	root apical meristem
RDN	root determined nodulation
RDH	root determined hypernodulation
RHC	root hair curling
RIC	rhizobia induced CLE gene
RLK	receptor like kinase
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
ROT4	rotundifolia4
RPK	receptor protein kinase

RR	response regulator
SAM	shoot apical meristem
SCR	S-locus cysteine rich proteins
SD	standard deviation
SDI	shoot derived inhibitor
SE	standard error
SKL	sickle
Sm	*Sinorhizobium meliloti*
snf	spontanous nodule formation
SOL2	suppressor of overexpression of LLP1-2
SOLi	Solution i
SP11	S-locus protein 11
Sr	*Sesbania rostrata*
SUNN	super numeric nodules
SYM	symbiosis
SYMRK	symbiosis receptor-like kinase
TAIR	the Arabidopsis information resource
TC	tentative consensus
TDIF	tracheary element differentiation inhibitory factor
T-DNA	transfer-DNA
TDR	TDIF receptor
TIGR	the institute for genomic research
TML	too much love
TOAD	toadstool
tRNA	transfer RNA
tZ	trans-zeatin
WOL	woodenleg
WOX	wus-related homeobox
wpi	weeks post inoculation
WT	wild type
WUS	wuschel

1

Scope and objectives

Nitrogen is one of the most important nutrients for plant development, because it is an essential component of nucleic and amino acids. However, for most plants, nitrogen sources are restricted to those available in the soil. Industrial nitrogen fertilizers can be applied, but their production and use is costly, energy-consuming and polluting. Legumes overcome this problem by interacting with soil-borne bacteria, rhizobia, resulting in root-based organs, called nodules, in which the rhizobia develop into nitrogen fixing bacteroids, which supply nitrogen to the host in exchange for energy-rich carbon sources and a protected niche. Many different processes are required to establish this interaction, making it an interesting yet complex research field. Apart from the nitrogen fixing symbiosis, research on root nodule formation also contributes to our understanding of processes of organogenesis and hormone signaling.

Research on nodule organogenesis has mainly focused on the role of the cell division hormones auxin and cytokinin. Much less is known about the involvement of peptide hormones, and more specifically CLAVATA3/EMBRYO SURROUNDING

REGION (CLV3/ESR or CLE) peptides.

The major objective was to characterize the function of nodulation-related CLE peptides during nodule organogenesis and in the control of nodule numbers during indeterminate nodulation on *Medicago truncatula*. This work especially focused on the role of two CLE peptide genes *MtCLE12* and *MtCLE13*, which are upregulated during nodulation and which encode structurally-related peptides. In addition, putative receptors of these nodulation-related CLE peptides were identified and characterized. Moreover, pathways and genes affected by *MtCLE12* and *MtCLE13* signaling were investigated in detail.

To elucidate differences in CLE peptide signaling between indeterminate and determinate nodulation and to have a more complete overview of the role of CLE peptides during nodulation, nodule-related CLE peptides and putative receptors were identified in soybean (*Glycine max*).

Finally, the interaction between cytokinin and CLE peptide signaling was investigated in more detail. Therefore, the influence of cytokinin activation via cytokinin riboside 5'-monophosphate phosphoribohydrolases, LONELY GUYs (LOGs), on nodule development was analyzed, as well as the putative involvement of CLE peptides in this pathway.

2

Plant peptides involved in nodulation

Virginie Mortier, Ulrike Mathesius, Marcelle Holsters and Sofie Goormachtig

In preparation

2.1 Nodulation: a macroscopic view

A symbiotic interaction of legume plants with rhizobia results in the development of novel root organs, nodules, the inner cells of which are colonized by bacteria, that fix nitrogen for the host. In return, the microsymbionts receive carbon sources and a protective niche. Nodulation is a sophisticated program that requires strictly coordinated bacterial infection and *de novo* organ formation. How this is achieved varies between different legume species and here we mainly focus on the Nod factor dependent root hair infection strategy and nodule development as observed in the model legume *Medicago truncatula*.

The symbiosis is initiated by a specific chemical signal exchange and recognition. When the rhizobia sense a specific array of flavonoid compounds secreted by the host, the expression of nodulation (nod) genes is switched on, resulting in the production and secretion of lipochitooligosaccharide signals, the Nod factors (NFs) (D'Haeze and Holsters, 2002). The NFs are sensed by LysM receptor-like-kinases (RLKs) located in the plasma membrane of susceptible root hairs and activate the nodulation process. The first responses observed take place within seconds to minutes after NF application and consist of plasma membrane depolarization and extracellular alkalinisation (Felle *et al.*, 1999). A few minutes later, Ca^{2+} oscillations are observed in and around the nucleus, and it is currently thought that this event is central in the activation of the epidermal and cortical responses and the coordination between both by triggering the expression of nodulin genes, classified early nodulin (ENOD) and late nodulin (LNOD), depending whether the induction of their expression precedes or accompanies nitrogen fixation (Miwa *et al.*, 2006; Sun *et al.*, 2007a; Talukdar *et al.*, 2009; Crespi and Galvez, 2000; Schultze and Kondorosi, 1998). Morphological changes are an arrest of root hair growth, followed by swelling and deformation of the root hair tip (Gage, 2004). As a result, the root hair will curl and entrap a rhizobial colony. This event is commonly referred to as root hair curling (RHC). Subsequently, the plant cell wall is locally degraded within the curl, followed by invagination of the plasma membrane. Cell wall material is deposited around the invaginated membrane ensuing the formation of a tubular structure, the infection thread, through which the bacteria are guided towards deeper root cell layers (Gage, 2004). The cortical cells respond to NFs by dedifferentiation and re-iteration of the cell cycle: while the inner cortical cells progress through the cell cycle and develop into a nodule primordium, the outer cortical cells arrest in G2 for pre-infection thread formation via cytoplasmic bridges

through which infection threads will pass (Yang *et al.*, 1994; van Spronsen *et al.*, 2001; Timmers, 2008). Once the infection threads reach the cells of the nodule primordium, the bacteria are released inside the cytoplasm via infection droplets, surrounded by a plant derived peribacteroid membrane. Within these new organelles, the symbiosomes, bacteria develop into bacteroids to fix nitrogen (Brewin, 2004; Kouchi *et al.*, 2010).

Two types of nodules have been intensively studied: determinate and indeterminate ones. While determinate nodules develop from division of outer cortical cells and in mature nodules all meristematic tissue has been consumed, indeterminate nodules arise from the inner cortex and are characterized by a persistent apical meristem (Crespi and Galvez, 2000; Ferguson *et al.*, 2010). Hence, mature indeterminate nodules are zonated with a meristem, an infection zone, a fixation zone and a senescence zone. The nodule parenchyma surrounds the central zone and contains the vascular tissue, via which nutrients are exchanged between both symbiotic partners (Van de Velde *et al.*, 2010).

2.2 Involvement of plant hormones in nodulation

There is ample evidence that the classical hormones cytokinin, auxin, abscissic acid, gibberellins, ethylene and jasmonic acid are all involved in the initiation and coordination of the nodulation process (Smit *et al.*, 1995a; Heidstra *et al.*, 1997; van Spronsen *et al.*, 2001; Mathesius *et al.*, 1998; van Noorden *et al.*, 2006; Boot *et al.*, 1999; Wasson *et al.*, 2006; Pacios-Bras *et al.*, 2003; Grunewald *et al.*, 2009; Murray *et al.*, 2007; Gonzalez-Rizzo *et al.*, 2006; Tirichine *et al.*, 2007; Frugier *et al.*, 2008; Oldroyd and Downie, 2008). The central hormone for nodule organogenesis is however cytokinin, as suggested by numerous experiments. The most convincing ones involved the *Lotus japonicus* knock-out mutant for the cytokinin receptor gene *LOTUS HISTIDINE KINASE1* (*LHK1*), *M. truncatula* transgenic plants with suppressed expression of *CYTOKININ RESPONSE1* (*CRE1*), or *Mtcre1* mutants, which were defective in nodule primordia formation (Gonzalez-Rizzo *et al.*, 2006; Murray *et al.*, 2007; Plet *et al.*, 2011). Similarly, the ectopic expression of a cytokinin degrading enzyme in *L. japonicus* resulted in reduced nodulation (Lohar *et al.*, 2004). Additionally, a *L. japonicus* gain-of-function mutant for the LHK1 receptor provoked spontaneous nodules, indicating that cytokinin signaling is both necessary and sufficient for nodule formation (Tirichine *et al.*, 2007). Next to cytokinin, also auxin is a prerequisite for nodule formation (Oldroyd and Downie, 2008; Ding and Oldroyd, 2009). In white clover (*Trifolium repens*), the auxin flow within the root vasculature was transiently inhibited at

the site of infection, leading to auxin accumulation in the cortical region where the nodule primordia form (Mathesius *et al.*, 1998). A reduction in auxin flow has been confirmed by radioactive auxin tracer experiments for *M. truncatula* and vetch (*Vicia faba*), but not *L. japonicus* (Boot *et al.*, 1999; Pacios-Bras *et al.*, 2003; van Noorden *et al.*, 2006; Wasson *et al.*, 2006). The involvement of auxin during early nodulation is also supported by the fact that application of synthetic auxin transport inhibitors, such as 1-naphthylphtalamic acid (NPA), lead to spontaneous nodule formation (Hirsch and Fang, 1994). Moreover, proteome studies in *M. truncatula* revealed a high overlap in protein changes in the roots 24 h after auxin or *Sinorhizobium meliloti* treatment (van Noorden *et al.*, 2007). A positive role for gibberellins during nodulation was suggested, by the reduced nodule number observed on a gibberellin biosynthetic mutant (Kawaguchi *et al.*, 1996; Ferguson *et al.*, 2005). Moreover, exogenous application of gibberellins rescued the phenotype by restoring the nodule number (Kawaguchi *et al.*, 1996; Ferguson *et al.*, 2005). A role of gibberellin in the intercellular invasion process of lateral root base nodulation in *Sesbania rostrata* has also been suggested (Lievens *et al.*, 2005). Abscissic acid (ABA) is believed to be involved in nodulation at the level of nodule initiation, where it would interfere with NF signaling, leading to a reduction in nodule number, and at the level of nodule development, where it can suppress cytokinin induced nodulation processes (Ferguson and Mathesius, 2003; Ding *et al.*, 2008). It has also been suggested that ABA plays a role at the latest stages of nodulation, because an increase in ABA levels is observed at the onset of nodule senescence (Ferguson and Mathesius, 2003).

High concentrations of ethylene inhibit nodulation, by exerting a negative effect at multiple levels during nodulation. First, ethylene suppresses the NF signaling pathway, either at or during NF induced calcium spiking, leading to inhibition of root hair deformation, shortening of the calcium spike period, blocking of bacterial infection and suppression of nodulin genes expression (Oldroyd *et al.*, 2001). In addition, ethylene has been shown to regulate infection thread formation and to dictate the positioning of nodules along the protoxylem poles independent of the NF signaling pathway (Penmetsa and Cook, 1997; Heidstra *et al.*, 1997). Indeed, 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase involved in the last step of ethylene biosynthesis is highly expressed at the protophloem poles (Heidstra *et al.*, 1997). Presumably an ethylene gradient is made with the lowest concentration located at the protoxylem poles, allowing nodulation at that place. Moreover, the addition of inhibitors of ACC synthase (AVG) and antagonists of ethylene action (Ag^+ ions) had a positive effect on nodule number

(Nukui *et al.*, 2000; Oldroyd *et al.*, 2001). Analysis of the ethylene insensitive *sickle* (*skl*) mutant confirmed the negative effect of ethylene on nodulation, as this mutant has a supernodulation and hyperinfection phenotype (Penmetsa and Cook, 1997; Penmetsa *et al.*, 2008). Also transgenic *L. japonicus* plants expressing a mutated ethylene receptor gene produce an elevated number of infection threads and nodule primordia (Nuku i *et al.*, 2004). Like ethylene, JA inhibits nodulation by suppressing calcium spiking and NF induced gene expression (Sun *et al.*, 2006; Hause and Schaarschmidt, 2009). Moreover, several lines of evidence indicate that JA signaling in the leaves is involved in the autoregulation of nodulation (AON) signal transduction pathway a process by which the plant controls the number of nodules (Kinkema and Gresshoff, 2008).

2.3 Peptide signals and nodulation

Peptide hormones have been extensively studied in animals where they act as the most common signal molecules to regulate and coordinate spatial and temporal expression of genes for the development of a multi-cellular organism. Peptide hormones consist of short stretches of amino acids (an informal dividing line is mostly set at 50 amino acids in length) that are mostly post-translationally modified, by for example glycosylation, arabinosylation and hydroxyprolination. Animal peptides are highly diverse in sequence and length, but members of the same family are highly conserved. While some peptides are derived from small ORFs and supposedly act within the cytoplasm, most peptides are derived from the C-terminal end of pro-proteins, often by cleavage at the level of dibasic amino acid residues. In addition, the presence of signal peptides at the N-terminal end of the preproproteins causes those peptides to be secreted in the extracellular medium where they act non-cell-autonomously as intercellular signaling molecules.

A restricted number of plant peptides have been identified until now mostly by chance and many are expected to be discovered by conducting more direct searches. The identified peptides play a role in various aspects of plant development, growth and defence.

Systemin, the first plant peptide hormone to be described, is involved in systemic defences triggered by insect attack or mechanical wounding (McGurl *et al.*, 1992; Pearce *et al.*, 1991). Systemin signalling has been thoroughly investigated and currently it is thought that the peptide acts as an enhancer of JA signalling in solanaceous species (Degenhardt *et al.*, 2010).

Phytosulfokines (PSKs), 5-amino acids long peptides with sulphated tyrosine residues, were the next peptides to be discovered. They regulate cellular dedifferentiation, proliferation and differentiation in suspension cultures of carrot, rice and *Asparagus officinalis* (Hanai *et al.*, 2000; Matsubayashi and Sakagami, 1996; Yang *et al.*, 2000a,b). Moreover, PSKs trigger tracheary element differentiation of cultured *Zinnia* mesophyll cells at nanomolar concentrations (Matsubayashi *et al.*, 1999). PSKs also promote various stages of plant growth, including adventitious bud and root formation, pollen germination and somatic embryogenesis (Hanai *et al.*, 2000; Igasaki *et al.*, 2003; Kobayashi *et al.*, 1999; Yamakawa *et al.*, 1998; Chen *et al.*, 2000).

The S-LOCUS CYSTEINE RICH PROTEINS (SCRs) and S-LOCUS PROTEIN11 (SP11) are involved in pollen self-incompatibility in *Brassicaceae* (Schopfer *et al.*, 1999; Kachroo *et al.*, 2002). POLARIS plays a role in root vascular patterning by maintaining responsiveness to exogenous auxin and cytokinin (Casson *et al.*, 2002). INFLORESCENSE DEFICIENT IN ABSCISSION (IDA) controls floral organ abscission (Butenko *et al.*, 2003).

Finally, peptides from the CLAVATA3/EMBRYO SURROUNDING REGION (CLE), EARLY NODULIN40 (ENOD40), RAPID ALKALINISATION FACTOR (RALF), DEVIL1/ROTUNDIFOLIA4 (DVL1/ROT4) and NODULE SPECIFIC CYSTEINE RICH (NCR) families carry out a range of different functions, but have in common that at least one of their members is involved in the process of nodulation. Here we will discuss these nodule-related peptides and how they control the nodulation process.

2.3.1 CLE peptides and nodulation

CLE peptides have been identified in the genome of monocotyledonous and dicotyledonous plants, in families that can consist of up to 40 members (Mitchum *et al.*, 2008; Cock and McCormick, 2001; Oelkers *et al.*, 2008). They were also found in the genome of the moss *Physcomitrella patens*, the green alga *Chlamydomonas reinhardtii* and the pteridophyte *Selaginella moelendorffii*, indicating that they occurred already at the early beginning of plant evolution (Oelkers *et al.*, 2008).

CLE peptides ($<$ 15 kDa) are 12 to 13 amino acids long and are cleaved from a 14 amino acids long conserved domain, located at or close to the carboxyl terminus of larger CLE proteins (Kondo *et al.*, 2006; Ito *et al.*, 2006; Ni and Clark, 2006). Post-translational modifications such as hydroxyprolination and arabinosylation have been reported (Kondo *et al.*, 2006; Ito *et al.*, 2006; Ohyama *et al.*, 2009). A hydrophobic sig-

nal peptide can be distinguished at the N-terminal site of most CLE proteins suggesting that the proteins are targeted to the secretory pathway and that the peptides act as intercellular signals (Sawa *et al.*, 2006). The region between the signal peptide and the CLE domain is highly variable and its sequence can be substituted by the sequence of other, unrelated proteins, without disrupting the function of the peptide (Ni and Clark, 2006). Despite variability in the core structure of the peptides, all members of the CLE family share certain structural parameters such as charge, length and hydrophilicity. The genomes of *Oryza sativa*, *M. truncatula*, *Tritium aestivum* and *Selaginella moelendorffii* encode CLE members with multiple CLE domains (Oelkers *et al.*, 2008; Mortier *et al.*, 2010). Processing of several CLE peptides from one pro-protein might be a mechanism to provide an amplification effect. These multiple CLE domains are probably the result of duplication events occurring during evolution.

CLE peptides are involved in balancing cell division and differentiation during several aspects of plant development. They control shoot and root meristem activity, apical dominance, organ size, vascular differentiation and nodulation (Brand *et al.*, 2000; Hirakawa *et al.*, 2008; Hobe *et al.*, 2003; Okamoto *et al.*, 2009; Mortier *et al.*, 2010). Interestingly, the only CLE peptide that was identified outside the plant kingdom is encoded in the genome of the cyst nematode *Heterodera glycines* (Wang *et al.*, 2001, 2011). This peptide, *HgSYV46*, is exclusively expressed in the dorsal esophageal gland cell of parasitic nematodes during syncitium formation in plants and is believed, after being injected in the plant root, to mimic endogenous CLE peptides to activate root cell proliferation for syncytial feeding cell formation (Wang *et al.*, 2001, 2011).

Due to a high level of functional redundancy observed between the different CLE members, the gene specific function of only a small number of CLE peptides (CLV3, TDIF and CLE40) was so far identified. CLV3, the best studied peptide of the CLE family, is involved in shoot apical meristem (SAM) homeostasis in Arabidopsis. *Clv3* mutants have an expanded SAM, fasciation of stems and leaves and supernumerary floral organs, caused by the accumulation of stem cells in the shoot apical and flower meristems (Clark *et al.*, 1995). Ectopic expression of *CLV3*, on the other hand, results in plants in which the SAM is not maintained and organogenesis from the shoot tip is arrested (Brand *et al.*, 2000). Hence, *CLV3* plays a key role in the suppression of stem cell proliferation in the SAM. Further studies have shown that *CLV3* is part of a cell non-autonomous negative feedback regulation loop, comprising amongst others, the homeodomain transcription factor *WUSCHEL* (*WUS*) (Brand *et al.*, 2000; Schoof *et al.*, 2000): *WUS*, which is expressed in the cells of the organizing centre, induces ex-

pression of *CLV3* in the overlying neighboring cells and confers their stem cell identity (Mayer *et al.*, 1998; Rojo *et al.*, 2002), while *CLV3* down-regulates *WUS* expression and thereby confines the size of the organizing centre (Schoof *et al.*, 2000; Lenhard and Laux, 2003). Recruitment of cells from the peripheral zone to the central zone and vice versa were observed as well, suggesting that *CLV3* is also involved in setting the boundary between both zones (Reddy and Meyerowitz, 2005; Muller *et al.*, 2006).

The CLV3 peptide is perceived by a complex interplay of membrane receptors. The peptide is directly bound by the leucine-rich-repeat RLK (LRR-RLK) CLV1, which can form homodimers or heterodimers in combination with their close homologs BARELY ANY MERISTEM1 (BAM1) or BAM2 (Trotochaud *et al.*, 1999, 2000; Ogawa *et al.*, 2008; Guo and Clark, 2010). In addition, genetic and physiological studies have recently demonstrated that the CLV1-unrelated LRR-RLK RECEPTOR PROTEIN KINASE2/TOADSTOOL2 (RPK2/TOAD2) and the LRR receptor-like-protein CLV2 interacting with CORYNE/SUPPRESSOR OF LLP1 2 (CRN/SOL2), a RLK lacking an extracellular domain, mediate CLV3-dependent SAM homeostasis in parallel to CLV1 (Miwa *et al.*, 2008; Muller *et al.*, 2008; Bleckmann *et al.*, 2010; Zhu *et al.*, 2010a,b; Guo *et al.*, 2010; Guo and Clark, 2010; Kinoshita *et al.*, 2010). However, different regulations might be observed throughout the plant kingdom as an evolutionary analysis suggested that CLV2 and CRN are restricted to vascular plants, while CLV1 and RPK2 are highly conserved (Miwa *et al.*, 2009; Sawa and Tabata, 2011). Still more receptors and putative CLE peptides might contribute to SAM homeostasis. Other components of the CLV-WUS pathway are a KINASE ASSOCIATED PROTEIN PHOSPHATASE (KAPP) and POLTERGEIST (POL) type 2C phosphatase which negatively regulate CLV signaling through interaction with CLV1 (Trotochaud *et al.*, 1999; Yu *et al.*, 2003). Next to their expression in the centre of the SAM, BAM receptors are also expressed at the flanks of the SAM and are proposed to sequester exterior CLV3-like CLE peptides thereby preventing their entrance in the core region of the SAM (Deyoung and Clark, 2008).

The signaling components for stem cell maintenance in the shoot and in the root apical meristems are relatively conserved, hence it is not surprising that CLE peptides were also shown to control root apical meristem homeostasis. Loss-of-function of the Arabidopsis *CLE40* caused roots to exhibit a slightly waving pattern, a reduced length and strong slant, indicative of a defect in gravity response (Hobe *et al.*, 2003). A reduction in root length was also reported in experiments in which CLE peptides were supplemented to the growth medium of Arabidopsis or *M. truncatula* seedlings or in

which *CLE* genes were ectopically expressed (Fiers *et al.*, 2005; Ito *et al.*, 2006; Oelkers *et al.*, 2008; Mortier *et al.*, 2010; Strabala *et al.*, 2006; Whitford *et al.*, 2008; Wang *et al.*, 2011). In these experiments, a high level of redundancy was observed between members with related sequences. Probably, the spatial and temporal expression patterns regulate the range of action of related CLE peptides. Indeed, expression analysis of Arabidopsis, soybean and *M. truncatula CLE* genes indicated different expression patterns for different *CLE* genes, often restricted to well defined tissues (Sharma *et al.*, 2003; Mortier *et al.*, 2010, 2011).

Another well-characterized CLE peptide, TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR (TDIF), isolated from *Zinnia elegans* cell cultures during *in vitro* experiments, suppresses tracheary element differentiation and controls the rate and orientation of vascular cell division (Ito *et al.*, 2006; Etchells and Turner, 2010). The sequence of the TDIF peptide is identical to CLE41 and CLE44 and highly homologous to CLE42 and CLE46. *In vitro* experiments performed with these Arabidopsis peptides indeed resulted in a suppression of xylem development, while the other CLE peptides tested did not exhibit TDIF activity (Ito *et al.*, 2006). Applying mature CLV3 peptides to the *Z. elegans* cell culture resulted in the opposite effect, the promotion of tracheary element cell differentiation (Ito *et al.*, 2006). Hence, based on these experiments, it was proposed that two counteracting pathways are involved in CLE signaling, one promoting and one inhibiting cell differentiation. Nevertheless, Whitford *et al.* (2008) reported synergistic actions between CLV3-like and TDIF-like peptides. Ectopic expression or exogenous application of TDIF-like peptides, but not of CLV3-like peptides, resulted in proliferation of vascular cells. However, when both types of peptides were simultaneously overexpressed, or ectopically applied onto *A. thaliana* seedlings, the effect of the TDIF gain-of-function was enhanced, resulting in a massive proliferation of vascular cells. Additional experiments revealed that this cell proliferation was auxin mediated (Whitford *et al.*, 2008).

Both during RAM homeostasis and vascular patterning, CLE peptides are perceived by membrane bound receptor like kinases. Arabidopsis CRINKLY4 (ACR4) is suggested as the receptor for CLE40 in the roots and would be involved in the suppression of the *WUS* homolog, *WUS-RELATED HOMEOBOX5* (*WOX5*), to maintain distal root stem cells (Stahl *et al.*, 2009; Stahl and Simon, 2009). In accordance, TDIF was shown to bind *in vitro* the PHLOEM INTERCALATED WITH XYLEM/TDIF RECEPTOR (PXY/TDR) receptor expressed in the procambial cells to inhibit vascular differentiation (Hirakawa *et al.*, 2008).

There are two main reasons to propose that CLE peptides might control nodule formation. Firstly, because of the similarity between the CLE peptide receptors CLV1, BAM1-3 and TDR, and the *M. truncatula* receptor SUPER NUMERIC NODULES (SUNN), and its orthologs of *L. japonicus*, pea and soybean (HYPERNODULATION ABERRANT ROOT FORMATION1 (HAR1), SYMBIOSIS29 (SYM29) and NODULE AUTOREGULATION RECEPTOR KINASE (NARK), respectively), all belonging to the subclass XI of the LRR-RLKs (Krusell *et al.*, 2002; Nishimura *et al.*, 2002a; Searle *et al.*, 2003; Schnabel *et al.*, 2005; Shiu and Bleecker, 2001). These legume receptors, although most similar to CLV1, seem not to control SAM organization but rather are involved in AON, a systemic mechanism that involves root to shoot signaling to control nodule number (Kosslak and Bohlool, 1984; Carroll *et al.*, 1985a,b; Delves *et al.*, 1986; Pierce and Bauer, 1983; Nutman, 1952; Magori and Kawaguchi, 2009). The similarity to CLV1 suggested that SUNN/HAR1/SYM29/NARK might perceive CLE peptides (Krusell *et al.*, 2002; Nishimura *et al.*, 2002a; Searle *et al.*, 2003; Schnabel *et al.*, 2005). Secondly, CLE peptides might be important to balance cell division and differentiation at the level of primordium formation, and during the formation and maintenance of nodule meristems. Indeed, in *L. japonicus*, soybean as well as in *M. truncatula*, *CLE* genes were identified with upregulated expression during nodulation (Figure 2.1) (Okamoto *et al.*, 2009; Mortier *et al.*, 2010, 2011; Reid *et al.*, 2011). Tissue specific expression analysis in *M. truncatula* revealed that the expression of two *CLE* genes, *MtCLE12* and *MtCLE13*, was linked to proliferation and differentiation during nodulation (Mortier *et al.*, 2010). *MtCLE13* expression was up-regulated early in nodule development and a high-to-low expression gradient, radiating from the inner towards the outer cortical cell layers was observed in a root cortical region defining the incipient nodule. At later stages, *MtCLE12* and *MtCLE13* were expressed in the meristem and early infection zone of mature, elongated nodules.

In the legumes, *L. japonicus*, *M. truncatula* and soybean, overexpression of nodule related CLE proteins, *LjCLE-RS1, LjCLE-RS2, MtCLE12, MtCLE13, GmRIC1, GmRIC2* and *GmNIC1*, resulted in the abolishment or strong reduction of nodulation (Okamoto *et al.*, 2009; Mortier *et al.*, 2010; Reid *et al.*, 2011). This effect involved long-distance signaling and was totally or partially dependent on SUNN/HAR1/NARK, suggesting that these CLE peptides might be involved in AON (Okamoto *et al.*, 2009; Mortier *et al.*, 2010; Reid *et al.*, 2011). In *M. truncatula* this inhibition was shown to occur at the level of the NF signal transduction (Mortier *et al.*, 2010).

Whether these CLE peptides directly bind SUNN/HAR1/NARK is currently not

known. Many studies have shown that SUNN/HAR1/NARK act in the shoot to provoke AON although the genes are also expressed in the root (Krusell *et al.*, 2002; Nishimura *et al.*, 2002a; Schnabel *et al.*, 2005; Searle *et al.*, 2003; Delves *et al.*, 1986; Jiang and Gresshoff, 2002; Francisco and Harper, 1995). Hence, because the peptide genes are expressed in the nodule, direct binding would indicate a long distance traveling of the CLE peptides from the root to the shoot. This would be a novel feature, as so far all characterized CLE peptides act within short distances from the cells by which they are produced (Fukuda *et al.*, 2007; Hirakawa *et al.*, 2008; Whitford *et al.*, 2008; Miwa *et al.*, 2009).

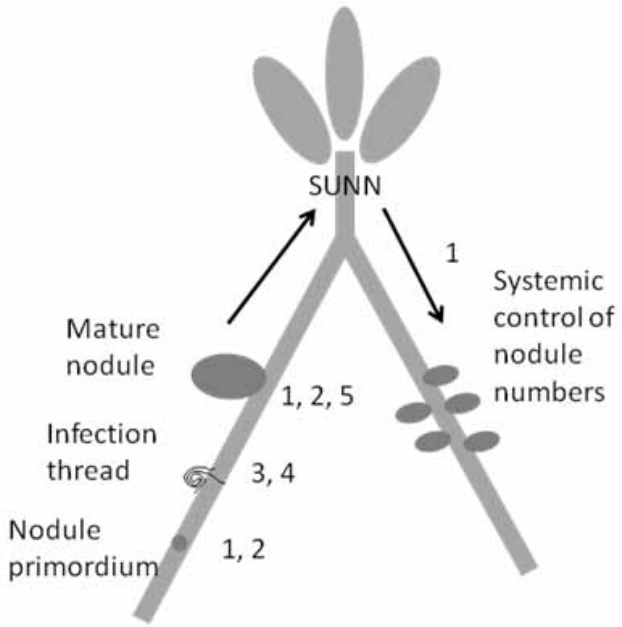

Figure 2.1: Schematic diagram of peptide involvement during nodulation. Peptides of the CLE (1), ENOD40 (2), RALF (3), DLV1/ROT4 (4) and NCR (5) families have roles at different stages of nodulation, including nodule primordium formation, infection thread development, nodule and bacteroid differentiation as well as in the systemic control of nodule numbers involving the receptor-like kinase, SUNN/NARK/HAR1/SYM29 acting in the shoot.

In *L. japonicus*, apart from HAR1, another shoot-controlled LRR-RLK, KLAVIER

(KLV), is involved in AON, as well as a CLV2 ortholog (Oka-Kira *et al.*, 2005; Krusell *et al.*, 2011). This suggests that complex receptor interactions exist in the shoot to control AON, which is in accordance to the CLV1, RPK2, CLV2 and CRN interactions observed to sustain SAM homeostasis and to the CLV2/CRN receptor complex, which is suggested to bind root CLE peptides (Meng *et al.*, 2010; Meng and Feldman, 2010). Although the *sunn* mutant did not show any SAM phenotypes, Lotus and pea *clv2* mutants and *klavier* have a shoot phenotype which is very similar to the phenotype reported for the Arabidopsis *clv1* and *clv2* mutants (Oka-Kira *et al.*, 2005; Miyazawa *et al.*, 2010; Krusell *et al.*, 2011). Hence, the same receptor complex might rule AON and SAM/RAM homeostasis but probably different plants will use different contributions of each receptor to induce different effects. Also the ACR4 and TDR/PXY receptors acting in the root apical meristem (RAM) and vascular tissue, respectively, might interact with currently unidentified receptors.

2.3.2 The ENOD40 peptide, a never ending story?

The ENOD40 gene has been isolated from soybean as an early upregulated gene encoding a short mRNA ($\pm$ 0.7 kb) lacking one long open reading frame (ORF) (Yang *et al.*, 1993). The gene is widely conserved across the plant kingdom and was found in several other legumes such as *M. truncatula*, *L. japonicus* and *Sesbania rostrata* but also in non legumes including monocots like rice, barley, rye grass, *Zea mays* and sorghum and dicots such as tobacco, tomato and citrus (Matvienko *et al.*, 1996; Kouchi *et al.*, 1999; Corich *et al.*, 1998; Compaan *et al.*, 2003; Larsen, 2003; Vleghels *et al.*, 2003; Gultyaev and Roussis, 2007). ENOD40 sequences carry two conserved nucleotide regions and several short small ORFs. The 10 to 13 amino acids long translation product of ORF A has been detected in *Medicago sativa* and soybean (Sousa *et al.*, 2001; Rohrig *et al.*, 2002). Moreover, during *in vitro* experiments with soybean, a second translation product (ORF B) has been detected, which is 24 to 27 amino acids long and partially overlapping with ORF A (Rohrig *et al.*, 2002). Next to a function for *ENOD40* as a peptide(s), *ENOD40* would also play a role as an un-translated, biologically active RNA molecule (Crespi *et al.*, 1994). This is supported by the highly conserved secondary structure of the transcript and the isolation of an *ENOD40* RNA binding protein (RBP1) in *M. truncatula* (Crespi *et al.*, 1994; Campalans *et al.*, 2004; Gultyaev and Roussis, 2007). Possibly, the *ENOD40* mRNA requires the ENOD40 peptide(s) for its stability and full activity.

The expression of *ENOD40* during nodulation has been widely studied in many legumes. It is expressed early after rhizobial infection, in the root pericycle opposite the protoxylem poles and in the dividing cortical cells of the nodule primordium (Crespi *et al.*, 1994; Kouchi and Hata, 1993; Yang *et al.*, 1993; Papadopoulou *et al.*, 1996; Takeda *et al.*, 2005). Also at later stages of nodulation, *ENOD40* expression was observed, more specifically in the pre-infection zone, in uninfected cells of the central tissue in mature nodules as well as in the pericycle of the nodule vascular bundles (Yang *et al.*, 1993; Papadopoulou *et al.*, 1996; Takeda *et al.*, 2005; Corich *et al.*, 1998). These nodulation-related expression patterns were reported for Phaseolus, soybean, *M. truncatula, M. sativa, L. japonicus* and *S. rostrata* (Papadopoulou *et al.*, 1996; Yang *et al.*, 1993; Compaan *et al.*, 2003; Takeda *et al.*, 2005; Gronlund *et al.*, 2005; Wan *et al.*, 2007; Corich *et al.*, 1998). The two *ENOD40* genes identified in *L. japonicus* are differentially regulated. They are both specifically upregulated during nodule organogenesis, but show different expression patterns upon rhizobial infection (Takeda *et al.*, 2005). Co-localization of different *ENOD40* genes within one species has also been reported for *M. sativa, M. truncatula, L. japonicus* and *T. repens* (Fang and Hirsch, 1998; Flemetakis *et al.*, 2000; Varkonyi-Gasic and White, 2002; Wan *et al.*, 2007).

Like CLE peptides, ENOD40 is possibly involved in control of cell division and differentiation for nodule primordium formation (Figure 2.1). Ectopic expression of *ENOD40* in *M. truncatula* induced extensive cortical cell divisions and accelerated nodulation (Charon *et al.*, 1997, 1999; Crespi *et al.*, 1994). The latter phenotype was the result of an increase in nodule primordium formation and a proliferation response of the region close to the roottip (Charon *et al.*, 1999). It is thought that ENOD40 is not an inducer of cell division per se, but rather that it relies on other factors for cell cycle activation and subsequent nodule primordium formation (Minami *et al.*, 1996; Mathesius *et al.*, 2000; Sousa *et al.*, 2001). In *M. truncatula*, downregulation of *ENOD40*, via co-suppression or RNAi resulted in a reduction in the number of nodules and nodule development was arrested (Crespi *et al.*, 1994; Charon *et al.*, 1999; Wan *et al.*, 2007). The same phenotype was reported for RNAi knock-down lines of *ENOD40* in *L. japonicus* (Kumagai *et al.*, 2006). Bacterial infection of root hairs was not affected illustrating that *ENOD40* is required for nodule initiation and organogenesis, but not for the early infection events (Kumagai *et al.*, 2006). In *M. truncatula*, impaired bacteroid development was also observed in the RNAi lines (Wan *et al.*, 2007). This could be a consequence of an imbalance between cell division and differentiation rather than that *ENOD40* is essential for bacteroid development per se. The expression analysis

together with the functional analysis thus demonstrates that *ENOD40* is involved in the establishment and differentiation of the nodule primordium and meristem. The fact that *ENOD40* is expressed in the pericycle opposite protoxylem poles prior to cortical cell divisions suggests that *ENOD40* might counteract the effects of ethylene to promote mitotic activation of root cortical cells for nodule formation (Heidstra *et al.*, 1997). Indeed, the *ENOD40* expression pattern is complementary to the expression pattern of ACC oxidase, a key enzyme in the synthesis of ethylene (Heidstra *et al.*, 1997; Dey *et al.*, 2004).

Perhaps interlinked with a role in controlling primordium development, *ENOD40* might function in nutrient mobilization. The ENOD40 peptides A and B from soybean have been shown to interact *in vitro* with the 93 kDa subunit of sucrose synthase (Rohrig *et al.*, 2002), and the covalent binding of peptide A to cystein 264 of sucrose synthase increased the enzyme cleavage activity (Rohrig *et al.*, 2004). These findings suggest a role for *ENOD40* in nutrient supply by controlling the breakdown of sucrose for nodule development (Gordon *et al.*, 1999).

ENOD40 transcripts were also detected outside developing nodules and gene homologs were identified in non-leguminous plants, suggesting a more general biological function. More specifically, *ENOD40* transcripts were found in stems, in the parenchyma cells surrounding the protoxylem, suggesting a role in vascular development (Kouchi *et al.*, 1999; Corich *et al.*, 1998). In addition, transcripts were found in developing flowers and lateral roots (Corich *et al.*, 1998; Varkonyi-Gasic and White, 2002; Vleghels *et al.*, 2003; Papadopoulou *et al.*, 1996). Since all these tissues are linked with cell division and differentiation, *ENOD40* may have a common underlying role in organ and tissue development. Indeed, ectopic expression of *ENOD40* in alfalfa affected the formation of somatic embryos under *in vitro* culture conditions (Crespi *et al.*, 1994), while its ectopic expression in *A. thaliana* caused a reduction in cell size (Guzzo *et al.*, 2005). This function is probably interlinked with phytohormone signaling pathways because ectopic expression of *ENOD40* in tobacco Bright Yellow-2 cells resulted in elongation growth by alteration of ethylene biosynthesis kinetics and changes in phytohormonal signaling were reported for *M. truncatula* and rice plants ectopically expressing *MtENOD40* (Dey *et al.*, 2004; Ruttink *et al.*, 2006). Despite these many studies, a detailed insight in *ENOD40* action is still not obtained and awaits further investigation.

2.3.3 RALF peptides involved in nodule organogenesis and infection

RALF is a 49 amino acids long peptide, processed from the C-terminus of its preproprotein, probably by cleavage at a dibasic amino acid motif (Pearce *et al.*, 2001; Bedinger *et al.*, 2010). At the N-terminus of the preproprotein, the presence of a signal peptide suggested extracellular secretion (Pearce *et al.*, 2001). Highly conserved homologs are present in various species throughout the plant kingdom, amongst which 39 members in *Arabidopsis thaliana* and 40 members in *M. truncatula* (Olsen *et al.*, 2002; Combier *et al.*, 2008; Pearce *et al.*, 2001). RALF peptide genes are expressed in various tissues including shoots, roots, flowers, leaves and nodules (Germain *et al.*, 2005; Pearce *et al.*, 2001; Combier *et al.*, 2008). RALF was originally discovered during the purification of tobacco systemin as a component that induces rapid alkalinisation of the medium, as well as rapid activation of MAPK activity in tobacco cells (Pearce *et al.*, 2001). In contrast to systemin, no defense responses were observed in tobacco cell cultures when supplied at nanomolar concentrations (Pearce *et al.*, 2001). RALF has been suggested to be involved in the regulation of root growth and development, as synthetic tomato RALF peptides, when supplied to tomato and Arabidopsis seedling, induced a small enlargement of meristematic cells and an immediate arrest of root growth (Pearce *et al.*, 2001). Knock-down of RALF expression in *Nicotiana attenuate*, resulted in plants with longer roots and abnormal root hair development (Wu *et al.*, 2007). According to Covey *et al.* (2010) RALF would be involved in pollen tube elongation. Finally, Combier *et al.* (2008) showed evidence for the involvement of a *M. truncatula RALF*, named *MtRALF1*, during nodulation (Figure 2.1).

Expression analysis confirmed that *MtRALF1* is transiently upregulated by Nod factor treatment (Combier *et al.*, 2008). A reduced number of nodules was observed on roots ectopically expressing *MtRALF1*, while the number of aborted infection threads, with a bulbous and sac-like phenotype, was strongly increased (Combier *et al.*, 2008). The few nodules that could form on the roots ectopically expressing *MtRALF1* had an abnormal development and remained small, with poor rhizobial colonization. These results suggest that *MtRALF1* might be involved in nodule development and rhizobial infection, more specifically during the initial progression of infection threads through the outer cortical cell layers. As both inner and outer cortical cells initiate the cell cycle upon inoculation, RALF peptides might be involved in a common step in cell cycle activation. Until now, two proteins were identified as possible components of

a RALF receptor complex (Scheer and Ryan, 1999). Further investigation of these proteins, and the identification of new RALF interacting partners need to be done to obtain a clear insight in the role of this peptide.

2.3.4 DVL1/ROT4

DVL1/ROT4 are peptides that are also derived from the C-terminus of larger prepro-proteins. In *A. thaliana*, a gene family of 23 members was identified and the peptides are derived from a conserved 29 amino acids long domain (Narita *et al.*, 2004; Wen *et al.*, 2004). The peptide genes were identified during screens for leaf shape and fruit mutants of *A. thaliana*. A dominant mutation of ROT4 (*rot4-1D*) resulted in plants with short leaves and floral organs, which was mainly caused by a decrease in cell proliferation (Narita *et al.*, 2004). *Dvl1-1D* plants exhibited shortened petioles and siliques, rounder leaves and moderately horned fruits (Wen *et al.*, 2004). Hence, ROT4 and DVL1 affect plant growth and development by regulating polar cell proliferation. The functions of the DVL1/ROT4 family members are expected to be redundant, as ectopic expression caused similar pleiotropic effects on plant development (Wen *et al.*, 2004). However, divergent expression patterns were reported for the family members: *ROT4* is expressed in young leaves and in the SAM; the expression of *DVL1* has been localized to leaves; and the other family members are mostly expressed in flowers and roots (Narita *et al.*, 2004; Wen *et al.*, 2004). In *M. truncatula*, 10 members of the *DVL1/ROT4* family were identified and expression analysis revealed that one of them, *MtDVL1*, is transiently upregulated after NF treatments during initial phases of nodulation (Figure 2.1) (Combier *et al.*, 2008). Ectopic expression of *MtDVL1* resulted in a reduction in nodule number and in a strong increase in the number of aborted infection threads in outer cortical cell layers, identical to what was observed for *MtRALF1* (Combier *et al.*, 2008). Nevertheless, in contrast to what was observed for *MtRALF1*, nodule development and nitrogen fixation were as normal. Based on all these phentoypes, *MtDVL1* is supposed to act during rhizobial infection at the initial progression of infection threads through the outer cortical cell layers. Because *DVL1* family members are involved in cell proliferation, Combier *et al.* (2008) suggest a role for *MtDVL1* during cellular events leading to the differentiation for pre-infection thread formation. While DVL1 peptides control polar cell proliferation in lateral organs, they might also play a role in the polar growth of root hairs and as such be indirectly linked to nodulation (Narita *et al.*, 2004; Wen *et al.*, 2004). Evidence for this hypothesis is still lacking,

but molecular analysis of root hair growth might reveal the answer.

2.3.5 The role of NCRs in symbiosome development

All the above mentioned peptides seem to affect cell division and differentiation in a certain way. Another group of peptides or small proteins, the NCR proteins, play an important role in controlling the microsymbiont during nodulation (Figure 2.1). NCRs are 30 to 50 amino acids long of which 4 or 6 amino acids are cysteine residues located at conserved positions in the C-terminus. The other amino acid residues are extremely divergent. A signal peptide at the N-terminus targets the proteins to the secretory pathway (Mergaert *et al.*, 2003). More than 300 NCR members were discovered until now and they all occur in the galegoid legumes such as *Medicago* spp. (Fedorova *et al.*, 2002; Graham *et al.*, 2004; Gyorgyey *et al.*, 2000; Mergaert *et al.*, 2003; Alunni *et al.*, 2007), *Pisum sativum* (Kardailsky *et al.*, 1993; Kato *et al.*, 2002; Scheres *et al.*, 1990), *Trifolium repens* (Crockard *et al.*, 2002), *Astragalus sinicus* (Chou *et al.*, 2006), *Galega orientalis* (Kaijalainen *et al.*, 2002) and *Vicia faba* (Fruhling *et al.*, 2000). NCRs are exclusively nodule specific, except for two members which were also expressed in mycorrhizal roots (Mergaert *et al.*, 2003). Different spatial and temporal expression patterns were observed for different NCR genes suggesting that NCRs play a role in different nodule tissues and cell types and at several stages of nodule organogenesis (Mergaert *et al.*, 2003). Many NCRs might work synergistically, as overlapping expression patterns were also observed. From the results of macro-array experiments NCRs seemed to be coregulated with a calmodulin (CaM)-like protein and two signal peptide peptidases (Mergaert *et al.*, 2003). Mergaert *et al.* (2003) therefore suggest that these genes might be involved in similar functions or pathways (Young, 2000). The exact function of the NCRs is not known, but Mergaert *et al.* (2003) and Alunni *et al.* (2007) suggested that they act as antimicrobial peptides especially recruited for bacteroid differentiation (Brogden, 2005). Indeed, NCRs were recently indicated to be involved in the terminal and irreversible differentiation of rhizobia to bacteroids in the *S. meliloti* - *M. truncatula* symbiosis (Van de Velde *et al.*, 2010).

Authors contributions

The main part of the work was done by V. Mortier. U. Mathesius, S. Goormachtig and M. Holsters were involved in revising the manuscript.

3

Never too many? How legumes control nodule numbers

Virginie Mortier, Marcelle Holsters and Sofie Goormachtig

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Abstract
Restricted availability of nitrogen compounds in soils, is often a major limiting factor for plant growth and productivity. Legumes circumvent this problem by establishing a symbiosis with soil-borne bacteria, called rhizobia that fix nitrogen for the plant. Nitrogen fixation and nutrient exchange take place in specialized root organs, the nodules, which are formed by a coordinated and controlled process that combines bacterial infection and organ formation. Because nodule formation and nitrogen fixation are highly energy consuming, legumes develop the minimal number of nodules required to ensure optimal growth. To this end, several mechanisms have evolved that adapt nodule formation and nitrogen fixation to the plant's needs and environmental conditions, such as nitrate availability in the soil. In this review, we give an updated view on the mechanisms that control nodulation.

3.1 Nodulation: coordinated bacterial infection and organ formation

Nodulation is initiated by a signal exchange between the symbiotic partners. The process starts when plant root exudates, mostly flavonoids, are perceived by the rhizobia activating the nodulation (*nod*) genes to produce and secrete the lipo-chitooligosaccharidic Nod Factors (NFs) (D'Haeze and Holsters, 2002). In turn, NFs are sensed by LysM-type receptor-like kinases (RLKs) in the root epidermis and trigger a signaling cascade to elicit infection and cortical cell division (Figure 3.1) (Madsen *et al.*, 2003; Radutoiu *et al.*, 2003; Amor *et al.*, 2003; Arrighi *et al.*, 2006; Jones *et al.*, 2007; Oldroyd and Downie, 2008). NF synthesis as well as nodule initiation is highly dependent on stringent structural requirements of both signal molecules. In most legumes studied, including the model legumes, the bacteria enter via root hairs. After perceiving the NFs, the root hairs respond with Ca^{2+} influx at the tip, immediately followed by Cl^{-} and K^{+} effluxes. These ion fluxes induce the deformation and curling of the root hairs (shepherd's crooks) to entrap a bacterial colony (Kijne *et al.*, 1992; Emons and Mulder, 2000). Only young growing root hairs in a restricted zone just above the roottip are responsive to NF perception (Bhuvaneswari *et al.*, 1981; Lerouge *et al.*, 1990). Local hydrolysis and invagination of the plasma membranes of root hair cells mark the development of intracellular infection threads, plant-derived tubular structures through which the rhizobia are guided towards the underlying cortical cell layers

(Turgeon and Bauer, 1985; Brewin, 1991; Kijne *et al.*, 1992). Ahead of the advancing threads, cells in the cortex are induced to dedifferentiate, divide and form a nodule primordium (Brewin, 1991; Truchet *et al.*, 1991; Mergaert *et al.*, 1993; Stokkermans and Peters, 1994). Depending on the legume host, the nodules will originate from either the inner or outer root cortex, resulting in indeterminate and determinate nodules, respectively. In *Medicago truncatula* (barrel medic), cylindrical indeterminate nodules develop, characterized by a distal, persistent meristem and various developmental zones. The meristem is followed by an infection zone with cells containing proliferating infection threads from which infection droplets release bacteria into the cytoplasm of the plant cells. During uptake, the bacteria are surrounded by a plant-derived peribacteroid membrane that together form organelle-like structures, called the symbiosomes. The bacteria in the symbiosomes differentiate into the nitrogen-fixing bacteroids of the fixation zone (Mylona *et al.*, 1995). Upon decay of the infected plant cells, a senescence zone is established (Perez Guerra *et al.*, 2010). In mature determinate nodules, all meristematic tissue has been consumed and the central tissue mainly consists of infected cells, where nitrogen fixation is taking place.

Several components of the NF signaling pathway have been identified. In *M. truncatula*, a leucine-rich-repeat (LRR) RLK, DOESN'T MAKE INFECTIONS2 (MtDMI2) and a nuclear potassium channel (MtDMI1) are active early in the pathway and trigger Ca^{2+} spiking in and around the nucleus (Oldroyd and Downie, 2004). In addition, orthologous genes with a similar function were found in other legumes as well (Endre *et al.*, 2002; Stracke *et al.*, 2002; Oldroyd and Downie, 2004; Imaizumi-Anraku *et al.*, 2005; Capoen *et al.*, 2005). Decoding of this Ca^{2+} signature by a Ca^{2+} calmodulin-binding protein (LjCCAMK and MtDMI3) and its interacting partner INTERACTING PROTEIN OF DMI3 (MtIPD3 and LjCYCLOPS) triggers the transcription factors NODULATION signaling PATHWAY1 (NSP1), NSP2, ETHYLENE-RESPONSIVE BINDING DOMAIN FACTOR REQUIRED FOR NODULATION1 (ERN1), and NODULE INCEPTION (NIN) (Schauser *et al.*, 1999; Catoira *et al.*, 2000; Borisov *et al.*, 2003; Oldroyd and Long, 2003; Gleason *et al.*, 2006; Andriankaja *et al.*, 2007; Marsh *et al.*, 2007; Middleton *et al.*, 2007; Oldroyd and Downie, 2008; Messinese *et al.*, 2007). Together, these transcription factors are involved in the activation of EARLY NODULATION (ENOD) genes that initiate infection and nodule primordium formation.

As long as rhizobia remain in the infection threads, they produce NFs that set off both cell autonomous and non-autonomous processes (Gage and Margolin, 2000; Gage,

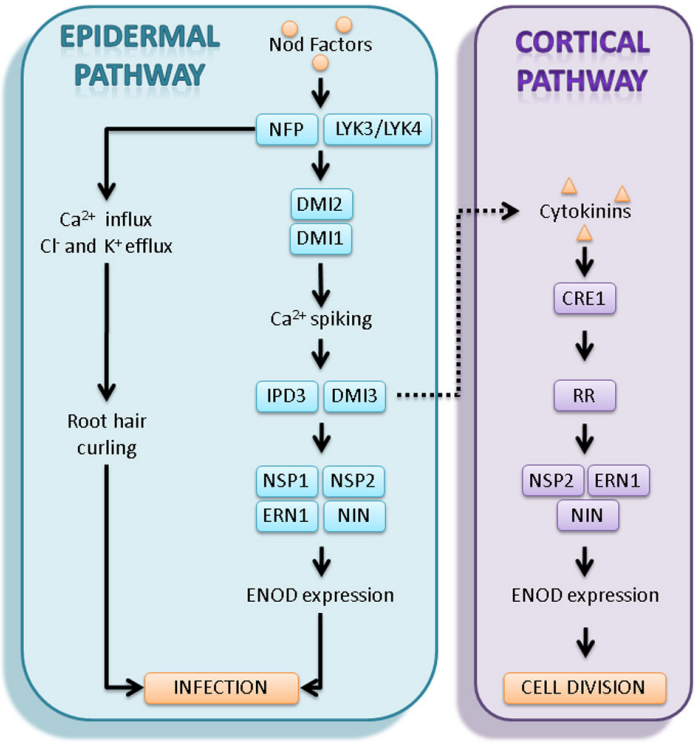

Figure 3.1: The epidermal and cortical Nod Factor (NF) signaling pathway in *Medicago truncatula*. NFs exuded by rhizobia are sensed by root epidermal cells, where they activate the LysM-type receptor like kinases NFP, LYK3 and LYK4. Receptor activation induces Ca^{2+} influx at the root hair tips, eventually resulting in root hair curling and rhizobial infection. Also the LRR-RLK DMI2 and the nuclear potassium channel DMI1 are active early in the epidermal pathway and trigger Ca^{2+} spiking in and around the nucleus. Decoding of this Ca^{2+} signature by the Ca^{2+} calmodulin-binding protein DMI3 and its interacting partner IPD3 results in the activation of the transcription factors NSP1, NSP2, ERN1 and NIN. Together, these transcription factors are involved in the activation of early nodulation (ENOD) genes to initiate the infection process. In parallel, a mobile signal, suggested to be cytokinin, is activated/produced by the epidermis downstream of DMI3 and is translocated to the cortex were it is sensed by the cytokinin receptor CRE1. Signaling via RRs results in the activation of the transcription factors ERN1, NSP2 and NIN, which activate the expression of ENOD genes to induce cortical cell divisions.

2004). Pre-infection threads and nodule primordia are formed from a distance by cell cycle induction. Pre-infection threads involve the redistribution of the cytoplasm to form cytoplasmic bridges through which the infection threads will pass. The cortical cells that develop pre-infection threads arrest cell cycling before the M phase (van Brussel *et al.*, 1992; Timmers *et al.*, 1999; van Spronsen *et al.*, 2001). Whether cells will develop pre-infection threads or will divide depends on their position within the root and on the legume species involved. In *M. truncatula*, cells of the outer cortex develop into pre-infection threads, while the inner cortical cells divide and make the primordium. Gradients of hormones and signals have been proposed to determine the response of each cell. The NF-dependent signal that activates cell division must be generated after Ca^{2+} spiking, because autoactivation of nodule development is induced when the autoinhibition domain of MtDMI3 is removed, revealing that a functional MtDMI3 is sufficient to induce primordium formation in the absence of rhizobia (Gleason *et al.*, 2006).

Many experiments have shown that cytokinin signaling is essential to induce nodule primordia (Crespi and Frugier, 2008; Frugier *et al.*, 2008). For an overview of the cytokinin signal transduction pathway we refer to Figure 3.2. The cortex-located LOTUS HISTIDINE KINASE1 (LjLHK1) and its orthologue CYTOKININ RESPONSE1 (MtCRE1) are involved in cytokinin perception and signaling through cytokinin response regulators (RRs) initiates nodule organogenesis (Gonzalez-Rizzo *et al.*, 2006; Lohar *et al.*, 2006; Murray *et al.*, 2007). Cytokinin signaling occurs downstream the NF signaling cascade and the transcription factors ERN1, NSP2 and NIN act downstream of cytokinin signaling to trigger cortical cell division (Tirichine *et al.*, 2007; Frugier *et al.*, 2008; Plet *et al.*, 2011).

Formation of nodule primordia is characterized by a rhizobia-induced, local accumulation of auxin at the site of nodule initiation in the inner cortex of *M. truncatula*. This local auxin accumulation is generated by an inhibition of the polar auxin transport (PAT) just below the infection site, but does not occur in *L. japonicus*, suggesting that determinate and indeterminate nodulation are differentially regulated (Mathesius *et al.*, 1998; Pacios-Bras *et al.*, 2003; van Noorden *et al.*, 2006). PAT is a highly regulated cell-to-cell transport and involves asymmetrically, cell membrane-localized auxin influx carrier proteins and auxin efflux carrier proteins (Huo *et al.*, 2006; Grunewald and Friml, 2010). Recently, *MtCRE1* has been shown to be essential to inhibit PAT in *M. truncatula* (Plet *et al.*, 2011).

Besides a local auxin accumulation, nodulation is also characterized by a rhizobia-

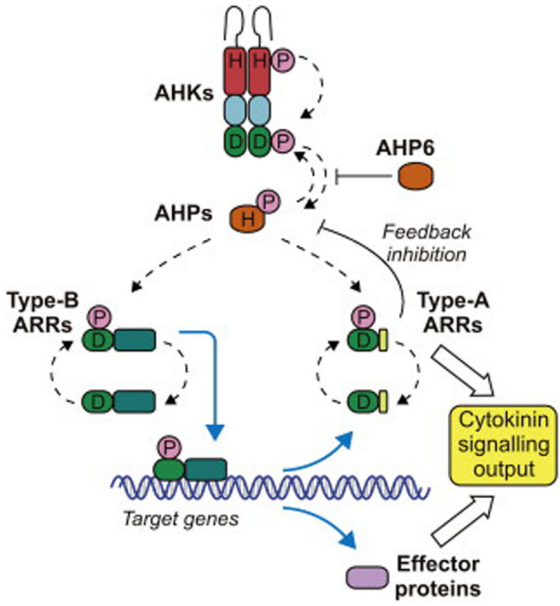

Figure 3.2: Overview of the cytokinin signal transduction pathway. Cytokinin signaling involves a phosphorelay mechanism, initiated by the autophosphorylation of transmembrane cytokinin receptors, designated ARABIDOPSIS HISTIDINE KINASES (AHKs), which subsequently transfer the phosphate group to ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEINS (AHPs). AHPs, on their turn, relay the signal to a group of ARABIDOPSIS RESPONSE REGULATORS (ARRs). Type A ARRs repress cytokinin signaling in a negative feedback loop, while type B ARRs are transcription factors regulating the expression of primary cytokinin response genes among which type A ARRs and effector proteins. Redrafted from Werner and Schmulling (2009).

induced long-distance inhibition of the PAT from the shoot to the root. Inhibition of this long-distance auxin transport seems to control the nodule number (van Noorden *et al.*, 2006). The role of auxin during nodulation has been suggested also by the elevated auxin levels in nodules, the differential expression of auxin response genes and by the induction of nodulin-expressing pseudonodules after the addition of PAT inhibitors (Hirsch *et al.*, 1989; Wu *et al.*, 1996; Ferguson and Mathesius, 2003; van Noorden *et al.*, 2006).

3.2 Different mechanisms control the nodulation process

From the start of the research on nodulation, it has been observed that nodulation is a strictly controlled process and that nodule formation and functioning in legumes are regulated by various mechanisms according to environmental conditions. This observation is not surprising because nodulation is not only an energy-consuming process but also requires the strict control of a bacterial invader (Tjepkema and Winship, 1980). As a result, when the environmental conditions are favorable, roots that are susceptible for nodulation will allow some infection threads to proceed while many others will be arrested in the epidermis or cortex (Gage, 2004). Moreover, the number of nodule primordia is restricted. After formation of the first primordia, new ones will be inhibited. As a consequence, under ideal circumstances, nodules occur together on a specific zone of the root on many legumes, such as *M. truncatula* and *L. japonicus*. Furthermore, environmental conditions, such as nitrate or ammonium availability affect the nodulation process and endogenous signals, such as carbon availability, determine the efficiency of the nodulation and nitrogen fixation processes. Here, we will mainly focus on the mechanisms that influence bacterial infection and primordium development to control nodule number and nodule growth.

3.2.1 Ethylene, abscissic acid and jasmonic acid as local, negative regulators of nodulation

The phytohormones ethylene, abscissic acid (ABA) and jasmonic acid (JA) negatively regulate nodulation by modifying epidermal responses because of their impact on the Ca^{2+} spiking event (Cho and Harper, 1993; Penmetsa and Cook, 1997; Suzuki *et al.*, 2004; Nakagawa and Kawaguchi, 2006; Sun *et al.*, 2006). NF-induced ethylene production inhibits nodulation, suggesting that a local negative feedback loop exists for controlling the nodule number (van Spronsen *et al.*, 1995). Indeed, ethylene suppresses the NF-signaling pathway either at or during NF-induced Ca^{2+} spiking, inhibiting root hair deformation, shortening the Ca^{2+} spike period, blocking bacterial infection and suppressing nodulin genes expression (Oldroyd *et al.*, 2001). Inhibitors of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (such as aminoethoxyvinylglycine [AVG]) and antagonists of ethylene actions (such as Ag^{+} ions) have been shown to enhance nodule formation (Nukui *et al.*, 2000; Oldroyd *et al.*, 2001), whereas

exogenous ethylene or one of its immediate precursors decreases nodule number (Nukui *et al.*, 2000). These findings have been confirmed by the identification of *sickle* (*skl*), an ethylene-insensitive hypernodulation mutant of *M. truncatula* that is affected in the orthologous gene of *Arabidopsis thaliana* ETHYLENE-INSENSITIVE2 (*EIN2*) (Penmetsa and Cook, 1997; Penmetsa *et al.*, 2003, 2008). The hypernodulation phenotype of *skl* mutants results in typical sickle-shaped roots and an uncontrolled growth of infection threads (Penmetsa and Cook, 1997; Penmetsa *et al.*, 2003, 2008). Transgenic *L. japonicus* plants expressing a mutated, probably dominant-negative ethylene receptor gene from either melon (*Cucumis melo*) or *Arabidopsis* produce a high number of infection threads and nodule primordia, similar to the *skl* phenotype (Nuku i *et al.*, 2004; Lohar *et al.*, 2009). In pea, exogenous addition of the cytokinin benzylaminopurine (BAP) stimulates the production of ethylene and results in a number of nodulation-related changes (Lorteau *et al.*, 2001). However, it is not clear whether there is a direct correlation between the actions of cytokinin and ethylene and the observed nodulation phenotypes (Lorteau *et al.*, 2001).

Ethylene also imposes the positioning of nodules along the root vasculature (Penmetsa and Cook, 1997; Heidstra *et al.*, 1997). In contrast to nodules on wild-type plants that preferentially arise opposite protoxylem poles, nodules on *skl* mutants and transgenic plants with hindered ethylene signaling are randomly distributed over the root circumference (Penmetsa and Cook, 1997; Penmetsa *et al.*, 2003, 2008). Transcripts encoding an ACC oxidase, involved in the last step of the ethylene biosynthesis, occur specifically in cells opposite protophloem poles. Based on these results and the negative influence of ethylene on cell division, an ethylene landscape in the root has been proposed, with the highest concentration in the inner cortical cells opposite protophloem poles and the lowest opposite the protoxylem poles, that restricts cell division to inner cortical cells opposite the protoxylem poles (Heidstra *et al.*, 1997).

Furthermore, ABA that negatively influences nodulation is believed to be involved in nodulation at two different levels. On the one hand, at the nodule initiation level, ABA would interfere with NF signaling and affect the nature of the NF-induced Ca^{2+} spiking, reducing the nodule number; on the other hand, at the nodule development level, ABA would suppress cytokinin-dependent organogenesis (Ferguson and Mathesius, 2003; Suzuki *et al.*, 2004; Ding *et al.*, 2008). This negative regulation of nodulation by ABA has been identified in *M. truncatula* and *L. japonicus* (Suzuki *et al.*, 2004; Liang *et al.*, 2007; Ding *et al.*, 2008; Biswas *et al.*, 2009).

Although the exogenous application of JA or methyl-JA (MeJA) affects *M. truncat-*

ula nodulation via Ca^{2+} spiking (Miwa *et al.*, 2006; Sun *et al.*, 2006), the involvement of JA during nodule development has recently been questionned because the differential expression of the JA-biosynthetic gene *ALLENE OXIDE CYCLASE* (*AOC*) did not vary between nodulated and non-inoculated roots (Zdyb *et al.*, 2011). Moreover, altered expression levels of *AOC*, via either overexpression or knock-down in transgenic roots, did not result in any nodulation phenotype (Zdyb *et al.*, 2011). These results indicate that the local production of JA is not involved in nodule development, but the translocation or release of JA should still be investigated. Moreover, a shoot-to-root movement of JA is not excluded.

3.2.2 Autoregulation of nodulation

In addition to local control mechanisms, split-root experiments have indicated that long-distance mechanisms exists that control nodule numbers (Kosslak and Bohlool, 1984). After inoculation of one half of the root system, nodulation was suppressed in the other half of the root system, even a few days after inoculation of the first half (Kosslak and Bohlool, 1984; Suzuki *et al.*, 2008). Such a long-distance feedback mechanism, whereby early nodulation events hinder further nodule development over the complete root system, has been designated autoregulation of nodulation (AON) (Nutman, 1952; Pierce and Bauer, 1983; Kosslak and Bohlool, 1984; Carroll *et al.*, 1985a,b; Delves *et al.*, 1986). Thanks to mutant analyses, general insights into AON have been obtained, but a molecular view on the mechanism is still lacking. Various split-root experiments have indicated that the signal for the systemic suppression is generated after root hair curling, but before nitrogen fixation and initiation of visible cortical and pericycle cell divisions (Kosslak and Bohlool, 1984; Mathews *et al.*, 1989a; Caetano-Anolles *et al.*, 1991; Sagan and Gresshoff, 1996; Suzuki *et al.*, 2008; Li *et al.*, 2009). When nodules are excised, a new round of nodulation is observed, suggesting that the AON signal is generated in nodules (Caetano-Anolles *et al.*, 1991). Furthermore, the capacity of primary nodules to inhibit further nodulation has been shown experimentally to be the highest in nitrogen-fixing mature nodules (Singleton and Kessel, 1987; Streeter and Wong, 1988; Caetano-Anolles *et al.*, 1991; Suzuki *et al.*, 2008; Li *et al.*, 2009). However, NFs alone can also activate AON, albeit at low levels (Li *et al.*, 2009). By means of plant mutants impaired at different stages of bacterial infection or cortical cell division, AON has been correlated with a signal associated with nodule primordium development rather than infection thread formation (Li *et al.*, 2009). Indeed,

AON is still active in the spontaneous nodulators, such as the autoactive *dmi3* and *lhk1* mutants, indicating that AON happens in the cortex downstream of the cytokinin signaling (Tirichine *et al.*, 2007; Okamoto *et al.*, 2009; Miyazawa *et al.*, 2010). Together, these results imply that AON signaling is activated in the cortex after NF-induced cytokinin signaling and that the strength of the signal depends on the number, the activity and the developmental age of the nodules (Pierce and Bauer, 1983; Caetano-Anolles *et al.*, 1991; Olsson *et al.*, 1989).

AON has been demonstrated in a variety of determinate and indeterminate nodules, including soybean (*Glycine max*) (Delves *et al.*, 1986; Olsson *et al.*, 1989), subterranean clover (*Trifolium subterraneum*) (Sargent *et al.*, 1987), alfalfa (Caetano-Anolles and Bauer, 1988; Caetano-Anolles *et al.*, 1991), *M. truncatula* (Schnabel *et al.*, 2005), white bean (*Phaseolus vulgaris*) (Park and Buttery, 1988), pea (Li *et al.*, 2009) and *L. japonicus* (Suzuki *et al.*, 2008). The stage at which nodulation is inhibited seemly differs for determinate and indeterminate nodulation. In indeterminate nodulation, AON prevents the formation of new nodule primordia, whereas in determinate nodulation suppression takes place at the level of nodule primordium development (Caetano-Anolles *et al.*, 1991).

3.2.3 Role of the shoot in AON

Hypernodulating (or supernodulating) mutants in soybean (NODULE AUTOREGULATION RECEPTOR KINASE [*GmNARK*], also known as NITRATE-TOLERANT SYMBIOSIS 1 [*NTS1*]), *L. japonicus* (HYPERNODULATION ABERRANT ROOT FORMATION 1 [*LjHAR1*]), *M. truncatula* (SUPER NUMERIC NODULES [*MtSUNN*]) and pea (*PsSYM29*) have led to the identification of orthologous LRR-RLKs that play a crucial role during AON (Table 3.1) (Duc and Messager, 1989; Sagan and Gresshoff, 1996; Krusell *et al.*, 2002; Nishimura *et al.*, 2002a; Searle *et al.*, 2003; Oka-Kira *et al.*, 2005; Schnabel *et al.*, 2005). These receptors consist of N-terminal LRR repeats, a single transmembrane domain and a C-terminal serine/threonine kinase domain and are leguminous homologs of *Arabidopsis CLAVATA1* (*CLV1*), which is part of a receptor complex that controls shoot meristem homeostasis (Clark *et al.*, 1997; Krusell *et al.*, 2002; Nishimura *et al.*, 2002a; Dievart *et al.*, 2003; Searle *et al.*, 2003; Schnabel *et al.*, 2005). Therefore, hereafter, we will refer to this group of legume proteins as CLV1-like RLKs. Mutants in CLV1-like RLKs have a normal sensitivity to ethylene (Krusell *et al.*, 2002; Nishimura *et al.*, 2002a; Searle *et al.*, 2003; Schnabel *et al.*,

2005). In contrast to *skl* mutants, which have densely packed nodules on a restricted area of the susceptible root zone, AON mutants carry numerous small nodules all over the roots (Penmetsa and Cook, 1997; Penmetsa *et al.*, 2003, 2008; Krusell *et al.*, 2002; Nishimura *et al.*, 2002a; Searle *et al.*, 2003; Schnabel *et al.*, 2005). Despite their high number of nodules, these hypernodulation mutants do not fix more nitrogen than wild type plants because the nitrogen fixation capacity per nodule is reduced (Carroll *et al.*, 1985a,b; Schuller *et al.*, 1988). Transcripts of genes encoding CLV1-like RLKs occur throughout the plant, more specifically in the vasculature (Nontachaiyapoom *et al.*, 2007; Krusell *et al.*, 2002; Nishimura *et al.*, 2002a; Schnabel *et al.*, 2005; Yamamoto *et al.*, 2000; Searle *et al.*, 2003). Reciprocal grafting experiments between mutant and wild-type plants have shown that the hypernodulation phenotype is determined by the shoot genotype (Delves *et al.*, 1986; Jiang and Gresshoff, 2002; Krusell *et al.*, 2002; Men *et al.*, 2002; Penmetsa *et al.*, 2003; Francisco and Harper, 1995; Nishimura *et al.*, 2002a).

Hence, a receptor complex similar to the one that perceives CLV3 in the *Arabidopsis* shoot apical meristem (SAM) to regulate stem cell homeostasis is active in legume shoots and controls the nodule number over long distances. Based on the results of these plant mutants, the current model for AON proposes that an unknown signal Q, produced in the roots in response to rhizobia, is translocated to the shoot where it is sensed by receptor complexes, including the CLV1-like RLKs, resulting in the formation/activation of an unknown 'shoot-derived inhibitor' (SDI), which is transmitted back to the roots to prevent excessive nodule formation (Figure 3.3). However, the nature of the Q and SDI signals are still unknown.

Mutants of CLV1-like RLKs are characterized by a short root phenotype and frequently by an elevated number of lateral roots in the presence and absence of rhizobia (Buzas and Gresshoff, 2007; Penmetsa *et al.*, 2003; Schnabel *et al.*, 2005; Wopereis *et al.*, 2000; Day *et al.*, 1986; Szczyglowski *et al.*, 1998). Reciprocal grafts between the *sunn* mutants and wild-type plants have revealed that the root phenotypes are also controlled by the shoot (Schnabel *et al.*, 2005). Additional phenotypes observed on the *clv1*-like RLK mutants include an increase in arbuscule formation and a lack of mycorrhizal autoregulation (Meixner *et al.*, 2005; Solaiman *et al.*, 2000), as well as hyperinfection by a parasitic root-knot nematode (Lohar and Bird, 2003).

The similarity to the *CLV1* gene of *Arabidopsis* is intriguing because no common phenotypes are shared between the *clv1* mutants and mutants of CLV1-like RLK-encoding genes of legumes. Nevertheless, other AON mutants do exhibit *clv1*-like

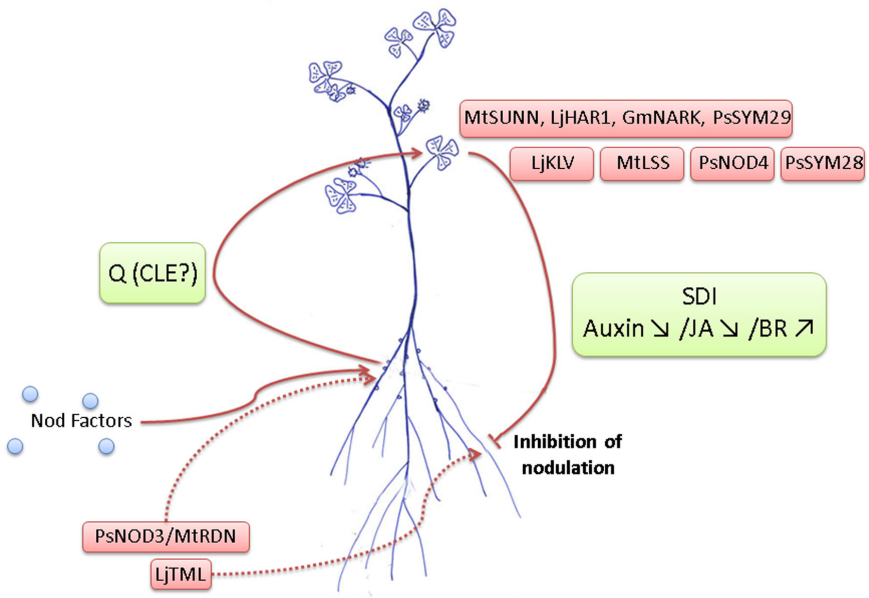

Figure 3.3: Model of autoregulation of nodulation (AON). Nod Factor-induced nodule development causes the production of an unknown signal Q, in the roots of leguminous plants which is translocated to the shoot where it induces the formation/activation of an unknown shoot controlled inhibition mechanism (SDI), which affects the roots to prevent excessive nodule formation. Perception of the Q signal in the shoot would be mediated by the LRR-RLKs MtSUNN, LjHAR1, GmNARK and PsSYM29. Also LjKLV, PsSYM28, PsNOD4 and MtLSS were shown to be essential in the shoot for proper AON. PsNOD3 and MtRDN are suggested to be involved in the roots in the earliest steps of AON, while LjTML is hypothesized to be involved in the root-perception of the SDI signal. The SDI mechanism remains elusive, but an upregulation of brassinosteroids (BR) or a downregulation of auxin or jasmonic acid (JA) might be involved.

phenotypes, of which two, *LjKlavier* (*klv*) and *Pssym28*, are affected in genes that encode proteins that are homologous to members of the same signaling complex as CLV1. The *klv* mutant, characterized by a shoot-determined hypernodulation phenotype, is altered in a gene encoding an LRR-RLK structurally unrelated to CLV1-like RLKs,

but closely homologous to RECEPTOR-LIKE PROTEIN KINASE2/TOADSTOOL2 (RPK2/TOAD2). Besides CLV1, the CLV2/CORYNE (CRN) and RPK2/TOAD2 receptor complexes have recently been found to also transmit the signal of the stem-cell-specific peptide hormone CLV3 in the *Arabidopsis* SAM (Oka-Kira *et al.*, 2005; Miyazawa *et al.*, 2010; Kinoshita *et al.*, 2010; Guo and Clark, 2010; Bleckmann *et al.*, 2010). The non-nodulation phenotypes exhibited by *klv* include fasciated stems and numerous extra organs (Oka-Kira *et al.*, 2005; Miyazawa *et al.*, 2010). Double mutant analysis has indicated that *KLV* and *HAR1* function in the same genetic pathway and biochemical analyses of their transient expression in *Nicotiana benthamiana* have revealed that KLV can interact with itself as well as with HAR1, suggesting that the proteins might form a receptor complex in *L. japonicus* (Miyazawa *et al.*, 2010). In accordance, the *Pssym28* mutant that, besides shoot-derived AON defects, also displays *clv1*-like phenotypes, has recently been found to be affected in the *Arabidopsis CLV2* homologue of pea that encodes a membrane-localized protein (Krusell *et al.*, 2011). A similar phenotype has been obtained in the *L. japonicus CLV2* mutants. *LjCLV2* and *HAR1* have overlapping expression patterns, but a direct protein-protein interaction could not be demonstrated (Krusell *et al.*, 2011). Hence, the same receptor complex might control AON and SAM homeostasis but different plants might use different contributions of each receptor to induce different effects. The *nodulation4* (*nod4*) mutant also exhibits shoot-dependent hypernodulation as well as non-symbiotic phenotypes (Table 3.1) (Duc and Messager, 1989; Sagan and Gresshoff, 1996; Sidorova and Shumnyi, 2003; Sidorova *et al.*, 2005), but the affected gene is currently unidentified. In addition, in *M. truncatula*, a supernodulator mutant has been characterized, *like sunn supernodulator* (*lss*), with a phenotype similar to that of *sunn* mutants. Although its origin is still not known, the mutation might involve an uncharacterized epigenetic modification at the *SUNN* locus (Schnabel *et al.*, 2010).

3.2.4 Role of the root in AON

In addition to the shoot-regulated hypernodulation mutants, other mutants have been identified, such as *too much love* (*tml*) from *L. japonicus*, *nodulation3* (*nod3*) from pea and *root determined nodulation* (*rdn*) from *M. truncatula* (Table3.1) that systemically inhibit nodulation and of which the hypernodulation phenotype is controlled by the root (Postma *et al.*, 1988; Magori and Kawaguchi, 2009). The molecular characteristics of the *TML, RDN* and *NOD3* genes remain to be elucidated, but approach-grafting

Table 3.1: Overview of hypernodulating mutants.

Mutant	Plant	Mutated gene	Shoot/root determined	Nitrate sensitive	Ethylene sensitive
sunn	*Mt*	LRR-RLK *SUNN*	shoot	no	yes
nts1/nark	*Gm*	LRR-RLK *NARK*	shoot	no	yes
har1	*Lj*	LRR-RLK *HAR1*	shoot	no	yes
sym29	*Ps*	LRR-RLK *SYM29*	shoot	no	yes
klavier	*Lj*	LRR-RLK *KLV*	shoot	partially	yes
sym28	*PS*	LRR-RLP *CLV2*	shoot	no	/
nod4	*Ps*	/	shoot	/	/
lss	*Mt*	/	shoot	no	yes
nod3	*Ps*	/	root	/	/
tml	*Lj*	/	root	/	yes
plenty	*Lj*	/	root	partially	yes
rdh1	*Lj*	/	root	yes	yes
rdn	*Mt*	/	root	/	/
skl	*Mt*	*EIN2*	root	yes	no
astray	*Lj*	bZIP TF	root	yes	yes

experiments have indicated that *Psnod3* functions at the onset of AON probably by producing or transporting Q in or to the roots (Li *et al.*, 2009). The same function has been proposed for *RDN* based on observation of *M. truncatula rdn* mutants (Frugoli *et al.*, unpublished data). Furthermore, *nod3* mutants are characterized by compact roots with increased secondary lateral roots (Postma *et al.*, 1988). In contrast, double-mutant analysis and inverted-Y grafting experiments have shown that *TML* functions locally and downstream of *HAR1*, suggesting that TML would rather be a receptor or a mediator of the shoot-derived SDI signal (Magori and Kawaguchi, 2009; Magori *et al.*, 2009). *Tml* mutants are characterized by an increased number of infection threads and nodules, which are small and cover a wide part of the roots (Magori *et al.*, 2009) and are not orthologous to *skl*, because they are not defective in the ethylene responses (Magori *et al.*, 2009). Finally, slightly shorter primary roots with fewer lateral roots are observed on *tml* mutants (Magori *et al.*, 2009). The molecular characterization and functional analysis of this *TML* gene will help to elucidate its exact role(s) and to get a deeper insight into the process of AON.

In contrast to the hypernodulators mentionned above, the root-determined hypernodulator mutant *plenty* has a moderate phenotype, because its nodule number is higher and its nodulation zone wider than those of wild-type plants, but not as enhanced as in *har1*, *klv* and *tml* of *L. japonicus* (Yoshida *et al.*, 2010). The nodules of the *plenty*

mutants are normal in size, in contrast to the reduced size observed on most AON hypernodulating mutants (Yoshida *et al.*, 2010). *Plenty* mutants are sensitive to ethylene, display some tolerance against external nitrates (Yoshida *et al.*, 2010) and the *PLENTY* gene might occur in a different pathway than the *HAR1*-mediated systemic regulation (Yoshida *et al.*, 2010). These observations, together with its unique phenotype, suggests that the *plenty* mutant forms a new category of hypernodulation mutants, possibly functioning independently of the AON pathway. The performance of split root experiments might be relevant to determine whether *PLENTY* involves long-distance signaling. *Plenty* mutants exhibit non-symbiotic phenotypes, such as a short root and shoot phenotype and an increased number of short lateral roots (Yoshida *et al.*, 2010). Whether the ROOT-DETERMINED HYPERNODULATION1 (RDH1) of *L. japonicus* is involved in long-distance signaling is also unclear (Ishikawa *et al.*, 2008; Yokota *et al.*, 2009). Characterization of LjRDH1 might be of great agricultural importance because it is the only hypernodulating mutant with normal growth, increased nitrogen-fixing activity and high seed and pod yields under optimal cultivation conditions (Ishikawa *et al.*, 2008; Yokota *et al.*, 2009).

3.2.5 Are CLE peptides the Q signals?

Until now, the nature of the root-derived AON signal molecule Q is still elusive. However, SUNN and its orthologues are phylogenetically related to known and putative CLV3/ESR-related (CLE) peptide receptors, such as CLV1, BAM1 and BAM2 and, hence, might perceive CLE peptides (Shiu and Bleecker, 2001; Ogawa *et al.*, 2008; Okamoto *et al.*, 2009; Guo and Clark, 2010), which are small secreted peptides composed of 12 to 13 conserved amino acids that are cleaved from the C-terminal end of CLE preproproteins (Wang and Fiers, 2010). In *Arabidopsis*, the CLE peptide family consists of 31 members and is involved in various developmental processes to balance cell division and differentiation (Sawa *et al.*, 2006; Ito *et al.*, 2006). The best characterized CLE peptide until now is CLV3 that is involved in SAM regulation through interaction with receptor complexes to which CLV1 belong. Signal transduction is carried out by a kinase-associated protein phosphatase (KAPP) that is phosphorylated by CLV1 and, in turn, dephosphorylates CLV1 (Williams *et al.*, 1997; Stone *et al.*, 1998; Trotochaud *et al.*, 1999). Two *KAPP* genes have been identified in the soybean genome and transphosphorylation activity between the CLV1-like RLK NARK and the two GmKAPP proteins has been demonstrated *in vitro*, hinting at a transduction path-

way in nodule regulation similar to that in SAM homeostasis (Miyahara *et al.*, 2008).

Several nodule-specific CLE peptide genes have been characterized in the genome of *L. japonicus* (*LjCLE-RS1* and *LjCLE-RS2*), *M. truncatula* (*MtCLE12* and *MtCLE13*) and soybean (*GmCLE14-GmCLE39* or *GmRIC1* and *GmCLE35-GmCLE37* or *GmRIC2*) (Okamoto *et al.*, 2009; Mortier *et al.*, 2010, 2011; Reid *et al.*, 2011). Functional analysis of the nodule-specific *GmCLE, LjCLE* and *MtCLE* genes revealed that these CLE peptides are good candidates for triggering AON (Okamoto *et al.*, 2009; Mortier *et al.*, 2010; Reid *et al.*, 2011). Indeed, ectopic expression of the structurally related genes *GmRIC1, GmRIC2, LjCLE-RS1, LjCLE-RS2, MtCLE12* and *MtCLE13* strongly reduced or abolished nodulation locally and systemically in a manner dependent on *NARK, HAR1* and *SUNN* in soybean, *L. japonicus* and *M. truncatula*, respectively (Chapter 5) (Okamoto *et al.*, 2009; Mortier *et al.*, 2010; Reid *et al.*, 2011; Saur *et al.*, 2011), whereas overexpression of *CLE* genes with a structurally unrelated CLE domain did not cause this effect (Okamoto *et al.*, 2009; Mortier *et al.*, 2010; Reid *et al.*, 2011). Interestingly, the expression of *MtCLE12* and *MtCLE13* (Mortier *et al.*, 2010) coincides with the activation and progression of AON that is initiated when the first nodule primordia are formed (Caetano-Anolles *et al.*, 1991; Li *et al.*, 2009), but, thus far, there is no proof that the peptides derived from these genes act as long-distance signals travelling from the developing nodules to the shoot, where SUNN and its orthologs are active. If this were the case, a long-distance root-to-shoot translocation of the peptides would have to occur. Until now, CLE peptides have been shown to act as short-distance signals (Fukuda *et al.*, 2007; Hirakawa *et al.*, 2008; Whitford *et al.*, 2008; Miwa *et al.*, 2009; Stahl and Simon, 2009). It is also possible that local CLE perception in the root triggers secondary signals that proceed to the shoot, where they provoke AON. The creation of fusion proteins might reveal whether the nodule-specific CLE peptides act as long or as short distance signaling molecules during AON. Finally, in soybean, nodule CLE peptides might be involved in the activation of AON, but not in its maintenance because potential AON-related *GmCLE* genes are only expressed in young nodules and not in mature nodules (Mortier *et al.*, 2011).

3.2.6 Systemic suppression of nodulation, a matter of hormonal regulations?

Although what happens downstream of the shoot-located AON receptor complex is still elusive, reciprocal grafting experiments with *L. japonicus har1* roots and *M. trun-*

catula wild-type shoots indicated that the AON signal is conserved among legume species (Lohar and VandenBosch, 2005). To elucidate potential downstream signals of NARK, a transcript profiling had been carried out with leaf material from either inoculated or uninoculated wild-type and *nark* supernodulating mutants in soybean plants (Kinkema and Gresshoff, 2008). The expression of JA-biosynthesis and response genes was downregulated in a systemic, NARK-reliant and rhizobia-dependent manner (Kinkema and Gresshoff, 2008). These results suggest that the shoot-specific downregulation of JA response genes by rhizobial inoculation might be one of the players inhibiting nodule formation during AON (Kinkema and Gresshoff, 2008; Hause and Schaarschmidt, 2009), as supported by the high level of JA measured in AON mutants (Seo *et al.*, 2007; Kinkema and Gresshoff, 2008), although it is still unclear whether these genes are critical for the AON shoot-to-root signaling. Shoot-specific suppression of JA biosynthesis as a mean to control nodule number has been insinuated as well by foliar application of a JA-biosynthesis inhibitor, propyl gallate to *M. truncatula*, *L. japonicus* and soybean plants that severely reduced the nodule number without affecting root growth (Sun *et al.*, 2006; Kinkema and Gresshoff, 2008). This negative effect on nodulation has been observed also in *har1* and *nark* mutant backgrounds, hinting again at an action downstream from the shoot-located receptor complex (Seo *et al.*, 2007; Kinkema and Gresshoff, 2008). In contrast, nodulation in *L. japonicus* has been reported to be strongly suppressed by 10^{-4} to 10^{-3} M shoot-applied MeJA (Nakagawa and Kawaguchi, 2006), but, because this experiment has never been repeated in other plant species, further investigation is needed.

Whether differential JA levels play an active role in shoot-to-root communication in AON is not known. Possibly, other hormones and signals act synergistically to control the nodule number. Indeed, several hormones, such as auxin (indole 3-acetic acid) and brassinosteroids (BRs) have also been associated with long-distance signaling pathways controlling nodule number in various legumes (Ferguson *et al.*, 2005; Nakagawa and Kawaguchi, 2006; Terakado *et al.*, 2006; van Noorden *et al.*, 2006). Foliar application of a BR member, brassinolide (BL) to the soybean hypernodulation mutant *En6500* (that is allelic to *nts1*) reduced nodule numbers (Terakado *et al.*, 2006) and increased the leaf concentration of spermidine, a polyamine, that itself also decreases nodule numbers upon foliar application (Terakado *et al.*, 2006). Hence, BL might be one of the steps in the autoregulation signaling, possibly by inducing the spermidine biosynthesis.

Nevertheless, auxin signaling and PAT might be the central regulators of the long-

distance control of nodulation and all signals mentioned above might impinge on this central hormone. Auxin cargo from the shoots to the roots has been shown to be reduced after inoculation in *M. truncatula* and this reduced auxin flow was absent in the *sunn-1* allele. In addition, the shoot-to-root auxin flux was stronger in *sunn-1* mutants than that of the wild type (van Noorden *et al.*, 2006). Hence, the AON-related shoot signaling complex might downregulate auxin fluxes upon inoculation. Interestingly, AON is seemingly not the only mechanism that controls the shoot-to-root transport of auxin. In the hypernodulating ethylene signaling mutant, *skl*, the auxin transported from shoot to root after inoculation is not inhibited anymore (Prayitno *et al.*, 2006). Thus, to obtain nodules, a certain shoot-to-root auxin flux must be sustained and several control mechanisms might force this central process to allow internal as well as environmental clues to act on nodulation. As a consequence, AON might involve changes in hormone homeostasis rather than in the production of a specific SDI signal. In *Arabidopsis*, overexpression of *CLE6*, related to the AON CLE peptides, can diminish the auxin signaling in the roots (Whitford *et al.*, 2008). Preliminary experiments in the lab hint at the same effect for *MtCLE12* and *MtCLE13* (S. Goormachtig, personal communication), although an opposite observation was recently published (Saur *et al.*, 2011). It is tempting to speculate that in the shoot, SUNN in combination with an unknown CLE peptide, reduces auxin fluxes and that the nodule-specific CLE peptides do the same in combination with root-localized receptors. The reason for the inability of CLE peptides to restrict the number of nodules in AON mutants might be that the action of the nodule-related CLE peptides might not be strong enough to overcome the high levels of auxin present in AON mutants.

Still, shoot-derived nodulation inhibitors might act together with reduced hormone flows. In soybean leaf extracts, a *GmNARK*-dependent SDI factor has been identified as a small (< 1 kDa), heat stable, NF-dependent component unlikely to be an RNA or a protein (Lin *et al.*, 2010). Whatever the SDI signal(s) might be, it(they) would be transported via phloem tissues, because it(they) are generally viewed as a conduit for long-distance communication (Lough and Lucas, 2006; Ruiz-Medrano *et al.*, 2001).

3.2.7 Perception of the return signal in the root

Little is known about the perception of the return signal in the roots. *TML* might play a central role, but awaits further characterization (Magori *et al.*, 2009). Double-mutant analysis and inverted-Y grafting experiments have shown that *TML* functions

locally and downstream of *HAR1*, suggesting a role as a receptor or a mediator of the shoot-derived SDI signal. Moreover, because rhizobial induction of *LjCLE-RS1* and *LjCLE-RS2*, the candidates of the AON-associated root-derived signal molecules, is still observed in the *tml* mutant, *TML* might not be involved in the generation of the root-derived signal (Magori *et al.*, 2009).

Recently, nodules on *sunn* mutant roots have been shown not only to be smaller, but also to have a higher growth rate capability than wild type nodules upon changes in environmental conditions (Jeudy *et al.*, 2010). This interesting observation needs further investigation and indicates that the AON pathway might control nitrogen fixation inside the nodules by regulating the meristem activity and, thus, the balance between growth and differentiation.

3.2.8 The negative effect of nitrate on nodulation, is there a link with AON and CLE peptide signaling?

High concentrations of nitrogen compounds inhibit nodule formation at different stages of the process, regardless of the plant age, nodule size or former inoculation events (Streeter, 1981, 1985; Carroll and Gresshoff, 1983; Carroll *et al.*, 1985a,b; Gibson and Harper, 1985). Via split-root experiments with *M. truncatula*, nitrate limitation has been demonstrated to result in both local and systemic regulation of nodulation that partially depended on *SUNN* (Jeudy *et al.*, 2010). This observation is in agreement with the nitrate-tolerant phenotype of the AON mutants, *har1, nark, sym29, sunn, klv* and *tml* that are able to nodulate in the presence of high levels of nitrate (Carroll *et al.*, 1985a,b; Sagan and Gresshoff, 1996; Schnabel *et al.*, 2005; Wopereis *et al.*, 2000; Penmetsa *et al.*, 2003; Oka-Kira *et al.*, 2005; Magori and Kawaguchi, 2009). These results imply that nitrate tolerance and AON might be linked, possibly by operating through a same 'control point' of nodule development (Kinkema *et al.*, 2006). In *L. japonicus*, *LjCLE-RS2* has been shown to be upregulated by rhizobial inoculation as well as by nitrate addition, suggesting that in this plant nitrate might suppress nodulation by activating AON via the induction of specific *CLE* genes (Okamoto *et al.*, 2009). Also in soybean, a nitrate-induced CLE peptide gene (*GmNIC1*) has been identified (Reid *et al.*, 2011).

Although the expression profiles and functional analysis hint at the involvement of AON in nitrate-mediated control on nodulation, the data should be interpreted with caution. Old literature clearly mentions that in AON mutants nodulation is only partially tolerant to nitrate addition and that application of very high concentrations of

nitrate blocks the nodule formation (Eskew *et al.*, 1989). Hence, it is equally possible that the nitrate tolerance of nodulation observed in AON mutants is only a secondary consequence of the mutation. Just as observed in *skl*, nitrate might also negatively regulate shoot-to-root auxin transport and, hence, increased nitrate concentrations would be needed to reduce the auxin level of AON mutants so as to inhibit nodulation. Because, in *Arabidopsis*, addition of CLE peptides might reduce the auxin responsiveness in the root vasculature (Whitford *et al.*, 2008), the nitrate-induced CLE peptides might act similarly to control nodule formation. Whether the shoot-located AON complex is involved must however still be demonstrated. In *A. thaliana*, the systemic inhibitory effect of nitrate on lateral root development has been associated with ABA signaling (Signora *et al.*, 2001; Zhang *et al.*, 2007). Although several ABA mutants are known to be impaired in nodulation, there are no indications for the involvement of ABA in systemic regulation of nodulation (Ding *et al.*, 2008; Tominaga *et al.*, 2009).

3.3 What remains to be discovered?

The identification of a certain number of mutants defective in nodule number has revealed that local as well as systemic signals are involved in the control of nodule numbers. These signals include, amongst others, phytohormones and possibly CLE peptides. In addition, several experiments have indicated that ethylene plays a local role in positioning of nodules around the root circumference. However, information is still lacking on how long-distance signaling pathways, including AON, regulate nodule numbers. Do specific AON signals exist or is AON the consequence of a change in hormone homeostasis? What is the nature of the signals and how are they decoded? Do several signaling pathways work in parallel during AON and impinge on PAT or the transport of other molecules? Are CLE peptides involved during AON, and where are they perceived? To which degree are nitrate inhibition of nodulation and AON intertwined? Does a mild inhibition of nodulation, as observed on the mutant astray, have an impact on AON (Nishimura *et al.*, 2002b)? The isolation and characterization of additional hypernodulating mutants, as well as reverse genetics and biochemical approaches might help us gain more insights into these interesting aspects of nodulation.

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Authors contributions

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4

CLE peptides control *Medicago truncatula* nodulation locally and systemically

Virginie Mortier, Griet Den Herder, Ryan Whitford, Willem Van de Velde, Stéphane Rombauts, Katrien D'haeseleer, Marcelle Holsters and Sofie Goormachtig

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Abstract
The CLAVATA3/embryo-surrounding region (CLE) peptides control the fine balance between proliferation and differentiation in plant development. We study the role of CLE peptides during indeterminate nodule development and identified 25 MtCLE peptide genes in the *Medicago truncatula* genome, of which two genes, *MtCLE12* and *MtCLE13*, had nodulation-related expression patterns that were linked to proliferation and differentiation. *MtCLE13* expression was up-regulated early in nodule development. A high-to-low expression gradient radiated from the inner toward the outer cortical cell layers in a region defining the incipient nodule. At later stages, *MtCLE12* and *MtCLE13* were expressed in differentiating nodules and in the apical part of mature, elongated nodules. Functional analysis revealed a putative role for *MtCLE12* and *MtCLE13* in autoregulation of nodulation, a mechanism that controls the number of nodules and involves systemic signals mediated by a leucine-rich repeat receptor-like kinase, SUNN, which is active in the shoot. When *MtCLE12* and *MtCLE13* were ectopically expressed in transgenic roots, nodulation was abolished at the level of the nodulation factor signal transduction and this inhibition involved long distance signaling. In addition, composite plants with roots ectopically expressing *MtCLE12* or *MtCLE13* had elongated petioles. This systemic effect was not observed in transgenic roots ectopically expressing *MtCLE12* and *MtCLE13* in a *sunn-1* mutant background, although nodulation was still strongly reduced. These results suggest multiple roles for CLE signaling in nodulation.

4.1 Introduction

In the symbiotic interaction between legume plants and rhizobia, root nodules develop within which the bacteria fix atmospheric nitrogen. Nodule development requires the spatio-temporal orchestration of developmental programs for infection and organ formation (Jones *et al.*, 2007). In *Medicago truncatula*, the microsymbiont *Sinorhizobium meliloti* enters via curled root hairs and transcellular infection threads. While infection is taking place, inner cortical and pericycle cells divide and form the nodule primordium. The infection threads penetrate primordium cells and bacteria are released into the plant cytoplasm within membrane-enclosed symbiosomes. Inside the symbiosomes, the bacteria differentiate into bacteroids and start the nitrogen fixation process (Jones *et al.*, 2007). Meanwhile, an apical meristem develops and provides

new cells for bacterial internalization. The nodules are of the indeterminate type and have a cylindrical shape.

The perception of bacterial signaling molecules, the nodulation factors (NFs), by specific LysM-type receptor-like kinases (RLKs) in the epidermis of the host plant, elicits various responses to allow root hair invasion and cell division (Madsen *et al.*, 2003; Radutoiu *et al.*, 2003, 2007; Jones *et al.*, 2007; Oldroyd and Downie, 2008). While the inner cortical cells divide, outer cortical cells arrest in the G2 phase of the cell cycle, resulting in cytoplasmic bridges, the preinfection threads, through which the infection threads grow (Yang *et al.*, 1994; van Spronsen *et al.*, 2001).

Opposite pre-existing and NF-induced signal gradients have been proposed to rule the cortical cell responses (Smit *et al.*, 1995a; Heidstra *et al.*, 1997; van Spronsen *et al.*, 2001). Uridine and ethylene are diffusive signals originating from the vasculature that have been identified as positive and negative regulators, respectively. In white clover (*Trifolium repens*), the auxin flow within the root vasculature was transiently inhibited at the site of infection, leading to auxin accumulation in the cortical region where the nodule primordia form (Mathesius *et al.*, 1998). A reduction in auxin flow has been confirmed by radioactive auxin tracer experiments for *M. truncatula* and vetch (*Vicia faba*), but not *Lotus japonicus* (Boot *et al.*, 1999; Pacios-Bras *et al.*, 2003; van Noorden *et al.*, 2006; Wasson *et al.*, 2006). Cytokinins are essential for nodule development because *L. japonicus* knockout mutants for the cytokinin receptor gene, *LHK1* or *M. truncatula* transgenic plants with suppressed expression of the ortholog, *CRE1*, were defective in nodule primordia formation (Gonzalez-Rizzo *et al.*, 2006; Murray *et al.*, 2007). Additionally, a *L. japonicus* gain-of-function mutant for the LHK1 receptor provoked spontaneous nodules, indicating that cytokinin signaling is both necessary and sufficient for nodule formation (Tirichine *et al.*, 2007).

Several components that link NF signaling to the initiation of cortical cell division have been identified. In *M. truncatula*, NF perception by LysM-type RLKs at the epidermis, activates a signaling cascade that is mediated by the leucine-rich repeat (LRR)-RLK, Doesn't Make Infections2 (DMI2), and the nuclear potassium channel, DMI1. This signaling cascade triggers Ca^{2+} spiking in and around the nucleus. Decoding of this Ca^{2+} signature by a Ca^{2+} calmodulin-binding protein (DMI3) results in the activation of the transcription factors, Nodulation Signaling Pathway 1 (NSP1), NSP2, Ethylene-Responsive binding domain Factor Required for Nodulation1 (ERN1), and Nodule Inception (NIN) (Schauser *et al.*, 1999; Catoira *et al.*, 2000; Borisov *et al.*, 2003; Oldroyd and Long, 2003; Gleason *et al.*, 2006; Andriankaja *et al.*, 2007; Marsh

et al., 2007; Middleton *et al.*, 2007; Oldroyd and Downie, 2008). NSP2, ERN1, and NIN also play a role downstream of the cytokinin signaling to trigger cortical cell division (Tirichine *et al.*, 2007; Frugier *et al.*, 2008; Plet *et al.*, 2011).

Nodule formation and functioning are energy-consuming processes and legumes have evolved several strategies to control the number of nodules. One such strategy, autoregulation of nodulation (AON) (Kosslak and Bohlool, 1984), is activated when the first nodules develop and involves systemic signals and shoot-controlled factors (Magori and Kawaguchi, 2009). Insight into this mechanism has been obtained by the identification of supernodulation mutants of soybean (*Glycine max*) (*nts-1*), *M. truncatula* (*sunn*), *L. japonicus* (*har1*), and pea (*Pisum sativum*) (*sym29*), which are each deficient in an LRR-RLK that is required in shoots for AON (Krusell *et al.*, 2002; Nishimura *et al.*, 2002a; Searle *et al.*, 2003; Schnabel *et al.*, 2005). Auxin might be implicated in this process because *sunn-1* mutants display a higher level of long-distance shoot-to-root auxin transport than wild type plants. Importantly, in contrast to the wild type, this transport is not reduced upon nodulation (van Noorden *et al.*, 2006).

The LRR-RLKs responsible for AON belong to the evolutionary clade of group XI RLKs (Shiu and Bleecker, 2001) that includes the Arabidopsis (*Arabidopsis thaliana*) receptors CLAVATA1 (CLV1), PXY-like 1-2 (PXL1-2), BARELY ANY MERISTEM 1-3 (BAM1-3), and the putative tracheary element differentiation inhibitory factor (TDIF) receptor (TDR/PXY) (Clark *et al.*, 1997; DeYoung *et al.*, 2006; Fisher and Turner, 2007; Hirakawa *et al.*, 2008; Ogawa *et al.*, 2008). These LRR-RLKs bind or putatively recognize CLV3/embryo-surrounding region (CLE) peptides (Hirakawa *et al.*, 2008; Ogawa *et al.*, 2008) that are a group of small (12-13 amino acids) secreted peptides derived from the C-terminal region of preproproteins (Mitchum *et al.*, 2008; Oelkers *et al.*, 2008). The Arabidopsis genome contains 32 gene family members, of which the best studied is CLV3, the peptide ligand for a CLV1-containing cell surface receptor complex. Recognition of the CLV3 peptide is important for stem cell homeostasis within the shoot apical meristem (SAM) (Ogawa *et al.*, 2008), whereas TDIF, a phloem secreted CLE peptide, binds *in vitro* the PXY/TDR receptor expressed in the procambial cells and inhibits vascular differentiation (Hirakawa *et al.*, 2008).

CLE peptides with related sequences exhibit redundancy as proven by similarity in gain-of-function phenotypes (Strabala *et al.*, 2006; Jun *et al.*, 2008). At least two groups of CLE peptides can be distinguished. Group-I peptides, exemplified by CLV3, result in premature root and shoot meristem growth arrest when exogenously applied or ectopically expressed, indicating that they are promoters of cellular differentiation.

Members of group-II, exemplified by TDIF, prevent cellular differentiation, as evidenced by the suppression of procambium-to-xylem transdifferentiation in Zinnia (*Zinnia elegans*) cell cultures and control the rate and orientation of vascular cell division (Ito *et al.*, 2006; Etchells and Turner, 2010). Although these studies would suggest two groups with opposing functions, synergistic actions between these groups of peptides have been demonstrated (Whitford *et al.*, 2008). In Arabidopsis, the proliferation of vascular precursor cells induced by the group-II CLE41 peptides is enhanced by the addition of CLE6 peptides that otherwise have an inhibiting effect on root growth when applied at high concentrations and thus belong to group-I. Moreover, genetic studies with *clv3*, *clv1*, and *bam* mutants reveal complex spatio-temporally controlled interactions between putative ligand/receptor complexes (Deyoung and Clark, 2008).

Cellular dedifferentiation and differentiation processes act at sequential stages of the nodule development. At nodule initiation, pericycle and cortical cells dedifferentiate and divide (van Brussel *et al.*, 1992; Timmers *et al.*, 1999; van Spronsen *et al.*, 2001). When sufficient cells encompassing the nodule primordia have formed, division ceases and the cells differentiate and become infected with rhizobia. Meanwhile, for indeterminate nodules, an apical meristem is established that supplies a constant pool of cells for bacterial infection. Hence, CLE peptides might not only be involved in AON, but also regulate the (de)differentiation processes that control nodule development.

We analyzed the role of CLE peptides in *M. truncatula* nodulation. By specialized BLAST searches, nine peptide genes were identified in the Mt2.0 release in addition to the 16 previously discovered (Cock and McCormick, 2001; Oelkers *et al.*, 2008). Two genes, designated *MtCLE12* and *MtCLE13*, were differentially expressed during nodule development. The expression patterns hint at roles during (de)differentiation throughout nodule development. Moreover, when *MtCLE12* and *MtCLE13* were ectopically overexpressed, an inhibition of nodulation by long-distance signaling was observed. Furthermore, transgenic plants bearing roots expressing *35S:MtCLE12* or *35S:MtCLE13* had elongated petioles, a systemic effect not observed in *sunn-1* mutants, although nodulation was still strongly hampered.

4.2 Results

4.2.1 Search for up-regulated *MtCLE* genes during nodulation

Besides the 16 putative *M. truncatula CLE* genes described (Cock and McCormick, 2001; Oelkers *et al.*, 2008), we searched for additional *MtCLE* genes in the expressed sequence tag (EST) database[1] and in the *M. truncatula* genomic data (Mt2.0) with a PAM30 tBLASTn homology-based algorithm to identify peptide sequences corresponding to the conserved CLE motif. Twenty-five *MtCLE* candidates were identified and designated *MtCLE1* to *MtCLE25* (Table 4.1). The corresponding CLE preproproteins varied in length between 45 and 221 amino acids and had a high level of sequence divergence outside the CLE motif. Except for MtCLE3, all proteins contained an N-terminal signal peptide as predicted by HMM signalP and neural networks (Bendtsen *et al.*, 2004). Two MtCLE candidates contained multiple CLE domains, namely MtCLE14 and MtCLE22 that had seven and three tandemly arranged CLE domains, respectively.

To determine tissue- or organ-specific expression, quantitative reverse-transcriptase (qRT)-PCR was carried out for each *MtCLE* candidate on cDNAs derived from root elongation zones, nodulated roots (1 month post inoculation), roottips, stems, SAMs, cotyledons, first leaves, and mature leaves. cDNA of root elongation zones was used as the reference tissue. Transcripts were detected for 15 of the 25 identified *MtCLE* genes; of which 10 required 30 or more PCR cycles, indicating low transcript levels or cell-specific expression. *MtCLE2*, *MtCLE4*, *MtCLE5*, *MtCLE11*, *MtCLE15*, and *MtCLE24* were mainly expressed in root tissues, *MtCLE1*, *MtCLE16*, and *MtCLE23* predominantly in shoot tissues, and *MtCLE6*, *MtCLE12*, and *MtCLE17* in several plant tissues (Figure 4.1A). For *MtCLE3* and *MtCLE13*, expression was restricted to stems and cotyledons and to nodulated roots, respectively, while for *MtCLE20* transcripts were detected in nodulated roots, leaves, and SAMs. Four *MtCLE* genes (*MtCLE12*, *MtCLE13*, *MtCLE16*, and *MtCLE20*) were more abundantly transcribed in nodulated than in control roots (Figure 4.1A). Biological repeats however only consistently confirmed the differential expression of *MtCLE12* and *MtCLE13*.

To study the temporal expression during nodule development, the relative transcript levels of each *MtCLE* gene was analyzed at 4, 6, 8, and 10 days postinoculation (dpi). The elongation zone of uninoculated roots, the nodule initiation site, was used as the

[1] http://www.tigr.org/tdb/tgi

Table 4.1: *MtCLE* identification. Identified *MtCLE* genes with their corresponding names in the general nomenclature and number of the bacterial artificial clone or of the expressed sequence tag. General nomenclature according to Cock and McCormick (2001) and Oelkers *et al.* (2008), who numbered each CLE member independently of the species origin and prefixed the numbers with 'CLE'.

M. truncatula nomenclature	General nomenclature	BAC clone, EST number
MtCLE1	CLE74	AW586793, BQ139113
MtCLE2	CLE67	AC137080.13, AC135319.28, AC147499.5, BI311733
MtCLE3	CLE69	BQ157494
MtCLE4	CLE71	AW329414
MtCLE5	CLE70	BM812815, BE999212
MtCLE6	CLE64	BF650504, BE941833, CX518468
MtCLE7		AC157502.1
MtCLE8	CLE73	AC151458.10, CX530629, CX530352
MtCLE9		AC155894.1
MtCLE10		AC157779.7
MtCLE11	CLE66	BQ122845
MtCLE12	CLE65	AC144893.15, AL381238, AL381237
MtCLE13	CLE39	AC144893.15, AL380420, AL380419
MtCLE14		AC155894.1
MtCLE15		AC151522.2
MtCLE16	CLE35	AC146785.12
MtCLE17	CLE72	AC123570.7, AC147960.8, AC147961.3, AY379776.1
MtCLE18	CLE36	AC146570.4
MtCLE19	CLE37	AC150244.2
MtCLE20		AC158501.3
MtCLE21	CLE38	CT009655_15.4
MtCLE22	CLE68	AC151522_11.4
MtCLE23		CU024869_31.4
MtCLE24		CR955009_22.5
MtCLE25		CU013514_50.4

reference tissue. *MtCLE12* expression was low at 4 dpi (Figure 4.1C), but increased until 6 dpi, and remained high until 10 dpi. *MtCLE13* transcript increased until 10 dpi (Figure 4.1D). As we were interested in the function of CLE peptides during nodulation, *MtCLE12* and *MtCLE13* were selected for further analysis and compared to *MtCLE4* given its root-specific expression (Figure 4.1, A and B).

As shown in Figure 4.2, the CLE peptide sequence of MtCLE12 and MtCLE13 differ by 4 amino acids. We analyzed the homology of MtCLE12, MtCLE13, and MtCLE4 to *A. thaliana* CLE peptides. MtCLE12 and MtCLE13 were most similar to

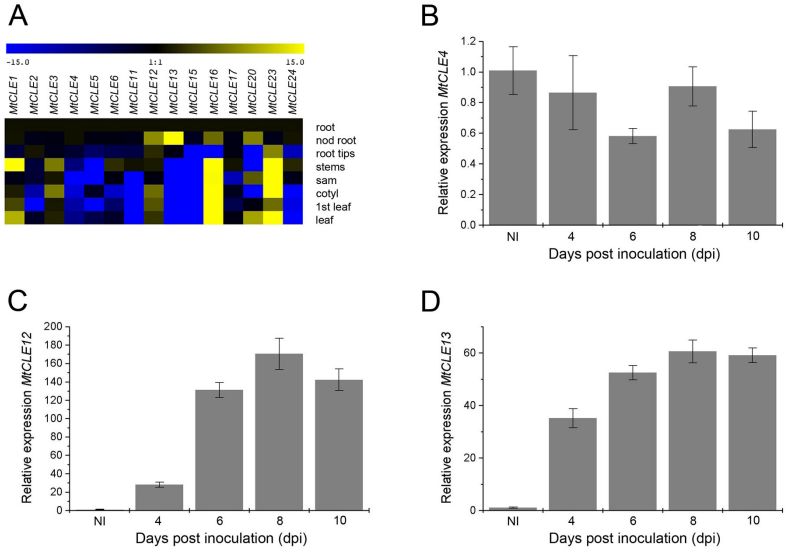

Figure 4.1: Expression analysis of *MtCLE* genes. A, Heat map of *MtCLE* expression in different tissues as measured by qRT-PCR. Samples are cDNA from root elongation zones (root), nodulated roots, 1 month post inoculation (nod root), roottips, stems, SAMs (sam), cotyledons (cotyl), first leaves (1st leaf), and mature leaves (leaf). B to D, Expression analysis of *MtCLE4*, *MtCLE12*, and *MtCLE13*, respectively, by qRT-PCR on cDNA samples of zone-I root tissues of uninoculated plants (NI) and at 4 dpi, 6 dpi, 8 dpi, and 10 dpi. Data and error bars represent means $\pm$ SD.

AtCLE1-AtCLE7 and MtCLE4 to AtCLE9/10. Upon *L. japonicus* nodulation, three *CLE* genes were described to be up-regulated (Okamoto *et al.*, 2009). Peptides derived from MtCLE12 and MtCLE13 differed by three and one amino acids (an A/G change at position 4) from the identical LjCLE-RS1 and LjCLE-RS2, respectively.

4.2.2 Effects of exogenous application of MtCLE4, MtCLE12, and MtCLE13 peptides on root growth and nodulation

To check the effect on root growth, we supplied 10 μM of chemically synthesized peptides, corresponding to the CLE domain of these three proteins (MtCLE4p, MtCLE12p, and MtCLE13p; see Materials and Methods page 74) exogenously to the primary roots

MtCLE12	**R L S P G G P N H I H N**
MtCLE13	**R L S P A G P D D P Q H N**
LjCLE1	R L S P G G P D P Q H N
LjCLE2	R L S P G G P D P Q H N
AtCLE7	R F S P G G P D P Q H H
AtCLE5/6	R V S P G G P D P Q H H
AtCLE2	R L S P G G P D P Q H H
AtCLE1/3/4	R L S P G G P D P R H H
AtCLE46	H K H P S G P N P T G N
AtCLE41/44	H E V P S G P N P I S N
AtCLE42	H G V P S G P N P I S N
LjCLE3	R V V P T G P N P L H N
MtCLE4	**R G V P S G A N P L H N**
AtCLE9/10	R L V P S G P N P L H N
AtCLE11	R V V P S G P N P L H H
AtCLE12	R R V P S G P N P L H H
AtCLE13	R L V P S G P N P L H H
AtCLE14	R L V P K G P N P L H N
AtCLE16	R L V H T G P N P L H N
AtCLE17	R V V H T G P N P L H N
AtCLE18	R Q I P T G P D P L H N
AtCLE19	R V I P T G P N P L H N
AtCLE20	R K V K T G S N P L H N
AtCLE21	R S I P T G P N P L H N
AtCLE22	R R V F T G P N P L H N
AtCLE25	R K V P N G P D P I H N
AtCLE26	R K V P R G P D P I H N
AtCLE27	R I V P S C P D P L H N
AtCLE40	R Q V P T G S D P L H H
AtCLE45	R R V R R G S D P I H N
AtCLV3	R T V P S G P D P L H H

Figure 4.2: Comparison of the CLE peptide sequence of *MtCLE4*, *MtCLE12*, and *MtCLE13* with the CLE peptide sequences from Arabidopsis *CLE* genes and of *LjCLE1*, *LjCLE2*, and *LjCLE3*. CLE peptide sequences homologous to *MtCLE12* and *MtCLE13* are framed in blue and those to *MtCLE4* in red.

of 2-day-old seedlings. Root length was measured after 8 days of growth. As controls, seedlings were grown on media either supplemented or not (no peptide) with a synthetic 16-amino-acid peptide corresponding to the C terminus of the Arabidopsis AGAMOUS protein. Primary root length for 16 plants per treatment was measured (Figure 4.3). After addition of MtCLE12p or MtCLE13p, an average root length of 91.0 $\pm$ 13.2 mm and 103.4 $\pm$ 5.8 mm was observed, respectively, which did not differ significantly from the average root length (101.9 $\pm$ 8.0 mm) with and without (97.0 $\pm$ 6.2 mm) addition of the control peptide. For the seedlings treated with MtCLE4p,

the average primary root length was 73.8 ± 8.0 mm, which is 24% shorter than the controls ($p < 0.001$, Linear mixed models). The difference in number of lateral roots per primary root was not statistically significant. Exogenous peptide application (10 μM up to 50 μM) had no effect on the nodule number.

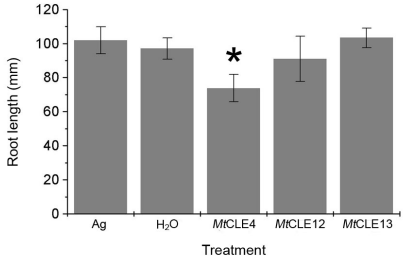

Figure 4.3: Effect of *in vitro* application of MtCLE4, MtCLE12, and MtCLE13 peptides on the root length of *M. truncatula*. The plants were grown for 6 days on plates containing 10 μM peptides (n = 39 for each treatment). As control, plants were not treated (H_2O) or treated with the AGAMOUS (Ag) peptide. Data and error bars represent means ± SD. Asterisk marks a statistically different group ($p < 0.001$, Linear mixed models).

4.2.3 *MtCLE12* and *MtCLE13* expression pattern in roots and developing nodules

Spatial expression patterns of *MtCLE12* and *MtCLE13* in roots and developing nodules was investigated by *promoter:GUS* analysis and *in situ* hybridizations. A 2-kb region upstream of *MtCLE12* and *MtCLE13* was isolated based on the available genomic data[2] and cloned 5' to the *uidA* gene. Transcriptional activation of the *uidA* gene was visualized by GUS staining.

No GUS staining was observed in uninoculated transgenic roots. At 3 dpi, *MtCLE13* expression was detected in cell clusters along root zones susceptible to rhizobial infection (Figure 4.4A). At that time point, some incipient nodule primordia were present, but not all were at the same stage on a single root. As a result, in-between the most developed primordia and the roottip, many more incipient nodulation events occurred and the closer to the roottip, the less developed the primordia. Inoculations with

[2] http://www.ncbi.nlm.nih.gov/

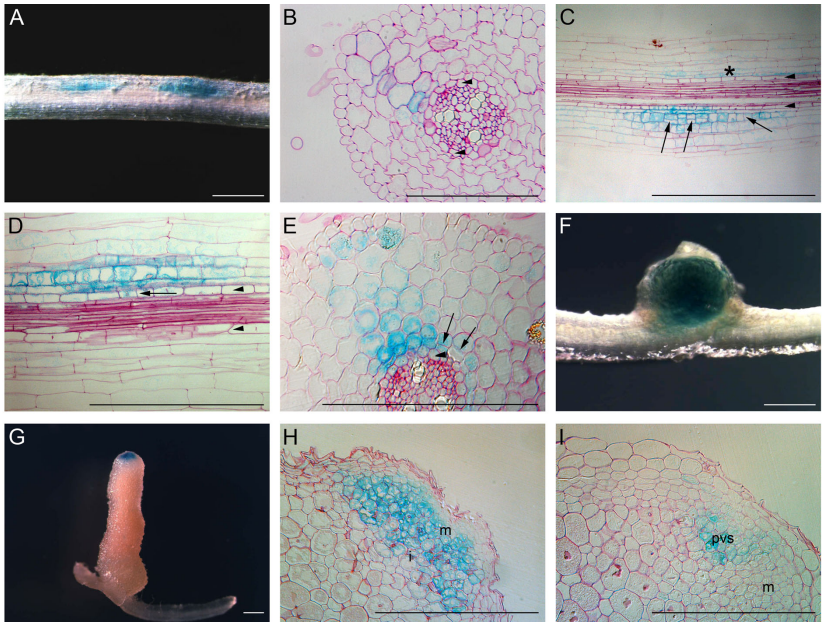

Figure 4.4: *MtCLE13* promoter activity during nodulation. A, Transgenic *pMtCLE13:GUS* root segment of the susceptible root zone I at 3 dpi. GUS staining was observed in patches along the root. B, Transversal section through a root segment at an initial stage of nodule formation when still no cell divisions occur. Blue staining is the highest in the inner cortical cells. Arrowheads indicate pericycle. C and D, Longitudinal sections through the root segment shown in (A). In the incipient nodulation event (indicated by an asterisk in (C)), staining is seen in inner cortical cells, but not in the pericycle cells. In slightly more developed nodule primordia, where cell divisions are visible in cortex and pericycle (examples marked by arrows in (C) and (D)), GUS staining is the strongest in the dividing cells of the cortex but also in the pericycle. E, Transversal section through a young developing nodule primordium at a stage similar to that in (D). Cell division is clearly visible in the cortex (arrows). The pericycle cell file is indicated by arrowheads in C, D and E. *pMtCLE13:GUS* is expressed in the cortex and at a low level in the pericycle, with the highest expression in the dividing inner cortical cells. F, A round, young nodule with GUS staining throughout the nodule tissue. G, GUS staining of a mature, elongated nodule. H, Longitudinal section through an elongated nodule. GUS staining is seen in the meristematic tissue and early infection zone. I, Longitudinal section through an elongated nodule, in which the expression is the highest in cells that presumably correspond to the provascular strands. Scale bars = 1 mm (A, B, D, F, and G) and 0.5 mm (C, E, H, and I). Abbreviations: m, meristem; i, infection zone; pvs, provascular strands.

Sm2011-mRFP, carrying the monomeric red fluorescent protein (mRFP), revealed that *pMtCLE13:GUS* was expressed only in regions of bacterial infection. Careful comparison of the infection events with the GUS staining patterns indicated that the *pMtCLE13:GUS* expression could be followed down to nodulation events in which only curled colonized root hairs were visible. Sections of these infected regions revealed that these clusters corresponded to early nodulation stages without or only with a few cell divisions. Closest to the roottip, the *pMtCLE13:GUS* expression was mainly localized in inner cortical cells (Figure 4.4, B and C), scattered in outer cortical cells (Figure 4.4B), and absent in the pericycle and vascular tissues (Figure 4.4, B and C). At positions where cell division was more pronounced in the inner cortex, the GUS staining pattern was more intense in the dividing cells (Figure 4.4, C-E). A decreasing gradient of *pMtCLE13:GUS* expression radiated from the inner to the outer cortical cells and pericycle (Figure 4.4, C-E). Expression was the highest in the dividing cortical cells, but dividing pericycle cells displayed GUS staining as well. In round, young nodules, GUS staining was seen throughout the central tissue (Figure 4.4F). In elongated nodules, it was restricted to the apical region, corresponding to the meristematic and early infection zones (Figure 4.4, G and H). Although mostly all the meristematic cells were blue, in some nodules, the expression was the highest in cells that corresponded to the provascular system (Figure 4.4I).

In situ hybridizations revealed a similar expression pattern with low transcript levels in the meristem and cells of the early infection zone (Figure 4.5). For some nodules, *MtCLE13* transcripts were more clearly detectable in cells of the provascular strands (Figure 4.5).

For *pMtCLE12:GUS*, no expression was detectable at the earliest stages of nodule development (Figure 4.6,A), but appeared in young round nodules (Figure 4.6,B). Later in nodule development, *pMtCLE12:GUS* was restricted to the apical zone of elongated nodules, similarly to *pMtCLE13:GUS* (Figure 4.6, C and D).

4.2.4 *MtCLE12* and *MtCLE13* expression in nodulation mutants

To investigate whether NF signaling is required for the induction of *MtCLE12* and *MtCLE13* expression, we analyzed the transcript levels by qRT-PCR before and after inoculation of *nin*, the *ERN1* mutant *branching infection threads1-1* (*bit1-1*), *nsp1*, *nsp2*, *dmi1*, *dmi2*, or *dmi3* mutants (Figure 4.7). The mutant lines did not develop nodules, except for *bit1-1*, which formed arrested primordia and infection foci (An-

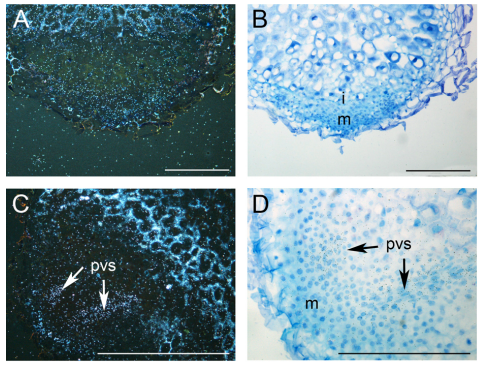

Figure 4.5: *MtCLE13* transcript accumulation in mature nodules by *in situ* hybridization. *MtCLE13* mRNA was localized by *in situ* hybridization with a *MtCLE13* antisense probe covering the complete open reading frame. Dark-field (A and C) and bright-field (B and D) microscopic images. Signal is seen as white and black dots in dark-field and bright-field micrographs, respectively. A and B, Detail of the meristematic zone of a mature nodule showing low signal in the meristem (m) and infection zone (i). C and D, Detail of a mature nodule in which the signal is localized in the provascular strands (pvs). Scale bars = 500 μm.

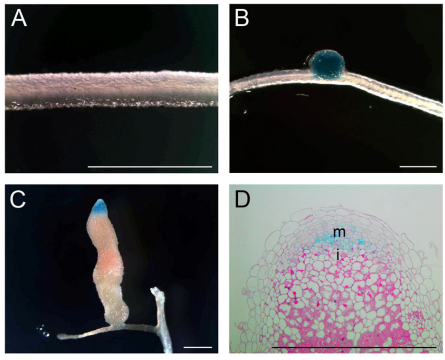

Figure 4.6: *MtCLE12* promoter activity during nodulation. A, *pMtCLE12:GUS* transgenic root segment of the susceptible root zone I at 3 dpi. No GUS staining is observed. B, GUS analysis of a young round nodule. *MtCLE12* is expressed throughout. C, GUS analysis of a mature elongated nodule. Blue staining is observed in the apical part. D, Longitudinal section through (C). Scale bars = 2 mm. Abbreviations: m, meristem; i, infection zone.

driankaja *et al.*, 2007; Middleton *et al.*, 2007). For *MtCLE12*, gene expression was induced upon inoculation in wild type *M. truncatula* roots (Figure 4.7A), but not in roots of the nodulation mutants, and likewise for *MtCLE13* transcripts, except in *bit1-1*, albeit less abundantly than upon wild type inoculation (Figure 4.7B). The expression levels of *MtCLE4* in the different mutants before and after inoculation were the same as those in inoculated wild type roots.

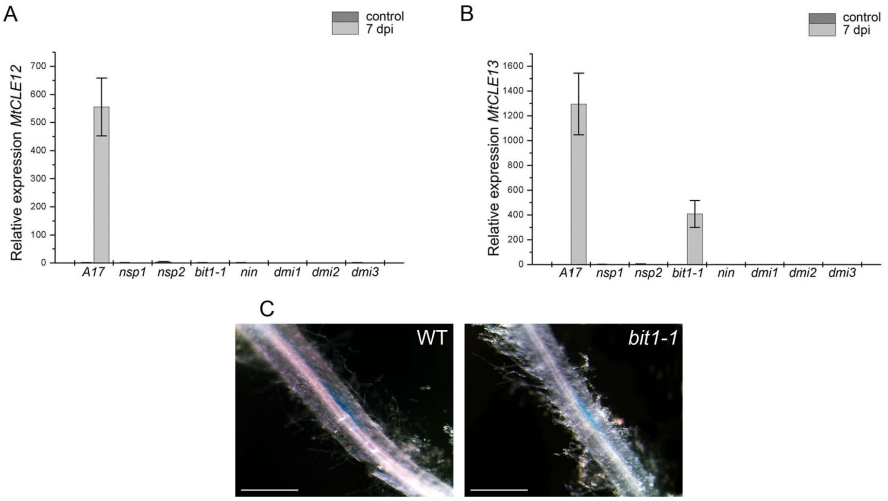

Figure 4.7: *MtCLE12* and *MtCLE13* expression in nodulation mutants. A and B, Expression analysis of *MtCLE12* and *MtCLE13* by qRT-PCR on cDNA samples of zone I root tissues of wild type plants (WT) and *nsp1*, *nsp2*, *bit1-1*, *nin*, *dmi1*, *dmi2*, or *dmi3* mutants, before inoculation (control) and at 7 dpi. C, *pMtCLE13:GUS* activity at 5 dpi in the roots of a wild type plant and in a *bit1-1* mutant. Data and error bars represent means $\pm$ SD. Scale bars = 1 mm.

As a confirmation of the qRT-PCR analysis, *pMtCLE13:GUS* transgenic roots were generated in each of the mutant backgrounds and analyzed at 5 dpi. Because nodulation events are not synchronysized in *M. truncatula*, consecutive early nodulation stages, ranging from stages with only a few cell divisions until young nodule primordia, could be observed along the root at 5 dpi. GUS staining revealed the typical *MtCLE13* cluster pattern in the wild type roots. The *bit1-1* mutant was the only mutant in which blue stained cortical regions were seen corresponding to infection (Figure 4.7C). The other mutants displayed no GUS staining. These data are in agreement with the qPCR

data and reveal that *MtCLE13* expression is linked with the NF induced cortical cell activation.

4.2.5 Induction of *MtCLE12* and *MtCLE13* transcription by auxin and cytokinin

A correct auxin/cytokinin balance is a prerequisite for nodule formation (Oldroyd and Downie, 2008; Ding and Oldroyd, 2009). To determine whether auxins and/or cytokinins affect *MtCLE12* and *MtCLE13* expression, 10^{-6} M indole-3-acetic acid (IAA) or 10^{-7} M 6-benzylaminopurine (BAP) was supplemented to the growth medium of 5-day-old seedlings. The roots of 18 seedlings were harvested under each condition after 0, 3, 6, 12, 24, and 96 h. Roots from plates without hormone addition were used as a negative control.

No significant differences in *MtCLE12* expression were detected in treated versus control roots. Auxin addition had no influence on the expression of *MtCLE13* (Figure 4.8), but in roots treated with 10^{-7} M BAP, *MtCLE13* transcripts were up-regulated after a 3-h treatment and levels further increased with extended treatment times (up to 24 h) (Figure 4.8).

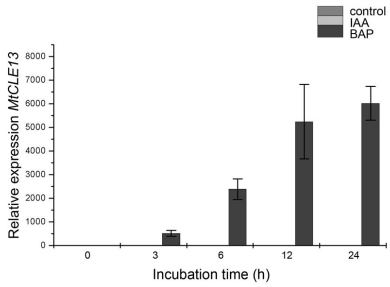

Figure 4.8: Influence of auxin and cytokinin on *MtCLE13* expression. qRT-PCR analysis of *MtCLE13* expression in cDNA samples of roots grown in the presence of 10^{-6} M auxin (IAA) or 10^{-7} M cytokinin (BAP). Growth medium without hormones was used for the control plants. Samples of 5-day-old plants were taken at 0 h, 3 h, 6 h, 12 h, and 24 h after hormone addition. Data and error bars represent means $\pm$ SD.

4.2.6 Effect of ectopic expression of *35S:MtCLE12* and *35S:MtCLE13* on nodulation

To analyze the function of *MtCLE4*, *MtCLE12*, and *MtCLE13*, composite plants were made that carried transgenic roots ectopically overexpressing one of the three *MtCLE* genes. Nodulation of these transgenic roots was assessed at 21 dpi. Nodulation of control transgenic roots (*35S:GUS*) resulted on average in 11 $\pm$ 4 nodules per root (Figure 4.9A). On the *35S:MtCLE4* transgenic roots, an average of 10 $\pm$ 4 nodules were counted. No nodules were detected on *35S:MtCLE12* and *35S:MtCLE13* transgenic roots (Figure 4.9A). qRT-PCR analysis confirmed the ectopic overexpression of the respective constructs. To determine at what stage these two CLE peptides affect nodulation, we investigated whether ectopic overexpression of *MtCLE12* and *MtCLE13* interferes with *S. meliloti* NF synthesis. For this purpose, the NF-overproducing strain Gmi6390:2011 (pMH682) (Roche *et al.*, 1991) was inoculated on *35S:MtCLE12* and *35S:MtCLE13* transgenic roots (Figure 4.10). Similar to results obtained with the wild type strain, nodulation was totally abolished, but was unaffected on *35S:MtCLE4* and control *35S:GUS* roots.

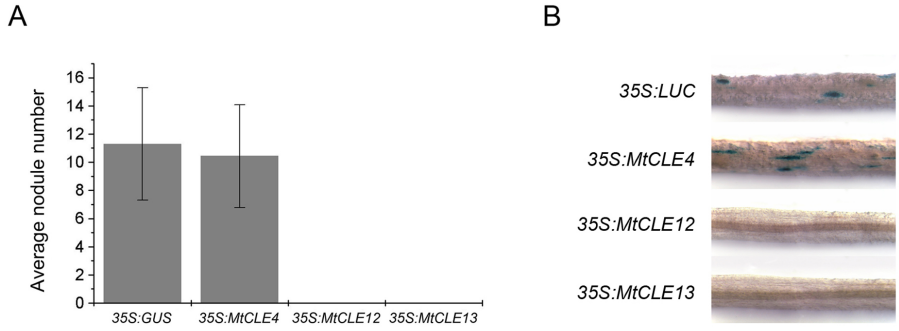

Figure 4.9: Inhibition of nodulation in *35S:MtCLE12* and *35S:MtCLE13* transgenic roots. A, Average nodule number on roots expressing *35S:GUS*, *35S:MtCLE4*, *35S:MtCLE12*, and *35S:MtCLE13* at 21 dpi (n = 28-66). Data and error bars represent means $\pm$ SD. B, *pMtENOD11:GUS* activity in roots expressing *35S:luciferase* (*LUC*), *35S:GUS*, *35S:MtCLE12*, and *35S:MtCLE13* at 5 dpi. No staining is visible in the *35S:MtCLE12* and *35S:MtCLE13* samples.

To assess whether early NF signaling events still take place in roots ectopically

overexpressing these *CLE* genes, we analyzed the transcription of the early marker, *ENOD11*, by using a GUS transcriptional reporter (p*ENOD11:GUS*), in *35S:MtCLE12* and *35S:MtCLE13* transgenic roots (Journet *et al.*, 2001) and compared it to transgenic roots ectopically overexpressing either firefly luciferase (*LUC*) or *MtCLE4*. The transgenic roots were inoculated and stained with GUS at 3 dpi. In the *35S:LUC* and *35S:MtCLE4* transgenic roots, GUS staining was observed in the epidermal cells at sites of incipient infection (Figure 4.9B), but not in *35S:MtCLE12* and *35S:MtCLE13* transgenic roots (Figure 4.9B). These results suggest that ectopic expression of *MtCLE12* and *MtCLE13*, but not of *MtCLE4*, inhibits nodulation at the very early stages of NF signal transduction, before the onset of *ENOD11* expression.

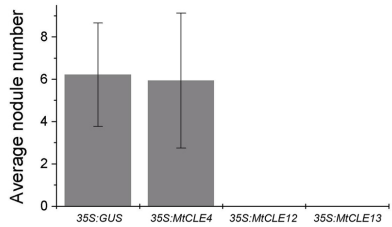

Figure 4.10: Average nodule number on roots ectopically expressing *GUS*, *MtCLE4*, *MtCLE12*, or *MtCLE13* at 11 dpi with a NF-overproducing *Sinorhizobium meliloti* strain. Average nodule number on plants with transgenic roots carrying the *35S:GUS*, *35S:MtCLE4*, *35S:MtCLE12* or *35S:MtCLE13* constructs at 11 dpi (n = 6 or 7). Inoculation was done with a NF-overproducing *Sinorhizobium meliloti* strain (GMI6390:2011(pMH682)). Data and error bars represent means ± SD.

4.2.7 Long-distance effect of *35S:MtCLE12* and *35S:MtCLE13* transgenic roots on wild type shoots

While analyzing the effect of *35S:MtCLE12* and *35S:MtCLE13* on the nodule number, we noticed that the petioles in these composite plants were longer than those in controls. To quantify this observation, we compared plants with *35S:MtCLE12*, *35S:MtCLE13* and as control *35S:GUS* and *35S:MtCLE4* transgenic roots. After growth in nitrogen-rich medium for 40 days after germination (DAG), the longest petiole on

each plant was measured (Figure 4.11A). For each construct, 60 plants were analyzed over three independent experiments. The average petiole length on control and *35S:MtCLE4* composite plants was 2.32 $\pm$ 0.36 cm and 2.21 $\pm$ 0.36 cm, respectively, whereas that on *35S:MtCLE12* and *35S:MtCLE13* composite plants was 3.06 $\pm$ 0.36 cm and 3.76 $\pm$ 0.36 cm, respectively ($p < 0.05$, Linear mixed models) [3]. To analyze whether ectopic expression of *MtCLE12* and *MtCLE13* would have a synergistic effect on the petioles of the wild type shoot, composite plants were made carrying transgenic roots containing both *35S:MtCLE12* and *35S:MtCLE13* constructs (*35S:MtCLE12/13*). The average petiole length was 3.44 $\pm$ 0.36 cm, which is not statistically different from the petiole length measured on composite plants with the *35S:MtCLE13* construct alone ($p > 0.05$, Linear mixed models) (Figure 4.11A). qRT-PCR was used to confirm the ectopic overexpression of each construct.

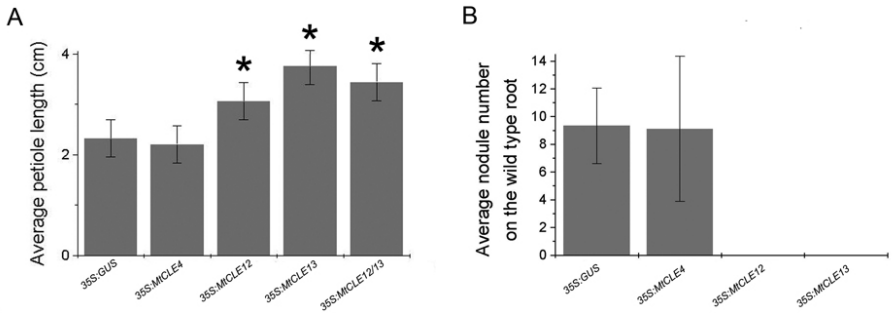

Figure 4.11: Long-distance effect of roots expressing *35S:MtCLE12* and *35S:MtCLE13* on petiole length and wild type root nodulation of composite plants. A, Graph represents the average petiole length of composite plants carrying *35S:GUS*, *35S:MtCLE4*, *35S:MtCLE12*, *35S:MtCLE13*, or *35S:MtCLE12* and *35S:MtCLE13* transgenic roots at 4 weeks post germination (n = 37-51). Data and error bars represent means $\pm$ SE. Asterisks mark groups statistically different from the control (*35S:GUS*) ($p < 0.05$, Linear mixed models). B, Average nodule number at 7 dpi on the wild type main roots of composite plants bearing additional transgenic *35S:GUS* (n = 65), *35S:MtCLE4* (n = 10), *35S:MtCLE12* (n = 12), or *35S:MtCLE13* (n = 71) roots. Data and error bars represent means $\pm$ SD.

A longer petiole length could either indicate a specific effect of MtCLE12 and MtCLE13 on petiole growth or an overall faster development. To distinguish between

[3] Petiole elongation was not observed anymore on *35S:MtCLE12* and *35S:MtCLE13* stable transformants. For these results, see page 128

these two hypotheses, developmental stages were assigned to each composite plant according to the method described by Bucciarelli *et al.* (2006). Results revealed no clear differences in the rate of composite plant development (Figure 4.12). However, plants with roots expressing *35S:MtCLE13* were out of the range of the control plants and, therefore deemed to be a little faster in development ($p < 0.05$, Linear mixed models). Leaf sizes of *35S:MtCLE12* and *35S:MtCLE13* composite plants, calculated with ImageJ[4], showed no statistically significant differences compared to controls.

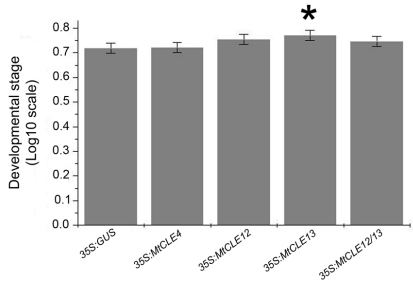

Figure 4.12: Log10 of the developmental stage of composite plants carrying roots ectopically expressing *GUS*, *MtCLE4*, *MtCLE12*, *MtCLE13*, or *MtCLE12* and *MtCLE13*. Developmental stage was measured according to Bucciarelli *et al.* (2006). Data and error bars represent means $\pm$ SE. Asterisk marks a group statistically different from the control (*35S:GUS*) ($p < 0.05$, Linear mixed models).

4.2.8 Long-distance effect of *35S:MtCLE12* and *35S:MtCLE13* transgenic roots on nodulation of wild type roots

In the *Agrobacterium rhizogenes* transgenic root assay, cotransformed transgenic roots were identified by screening for green fluorescent protein (GFP) roots (see Materials and Methods page 76). Often non-GFP-expressing roots grow on the same composite plant, suggesting that these roots are not cotransformed and contain only endogenous *A. rhizogenes* T-DNA(s). We repeatedly saw no nodules on these roots when the composite plants carried *35S:MtCLE12* and *35S:MtCLE13* transgenic roots. These observations indicated that the expression of *35S:MtCLE12* or *35S:MtCLE13* in roots,

[4] http://rsb.info.nih.gov/ij/

might have a negative systemic effect on the nodulation of roots that do not express the constructs, but grow on the same plant. Therefore, genomic DNA was prepared from GFP-fluorescent and nonfluorescent roots and the presence of the *GFP* gene was analyzed by PCR. In some composite plants (Figure 4.13B), nonfluorescent roots still contained the *GFP* gene. Therefore, these observations did not unequivocally demonstrate a negative systemic influence of *35S:MtCLE12* or *35S:MtCLE13* on nodulation.

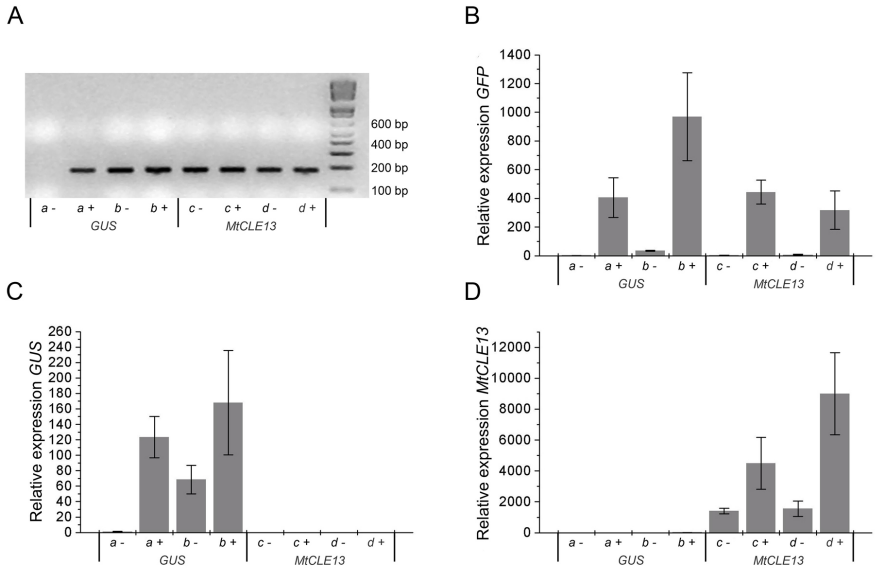

Figure 4.13: Detailed genotypic analysis of transgenic roots obtained by *A. rhizogenes* transformation. Jemalong J5 plants were transformed with either *35S:GUS* (plants *a* and *b*) or *35S:MtCLE13* (plants *c* and *d*). Roots with (+) and without (-) GFP fluorescence were kept on the plants and harvested separately at 7 dpi. gDNA as well as cDNA were prepared from these samples. A, Gel electrophoresis of PCR products obtained with GFP-specific primers on the gDNA samples of these roots. B-D, Expression analysis of *GFP*, *GUS*, and *MtCLE13* by qRT-PCR on the cDNA samples of the roots.

With a different procedure (see Materials and Methods page 76), composite plants were generated with small transgenic roots, while the wild type root was kept intact. At 7 dpi with *Sm2011*-GFP, a Nod$^-$ phenotype was observed on the primary wild type root of *35S:MtCLE12* and *35S:MtCLE13* composite plants (Figure 4.11B) and

on average nine nodules were counted on the primary wild type roots of *35S:MtCLE4* and *35S:GUS* control plants. Given that plants were grown in an aeroponic system that confined the roots of all the plants in the same compartment, we can rule out that this phenotype is the result of peptide diffusion. These data show that ectopic overexpression of *MtCLE12* and *MtCLE13* not only abolishes nodulation locally in transgenic roots, but also systemically in nontransformed roots of the same plant.

4.2.9 Analysis of the long-distance responses in the *sunn-1* mutant background

Nodulation is under the control of AON, a systemic response that involves shoot-controlled factors (Magori and Kawaguchi, 2009). Because of the long-distance responses observed when *MtCLE12* and *MtCLE13* are ectopically overexpressed, we investigated whether *MtCLE12* and *MtCLE13* are involved in AON and might be perceived by the SUNN receptor. Therefore, we tested whether the long-distance effects provoked by *35S:MtCLE13* expression on petiole length and on nodulation could be observed in the *sunn-1* mutant background (Schnabel *et al.*, 2005).

In contrast to the results in a wild type background, the petiole length did not elongate in composite *sunn-1* mutant plants containing roots expressing *35S:MtCLE13* (Figure 4.14A). The average petiole length was 2.7 $\pm$ 0.7 cm, which is comparable to that of control plants (2.4 $\pm$ 0.7 cm) (Figure 4.14A).

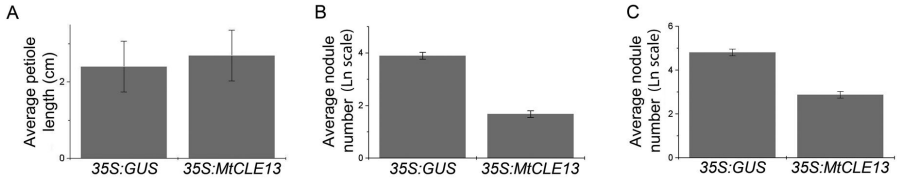

Figure 4.14: Local and systemic responses in the *sunn-1* mutant. A, Average length of the longest petiole of composite plants with roots ectopically expressing either *35S:GUS* or *35S:MtCLE13* at 4 weeks post germination (n = 12). Data and error bars represent means $\pm$ SE. B, Ln (logaritmus naturalis) of the nodule number at 7 dpi on *35S:GUS* or *35S:MtCLE13* transgenic roots (n = 10-12). C, Ln of the nodule number at 7 dpi on the wild type main roots of plants bearing *35S:GUS* or *35S:MtCLE13* transgenic roots (n = 17-24). Data and error bars represent means $\pm$ SE.

On average, only 5.3 nodules occurred on *sunn-1,35S:MtCLE13*-transformed roots

versus 49.0 nodules on the *sunn-1,35S:GUS* roots (Figure 4.14B). The long-distance repression of nodulation was investigated in the *sunn-1* mutant background with the 'hypocotyl stabbing method' on plants growing under aeroponic conditions. Nodule numbers were counted at 7 dpi with *Sm2011-GFP*. Nontransformed primary roots of control plants had on average 74.0 nodules versus 17.6 nodules on the main root of *sunn-1* plants that expressed *35S:MtCLE13* in transgenic roots (Figure 4.14C). Together, these analyses in the *sunn-1* mutant background revealed that the petiole elongation was *SUNN* dependent, while the repression of nodulation was only partially dependent on *SUNN*.

4.3 Discussion

4.3.1 CLE family in *M. truncatula*

With specialized BLAST searches, 25 *MtCLE* genes were identified in the Mt2.0 release of the *M. truncatula* genome that represents approximately 60% of the genome and more genes are expected upon completion of the genome sequencing. A more accurate picture of the size of the *CLE* gene family derives from the analysis of *L. japonicus* and Arabidopsis. The genome of *L. japonicus* is comparable in size to that of *M. truncatula* (470 Mbp) and currently 91.3% of its genome has been sequenced. Thus far, 39 *CLE* genes have been identified in *L. japonicus* (Sato *et al.*, 2008; Okamoto *et al.*, 2009), which is equivalent to the 32 *CLE* genes in the completely sequenced Arabidopsis genome (157 Mbp) (Cock and McCormick, 2001; Oelkers *et al.*, 2008).

qRT-PCR detection of 15 *MtCLE* transcripts revealed six to be expressed specifically in roots, three in shoots, one in nodules, and five across several tissues, hinting at a CLE peptide involvement in a multitude of developmental processes throughout the plant (Mitchum *et al.*, 2008). For the remaining 10 *MtCLE* genes, no transcripts were detected because either they are very lowly expressed and/or respond to specific biotic or abiotic stimuli, or they have low abundant cell-type specific expression, or are pseudogenes.

As we were interested in CLE peptide function during nodulation, *MtCLE12* and *MtCLE13* were selected for further analysis because they were up-regulated in nodulated roots. The CLE domain sequences of MtCLE12 and MtCLE13 are very similar, which is indicative of redundant functions, but the gene expression patterns, although partially overlapping, are not identical. For instance, the *MtCLE13* expression is spe-

cific for nodulation, while *MtCLE12* transcripts occur also at low levels in roottips, cotyledons, and first leaves. Moreover, upon inoculation, the *MtCLE13* expression is up-regulated much earlier than that of *MtCLE12*. The promoter of *MtCLE13* functions in the NF-activated cortical cells, its activity is later restricted to the nodule primordium, and is maintained through nodule maturity, in the apical meristematic zone. In contrast, the *MtCLE12* promoter activity occurs first in young round nodules and, similarly to that of *MtCLE13*, is later restricted to the apical zone. Finally, the expression of *MtCLE13* is induced rapidly by cytokinin, while that of *MtCLE12* is unaffected.

In the genome of *L. japonicus*, three *CLE* genes have been identified to be up-regulated by rhizobial inoculation (Okamoto *et al.*, 2009). *LjCLE-RS1* and *LjCLE-RS2* have a high degree of similarity with the CLE domain of *MtCLE12* and *MtCLE13*, but no nodulation-related *MtCLE* gene was found with a CLE domain similar to that of *LjCLE3*. Interestingly, *LjCLE-RS1* and *LjCLE-RS2* are also up-regulated at early stages of nodulation. Based on sequence similarity and expression profiles, *MtCLE13* and *LjCLE-RS1/LjCLE-RS2* might exert a comparable function during indeterminate and determinate nodule development, respectively.

Studies on the putative CLE peptides of Arabidopsis (Ito *et al.*, 2006; Strabala *et al.*, 2006; Ni and Clark, 2006; Whitford *et al.*, 2008) have shown a direct relationship between CLE domain sequence and induced phenotypes. MtCLE12 and MtCLE13 are most similar to a group less characterized Arabidopsis CLE peptides (AtCLE1 to AtCLE7), that are broadly produced with higher activity levels in the root (Sharma *et al.*, 2003; Ito *et al.*, 2006). Exogenous peptide addition did not suppress procambial to xylem cell transdifferentiation in a Zinnia cell culture while application of high, but not low, concentrations of peptides resulted in primary root meristem arrest (Ito *et al.*, 2006; Strabala *et al.*, 2006; Kinoshita *et al.*, 2007; Whitford *et al.*, 2008). Furthermore, ectopic overexpression of this group of peptides resulted in root elongation, mild *wus* loss-of-function phenotypes, mild distorted leaves, and dwarfing in later growth stages (Strabala *et al.*, 2006). Upon exogenous peptide addition or ectopic overexpression in transgenic roots of *MtCLE12* and *MtCLE13*, neither root growth arrest, nor enhanced root elongation were observed. Because root growth analysis of *A. rhizogenes* generated roots is technically difficult, phenotypic analysis of transgenic plants carrying heritable *35S:MtCLE12* or *35S:MtCLE13* constructs might help to decipher whether these CLE peptides do indeed induce root growth defects. The CLE domain sequence of *MtCLE4* is most highly homologous to type-I Arabidopsis CLE peptides, to which CLV3 belongs. This class of CLE peptides causes root meristem arrest upon exogenous

application or ectopic overexpression. As expected, exogenous MtCLE4p application inhibited root growth by 24%.

4.3.2 Nodule-related *MtCLE12* and *MtCLE13* expression is linked with differentiation and dedifferentiation processes

If *MtCLE13* were to be involved in nodulation, its transcription would depend on early nodulation signaling components. Quantitative detection of *MtCLE13* transcripts, across different nodulation mutants revealed that functional DMI1, DMI2, DMI3, NSP-1, NSP2, and NIN, but not ERN1, proteins, are necessary for *MtCLE13* expression. Interestingly, the *ERN1* mutant, *bit1-1*, is the only mutant in which cell division is initiated and small arrested primordia are observed, indicative for active NF signaling toward the cortex and pericycle (Andriankaja *et al.*, 2007; Middleton *et al.*, 2007). *MtCLE13* transcript expression was quickly induced by cytokinin, but not by auxin, the former being the most important hormone for primordium initiation (Gonzalez-Rizzo *et al.*, 2006; Murray *et al.*, 2007; Tirichine *et al.*, 2007). These data show that *MtCLE13* transcript expression is positioned downstream of early NF signaling components and suggest a role downstream to cytokinin perception in the control of organ development.

pMtCLE13:GUS analysis indicated that *MtCLE13* transcripts are expressed at very early stages of infection within nodulation-susceptible zones of the root cortex. The expression pattern is reminiscent of the signal gradient hypothesis in which opposing signal gradients from the vasculature and from the NF signaling at the epidermis would delimit the cellular landscape to form a nodule (Smit *et al.*, 1995a; Heidstra *et al.*, 1997; van Spronsen *et al.*, 2001). Because *MtCLE13* is induced by cytokinin, its expression pattern presumably reflects an internal cytokinin gradient in the cortex. Consequently, MtCLE13 peptides might serve as subsequent intercellular signals to control downstream responses. Once cell division was initiated, the *MtCLE13* expression was the strongest in the dividing cortical and pericycle cells. CLE peptides have been shown to regulate the balance between cell division and differentiation (Simon and Stahl, 2006; Kondo *et al.*, 2006; Ito *et al.*, 2006; Whitford *et al.*, 2008). Therefore, the MtCLE13 peptide gradient might maintain cell division and/or cell identity during the course of nodule development. Analysis of downstream responses to MtCLE13 peptide overexpression via genome-wide expression analysis might provide further insight into MtCLE13 function.

The expression of *MtCLE12* is also correlated with cell division and differentiation.

Expression of *MtCLE12* during nodule development was first observed throughout the mature primordium, where also *MtCLE13* expression was detected. The late expression pattern of *MtCLE12* was confirmed by the absence of expression in the inoculated nodulation mutants, because none of the tested mutants developed nodule primordia of a stage corresponding with the *MtCLE12* expression. By which trigger *MtCLE12* expression is induced is thus far unknown, but application of neither cytokinin nor auxin could activate *MtCLE12* expression. The transcription factor *MtHAP2-1*, located in nodule meristematic tissues, is essential for meristem differentiation (Combier *et al.*, 2006). It would be interesting to determine whether a functional *MtHAP2-1* is necessary for *MtCLE12* induction or whether *MtCLE12* regulates *MtHAP2-1* expression.

In mature nodules, both *MtCLE12* and *MtCLE13* are expressed apically, in a zone comprising meristematic cells and cells of the early infection zone. In some nodules, *MtCLE13* expression was higher in the provascular strands of the nodule meristem than that in other meristematic cell types. Expression of *MtCLE12* and *MtCLE13* in the nodule apex again suggests a regulatory role for their encoded peptides in nodule meristem cell proliferation and/or differentiation.

4.3.3 MtCLE12, MtCLE13, or a peptide with a related sequence might control nodule number

The lack of nodule development in transgenic roots ectopically overexpressing either *MtCLE12* or *MtCLE13* hints at a role for CLE signaling in controlling nodule number. Nodulation on *35S:MtCLE12* and *35S:MtCLE13*, but not on *35S:MtCLE4*, roots was totally abolished at the level of NF perception: no *MtENOD11* expression and no developing nodules were seen upon inoculation of transgenic roots. This suppressive effect on nodulation was not phenocopied by exogenous application of synthetic peptides. One possible reason could be related to a role for posttranslational hydroxyprolination and subsequent arabinosylation on CLE peptide bioactivity, as suggested for Arabidopsis CLE peptides AtCLE1 till AtCLE7 (Strabala *et al.*, 2006; Ohyama *et al.*, 2009). Moreover, CLV3 arabinosylation has been found to be critical for high-affinity binding to the CLV1 ectodomain (Ohyama *et al.*, 2009).

Nodule number is controlled by different processes. Our results suggest that CLE peptides are specifically involved in AON because nodule development was inhibited systemically in wild type roots of composite plants containing roots ectopically overexpressing either *MtCLE12* or *MtCLE13*. Moreover, ectopic expression of *MtCLE12*

and *MtCLE13* in roots promoted petiole elongation in wild type shoots of these composite plants. Taken together, these results demonstrate that MtCLE12 and MtCLE13 peptides can activate physiological responses at significant distances from the site of transgene expression. In a *sunn-1* mutant background, no petiole elongation was observed, but the nodule number was strongly suppressed instead of a complete nodule development inhibition, like that observed in wild type primary roots. The *sunn-1* mutant might possess residual SUNN activity that could potentially allow MtCLE12 or MtCLE13 perception and downstream signaling at a level sufficient for only a mild suppression of nodulation, but insufficient for promotion of petiole elongation. The ectopic expression of *LjCLE-RS1* or *LjCLE-RS2* of *L. japonicus* strongly reduces nodulation locally and systemically (Okamoto *et al.*, 2009). This inhibitive effect on nodulation was abolished in the *hypernodulating1-4* (*har1-4*) mutant roots, *HAR1* being an orthologous gene to *SUNN* (Okamoto *et al.*, 2009). The phenotypic differences between mutants could potentially be attributed to differences in allele functionality, because *har1-4* bears a missense mutation in the LRR ectodomain and *sunn-1* is mutated in the kinase domain (Kawaguchi *et al.*, 2002; Krusell *et al.*, 2002; Nishimura *et al.*, 2002a; Schnabel *et al.*, 2005). Accordingly, a missense mutation in the LRR ectodomain of CLV1 has a stronger phenotype than a mutation in the kinase domain (Dievart *et al.*, 2003). Moreover, the *har1-4* mutant allele causes a more severe nodulation phenotype than the *har1-5* allele, which is mutated in the intracellular kinase domain (Kawaguchi *et al.*, 2002; Krusell *et al.*, 2002; Nishimura *et al.*, 2002a; Schnabel *et al.*, 2005). Thus, the differences in phenotype might simply be due to residual activities of the mutant protein or to a potentially dominant negative effect on interacting receptors, as for mutant alleles of CLV1 (Dievart *et al.*, 2003).

Because SUNN belongs to the class XI of LRR-RLKs to which a few CLE peptide receptors belong (PXY/TDR, CLV1), it is tempting to propose that SUNN might be part of the receptor complex for MtCLE12 or MtCLE13. However, grafting studies have shown that SUNN and its orthologs are active in the shoot to invoke AON via a shoot-localized mechanism (Nutman, 1952; Delves *et al.*, 1986; Krusell *et al.*, 2002; Nishimura *et al.*, 2002a; Searle *et al.*, 2003; Schnabel *et al.*, 2005). Consequently, if the MtCLE12 and MtCLE13 peptides, which are produced upon nodulation in the root, could be perceived by SUNN, long-distance root-to-shoot translocation of the peptides would have to occur, which is in contradiction to the short-distance signaling activities proposed for many CLE peptides (Fukuda *et al.*, 2007; Hirakawa *et al.*, 2008; Whitford *et al.*, 2008; Miwa *et al.*, 2009; Stahl *et al.*, 2009).

Alternatively, the various CLE peptide bioactivities observed upon ectopic overexpression of *MtCLE12* or *MtCLE13* in roots might mimic the signaling of structurally related CLE peptides that are produced in the shoot, the place of action of SUNN and its orthologs. Because the strong *35S* promoter is expressed in the root vasculature, the ectopically produced CLE peptides might be systemically spread throughout the plant and be perceived by the SUNN protein in the shoot. As the SUNN proteins might also be produced in the root vasculature (Schnabel *et al.*, 2005; Nontachaiyapoom *et al.*, 2007), we cannot rule out that the misexpressed CLE proteins in the root vasculature are locally perceived by SUNN to provoke the observed long distance effects. The shoot-expressed *CLE* gene might be *MtCLE12* itself, because expression analysis has revealed that *MtCLE12* is also expressed in first leaves and cotyledons, the site of SUNN activity. In this scenario, MtCLE12 and MtCLE13 activity within the nodule would not be linked with AON, but rather with balancing proliferation and differentiation as supported by the observed expression patterns.

It is equally possible that MtCLE12 and MtCLE13 perception occurs locally in the root, resulting in secondary signals that travel to the shoot where they are recognized to provoke AON and petiole elongation. Interestingly, the expression of *MtCLE12* and *MtCLE13* coincide with the activation and progression of AON that is first initiated when the first nodule primordia are formed and is strengthened as more nodules develop (Nutman, 1952; Delves *et al.*, 1986; Takats, 1990; Wopereis *et al.*, 2000; Krusell *et al.*, 2002; Nishimura *et al.*, 2002a; Searle *et al.*, 2003; Schnabel *et al.*, 2005; Li *et al.*, 2009). Besides the long-distance effect on nodulation, *35S:MtCLE12* and *35S:MtCLE13* transgenic roots induced elongation of the petioles of the composite plants. Insights into petiole growth might hint at downstream responses of MtCLE12 and MtCLE13 signaling. Petiole length elongation is influenced by auxin, ethylene, abscisic acid, and gibberellins (Cox *et al.*, 2004; Millenaar *et al.*, 2009; Pierik *et al.*, 2009).

In conclusion, CLE peptides most probably play different roles in nodulation. Expression patterns hint at roles during cellular differentiation processes, both at the onset of nodulation and later during nodule meristem development and subsequent homeostasis. Moreover, intertwined or not, the functional analyses imply a role for *MtCLE* peptides in AON. In Arabidopsis, the CLE peptide signaling is intricate and mediated by different receptor complexes (Miwa *et al.*, 2009; Stahl *et al.*, 2009). *In silico* analysis of the *M. truncatula* genome has revealed additional genes that belong to the class XI of LRR-RLKs and expression profiling indicates that they are up-regulated in the

nodule. Future studies will investigate the individual roles of each of these peptides and their corresponding receptors during the nodulation process and will provide a valuable insight into nodule development, into nodule cell-type determination, and regulation of nodule number.

4.4 Materials and methods

Biological material

M. truncatula Gaertn. cv. Jemalong A17 and J5 as well as *nin*, *bit1-1*, *nsp1*, *nsp2*, *dmi1*, *dmi2*, *dmi3*, and *sunn-1* mutants (Catoira *et al.*, 2000; Oldroyd and Long, 2003; Marsh *et al.*, 2007; Middleton *et al.*, 2007) and *pENOD11:GUS* transgenic seeds (Journet *et al.*, 2001) were grown and inoculated as described (Mergaert *et al.*, 2003). *Sinorhizobium meliloti 1021*, *Sm1021* pHC60-GFP (Cheng and Walker, 1998), *Sm1021* pQE81-dsRedT3 (Bevis and Glick, 2002), *Sm2011* pBHR-mRFP (Smit *et al.*, 2005), *Sm2011* pHC60-GFP (Cheng and Walker, 1998), and *Sm2011* pMH682-Gmi6390 (Roche *et al.*, 1991) were grown at 28°C in yeast extract broth medium (Vervliet *et al.*, 1975), supplemented with 10 mg L^{-1} tetracycline for the *Sm1021* pHC60-GFP, *Sm1021* pQE81-dsRedT3, *Sm2011* pBHR-mRFP, *Sm2011* pHC60-GFP, and *Sm1021* pMH682-Gmi6390 strains.

PCR fragments corresponding to the full-length open reading frames of *MtCLE4*, *MtCLE12*, and *MtCLE13* were amplified from *M. truncatula* cDNA and cloned in pB7WG2D driven by the CaMV 35S promoter (De Loose *et al.*, 1995; Karimi *et al.*, 2002). The vector pK7m34GW2-8m21GW3D was used for the simultaneous ectopic expression of *MtCLE12* and *MtCLE13* (Karimi *et al.*, 2007). For *promoter:GUS* analysis, a 2-kb region upstream of *MtCLE12* and *MtCLE13* was isolated from genomic DNA based on the available genomic data[5]. The promoters were fused to the *uidA* gene in pKm43GWRolDC1 (Karimi *et al.*, 2002). Primers used for amplification are presented in Table 4.2.

For the qRT-PCR analysis, *M. truncatula* J5 plants were grown *in vitro* in square Petri dishes (12 × 12 cm) on nitrogen-poor SOLi agar (Blondon, 1964). After 7 days, roottips, SAMs, cotyledons, and first leaves were harvested, mature leaves, stems, and roots from 1-month-old plants grown in perlite and watered with nitrogen-poor SOLi medium and nodulated roots after 1 month from plants inoculated with *Sm1021*

[5] http://www.ncbi.nlm.nih.gov/

pHC60-GFP. For the analysis of temporal expression during nodulation, nodules were harvested 4 to 10 dpi from plants grown in pouches, watered with nitrogen-poor SOLi medium, and inoculated with *Sm1021* pHC60-GFP. Infection threads were visible from 4 dpi on, nodule primordia at 6 dpi, and small nodules at 8 dpi. Two days later, at 10 dpi, slightly bigger nodules were observed. Tissue was collected by visualizing the green fluorescent bacteria under a stereomicroscope MZFLII (Leica Microsystems, Wetzlar, Germany) equipped with a blue-light source and a Leica GFP Plus filter set (λ_{ex} = 480/40; λ_{em} = 510 nm LP barrier filter). The zones I of uninoculated roots were isolated at the same developmental stage as the 4 dpi stage.

In silico identification of *M. truncatula CLE* genes

BLAST searches were done at The Insititue for Genomic Research (TIGR[6]) or at the National Center for Biotechnology Information[7]. The CLE family was identified by repetitive searches similar to those conducted by Cock and McCormick (2001) Repetitive searches were done at Medicago Gene Index (MGI) at the Dana-Farber Cancer Institute (Release 9.0) first with the tBLASTn PAM30 algorithm for the Arabidopsis CLE box consensus (RXXPXXPXPXH). The first identified sequence, MtCLE1 (Genbank EST AW586793), that was confirmed to encode a MtCLE-like peptide was based on the predicted peptide length (<150 amino acids), the presence of a C-terminally localized CLE box, and an N-terminal signal peptide as predicted by HMM signalP and neural networks (Bendtsen *et al.*, 2004). This first sequence was used to repeat the same search, each time with an additional homologous CLE box sequence, until no unknown family members were found in the EST data. These CLE box sequences were used in the same iterative BLAST searches to identify additional putative CLE peptides from the partially completed genomic sequence (Mt2.0). Sequences were aligned with AlignX within the VectorNTI Advance v.10 suite of programs[8].

RNA extraction, cDNA synthesis, and qRT-PCR analysis

Total RNA was isolated with the RNeasy Plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After a DNAse treatment, the samples were purified through NH_4Ac (5 M) precipitation, quality controlled, and quantified

[6] http://www.tigr.org/tdb/tgi/
[7] http://www.ncbi.nlm.nih.gov/BLAST/
[8] http://www.invitrogen.com

with a Nanodrop spectrophotometer (Isogen, Hackensack, NJ). RNA (2 μg) was used for cDNA synthesis with the Superscript Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA). The samples were diluted 50 times and stored at -20°C until further use. The qRT-PCR experiments were done on a LightCycler 480 (Roche Diagnostics, Brussels, Belgium) and SYBR Green was used for detection. All reactions were done in triplicate and averaged. The total reaction volume was 5 μl (2.5 μl master mix, 0.25 μl of each primer [5 μM] and 2 μl cDNA). Cycle threshold (C_T) values were obtained with the accompanying software and data were analyzed with the $2^{-\triangle\triangle C_t}$ method (Livak and Schmittgen, 2001). The relative expression was normalized against the constitutively expressed 40S ribosomal S8 protein (TC100533, MGI). Primers used (Table 4.2) were unique in the MGI version 9.0 and the Medicago EST Navigation System database (Journet *et al.*, 2002). Each experiment was repeated at least three times with independent biological tissue.

Statistical analysis

To estimate the genotype effects on developmental stage, petiole length, and root length, the linear mixed model (random terms underlined) $y = \mu + Genotype + Experiment + \varepsilon$, was fitted to the data, where y represents the variable, μ is the overall mean, *Genotype* the fixed genotype effect, *Experiment* random experimental effects, and ε the random error. Statistical significance of genotype effects was assessed by a Wald test. In the case of the nodule number in *sunn-1* plants, a generalized linear mixed model (GLMM) of the form $y = \mu + Genotype + Experiment + \varepsilon$ with a Poisson distribution and a logarithmic link, was fitted to the data. Again, the statistical significance of genotype effects was assessed by a Wald test. All analyses were done with Genstat[9].

In vitro application of MtCLE synthetic peptides, auxins, and cytokinins

Peptides (AGAMOUS, APNNHHYSSAGRQDQT; MtCLE4, KRGVpSGANPLHNR; MtCLE12, DRLSpGGpNHIHN; and MtCLE13, DRLSpAGpDPQHNG; lowercase p indicate hydroxylated prolines), with a purity greater than 89% (ServiceXS, Leiden, The Netherlands), were dissolved in a filter-sterilized sodium phosphate buffer (pH

[9] http://www.vsni.co.uk/software/genstat/

Table 4.2: Primers used in the analysis

Gene	Sense primer	Antisense primer
Gateway cloning		
ORF-MtCLE4	ATGCTAACAAGTGAAATGAGCG	CTACCGATTATGCAGAGGATTAGC
ORF-MtCLE12	ATGGAGAATTCAAATAAAGTGCCAAT	TTAGTTATGTATATGGTTTGGTCCAC
ORF-MtCLE13	ATGGGCCGGTATACAACC	CTACTTTCTTGGTGGTGATCT
promoter-MtCLE12	TGTAATGTGATGGTGGACTT	CATGCAAAGAACAAGAAGTTGG
promoter-MtCLE13	TCATTCCTAGTAGAACGGCG	GCCCATGTGTGATTTTAATC
qRT-PCR amplification		
MtCLE1	TGGTGGGGTTTGTTTGTTTTATGG	GTCTTGTAGTTGTTTGTCCTGTTCC
MtCLE2	ATGAATGTGAATGTTCTCTCTAGGC	GGTTGTCTATATTTCCTTGCTCTCC
MtCLE3	GGTTCAAGCAACAAGAATGAAACAC	AGGATCTGGGCAGCTTGGTAC
MtCLE4	AATTTCACAAGTTCTGCTTCATCGC	TGGCACACCTCTCTTGTCTTCC
MtCLE5	AATGCAAGCTACCACGTCTCG	GCAGAGGATTTGGTCCAGAAGG
MtCLE6	TCTATCTCACCAACCAACAACAACC	TCCAAATTCTTTTCCAGCTCCATCC
MtCLE7	AACTGTGTTTTGTTATTCTTCTTC	AGATGATGATGATTTGAATGAACC
MtCLE8	TTCTCAAAGTAGGAAAATGTTAGC	CGATGATGAAGACGACTATGG
MtCLE9	GCTCGCTATGTTTCCTTCAC	AGAGATGGACTTAGATTATTCTGC
MtCLE10	GCACATGGAAGAAAGAACAAGAGTC	CCCCTGGAGTAGATGTATGTGAAAC
MtCLE11	TGTTAGCTTCTCAACCATACATTGC	TGAAGGTGAAGGTGCCAAATCC
MtCLE12	CAACGTCTCTTGCATGAGTTAATGG	ACCTGGTGAAAGCCTATCTCCTG
MtCLE13	CCGAAGCCTTCTACAGAAACTACG	TCTTGGTGGTGATCTTCCATTATGC
MtCLE14	ACACCACCACAATTCCTTAC	ATATGATGAACATTCTCAGATAGC
MtCLE15	CAGTGGTTGGAGAGTTGAGAAAGG	TCAAGGGTTTTCAGGCTTAATAGGG
MtCLE16	AACACTCAAAACAACCAACAACAACATC	ACTCTTCTCTTCTCAGCACCAAATC
MtCLE17	TCTCAAGCCCTTCTTCTACTTC	TCCTGTAGGCACTTTGCGTTTC
MtCLE18	TTCACATTCTACTATGGCTATCTC	ATGATGACTTGCTTGTATGATTTG
MtCLE19	TTGATACTTTTCTTTTCTTGTTGG	TTGAAACTGACATCACTTCT
MtCLE20	TGTTACCACTTCATCACGGCATC	TGTGGCTCAACAACTGAAGACTC
MtCLE21	GCTAAAGGAGGTTCAAGACAG	GTAAAGGATTAGGACCAGTGTAG
MtCLE22	TCATCCCTACCTCTGTCATTATAC	ATCCTTGCTTACCAACCTTCC
MtCLE23	CTTCCTTACACCTACATCTTACAC	TTACCTCTGCTGCTGATTGG
MtCLE24	AAGAAGAGTTCGGCTAATGTC	CTGTTGTGAATAGGATCTGACC
MtCLE25	CACTCAAACATGATGATGAACAAG	TGAATAGGATCTGGTCCATTAGG
40S Ribos	GCCATTGTCCAAGTTTGATGCTG	TTTTCCTACCAACTTCAAAACACCG
GFP	ACGGCATCAAGGTGAACTTC	GTGCTCAGGTAGTGGTTGTC
GUS	CTACACCACGCCGAACAC	CACCACCTGCCAGTCAAC

6, 50 mM [43.5 mM NaH_2PO_4 and 6.2 mM Na_2HPO_4]). Two-day-old Jemalong J5 seedlings were grown *in vitro* in square Petri dishes (12 $\times$ 12 cm) on agar HP 696-7470 (Kalys, Bernin, France) containing the SOLi medium supplemented with 1 mM NH_4NO_3 and 10 μM peptides (Fiers *et al.*, 2005). The plants were cultured at 25°C, 16-h photoperiod, and 70 μE $m^{-2}m^{-1}$ light intensity per day. The roots were covered with aluminum foil for light protection. After 8 days of growth, the root length of 16 plants under each condition was measured from the roottip of the primary root to the

base of the hypocotyls with the ImageJ 1.40b program[10]. The experiment was repeated three times with comparable results.

Auxins (10^{-6} M IAA) and cytokinins (10^{-7} M BAP) were diluted in dimethylsulfoxide and supplemented to the medium of 5-day-old, *in vitro*-grown plants. As a control, plants were grown without supplemented hormones. The growth conditions of the seedlings were the same as above. After 0 h, 3 h, 6 h, 12 h, and 24 h of incubation, the roots of 18 plants under each condition were harvested and analyzed by qRT-PCR. The experiment was repeated twice with comparable results.

Agrobacterium rhizogenes-mediated transgenic root transformation

The protocol was adapted from Boisson-Dernier *et al.* (2001). Approximately 48 h after germination, the radicle was sectioned at 5 mm from the roottip with a sterile scalpel. Sectioned seedlings were infected by coating the freshly cut surface with the binary vector-containing *A. rhizogenes* Arqua1 strains. The *A. rhizogenes* strain was grown at 28°C for 2 days on solid yeast extract broth medium with the appropriate antibiotics (Quandt *et al.*, 1993). The infected seedlings were placed on agar (Kalys) containing the SOLi medium, supplemented with 1 mM NH_4NO_3, in square Petri dishes (12 × 12 cm) placed vertically for 5 days at 20°C, 16-h photoperiod, and 70 μE $m^{-2}m^{-1}$. Subsequently, plants were placed on the same medium between brown paper at 25°C and under identical light conditions. One and 2 weeks later, plants were screened for transgenic roots, characterized by GFP fluorescence with a stereomicroscope MZFLII (Leica Microsystems) equipped with a blue-light source and a Leica GFP plus filter set. One main transgenic root was retained per composite plant. Four weeks after infection, plants were transferred to an aeroponic system, pouches, or perlite-containing pots and incubated with SOLi medium. Three to 7 days after planting, composite plants were inoculated. The petiole lengths were measured on plants grown under the same conditions, but incubated with N-containing ISV medium[11]. Forty days after germination, the longest petiole of each plant was scored by ImageJ[12].

In some experiments, the main root was kept on the juvenile plant and infected by stabbing the hypocotyls with a fine needle containing an *A. rhizogenes* culture and cotransformed as described above, after which the plants were grown for 2 weeks at 25°C, with a 16-h photoperiod and 70 μE $m^{-2}m^{-1}$. After the plants had been transferred to

[10] http://rsb.info.nih.gov/ij/
[11] http://www.isv.cnrs-gif.fr/ISV/embo01/manuels/pdf/module1.pdf
[12] http://rsb.info.nih.gov/ij/

an aeroponic system for 7 days, nodulation was analyzed on the main, untransformed root of plants bearing GFP-positive hairy roots.

Histochemical localization of GUS activity

GUS activity in cotransformed roots and nodules was analyzed using 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid as substrate (Van den Eede *et al.*, 1992). Roots and nodules were vacuum infiltrated during 20 min and subsequently incubated in GUS buffer at 37°C. Incubation lasted 3.5 h and 7 h for *pMtCLE13-GUS* and *pMtCLE12-GUS*, respectively. After staining, root nodules were fixed, dehydrated, embedded with the Technovit 7100 kit (Heraeus Kulzer, Wehrheim, Germany), according to the manufacturer's instructions, and sectioned with a microtome (Reichert-Jung, Nussloch, Germany). The 3-μm thick sections were mounted on coated slides (Sigma-Aldrich, St. Louis, MO). For tissue-specific staining, sections were submerged in a 0.05% (w/v) ruthenium red solution (Sigma-Aldrich, St. Louis, MO), washed in distilled water, and dried. Finally, sections were mounted with Depex (BDH Chemicals, Poole, England). Photographs were taken with a Diaplan microscope equipped with bright- and dark-field optics (Leitz, Wetzlar, Germany). GUS activity of *pENOD11:GUS* roots was visualized after 7 h of incubation.

In situ hybridization

Ten-μm sections of paraffin-embedded nodules were hybridized as described (Goormachtig *et al.*, 1997). Nodules were harvested, incubated in fixation buffer, and maintained for twice 15 min under vacuum. A ^{35}S-labeled antisense probe against the complete open reading frame of *MtCLE13* was produced according to standard procedures (Sambrook *et al.*, 1989). The probe was cloned into pBlueScript KS^{+} (Stratagene, Madison, WI) and further digested with HindIII restriction enzyme to yield templates for radioactive antisense probe production with T3 RNA polymerase (Invitrogen).

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Authors Contributions

The main part of this work was done by V. Mortier. G. Den Herder was involved in the first expression analyses. The bioinformatic analysis was performed by S. Rombauts, W. Van de Velde and R. Whitford. K. D'haeseleer helped with cloning the open reading frames and promotors of the *MtCLE4*, *MtCLE12* and *MtCLE13* genes. S. Goormachtig and M. Holsters were involved in designing the research and revising the manuscript.

Addendum Chapter 4

Search for nitrate-induced *MtCLE* genes

Virginie Mortier, Cindy Martens, Stéphane Rombauts, Marcelle Holsters and Sofie Goormachtig

In preparation

4.5 Introduction

Nodulation is, amongst others, controlled by environmental signals such as nitrate availability in the soil. High nitrate concentrations inhibit nodule formation at different levels and local, as well as systemic influences have been reported (Carroll and Gresshoff, 1983; Carroll and Mathews, 1990; Hinson, 1975; Day *et al.*, 1989; Francisco and Akao, 1993; Ruffel *et al.*, 2008; Jeudy *et al.*, 2010; Streeter, 1981, 1985; Carroll and Gresshoff, 1983; Carroll *et al.*, 1985a,b; Gibson and Harper, 1985). Nitrate regulation is suggested to be partially mediated via AON, as hypernodulation mutants exhibit a nitrate tolerant phenotype (Schnabel *et al.*, 2005; Kinkema *et al.*, 2006; Barbulova *et al.*, 2007; Jeudy *et al.*, 2010). In agreement, in both *L. japonicus* and soybean group-III CLE peptide genes were identified that are upregulated by nitrate and which reduce nodulation by ectopic expression in a *NARK*- or *HAR1*-dependent way (Okamoto *et al.*, 2009; Gresshoff *et al.*, 2009; Reid *et al.*, 2011). Whether AON is involved in local and/or systemic nitrate effects is still under debate but in summary, nitrate regulation, AON and CLE peptide signaling seem to be intertwined and the interactions might be different in various legumes.

We analyzed the interaction between CLE peptides and nitrate control of nodule number but so far could not find a nitrate-induced *MtCLE* gene. In addition, we observed, in our set-up, that inhibition of nodulation by the addition of nitrate involves long distance mechanisms which are independent of *SUNN*, as *sunn-4* was still very sensitive to 10 μM of nitrate. Finally, in our search for nitrate-upregulated genes, we identified six additional *MtCLE* genes in the latest available *M. truncatula* genome of which *MtCLE26, MtCLE28* and *MtCLE31* are induced upon nodulation, suggesting that nodulation is governed by many CLE peptides that act in a redundant way.

4.6 Results

4.6.1 Involvement of *SUNN* in nitrate control of nodulation

It was previously shown that the nitrate inhibition of nodulation might act via *SUNN* or its orthologs (Carroll *et al.*, 1985a,b; Streeter and Wong, 1988; Barbulova *et al.*, 2007; Jeudy *et al.*, 2010). In agreement, in respectively *L. japonicus* and soybean, the expression of the *CLE* genes, *LjCLE-RS2* and *GmNIC1*, was upregulated by exogenous addition of nitrate (Okamoto *et al.*, 2009; Reid *et al.*, 2011). We therefore investigated

whether *M. truncatula CLE* genes could be found from which the expression is induced by nitrate. To do so, we re-examined the effect of nitrate on nodulation in wild type and *sunn-4* plants and investigated whether there was an *MtCLE* gene for which the expression was regulated by the addition of nitrate.

First, we investigated the minimal nitrate concentration needed to inhibit nodulation. Therefore, 2-day-old seedlings were grown for five days on growth medium supplemented with 0, 1, 3, 5, 10 or 30 mM KNO_3 after which they were inoculated with *Sm2011* pHC60-GFP. Nodule number was assessed at 12 dpi. The average nodule number on the plants grown in the presence of 1 or 3 mM KNO_3 was similar to the results obtained for the control plants (0 mM KNO_3) (Figure 4.15A). Addition of 5 mM KNO_3 resulted in a strong reduction in nodule number, as only 0.43 ± 0.20 nodules were counted on these plants, while 3.93 ± 0.77 nodules were observed on control plants (Figure 4.15A). A complete inhibition of nodulation was observed on plants grown in the presence of 10 or 30 mM KNO_3 (Figure 4.15A).

To analyze whether the inhibitory effect involves long distance signaling, a split-root-system was set up making use of square-shaped plates (12 x 12 cm) that were subdivided by an aluminium foil into two sections of which one compartment contained nitrogen poor growth medium, while the growth medium of the other half was supplemented with 10 mM KNO_3 (Figure 4.15B). Control plants were grown on plates containing nitrogen poor medium on both sides.

M. truncatula J5 seedlings were cut at the roottip to induce branching and 2 weeks later transferred to the split-root-system in a way that the roots were evenly distributed to both compartments of the plate. One week later, the plants were inoculated with *Sm2011* pBHR-mRFP and the average nodule number on each side of the plates was counted at 7 dpi (Figure 4.15C). The control plants revealed an average nodule number of 6.77 ± 0.36 on either the left or the right section of the plate. In contrast, on plants from which half of the root system was grown in the presence of 10 mM KNO_3, nodule number was greatly reduced both on the roots grown in the presence and absence of nitrate ($p < 0.001$, Regression analysis). The average nodule number on the nitrate-free side was 0.72 ± 0.12 and on the nitrate-rich side 0.03 ± 0.02. These results clearly indicate that inhibition of nodulation by the addition of nitrate involves long distance mechanisms. This is in agreement with previously published data (Krusell *et al.*, 2002; Nishimura *et al.*, 2002a; Searle *et al.*, 2003; Schnabel *et al.*, 2005; Oka-Kira *et al.*, 2005; Sagan and Gresshoff, 1996; Jeudy *et al.*, 2010).

In order to control whether the systemic inhibition of nodulation by nitrogen is

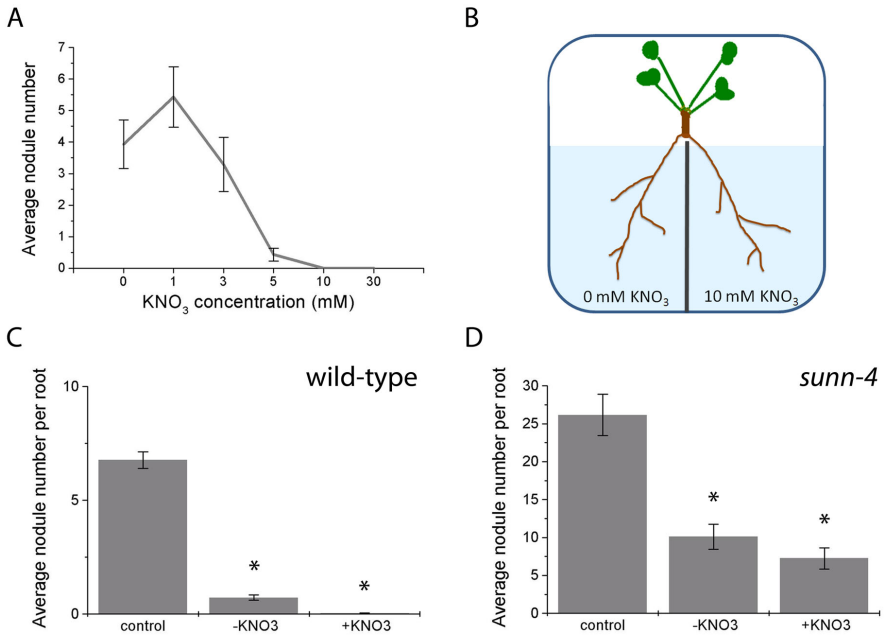


Figure 4.15: Influence of nitrate on nodulation in *M. truncatula*. A, Average nodule number on plants grown in the presence of 0, 1, 3, 5, 10, or 30 mM KNO_3 at 12 dpi with *Sm2011* pBHR-mRFP (n = 14). B, For split-root analyses plates were subdivided by aluminium foil into two sections of which one compartment contained nitrogen poor growth medium (0 mM KNO_3) and the other compartment growth medium supplemented with 10 mM KNO_3. C and D, Systemic inhibition of nodulation by nitrogen on wild type and *sunn-4* plants. Average nodule number on half of the roots of control plants grown in the absence of nitrogen (control) and of plants from which one root was grown in the absence of nitrogen (-KNO_3) and the other in the presence of 10 mM KNO_3 (+KNO_3) at 7 dpi. C, Experiment performed with J5 wild type plants (n = 30-39). D, Experiment performed with *sunn-4* mutant plants (n = 23-24). Asterisks indicate statistically significant differences in comparison to control roots ($p < 0.001$, Regression analysis). Data and error bars represent means $\pm$ SE. All experiments were repeated twice with comparable results. The total mean of both biological repeats is represented in the graph.

SUNN-dependent, we repeated the experiment in a *sunn-4* mutant background (Figure 4.15D). On the roots grown in the presence of 10 mM KNO_3, rather than a complete inhibition of nodulation a reduction in nodule number (7.25 $\pm$ 1.40) was observed as compared to the control plants (26.16 $\pm$ 2.72) ($p < 0.001$, Regression analysis).

On average 10.12 $\pm$ 1.66 nodules were counted on the part of the root grown under nitrogen poor conditions when the other root part was growing under nitrogen rich conditions ($p < 0.001$, Regression analysis). Thus, a reduction in nodule number was observed locally as well as systemically by the addition of nitrate to *sunn-4* plants. These results suggest that the long distance effect of nitrate on nodule number is only partially dependent on *SUNN*.

4.6.2 Search for *M. truncatula CLE* genes upregulated by nodulation or nitrate

With the release of the Mt3.0 version of the *M. truncatula* genomic sequence, which represents about 80 % of the genome, we searched for additional *MtCLE* genes on top of the 25 *MtCLE* genes previously identified (Okamoto *et al.*, 2009; Reid *et al.*, 2011). Using a PAM30 tBLASTn homology-based algorithm, six additional *MtCLE* genes were identified, which were designated *MtCLE26* till *MtCLE31*. Their preproproteins vary in length between 64 and 108 amino acids (Table 4.3) and show a high level of sequence divergence outside the CLE motif (Figure 4.16). For all proteins, an N-terminal signal peptide or signal anchor is predicted by HMM signalP and neural networks (Bendtsen *et al.*, 2004). None of the genes contain an intron (Table 4.3).

Table 4.3: Overview of the MtCLE26 till MtCLE31 peptide genes and derived MtCLE peptides.

M. truncatula nomenclature	CLE domain sequence	Signal peptide	Chromosome number	Length (amino acid)	Intron
MtCLE26	RLSPGGPDPRHH	Yes	2	78	No
MtCLE27	RLSPGGPDRHHN	Yes	7	108	No
MtCLE28	RKVPSCPDPLHN	Yes	5	84	No
MtCLE29	RKVPTGPDPLHH	Yes	2	64	No
MtCLE30	RLVPSGPNPLHN	Yes	4	92	No
MtCLE31	RVVPTGPNPLHN	Yes	8	88	No

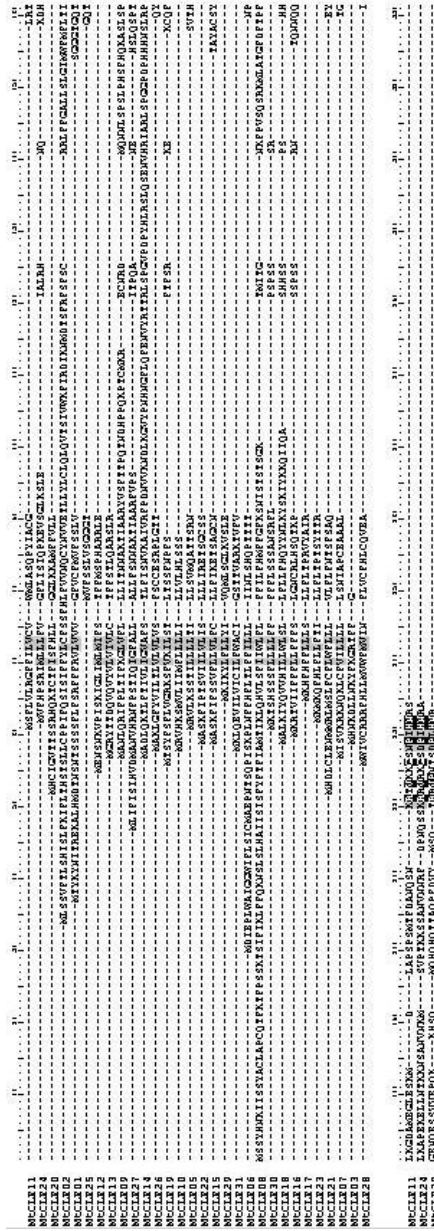
Next, a tree-based alignment was made with the CLE domains of all *M. truncatula* and Arabidopsis *CLE* genes as well as the nodulation-specific *CLE* genes of *L. japonicus* and soybean (Oelkers *et al.*, 2008; Okamoto *et al.*, 2009; Mortier *et al.*, 2010, 2011). 24 *MtCLE* genes encode CLE peptides that fall in one class, designated group-I, and exemplified by CLV3 (Figure 4.17). 2 *MtCLE* genes encode peptides similar to TDIF/CLE41/CLE44 and are designated group-II. Two additional *MtCLE* genes

(*MtCLE26* and *MtCLE27*), encode peptides that are most homologous to the peptides of the genes *MtCLE12, MtCLE13, MtCLE14, LjCLE-RS1, LjCLE-RS2, GmCLE14-GmCLE39/GmRIC1, GmCLE35-GmCLE37/GmRIC2* and *GmNIC1* that are involved in AON and to Arabidopsis *CLE1* to *CLE7* (Okamoto *et al.,* 2009; Mortier *et al.,* 2010, 2011; Reid *et al.,* 2011; Van de Velde et al., unpublished results). This group was named group-III (Mortier *et al.*, 2011). To facilitate the comparison of the amino acids between CLE domains, they were numbered as described by Oelkers *et al.* (2008) with the zero position assigned to the conserved glycine (G) residue located at the center of the CLE motif and the positions of the other amino acids numbered relative to this G. The peptide sequences derived from MtCLE26 and MtCLE27 are very similar to that of the nodulation-related CLE peptides, LjCLE-RS1/LjCLE-RS2 and MtCLE13 (Figure 4.17). The sequences of the MtCLE26 and MtCLE27 peptides only differed at 3 or 4 amino acid positions with MtCLE12 and MtCLE13, and 2 amino acid positions with LjCLE-RS1/LjCLE-RS2 (Okamoto *et al.*, 2009; Mortier *et al.*, 2010). The conserved pattern of the residues in each group is shown in a WebLogo representation (Figure 4.17).

To study the temporal expression during nodule development, the relative transcript levels of the newly identified *MtCLE* genes was analyzed at 4, 6, 8, and 10 dpi. The elongation zone of uninoculated roots, the nodule initiation site, was used as the reference tissue. For *MtCLE26, MtCLE28*, and *MtCLE31*, expression was low at 4 dpi, started to rise at 6 dpi, and remained high until 10 dpi (Figure 4.18, A-C). For *MtCLE27, MtCLE29* and *MtCLE30* no expression could be measured in any of the tested samples (data not shown). In addition to the previously published *MtCLE12* and *MtCLE13* genes, three more *CLE* genes are induced upon inoculation (Mortier *et al.*, 2010). From these five nodulation induced *CLE* genes, three belong to group-III (*MtCLE12, MtCLE13* and *MtCLE26*), while another 2 (*MtCLE28* and *MtCLE31*) are members of group-I.

To control whether nitrate addition has an influence on *MtCLE* expression, we performed a qRT-PCR analysis on cDNA of roots treated with different concentrations of

Figure 4.16 ***(facing page)*:** Multiple alignment of MtCLE preproproteins. Those amino acids which share at least 60 % similarity throughout the alignment are shaded. The darker the shading is, the more conserved is the amino acid. The conserved domain at the C-terminal end corresponds with the CLE domain, while at the N-terminal end, a few amino acids are conserved in the signal peptide.

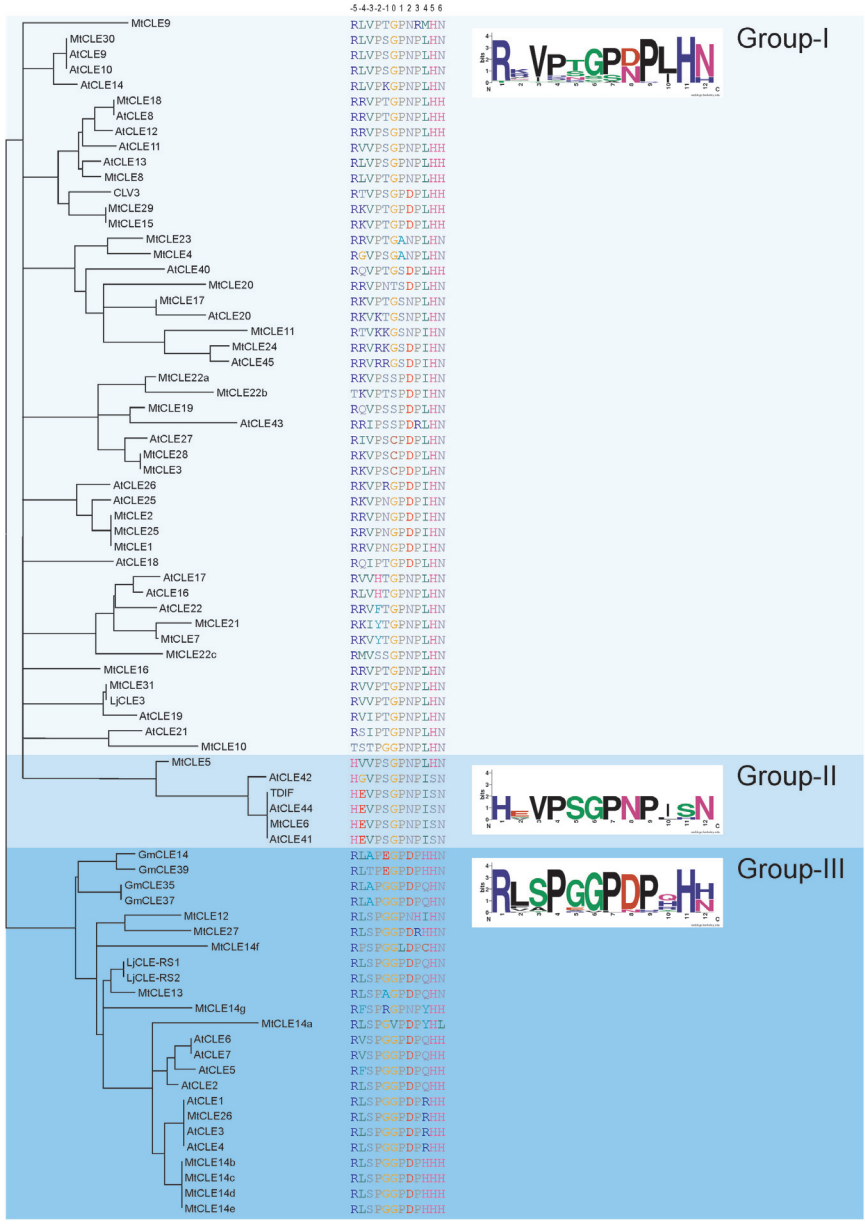


KNO_3. 0, 1, 5, 10 or 30 mM KNO_3 was supplemented to the growth medium of 7-d-old plants. The roots of 18 plants were harvested under each condition 7 days later and the relative expression level of all *MtCLE* genes was assessed. No significant differences were observed for any of the tested genes (Supplemental data, Table 4.5).

4.7 Discussion

The negative effect of nitrate is tightly connected to AON (Carroll *et al.*, 1985a,b; Sagan and Gresshoff, 1996; Schnabel *et al.*, 2005; Wopereis *et al.*, 2000; Penmetsa *et al.*, 2003; Oka-Kira *et al.*, 2005; Magori and Kawaguchi, 2009). We re-analyzed the effect of nitrate on nodulation in wild type and *sunn-4* mutants. A complete inhibition of nodulation on wild type plants was achieved by the addition of 10 mM KNO_3 and split-root experiments indicated that this effect involves systemic signals, in accordance to previously published data (Jeudy *et al.*, 2010; Carroll *et al.*, 1985a,b; Sodek and Moura Silva, 1996; Eskew *et al.*, 1989). Nodulation was also more strongly inhibited in the nitrogen-rich roots, than in the nitrogen-deprived roots. Jeudy *et al.* (2010), explained this effect because a local inhibition acts on top of a systemic inhibition. However, it is equally possible that addition of nitrate activates a concentration gradient of a nodule-inhibiting compound, which is strongest at the place of nitrate addition. Interestingly, nitrate-induced inhibition of nodulation, locally as well as systemically, was reduced in the *sunn-4* mutant. Many scientists have explained this partial tolerance by the fact that nitrate-mediated inhibition might involve the AON pathway. *Sunn-4* mutants are very strong mutants and in our condition, there was still a clear inhibition of nodulation, locally as well as systemically after nitrate addition. Eskew *et al.* (1989) described already in 1991 that nodulation of AON mutants is only partially tolerant to nitrate, because very high concentrations of nitrate were able to severely reduce nodule numbers on AON mutants. Hence, it might equally be possible that the lower sensitivity is a consequence of changes in hormone balances in the *sunn* mutants, whereby auxin levels might be the central actor. If nitrate addition would inhibit nodule formation via reducing the auxin levels in the root, higher nitrate concentrations would be required

Figure 4.17 ***(facing page)*:** Tree-based alignment of the CLE domain encoded by all *MtCLE* genes and of the CLE domain of all Arabidopsis *CLE* genes as well as the nodulation-specific *CLE* genes of *L. japonicus* and soybean.


-5 -4 -3 -2 -1 0 1 2 3 4 5 6
MtCLE9 RLVPTGPNRMHN
MtCLE30 RLVPSGPNPLHN
AtCLE9 RLVPSGPNPLHN
AtCLE10 RLVPSGPNPLHN
AtCLE14 RLVPKGPNPLHN
MtCLE18 RRVPTGPNPLHH
AtCLE8 RRVPTGPNPLHH
AtCLE12 RRVPSGPNPLHH
AtCLE11 RVVPSGPNPLHH
AtCLE13 RLVPSGPNPLHH
MtCLE8 RLVPTGPNPLHH
CLV3 RTVPSGPDPLHH
MtCLE29 RKVPTGPDPLHH
MtCLE15 RKVPTGPDPLHH
MtCLE23 RRVPTGANPLHN
MtCLE4 RGVPSGANPLHN
AtCLE40 RQVPTGSDPLHH
MtCLE20 RRVPNTSDPLHN
MtCLE17 RKVPTGSNPLHN
AtCLE20 RKVKTGSNPLHN
MtCLE11 RTVKKGSNPIHN
MtCLE24 RRVRKGSDPIHN
AtCLE45 RRVRRGSDPIHN
MtCLE22a RKVPSSPDPIHN
MtCLE22b TKVPTSPDPIHN
MtCLE19 RQVPSSPDPLHN
AtCLE43 RRIPSSPDRLHN
AtCLE27 RIVPSCPDPLHN
MtCLE28 RKVPSCPDPLHN
MtCLE3 RKVPSCPDPLHN
AtCLE26 RKVPRGPDPIHN
AtCLE25 RKVPNGPDPIHN
MtCLE2 RRVPNGPDPIHN
MtCLE25 RRVPNGPDPIHN
MtCLE1 RRVPNGPDPIHN
AtCLE18 RQIPTGPDPLHN
AtCLE17 RVVHTGPNPLHN
AtCLE16 RLVHTGPNPLHN
AtCLE22 RRVFTGPNPLHN
MtCLE21 RKIYTGPNPLHN
MtCLE7 RKVYTGPNPLHN
MtCLE22c RMVSSGPNPLHN
MtCLE16 RRVPTGPNPLHN
MtCLE31 RVVPTGPNPLHN
LjCLE3 RVVPTGPNPLHN
AtCLE19 RVIPTGPNPLHN
AtCLE21 RSIPTGPNPLHN
MtCLE10 TSTPGGPNPLHN
MtCLE5 HVVPSGPNPLHN
AtCLE42 HGVPSGPNPISN
TDIF HEVPSGPNPISN
AtCLE44 HEVPSGPNPISN
MtCLE6 HEVPSGPNPISN
AtCLE41 HEVPSGPNPISN
GmCLE14 RLAPEGPDPHHN
GmCLE39 RLTPEGPDPHHN
GmCLE35 RLAPGGPDPQHN
GmCLE37 RLAPGGPDPQHN
MtCLE12 RLSPGGPNHIHN
MtCLE27 RLSPGGPDRHHN
MtCLE14f RPSPGGLDPCHN
LjCLE-RS1 RLSPGGPDPQHN
LjCLE-RS2 RLSPGGPDPQHN
MtCLE13 RLSPAGPDPQHN
MtCLE14g RFSPRGPNPYHH
MtCLE14a RLSPGVPDPYHL
AtCLE6 RVSPGGPDPQHH
AtCLE7 RVSPGGPDPQHH
AtCLE5 RFSPGGPDPQHH
AtCLE2 RLSPGGPDPQHH
AtCLE1 RLSPGGPDPRHH
MtCLE26 RLSPGGPDPRHH
AtCLE3 RLSPGGPDPRHH
AtCLE4 RLSPGGPDPRHH
MtCLE14b RLSPGGPDPHHH
MtCLE14c RLSPGGPDPHHH
MtCLE14d RLSPGGPDPHHH
MtCLE14e RLSPGGPDPHHH
Group-I
Group-II
Group-III

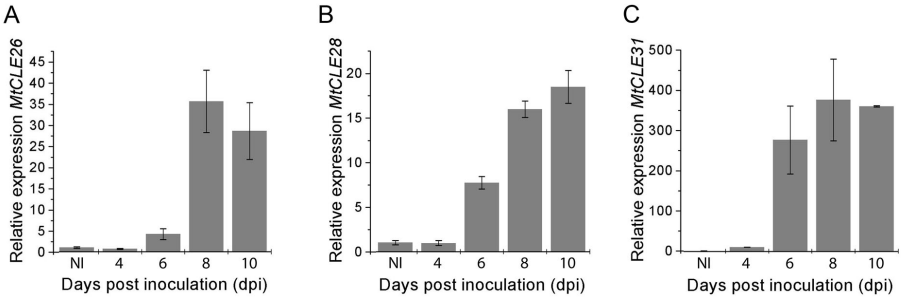


Figure 4.18: Expression analysis of *MtCLE26* (A), *MtCLE28* (B), and *MtCLE31* (C), by qRT-PCR on cDNA samples of zone-I root tissues of uninoculated plants (NI) and at 4, 6, 8 and 10 dpi. Data and error bars represent means ± SD. The experiment was repeated twice with comparable results.

in *sunn* mutants to reduce the high auxin levels sufficiently to block nodule formation. In Arabidopsis, a link between nitrate and auxin transport has recently been resolved (Krouk *et al.*, 2010; Beeckman and Friml, 2010).

The nitrate effect might act via CLE peptides because in *L. japonicus* as well as in soybean, nitrate-induced CLE peptide genes were identified, which inhibit nodulation in a *HAR1/NARK*-dependent way (Okamoto *et al.*, 2009; Reid *et al.*, 2011). In the recently released *M. truncatula* genomic data (Mt3.0 version) 6 more *CLE* genes (*MtCLE26* to *MtCLE31*) were identified by specialized BLAST searches, besides the 25 (*MtCLE1* to *MtCLE25*) identified before (Mortier *et al.*, 2010). However, none of these *MtCLE* genes were nitrate-upregulated. As the Mt3.0 version represents about 80 % of the genome, more *CLE* genes are expected to be found upon completion of the genome sequencing. In the genome of *L. japonicus*, which is comparable in size to that of *M. truncatula* (470 Mb) and which has currently been sequenced for 91,3 %, 39 *CLE* genes were identified (Sato *et al.*, 2008; Okamoto *et al.*, 2009).

In Arabidopsis, gain-of-function analysis has shown that there is a correlation between the function of the CLE peptide and its sequence (Hobe *et al.*, 2003; Ni and Clark, 2006). Based on the sequences of the CLE motif, CLE peptides have been divided in three groups (Ito *et al.*, 2006; Mortier *et al.*, 2011). 24 MtCLE peptides were found to belong to group-I, exemplified by CLV3 and which are promoters of cellular differentiation (Ito *et al.*, 2006); 2 MtCLE peptides are related to group-II, exemplified by TDIF and which prevent cellular differentiation and control the rate and orientation

of vascular cell division (Ito *et al.*, 2006; Etchells and Turner, 2010). Finally, 5 MtCLE peptides belong to group-III, to which the *M. truncatula, L. japonicus* and soybean CLE peptides belong, from which the expression is enhanced during nodulation and which affect nodulation after ectopic overexpression (Mortier *et al.*, 2010, 2011; Okamoto *et al.*, 2009; Reid *et al.*, 2011). The newly identified group-III CLE peptide genes, *MtCLE26* and *MtCLE27*, might possibly exert a nodulation-related function. Indeed, for *MtCLE26*, transcripts were detected by qRT-PCR analysis from 6 dpi on, similar to the group-III *MtCLE12* and *MtCLE13* genes (Mortier *et al.*, 2010). In contrast, *MtCLE27* transcripts were not detected during early nodulation (4 till 10 dpi). Still, an enhanced transcript level was found in whole root samples carrying 1-month-old nodules compared to uninoculated roots. Because this expression pattern was not detected in RNA derived from separate nodules of the same age (data not shown), these results might indicate that *MtCLE27* is expressed in the nodulated root tissue rather than in the nodules. This would be a new expression pattern for nodulation-related CLE peptides, but *promoter:GUS* analysis has to be executed to confirm this result. Also, expression of the group-I type *MtCLE28* and *MtCLE31* genes was upregulated during nodulation. Group-I CLE peptides are known to cause consumption of the root meristem upon ectopic addition or overexpression (Okamoto *et al.*, 2009; Mortier *et al.*, 2010, 2011; Reid *et al.*, 2011). Hence, it seems that a versatile group of *CLE* genes are activated during nodulation. Also in soybean nodulation, 6 *GmCLE* genes, from which 5 had a second copy in the genome, are induced during nodulation (Mortier *et al.*, 2011). We have previously shown that *MtCLE13* expression coincides with nodule primordium formation and that, together with *MtCLE12*, the gene is expressed in the apical meristematic part of the nodule (Mortier *et al.*, 2010). It will be interesting to analyze the expression patterns of the newly identified nodulation-related *CLE* genes. Moreover, ectopic expression might shed a light on the exact role of *MtCLE26, MtCLE27, MtCLE28, MtCLE29, MtCLE30* and *MtCLE31* in nodulated roots.

4.8 Materials and methods

Biological material

M. truncatula Gaertn. cv. Jemalong A17 and J5 as well as *sunn-4* mutants (Schnabel *et al.*, 2005) were grown and inoculated as described (Mergaert *et al.*, 2003). *Sinorhizobium meliloti 1021* pHC60-GFP (Cheng and Walker, 1998) and *Sm2011* pBHR-mRFP

(Smit *et al.*, 2005) were grown at 28°C in yeast extract broth medium (Vervliet *et al.*, 1975), supplemented with 10 mg L^{-1} tetracycline.

For the analysis of temporal expression during nodulation, nodules were harvested 4 to 10 dpi from plants grown in pouches, watered with nitrogen-poor SOLi medium, and inoculated with *Sm1021* pHC60-GFP. Infection threads were visible from 4 dpi on, nodule primordia at 6 dpi, and small nodules at 8 dpi. Two days later, at 10 dpi, slightly bigger nodules were observed. Tissue was collected by visualizing the green fluorescent bacteria under a stereomicroscope MZFLII (Leica Microsystems, Wetzlar, Germany) equipped with a blue-light source and a Leica GFP Plus filter set (λ_{ex} = 480/40; λ_{em} = 510 nm LP barrier filter). The zones I of uninoculated roots were isolated at the same developmental stage as the 4 dpi stage.

In silico identification of additional *M. truncatula CLE* genes

The *in silico* identification of additional *M. truncatula CLE* genes (Mt3.0) was based on the previously identified *MtCLE* genes (*MtCLE1* to *MtCLE25*) and performed as described by Mortier *et al.* (2010).

RNA extraction, cDNA synthesis, and qRT-PCR analysis

The RNA extraction, cDNA synthesis, and qRT-PCR analysis were performed as described by Mortier *et al.* (2010). Primers used (Table 4.4 and Mortier *et al.* (2010)) were unique in the MGI version 9.0 and the Medicago EST Navigation System database (Journet *et al.*, 2002). Each experiment was repeated at least two times with independent biological tissue.

Table 4.4: Primers used in the analyses.

Gene	Sense primer	Anti-sense primer
MtCLE26	CTCGTCCACTTGGTACTACTC	TCAATGGTGCCTTGGATCTG
MtCLE27	CACCAAGAACCAACAGAATAATC	GGACCTCCTGGACTTAACC
MtCLE28	GGCATTGTAGATAAGAAGGGTAAG	GGAGAGGATCTGGACAACTTG
MtCLE29	GACAGTGGTTGGAGAGTTGAG	AATAGGGTTGCCAATGTTATGATG
MtCLE30	TTCTTCTTCCTCTCCTCATCAG	GTCTCTTGTCTACACCATATCTTG
MtCLE31	CAGTAGCAGTGTGGCAGTAC	CGTGTTGTTGTAGCAAGAAGAC
40S	GCCATTGTCCAAGTTTGATGCTG	TTTTCCTACCAACTTCAAAACACCG

In vitro application of nitrogen

For the *in vitro* application of nitrogen 1, 5, 10 and 30 mM KNO_3 were supplemented to the SOLi medium of *in vitro*-grown plants. As a control, plants were grown without supplemented nitrogen. The plants were cultured at 25°C, 16-h photoperiod, and 70 μE $m^{-2}m^{-1}$ light intensity per day. For qRT-PCR analysis, 7-day-old seedlings were treated with KNO3 for 7 days, after which the roots of 18 plants under each condition were harvested. For nodule numbers, 2-day-old seedlings were treated with KNO_3 for 5 days, prior to inoculation with *Sm2011* pHC60-GFP, as well as during nodulation. Nodulation was assessed 12 days later. The experiment was repeated twice with comparable results.

Statistical analysis

The following generalized linear model (GLM) $Y_{ijk} = \ + genotype_j + experiment_k + error_{ijk}$ (Regression analysis) was fitted to the nodule number, partitioning phenotypic variation into fixed genotype and experiment effects and random error effects. Y_{ijk} is the phenotype of the i-th plant from the genotype j analyzed in experiment k; μ is the overall mean of the phenotypes obtained for all lines considered. Because the data has a Poisson distribution, a logarithm base e link function was incorporated. All analyses were done by means of the GenStat software[13].

[13] http://www.vsni.co.uk/software/genstat/

4.9 Supplemental data

Table 4.5: Influence of KNO_3 on the expression of *MtCLE* genes. qRT-PCR analysis was done on cDNA samples of roots grown for 7 days in the presence of 0, 1, 5, 10 or 30 mM KNO_3. The qRT-PCR analysis was done in triplicate. The average of the relative expression levels of each gene (AV) and the standard deviation (SD) were calculated. The relative expression levels were defined by the $2^{-\triangle\triangle C_t}$ method (Livak and Schmittgen, 2001). For *MtCLE13, MtCLE15, MtCLE20, MtCLE23, MtCLE27, MtCLE30* and *MtCLE31* no expression was measured. For the remaining *MtCLE* genes, which are not listed in the table, no descent primers were designed (Mortier *et al.*, 2010).

	0 mM		1 mM		5 mM		10 mM		30 mM	
Gene	AV	SD	AV	SD	AV	SD	AV	SD	AV	SD
MtCLE1	1.03	0.28	1.93	0.17	1.01	0.06	1.26	0.21	1.11	0.04
MtCLE2	1.01	0.21	0.93	0.05	0.57	0.09	0.51	0.14	0.91	0.09
MtCLE3	1.05	0.40	1.58	0.27	0.89	0.02	1.00	0.04	1.23	0.06
MtCLE4	1.03	0.30	0.89	0.05	0.55	0.01	0.51	0.06	0.63	0.01
MtCLE5	1.01	0.13	0.77	0.34	0.79	0.06	0.64	0.24	1.56	0.18
MtCLE6	1.05	0.41	1.16	0.14	0.70	0.03	0.77	0.07	0.93	0.05
MtCLE11	1.01	0.18	1.28	0.39	0.67	0.15	0.71	0.18	0.62	0.10
MtCLE12	1.12	0.68	0.91	0.07	0.55	0.09	0.87	0.99	0.94	0.07
MtCLE16	1.13	0.60	0.53	0.15	2.26	1.29	2.02	1.55	2.11	0.66
MtCLE17	1.03	0.34	1.35	0.19	1.01	0.11	0.92	0.09	1.02	0.09
MtCLE24	1.03	0.32	1.04	0.02	0.65	0.10	0.65	0.12	0.56	0.03
MtCLE26	1.21	0.95	0.36	0.63	2.41	2.10	0.30	0.51	1.16	0.69
MtCLE28	1.04	0.36	1.32	0.69	0.90	0.34	0.61	0.17	0.55	0.22
MtCLE29	1.13	0.66	0.75	0.71	1.97	1.13	1.10	1.01	0.32	0.56

Acknowledgements

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Authors contributions

The main part of this work was performed by V. Mortier. The bioinformatic and phylogenetic analysis were performed by S. Rombauts and C. Martens, respectively. S. Goormachtig and M. Holsters were involved in designing the research and revising the manuscript.

5

CLE peptides control nodule number and nodule meristem activity in *Medicago truncatula*

Virginie Mortier, Marcelle Holsters and Sofie Goormachtig

In preparation

Abstract
CLE genes encode small peptides involved in the balance between cell division and differentiation throughout plant development, including nodulation. Previously, we have reported that two *CLE* genes, *MtCLE12* and *MtCLE13*, might be involved in nodule organogenesis and autoregulation of nodulation in *Medicago truncatula*. Here we confirm this observation via RNAi analysis and also show that, for autoregulation of nodulation, *MtCLE12* and *MtCLE13* might act in the same pathway as the leucine-rich-repeat receptor-like-kinase SUNN that is potentially involved in CLE peptide binding. Moreover, the essential nodulation hormone cytokinin induces an *MtCLE13* expression pattern which is similar to that observed after nodulation and dependent on the nodulation components *NIN* and *MtCRE1*. These results suggest that several CLE peptides are involved in several aspects of nodulation.

5.1 Introduction

Legume plants escape nitrogen limitations of soils by entering a symbiosis with rhizobia. During this interaction, a controlled exchange of nitrogen compounds and dicarboxylic acids occurs between both symbiotic partners in newly formed root organs, the nodules. The legume-rhizobia symbiosis is initiated by an exchange of chemical signals of which the rhizobial lipo-chitooligosaccharides, the Nod Factors (NFs), are essential to activate two major developmental plant pathways (Jones *et al.*, 2007; Ferguson *et al.*, 2010). One pathway allows bacterial infection and is derived from the ancient arbuscular mycorrhizal symbiosis, while the other pathway reactivates cortical cell divisions to enable nodule primordium formation (Jones *et al.*, 2007; Ferguson *et al.*, 2010).

In most legumes studied so far, the bacteria enter via root hairs. After sensing the NFs, the root hairs respond with Ca^{2+} influx at the tip, immediately followed by Cl^{-} and K^{+} effluxes. These ion fluxes induce curling of the root hair to entrap a bacterial colony. Inside the root hair, an infection thread is formed via inverted tip growth that involves targeted exocytosis of membrane and cell wall material. This infection thread guides the bacteria towards deeper cell layers. Meanwhile cortical cells re-initiate cell division and form the nodule primordium. The infection threads proceed through several cell layers to reach the cells of the nodule primordium, where the bacteria are released in membrane enclosed compartments, the symbiosomes, and differentiate into

nitrogen fixing bacteroids. Eventually, nodules are subjected to natural senescence during which both symbiotic partners decay. This process involves massive protein degradation for nutrient remobilization (Van de Velde *et al.*, 2006). In *Medicago truncatula*, natural nodule senescence is initiated in single infected cells located at the center of the nodule fixation zone and a conical shaped senescence front moves towards the apex (Perez Guerra *et al.*, 2010). The phytohormones ethylene, gibberellins and abscissic acid (ABA) and reactive oxygen species (ROS) are believed to play a role in the process (Gonzalez *et al.*, 2001; Ferguson and Mathesius, 2003; Van de Velde *et al.*, 2006; Puppo *et al.*, 2005; Loscos *et al.*, 2008).

Several components of the early nodulation signaling pathway have been identified. In the model legume *M. truncatula*, NF recognition by the putative NF receptors (*MtNFP, MtLYK3/MtLYK4*) (Madsen *et al.*, 2003; Radutoiu *et al.*, 2003; Amor *et al.*, 2003; Arrighi *et al.*, 2006) located at the epidermis activate a signaling cascade, which is mediated by a leucine-rich-repeat receptor-like-kinase (LRR-RLK) DOESN'T MAKE INFECTIONS2 (*MtDMI2*) and a nuclear potassium channel (*MtDMI1*) and during which Ca^{2+} spiking in and around the nucleus of the root hair is essential (Endre *et al.*, 2002; Stracke *et al.*, 2002; Oldroyd and Downie, 2004; Imaizumi-Anraku *et al.*, 2005). Decoding of this Ca^{2+} signature by a Ca^{2+} calmodulin-binding protein (*MtDMI3*) and some accompanying proteins activates several transcription factors such as the NODULATION SIGNALING PATHWAY1 (NSP1), NSP2, ETHYLENE-RESPONSIVE BINDING DOMAIN FACTOR REQUIRED FOR NODULATION1 (ERN1), and NODULE INCEPTION (NIN), leading to the initiation of the nodulation process (Schauser *et al.*, 1999; Catoira *et al.*, 2000; Borisov *et al.*, 2003; Oldroyd and Long, 2003; Gleason *et al.*, 2006; Andriankaja *et al.*, 2007; Marsh *et al.*, 2007; Middleton *et al.*, 2007; Oldroyd and Downie, 2008).

Cytokinins play a central role in nodule organ formation and possibly also in the control of infection and the synchronization of the early responses (Murray *et al.*, 2007; Tirichine *et al.*, 2007; Gonzalez-Rizzo *et al.*, 2006; Frugier *et al.*, 2008). Nodule organogenesis might result amongst others from the activation of the *L. japonicus* LOTUS HISTIDINE KINASE (LjLHK1) or its *M. truncatula* ortholog CYTOKININ RESPONSE1 (MtCRE1), and subsequent signaling through cytokinin RESPONSE REGULATORS (RRs) (Gonzalez-Rizzo *et al.*, 2006; Murray *et al.*, 2007; Lohar *et al.*, 2006). The central role of cytokinin during nodulation was suggested by numerous experiments. *L. japonicus* knock-out mutants for the cytokinin receptor gene, LHK1, *M. truncatula* transgenic plants with suppressed expression of *CRE1*, or *Mtcre1* mu-

tant plants were defective in nodule primordia formation (Gonzalez-Rizzo *et al.*, 2006; Murray *et al.*, 2007; Plet *et al.*, 2011). Similarly, the ectopic expression of a cytokinin degrading enzyme in *L. japonicus* resulted in reduced nodulation (Lohar *et al.*, 2004). Additionally, a gain-of-function mutation of the LHK1 receptor provoked spontaneous nodules, indicating that cytokinin signaling is both necessary and sufficient for nodule formation (Tirichine *et al.*, 2007). Also a proper auxin balance is a prerequisite for nodule formation (Oldroyd and Downie, 2008; Ding and Oldroyd, 2009). In white clover (*Trifolium repens*), the auxin flow within the root vasculature was transiently inhibited at the site of infection, leading to auxin accumulation in the cortical region where the nodule primordia form (Mathesius *et al.*, 1998). A reduction in auxin flow has been confirmed by radioactive auxin tracer experiments for *M. truncatula* and vetch (*Vicia faba*), but not *L. japonicus* (Boot *et al.*, 1999; Pacios-Bras *et al.*, 2003; van Noorden *et al.*, 2006; Wasson *et al.*, 2006).

As nodule formation and nitrogen fixation are highly energy consuming processes, legumes restrict nodules to the minimal number required for optimal nitrogen supply. Several mechanisms have evolved to control nodulation at different levels and in function of diverse physiological and environmental parameters. One such mechanism, called autoregulation of nodulation (AON), involves a long-distance feedback mechanism (Kosslak and Bohlool, 1984; Carroll *et al.*, 1985a,b; Delves *et al.*, 1986; Pierce and Bauer, 1983; Nutman, 1952). A current model for AON states that, an unknown signal Q, produced in the roots in response to rhizobial infection, is translocated to the shoot where it induces the formation/activation of an unknown 'shoot derived inhibitor' (SDI), which is transmitted to the roots to inhibit subsequent nodule initiation (Nishimura *et al.*, 2002a). Although the molecular characteristics of the Q and SDI signal remain unknown, several AON-deficient mutants have been identified, amongst which *super numeric nodules* (*sunn*) in *M. truncatula* (Krusell *et al.*, 2002; Nishimura *et al.*, 2002a; Searle *et al.*, 2003; Schnabel *et al.*, 2005; Oka-Kira *et al.*, 2005; Duc and Messager, 1989; Sagan and Gresshoff, 1996). *Sunn* is characterized by a hypernodulation (or supernodulation) phenotype, with numerous, rather small nodules developing over a wider range of the roots as compared to wild type plants. Molecular characterization indicated that a gene encoding a CLAVATA1 (CLV1)-like LRR-RLK was affected in *sunn* mutants and in its orthologs in pea, soybean and *L. japonicus* (Krusell *et al.*, 2002; Nishimura *et al.*, 2002a; Searle *et al.*, 2003; Schnabel *et al.*, 2005). Although transcripts of *SUNN* were detected throughout the plants (Nontachaiyapoom *et al.*, 2007; Schnabel *et al.*, 2005), reciprocal grafting experiments between *sunn* mutants

and wild type plants have shown that the hypernodulation phenotype is determined by the shoot genotype (Schnabel *et al.*, 2005).

Due to structural similarities between SUNN and CLV1, SUNN is suggested to bind CLV3/EMBRYO SURROUNDING REGION (CLE) peptides (Clark *et al.*, 1997; Fletcher *et al.*, 1999; Kondo *et al.*, 2006; Ogawa *et al.*, 2008). *CLE* genes are involved in shoot and root apical meristem (SAM and RAM, respectively) homeostasis, vascular differentiation and nodulation (Fletcher *et al.*, 1999; Fiers *et al.*, 2005; Hirakawa *et al.*, 2010a; Ito *et al.*, 2006; Okamoto *et al.*, 2009; Mortier *et al.*, 2010, 2011; Reid *et al.*, 2011). The CLE peptides are 12 to 13 amino acids long and are cleaved from a 14 amino acids long conserved CLE domain, located at or close to the carboxyl terminus of the CLE preproproteins (Kondo *et al.*, 2006; Ito *et al.*, 2006; Ni and Clark, 2006; Mitchum *et al.*, 2008; Oelkers *et al.*, 2008). A hydrophobic signal peptide at the N-terminal site of most CLE proteins suggests that the peptides are targeted to the secretory pathway and act as intercellular signals (Sawa *et al.*, 2006; Oelkers *et al.*, 2008). The region between the signal peptide and the CLE domain is highly variable and would have no specific function (Ni and Clark, 2006). *CLE* genes have been identified in the genome of monocotyledonous and dicotyledonous plants, in families that can consist of up to 40 members (Cock and McCormick, 2001; Oelkers *et al.*, 2008; Mitchum *et al.*, 2008). Expression analysis in Arabidopsis and *M. truncatula* indicated many redundant but also tissue specific expression patterns (Sharma *et al.*, 2003; Mortier *et al.*, 2010). In addition, a high level of redundancy is observed between members with related sequences in gain-of-function analyses (Strabala *et al.*, 2006; Jun *et al.*, 2008). Based on sequence comparison, at least three groups of CLE peptides can be distinguished (Strabala *et al.*, 2006; Jun *et al.*, 2008; Mortier *et al.*, 2010, 2011). Group-I peptides, exemplified by CLV3, result in premature root and shoot meristem growth arrest when exogenously applied or ectopically expressed, indicating that they are promoters of cellular differentiation. Members of group-II, exemplified by TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR (TDIF), prevent cellular differentiation, as evidenced by the suppression of procambium-to-xylem transdifferentiation in Zinnia (*Zinnia elegans*) cell cultures, and control the rate and orientation of vascular cell division (Ito *et al.*, 2006; Etchells and Turner, 2010). Although these studies would suggest two groups with opposing functions, synergistic actions between these groups of peptides have been demonstrated (Whitford *et al.*, 2008). No function was assigned yet to the group-III members of Arabidopsis, as functional analysis resulted in conflicting data (Ito *et al.*, 2006; Meng *et al.*, 2010). How-

ever, in *M. truncatula, L. japonicus* and soybean nodulation-related CLE peptides were identified, which belong to group-III (Mortier *et al.*, 2010, 2011; Okamoto *et al.*, 2009; Reid *et al.*, 2011). Gain-of-function analysis of the nodule-related *GmCLE* (*GmRIC1* and *GmRIC2*), *LjCLE* (*LjCLE-RS1* and *LjCLE-RS2*) and *MtCLE* (*MtCLE12* and *MtCLE13*) genes suggested these CLE peptides to be good candidates for activating AON (Okamoto *et al.*, 2009; Mortier *et al.*, 2010; Reid *et al.*, 2011). Ectopic expression of these *CLE* genes strongly reduced or abolished nodulation locally and systemically in a *HAR1-, NARK*- and partially *SUNN*-dependent way (Okamoto *et al.*, 2009; Reid *et al.*, 2011). So far, inhibition of nodulation was specific for group-III genes because overexpression of *CLE* genes with a structurally unrelated CLE domain (*MtCLE4* and *LjCLE3* of group-I) did not induce this effect (Okamoto *et al.*, 2009; Mortier *et al.*, 2010, 2011; Reid *et al.*, 2011). Interestingly, the expression of *MtCLE13* coincided with the activation and progression of AON (Caetano-Anolles *et al.*, 1991; Li *et al.*, 2009; Mortier *et al.*, 2010). So far, however, it is not proven that these group-III peptides might be the long-distance signals that travel from the developing nodules where they are expressed, to the shoot where SUNN and its orthologs are active for AON.

Because they are expressed at the onset of nodule primordium formation and in the nodule meristem, CLE peptides might also influence the balance between proliferation and differentiation during nodule organogenesis, in analogy to what was seen for other CLE peptides in the SAM and RAM homeostasis and during vascular development (Stahl *et al.*, 2009; Mortier *et al.*, 2010; Hirakawa *et al.*, 2010b; Guo *et al.*, 2010). In agreement, cytokinin could trigger *MtCLE13, GmCLE14-GmCLE39* and *GmCLE35-GmCLE37* expression in respectively *M. truncatula* and soybean, but a detailed connection between cytokinin and CLE peptides during nodulation is currently lacking. In the regulation of protoxylem formation, a cross-talk between *CLE* genes and cytokinin signaling pathways was suggested through negative regulation of type A RRs (Kondo *et al.*, 2011).

We unraveled the interaction between *MtCLE13* expression and cytokinin signaling and show that the cytokinin induction of *MtCLE13* is dependent on *NIN* and *MtCRE1* and that the expression pattern overlaps with the expression pattern reported for *MtCLE13* during nodulation (Mortier *et al.*, 2010). In addition, we show that the effect of *MtCLE13* ectopic expression on nodulation is dependent on *SUNN*, but that differences in allele functionality exist between the different *sunn* mutants. Finally, we observed that simultaneous knock-down of *MtCLE12* and *MtCLE13* results in an elevated nodule number and premature nodule senescence of infected cells of mature nodules.

5.2 Results

5.2.1 *MtCLE13* induction by cytokinin

Promoter:GUS analysis has previously shown that *MtCLE13* is activated during early nodulation in the cortex as a gradient, with the highest expression in the inner cortex, where cell division is taking place (Mortier *et al.*, 2010). To see whether cytokinin signaling is involved in this expression pattern, the 6-benzylaminopurine (BAP)-induced *MtCLE13* expression pattern was studied at a microscopic level (Figure 5.1, A-C). As shown in Figure 5.1A, before BAP supplementation, no GUS staining is seen. Twenty-four hours after BAP supplementation a gradient of *pMtCLE13:GUS* expression was observed, with the highest expression in the inner cortical cells and a lower expression in the outer cortical cells (Figure 5.1B). Also expression in the pericycle and endodermis was observed (Figure 5.1C). This expression pattern more or less matches the expression pattern reported for *MtCLE13* during early nodulation (Mortier *et al.*, 2010). In addition, BAP also induced *pMtCLE13:GUS* expression in the vascular tissue (Figure 5.1C).

Next, we analyzed if the components of the early NF signaling pathway are required for the induction of *MtCLE13* expression by cytokinin. The transcript level of *MtCLE13* before and after addition of 10^{-7} M BAP in *nfp, dmi3, bit1-1, nsp1, nsp2, cre1-1* and *nin-1* mutants was analyzed by qRT-PCR. As shown in Figure 5.1D, *MtCLE13* expression was induced after addition of cytokinin in all nodulation mutants, except in the *cre1-1* and *nin-1* mutant. This indicates that not only the cytokinin receptor MtCRE1 but also the transcription factor NIN are essential for *MtCLE13* induction by cytokinin.

To confirm this result, *pMtCLE13:GUS* transgenic roots were generated in wild type (A17), *cre1-1* and *nin-1* plants and analyzed 24 hours after supplementation of 10^{-7} M BAP. In wild type plants treated with BAP, *MtCLE13* expression was seen in the elongation zone of the root, just above the roottip (Figure 5.1E). This expression pattern was not seen in the *cre1-1* and *nin-1* mutants treated with cytokinin (Figure 5.1E). Consequently, these results corroborate the result of the qRT-PCR analysis, and confirm that the induction of *MtCLE13* expression by BAP is dependent on *MtCRE1* and *NIN*.

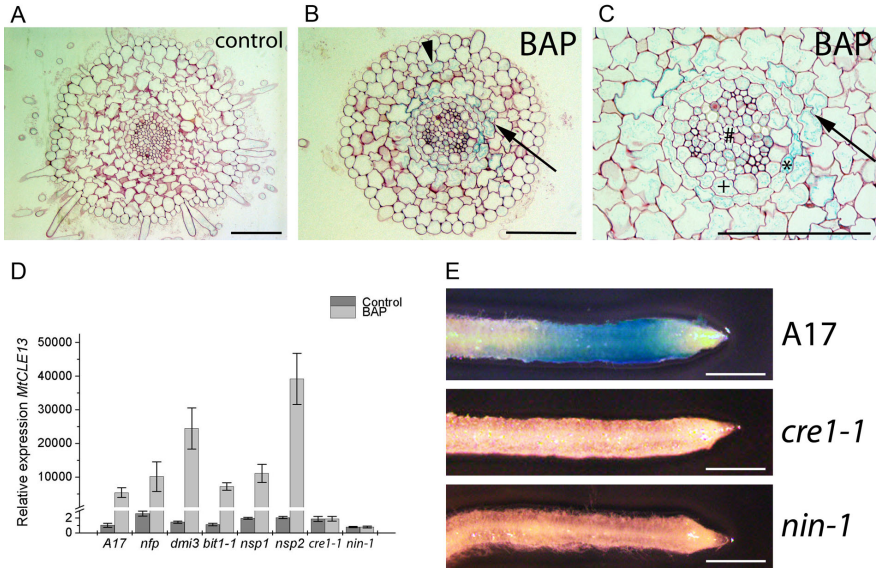

Figure 5.1: *MtCLE13* expression in nodulation mutants treated with 10^{-7} M cytokinin (BAP). A, Transverse sections through a root segment of A17 plants transformed with *pMtCLE13:GUS* before supplementation of BAP. No GUS staining is seen. B and C, Twenty-four hours after BAP supplementation a gradient of *pMtCLE13:GUS* expression is observed, with the highest expression in the inner cortical cells (arrow) and a lower expression in the outer cortical cells (arrowhead). Also in the pericycle (cross), endodermis (asterisk) and vascular tissue (hash), a faint blue color is seen. C is a magnification of the microscopic image represented in B. The section comes from the most mature part (laying distally from the roottip) of the blue stained region depicted in E. D, *MtCLE13* expression in wild type (A17), *nfp, dmi3, bit1-1, nsp1, nsp2, cre1-1* or *nin-1* plants as measured by qRT-PCR analysis on cDNA samples of untreated roots (control) and roots treated for 24 h with BAP. A cutoff was set at Ct value ≥ 35. Data and error bars represent means $\pm$ SD. The experiment was repeated twice with comparable results. E, *pMtCLE13:GUS* activity in roots of wild type (A17), *cre1-1* and *nin-1* plants treated for 24 h with BAP (n = 15-20). *MtCLE13* expression is visualized just above the roottip of treated wild type plants. No staining is visible in the roots of *cre1-1* and *nin-1* plants. Bars = 0.25 mm (A-C) and 1 mm (E).

5.2.2 *SUNN* dependence of *35S:MtCLE13*-induced inhibition of nodulation

Previous results have shown that ectopic expression of the group-III peptide genes, *MtCLE12* and *MtCLE13*, resulted in an inhibition of nodulation in a systemic and partially

SUNN-dependent manner (Mortier *et al.*, 2010). These data were in disagreement with the results obtained for *L. japonicus* and soybean (Okamoto *et al.*, 2009; Reid *et al.*, 2011). Because *sunn-1*, the *SUNN* mutant allele used in the experiment, was shown to be a weak allele (Schnabel *et al.*, 2010), we repeated the experiments using the other 3 mutant alleles available for *SUNN* (*sunn-2* to *sunn-4*) (Schnabel *et al.*, 2005). In contrast to *sunn-1* and *sunn-2*, carrying a missense mutation, *sunn-3* and *sunn-4* contain a nonsense mutation (Schnabel *et al.*, 2005). In a first set-up to control whether differences in allele functionality can be observed between these different *sunn* mutants, we analyzed whether differences in nodule number could be seen between the different *sunn* mutants. Several plants of each *sunn* mutant were grown in an aeroponics system, inoculated with *Sm2011*-GFP, and nodulation was assessed 14 days later. Nodulation of control roots (wild type A17) resulted on average in 6.65 $\pm$ 0.90 nodules, which is statistically different from the nodule number counted on *sunn-1* (21.00 $\pm$ 1.62), *sunn-2* (27.88 $\pm$ 1.86), *sunn-3* (33.43 $\pm$ 1.98), and *sunn-4* (31.54 $\pm$ 1.96) mutant plants ($p < 0.001$, Regression analysis) (Figure 5.2A). The variations in nodule number observed between the four *sunn* mutants were not consistently seen in different repeats (compare for instance Figure 5.2A with Figure 5.2B).

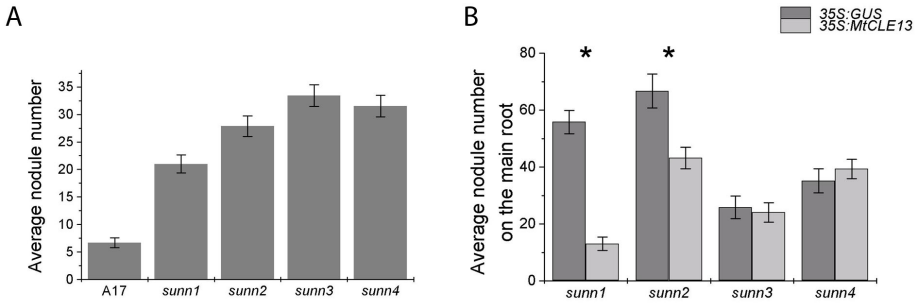

Figure 5.2: Nodule number on sunn mutant plants. A, The average nodule number counted at 14 dpi on wild type (A17), *sunn-1, sunn-2, sunn-3*, and *sunn-4* plants (n between 41 and 46). B, The average nodule number at 14 dpi on the wild type main roots of *sunn-1, sunn-2, sunn-3,* and *sunn-4* mutant plants carrying additional *35S:GUS* or *35S:MtCLE13* transgenic roots (n between 19 and 37). Asterisks indicate statistically significant differences between the *35S:GUS* and *35S:MtCLE13* lines ($p < 0.001$, Regression analysis). Data and error bars represent means $\pm$ SE. Both experiments were repeated twice with comparable results. The total mean of both biological repeats is represented in the graph.

Next, we controlled the effect of ectopic expression of *MtCLE13* on nodule number of non transformed wild type roots. In wild type plants, the ectopic expression of *MtCLE13* results in an inhibition of nodulation (Mortier *et al.*, 2010). For *sunn-1* and *sunn-2*, nodule numbers on wild type roots of plants carrying *35S:MtCLE13* transgenic roots were reduced, as compared to control roots (*35S:GUS*) (Figure 5.2B). For *sunn-1*, nodule number decreased on average, from 55.80 $\pm$ 2.33 to 13.03 $\pm$ 4.14 nodules and for *sunn-2* from 66.72 $\pm$ 6.01 to 43.14 $\pm$ 3.77 nodules (Figure 5.2B). The reduction in nodule number in a *sunn-1* mutant background has been described previously (Mortier *et al.*, 2010). No statistically significant differences in nodule number could be observed for *sunn-3* (*35S:GUS*: 25.84 $\pm$ 3.99; *35S:MtCLE13*: 24.01 $\pm$ 3.39) and *sunn-4* mutants (*35S:GUS*: 35.15 $\pm$ 4.25; *35S:MtCLE13*: 39.34 $\pm$ 3.40) (Figure 5.2B). Together, these data demonstrate that the inhibition of nodulation by *MtCLE13* overexpression is indeed dependent on *SUNN* and that differences in allele functionality exist between the different *sunn* mutants.

5.2.3 Effect of knock-down of *MtCLE12* and *MtCLE13* expression on nodulation

To investigate the effect of knock-down of *MtCLE12* and *MtCLE13* on the nodulation process, we created RNAi hairpin constructs and generated composite plants using *Agrobacterium rhizogenes* transformation.

For *MtCLE12* we made use of the complete open reading frame (ORF) (246 bp), but mutated the startcodon from ATG to ATA to avoid the production of MtCLE12 from the hairpin construct. Next, the expression level of *MtCLE12* was measured by qRT-PCR analysis on cDNA samples of transgenic roots expressing either *35S:GUS* (control) or a hairpin construct to induce knock-down of *MtCLE12* (RNAi *MtCLE12*) and from the same lines nodule numbers were counted at 21 days post inoculation (dpi). Of the 12 knock-down lines analyzed, 6 had a lower level of *MtCLE12* expression than any of the control lines (Figure 5.3A). However, the lines with the lowest expression of *MtCLE12* carried a nodule number comparable to the nodule numbers found on the control plants (Figure 5.3B).

Next, we investigated whether knock-down of *MtCLE12* resulted in differences in nodule development. Therefore, the nodules of the lines which had the lowest level of *MtCLE12* expression (RNAi *MtCLE12* 2, 4 and 8) were sectioned and subjected to microscopic analysis. No differences in nodule development were observed between

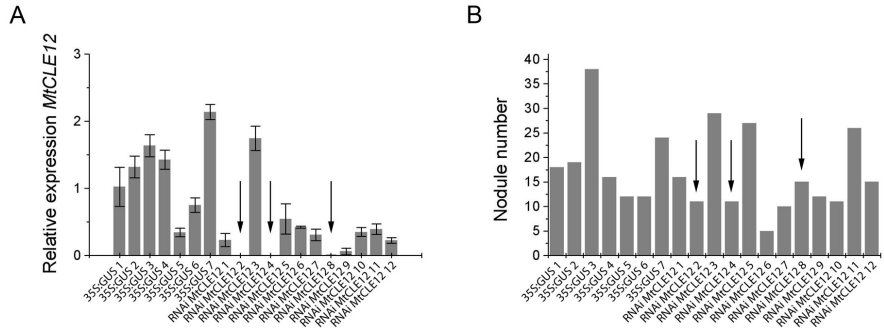

Figure 5.3: Effect of *MtCLE12* knock-down on nodule number. A, qRT-PCR analysis of *MtCLE12* expression in cDNA samples of transgenic roots expressing either *35S:GUS* (control) or a *MtCLE12* hairpin construct (RNAi *MtCLE12*). B, Nodule number at 21 dpi of the same lines as shown in A. Arrows indicate lines with lowest level of *MtCLE12* expression. Data and error bars represent means ± SD.

nodules of control lines (Figure 5.4, A-C) and of RNAi *MtCLE12* lines (Figure 5.4, D-F).

The experiments were repeated to analyze the effect of *MtCLE13* knock-down on nodulation. To perform the experiment, a 188 bp long fragment was selected, which is located in the *MtCLE13* ORF, but which neither includes the start codon nor the CLE domain sequence. The experiment was carried out in an identical way to what is described for the knock-down of *MtCLE12*. To check the expression levels, RNA was prepared from inoculated control and RNAi roots. These results show that there was no knock-down of *MtCLE13*.

As a high level of redundancy has previously been observed between CLE peptides with similar sequences, a hairpin construct was designed which should downregulate *MtCLE12* as well as *MtCLE13* (Strabala *et al.*, 2006; Jun *et al.*, 2008). To do so, the sequences used for the knock-down of *MtCLE12* and *MtCLE13* separately, were fused and subsequently cloned in one hairpin construct. Next, composite plants were inoculated with *Sm2011* pBHR-mRFP and nodule number was assessed at 9 dpi. An average of 6.46 ± 0.77 nodules were counted on *35S:GUS* control roots, while RNAi *MtCLE12/13* plants bared on average 21.18 ± 1.56 nodules ($p < 0.001$, Regression analysis) (Figure 5.5).

To confirm the nodulation phenotype, the *MtCLE12* and/or *MtCLE13* expression

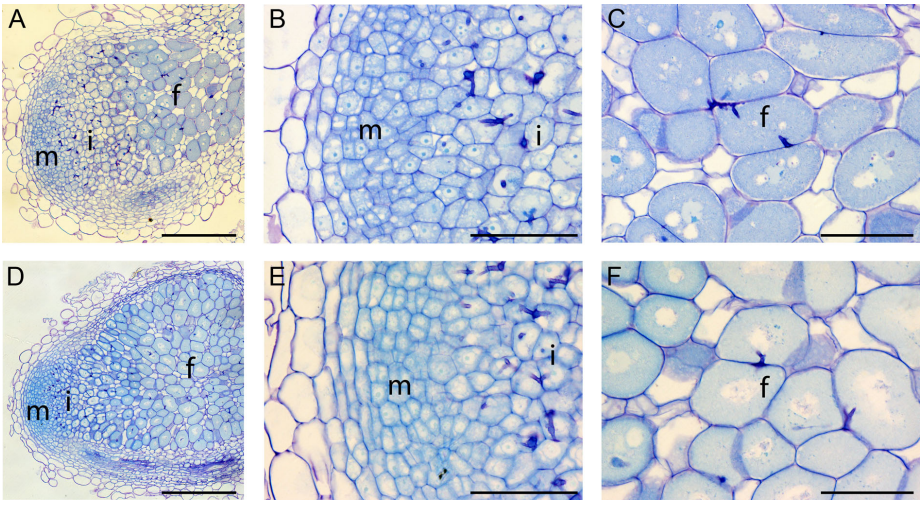

Figure 5.4: Analysis of *MtCLE12* knock-down on nodule development. Bright-field, microscopical analysis of nodules expressing either *35S:GUS* or an RNAi *MtCLE12* hairpin construct at 21 dpi. A, longitudinal section through a nodule ectopically expressing *35S:GUS* (control). B, Detail of meristem and infection zone of the nodule represented in A. C, Detail of the fixation zone of the nodule represented in A. D, Longitudinal section through a nodule ectopically expressing a *MtCLE12* hairpin construct. E, Detail of meristem and infection zone of the nodule represented in D. F, Detail of the fixation zone of the nodule represented in D. The sections were stained with toluidine blue. m, meristem; i, infection zone; f, fixation zone. Bars = 0.5 mm (A and D), 0.2 mm (B and E) and 50 m (C and F).

levels were measured and correlated with nodule number. Because *MtCLE13* is specifically expressed during nodulation, a pool of 10 nodules of *35S:GUS* (control) or *MtCLE12/13* knock-down lines were taken. For *MtCLE13*, a lower level of expression was measured in all RNAi *MtCLE12/13* lines as compared to the *35S:GUS* lines (Figure 5.6A). The same result was obtained for the expression of *MtCLE12*, except in one sample (RNAi *MtCLE12/13 2*), in which an expression level was measured similar to the expression level in the control plants (Figure 5.6A). Comparing the expression levels with nodule numbers revealed that the only plant without downregulation of *MtCLE12* (RNAi *MtCLE12/13 2*) has a nodule number (nn = 6) comparable to the nodule number on control plants (nn = 6 or 7), while the other RNAi *MtCLE12/13* roots carried more nodules (nn = 18.8 $\pm$ 2.4 on average) (Figure 5.6B).

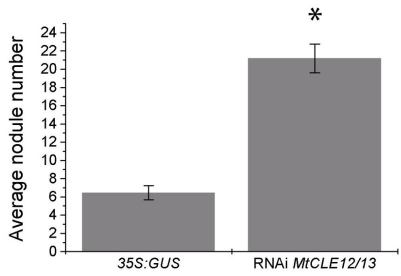

Figure 5.5: Nodule number on transgenic roots carrying an RNAi hairpin construct knocking-down *MtCLE12* as well as *MtCLE13* (RNAi *MtCLE12/13*). Average nodule number on roots expressing either *35S:GUS* (control) or RNAi *MtCLE12/13* at 9 dpi (n between 35 and 41). Data and error bars represent means $\pm$ SE. Asterisk indicates a statistically significant difference in comparison to control roots ($p < 0.001$, Regression analysis). The experiment was repeated twice with comparable results. The total mean of both biological repeats is represented in the graph.

Next, we investigated whether the simultaneous knock-down of *MtCLE12* and *MtCLE13* resulted in a phenotype at the level of nodule development. Fourteen day-old nodules of RNAi lines which had a low level of *MtCLE12* and *MtCLE13* expression were sectioned and analyzed by light microscopy. Upon toluidine blue staining, senescing cells are more purple and non-senescing cells more blue (Van de Velde et al., 2006). In the nodules of RNAi *MtCLE12/13* lines senescence was observed in half of the infected cells (Figure 5.7, D and F), while almost no senescence was observed in control nodules (*35S:GUS*) of the same age (Figure 5.7, A and C). Via qRT-PCR analysis we measured the expression level of *MtHAP2-1* in the different cDNA samples. *MtHAP2-1* transcripts are most abundant in the nodule meristematic zone, and RNAi and *in situ* hybridization indicated a role during nodule organogenesis, probably via the control of the nodule meristem function (Combier *et al.*, 2006). A lower level of *MtHAP2-1* was measured in most cDNA samples of RNAi *MtCLE12/13* lines (RNAi *MtCLE12/13* 1, 3, 4 and 5), indicative of a smaller meristem and thus of an abnormal nodule developmental process (Figure 5.6C).

In conclusion, the simultaneous knock-down of *MtCLE12* and *MtCLE13* results in an elevation of nodule number and a higher level of nodule senescence in infected cells of mature nodules.

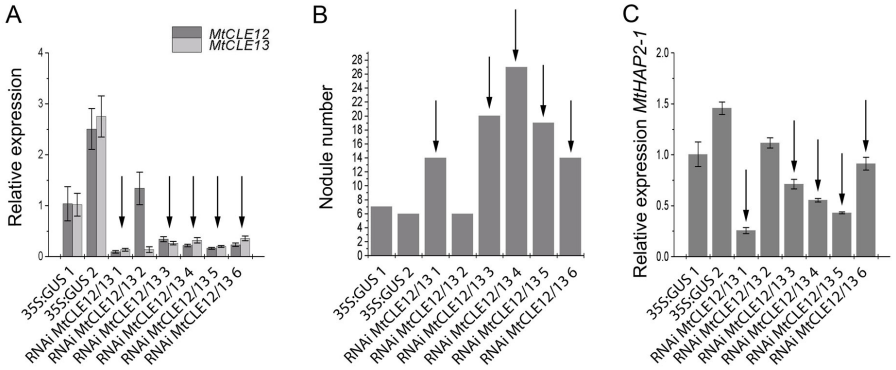

Figure 5.6: Analysis of the effect of knock-down of *MtCLE12* and *MtCLE13* on nodule number. A, qRT-PCR analysis of *MtCLE12* and *MtCLE13* expression in cDNA samples of transgenic roots expressing either *35S:GUS* (control) or an hairpin construct to induce knock-down of both *MtCLE12* and *MtCLE13* (RNAi *MtCLE12/13*). B, Nodule number at 9 dpi on the roots of the plants mentioned in A. C, qRT-PCR analysis of *MtHAP2-1* expression in cDNA samples mentioned in A. Arrows indicate lines with low levels of both *MtCLE12* and *MtCLE13* expression. Data and error bars represent means ± SD.

5.2.4 Analysis of the knock-down of *MtCLE12* and *MtCLE13* in a *sunn-4* mutant background

Previous experiments have shown that the ectopic expression of *MtCLE13* results in an inhibition of nodule formation and that this phenotype is dependent on *SUNN* (Mortier *et al.*, 2010). To check whether the hypernodulation phenotype observed during simultaneous knock-down of *MtCLE12* and *MtCLE13* interacts with that of *sunn* mutants, the RNAi experiment was repeated in a *sunn-4* mutant background. None of the analyzed knock-down lines had a nodule number that was more elevated than the nodule number of any of the control lines (*35S:GUS*) (Figure 5.8B). More specifically, although downregulation of *MtCLE12* and *MtCLE13* was measured in some samples (RNAi *MtCLE12/13* 4, 7, and 11) (Figure 5.8A), these low levels of expression could not be correlated with higher nodule numbers compared to nodule numbers found in control lines (Figure 5.8B). These results indicate that no enhancement of the hypernodulation phenotype is obtained by the simultaneous downregulation of *MtCLE12* and *MtCLE13* indicating that *MtCLE12, MtCLE13* and *SUNN* act in the same pathway.

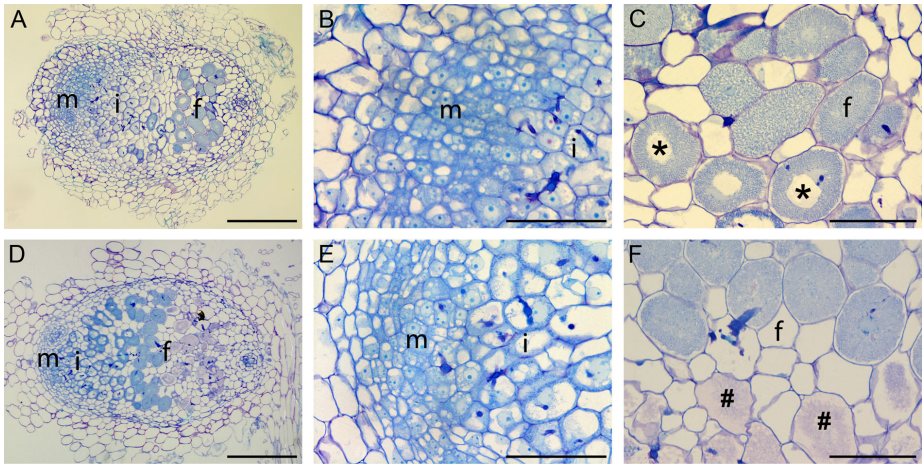

Figure 5.7: Microscopic analysis of nodules derived from *MtCLE12/MtCLE13* RNAi lines at 14 dpi. A, Longitudinal section through a nodule ectopically expressing *35S:GUS* (control). B, Detail of meristem and infection zone of the nodule represented in A. C, Detail of the fixation zone of the nodule represented in A. Large infected cells that are totally filled with symbiosomes are observed (asterisks). D, Longitudinal section through a nodule ectopically expressing RNAi *MtCLE12/MtCLE13*. E, Detail of meristem and infection zone of the nodule represented in D. F, Detail of the fixation zone of the nodule represented in D. Senescence is observed in infected cells located proximal to the root (hashes). The sections were stained with toluidine blue. m, meristem; i, infection zone; f, fixation zone. Bars = 0.5 mm (A and D), 0.2 mm (B and E) and 50 μm (C and F).

5.3 Discussion

So far, CLE peptide signaling is related to the balance between cell proliferation and differentiation. During nodulation, cytokinin signaling is essential for the re-initiation of cortical cell division (Gonzalez-Rizzo *et al.*, 2006; Murray *et al.*, 2007; Tirichine *et al.*, 2007). A role for *MtCLE13* downstream of cytokinin in the control of nodule organ development has been suggested because *MtCLE13* transcript expression is quickly induced by cytokinin and because *MtCLE13* expression was dependent on the early NF signaling components (Mortier *et al.*, 2010). In addition, by making use of *promoter:GUS* fusion, *MtCLE13* was localized in the cortex, at the onset or even before visible cell divisions were observed, in a gradient with the highest expression in the inner cortex and a lower expression in the outer cortex (Mortier *et al.*, 2010). Mi-

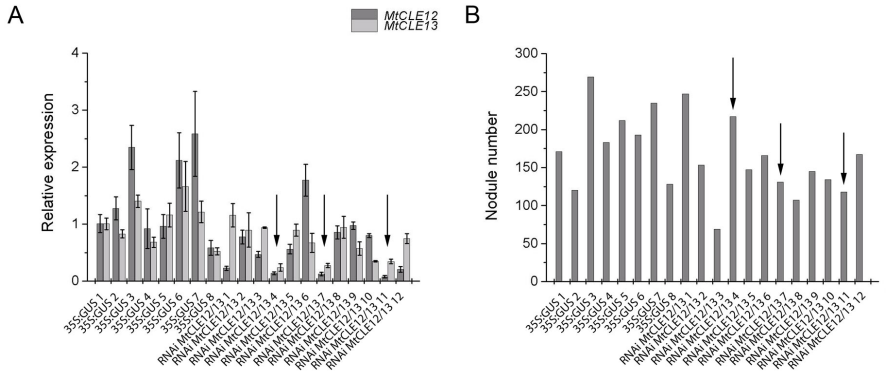

Figure 5.8: Analysis of the effect of knock-down of *MtCLE12* and *MtCLE13* on nodule number in a *sunn-4* mutant background. A, qRT-PCR analysis of *MtCLE12* and *MtCLE13* expression in cDNA samples of transgenic roots expressing either *35S:GUS* (control) or a hairpin construct to induce knock-down of both *MtCLE12* and *MtCLE13* (RNAi *MtCLE12/13*). Data and error bars represent means ± SD. B, Nodule number at 7 dpi on the roots of the plants mentioned in A. Arrows indicate lines with lowest level of both *MtCLE12* and *MtCLE13* expression.

croscopic analysis of *pMtCLE13:GUS* root sections of BAP-treated plants revealed that the observed expression pattern highly overlapped with the expression pattern reported for *MtCLE13* during early nodulation (Mortier *et al.*, 2010). Hence, the *MtCLE13* expression pattern might visualize the cytokinin landscape of the root after rhizobial inoculation. The *MtCLE13* expression pattern might also reflect the responsiveness of the *M. truncatula* root cells towards cytokinin. Expression was only observed in the cortical region of the elongation zone of the root, which is the only region of the root which is susceptible for nodulation. Hence, susceptibility to cytokinin might be one of the ways that controls the responsiveness of the root to nodule formation. To further link cytokinin signaling with *MtCLE13* expression during nodulation, we investigated whether components of the early NF signaling pathway are essential for BAP-induced *MtCLE13* expression. Interestingly, the expression was not dependent on *NFP, DMI3, ERN1, NSP1* and *NSP2*, but was dependent on the putative transcriptional regulator *NIN* and the cytokinin receptor *MtCRE1*, for which a role during cytokinin signaling and nodulation has previously been described (Frugier *et al.*, 2008). *NIN* was also shown to be essential downstream of cytokinin for primordium activation (Plet *et al.*, 2011). Hence, the expression of *MtCLE13* during nodulation is positioned in the corti-

cal signaling cascade.

Previous results suggested the involvement of *MtCLE13* in nodule organogenesis and in the control of nodule numbers, possibly via the AON mechanism. Accordingly, ectopic expression of group-III nodulation-related CLE peptides abolished nodulation by a long distance mechanism and this inhibition was dependent on the LRR-RLKs HAR1 and NARK in respectively, *L. japonicus* and soybean (Okamoto *et al.*, 2009; Reid *et al.*, 2011). However, for *M. truncatula*, the effect was only partially dependent on the orthologous LRR-RLK SUNN (Mortier *et al.*, 2010). Because the mutant allele *sunn-1*, used in the experiments, was shown to be a weak allele, the experiments were repeated with three other available alleles, *sunn-2, sunn-3* and *sunn-4* (Schnabel *et al.*, 2005, 2010). An initial experiment revealed that all four *sunn* mutants exhibit a supernodulation phenotype. Ectopic overexpression of *MtCLE13* resulted in an inhibition of nodulation on the main, untransformed roots of *sunn-1* and *sunn-2* mutants, but not of *sunn-3* and *sunn-4* mutants. *Sunn-1* and *sunn-2* are caused by missense mutations, while *sunn-3* and *sunn-4* are nonsense mutations, suggesting that especially the *sunn-4* allele, that is truncated between the signal peptide and the LRR domain, might be real a knock-out mutant (Schnabel *et al.*, 2005). Phenotypic differences between different alleles of mutants in *CLV1*-like LRR-RLKs have been reported previously (Kawaguchi *et al.*, 2002; Krusell *et al.*, 2002; Nishimura *et al.*, 2002a; Schnabel *et al.*, 2005). Interestingly, our observations are opposite to the observations for *CLV*, from which nonsense mutations are much weaker than missense mutations (Dievart *et al.*, 2003). Since SUNN probably belongs to a large receptor complex just like CLV1 and HAR1, these differences might illustrate differences in the relative contribution of orthologous components in different plant species. Together, these experiments indicate in accordance to the *HAR1*- and *NARK*-dependence for nodule inhibition by overexpression of *LjCLE-RS1/LjCLE-RS2* and *GmRIC1/GmRIC2*, that *MtCLE13* acts upstream of *SUNN* to inhibit nodulation and that, *sunn-3* and *sunn-4* are much more stronger alleles compared to *sunn-1* and *sunn-2*. This is in agreement with Schnabel *et al.* (2010) who reported stronger nodulation-related phenotypes for *sunn-4* than for *sunn-1*.

CLE peptides are acting redundantly and this hampers knock-out analysis. So far in Arabidopsis, only mutants in *CLV3* and *CLE40* resulted in phenotypic differences (Clark *et al.*, 1995; Hobe *et al.*, 2003). *MtCLE12* and *MtCLE13* might also act redundantly because they have overlapping expression patterns from the primordium stage on (Mortier *et al.*, 2010). In agreement, knock-down of *MtCLE12* did not result in

any phenotype, neither at the level of nodule number nor at the level of nodule development. On the other hand, simultaneous knock-down of *MtCLE12* and *MtCLE13* resulted in an increase in nodule number and the nodules underwent premature nodule senescence. In the results shown here, one line was found in which the expression of *MtCLE13*, but not of *MtCLE12*, was down-regulated. This line carried a normal number of nodules and hence might be preliminary evidence that the peptides act in a redundant way. RNAi of *MtCLE13* will be repeated with new constructs in order to fully address this question.

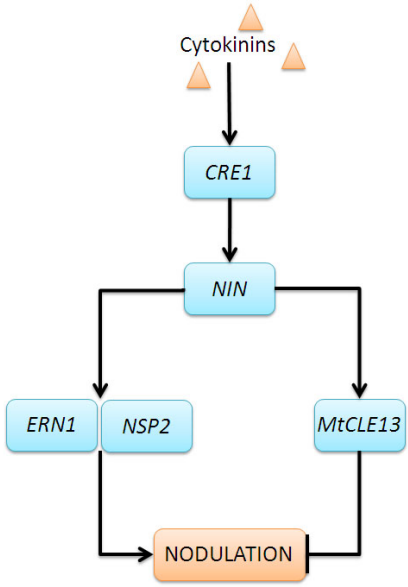

Figure 5.9: Cytokinin activation of *MtCLE13* was shown to be *CRE1*- and *NIN*-dependent, but *NSP2*-independent, suggesting that two parallel pathways are activated in the root cortex by cytokinins. One pathway is required for the activation of *NSP2* and *ERN1*, resulting in cortical cell divisions, while the other pathway involves *MtCLE13*, which is suggested to inhibit nodulation.

The enhanced number of nodules observed in the simultaneous knock-down of *MtCLE12* and *MtCLE13* expression confirms that they negatively regulate nodule number (Mortier *et al.*, 2010). In addition, the phenotype was not observed in the *sunn-4* supernodulation mutant, indicative that *MtCLE12, MtCLE13* and *SUNN* might act in the same pathway. Although *SUNN* is expressed in the vascular tissue of both roots and

shoots, grafting experiments have shown that *SUNN* acts in the shoot for AON. This has raised the hypothesis that the group-III CLE peptides from the incipient nodules, might travel to the shoot, to be bound by SUNN to induce AON. Currently, CLE peptides are known to act as short-distance molecules and although the long-distance hypothesis is appealing, it raises many questions as to how the CLE peptides might reach the vasculature to be transported. In wild type plants, nodule inhibition is associated with a reduction in polar auxin flow from the shoot to the root, and this is not observed anymore in *sunn-1* mutants (van Noorden *et al.*, 2006). In addition, *sunn-1* mutants have a higher auxin flow from the shoot to the root before inoculation (van Noorden *et al.*, 2006). This high auxin landscape might be the underlying cause of the high nodule number (van Noorden *et al.*, 2006). In Arabidopsis, application of CLE peptides was shown to reduce auxin signaling in the vascular system (Whitford *et al.*, 2008). Hence, it is possible that the nodulation-related CLE peptides act by suppressing the auxin levels in the root to inhibit nodulation and that the phenotype is absent in the *sunn* mutants because their effect is not strong enough to overrule the high auxin levels of the *sunn* mutants. Strikingly, the number of nodules observed on the *MtCLE12/MtCLE13* RNAi lines was 10 times lower than the number observed on the *sunn-4* mutants. These differences might be explained because other nodulation-induced CLE peptides influence AON or because knock-down does not completely abolish expression. It might also indicate that the nodulation-related CLE peptides are perceived locally in the root and act separately from the *SUNN* pathway in the shoot on the same process, e.g. auxin transport in the vasculature, to control nodule number. What the receptors are in the root is unknown, but root-located SUNN is a possible candidate.

Besides the higher nodule number, RNAi *MtCLE12/MtCLE13* nodules also underwent premature senescence. This is an intriguing phenotype because the CLE peptides were shown to be expressed at the apical part of the nodule. However, this phenotype might be a consequence of an imbalance between cell division and differentiation leading to aberrant differentiation of the infected cells resulting in senescence. Indeed, expression analysis of the nodule meristem marker gene *MtHAP2-1* in the nodules of RNAi *MtCLE12/MtCLE13* lines, indicated consumption of the nodule meristem, confirming an imbalance between cell proliferation and differentiation. Interestingly, also in *ENOD40* RNAi lines, nodule senescence was observed (Wan *et al.*, 2007). Hence these results might indicate that in addition to being involved in AON, the CLE peptides might control the meristem of the nodule in agreement with their function during vascular development and in the meristematic tissues of shoots and roots.

Together, we have shown that during nodulation *MtCLE13* requires cytokinin signaling in the root. Cytokinin activation of *MtCLE13* was shown to be *CRE1*- and *NIN*-dependent, but *NSP2*- and *ERN1*-independent, suggesting that two parallel pathways are activated in the root cortex by cytokinins (Figure 5.9). One pathway is required for the activation of *NSP2* and *ERN1*, resulting in the activation of cortical cell divisions (Plet *et al.*, 2011), while the other pathway involves *MtCLE13*, which is suggested to inhibit nodulation. Indeed, RNAi analysis confirmed that at least the group-III peptides, MtCLE12 and MtCLE13, act to control nodule number and that the mechanism used, interacts with the pathway activated by the shoot-localized SUNN. In addition, RNAi analysis gave the first evidence that the nodulation-related CLE peptides might control the nodule meristem. Whether *SUNN* is required for this process is currently not known, but an interesting hypothesis to test.

5.4 Materials and methods

Biological material

M. truncatula Gaertn. cv. Jemalong A17 and J5 as well as *nin-1, bit1-1, nsp1, nsp2, dmi3, nfp, cre1-1, sunn-1, sunn-2, sunn-3* and *sunn-4* mutants (Catoira *et al.*, 2000; Oldroyd and Long, 2003; Marsh *et al.*, 2007; Middleton *et al.*, 2007; Schnabel *et al.*, 2005; Gonzalez-Rizzo *et al.*, 2006) were grown and inoculated as described (Mergaert *et al.*, 2003). *Sinorhizobium meliloti 1021* pHC60-GFP (Cheng and Walker, 1998) and *Sm2011* pBHR-mRFP (Smit *et al.*, 2005) were grown at 28°C in yeast extract broth medium (Vervliet *et al.*, 1975), supplemented with 10 mg L^{-1} tetracycline.

For *promoter:GUS* analysis, a 2-kb region upstream of *MtCLE13* was isolated from genomic DNA based on the available genomic data[14]. The promoter was fused to the *uidA* gene in pKm43GWRolDC1 (Karimi *et al.*, 2002). Primers used for amplification are presented in Table 5.1. For RNAi analysis, the selected sequences were obtained via PCR on nodule cDNA and cloned into the Gateway vector pDONR221. LR reaction of the latter vectors with pK7GWiWG2D (Karimi *et al.*, 2002) resulted in the RNAi hairpin constructs. For the hairpin construct which should downregulate *MtCLE12* as well as *MtCLE13*, both individual sequences were fused by use of the USER enzyme (New England Biolabs, Ipswich, MA) prior to Gateway cloning.

[14] http://www.ncbi.nlm.nih.gov/

Table 5.1: Primers used in the analyses.

Gene	Sense primer	Anti-sense primer
qRT-PCR		
MtCLE4	AATTTCACAAGTTCTGCTTCATCGC	TGGCACACCTCTCTTGTCTTCC
MtCLE12	CAACGTCTCTTGCATGAGTTAATGG	ACCTGGTGAAAGCCTATCTCCTG
MtCLE13	CCGAAGCCTTCTACAGAAACTACG	TCTTGGTGGTGATCTTCCATTATGC
RNAi *MtCLE12*	AAACTCACACATCTTGTTTCG	CGAGGATTTGTTGCTTACTTAG
RNAi *MtCLE13*	TTTTGGGGTGATTTTGTTATGG	GGAGAAGGAATTAAATACCACTAC
40S	GCCATTGTCCAAGTTTGATGCTG	TTTTCCTACCAACTTCAAAACACCG
Gateway cloning		
promoter-MtCLE13	TCATTCCTAGTAGAACGGCG	GCCCATGTGTGATTTTAATC
RNAi *MtCLE12*	ATAGAGAATTCAAATAAAGTGCC	TTAGTTATGTATATGGTTTGGTCCAC
RNAi *MtCLE13*	TCCTGAGATATCCCTAACTTGTTT	GCCGGTATACAACCGATCAA

For the analysis of temporal expression during nodulation, nodules were harvested 4 to 10 dpi from plants grown in pouches, watered with nitrogen-poor SOLi medium, and inoculated with *Sm1021* pHC60-GFP. Infection threads were visible from 4 dpi on, nodule primordia at 6 dpi, and small nodules at 8 dpi. Two days later, at 10 dpi, slightly bigger nodules were observed. Tissue was collected by visualizing the green fluorescent bacteria under a stereomicroscope MZFLII (Leica Microsystems, Wetzlar, Germany) equipped with a blue-light source and a Leica GFP Plus filter set (λ_{ex} = 480/40; λ_{em} = 510 nm LP barrier filter). The zones I of uninoculated roots were isolated at the same developmental stage as the 4 dpi stage.

RNA extraction, cDNA synthesis, and qRT-PCR analysis

Total RNA was isolated with the RNeasy Plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After a DNAse treatment, the samples were purified through NH_4Ac (5 M) precipitation, quality controlled, and quantified with a Nanodrop spectrophotometer (Isogen, Hackensack, NJ). RNA (2 μg) was used for cDNA synthesis with the Superscript Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA). The samples were diluted 50 times and stored at -20°C until further use. The qRT-PCR experiments were done on a LightCycler 480 (Roche Diagnostics, Brussels, Belgium) and SYBR Green was used for detection. All reactions were done in triplicate and averaged. The total reaction volume was 5 μl (2.5 μl master mix, 0.25 μl of each primer [5 μM] and 2 μl cDNA). Cycle threshold (C_T) values were obtained with the accompanying software and data were analyzed with the $2^{-\triangle\triangle C_t}$ method (Livak

and Schmittgen, 2001). The relative expression was normalized against the constitutively expressed 40S ribosomal S8 protein (TC100533, MGI). Primers used (Table 5.1 were unique in the MGI version 9.0 and the Medicago EST Navigation System database (Journet *et al.*, 2002). Each experiment was repeated at least two times with independent biological tissue.

In vitro application of cytokinin

For analysis of GUS activity, cytokinins (10^{-7} M BAP) were diluted in dimethylsulfoxide and supplemented to the medium of 1-month-old, *pMtCLE13:GUS* transformed and *in vitro*-grown plants. As a control, plants were grown without supplemented hormones. The plants were cultured at 25°C, 16-h photoperiod, and 70 μE $m^{-2}m^{-1}$ light intensity per day. After 24 h of incubation, the roots of approximately 18 plants were harvested and GUS activity was analysed. The experiment was repeated twice with comparable results. For qRT-PCR analysis, cytokinins (10^{-7} BAP) were diluted in dimethylsulfoxide and supplemented to the medium of 7-day-old, *in vitro*-grown plants. As a control, plants were grown without supplemented hormones. The growth conditions of the seedlings were the same as above. After 24 h of incubation, the roots of approximately 18 plants were harvested and analyzed by qRT-PCR analysis. The experiment was repeated twice with comparable results.

Agrobacterium rhizogenes-mediated transgenic root transformation

The protocol was adapted from Boisson-Dernier *et al.* (2001) and performed as described by Mortier *et al.* (2010).

Histochemical localization of GUS activity and Technovit embedding

GUS activity in cotransformed roots of *pMtCLE13:GUS* plants was analyzed using 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid as substrate (Van den Eede *et al.*, 1992). Roots were vacuum infiltrated during 20 min and subsequently incubated in GUS buffer at 37°C. Incubation lasted 4 h. After staining, roots and root nodule primordia were fixed, dehydrated, embedded with the Technovit 7100 kit (Herqeus Kulzer, Wehrheim, Germany), according to the manufacturer's instructions, and sectioning with a microtome (Reichert-Jung, Nussloch, Germany). The 3-μm thick sections were

mounted on coated slides (Sigma-Aldrich, St. Louis, MO). For tissue-specific staining, sections were submerged in a 0.05 % (w/v) ruthenium red solution (Sigma-Aldrich, St. Louis, MO), washed in distilled water, and dried. Finally, sections were mounted with Depex (BDH Chemicals, Poole, England). Photographs were taken with a Diaplan microscope equipped with bright- and dark-field optics (Leitz, Wetzlar, Germany). The sections made during knock-down analysis were submerged in a 0.5 % toluidine blue solution.

Statistical analysis

The following generalized linear model (GLM) $Y_{ijk} = \ + genotype_j + experiment_k + error_{ijk}$ (Regression analysis) was fitted to the nodule number, partitioning phenotypic variation into fixed genotype and experiment effects and random error effects. Y_{ijk} is the phenotype of the i-th plant from the genotype j analyzed in experiment k; μ is the overall mean of the phenotypes obtained for all lines considered. Because the data has a Poisson distribution, a logarithm base e link function was incorporated. All analyses were done by means of the GenStat software[15].

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Authors contributions

The main part of this work was performed by V. Mortier. S. Goormachtig and M. Holsters were involved in designing the research and revising the manuscript.

[15] http://www.vsni.co.uk/software/genstat/

6

Insight in gain-of-function of two nodulation-related *CLE* genes in *M. truncatula*

Virginie Mortier, Christa Verplancke, Marcelle Holsters and Sofie Goormachtig

In preparation

Abstract
CLE peptides are known to be involved in determinate and indeterminate nodulation. Ectopic overexpression of certain members of the CLE family, amongst which *MtCLE12* and *MtCLE13* of *Medicago truncatula*, results in a local and systemic inhibition of nodulation. Until now, little was known about the molecular pathways and hormonal/physiological processes activated by the nodulation-related *CLE* genes. Via qRT-PCR analysis and micro-array analysis of the roots of chimeric *M. truncatula* plants, we attempted to elucidate the effect of ectopic expression of *MtCLE13* on the transcriptome. Moreover, to have a more complete view of the gain-of-function phenotypes of *35S:MtCLE12/13*, morphological changes were assessed on stable transformed plants. Here, we report that ectopic expression of *MtCLE12* and *MtCLE13* results in inhibition of nodulation and in a slight reduction of root length. In addition, we provide preliminary evidence that the *MtCLE13* might act locally and systemically via the downregulation of the Nod factor receptor gene *NFP*. Finally, we observed that *MtCLE13* mRNAs can spread systemically in the root system.

6.1 Introduction

Many plants are dependent on symbiotic interactions with bacteria or fungi for their survival on nutrient poor soils. The majority of land plants establish an intracellular root symbiosis with arbuscular mycorrhizal fungi, resulting in an improved water and phosphate uptake (Harrison *et al.*, 1999; Kistner and Parniske, 2002; Hata *et al.*, 2010). In nitrogen-limited conditions, many leguminous plants interact with rhizobia, resulting in the formation of nodules, small, root-based organs in which the bacteria fix nitrogen for the plant (Geurts and Bisseling, 2002; Ferguson *et al.*, 2010). In return, the legumes provide the rhizobia with carbon and a protective niche. During both arbuscular mycorrhization and nodulation, the microsymbionts secrete lipochitooligosaccharides that are perceived by the host and activate a common signaling cascade involving, amongst others, the 'common SYMBIOSIS (SYM) genes' *DOESN'T MAKE INFECTIONS1 (DMI1), DMI2* and *DMI3* and Ca^{2+} spiking (Limpens *et al.*, 2003; Ferguson *et al.*, 2010; Maillet *et al.*, 2011). For a detailed overview of the Nod Factor (NF) signaling pathway we refer to Chapter 3. A difference in calcium spike frequency is observed during nodulation and arbuscular mycorrhization and is suggested to provide enough flexibility to activate either process (Kosuta *et al.*, 2008).

As for most plant developmental processes, nodulation is governed by phytohormone signals, which may act antagonistically or synergistically in a concentration-dependent manner. The phytohormones auxin, cytokinin, gibberellin, abscissic acid (ABA), ethylene and jasmonic acid (JA) are suggested to be involved in the initiation and coordination of the nodulation process (Ferguson and Mathesius, 2003; Hirsch and Fang, 1994). Downstream of NF perception, cytokinin signaling is activated in the cortex for nodule organogenesis (Frugier *et al.*, 2008; Crespi and Frugier, 2008; Gonzalez-Rizzo *et al.*, 2006; Murray *et al.*, 2007; Tirichine *et al.*, 2007; Lorteau *et al.*, 2001; Dehio and de Bruijn, 1992; Bauer *et al.*, 1996; Hirsch and LaRue, 1997). There, cytokinin is perceived by the histidine kinase cytokinin receptor CYTOKININ RESPONSE1 (CRE1) (Gonzalez-Rizzo *et al.*, 2006; Eckardt, 2006). Signaling via the type-A RESPONSE REGULATOR4 (RR4) and the type-B RR1 results in the activation of the transcription factors NODULATION SIGNALING PATHWAY2 (NSP2), ETHYLENE-RESPONSIVE BINDING DOMAIN FACTOR REQUIRED FOR NODULATION1 (ERN1), and NODULE INCEPTION (NIN) (Tirichine *et al.*, 2007; Frugier *et al.*, 2008; Plet *et al.*, 2011). These transcription factors are involved in the activation of early nodulation (ENOD) genes to induce cortical cell divisions (Gonzalez-Rizzo *et al.*, 2006). Cytokinin signaling through the CRE1 pathway also regulates the expression and accumulation of the auxin efflux carrier PIN proteins, leading to an inhibition of polar auxin transport (PAT), which is suggested to be crucial for nodule primordium formation in indeterminate nodules (Plet *et al.*, 2011; van Noorden *et al.*, 2006; Mathesius *et al.*, 1998; Boot *et al.*, 1999; Ferguson and Mathesius, 2003; Wasson *et al.*, 2006). PAT is a highly regulated cell-to-cell transport and involves asymmetrically positioned, cell membrane-localized auxin influx carrier proteins (AUXs) and PIN auxin efflux proteins (Huo *et al.*, 2006; Grunewald and Friml, 2010). Presumably as a result of PAT inhibition, nodulation on *M. truncatula* is associated with auxin accumulation in the root cortex at the infection site (Mathesius *et al.*, 1998; Boot *et al.*, 1999; Ferguson and Mathesius, 2003; van Noorden *et al.*, 2006; Wasson *et al.*, 2006).

The role of gibberellins, abscissic acid (ABA) and jasmonates during nodulation is not so clear yet. Gibberellins would play a positive role during nodulation because a mutation in a gibberellin biosynthetic gene results in a reduction in nodule number, while the exogenous application of gibberellins rescues the phenotype by restoring the nodule number (Kawaguchi *et al.*, 1996; Ferguson *et al.*, 2005). Abscissic acid (ABA) is proposed to act negatively on nodulation because it interferes with NF signaling and at the same time, might suppress cytokinin signaling (Phillips, 1971; Bano and Harper,

2002; Ferguson and Mathesius, 2003; Ding *et al.*, 2008). Also ethylene inhibits nodulation, by suppressing the NF signaling pathway, by restricting infection thread formation and by inhibiting nodule primordium formation along the protophloem poles (Penmetsa and Cook, 1997; Heidstra *et al.*, 1997). Although the exogenous application of JA or methyl-JA (MeJA) has been shown to affect *M. truncatula* nodulation (Miwa *et al.*, 2006; Sun *et al.*, 2006), JAs probably do not to act locally in the root, but might be involved in autoregulation of nodulation (AON) (Zdyb *et al.*, 2011). AON involves a long-distance feedback mechanism, whereby early infection events act to suppress excessive nodule development. Central in this response is a shoot-acting leucine-rich-repeat receptor-like-kinase (LRR-RLK), called SUPER NUMERIC NODULES (MtSUNN), HYPERNODULATION ABERRANT ROOT FORMATION1 (LjHAR1), NODULE AUTOREGULATION RECEPTOR KINASE (GmNARK) or SYMBIOSIS29 (PsSYM29) (Kosslak and Bohlool, 1984; Carroll *et al.*, 1985a,b; Delves *et al.*, 1986; Pierce and Bauer, 1983; Nutman, 1952). Transcript profiling analysis of leaf material from either inoculated or uninoculated wild type and *nark* soybean plants revealed a differential expression for JA biosynthesis and response genes in a systemic, *NARK*-reliant and rhizobia-dependent manner (Kinkema and Gresshoff, 2008; Hause and Schaarschmidt, 2009). Hence, a shoot-specific downregulation of JA response genes by rhizobial inoculation is suggested to mediate AON, at least in legumes developing determinate nodules (Kinkema and Gresshoff, 2008; Hause and Schaarschmidt, 2009). The high level of JA measured in the AON mutants, *har1* and *nark*, further supports this hypothesis, as well as the fact that MeJA application to the shoots of *L. japonicus* plants diminishes nodulation (Nakagawa and Kawaguchi, 2006; Seo *et al.*, 2007; Kinkema and Gresshoff, 2008). In addition, AON would also involve an inhibition of long-distance PAT from the shoots to the roots in *M. trunculata*, limiting auxin accumulation at infection sites and likewise reducing additional nodule formation (Thimann, 1937; Morris and Thomas, 1978; Mathesius *et al.*, 1998; van Noorden *et al.*, 2006; Prayitno *et al.*, 2006).

To make the nodule organ, the NF signaling pathway activates the cell cycle in the cortical cells, resulting in both nodule primordia development and pre-infection thread formation, cytoplasmic bridges through which the infection threads grow, that guide the bacteria towards the recipient cells (Yang *et al.*, 1994; van Spronsen *et al.*, 2001; Jones *et al.*, 2007). Mature indeterminate nodules, such as occurring on *M. truncatula*, are characterized by a persistent meristem, which continuously provides new cells for bacterial internalization (Ferguson *et al.*, 2010). Several peptide hormones, amongst

which CLAVATA3/embryo surrounding region related peptides (CLV3/ESR or CLE), are involved during nodulation (Okamoto *et al.*, 2009; Mortier *et al.*, 2010, 2011; Reid *et al.*, 2011). CLE peptides are short distance signaling peptides, which are 12 to 13 amino acids long and derived from the C-terminal region of pre-proproteins (Mitchum *et al.*, 2008; Oelkers *et al.*, 2008). Their function is mostly associated with balancing cell proliferation and differentiation during plant development, more specifically during shoot apical meristem (SAM) and root apical meristem (RAM) homeostasis and tracheary element differentiation (Fletcher *et al.*, 1999; Rojo *et al.*, 2002; Hobe *et al.*, 2003; Kondo *et al.*, 2006; Mortier *et al.*, 2010). Functional redundancy between CLE ligands has been suggested, as ectopic expression of *CLE* genes or exogenous application of CLE peptides, indicated that structurally related CLE peptides often cause similar and pleiotropic phenotypes, such as increased rosette growth, root growth inhibition or stimulation, dwarfing, SAM arrest, lack of apical dominance and asymmetric leaf development (Fiers *et al.*, 2005; Strabala *et al.*, 2006; Ni and Clark, 2006; Kinoshita *et al.*, 2007; Jun *et al.*, 2008). CLE peptides were also proposed to control the cell division and differentiation processes during nodule formation (Mortier *et al.*, 2010). In addition, a group of structurally related CLE peptides, here designated the AON peptides, might control nodule number and activate the AON process (Okamoto *et al.*, 2009; Mortier *et al.*, 2010, 2011; Reid *et al.*, 2011). Because SUNN and its orthologs in other legumes are highly similar to the CLV3 peptide receptor CLV1, it was suggested that the root-expressed AON CLE peptides are the ligands for SUNN in the shoot to activate AON (Shiu and Bleecker, 2001; Okamoto *et al.*, 2009; Krusell *et al.*, 2002; Nishimura *et al.*, 2002a; Searle *et al.*, 2003; Schnabel *et al.*, 2005). In *M. truncatula*, two *CLE* genes, *MtCLE12* and *MtCLE13*, were investigated in more detail (Mortier *et al.*, 2010). Tissue specific expression analysis revealed that the expression of both genes is linked to proliferation and differentiation during nodulation (Mortier *et al.*, 2010). Ectopic expression of *MtCLE12* and/or *MtCLE13*, via *Agrobacterium rhizogenes* mediated hairy root transformation, resulted in a *SUNN*-dependent inhibition of nodulation, that involved long distance signaling (Mortier *et al.*, 2010) (Chapter 4 and 5). The inhibition was suggested to occur at the level of the NF signal transduction pathway, as the early nodulation gene *ENOD11* was no longer activated upon rhizobial inoculation. In addition, elongated petioles were observed on composite plants with roots ectopically expressing *MtCLE12* and/or *MtCLE13*, suggesting long-distance communication between root and shoot.

To further analyze the local and systemic molecular pathways or hormonal/physi-

ological processes that are activated by *MtCLE12* or *MtCLE13*, the effect on the transcriptome of ectopic *MtCLE13* expression was investigated by qRT-PCR analysis and micro-arrays. Moreover, morphological changes were assessed in stable transformed gain-of-function plants, as these are more suitable for the analysis of effects on root hair deformations, than the previously used *A. rhizogenes*-mediated transformants (Mortier *et al.*, 2010). Here, we report that ectopic expression of *MtCLE12* and *MtCLE13* results in inhibition of nodulation and in a slight reduction of root length. In addition, we provide preliminary evidence that the AON peptides might act via the downregulation of genes involved in nodule formation among which the gene encoding the NF receptor *NFP*.

6.2 Results

6.2.1 Effect of ectopic expression of *MtCLE12* and *MtCLE13* on nodule development in stable transgenic lines

Composite plants carrying transgenic roots ectopically expressing *MtCLE12* or *MtCLE13* were made previously by *A. rhizogenes* transformation (Mortier *et al.*, 2010). These plants showed an elongation of the wild type petioles and an inhibition of nodulation on the transgenic roots as well as on the wild type roots (Mortier *et al.*, 2010). However, it was impossible to determine whether the inhibition of nodulation was caused by aberrant root hair morphology, because root hair deformations are difficult to asses on *A. rhizogenes*-transformed plants. As an alternative, stable transgenic plants were made by *A. tumefaciens* transformation (see Materials and Methods page 148). After Southern hybridization and *in vitro* culture, seeds of two independent *35S:MtCLE12* lines (*MtCLE12-1* and *MtCLE12-14*) and one *35S:MtCLE13* line (*MtCLE13-13*) were selected for further analysis. *35S:LUCIFERASE-1* (*35S:LUC-1*) was used as a control. QRT-PCR analysis confirmed the ectopic expression of the respective constructs (Figure 6.1).

To control the effectiveness of the constructs and the transformation event, the nodulation phenotype of these transgenic lines was first investigated and compared to the results of the *A. rhizogenes*-transformed plants (Mortier *et al.*, 2010). Roots of *35S:LUC-1, 35S:MtCLE12-1, 35S:MtCLE12-14* and *35S:MtCLE13-13* stable transformants were infected with *Sm2011* pBHR-mRFP and nodule number was assessed at 7 days post inoculation (dpi). Nodulation of control plants (*35S:LUC-1*) resulted on aver-

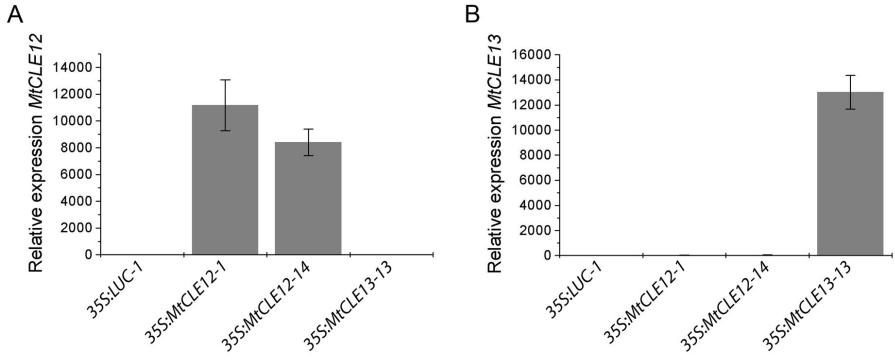

Figure 6.1: *MtCLE* expression in the roots of *MtCLE* transgenic lines. A, Relative expression of *MtCLE12*. Ectopic expression is observed in the *35S:MtCLE12-1* and *35S:MtCLE12-14* lines. B, Relative expression of *MtCLE13*. Ectopic expression is observed in the *35S:MtCLE13-13* line. *35S:LUC-1* is included as a control line. Data and error bars represent means $\pm$ SD.

age in 4.18 ± 0.18 nodules per root, while no nodules were detected on *35S:MtCLE12-1, 35S:MtCLE12-14* and *35S:MtCLE13-13* plants, similar to what was reported for chimeric plants ($p < 0.001$, Regression analysis) (Figure 6.2A) (Mortier *et al.*, 2010). Previous experiments have indicated that the ectopic expression of *MtCLE13* inhibits epidermal responses during nodulation (Mortier *et al.*, 2010). In agreement, no root hair deformations were observed after inoculation. Fluorescence microscopy revealed neither infection threads, nor microcolony formation. Because changes in root hair structure might cause nodulation defects (reviewed by Gage (2004)), we analyzed the structure of the root hairs. Root hairs of 15 plants of each line, grown on plates, were analyzed. No differences in root hair formation were observed (Figure 6.2B). We next analyzed whether cortical nodulation markers might be activated upon inoculation of *35S:MtCLE12* or *35S:MtCLE13* plants. The expression level of *NIN* and *MtRR4* was measured at 6 dpi in wild type roots and roots of the stabile transformant *35S:MtCLE13-13*. The expression level in non-inoculated wild type roots was taken as a reference. In wild type plants, an upregulation during nodulation was observed for both genes, but this upregulation was not observed in *35S:MtCLE13-13* plants (Figure 6.2, C-D). Hence, ectopic expression of *MtCLE13* interferes with activation of the cortical marker genes. Both epidermal and cortical signaling is inhibited by ectopic overexpression of *MtCLE13*.

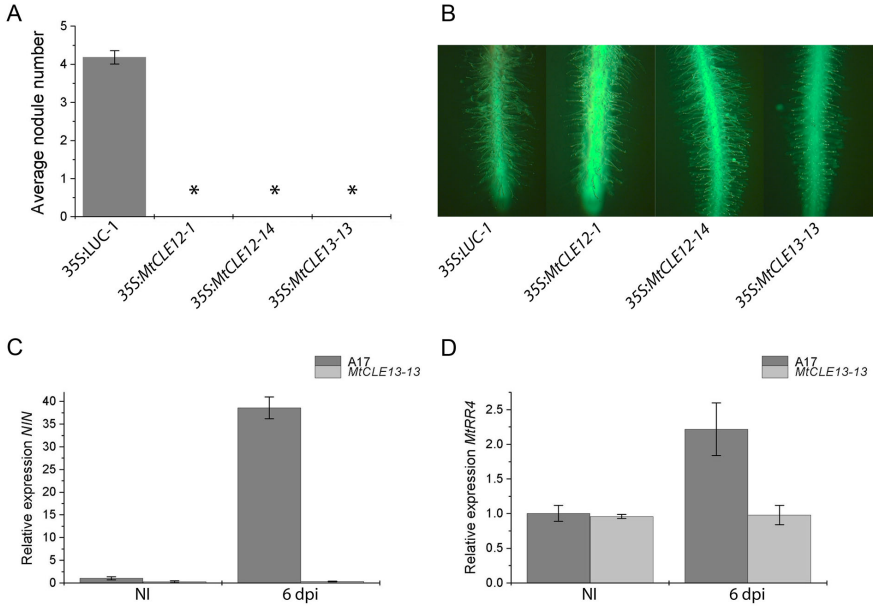

Figure 6.2: Inhibition of nodulation in *35S:MtCLE12-1, 35S:MtCLE12-14* and *35S:MtCLE13-13* stable transformants. A, Average nodule number on the roots of stable transformants expressing *35S:LUC-1, 35S:MtCLE12-1, 35S:MtCLE12-14* or *35S:MtCLE13-13* at 7 dpi (n = 18-73). Asterisks indicate statistically significant differences in comparison to *35S:LUC-1* stable transformants ($p < 0.001$, Regression analysis). Data and error bars represent means ± SE. The total mean of two biological repeats is represented in the graph. B, Root hairs located close to the roottip of stable transformants expressing *35S:LUC-1, 35S:MtCLE12-1, 35S:MtCLE12-14* or *35S:MtCLE13-13*. C and D, Relative expression of the cortical marker genes *NIN* (C) and *MtRR4* (D) in *35S:MtCLE13-13* stable transformants during nodulation. Expression analysis by qRT-PCR on cDNA samples of zone I root tissues of wild type plants (A17) and *35S:MtCLE13-13* transgenic plants before inoculation (NI) and at 6 days post inoculation (6 dpi). Data and error bars represent means ± SD.

6.2.2 Effect of ectopic expression of *MtCLE12* and *MtCLE13* on root development, petiole length and leaf area

To investigate the effect of ectopic expression of *MtCLE12* and *MtCLE13* on root growth, the root length of 7-day-old plants was calculated with ImageJ[16]. The root

[16] http://rsb.info.nih.gov/ij/

length of control plants (*35S:LUC-1*) was on average 11.84 $\pm$ 0.83 cm, while the roots of *35S:MtCLE12-1, 35S:MtCLE12-14* and *35S:MtCLE13-13* plants were respectively 9.77 $\pm$ 0.99 cm, 9.54 $\pm$ 0.77 cm and 7.01 $\pm$ 0.73 cm (Figure 6.3A). Hence, the roots of the *35S:MtCLE12* and *35S:MtCLE13* plants are statistically shorter than those of the control plants ($p < 0.05$, ANOVA). Next, we analyzed whether the reduction in root length is accompanied by a difference in root diameter or root structure. Therefore, root material was harvested for sectioning 1 to 2 cm above the roottips of these stable transformed plants. No differences were observed between the root sections of *35S:MtCLE12* or *35S:MtCLE13* plants, as compared to root sections of control plants (*35S:LUC-1*) (Figure 6.3D).

Composite plants with transgenic roots ectopically overexpressing either *MtCLE12* and/or *MtCLE13* have elongated petioles as compared to control plants (*35S:GUS*) (Mortier *et al.*, 2010). To analyze whether this phenotype is observed on stable transgenic plants, the average petiole length of the 4^{th} leaflet of *35S:LUC-1, 35S:MtCLE12-1, 35S:MtCLE12-14* and *35S:MtCLE13-13* stable transformants was assessed after 1 month of growth. The average petiole length of control plants (*35S:LUC-1*) was on average 5.06 $\pm$ 0.20 cm long (Figure 6.3B). No statistically significant different values were measured for *35S:MtCLE12-1* (5.10 $\pm$ 0.20 cm), *35S:MtCLE12-14* (4.65 $\pm$ 0.19 cm) or *35S:MtCLE13-13* (4.78 $\pm$ 0.21 cm) (Figure 6.3B).

During the same experiment, the total leaf area of all leaflets present on 1-month-old plants was measured. Again, no statistically relevant differences were seen between control plants and *MtCLE* overexpressing lines (Figure 6.3C). The total leaf area calculated for *35S:LUC-1* plants is 9.71 $\pm$ 0.48 cm^2, for *35S:MtCLE12-1* plants 8.45 $\pm$ 0.48 cm^2, for *35S:MtCLE12-14* plants 8.18 $\pm$ 0.46 cm^2 and for *35S:MtCLE13-13* plants 8.56 $\pm$ 0.50 cm^2 (Figure 6.3C).

To conclude, the ectopic expression of *MtCLE12* or *MtCLE13* in stable transformed lines affected nodulation and root growth, but had no effect on leaf and petiole growth.

6.2.3 Effect of ectopic expression of *MtCLE12* and *MtCLE13* on mycorrhization

Next, the effect of ectopic expression of *MtCLE12* and/or *MtCLE13* on mycorrhization was investigated (in collaboration with Bettina Hause, Germany). Therefore, 6 plants of each line (*35S:LUC-1, 35S:MtCLE12-1, 35S:MtCLE12-14* and *35S:MtCLE13-13*) were inoculated with *Glomus intraradices* and analyzed 3 weeks later. All plants

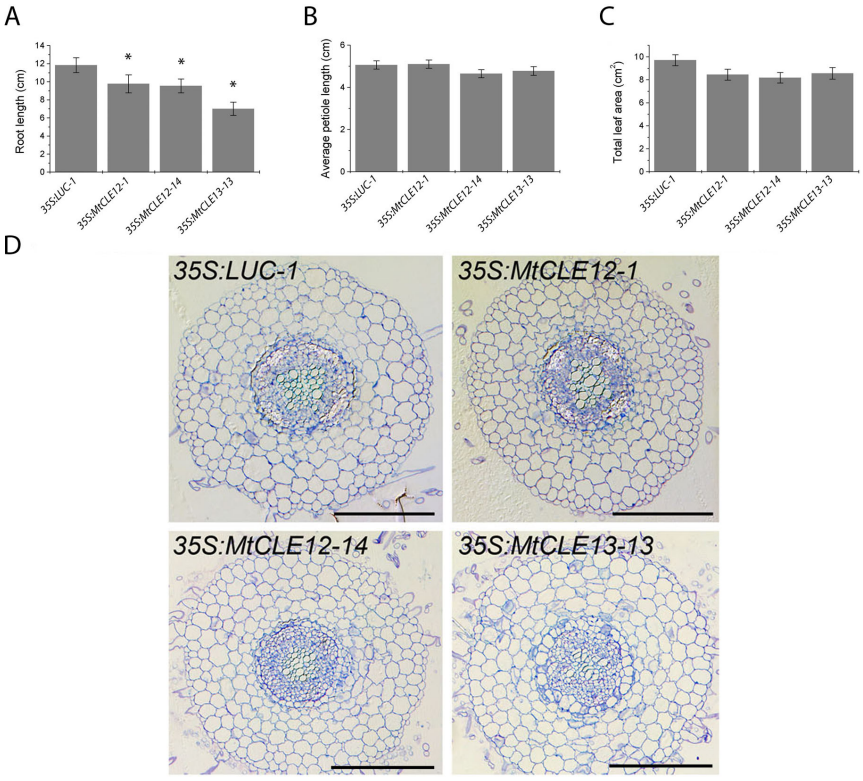

Figure 6.3: Root and shoot analysis of *35S:LUC-1, 35S:MtCLE12-1, 35S:MtCLE12-14*, and *35S:MtCLE13-13* stable transformed *M. truncatula* plants. A, Average root length. The plants were grown for 7 days in pouches, after which the average root lengths were calculated with ImageJ (n = 20-54). Asterisks indicate statistically significant differences in comparison to *35S:LUC-1* stable transformants ($p < 0.05$, ANOVA). B, Average petiole length of the 4^{th} leaflet. C, Total leaf area. For the analyses represented in B and C, plants were grown for 1 month in perlite, after which the average petiole length and the total leaf area were calculated with ImageJ (n = 13). The small differences observed in the graphs are not statistically relevant ($p > 0.05$, ANOVA). A-C, Data and error bars represent means $\pm$ SE. All experiments were repeated at least twice with comparable results. The total mean of the different repeats are represented in the graphs. D, Transversal root sections of *35S:LUC-1, 35S:MtCLE12-1, 35S:MtCLE12-14* and *35S:MtCLE13-13* stable transformed *M. truncatula* plants. Sections were made of root material harvested 1 to 2 cm above the roottip and were stained with toluidine blue. Bars = 0.5 mm.

showed mycorrhization with a similar degree of colonization between the different lines (Figure 6.4). Large differences were observed between individual plants of *35S:-MtCLE12-14* and *35S:MtCLE13-13* resulting in large error bars.

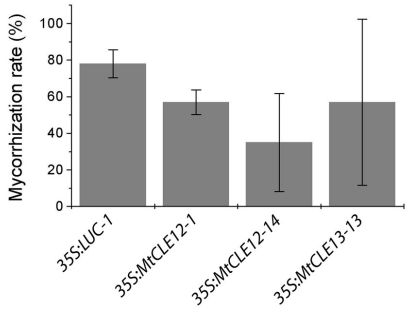

Figure 6.4: Effect of the ectopic expression of *MtCLE12* or *MtCLE13* on mycorrhization. The mycorrhization rate was measured on *35S:LUC-1* (control), *35S:MtCLE12-1, 35S:MtCLE12-14* and *35S:MtCLE13-13* stable transformants, 3 weeks after inoculation (n = 6; 1 biological repeat). Data and error bars represent means ± SD.

6.2.4 A gene-specific approach to study effects of *35S:MtCLE13*

So far, no molecular mechanisms are known that are controlled by *MtCLE12* or *MtCLE13*. As initial analysis, a gene specific approach was taken and the expression level of a selection of marker genes, which were shown in literature to be involved in nodulation, in AON or in different hormone treatments, was measured by qRT-PCR analysis. Roots of approximately 15 chimeric plants with *35S:GUS* (control) or *35S:MtCLE13* transgenic roots were pooled for cDNA preparation. The experiment was repeated 4 times on biologically independent material, and only those genes which are at least 2 fold up- or downregulated in the four experiments were taken as relevant. The list of all markers tested is indicated in Table 6.1.

First, we controlled whether the expression of the early signaling components *MtLYR3, NFP, NSP1, NSP2, ERN1, DMI1, DMI2, DMI3, NIN* and *ENOD11* was changed upon ectopic expression of *MtCLE13*. For *NIN, ENOD11, DMI1* and *DMI2*, no expression was measured in any of the tested samples. The remaining genes were not differentially regulated by the ectopic expression of *MtCLE13* as compared to the con-

Table 6.1: List of all marker genes that were measured in *35S:MtCLE13* overexpression lines by qRT-PCR analysis.

Gene	Annotation	Gene	Annotation
Early signaling components		**Hormonal responses**	
LYR3	MtD20757	AUX-responsive protein IAA14	TC125759
NFP	DQ496250	Auxin response factor 3	TC125184
NSP1	AJ972478	PIN1	AY115836
NSP2	AJ832138	PIN2	AY115837
ERN	EF396330	LAX1	AY115841
DMI1	AY497771	LAX2	AY115843
DMI2	TC112575	LAX3	AY115842
DMI3	AY496049	Isoflavone reductase homolog 1	TC118130
NIN	AM774003	Naringenin-CHS 2	TC119414
ENOD11	AJ297721	CRE1	TC109250
		MtRR4	TC103991
Cell cycle regulation		Cytokinin-specific binding protein	TC124213
MtcycB1	AC169076	MtIPT	TC123012
MtcycD3	TC122361	Cytokinin synthase	TC112199
		Gibberellin-regulated protein	TC138046
		ABA-activated protein kinase	TC108117
		Ehtylene-responsive element-binding factor3	TC136350
		Myc2-like protein	TC126060
		Putative cytochrome P450	TC114056
		Zym containing protein	TC114737

trol plants (*35S:GUS*) (data not shown), except for *NFP*, for which a downregulation was observed (Figure 6.5).

In a next step, we analyzed whether hormonal responses were affected by ectopic expression of *35S:MtCLE13*. There is ample evidence that the classical hormones auxin, cytokinin, abscissic acid, gibberelin, ethylene, and jasmonic acid are involved in the initiation and coordination of the nodulation process (Ferguson and Mathesius, 2003; Hirsch and Fang, 1994). We therefore looked for markers of the different hormones available in literature and controlled their expression in our analysis. The expression levels of two auxin responsive genes, AUX responsive protein indole-3-acetic acid inducible14 (IAA14) and auxin response factor 3 were measured (Fei and Vessey, 2009), as well as two *PIN* (*PIN1* and *PIN2*) and three *LAX* (*LAX1, LAX2* and *LAX3*) genes, which were shown to be involved in nodulation, were included (de Billy *et al.*, 2001; Schnabel and Frugoli, 2004). None of these markers showed a differential regulation upon *MtCLE13* overexpression. In addition, two genes were selected which might be involved in the synthesis of flavonoids that possibly influence auxin transport, an isoflavone reductase homolog 1 and a naringenin-chalcone synthase (CHS) (Wasson *et al.*, 2006, 2009). Also no change in expression of those

genes was caused by ectopic overexpression of *MtCLE13*. Next, we analyzed the expression levels of five cytokinin-responsive genes: the cytokinin signaling genes: *CRE1, MtRR4* and a cytokinin-specific binding protein; and the cytokinin biosynthesis genes: isopentenyltransferase (*MtIPT*) and a cytokinin synthase (Fei and Vessey, 2009). No change in expression of those genes was seen after overexpression. We also analyzed different markers for the other hormones: gibberellins, abscissic acid, ethylene and jasmonic acid. Here again, no changes in expression level were observed. Finally, since CLE peptides interfere with cell division and differentiation, we controlled whether there was an influence on cell cycle markers. The closest *M. truncatula* homologs of *cyclinB1* (*cycB1)* and *cycD3* were retrieved by tBLASTx analysis (*MtcycB1*: AC169076.2, NCBI; *MtcycD3*: TC122361, TIGR) and included in the qRT-PCR analysis. The expression level of both markers was similar in the *35S:GUS* and *35S:MtCLE13* lines.

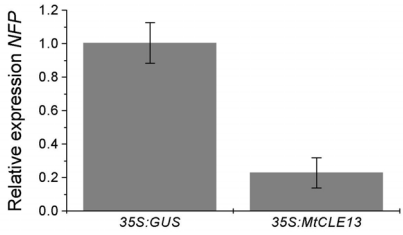

Figure 6.5: *NFP* expression in *35S:GUS* (control) and *35S:MtCLE13* transgenic roots. Expression analysis of *NFP* by qRT-PCR on cDNA samples of zone I root tissues of uninoculated *35S:GUS* (control) or *35S:MtCLE13* transgenic roots, harvested at 40 days post germination. Data and error bars represent means $\pm$ SD.

To conclude, based on our qRT-PCR analysis, of all the markers tested, only the NF receptor *NFP* was downregulated by the ectopic expression of *MtCLE13*.

6.2.5 A micro-array analysis to study effects of *35S:MtCLE13*

To identify *MtCLE13* target genes, a micro-array analysis was performed. The transcriptome of transgenic roots from non inoculated *35S:GUS* (control) and *35S:MtCLE13* chimeric plants was compared by hybridization with the *Medicago* genome

Affymetrix chips, containing over 61200 probe sets (of which 32167 from *M. truncatula*) (Barnett et al., 2004). Three biological repeats were performed. This analysis resulted in the identification of 17 *M. truncatula* genes that are differentially expressed (minimum 2 fold up or down regulated, $p < 0.05$) between control and *35S:MtCLE13* roots (Table 6.2).

Table 6.2: List of genes which were minimum 2 fold up or downregulated in *35S:MtCLE13* roots compared to *35S:GUS* (control) roots, as determined by the *Medicago* genome Affymetrix chip ($p < 0.05$).

Probe set ID	x fold up/down regulated	Target description	Annotation
Mtr.19174.1.S1_at	888.7 up	MtCLE13	AC144893_10.1(11)
Mtr.28373.1.S1_at	11.5 up	Kelch-repeat containing F-box protein (B)	BG644495
Mtr.9802.1.S1_at	5.4 up	Kelch-repeat containing F-box protein (A2)	TC104433
Mtr.13058.1.S1_at	4.7 up	Kelch-repeat containing F-box protein (A1)	TC96809
Mtr.35456.1.S1_at	2.8 up	High affinity nitrate transporter	TC103501
Mtr.35815.1.S1_at	2.7 up	Germin-like protein	TC95760
Mtr.3265.1.S1_at	2.5 up	Hypothetical protein (A)	TC112497
Mtr.6317.1.S1_at	2.5 up	Hypothetical protein (E)	BQ138801
Mtr.26826.1.S1_at	2.3 up	Hypothetical protein (B)	AJ501727
Mtr.14816.1.S1_at	2.2 up	Copper binding protein	AC119415_13.1(11)
Mtr.37565.1.S1_at	2.1 down	2-oxoglutarate-dependent dioxygenase (ODD)	TC100930
Mtr.46917.1.S1_at	3.2 down	Nicotianamine syntase	AC149474_1.1(11)
Mtr.15789.1.S1_at	3.3 down	NFP	AC126779_3.2(11)
Mtr.25025.1.S1_s_at	3.5 down	LYK4	AY372407(11)
Mtr.15758.1.S1_at	3.7 down	Close homolog of LYK5	AC123570_22.1(11)
Mtr.11343.1.S1_at	3.7 down	Hypothetical protein (C)	TC109649
Mtr.10837.1.S1_at	4.7 down	Hypothetical protein (D)	TC108074

The first hit retrieved corresponds with a 888.7 fold upregulation of *MtCLE13*, validating the proper set-up of our experiment. In order to confirm the results of the micro-array analysis, a qRT-PCR analysis was performed on cDNA samples of *35S:GUS* and *35S:MtCLE13* transgenic roots grown and harvested under the same conditions as the plant material used for the micro-array analysis (Figure 6.6). The differential regulation of all potential *MtCLE13* target genes could be confirmed, with the exception of one gene encoding a hypothetical protein (B), which was upregulated by the ectopic expression of *MtCLE13* according to the micro-array data, but downregulated according to the results of the qRT-PCR analysis (Figure 6.6). For hypothetical protein (A) no qRT-PCR primers could be designed using the program 'Beacon designer'[17].

A down-regulation was measured for *NFP*, as well as for *LYK4* and a gene, similar to *LYK5* (Arrighi *et al.*, 2006; Limpens *et al.*, 2003; Oldroyd and Downie, 2008). Other

[17] http://www.premierbiosoft.com

downregulated genes encoded a nicotianamine synthase (*NAS*), and a 2-oxoglutarate-dependent dioxygenase (ODD). Also two kelch-repeat containing F-box proteins belong to the potential *MtCLE13* targets, as their genes were upregulated by the ectopic expression of *MtCLE13*. Initially, three hits were retrieved corresponding to kelch-repeat containing F-box proteins (A1, A2 and B), but further sequence analysis revealed that two of them belong to the same gene (Mtr13058.1.S1_at (A1) and Mtr. 9802.1.S1_at (A2)). An upregulation was also measured for a high affinity nitrate transporter, a copper binding protein and a germin-like protein. The remaining 5 genes with a differential expression pattern in *35S:MtCLE13* transgenic roots as compared to *35S:GUS* transgenic roots, encoded hypothetical proteins (A to E).

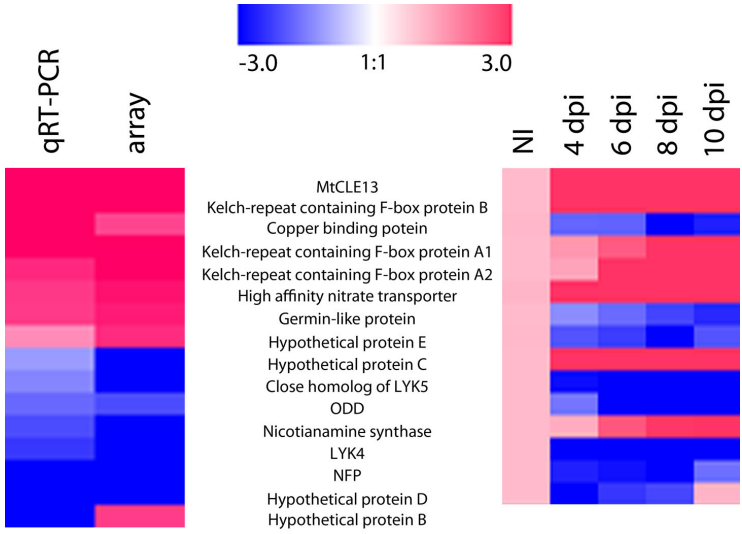

Figure 6.6: Left: heatmap of the expression of *MtCLE13* target genes by qRT-PCR and (micro)-array analysis on cDNA samples of root tissues of chimeric plants expressing *35S:MtCLE13*. cDNA of roots expressing *35S:GUS* was used as the reference tissue (not represented in heatmap). Right: heatmap of relative expression of *MtCLE13* target genes by qRT-PCR on cDNA samples of zone I root tissues of uninoculated plants (NI) and at 4, 6, 8 and 10 dpi. NI was used as the reference tissue.

In a next step, the temporal expression of the *MtCLE13* target genes during nodule development was studied by analyzing the relative transcript levels at 4, 6, 8, and 10 days postinoculation (dpi). The elongation zone of uninoculated roots, the nodule initi-

ation site, was used as the reference tissue. As shown in Figure 6.6, the genes encoding the kelch-repeat containing F-box proteins are upregulated during early nodulation, as well as the high affinity nitrate transporter, hypothetical protein (C) and the nicotianamine synthase. All other genes were downregulated, and for hypothetical protein (D) a transient expression pattern was observed, with a lower expression level than the reference tissue in the cDNA samples of 4 till 8 dpi, and a higher level of expression at 10 dpi (Figure 6.6).

6.2.6 *NFP* promoter activity in *35S:MtCLE13* transgenic roots

The results of the qRT-PCR analysis and the micro-array analysis indicate that the ectopic expression of *MtCLE13* results in the downregulation of *NFP*. To corroborate these observations, stable *pNFP:GUS* plants were transformed with the *35S:MtCLE13* construct by use of *A. rhizogenes*, resulting in chimeric plants expressing both *pNFP:-GUS* and *35S:MtCLE13* in their roots. As a control, *pNFP:GUS* plants were transformed with *35S:LUCIFERASE* (*35S:LUC)*. In uninoculated control roots a constitutive GUS activity was observed, as described by Arrighi *et al.* (2006) (Figure 6.7). The level of *NFP* expression seemed to be slightly lower in *35S:MtCLE13* roots than in *35S:LUC* roots. In addition, the *pNFP:GUS* expression seemed to have shifted to cells closer to the roottips in the *35S:MtCLE13* samples (Figure 6.7). *pNFP:GUS* activity was also analyzed during nodulation. As the ectopic expression of *MtCLE13* results in an inhibition of nodulation, we focused the experiment on the earliest stages of nodulation (Mortier *et al.*, 2010). The plants were inoculated with *Sm2011* pBHR-mRFP and at 5 dpi, those zones of *35S:LUC* roots harboring infection threads, as visualized by mRFP fluorescence, were harvested and subjected to GUS analysis. As no infection threads are observed on *35S:MtCLE13* transgenic roots, root zones susceptible to rhizobial infection were harvested on these plants (Mortier *et al.*, 2010). In *35S:LUC* roots, GUS activity was visualized in the cortex as broad stained regions corresponding with early nodulation stages (Figure 6.7). No GUS staining was seen in *35S:MtCLE13* roots (Figure 6.7).

These results support the observation that ectopic expression of *MtCLE13* results in a downregulation of *NFP* under uninoculated conditions and that the typical *NFP* expression pattern observed after inoculation is absent in inoculated *MtCLE13* roots.

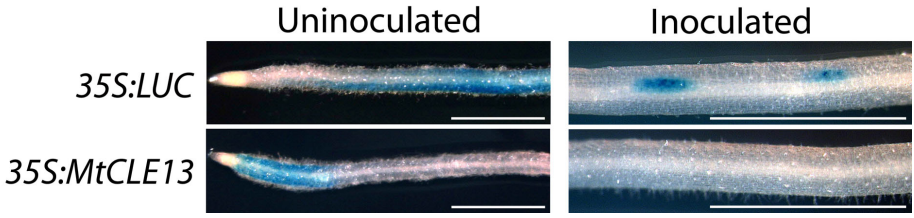

Figure 6.7: *NFP* expression in roots ectopically expressing *MtCLE13* before and at 5 dpi. *pNFP:GUS* activity in roots ectopically expressing either *LUC* (control) or *MtCLE13* (n = 15-20). Before inoculation, the level of *NFP* expression detected in lateral roots is slightly higher in *35S:LUC* roots than in *35S:MtCLE13* roots. Moreover, the expression of *NFP* is shifted towards the roottip in *35S:MtCLE13* transgenic roots. At 5 dpi, no *NFP* expression is detected in the nodule susceptible zone of *35S:MtCLE13* transgenic roots, while on *35S:LUC* roots, GUS activity is visualized in the cortex as broad stained regions corresponding with early nodulation stages. Bars = 2 mm.

6.2.7 Long distance effect of *35S:MtCLE13* transgenic roots on the expression of potential *MtCLE13* target genes in wild type roots

Ectopic overexpression of *MtCLE13* results in an inhibition of nodulation by long-distance signaling (Mortier *et al.*, 2010). To elucidate whether the differential expression of potential *MtCLE13* target genes is also regulated over a long distance, composite plants were generated that contained small *35S:MtCLE13* transgenic roots besides the wild type root (see Materials and Methods page 147). Control plants carried wild type roots and transgenic roots carrying the *35S:GUS* construct. Because *MtCLE13* expression is induced upon nodulation, only those genes from which the expression was either up- or down regulated by both *35S:MtCLE13* expression and nodulation were retained for further analysis. The expression of the genes encoding the kelch-repeat containing F-box proteins, the high affinity nitrate transporter, the hypothetical protein D, *NFP, LYK4*, the *LYK5* homolog and the ODD was measured. So far only one biological repeat of this analysis was performed. *NFP* and the ODD gene were slightly downregulated, while the expression level of the gene encoding the kelch-repeat containing F-box protein B was higher in the main root of composite plants carrying additional *35S:MtCLE13* roots, compared to the level found when the composite plants carried additional control roots (*35S:GUS*) (Figure 6.8, A-C). The expression of the

high affinity nitrate transporter, the kelch-repeat containing F-box protein A, the hypothetical protein D, *LYK4* and the *LYK5* homolog was not changed in the main root when the composite plants carried additional *35S:MtCLE13* roots.

The long distance inhibition of nodulation, observed during ectopic expression of *MtCLE13*, is *SUNN*-dependent (see chapter 5). To analyze whether the long distance effect of *35S:MtCLE13* on the differential regulation of its target genes is also *SUNN*-dependent, we repeated the qRT-PCR experiment on material harvested from chimeric *sunn-4* mutants. The long distance differential expression of *NFP* and the genes encoding the ODD and the kelch-repeat containing F-box protein B caused by the ectopic expression of *MtCLE13* was not observed anymore in chimeric *sunn-4* mutants (Figure 6.8, A-C).

As a control, the expression of *MtCLE13* was also tested in these tissues and surprisingly, a higher level of *MtCLE13* transcripts was measured in the wild type main root of plants bearing additional *35S:MtCLE13* transgenic roots (Figure 6.8D). We next tested whether these transcripts were derived from the transgene construct or from the endogene using primers that specifically recognized the transgene transcripts. QRT-PCR analysis indicated that the *MtCLE13* transcripts detected were derived from the transgenic construct (Figure 6.8E). However, the amount of *MtCLE13* transcripts detected by qRT-PCR analysis in these wild type main roots is low (Ct value 30), compared to the amount detected in *35S:MtCLE13* transformed roots (Ct value 20). To control whether this effect was specific for *35S:MtCLE13*, the expression of *GUS* was measured in non-transgenic roots and shoots of composite plants carrying additional *35S:GUS* roots, but no expression was detected. Similarly, because the transgenic roots also contain the *pRolD:GFP* construct, the expression of *GFP* was tested in the different samples. *GFP* transcripts could only be detected in the transgenic roots itself, but not in the wild type roots or shoots. These results indicate that harvesting of the wild type main root has been done properly and that mRNA of *MtCLE13* might move across root tissues. Next, we analyzed whether *MtCLE13* transcripts could be detected in the shoot. As shown in Figure 6.8E, *35S:MtCLE13* transcripts were not detected in the shoot tissues.

Next, to analyze whether the upregulation of *MtCLE13* is *SUNN*-dependent, we repeated the qRT-PCR experiment on material harvested from plants with a *sunn-4* mutant background. An upregulation of *MtCLE13* was still observed in the wild type main roots of chimeric plants bearing additional *35S:MtCLE13* transgenic roots (Figure 6.8D). qRT-PCR analysis with transgene specific primers indicated that the *MtCLE13*

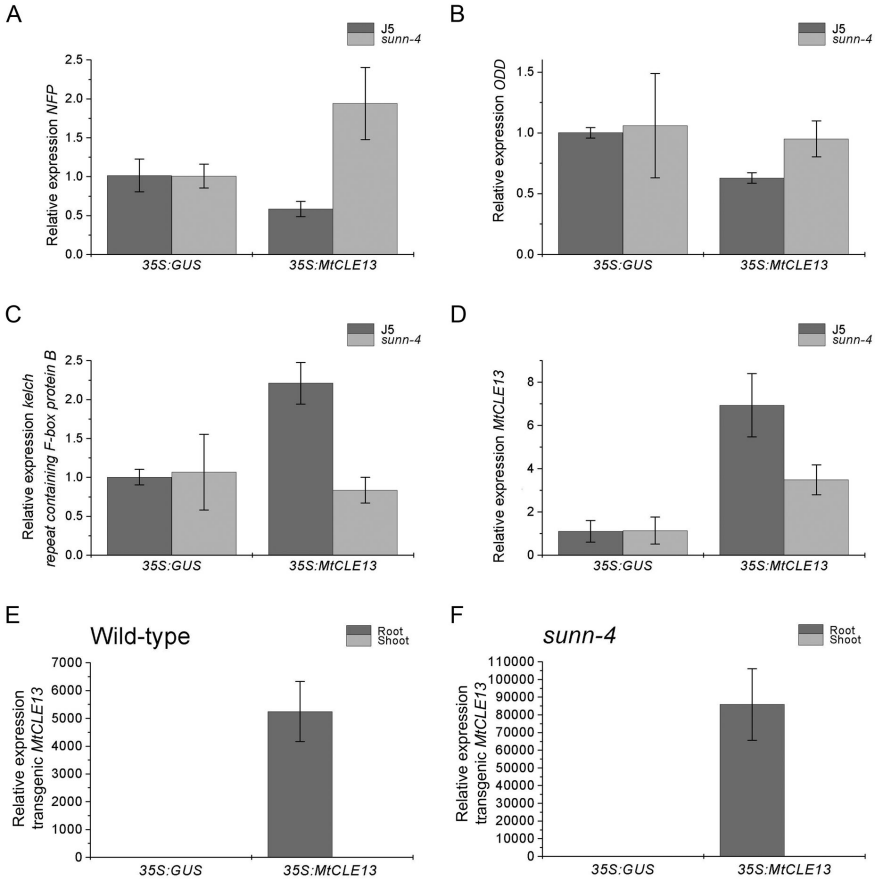

Figure 6.8: Long-distance effect of *35S:MtCLE13* transgenic roots. A-D, *NFP* (A), *ODD* (B), *Kelch-repeat containing F-box protein B* (C) and *MtCLE13* (D) expression analysis by qRT-PCR on cDNA samples of uninoculated wild type main roots of chimeric plants bearing additional *35S:GUS* (control) or *35S:MtCLE13* transgenic roots in a wild type (J5) or *sunn-4* mutant background at 40 days post germination. E-F, Expression analysis of the transcript of *35S:MtCLE13* by qRT-PCR on cDNA samples of uninoculated wild type main roots and wild type shoots of chimeric plants bearing additional *35S:GUS* (control) or *35S:MtCLE13* transgenic roots at 40 days post germination. E, In wild type plants (J5). F, In a *sunn-4* mutant background. Data and error bars represent means ± SD. All experiments were performed once.

transcripts derived from the transgenic roots can be detected in the wild type roots of *sunn-4* mutants, but not in shoots, indicating that the movement of *MtCLE13* transcripts across roots is independent of *SUNN* (Figure 6.8F).

Together, ectopic expression of *MtCLE13* seems to result in a systemic and *SUNN*-dependent differential regulation of the genes encoding NFP, the kelch-repeat containing F-box protein (B) and the ODD, but not of the genes encoding the high affinity nitrate transporter, the kelch-repeat containing F-box protein A, the hypothetical protein D, LYK4 and the LYK5 homolog. Moreover, we noticed a systemic, root-specific and *SUNN*-independent spreading of *MtCLE13* transcripts.

6.3 Discussion

Two structurally related *CLE* genes, *MtCLE12* and *MtCLE13*, upregulated during nodulation, were shown to negatively regulate nodulation because ectopic expression abolished nodulation and a combined RNAi resulted in the development of more nodules (Mortier *et al.*, 2010) (see chapter 4 and 5). The peptides interfered at an early stage of nodulation because expression of *ENOD11*, an early epidermal nodulation marker, was not observed upon rhizobial inoculation of plants ectopically expressing either CLE peptide gene (Mortier *et al.*, 2010).

To enable careful analysis of the early nodulation events, stably transformed *35S:-MtCLE12* and *35S:MtCLE13* plants were made. In agreement with the results obtained for the composite plants, also these stable transgenic lines were devoid of nodules. Microscopic analysis revealed no differences in root hair morphology between control and *35S:MtCLE* transgenic lines. After inoculation, neither micro-colony nor infection thread formation was detected, and the marker genes for cortical nodulation responses, *MtRR4* and *NIN*, were not switched on in *35S:MtCLE12* or *35S:MtCLE13* roots. These results confirm that ectopic expression of *MtCLE12* or *MtCLE13* inhibits nodulation at the level of the NF signaling pathway from a very early stage on, before the appearance of any epidermal or cortical responses.

To analyze how this inhibition works, both a gene-specific and a micro-array based transcription analysis was done. Interestingly, the approaches revealed that ectopic expression of *MtCLE13* resulted in a downregulation of the NF receptor *NFP* and two homologous genes, *LYK4* and a close homolog of *LYK5*, but not of other NF signaling genes like *LYR3, NSP1, NSP2, ERN1, DMI1, DMI2, DMI3, NIN* and *ENOD11*. Analysis of *pNFP:GUS* expression revealed that, before inoculation, the gene is ex-

pressed in the epidermal cells of the root zone which is susceptible for nodulation (Arrighi *et al.*, 2006). Histochemical localization of GUS activity in uninoculated, *35S:MtCLE13* transformed roots of *pNFP:GUS* plants revealed a slight downregulation of *NFP* expression in epidermal cells compared to the level observed in control roots. In addition, a shift of *NFP* expression was seen from the cells of the root susceptible zone towards the cells of the roottip. The lack of *NFP* expression in the susceptible zone might explain the absence of nodulation observed in *35S:MtCLE13* lines. Hence, in the cells susceptible for nodulation, *NFP* expression is repressed by the action of *MtCLE13*. Because structurally related CLE peptides act in a redundant way in gain-of-function analyses, the same might be true for *MtCLE12* overexpressing lines.

At the onset of cortical cell division during nodulation, *MtCLE13* expression is observed as a gradient with the highest expression in the inner cortex and the lowest expression in the outer cortical cells (Mortier *et al.*, 2010). Based on the results obtained by the gain-of-function analysis, this *MtCLE13* expression pattern might lead to a subsequent downregulation of *NFP* to limit additional nodule formation in adjacent root zones. In agreement, qRT-PCR analysis revealed that *NFP* expression is down-regulated early after nodule initiation. At first sight, this is in contradiction to the results obtained by Arrighi *et al.* (2006), who reported a local induction of *NFP* expression in cells of the infection track, in nodule primordium cells and later on in the apical region of developing nodules. In our qRT-PCR analysis however the complete root susceptible zone was harvested. Hence, the tissue analyzed by qRT-PCR also contained epidermal tissue and the observed downregulation of *NFP* might be due to the fact that the reduction of *NFP* expression in the epidermis may overrule the upregulation observed in infection thread containing cells and cells of the nodule primordium. To further confirm these results, *NFP* expression in epidermal cells of inoculated roots should be carefully analyzed by *pNFP:GUS* or *pNFP:GFP* analysis. These experiments might be very difficult to perform as the high expression seen in infected cells might hinder analysis of expression levels in epidermal cells. Moreover, it might be interesting to analyze NFP protein levels.

Previously it has been shown via gain-of-function analysis that the inhibitory effect of AON peptides on nodulation involves long-distance signaling and is mediated by the AON LRR-RLK SUNN and its orthologs in other legumes (Krusell *et al.*, 2002; Nishimura *et al.*, 2002a; Searle *et al.*, 2003; Schnabel *et al.*, 2005; Oka-Kira *et al.*, 2005). A preliminary qRT-PCR analysis using a split-root approach indicated that the downregulation of *NFP* expression but not of two other *LYK* genes by *35S:MtCLE13*

would also be systemic and *SUNN*-dependent (Mortier *et al.*, 2010). The effect of *MtCLE13* seems to be stronger in the roots ectopically expressing *MtCLE13* than in the non expressing roots. Indeed, in contrast to *NFP*, the expression of the other *LYK* genes was not modulated in the distant roots by *35S:MtCLE13* expression. Together, these preliminary data indicate that AON might affect the expression of the NF receptor *NFP* to control nodule number. To further support this hypothesis, split-root experiments with *pNFP:GUS* plants will be performed to analyze the effect of nodulation on one side of the roots on the expression of *NFP* on the other side of the root.

In addition to the genes encoding the LysM-type receptors, other possible target genes for *MtCLE13* signaling were detected. QRT-PCR analysis revealed that several marker genes, involved in cell division, hormonal responses or nodulation, were not differentially regulated by the ectopic expression of *MtCLE13*. However, array hybridization revealed that besides the three *LYK* genes, the expression of 11 other genes was differentially modulated by *35S:MtCLE13* expression. A gene, encoding a kelch-repeat containing F-box protein and an ODD, were respectively up- and downregulated by the ectopic expression of *MtCLE13* in a putatively systemic and *SUNN*-dependent way, suggesting that they might be involved in the same pathway as *NFP* to inhibit nodulation. In agreement with their *35S:MtCLE13* expression pattern, these genes were respectively up- and downregulated during nodulation. Kelch-repeat containing F-box proteins are subunits of E3 ubiquitin ligase complexes, which specify protein substrates for degradation by the 26S proteasome (Pickart, 2001; Koepp *et al.*, 2001; Adams *et al.*, 2000; Sun *et al.*, 2007b). Ubiquitination is also involved in receptor internalization, leading either to endocytosis of the receptor or to receptor recycling (Acconcia *et al.*, 2009; Yee and Goring, 2009). The kelch-repeat containing F-box proteins activated by *MtCLE13* could therefore control NFP internalization, hence diminishing NFP activation and nodule initiation. Only a few Arabidopsis kelch-repeat containing F-box proteins have been analyzed in more detail, and all of them were involved in circadian control (Adams *et al.*, 2000; Yasuhara *et al.*, 2004). Until now, nothing has been reported about kelch-repeat containing F-box proteins acting specifically during nodulation. It will therefore be interesting to further study the role of this gene. The proteins of the ODD superfamily are involved in many aspects of plant metabolism, including the biosynthesis of gibberellins, flavonoids and flavonoid derivatives (Prescott and John, 1996; Hedden and Phillips, 2000). It will be interesting to unravel the function of this ODD protein in CLE signaling. Related genes of the ODD superfamily were identified in soybean and were proposed to play a role in nodule senescence (Webb

et al., 2008).

In addition, a gene encoding a high affinity nitrate transporter and a second kelch-repeat containing F-box protein were upregulated in *35S:MtCLE13* roots compared to control roots. Moreover, the expression of both genes was upregulated during nodulation, indicating that they might be targets for *MtCLE13* signaling. Like for *MtLYK4* and *MtLYK5*, we found no indications for a long distance control of the expression of these genes, suggesting only a local regulation by *MtCLE13*. Again, the absence of changes in expression at long distances might reflect the possibility that the effect of *35S:MtCLE13* is stronger in the local root compared to the systemic roots. Alternatively, RNAi has shown that the CLE peptides also control the differentiation of the central tissue cells. Hence, functional analysis should reveal whether the *MtCLE13* target genes play a role in the latter process.

One of the members of the nitrate transporter (NRT) family is the *LATERAL ROOT DEFECTIVE/NUMEROUS INFECTION THREADS, POLYPHENOLICS* (*LATD/NIP*) gene encoded by *M. truncatula* (Yendrek *et al.*, 2010; Harris and Dickstein, 2010). *LATD/NIP* regulates root architecture and symbiotic nodule formation (Yendrek *et al.*, 2010; Harris and Dickstein, 2010). The nodulation-specific functions of *LATD/NIP* are related to rhizobium infection and nodule meristem function (Yendrek *et al.*, 2010; Harris and Dickstein, 2010). Also for *MtCLE13*, a function during nodule meristem formation/maintenance has been suggested (Mortier *et al.*, 2010). The expression of *LATD/NIP* in roottips is upregulated by cytokinin and downregulated by auxin and ABA (Yendrek *et al.*, 2010; Harris and Dickstein, 2010). The upregulation of *LATD/NIP* expression by cytokinin suggests that it might act in the cortical pathway leading to cell divisions for primordium formation, similar to what was suggested for *MtCLE13* (see chapter 4 and 5). Whether the high affinity nitrate transporter upregulated by *MtCLE13* exerts a similar function as *LATD/NIP* is currently not known but is an interesting hypothesis to test.

Genes encoding a copper binding protein and a germin-like protein were upregulated by the ectopic expression of *MtCLE13*, but downregulated during early nodulation. In addition, nicotianamine synthase was downregulated by ectopic expression of *MtCLE13*, but upregulated during nodulation. It is difficult to envisage the relation between *MtCLE13* signaling and the expression of these genes. The effect might be secondary and might possibly involve cell-non-autonomous effects. Functional analysis by overexpression or RNAi might reveal how the interactions work. Although it is very precocious to speculate about the function of the encoded proteins during nodu-

lation, nicotianamine synthase might be involved in the intercellular transport of iron (Fe) in dicotyledonous plants (Ling *et al.*, 1999; Pich *et al.*, 2001; Takahashi *et al.*, 2003). Fe mobilization is essential in nodules as it is a component of leghemoglobin and the nitrogenase complex, both involved in nitrogen fixation (Ragland and Theil, 1993). Nodule specific expression of nicotianamine synthase has been observed in *Lotus japonicus* (Hakoyama *et al.*, 2009). Hence, *MtCLE13*, which is expressed in developing nodules and in the apical part of mature nodules, might possibly restrict the expression of nicotianamine synthase to the fixation zone of mature nodules, the place where the nitrogenase enzyme is active.

At last, the expression of three more hypothetical proteins was modulated by *35:MtCLE13*. One of these, encoding hypothetical protein D, is downregulated by the ectopic expression of *MtCLE13* and also transiently downregulated during nodulation. This gene might be a specific target of *MtCLE13* at the early stages of nodulation, during primordium development. Functional analysis should reveal its function.

Finally, in the experiments to control long distance effects, a low expression level of *MtCLE13* transcripts was found back in the non-transgenic roots. The transcripts were derived from the transgenic construct and the effect was specific for *MtCLE13*, as no transcripts of the *GUS* and *GFP* marker were detected. Hence, it seems that in the gain-of-function analysis a small portion of the *MtCLE13* transcripts spread systemically in the roots but not towards the shoot in a *SUNN*-independent way. This is a very preliminary indication but sheds new light on how the AON peptides might control nodulation. Long distance movement of mRNAs is an intriguing and largely unexplored field. In the phloem saps, many mRNAs, especially small RNAs, have been found back that might control whole plant homeostasis (Yoo *et al.*, 2004; Chitwood and Timmermans, 2010; Kalantidis *et al.*, 2008). In-depth analysis to check whether this *MtCLE13* movement also occurs during nodulation is very tempting, as CLE peptides are mostly regarded as short-distance signals (Fukuda *et al.*, 2007; Hirakawa *et al.*, 2008; Whitford *et al.*, 2008; Miwa *et al.*, 2009). However, via movement of mRNA molecules, the peptides might act at long distances.

The preliminary experiments also showed that the *MtCLE13* mRNA did not move towards the shoot. Analysis of the stable *35S:MtCLE12* and *35:MtCLE13* lines also indicates that ectopic expression did not result in any shoot phenotype. No differences in leaf sizes were observed between control and *35S:MtCLE12* or *35S:MtCLE13* plants. In addition, in contradiction to the elongated petioles that were observed on *A. rhizogenes*-mediated composite plants ectopically expressing *MtCLE12* or *MtCLE13* in

the roots (Mortier *et al.*, 2010), the petiole length of *35S:MtCLE12* and *35S:MtCLE13* stable transgenic plants did not differ from control plants. This discrepancy might be explained in different ways. It is well-known that *A. rhizogenes*-induced transgenic roots have an altered hormone balance. This change in balance might add up with the effect of ectopic *MtCLE12* or *MtCLE13* expression to cause the elongation of the petioles. However, this hypothesis was not reflected by the results of the transcriptome analysis, as no changes in expression of hormone-specific genes were observed. Alternatively, the petiole elongation might be a cell-non-autonomous effect that is abolished when the CLE peptides are also expressed in the cells of the shoot.

In contrast to the shoot, an effect of *35S:MtCLE13* expression was seen on the root length. The MtCLE12 and MtCLE13 peptides are most homologous to the CLE1 till CLE7 peptides of Arabidopsis (Mortier *et al.*, 2010). Functional analysis of this group of CLE peptides resulted in conflicting data concerning the effect on root length. According to Kinoshita *et al.* (2007) and Meng *et al.* (2010) exogenous addition of CLE1 to CLE7 peptides to the growth medium of *A. thaliana* seedlings did not result in any root phenotype, however, at high peptide concentrations Ito *et al.* (2006) observed an inhibition of root growth. In case of ectopic expression of the *CLE1* till *CLE7* genes, root elongation was reported by Strabala *et al.* (2006), while Whitford *et al.* (2008) reported root meristem consumption specifically for *CLE6*. Exogenous addition of MtCLE12 and MtCLE13 peptides did not result in any root-related phenotype (Mortier *et al.*, 2010). However, stable ectopic expression caused a reduction in root length. These opposite results might be explained by the high level of redundancy that is observed between CLE peptides (Fiers *et al.*, 2005; Strabala *et al.*, 2006; Ni and Clark, 2006; Kinoshita *et al.*, 2007; Jun *et al.*, 2008). Possibly, MtCLE12 and MtCLE13 might bind with very low efficiency to receptors of unrelated CLE peptides which are putatively involved in root development. As a result, only high levels of MtCLE12 and MtCLE13 peptides, such as observed during ectopic expression would be able to activate the receptor sufficiently to trigger downstream responses. At the microscopical level, no phenotypical changes were observed in *35S:MtCLE12* or *35S:MtCLE13* roots, which is in accordance to the absence of a root developmental phenotype reported for the ectopic expression of the homologous *CLE6* gene in *A. thaliana* (Whitford *et al.*, 2008). Together, these results suggest that very high levels of *MtCLE12* and *MtCLE13* expression might have an effect on root length, similar to what was described by Whitford *et al.* (2008).

Legumes also establish a symbiosis with arbuscular mycorrhizal fungi and both

symbioses are initiated by a similar signaling pathway. Moreover, the *SUNN*-mediated AON pathway was suggested to be also involved in restricting arbuscule formation (Amiour *et al.*, 2006). Because the negative effect of AON peptides on nodulation also involves SUNN, we analyzed whether ectopic expression of *MtCLE12* and *MtCLE13* affected mycorrhization. No differences in mycorrhization rate were observed, suggesting these CLE peptides not to be involved in the mycorrhization pathway. Accordingly, mycorrhization did not induce the expression of the soybean *GmNIC1, GmRIC1* and *GmRIC2 CLE* genes, which have a CLE domain highly homologous to that of *MtCLE12* and *MtCLE13* (Reid *et al.*, 2011).

Together, this study revealed the first molecular insights in how peptides might control nodulation. The interaction with *NFP* is very intriguing and indicates that the repression of the NF receptor transcription might be one of the targets used to control nodule number. Functional analysis of the other identified *MtCLE13* target genes might further reveal more aspects of the repression mechanism. Also the observation that the *MtCLE13* mRNAs are traveling systemically in the root system is an intriguing piece of the puzzle to be solved.

6.4 Materials and methods

Biological material

M. truncatula Gaertn. cv. Jemalong A17 and J5, as well as *pNFP:GUS* (Arrighi *et al.*, 2006) and *sunn-4* (Schnabel *et al.*, 2005) were grown and inoculated as described (Mergaert *et al.*, 1993). *Sm2011* pHC60-GFP (Cheng and Walker, 1998), and *Sm2011* pBHR-mRFP (Smit *et al.*, 2005) were grown at 28°C in yeast extract broth medium (Vervliet *et al.*, 1975), supplemented with 10 mg L^{-1} tetracycline.

For the qRT-PCR analysis, *M. truncatula* J5 plants were grown *in vitro* in square Petri dishes (12 x 12 cm) on nitrogen-poor SOLi agar (Blondon, 1964). For the analysis of temporal expression during nodulation, nodules were harvested 4 to 10 dpi from plants grown in pouches, watered with nitrogen-poor SOLi medium, and inoculated with *Sm1021* pHC60-GFP. Infection threads were visible from 4 dpi on, nodule primordia at 6 dpi, and small nodules at 8 dpi. Two days later, at 10 dpi, slightly bigger nodules were observed. Tissue was collected by visualizing the green fluorescent bacteria under a stereomicroscope MZFLII (Leica Microsystems, Wetzlar, Germany) equipped with a blue-light source and a Leica GFP Plus filter set (λ_{ex} = 480/40; λ_{em} =

510 nm LP barrier filter). The zones I of uninoculated roots were isolated at the same developmental stage as the 4 dpi stage.

RNA extraction, cDNA synthesis, and qRT-PCR analysis

Total RNA was isolated with the RNeasy Plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After a DNAse treatment, the samples were purified through NH_4Ac (5 M) precipitation, quality controlled, and quantified with a Nanodrop spectrophotometer (Isogen, Hackensack, NJ). RNA (2 μg) was used for cDNA synthesis with the Superscript Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA). The samples were diluted 50 times and stored at -20°C until further use. The qRT-PCR experiments were done on a LightCycler 480 (Roche Diagnostics, Brussels, Belgium) and SYBR Green was used for detection. All reactions were done in triplicate and averaged. The total reaction volume was 5 μl (2.5 μl master mix, 0.25 μl of each primer [5 μM] and 2 μl cDNA). Cycle threshold (C_T) values were obtained with the accompanying software and data were analyzed with the $2^{-\triangle\triangle C_t}$ method (Livak and Schmittgen, 2001). The relative expression was normalized against the constitutively expressed 40S ribosomal S8 protein (TC100533, MGI). Primers used (Table 6.3) were unique in the MGI version 9.0 and the Medicago EST Navigation System database (Journet *et al.*, 2002). The qRT-PCR primers 'transcript of *35S:MtCLE13*' specifically detect the transcript of *35S:MtCLE13*, while discriminating the endogenous expression of *MtCLE13*, as the forward primer binds in the open reading frame (ORF) of *MtCLE13*, while the reverse primer is complementary to the *35S* terminator, which is the terminator of the *35S:MtCLE13* construct.

Agrobacterium rhizogenes-mediated transgenic root transformation

The protocol was adapted from Boisson-Dernier *et al.* (2001). Approximately 48 h after germination, the radicle was sectioned at 5 mm from the roottip with a sterile scalpel. Sectioned seedlings were infected by coating the freshly cut surface with the binary vector-containing *A. rhizogenes* Arqua1 strains. The *A. rhizogenes* strain was grown at 28°C for 2 days on solid yeast extract broth medium with the appropriate antibiotics (Quandt *et al.*, 1993). The infected seedlings were placed on agar (Kalys) containing the SOLi medium, supplemented with 1 mM NH_4NO_3, in square Petri dishes (12 12 cm) placed vertically for 5 days at 20°C, 16-h photoperiod, and 70 μE $m^{-2}m^{-1}$. Subsequently, plants were placed on the same medium between brown paper at 25°C

and under identical light conditions. One and 2 weeks later, plants were screened for transgenic roots, characterized by GFP fluorescence with a stereomicroscope MZFLII (Leica Microsystems) equipped with a blue-light source and a Leica GFP plus filter set. One main transgenic root was retained per composite plant. Four weeks after infection, plants were transferred to an aeroponic system, pouches, or perlite-containing pots and incubated with SOLi medium. Three to 7 days after planting, composite plants were inoculated.

To check the long distance effect of *MtCLE13*, the main root was kept on the juvenile plant and infected by stabbing the hypocotyls with a fine needle containing an *A. rhizogenes* culture and cotransformed as described above, after which the plants were grown for 2 weeks at 25°C with a 16-h photoperiod and light at 70 μE $m^{-2}m^{-1}$. Next, the plants were grown in an aeroponic system, supplemented with the nitrogen minimal medium Soli (Blondon, 1964). At the age of 40 days post germination, the wild type roots of approximately 20 plants were harvested and pooled for RNA isolation, which was subsequently used for cDNA synthesis. qRT-PCR analysis was performed twice on biologically independent material.

Agrobacterium tumefaciens stable transformation and shoot regeneration

M. truncatula Jemalong 5 young leaves from 4 week-old *in vitro* grown plants were used for *A. tumefaciens*-mediated transformation which was performed as described by Primard Brisset *et al.* (2005). Binary vectors pB7WG2D-*35S:MtCLE4*, pB7WG2D-*35S:MtCLE12*, pB7WG2D-*35S:MtCLE13* and pB7WG2D-*35S:Luciferase* was electroporated in *A. tumefaciens* AGLO and a single colony was isolated after selection on YEB supplemented with 100 mg/L rifampicin and 100 mg/L spectinomycin. After plant transformation and cocultivation, transgenic tissue was selected on subsequently SHb3a (incubation), AgCIM (callogenesis), AgEIM (pro-embrygenesis), AgEMB (embryogenesis) and plant medium (plant development and rooting). All media, except the plant medium, contained between 200 to 800 mg/L augmentine during callogenesis and (pro) embryogenesis stages and 3 mg/L basta (Trinh *et al.*, 1998; Chabaud *et al.*, 2003). *In vitro* regenerated transgenic shoots were further grown on plates for 1 month and then planted in earth and sand (3:1) in the growth chamber or shoot regenerated on PDM medium. Shoot regeneration was performed by cutting the shoot tip at the fourth

internode from the shoot apex and transferring the sectioned shoot on PDM medium[18]. The root length, petiole length and total leaf area was scored by ImageJ[19]. Root hairs were analyzed using a stereomicroscope MZFLII (Leica Microsystems, Wetzlar, Germany).

Histochemical localization of GUS activity

GUS activity in cotransformed roots and nodule primordia of *pNFP:GUS* plants was analyzed using 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid as substrate (Van den Eede *et al.*, 1992). Roots and nodule primordia were vacuum infiltrated during 20 min and subsequently incubated in GUS buffer at 37°C. Incubation lasted 4 h. After staining, roots and root nodule primordia were fixed and dehydrated in 70 % ethanol. Photographs were taken with a stereomicroscope MZFLII (Leica Microsystems, Wetzlar, Germany).

Technovit sectioning

Transgenic roots were fixed and dehydrated before embedding with the Technovit 7100 kit (Heraeus Kulzer, Wehrheim, Germany), according to the manufacturer's instructions, and sectioning with a microtome (Reichert-Jung, Nussloch, Germany). The 3-μm thick sections were mounted on coated slides (Sigma-Aldrich, St. Louis, MO), and submerged in a 0.5 % toluidine blue solution, washed in distilled water, and dried. Finally, sections were mounted with Depex (BDH Chemicals, Poole, England). Photographs were taken with a Diaplan microscope equipped with bright- and dark-field optics (Leitz, Wetzlar, Germany).

Gene-specifc approach

Roots of approximately 15 chimeric plants with *35S:GUS* (control) or *35S:MtCLE13* transgenic roots were made by *A. rhizogenes* transformation and grown in an aeroponic system, supplemented with the nitrogen minimal medium Soli (Blondon, 1964). At the age of 40 days post germination, the transgenic roots of approximately 15 plants were harvested and pooled for RNA and cDNA preparation.

[18] http://www.isv.cnrsgif.fr/embo01/manuels/pdf/module2.pdf
[19] http://rsb.info.nih.gov/ij/

Micro-array analysis

Chimeric plants with *35S:GUS* or *35S:MtCLE13* transgenic roots were made by *A. rhizogenes* transformation and grown in an aeroponic system, supplemented with the nitrogen minimal medium Soli (Blondon, 1964). At the age of 40 days post germination, the transgenic roots of approximately 15 plants were harvested and pooled for RNA isolation and probe preparation. Samples were hybridized to the Gene Chip Medicago genome arrays (Affymetrix, www.affymetrix.com). RNA processing, hybridization, washing and scanning of the arrays were carried out at the MicroArray Facility (Leuven, VIB). Medicago Affymetrix genome array data were preprocessed using the robust multiarray averaging (RMA) algorithm (Irizarry *et al.*, 2003), which involves three steps: background adjustment of RMA convolution, quantile normalization and summarization, using the median polish algorithm - where the median values per probe set, adjusted for slide differences, are calculated. On the basis of an empirical Bayes moderated *t*-statistic for the contrasts (Smyth, 2004), as implemented in the Bioconductor package *LIMMA*, p-values were calculated to measure differential expression between *35:GUS* (control) transgenic roots and *35S:MtCLE13* transgenic roots. P-values were then corrected for multiple testing problems using Benjamini and Hochberg to control false discovery rate. Treshold values for differential expression were set at 1.93 (log scale 0.95) and minimal p-values was set at 0.05.

Southern analysis

M. truncatula genomic DNA was extracted from young leaves using the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol. 10 μg was digested with the restriction enzyme HindIII according to standard protocols (Sambrook *et al.*, 1989) and were separated on a 1 % agarose gel. The DNA was denaturated and transferred to positively charged nylon menbranes (Amersham Hybond-N^+, GE Healthcare). The probes were synthesized via PCR using Taq recombinant polymerase (Invitrogen) and Dig labelled by use of the PCR Dig probe synthesis kit (Roche Diagnostics GmbH). After cross-linking the gDNA via UV light, the filter was hybridized with the Dig labelled probe at 42°C, overnight in Dig easy hyb hybridization buffer (Roche). After washing the filter (2x SSC, 0.1 % SDS and 1xSSC, 0.1 % SDS, 65C, 15', the hybridized membrane was analyzed by chemiluminiscent detection (Antidigoxigenin-AP binding and CDP-star substrate (Roche)) on a light sensitive film.

Statistical analysis

The following generalized linear model (GLM) $Y_{ijk} = \ + genotype_j + experiment_k + error_{ijk}$ (Regression analaysis) was fitted to the nodule number, partitioning phenotypic variation into fixed genotype and experiment effects and random error effects. Y_{ijk} is the phenotype of the i-th plant from the genotype j analyzed in experiment k; μ is the overall mean of the phenotypes obtained for all lines considered. Because the data has a Poisson distribution, a logarithm base e link function was incorporated. In the case of root length, petiole length and total leaf area, an analysis of variance (ANOVA) was performed. All analyses were done by means of the GenStat software[20].

Mycorrhization assay

M. truncatula cv. Jemalong A 17 seeds were scarified by incubation in concentrated H_2SO_4 for 10 min, washed with water and surface-sterilized in 1:6-diluted sodium hypochlorite solution (12 % Cl) (Roth, Karlsruhe, Germany) for 5 min. After washing, the seeds were placed on wet filter paper and germinated at 4°C in the dark for two days, at RT in the dark for one day and at RT in the light for one day. Seedlings were transferred into in pots filled with expanded clay of 2 to 5 mm particle size (Original Lamstedt Ton; Fibo ExClay, Lamsted, Germany). Plants were cultivated in a phytochamber at a 16 h/8 h cycle (22°C/18°C) at 375 μM light intensity and watered with distilled water and fertilized once per week with 10 ml 10x Long Ashton (20 % phosphate).

At time of transfer into expanded clay, seedlings were inoculated with the AM fungus *Glomus intraradices* Schenk & Smith isolate 49 (Maier *et al.*, 1995) by transfer to clay substrate containing 15 % (v/v) inoculum. The inoculum was enriched in propagules by cultivation with mycorrhizal leek (*Allium porrum* cv. Elefant).

The mycorrhizal structures in the root pieces were stained according to (Vierheilig *et al.*, 1998) using 5 % (v/v) ink (Sheaffer Skrip jet black, Sheaffer Manufacturing, Madison, USA) in 2 % (v/v) acetic acid and analyzed using a stereomicroscope. The degree of mycorrhization was determined from five plants percent colonization of root length.

[20] `http://www.vsni.co.uk/software/genstat/`

Table 6.3: Primers used in the analyses.

Gene	Sense primer	Anti-sense primer	Reference
MtCLE13	CCGAAGCCTTCTACAGAAACTACG	TCTTGGTGGTGATCTTCCATTATGC	
CRE1	TATGGATTCTTCTGATGGCTCTG	ACACTAACACTGAATTGGTCTTG	Gonzales-Rizzo *et al*., 2006
NSP1	GCAAGGCTATAAGCAAGGTAC	ATAAGGAAGAACTACATTGTGGTG	
NSP2	GACACACTTGCTGCTTTCCA	AATGCGGTTATCCGAAGATG	Gonzales-Rizzo *et al*., 2006
ENOD11	ATCCCACAATATGCCTCCAA	AGGAAGTGGTGGCTTTAGCA	Gonzales-Rizzo *et al*., 2006
NIN	GGAAGATTGAGAGGGGAAGCTT	GCAATGTGGGGATTTAGAGATT	Gonzales-Rizzo *et al*., 2006
ERN	ACAAGCAATGATGATAACAAGGAG	CTTCGCCATATCCAATAACTGAG	
NFP (qRT-PCR)	GGCTTCTATGTTACTGGCTTTTG	CTGTGGATTCTCCTCCTTCATG	
LYR3	CTCTTCTTCTTCTTCTTCGTCAGCA	GGTATGCTTGGCATGTTTGAGTTT	Arrighi *et al*., 2006
DMI1	CGGCTCTAAGAATTGGTTACTC	AGTTTCTAAATGTGCTATCCTCTG	
DMI2	GTATTTCCATCCACTCTATGTTTC	GGACTTGGCAAATGATATGATATG	
DMI3	GACAACAACCGTGATGGAAC	AAAGCCCTGAGCATGGATG	
PIN1	ACAAAGACATAATCAATGGCTATC	CAATGTGACGACACTGTGG	
PIN2	GCTGTTACTGAATTGATTGAGAAC	CTCCTCCTTCCAAGTCCATC	
LAX1	TTCACACTTACCCTACCATCTG	AGGCATTACGAGGAAGAAGAG	
LAX2	TCACCTCAACCTCAACATAGAAG	ACAACACACAGCCAGCAC	
LAX3	AAACATTTGGGCTATTTGCTAAG	GCCAATAATCTCTTTACACACATG	
MtcycB1	TTACTCCGAAGAACAGTTGATGG	GCAGCAGGTAGCAGAAGATTC	
MtcycD3	AAGGTGGATGAATGTGGGAAG	GGCTCTGGTGAAGATGATACTG	
MtIPT	TGTCCGCCGTCAGAAGAG	TCACTCACGAATGTTGTTACTATC	
MtRR4	ATGCTTTTGTTCCGGGTTTA	CTGCACCTTCCTCCAAACAT	Gonzales-Rizzo *et al*., 2006
ABA-activated protein kinase	ATTCAGAGGGTTCTCAGTGTCC	GCAGGGTCAAAAACAAAGATTC	Fei and Vessey, 2009
Gibberellin-regulated protein	ATTCACAGGGTTCTCTTCTTCG	TGGCACACAGTTACATCTTCTG	Fei and Vessey, 2009
Ehtylene-responsive element-binding factor3	GCAAGAGTTTGGCTCGGAAC	CTGCTGCGTCAAGATTATGGTA	Fei and Vessey, 2009
AUX-responsive protein IAA14	GCCCAAGGAATGATAGACTTC	ATCTCCCACGAGCATCCA	Fei and Vessey, 2009
Auxin response factor 3	GTGGACCCTCTCCTCTCTTCAG	AATGGTGGCAAGGTAATAGCAT	Fei and Vessey, 2009
Cytokinin-specific binding protein	TCAACACCCAAACAACACTGA	ACCCTCCATCCCCTTCAAT	Fei and Vessey, 2009
Cytokinin synthase	GCAACCTTAGCCCTTCACAA	CGACTTTGGTGGGAGACTTT	Fei and Vessey, 2009
Naringenin-CHS 2	TGGACAAGCACTATTTGGAGAT	TCAATGGCACCTTCACTATCAG	Fei and Vessey, 2009
Isoflavone reductase homolog 1	TTGAAGCCAAAACAAACCTCTT	ACGATGAAACAAACACAAATGG	Fei and Vessey, 2009
Myc2-like protein	ATGACCACCTCCGACATC	TCTTGATTGAATAGAGACTGTTG	Goossens *et al*., unpublished
Putative cytochrome P450	TCACCGCAGGCTTCAATG	CGATCGCTTCATAGACAAC	Goossens *et al*., unpublished
Zym containing protein	ACACTTTCATTCTTATTACAACGC	ATATTCCGATGAGGTAGACATAG	Goossens *et al*., unpublished
Kelch-repeat containing F-box protein (A1)	TTTGGCGATTTATACTTGTTGTC	CCATCATCACAGCACAGTTC	
Kelch-repeat containing F-box protein (A2)	GCGGCGAAAGCAAATTGG	GCTGTAATCTGATTGAATCCTTGG	
Close homolog of LYK5	AGTTTAGTTGCTGCTGGAATATG	GGTGACATTGAATCTTCTGGTG	
Kelch-repeat containing F-box protein (B)	TCTGGTGACAATGGTTCCTC	AAGACATGGTAATGGTAGTAGAAC	
NFP (micro-array)	TGGCAGGATAATGACAATGTTAC	GTTGAAGCAGTGAAGTTATGATTG	
LYK4	CCAATGCTACAGAGAACTTCAG	GCTTGCGTCTTCATCTTCTTG	
High affinity nitrate transporter	TTGGCTGAATGGAACTCAAAG	TTGTGGTGGTGTAACATCTTC	
Nicotianamine syntase	TCTGTGTGCGTGTGTCTAAG	CAAGGTTTGAGTGTGTCTAAGC	
Hypothetical protein (A)	TTATTCTGATCCTCCTCCTAATGC	CCCTTATTGTCTGACTTCTTTGAG	
Hypothetical protein (B)	TTGCTTGAACCTAACATTTGTATC	TAGTATGTGCAGGAGGAGAAC	
Copper binding protein	GAAGCAGAAGGAGAGCCTATAC	CTCAGCACAAGCCTCACAG	
Hypothetical protein (C)	TGCCGTCTTCACCACTATATTC	TCTTCTTCCGTTGATCTCTGAC	
Hypothetical protein (D)	ATTTCAACTCCTGGTCTACTCG	CCGCTCCTCCTCCATCAC	
Germin-like protein	TGTTGCTATTGCTGGACTTAG	ATCAATTATGCTCTTATCCACTTG	
Hypothetical protein (E)	TCAGAGTATCAAGGATGGTTGTC	GCATGAAAGCAATAGGAATAACTG	
ODD	TCAACCAATTACCGACTCAGG	CATCATCTCTAAACCTTCCACTTG	
Transcript of 35S:MtCLE13	CCTCAGCATAATGGAAGATCAC	AGCGAAACCCTATAAGAACCC	
GUS	CTACACCACGCCGAACAC	CACCACCTGCCAGTCAAC	
GFP	ACGGCATCAAGGTGAACTTC	GTGCTCAGGTAGTGGTTGTC	
40S Ribos	GCCATTGTCCAAGTTTGATGCTG	TTTTCCTACCAACTTCAAAACACCG	

6.5 Supplemental data

Table 6.4: Numeric data of heatmap represented left in Figure 6.6.

Gene	qRT-PCR	array
Kelch-repeat containing F-box protein (B)	69.4	11.5
Copper binding protein	4.7	2.2
Kelch-repeat containing F-box protein (A1)	4.1	4.7
Kelch-repeat containing F-box protein (A2)	2.5	5.4
High affinity nitrate transporter	2.4	2.8
Germin-like protein	2.3	2.7
Hypothetical protein (E)	1.4	2.5
Hypothetical protein (C)	-1.2	-3.7
Close homolog of LYK5	-1.4	-3.7
ODD	-1.8	-2.1
Nicotianamine syntase	-2.1	-3.2
LYK4	-2.4	-3.5
Hypothetical protein (B)	-4.2	2.3
NFP	-5.9	-3.3
Hypothetical protein (D)	-12.3	-4.7

Table 6.5: Numeric data of heatmap represented right in Figure 6.6.

Gene	NI	4 dpi	6 dpi	8 dpi	10 dpi
High affinity nitrate transporter	1.09	8.76	13.04	10.97	137.69
Kelch-repeat containing F-box protein (B)	1.00	7.27	16.45	9.49	24.08
Hypothetical protein (C)	1.02	3.63	8.20	5.64	15.14
Kelch-repeat containing F-box protein (A1)	1.00	1.53	2.43	3.80	8.17
Kelch-repeat containing F-box protein (A2)	1.01	1.36	3.32	5.44	8.11
Nicotianamine syntase	1.02	1.21	2.51	2.95	9.07
Hypothetical protein (D)	1.00	-4.89	-2.36	-2.17	1.13
Germin-like protein	1.01	-1.35	-1.75	-2.20	-2.53
Copper binding protein	1.06	-1.81	-1.84	-5.07	-2.63
Hypothetical protein (E)	1.03	-2.00	-2.28	-3.87	-2.00
NFP	1.01	-2.63	-2.80	-3.92	-1.69
ODD	1.01	-1.62	-6.08	-4.32	-5.81
Close homolog of LYK5	1.01	-2.85	-3.71	-5.15	-3.19
Hypothetical protein (B)	1.00	-11.45	-12.93	-11.69	-3.46
LYK4	1.01	-4.54	-7.15	-12.64	-9.92

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Authors contributions

The main part of this work was performed by V. Mortier. C. Verplancke performed the *Agrobacterium tumefaciens* transformations. S. Goormachtig and M. Holsters were involved in designing the research and revising the manuscript.

7

Receptor-like kinases involved in nodulation on *Medicago truncatula*

Virginie Mortier, Carolien De Cuyper, Stéphane Rombauts, Marcelle Holsters and Sofie Goormachtig

In preparation

Abstract
CLE peptides are short-distance signaling molecules, involved in balancing cell division and differentiation during numerous aspects of plant development. They are mostly recognized by several receptor complexes working in parallel. Nearly all components of these receptor complexes are leucine-rich-repeat receptor-like-kinase (LRR-RLK) of subclass XI. We searched for putative nodule-specific CLE peptide receptors belonging to this subclass XI. Two candidate genes, *MtRLK1* and *MtRLK2*, were initially identified. In-depth phylogenetic and expression analysis of *MtRLK2*, rather hints at a role during plant immunity. On the other hand, *promoter:GUS* and qRT-PCR analysis of *MtRLK1*, a close homolog of *BAM3*, confirmed its nodulation-related expression pattern. In addition, the *MtRLK1* expression pattern is lying adjacent to and is partially overlapping with the expression patterns of nodulation-related CLE peptides, confirming that MtRLK1 might act as a CLE ligand receptor to control stem cell homeostasis in the nodule meristem. We also investigated the expression of *SUNN*, a LRR-RLK of subclass XI involved in autoregulation of nodulation, during nodulation and observed that the gene is only expressed at a low level in the vasculature of the root. An analogous expression pattern was observed for *MtRLK1*, suggesting that these receptors might interact in the root vasculature.

7.1 Introduction

Plant growth relies on meristem-located stem cells from which most plant tissues and organs are post-embryonically formed (Byrne *et al.*, 2003; Carles and Fletcher, 2003; Gross-Hardt and Laux, 2003). Growth is mainly organized at the shoot- and the roottip, in respectively the shoot and root apical meristem (SAM, respectively RAM). Plants tightly control the equilibrium between cell division and differentiation at those sites in order to keep a specific number of dividing stem cells to sustain indeterminate growth and at the same time to provide enough cells for differentiation into the appropriate tissues and organs (Hobe *et al.*, 2001; Fletcher, 2002; Carles and Fletcher, 2003). CLE peptides are small, secreted, intercellular peptides that act as short-distance signaling molecules in a non-cell-autonomous manner to balance proliferation and differentiation not only at the SAM and RAM but also during vascular development and during nodulation (Brand *et al.*, 2000; Hirakawa *et al.*, 2008; Hobe *et al.*, 2003; Okamoto *et al.*, 2009; Mortier *et al.*, 2010, 2011; Reid *et al.*, 2011). CLE ligands are sug-

gested to bind an array of plasma membrane located receptor complexes (Zhu *et al.*, 2010a,b; Bleckmann *et al.*, 2010; Guo *et al.*, 2010; Ogawa *et al.*, 2008; Ohyama *et al.*, 2009; Kinoshita *et al.*, 2010; Stahl *et al.*, 2009; Meng and Feldman, 2010). For instance at the SAM in Arabidopsis, the stem cell maintenance and organ specification is balanced by a negative feedback-loop including the CLE peptide ligand CLV3 (Fletcher *et al.*, 1999; Ni and Clark, 2006; Kondo *et al.*, 2006), the leucine-rich repeat receptor like kinase (LRR-RLK) CLAVATA1 (CLV1) (Dievart *et al.*, 2003; Clark *et al.*, 1997) and its close homologs BARELY ANY MERISTEM1 (BAM1), BAM2 and BAM3 (Deyoung and Clark, 2008; DeYoung *et al.*, 2006), the LRR receptor-like-protein (LRR-RLP) CLV2 (Jeong *et al.*, 1999; Kayes and Clark, 1998), the transmembrane kinase-related protein CORYNE/SUPPRESSOR OF OVEREXPRESSION OF LLP1-2 (CRN/SOL2, hereafter CRN) (Casamitjana-Martinez *et al.*, 2003; Muller *et al.*, 2008; Miwa *et al.*, 2008), the CLV1-unrelated LRR-RLK RECEPTOR-LIKE PROTEIN KINASE2/TOADSTOOL2 (RPK2/TOAD2, hereafter RPK2) (Kinoshita *et al.*, 2010; Sawa and Tabata, 2011), the KINASE ASSOCIATED PROTEIN PHOSPHATASE (KAPP) (Williams *et al.*, 1997; Stone *et al.*, 1998), the type 2C protein phosphatases POLTERGEIST (POL) and POL-LIKE 1 (PLL1) (Gagne and Clark, 2010; Song *et al.*, 2006, 2008) and the homeodomain transcription factor WUSCHEL (WUS) (Schoof *et al.*, 2000; Mayer *et al.*, 1998; Brand *et al.*, 2000).

The SAM is organized in several domains and layers and central are a group of dividing stem cells that are distributed over three layers and that give rise to particular organs (Schoof *et al.*, 2000; Hobe *et al.*, 2001; Carles and Fletcher, 2003). The CLV3 peptides, expressed in the outermost L1 layer and middle L2 layer of the SAM, are secreted in the extracellular medium and diffuse to the adjacent, more inside located L3 layer. There, they are sequestered by plasma membrane-localized receptors (CLV1, BAM1, BAM2, CLV2, CRN and RPK2), generating a signal that is relayed through KAPP, POL and PLL1, and which results in *WUS* suppression in the organizing centre (OC), a small group of non dividing cells, located in the L3 layer, which control stem cell maintenance. *WUS*, in turn, has a positive effect on *CLV3* expression (Brand *et al.*, 2000; Schoof *et al.*, 2000; Reddy *et al.*, 2004; Reddy and Meyerowitz, 2005). As a consequence, in *A. thaliana, clv1, clv2* and *clv3* loss-of-function mutants cause an increased *WUS* expression domain, resulting in additional stem cell accumulation, ensuing an enlargement of the SAM, increased floral organ number and altered phyllotaxy (Clark *et al.*, 1993, 1995; Kayes and Clark, 1998; Brand *et al.*, 2000; Schoof *et al.*, 2000; Laux *et al.*, 1996; Wang and Fiers, 2010). In *wus* mutants the identity

of the neighboring stem cells is lost, resulting in cell differentiation, which ensues the consumption of the SAM and termination of organogenesis (Laux *et al.*, 1996).

CLV1 and CLV2 are not the only receptors for CLV3 and different receptor complexes seem to act together for SAM homeostasis. Unraveling the specific composition of the receptor complexes that bind CLV3 has turned out to be a tedious work. Initial experiments involving *clv1* mutants revealed that *clv1* missense alleles are dominant-negative, while *clv1* null alleles are weak in phenotype, suggesting that other receptors function in parallel to CLV1 (Dievart *et al.*, 2003). Co-immunoprecipitation (co-IP) via transient expression in tobacco but also via *in vivo* experiments in Arabidopsis meristem cells confirmed the existence of different receptor complexes, such as CLV1 homomultimers, heteromultimers of CLV1 with BAM1 and BAM2, and CLV2/CRN heteromultimers, being involved independently and with a similar ligand affinity in CLV3 peptide perception (Zhu *et al.*, 2010a,b; Bleckmann *et al.*, 2010; Guo *et al.*, 2010; Ogawa *et al.*, 2008; Ohyama *et al.*, 2009). CRN and CLV2 must interact to form a functional receptor complex, as CRN is lacking a distinct extracellular domain, while CLV2 is devoid of a kinase domain. Receptor complexes consisting of CLV1-CRN-CLV2 and CLV1-CRN were previously suggested (Zhu *et al.*, 2010a,b; Bleckmann *et al.*, 2010), but the interactions between the components of these complexes were later on shown to be weak in transient systems and absent *in vivo* (Guo *et al.*, 2010). Recently, also a RPK2 homodimer has been shown to transmit the CLV3 signal in the Arabidopsis SAM independently from CLV1/CLV1, CLV1/BAM and CLV2/CRN (Kinoshita *et al.*, 2010). The biochemical interaction between CLV3 and RPK2 could however not be shown (Kinoshita *et al.*, 2010). The BAM receptors were initially suggested to act opposite to CLV1, to promote meristem growth, as reduced SAMs were observed on *bam1/bam2* double mutants, and an arrest of SAM growth on BAM triple mutants (*bam1/bam2/bam3*) (DeYoung *et al.*, 2006). However, *CLV1* can fully rescue *bam1* and *bam2* mutants when broadly expressed, while *BAM1* and *BAM2* can partially rescue *clv1* mutants when specifically expressed in the meristem, implying a common function (DeYoung *et al.*, 2006). In contrast to the restricted expression pattern of *CLV1* in the SAM centre, *BAM1* and *BAM2* exhibit weak expression in the SAM centre and high expression at the meristem flanks (Clark *et al.*, 1997; DeYoung *et al.*, 2006). Hence, based on these expression patterns and phenotypes of double mutants, a new hypothesis was put forward, in which the BAM1 and BAM2 receptors are suggested to be redundant to CLV1 for the stem cell regulation in the meristem centre, but have additional roles at the meristem flanks to sequester CLV3-like ligands that are

expressed outside the SAM, in order to inhibit these ligands to enter the stem cells/OC region (Deyoung and Clark, 2008). While *CLV1* expression is restricted to the SAM, *CLV2, CRN, RPK2, BAM1, BAM2* and *BAM3* are more broadly expressed throughout the plant, where they regulate numerous developmental processes (Kayes and Clark, 1998; Strabala *et al.*, 2006; Muller *et al.*, 2008; Mizuno *et al.*, 2007; Nodine *et al.*, 2007; Clark *et al.*, 1993; DeYoung *et al.*, 2006; Hord *et al.*, 2006).

In the RAM of Arabidopsis, an analogous pathway is observed as the one described above for the SAM, to fine tune the balance between stem cell differentiation and maintenance (Stahl *et al.*, 2009). A quiescent center (QC), which is the functional equivalent of the SAM-located OC, comprises four cells and is surrounded by stem cells, also called initials, which give rise to the different root tissues (Sarkar *et al.*, 2007). Recently, Stahl and Simon (2009) reported on the existence of a CLE40/ARABIDOPSIS CRINKLY4 (ACR4)/WOX5 pathway controlling distal stem cell regulation in Arabidopsis root meristems. Reduction of *CLE40* levels expanded the *WOX5* expression domain, resulting in distal stem cell accumulation, while increased levels of *CLE40* promoted distal stem cell differentiation by reduced *WOX5* expression (Stahl *et al.*, 2009). *CLE40* signaling in the roots was shown to be independent of *CLV2* (Stahl *et al.*, 2009), but dependent on *ACR4*, encoding a RLK which is mainly expressed in the columella stem cells (De Smet *et al.*, 2008; Stahl *et al.*, 2009). Like *CLE40, ACR4* is suggested to be involved in a negative regulation of distal stem cell fate, as *acr4* mutants carry additional layers of distally located columella stem cells (De Smet *et al.*, 2008; Stahl *et al.*, 2009). Moreover, the absence of a synergistic phenotype when supplying CLE40 peptides to *acr4* mutants implies that *ACR4* and *CLE40* are acting in the same pathway (Stahl *et al.*, 2009). Finally, ectopic CLE40 peptides would have a positive effect on *ACR4* expression (Stahl *et al.*, 2009; Stahl and Simon, 2009). According to Stahl and Simon (2009), this upregulation of *ACR4* by *CLE40*, might be necessary to sequester all available CLE40 peptides and consequently protect *WOX5* expression in the QC niche cells, thereby maintaining the surrounding stem cells. In addition to *CLE40, CLE14* and *CLE20* are suggested to be involved in RAM maintenance. These CLE peptide genes are expressed outside the RAM in highly differentiated root cells, and their influence on RAM homeostasis was suggested to be CLV2/CRN-dependent, but CLV1-independent (Casamitjana-Martinez *et al.*, 2003; Miwa *et al.*, 2008; Meng *et al.*, 2010; Meng and Feldman, 2010, 2011; Muller *et al.*, 2008). This indicates that CLV2 and CRN might act cooperatively in mediating CLE signaling not only in the shoot but in certain cases also in the roots. Hence, like in the SAM, several receptor

complexes, including ACR4, CLV2 and CRN, are involved in *CLE*-regulated control of RAM homeostasis.

A CLE peptide mediated signaling pathway also controls the balance between proliferation of (pro)cambial cells and differentiation into xylem tissue during secondary growth of vascular tissue (Evert, 2006; Hirakawa *et al.*, 2008, 2010a,b). TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR (TDIF), a phloem-expressed CLE peptide with an identical amino acid sequence as CLE41 and CLE44 of Arabidopsis, signals through the procambial cell-located LRR-RLK of subclass XI TDIF RECEPTOR/PHLOEM INTERCALATED WITH XYLEM (TDR/PXY, hereafter TDR) to regulate *WOX4* expression in the procambium and cambium, resulting in the inhibition of tracheary element differentiation. Hence, these CLE peptides inhibit cellular differentiation which is opposite to the function of *CLV3* in the SAM and *CLE14, CLE20* and *CLE40* in the RAM (Kubo *et al.*, 2005; Ito *et al.*, 2006; Fisher and Turner, 2007; Hirakawa *et al.*, 2008, 2010a). In contrast to the CLE peptide mediated repression of *WUS/WOX5* expression observed in the SAM/RAM, TDIF application results in an upregulation of *WOX4* in a TDR-dependent manner (Hirakawa *et al.*, 2010a).

Legumes plants have particular secondary root organs, the nodules, which harbor symbiotic nitrogen fixing rhizobia. Nodules develop after re-initiation of cell division in the cortical and pericycle cells (Oldroyd and Downie, 2008; Ferguson *et al.*, 2010). The nodule primordium is colonized by rhizobia, resulting in cells in which the bacteria reside and fix nitrogen. Many legumes, amongst which model legume *Medicago truncatula*, carry indeterminate nodules with an apical meristem that continuously provides new cells that are infected by bacteria and in which nitrogen fixation is taking place (reviewed by (Oldroyd and Downie, 2008; Ferguson *et al.*, 2010).

Although the location/existence of an OC/QC in the nodule meristem is currently unknown, the coordination of cell proliferation and differentiation is generally assumed to be similar to the CLE/RLK/WOX pathway observed during SAM, RAM and vascular meristem maintenance. Based on expression patterns and functional analysis, two *M. truncatula CLE* genes, *MtCLE12* and *MtCLE13*, were suggested to be involved in nodule organogenesis and autoregulation of nodulation (AON) (Mortier *et al.*, 2010) (see chapter 4). The AON mechanism prevents the uncontrolled growth of nodules and involves a long-distance feedback mechanism, whereby early infection events suppress subsequent nodule initiation (Kosslak and Bohlool, 1984; Carroll *et al.*, 1985a,b; Delves *et al.*, 1986; Pierce and Bauer, 1983; Nutman, 1952). A shoot localized receptor complex, with components that are similar to receptors of CLV3 in Arabidop-

sis, controls AON. The first identified component of this complex was the *M. truncatula* SUPER NUMERIC NODULES (SUNN, or HYPERNODULATION ABERRANT ROOT FORMATION1 (HAR1) in *Lotus japonicus*, NODULE AUTOREGULATION RECEPTOR KINASE (NARK) in soybean and SYMBIOSIS29 (SYM29) in pea), a LRR-RLK of subclass XI and closest legume homolog of Arabidopsis CLV1 (Nishimura *et al.*, 2002a; Schnabel *et al.*, 2005; Okamoto *et al.*, 2009; Mortier *et al.*, 2010). Recently, the Lotus and pea *CLV2*-homologs, as well as *KLAVIER* (*KLV*), encoding a LRR-RLK closely homologous to Arabidopsis *RPK2*, were added, suggesting that, similar to SAM and RAM homeostasis, several receptor complexes are involved in the shoot control of nodulation (Kinoshita *et al.*, 2010; Krusell *et al.*, 2011).

What the signal is that binds this receptor complex is unknown but it is with high probability a CLE peptide. It has been proposed that the CLE peptide might come from the developing nodules in the root. Functional analysis in *L. japonicus*, *M. truncatula* and soybean, indeed identified a group of structurally related CLE peptides that are expressed in the nodules as good candidates because ectopic expression abolishes or reduces nodulation in a *HAR1/SUNN/NARK* dependent way (Okamoto *et al.*, 2009; Mortier *et al.*, 2010, 2011; Reid *et al.*, 2011). However, long- distance travel of these CLE peptides have not been shown and since the functional data involves ectopic expression analysis and because CLE peptides are currently proposed to be short distance signals, it is equally possible that different CLE peptide/receptor complexes act in the root and the shoot to control nodule number.

We searched for putative nodule-specific CLE peptide receptors based on the observation that many known and putative CLE peptide receptors such as CLV1, BAM1, BAM2, BAM3 and TDR belong to subclass XI of LRR-RLKs (Shiu and Bleecker, 2001). A primary analysis identified two possible candidate genes, *MtRLK1* and *MtRLK2*. However, a more in-depth phylogenetic analysis revealed that *MtRLK2* is more closely related to the genes encoding LRR-RLKs of subclass VII, suggesting a possible role in plant immunity, while *MtRLK1* is closely homologous to the Arabidopsis *BAM3*. Both *MtRLK1* and *MtRLK2* are upregulated from 6 days post inoculation on and their expression is positioned downstream of most early NF signaling components. In addition, the expression pattern of *MtRLK1* is lying adjacent to and is partially overlapping with the expression patterns of *MtCLE12* and *MtCLE13*, confirming that it might act as a receptor for MtCLE12 and MtCLE13 ligands. Finally, we also investigated the expression of *SUNN* in nodules and could confirm previous data indicating that the gene is not expressed during nodulation but has a low expression level in the vasculature of

the root.

7.2 Results

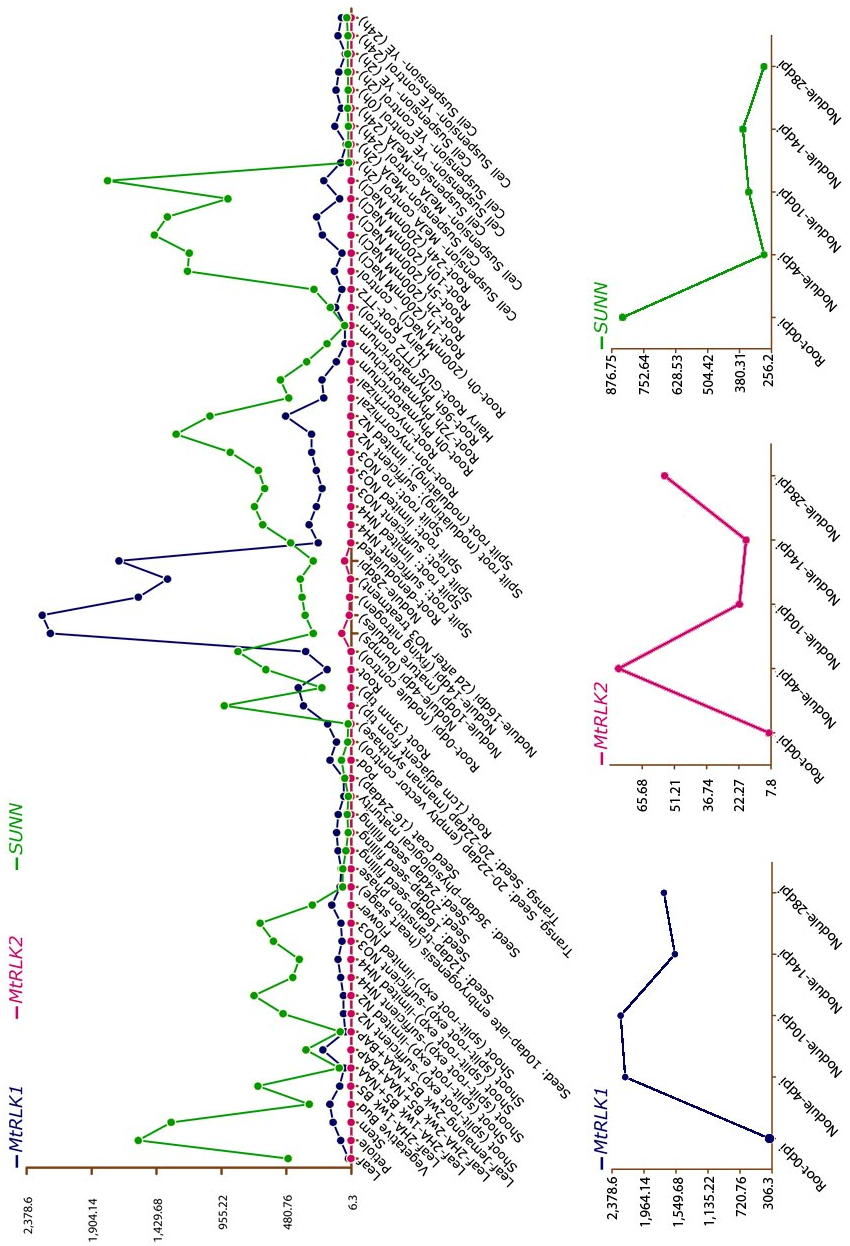
7.2.1 Search for *SUNN*-related RLKs involved in nodulation

Many CLE receptors, such as CLV1, TDIF and possibly BAM1, BAM2 and BAM3, belong to the subclass XI of LRR-RLKs (Shiu and Bleecker, 2001; DeYoung *et al.*, 2006; Deyoung and Clark, 2008). To find putative CLE peptide receptors expressed in developing nodules, we looked for all LRR-RLK homologs of subclass XI from the *M. truncatula* genomic data (Mt3.0) and analyzed the *in silico* expression patterns using the GeneAtlas tool[21].

To identify the subclass XI LRR-RLKs from *M. truncatula*, an *in silico* pipeline was used which is based on the HMMer software (Rombauts S. and Van de Peer Y., in preparation) that is more sensitive and specific than BLAST or PSI-BLAST analyses (Eddy, 2009). Primarily, Hidden Markov Models (HMMs) were constructed based on a multiple alignment of all known Arabidopsis LRR-RLKs of subclass XI but also of subclass XIII. The subclass XIII was included in the analysis because phylogenetic analysis revealed that this group is closely related to the subclass XI of RLKs (Shiu and Bleecker, 2001). All conserved regions in the alignment (domains) from at least 6 amino acids were taken into consideration. In a next step, the whole *M. truncatula* proteome (Mt3.0) and all known Arabidopsis LRR-RLKs, were screened for the presence of those HMMs. The scores for each HMM, received from the HMMer software, were normalized in function of the length of each individual domain to allow domains to contribute equally to the final score. The final scores were next ordered in vectors per gene and stored in a matrix. By applying hierarchical clustering (Cluster 3.0) on this matrix, genes with highly correlated vectors were grouped together (de Hoon *et al.*,

[21] `http://mtgea.noble.org/v2/`

Figure 7.1 ***(facing page)*****:** *In silico* expression profiles of *MtRLK1, MtRLK2* and *SUNN*, retrieved from the Medicago Gene Expression Atlas from the Noble foundation. Tissues analyzed are indicated below the diagrams. The three diagrams at the bottom of the page are magnifications of the top diagram and represent the expression profiles of *MtRLK1, MtRLK2* and *SUNN* in nodulation-specific tissues.

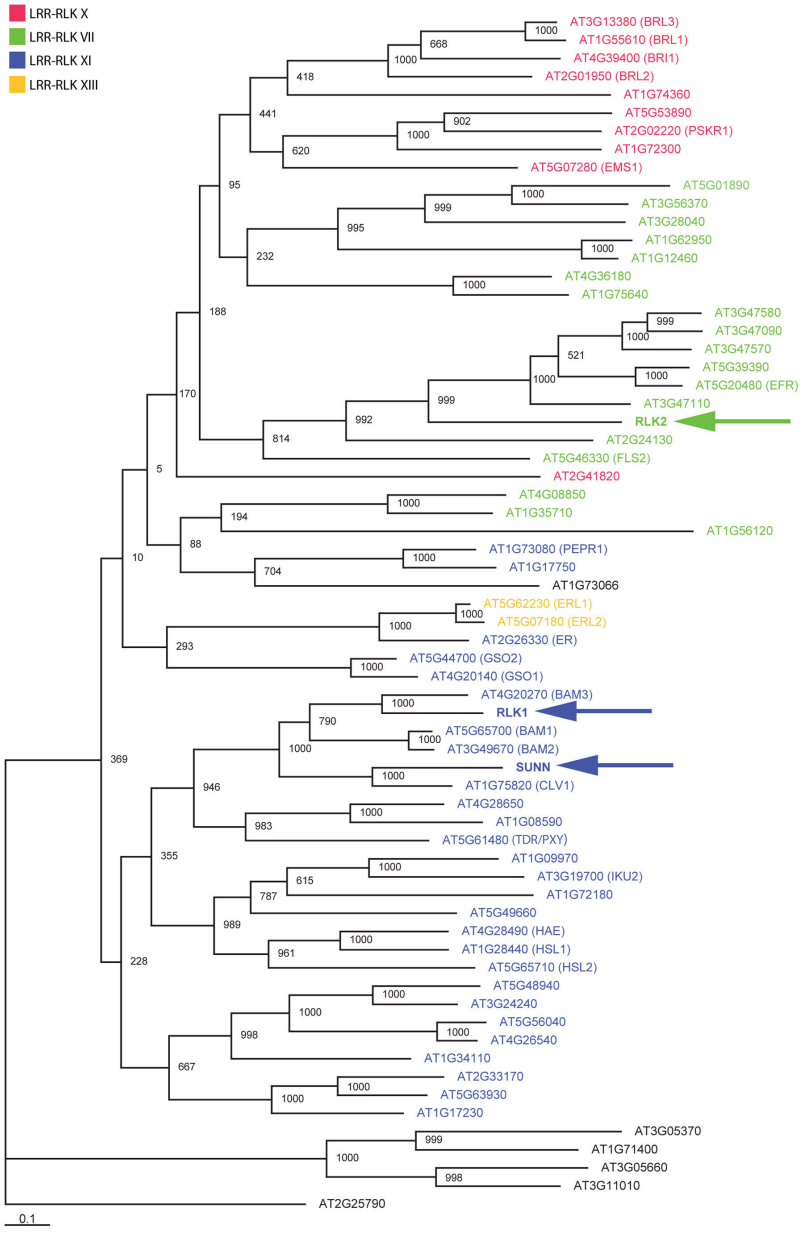

MtRLK1
MtRLK2
SUNN
2,378.6
1,904.14
1,429.68
955.22
480.76
6.3
Root-0dpi
Nodule-4dpi
Nodule-10dpi
Nodule-14dpi
Nodule-28dpi
65.68
51.21
36.74
22.27
7.8
876.75
752.64
628.53
504.42
380.31
256.2
2,378.6
1,964.14
1,549.68
1,135.22
720.76
306.3

2004). The *M. truncatula RLK* genes that clustered together with *CLV1* and *BAM1-3* were taken as primary candidates.

From this analysis, 33 sequences were retained and for 16 of those, an expression pattern was available at the Medicago Gene Expression Atlas from the Noble Foundation (Supplemental data; Table 7.2). This analysis revealed that two of these 16 genes were upregulated during nodulation (Figure 7.1, A and B); the genes were designated *MtRLK1* (Mtr.4752.1.S1_at) and *MtRLK2* (Mtr.1974.1.S1_at). According to the Medicago Gene Expression Atlas, a basal *MtRLK1* expression was seen in roots and the expression level in developing nodules was higher than in roots. This was true for all nodulated tissues available. *MtRLK2* expression was only detected in nodulated tissues especially in the sample of 4 days post-inoculation. As a comparison, the expression of *SUNN* was analyzed. As shown in Figure 7.1C, the *SUNN* (Mtr.46819.1.S1_s_at) transcript level is lower in nodulated samples compared to root samples (Figure 7.1C). Moreover, the *SUNN* transcripts are seen in a wide range of tissues including leaves, petioles, stems and roots (Figure 7.1C).

To confirm whether the identified *MtRLKs* belong to subclass XI, a phylogenetic analysis was performed (Figure 7.2). *A. thaliana* sequences homologous with *MtRLK1, MtRLK2* and *SUNN* were retrieved from the TAIR9 CDS databank using the tBLASTx algorithm and aligned to the 3 *M. truncatula RLKs*. Next, a phylogenetic tree was designed according to the 'maximum likelihood' principle (Guindon and Gascuel, 2003) using homologous regions throughout the full length protein sequences. The AtLRR-RLK AT2G25790 was chosen as an outgroup. Bootstrap values were based on 1000 reconstructions. The resulting tree is shown in Figure 7.2. For each Arabidopsis RLK, the subclass is indicated using a color code, that was assigned by Shiu and Bleecker (2001) based on a phylogenetic analysis of the kinase domains. Comparison of the color code distribution with the tree organization shows that a similar phylogenetic relation is obtained when the complete amino acid sequences or only the kinase domains

Figure 7.2 ***(facing page)*****:** Phylogenetic tree of MtRLK1, MtRLK2, SUNN and their *A. thaliana* homologs. The phylogenetic tree is based on the full amino acid sequences and build according to the 'maximum likelihood' principle. The LRR-RLK AT2G25790 was chosen as an outgroup, and bootstrap values were based on 1000 reconstructions. Genes marked in black do not belong to any of these groups. Color-marking refers to the 13 subclasses of LRR-RLKs identified in the genome of *A. thaliana* as identified by Shiu and Bleecker (2001). Arrows mark location of MtRLK1, MtRLK2 and SUNN in the phylogenetic tree.


LRR-RLK X
LRR-RLK VII
LRR-RLK XI
LRR-RLK XIII
AT3G13380 (BRL3)
AT1G55610 (BRL1)
AT4G39400 (BRI1)
AT2G01950 (BRL2)
AT1G74360
AT5G53890
AT2G02220 (PSKR1)
AT1G72300
AT5G07280 (EMS1)
AT5G01890
AT3G56370
AT3G28040
AT1G62950
AT1G12460
AT4G36180
AT1G75640
AT3G47580
AT3G47090
AT3G47570
AT5G39390
AT5G20480 (EFR)
AT3G47110
RLK2
AT2G24130
AT5G46330 (FLS2)
AT2G41820
AT4G08850
AT1G35710
AT1G56120
AT1G73080 (PEPR1)
AT1G17750
AT1G73066
AT5G62230 (ERL1)
AT5G07180 (ERL2)
AT2G26330 (ER)
AT5G44700 (GSO2)
AT4G20140 (GSO1)
AT4G20270 (BAM3)
RLK1
AT5G65700 (BAM1)
AT3G49670 (BAM2)
SUNN
AT1G75820 (CLV1)
AT4G28650
AT1G08590
AT5G61480 (TDR/PXY)
AT1G09970
AT3G19700 (IKU2)
AT1G72180
AT5G49660
AT4G28490 (HAE)
AT1G28440 (HSL1)
AT5G65710 (HSL2)
AT5G48940
AT3G24240
AT5G56040
AT4G26540
AT1G34110
AT2G33170
AT5G63930
AT1G17230
AT3G05370
AT1G71400
AT3G05660
AT3G11010
AT2G25790
0.1

are taken for the analysis. Likewise the same subclass division can be used here as the one used by Shiu and Bleecker (2001). As previously shown, *SUNN* is the closest related to *CLV1* from Arabidopsis (Schnabel *et al.*, 2005). Also *MtRLK1* is closely related to *CLV1*. *MtRLK1* is the closest relative of *BAM3* and belongs together with *CLV1, BAM1, BAM2* and *SUNN* to the same branch of the phylogenetic tree (Figure 7.2). On the other hand, *MtRLK2* shares the highest similarity with the LRR-RLKs of subclass VII, to which FLAGELLIN SENSITIVE2 (FLS2) and EF-TU RECEPTOR (EFR) belong (Figure 7.2).

7.2.2 QRT-PCR analysis of *RLK1*, *RLK2* and *SUNN* during nodulation and in different tissues

In order to confirm the *in silico* expression data retrieved from the Medicago Gene Expression Atlas from the Noble foundation, the temporal expression of *MtRLK1, MtRLK2* and *SUNN* was studied during nodule development, by analyzing the relative transcript levels at 4, 6, 8, and 10 days post inoculation (dpi). The elongation zone of uninoculated roots, the nodule initiation site, was used as the reference tissue. *MtRLK1* transcripts increased from 6 dpi until 10 dpi (Figure 7.3A). *MtRLK2* expression was low at 4 dpi, but increased at 6 dpi, and remained high until 10 dpi (Figure 7.3B). The expression level of *SUNN* was lower in all nodulation related tissues compared to the level observed in roots (Figure 7.3C). These results broadly confirm the *in silico* expression data.

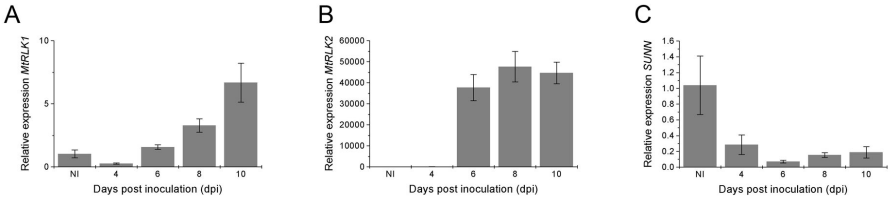


Figure 7.3: Expression analysis during nodulation. Expression analysis of *MtRLK1, MtRLK2* and *SUNN* by qRT-PCR on cDNA samples of zone I root tissues of uninoculated plants (NI) and at 4, 6, 8, and 10 dpi. The elongation zone of uninoculated roots, the nodule initiation site, was used as the reference tissue. Data and error bars represent means ± SD. The experiment was repeated twice with comparable results.

To determine tissue- or organ-specific expression, quantitative reverse-transcriptase

(qRT)-PCR was carried out for *MtRLK1, MtRLK2* and *SUNN* on cDNAs derived from root elongation zones, leaves, stems, roottips, shoot apical meristems (SAMs), first leaves, and cotyledons grown in nitrogen poor, nitrogen rich or nodulated conditions. The root elongation zone of roots grown in nitrogen rich conditions was used as the reference tissue. *MtRLK1* and *SUNN* transcripts were found in all material tested and thus have a general expression pattern (Figure 7.4, A and C). For *MtRLK1*, the highest expression is measured in root and stem tissues, while for *SUNN*, the highest expression was seen in stems. The expression of *MtRLK2* is specific for nodulated root tissues (Figure 7.4B).

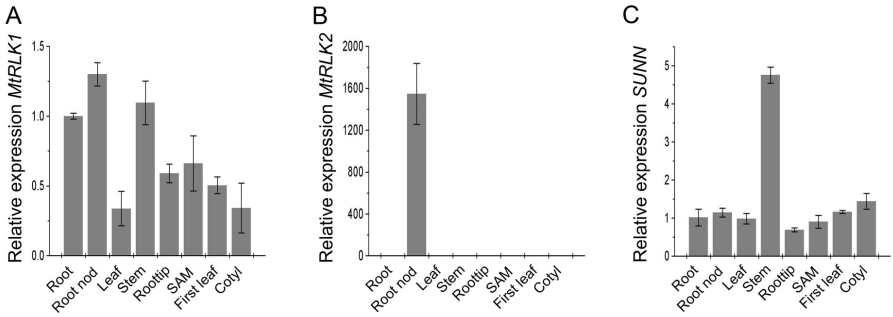


Figure 7.4: Expression analysis in different plant tissues. Expression analysis of *MtRLK1, MtRLK2* and *SUNN* respectively, by qRT-PCR on cDNA samples of root elongation zones (Root), leaves, stems, roottips, shoot apical meristems (SAMs), first leaves and cotyledons (cotyl) grown in nitrogen rich conditions, and of 1-month-old nodulated roots (Root nod). The root elongation zone of roots grown in nitrogen rich conditions was used as the reference tissue. A cutoff was set at Ct value ≥ 35. Data and error bars represent means $\pm$ SD.

7.2.3 *RLK1*, *RLK2* and *SUNN* expression pattern in roots and developing nodules

Spatial expression patterns of *MtRLK1, MtRLK2* and *SUNN* in roots and developing nodules were investigated by *promoter:GUS* analysis and *in situ* hybridizations. A 2-kb region upstream of *MtRLK1, MtRLK2* and *SUNN* was isolated based on the available genomic data[22] and cloned 5' to the *uidA* gene. Transcriptional activation of the *uidA*

[22] http://www.ncbi.nlm.nih.gov/

gene was visualized by GUS staining.

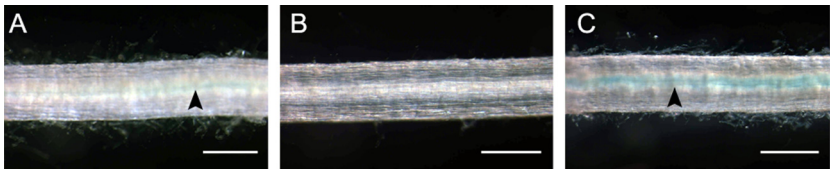
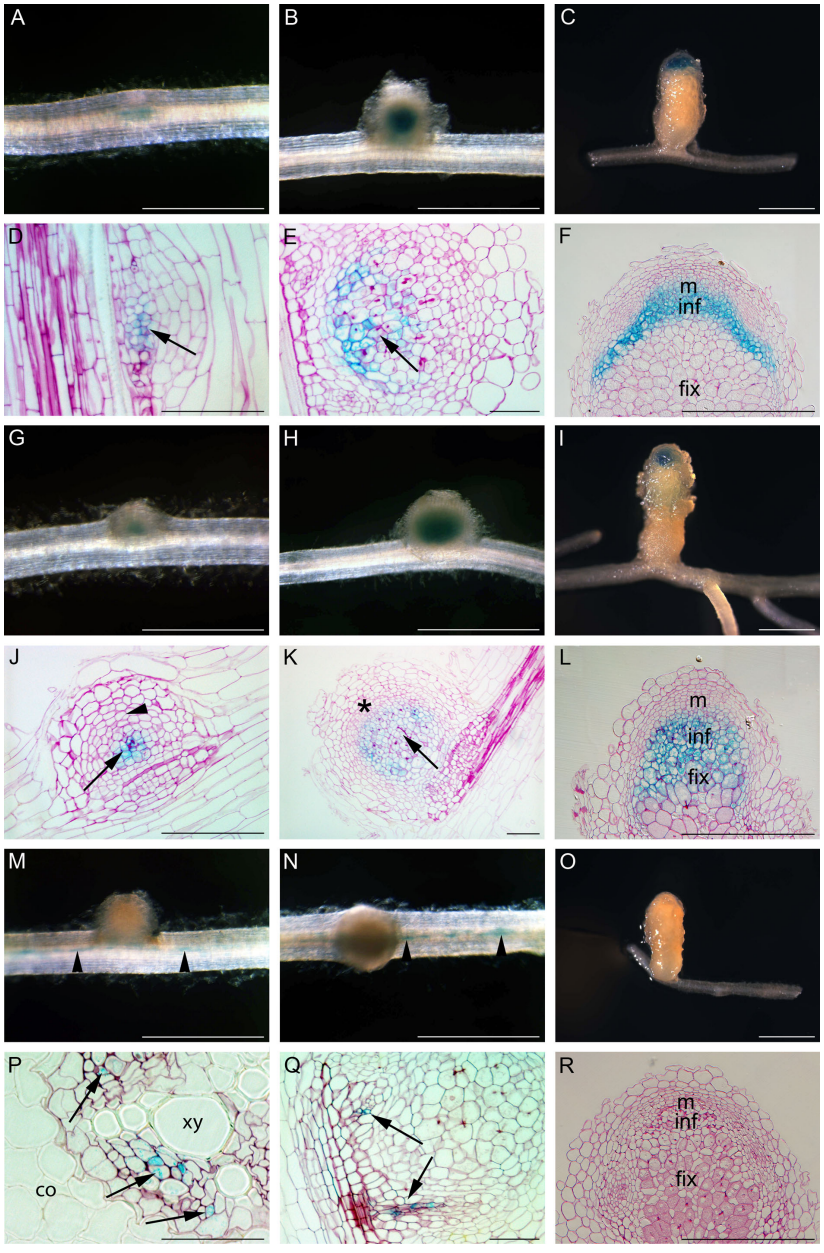


Figure 7.5: *Promoter:GUS* activity of *MtRLK1, MtRLK2* and *SUNN* in uninoculated roots. For *MtRLK1* (A) a faint and for *SUNN* (C) a bright blue staining was observed in the root vascular tissue (arrowheads). B, No blue staining was observed in the *pMtRLK2:GUS* transformed root samples. Bars = 0.5 mm.

In the vascular tissue of uninoculated roots, a weak and a strong blue staining was

Figure 7.6 *(facing page)*: *MtRLK1, MtRLK2* and *SUNN* promoter activity during nodulation. A-F, *Promoter:GUS* activity of *MtRLK1* in nodule primordia (A and D), young nodules (B and E) and mature nodules (C and F). D to F are bright-field pictures of longitudinal sections through the nodules represented in A to C, respectively. In nodule primordia, weak GUS expression is observed at the base of the primordium (D, arrow). In slightly older nodules, blue staining is observed in the central tissue in a zone comprising the infection and fixation zone (E, arrow). In mature nodules, expression is restricted to the apical zone of elongated nodules, with the highest expression in the lower part of the meristem and infection zone. G-L, *Promoter:GUS* activity of MtRLK2 in nodule primordia (G and J), young nodules (H and K) and mature nodules (I and L). J to L are bright-field pictures of longitudinal sections through the nodules represented in G to I, respectively. In nodule primordia blue coloring was observed in infection threads containing differentiating cells (J, arrow), but not in the outer cortical cells (J, arrowhead). In young nodules, blue coloring was observed in the central tissue in a zone comprising the infection and fixation zone (K, arrow). A weak expression was observed in the pre-infection zone, but not in the meristem (K, asterisk). In mature nodules expression is detected in the infection zone and the upper part of the fixation zone, but not in the meristematic tissue (L). M-R, *Promoter:GUS* activity of *SUNN* in root vascular tissue (P), young nodules (M, N and Q) and mature nodules (O and R). P is a bright-field picture of a section through a root vascular tissue. Q and R are bright-field pictures of longitudinal sections through the nodules represented in N and O, respectively. Blue coloring was observed in the phloem of the root vascular bundles (P, arrows; M and N, arrowheads) and at the base of the nodule vascular tissue (Q, arrows). No expression was detected in the meristem, infection and fixation zone of mature nodules (Q and R). All sections were counterstained with 0.05 % ruthenium red. m, meristem; inf, infection zone; fix, fixation zone; xy, xylem; co, cortex; Bars = 2 mm (A-C, G-I and M-O), 0.4 mm (D-E, J-K and Q), 0.1 mm (P) and 1 mm (F, L and R).


A
B
C
D
E
F
m
inf
fix
G
H
I
J
K
*
L
m
inf
fix
M
N
O
P
xy
co
Q
R
m
inf
fix

observed for *MtRLK1* and *SUNN*, respectively (Figure 7.5, A and C). For *MtRLK2* no GUS expression could be detected in the roots (Figure 7.5B).

At early stages after inoculation, *pMtRLK1:GUS* activity was observed within the incipient nodule primordia (Figure 7.6, A and D). Sectioning revealed that it were the cells derived from the pericycle/endodermis that stained blue. At a later stage, when infection threads were observed within the primordium, GUS staining was still mostly seen in the part of the primordium located near the vascular tissue. Later in nodule development, in mature nodules with an apical meristem, *pMtRLK1:GUS* was restricted to the apical zone of elongated nodules, with the highest expression in the lower part of the meristem and infection zone (Figure 7.6, C and F).

Also *MtRLK2*-related GUS staining was seen in primordia at very early stages (Figure 7.6G). Sectioning revealed that *pMtRLK2:GUS* was only observed in infection threads containing cells (Figure 7.6J). In round, young nodules, *pMtRLK2:GUS* staining was seen throughout the central tissue, comprising the infection and young fixation zone, (Figure 7.6, H and K). No *pMtRLK2:GUS* expression was seen in the meristem (Figure 7.6K). Also in mature nodules expressing *pMtRLK2:GUS*, GUS staining was observed in the infection zone and the upper part of the fixation zone, but not in the meristematic tissue (Figure 7.6, I and L).

Expression of *SUNN* was observed in the phloem of the root vascular bundles (Figure 7.6, M, N and P) and at the base of the nodule vascular tissue (Figure 7.6Q). Blue staining was not seen in the cortical tissue of young nodules (Figure 7.6, M, N and Q), neither in the meristem, infection and fixation zone of mature nodules (Figure 7.6, O and R).

In situ hybridizations to detect the transcript accumulation of *MtRLK1* and *MtRLK2* revealed an expression pattern broadly similar to the results of the *promoter:GUS* analysis. Transcripts of *MtRLK1* were mostly detected in the infection zone, but also slightly in the pre-infection zone and meristematic tissue (Figure 7.7, A and B). For *MtRLK2*, transcript levels were located not only in the infection and fixation zone, but also at a very low level in the meristem (Figure 7.7, C and D).

7.2.4 Contribution of nodulation-related components and hormones for *RLK1*, *RLK2* and *SUNN* expression

To investigate whether NF signaling components are required for the expression of *MtRLK1, MtRLK2* and *SUNN*, we analyzed their transcript levels by qRT-PCR before

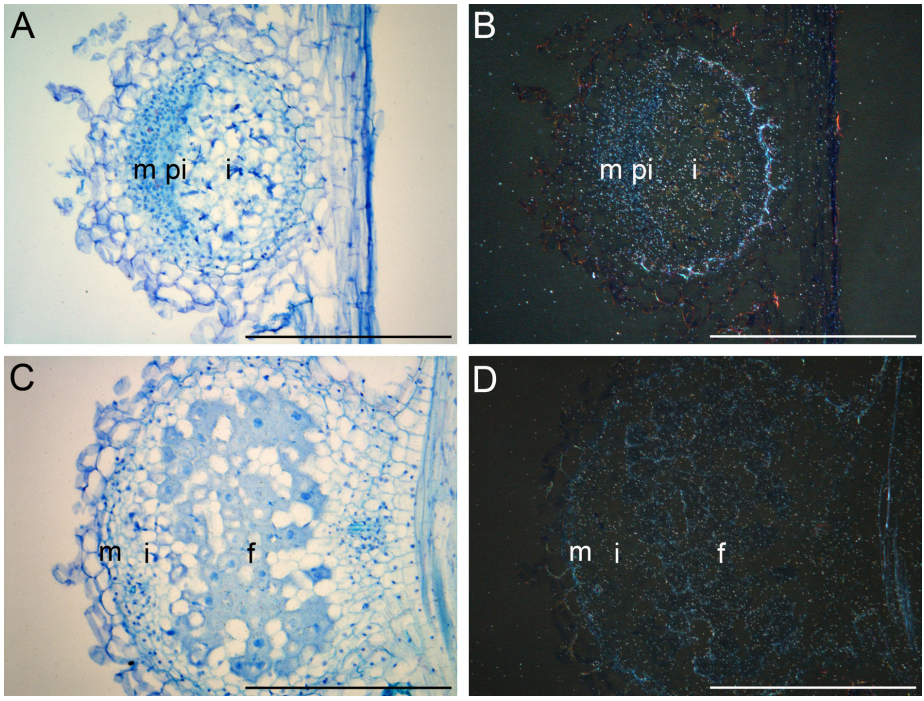


Figure 7.7: *MtRLK1* and *MtRLK2* transcript accumulation in nodules by using *in situ* hybridizations. Microscopical images of *in situ* hybridizations on sections of young nodules. On the bright field picture (A and C), signal is seen as black spots, on the dark field picture (B, D), signal is seen as white spots. A-B, Detection of *MtRLK1* transcripts. *MtRLK1* transcripts are mostly seen in the infection zone, but also in the pre-infection zone and in the meristematic tissue close to the pre-infection zone. C-D, Detection of *MtRLK2* transcripts, mostly in the infection and fixation zone, but also slightly in the meristem. Sections were counterstained with toluidine blue. f, fixation zone; i, infection zone; pi, pre-infection zone; m, meristem. Bars = 0.5 mm.

and after inoculation of *nin*, the *ERN1* mutant *branching infection threads1-1 (bit1-1), nsp1, nsp2, dmi1, dmi2,* and *dmi3* mutants (Figure 7.8). The mutant lines did not develop nodules, except for *bit1-1*, which formed arrested primordia and infection foci (Andriankaja *et al.*, 2007; Middleton *et al.*, 2007). For *MtRLK1*, gene expression was upregulated upon inoculation in wild type *M. truncatula* roots (Figure 7.8A). In the roots of the nodulation mutants this effect was not observed, indicating that the expres-

sion of *MtRLK1* is dependent on early nodulation signaling components (Figure 7.8A). A similar result was obtained for *MtRLK2*, except in *bit1-1*, where an upregulation after inoculation was still observed, albeit less abundantly than in wild type plants (Figure 7.8B). To confirm the qRT-PCR analysis, *pMtRLK2:GUS* transgenic roots were generated in an *ERN1* mutant background and analyzed at 5 dpi. GUS staining revealed the typical *MtRLK2* expression pattern in the wild type roots and in the *bit1-1* mutant, blue stained regions were seen corresponding to arrested infection foci (Figure 7.8D). These data are in agreement with the qRT-PCR data. The reduction in *SUNN* expression observed after inoculation of wild type plants was also measured in the nodulation mutants *nsp1, nsp2, bit1-1, nin-1* and *dmi1* but not in the mutants *dmi2* and *dmi3* (Figure 7.8C).

Auxin and cytokinin are important hormones for nodule formation (Oldroyd and Downie, 2008; Ding and Oldroyd, 2009). To determine whether auxins and/or cytokinins affect *MtRLK1, MtRLK2* and *SUNN* expression, 10^{-6} M indole-3-acetic acid (IAA) or 10^{-7} M 6-benzylaminopurine (BAP) was supplemented to the growth medium of 5-day-old seedlings. The roots of 18 seedlings were harvested under each condition after 0, 3, 6, 12, 24, and 96 h. Roots from plates without hormone addition were used as a negative control. No significant differences in *MtRLK1* or *SUNN* expression were detected in treated versus control roots (data not shown). For *MtRLK2* no expression was detected in any of the tested samples (data not shown).

7.2.5 Effect of *MtRLK1* RNAi on nodulation

To investigate the effect of knocking down *MtRLK1* expression on the nodulation process, a RNAi hairpin construct was made and introduced into transgenic roots by *Agrobacterium rhizogenes* Arqua1 transformation. To do so, a 205 bp long fragment, located in that part of the open reading frame (ORF) encoding the extracellular domain, was selected. Next, composite plants with transgenic roots were made, grown in aeroponics, inoculated with *Sm2011* pBHR-mRFP and analyzed at 14 dpi. The expression level of *MtRLK1* was measured by qRT-PCR analysis on cDNA samples of a pool of 10 nodules harvested on transgenic roots expressing either *35S:GUS* (control) or a hairpin construct inducing the knock-down of *MtRLK1* expression (RNAi *MtRLK1*). Unfortunately, we were not able to demonstrate an efficient knock-down of *MtRLK1* expression (Figure 7.9). Possibly, the fragment is too short to activate the RNAi machinery. Therefore, the experiment will be repeated with a longer RNAi fragment.

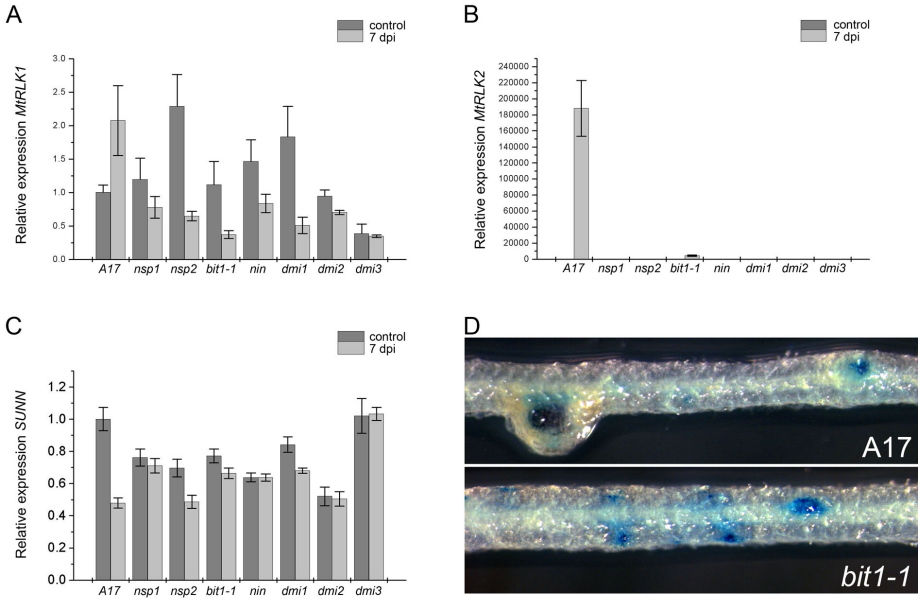


Figure 7.8: *MtRLK* expression in nodulation mutants. A, B and C, Expression analysis of *MtRLK1, MtRLK2* and *SUNN*, respectively, by qRT-PCR on cDNA samples of zone I root tissues of wild type plants (WT) and *nsp1, nsp2, bit1-1, nin, dmi1, dmi2,* or *dmi3* mutants, before inoculation (control) and at 7 dpi. A cutoff was set at Ct value ≥ 35. D, *pMtRLK2:GUS* activity at 5 dpi in the roots of a wild type plant (A17) and in a *bit1-1* mutant. Blue stained cortical regions are observed corresponding to infection. The experiments were repeated twice with comparable results. Data and error bars represent means $\pm$ SD.

7.3 Discussion

CLE peptides are mostly recognized by different receptor complexes of which many members belong to the LRR-RLKs of subclass XI (CLV1, BAM1, BAM2, BAM3, SUNN and orthologs) (Shiu and Bleecker, 2001). In our search for putative receptors of MtCLE12 and MtCLE13 ligands in the genome of *M. truncatula*, we identified two new receptor candidates, *MtRLK1* and *MtRLK2*. Although both *MtRLK1* and *MtRLK2* were initially identified as LRR-RLK of subclass XI, a more in-depth phylogenetic analysis revealed that *MtRLK2* is actually closer related to the LRR-RLKs of subclass VII, to which the pattern recognition receptors (PRRs) FLAGELLIN SENSITIVE 2

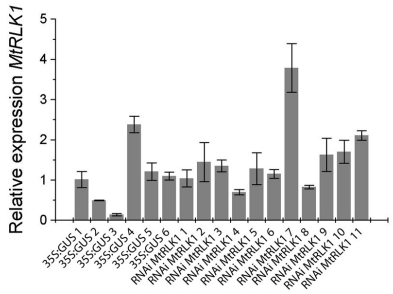


Figure 7.9: Analysis of the knock-down of *MtRLK1*. qRT-PCR analysis of *MtRLK1* expression in cDNA samples of nodules harvested on transgenic roots expressing either *35S:GUS* (control) or a hairpin construct to induce knock-down of *MtRLK1* expression (RNAi *MtRLK1*). Data and error bars represent means ± SD.

(FLS2) and EF-TU RECEPTOR (EFR) from Arabidopsis belong. PRRs are involved in plant immunity by the recognition of pathogen associated molecular patterns (PAMPs), ensuing the activation of a mitogen-activated protein kinase (MAPK) signaling pathway to trigger immunity (Jones and Dangl, 2006). Hence, based on its phylogenetic position, MtRLK2 is suggested to act as a PRR. The expression pattern detected by qRT-PCR, *promoter:GUS* analysis and *in situ* hybridization fits with a role during invasion. *MtRLK2* was solely expressed in nodulated roots and was not detected in other plant tissues. In addition, at the cellular level, *MtRLK2* expression was seen in cells that surrounded invading infection threads, not only early at the infection sites, but also later in the cortex and finally in the infection zone and young fixation zone of the mature nodule. It is well known that rhizobial invasion is accompanied by plant-derived defense responses to prevent the uncontrolled growth of rhizobia and/or to avoid co-invasion of opportunistic or pathogenic bacteria. In addition, the defense responses might contribute positively to support infection thread formation (Arrighi *et al.*, 2006; Smit *et al.*, 2007; Oldroyd and Downie, 2008). *MtRLK2* might be involved in activating those defense responses specifically during infection thread development, as *MtRLK2* expression was not detected anymore in cells of the mature fixation zone. What triggers the expression of *MtRLK2* is not known but it might be of bacterial origin. This is supported by the invasion-specific expression pattern. In agreement, addition of cytokinin and auxin could not trigger the expression. Moreover, the *MtRLK2* expression was dependent on most components of the early NF signaling pathway, including *DMI1,*

DMI2, DMI3, NSP1, NSP2, and *NIN*. Only in the *ERN* mutant, *bit1-1*, an upregulation of *MtRLK2* was measured. *Bit1-1* is the only mutant tested that allows primary invasion and cell division (Andriankaja *et al.*, 2007; Middleton *et al.*, 2007), and *MtRLK2* was associated with sites of bacterial invasion. Whether the bacterial Nod factors can activate *MtRLK2* expression or whether other bacterial components are involved, is currently not known, but interesting to test in the future. In addition, functional analysis, including RNAi and ectopic expression might reveal its precise role in nodulation.

A role for *MtRLK2* as CLE ligand receptor, although not plausible because of the expression patterns and phylogenetic analysis, can yet not be excluded because several CLE peptide receptors are known, which do not belong to subclass XI of LRR-RLKs (Jeong *et al.*, 1999; Kayes and Clark, 1998; Miwa *et al.*, 2008; Muller *et al.*, 2008; Hirakawa *et al.*, 2008; Stahl *et al.*, 2009). On the other hand, *MtRLK1*, like *SUNN*, is more likely to function as a CLE-ligand receptor because it is closely related to the members of subclass XI of LRR-RLKs. More particularly, the phylogenetic analysis showed that *MtRLK1* shares the highest similarity with *A. thaliana BAM3* while, as already known, *SUNN* is the closest *M. truncatula* homolog of *CLV1* (Schnabel et al., 2005). Both *BAM3* and *CLV1* function in SAM homeostasis by regulating stem cell identity (DeYoung *et al.*, 2006; Deyoung and Clark, 2008), suggesting that *MtRLK1* might be involved in controlling the balance between cell proliferation and differentiation within the nodule. Nevertheless, functional analyses need to be done as homologous genes do not always carry out identical functions; e.g. *sunn* mutants do not exhibit a *clv1* phenotype, but are characterized by a supernodulation phenotype (Schnabel *et al.*, 2005).

The question arises whether both *SUNN* and *MtRLK1* might be receptors of *MtCLE12* and *MtCLE13*. So far, CLE peptides are known to act as short distance signals that are perceived in a cell-non autonomous manner by neighboring cells (Fukuda *et al.*, 2007; Hirakawa *et al.*, 2008; Whitford *et al.*, 2008; Miwa *et al.*, 2009; Stahl *et al.*, 2009). Both *SUNN* and *MtRLK1* are expressed in the vascular tissue of the root but in contrast to *SUNN*, which is downregulated upon inoculation, the transcript level of *MtRLK1* raised from 6 dpi on. The *MtRLK1* upregulation coincided with the expression of *MtCLE12* and *MtCLE13* (Mortier *et al.*, 2010). Moreover, at the onset of the nodulation process, *MtRLK1* transcripts were detected in the inner cells of the developing nodules. Later on in mature nodules, *MtRLK1* expression was seen in part of the meristem and in the infection zone. This expression pattern is lying adjecent to and is partially overlapping with the expression pattern of *MtCLE12* and *MtCLE13* (Figure 7.10) (Mortier *et al.*, 2010). Hence, if CLE peptides act as short-distance signal-

ing molecules, *MtRLK1* might be a receptor of the MtCLE12 and MtCLE13 peptides (Clark *et al.*, 1997; Ogawa *et al.*, 2008; Stahl *et al.*, 2009; Hirakawa *et al.*, 2010b). The analysis of expression in the different nodulation mutants indicated that all early nodulation components tested are needed for nodulation-triggered *MtRLK1* expression. Even in *bit1-1*, in which arrested nodule primordia are detected, *MtRLK1* expression was not seen. This is in contrast to *MtCLE13* from which promoter activity was already seen before or at the onset of primordium formation. Hence, *MtRLK1* expression is switched on after *MtCLE13* expression, but before *MtCLE12* expression. By which trigger is unknown but the expression seems independent of auxin or cytokinin addition (Gonzalez-Rizzo *et al.*, 2006; Murray *et al.*, 2007; Tirichine *et al.*, 2007).

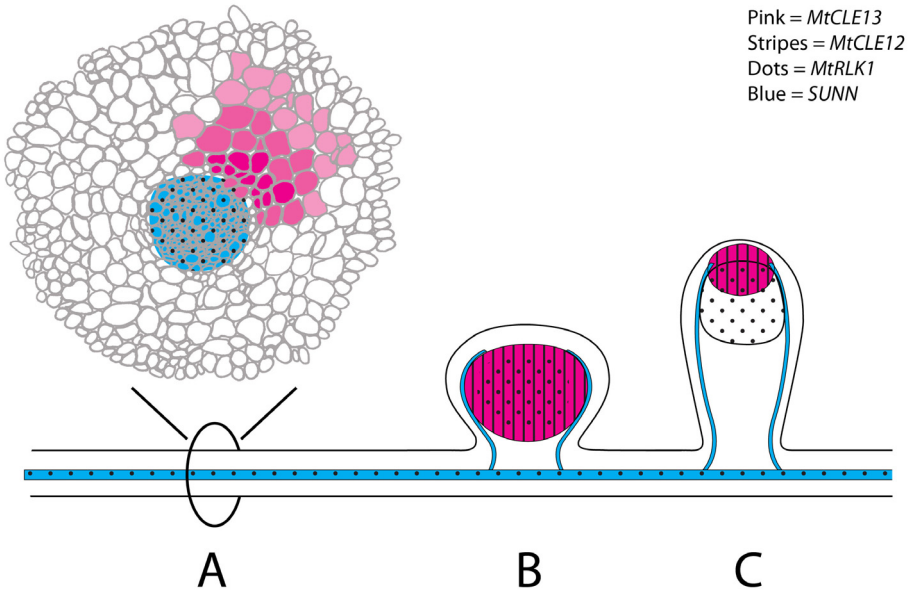


Figure 7.10: Expression pattern of CLE peptides (MtCLE12 and MtCLE13) and putative CLE peptide receptors (SUNN and MtRLK1) involved at different stages during nodulation on *M. truncatula*. A nodule initiation, B nodule primordium, C mature indeterminate nodule.

The expression pattern of *SUNN* is less supportive for short-distance signaling via the MtCLE12 and MtCLE13 ligands. The *SUNN* transcripts were solely detected in the vasculature of the root and of the basal side of the nodule. This is a few cell

layers away from the site where *MtCLE12* and *MtCLE13* expression are detected. In addition, the expression was decreasing as nodulation progressed, which is opposite to the expression pattern of *MtCLE12* and *MtCLE13*. Still, a function for *SUNN* as a long-distance receptor cannot be ruled out. There was a large variation in basal expression level between the different nodulation mutants for both *MtRLK1* and *MtSUNN*. Hence, an unknown component might modulate the expression levels of both genes in the vascular tissue of the root.

MtCLE12 and *MtCLE13* are supposed to have a dual role in nodulation: to control nodule number and to control nodule homeostasis (Mortier *et al.*, 2010). *MtCLE12* and *MtCLE13* were suggested to be involved during AON, as ectopic expression of these genes resulted in *SUNN*-dependent inhibition of nodulation and RNAi resulted in *SUNN*-dependent increase in nodule number (Mortier *et al.*, 2010) (see chapter 5). Grafting experiments have shown that although *SUNN* is expressed in the root, it is the shoot-expressed *SUNN* that controls nodule number (Penmetsa *et al.*, 2003; Francisco and Harper, 1995). Similar observations were made for soybean and *L. japonicus* (Jiang and Gresshoff, 2002; Krusell *et al.*, 2002; Nishimura *et al.*, 2002a). Based on these experiments it has been proposed that the nodulation-related CLE peptides might migrate to the shoot to act as ligands for *SUNN* and its orthologs. However, long distance movement of CLE peptides have not been demonstrated yet and so far it is not known whether root or shoot expressed *SUNN* is needed for the *MtCLE12* or *MtCLE13* overexpression phenotype. Interestingly, *MtRLK1* shares an analogous expression pattern with *SUNN* in the root. A weak and strong blue staining was observed in the root vascular tissue for *MtRLK1* and *SUNN*, respectively. A similar expression pattern was reported for the *SUNN* homologs, *HAR1* from *L. japonicus* and *NARK* from *Glycine max* (Nontachaiyapoom *et al.*, 2007). This suggests that in the vasculature, MtRLK1 might act as an interacting partner of SUNN. In agreement, in the SAM and the RAM several homo- and heteromultimers were previously shown to be involved in stem cell homeostasis through CLE peptide signaling (Zhu *et al.*, 2010a; Bleckmann *et al.*, 2010; Guo and Clark, 2010; Miwa *et al.*, 2008; Muller *et al.*, 2008; Stahl *et al.*, 2009; Stahl and Simon, 2009). Functional analysis needs to be done to unequivocally show the function of *MtRLK1*. Unfortunately, we were not able to demonstrate an efficient knock-down of *MtRLK1* expression so far. Possibly, the fragment used was too short to efficiently activate the RNAi machinery. The experiment should be repeated with a longer RNAi fragment.

As suggested by their expression pattern, *MtCLE12* and *MtCLE13*, might control

the balance between cell division and differentiation during nodulation. In addition, RNAi analysis has shown that down-regulation of *MtCLE12* and *MtCLE13* expression resulted in nodules with an early senescence zone, indicative for a change in differentiation stage of the nodule cells. During early nodulation, expression of *MtRLK1* is also linked with cell differentiation as the expression was detected in nodule primordia, and later on in the apical region of the nodule comprising meristematic cells, infection zone cells and cells of the early fixation zone. This expression pattern is similar to the expression patterns of *BAM1* and *BAM2* in differentiating cells on the meristem flanks of the SAM (DeYoung *et al.*, 2006). Hence, MtRLK1 might be the receptor of MtCLE12 and MtCLE13 ligands to control cell division and differentiation in the nodule. A gradient of MtCLE12 and MtCLE13 peptides with the highest concentration in the pre-infection/infection zone might control the transition of the meristem descendants into cells of the fixation zone through MtRLK1 signaling (Figure 7.10) (Mortier *et al.*, 2010). Combined with the results of the RNAi analysis (see chapter 5), these data suggest that *MtCLE12* and *MtCLE13* are restraining cell differentiation, similar to what was reported for the CLE peptide TDIF (Hirakawa *et al.*, 2008, 2010b). However, in contrast to *TDIF*, the signal generated downstream of *MtCLE12/MtCLE13* would not only be produced in stem cells, but also in differentiating cells, the places where *MtRLK1* transcripts were detected. Possibly, restraining of cell differentiation by *MtCLE12* and *MtCLE13* is needed to sustain a positive environment in differentiating cells, to allow efficient bacterial invasion. For *BAM3*, the closest homolog of *MtRLK1*, an opposite role has been reported, more precisely in cell differentiation during pollen formation and the development of the vascular system (DeYoung *et al.*, 2006; Deyoung and Clark, 2008).

Whatever the function, there is an urgent need for functional analysis of both receptors identified in this study to demonstrate their possible function. It seems that we have identified another receptor-like-kinase that might be involved in CLE peptide signaling during nodulation. The CLE peptide signaling gets very complex and different receptor complexes might be located in different cells to interprete the CLE peptide landscape and translate it in the proper outcomes. Although the functional analysis might be hampered by the redundant action of the CLE peptides and receptors, many experiments can be done to further understand how the CLE peptides act. For instance, translational fusions should confirm that MtCLE12 and/or MtCLE13 peptides are secreted in the nodule infection zone, where *MtRLK1* is expressed. In addition, biochemical analysis should be performed to analyze the interactions between *MtRLK1* and *SUNN*, or

other possible candidates of the CLE peptide receptor complex.

7.4 Materials and methods

Biological material

Medicago truncatula Gaertn. cv. Jemalong A17 and J5 as well as *nin, bit1-1, nsp1, nsp2, dmi1, dmi2,* and *dmi3* mutants (Catoira *et al.*, 2000; Oldroyd and Long, 2003; Marsh *et al.*, 2007; Middleton *et al.*, 2007) were grown and inoculated as described (Mergaert *et al.*, 2003). *Sinorhizobium meliloti* 1021, *Sm1021* pHC60-GFP (Cheng and Walker, 1998), and *Sm2011* pBHR-mRFP (Smit *et al.*, 2005) were grown at 28°C in yeast extract broth medium (Vervliet *et al.*, 1975), supplemented with 10 mg L^{-1} tetracycline for the *Sm1021* pHC60-GFP and *Sm2011* pBHR-mRFP strains.

For *promoter:GUS* analysis, a 2-kb region upstream of *MtRLK1* (CT485797), *MtRLK2* (CR931811) and *SUNN* (AY769943) was isolated from genomic DNA based on the available genomic data[23]. The promoters were fused to the *uidA* gene in pKm43-GWRolDC1 (Karimi *et al.*, 2002). Primers used for amplification are presented in Table 7.1.

Table 7.1: Primers used in the analyses.

Gene	Sense primer	Anti-sense primer
Gateway cloning		
promoter-MtRLK1	ATTATGCACCCAACACGAAA	CATTATTTGTGGAATAACTAGC
promoter-MtRLK2	ATGACGTGGCCTACATGACA	GACATGAAAAGGCTTTGAGAAA
promoter-SUNN	TCCCTTTCACACACAAAATGA	CATCTTTCTCTTTTGCTCAGTTTT
RNAi *MtRLK1*	GCAAACTGTGGTTTGAAAGG	TGTGAGTTCGCGAAGATTTG
qRT-PCR amplification		
MtRLK1	GCCAGTGGGAGATTTT	GGTCGTTCCACACTTTGTTCC
MtRLK2	CATCTTGAATGTGAGCAATCG	GAGAGGTGTCGTCAATAACTG
SUNN	GCTCCTACGGCTACATTGC	ACTGGCTTCCTTCCTATTATCAG
40S Ribos	GCCATTGTCCAAGTTTGATGCTG	TTTTCCTACCAACTTCAAAACACCG
***in situ* hybridization**		
MtRLK1	GCCAGTGGGAGATTTTGGTGAAG	AAGCCAAGCCAAGGTATGC
MtRLK2	TGGACTTGCTTGTTCAGTGG	TTGTTGCGAACCAAACTCAG

For the qRT-PCR analysis, *M. truncatula* J5 plants were grown *in vitro* in square Petri dishes (12 × 12 cm) on nitrogen-poor SOLi agar (Blondon, 1964). After 7 days,

[23] http://www.ncbi.nlm.nih.gov/

roottips, SAMs, cotyledons, and first leaves were harvested, as well as mature leaves, stems, and roots from 1-month-old plants grown in perlite under nitrogen-rich conditions (*Sm1021* pHC60-GFP) and 1-month-old nodulated roots. For the analysis of temporal expression during nodulation, susceptible root zones were harvested 4 to 10 dpi from plants grown in pouches, watered with nitrogen-poor SOLi medium, and inoculated with *Sm1021* pHC60-GFP. Infection threads were visible from 4 dpi on, nodule primordia at 6 dpi, and small nodules at 8 dpi. Two days later, at 10 dpi, slightly bigger nodules were observed. Tissue was collected by visualizing the green fluorescent bacteria under a stereomicroscope MZFLII (Leica Microsystems, Wetzlar, Germany) equipped with a blue-light source and a Leica GFP Plus filter set ((λ_{ex} = 480/40; λ_{em} = 510 nm LP barrier filter). The zone I of uninoculated roots was isolated at the same developmental stage as the 4 dpi stage. For the analysis of *MtRLK1, MtRLK2* and *SUNN* expression in nodulation mutants, plants were grown in an aeroponic system. Half of the plants were harvested at the age of 6 days, while the other half of the plants was inoculated with *Sm2011* pBHR-mRFP and was harvested 7 days later.

Phylogenetic analysis

MtRLK1, MtRLK2 and *SUNN* homologs from *A. thaliana* were retrieved from the TAIR9 CDS databank using the tBLASTx algorithm[24]. The amino acid homologs of these sequences were downloaded from NCBI[25] and put together in a multiFASTA file. By use of the 'Multiple Sequence Comparison by Log-Expectation' (MUSCLE) (Edgar, 2004), a multiple sequence alignment was designed. Highly similar or divergent domains were deleted with the help of BioEdit7. Next, the PhyML program (Guindon and Gascuel, 2003) was applied to design a phylogenetic tree, based on the complete amino acid sequences of MtRLK1, MtRLK2, SUNN and their *A. thaliana* homologs and build according to the 'maximum likelihood' principle. Bootstrap values were based on 1000 reconstructions. We made use of Treeview1.6.6 for visualization (Page, 1996).

RNA extraction, cDNA synthesis, and qRT-PCR analysis

Total RNA was isolated with the RNeasy Plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After a DNAse treatment, the samples

[24] http://www.arabidopsis.org/index.jsp
[25] http://www.ncbi.nlm.nih.gov/

were purified through NH_4Ac (5 M) precipitation, quality controlled, and quantified with a Nanodrop spectrophotometer (Isogen, Hackensack, NJ). RNA (2 μg) was used for cDNA synthesis with the Superscript Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA). The samples were diluted 50 times and stored at -20°C until further use. The qRT-PCR experiments were done on a LightCycler 480 (Roche Diagnostics, Brussels, Belgium) and SYBR Green was used for detection. All reactions were done in triplicate and averaged. The total reaction volume was 5 μl (2.5 μl master mix, 0.25 μl of each primer [5 μM] and 2 μl cDNA). Cycle threshold (C_T) values were obtained with the accompanying software and data were analyzed with the $2^{-\triangle\triangle C_t}$ method (Livak and Schmittgen, 2001). The relative expression was normalized against the constitutively expressed 40S ribosomal S8 protein (TC100533, MGI). Primers used (Table 7.1) were unique in the MGI version 9.0 and the Medicago EST Navigation System database (Journet *et al.*, 2002).

In vitro application of auxins, and cytokinins

Auxins (10^{-6} M IAA) and cytokinins (10^{-7} M BAP) were diluted in dimethylsulfoxide and supplemented to the medium of 5-day-old, *in vitro*-grown plants. As a control, plants were grown without supplemented hormones. The plants were cultured at 25°C, 16-h photoperiod, and 70 μE $m^{-2}m^{-1}$ light intensity per day. The roots were covered with aluminum foil for light protection. After 0, 3, 6, 12, 24 and 96 h of incubation, the roots of 18 plants under each condition were harvested and analyzed by qRT-PCR. The experiment was repeated twice with comparable results.

Agrobacterium rhizogenes-mediated transgenic root transformation

The protocol was adapted from Boisson-Dernier *et al.* (2001). Approximately 48 h after germination, the radicle was sectioned at 5 mm from the roottip with a sterile scalpel. Sectioned seedlings were infected by coating the freshly cut surface with the binary vector-containing *A. rhizogenes* Arqua1 strains. The *A. rhizogenes* strain was grown at 28°C for 2 days on solid yeast extract broth medium with the appropriate antibiotics (Quandt *et al.*, 1993). The infected seedlings were placed on agar (Kalys) containing the SOLi medium, supplemented with 1 mM NH_4NO_3, in square Petri dishes (12 $\times$ 12 cm) placed vertically for 5 days at 20°C, 16-h photoperiod, and 70 μE $m^{-2}m^{-1}$. Subsequently, plants were placed on the same medium between brown papers at 25°C and under identical light conditions. One and 2 weeks later, plants

were screened for transgenic roots, characterized by GFP fluorescence with a stereomicroscope MZFLII (Leica Microsystems) equipped with a blue-light source and a Leica GFP plus filter set. One main transgenic root was retained per composite plant. Four weeks after infection, plants were transferred to an aeroponic system, pouches, or perlite-containing pots and incubated with SOLi medium. Three to 7 days after planting, composite plants were inoculated.

Histochemical localization of GUS activity

GUS activity in cotransformed roots and nodules was analyzed using 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid as substrate (Van den Eede *et al.*, 1992). Roots and nodules were vacuum infiltrated during 20 min and subsequently incubated in GUS buffer at 37°C. Incubation lasted 6, 8 and 10 h for *pMtRLK1-GUS, pMtRLK2-GUS* and *pSUNN-GUS*, respectively. After staining, roots and root nodules were fixed, dehydrated, embedded with the Technovit 7100 kit (Heraeus Kulzer, Wehrheim, Germany), according to the manufacturer's instructions, and sectioned with a microtome (Reichert-Jung, Nussloch, Germany). The 3-μm thick sections were mounted on coated slides (Sigma-Aldrich, St. Louis, MO). For tissue-specific staining, sections were submerged in a 0.05 % (w/v) ruthenium red solution (Sigma-Aldrich, St. Louis, MO), washed in distilled water, and dried. Finally, sections were mounted with Depex (BDH Chemicals, Poole, England). Photographs were taken with a Diaplan microscope equipped with bright- and dark-field optics (Leitz, Wetzlar, Germany).

In situ hybridization

Ten-μm sections of paraffin-embedded nodules were hybridized as described (Goormachtig *et al.*, 1997). Nodules were harvested, incubated in fixation buffer, and maintained for twice 15 min under vacuum. A ^{35}S-labeled antisense probe against a part of the open reading frame and the 3' UTR of *MtRLK1* and *MtRLK2* was produced according to standard procedures (Sambrook *et al.*, 1989). Primers used for probe amplification are listed in Table 7.1. The probes were cloned into pCRII-TOPO (Invitrogen, Carlsbad, CA) and further digested with BamHI restriction enzyme to yield templates for radioactive antisense probe production with T7 RNA polymerase (Invitrogen).

7.5 Supplemental data

Table 7.2: Overview of 33 *MtRLK* genes retrieved from the analysis described on page 164.

Gene	BAC	MT3.5	identity	chrom	E/H
MtRLK1	CT485797_34.5	Medtr5g090100.1	100	5	E
MtRLK2	CR931811_16.4	Medtr5g019070.1	99.7	5	E
MtRLK3	AC158497_52.4	Medtr2g014560.1	100	2	E
MtRLK4	CT009540_33.5	Medtr3g092390.1	100	3	E
MtRLK5	CU302336_1.4	Medtr5g026010.1	100	5	E
MtRLK6	CT009540_14.5	Medtr3g092420.1	100	3	H
MtRLK7	AC203530_15.3	Medtr5g087090.1	100	8	E
MtRLK8	CU302336_20.4	Medtr5g025890.1	98.53	5	E
MtRLK9	CU302345_22.3	Medtr5g025860.1	96.37	5	E
MtRLK10	CU302345_21.3	Medtr5g025850.1	97.82	5	H
MtRLK11	AC175312_46.5	Medtr1g090520.1	99.78	1	E
MtRLK12	AC174346_49.5	Medtr6g036780.1	87.77	6	H
MtRLK13	AC174346_53.5	Medtr6g036840.1	81.91	6	E
MtRLK14	CT030165_27.5	Medtr7g067530.1	99.89	3	E
MtRLK15	AC149574_15.4	Medtr8g066700.1	100	8	H
MtRLK16	CT027665_52.4	Medtr5g044680.1	97.62	5	E
MtRLK17	AC140550_56.4	Medtr2g005810.1	99.86	2	H
MtRLK18	AC203555_16.4	Medtr4g032320.1	91.9	4	E
MtRLK19	AC171265_41.5	Medtr5g082980.1	93.56	1	E
MtRLK20	CT009479_61.4	Medtr4g016910.1	100	3	E
MtRLK21	CU302345_20.3	Medtr5g025840.1	97.68	5	H
MtRLK22	CT962508_27.4	Medtr3g070220.1	100	3	H
MtRLK23	CT009479_57.4	Medtr4g016800.1	30.88	3	H
MtRLK24	CR955004_34.4	Medtr5g014700.1	100	5	E
MtRLK25	AC141862_30.4	Medtr4g097880.1	99.56	8	E
MtRLK26	AC154036_26.4	Medtr8g089210.1	90	4	H
MtRLK27	AC154036_25.4	Medtr8g089200.1	99.9	4	E
MtRLK28	CU302336_10.4	Medtr5g025950.1	98.79	5	H
MtRLK29	AC144729_4.4	Medtr5g082920.1	100	5	H
MtRLK30	CU302336_2.4	Medtr5g026000.1	100	5	H
MtRLK31	AC121244_30.5	Medtr4g070950.1	100	4	E
MtRLK32	CT009479_7.4	Medtr4g016780.1	93.76	3	E
MtRLK33	AC146559_20.4	Medtr1g079520.1	98.93	1	H

The sequences can be retrieved from: `http://www.medicagohapmap.org/`
BAC: number of the gene on the BAC clones
MT3.5: IMGAG annotation of the gene (version MT3.5)
Identity: compared between original sequence and sequence of IMGAG annotation
E/H: data supported by EST (E) or homology (H)

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Authors contributions

C. De Cuyper performed most of the expression analyses, as well as the phylogenetic analysis during her masters thesis, as a student of V. Mortier. S. Rombauts was involved in the bioinformatic analysis. S. Goormachtig and M. Holsters were involved in designing the research and revising the manuscript.

8

Involvement of *MtLOG* genes during indeterminate nodulation on *Medicago truncatula*

Virginie Mortier, Ulrike Mathesius, Petr Tarkowski, Marcelle Holsters and Sofie Goormachtig

In preparation

Abstract
Cytokinin signaling is a prerequisite for nodule formation. However, it is not clear whether NF signaling involves *de novo* cytokinin synthesis, cytokinin activation or relocation. We addressed this question via the analysis of *Medicago truncatula LOG* genes, encoding cytokinin riboside 5'-monophosphate phosphoribohydrolases involved in the direct activation of cytokinins. Two *MtLOG* genes, *MtLOG1* and *MtLOG2*, were found, the expression of which is upregulated during nodulation in a Nod Factor-dependent way. Histochemical analysis of *pMtLOG1:GUS* transgenics as well as functional analysis by RNAi and overexpression indicated an important role during nodulation and lateral root formation. *pMtLOG1:GUS* analysis indicated expression in root primordia, in dividing cortical cells of nodule primordia and later in the meristematic and early infection zone of mature nodules. Ectopic expression of *MtLOG1* resulted in plants with reduced nodule numbers, and premature nodule senescence. This inhibition of nodule formation was local and *SUNN*-independent, but might involve the upregulation of *MtCLE12* and *MtCLE13*, two *M. truncatula CLE* genes, which are involved during nodulation. Moreover, ectopic expression of *MtLOG1* resulted in reduced lateral root formation, enlargement of the root vascular tissue and shortening of the primary root in a *Mtcre1*-dependent manner. The involvement of *MtLOG* genes during nodule initiation and lateral root emergence was confirmed by the simultaneous downregulation of *MtLOG1* and *MtLOG2*. Together, these results suggest that the release of free cytokinins via MtLOGs accounts for proper nodule primordium development, nodule meristem functioning and partially for the negative regulation of lateral root formation.

8.1 Introduction

Cytokinins are involved in several physiological and developmental plant processes, amongst which lateral root formation, meristem activity, cell division, leaf senescence, nutritional signaling, germination, nodulation, circadian rhythms and stress tolerance (Mok, 1994; Sakakibara, 2006; Riefler *et al.*, 2006; Gonzalez-Rizzo *et al.*, 2006; Hana no *et al.*, 2006; Murray *et al.*, 2007; Tirichine *et al.*, 2007; Argueso *et al.*, 2009; Werner and Schmulling, 2009). Cytokinins are mobile molecules that can function as autocrine, paracrine or long-distance signals. The active forms of cytokinins consist of N^6-substituted adenine derivatives, such as ($\triangle^2$-isopentenyl)adenine (iP), *trans*-zeatin

(tZ) and *cis*-zeatin (cZ) (Mok and Mok, 2001; Sakakibara, 2006).

Cytokinin homeostasis is maintained by a fine-tuned balance between synthesis, catabolism and storage of inactive forms (Figure 8.1) (Sakakibara, 2006; Werner *et al.*, 2003). TransferRNA-isopentenyltransferases (tRNA-IPTs) act during the formation of cZ, a cytokinin for which no functional relevance was yet shown in Arabidopsis (Miyawaki *et al.*, 2006), while adenosine phosphate-isopentenyltransferases (IPTs) catalyze the synthesis of iP and tZ precursors (Kakimoto, 2001; Takei *et al.*, 2001; Sakamoto *et al.*, 2006). The cytokinin precursors are converted into active cytokinins by a two-step pathway or directly in a one-step reaction (Chen, 1997; Kurakawa *et al.*, 2007). In the two-step pathway, cytokinin riboside 5'-monophosphates are converted, by currently unknown enzymes, into the corresponding nucleosides and subsequently into active cytokinin nucleobases. The direct activation pathway, on the other hand, is mediated by a cytokinin riboside 5'-monophosphate phosphoribohydrolase, designated LONELY GUY (LOG), and allows immediate conversion to the active cytokinin nucleobases iP and tZ (Kurakawa *et al.*, 2007). Inactivation of cytokinins is achieved through either conjugation or degradation, by cytokinin glycosyltransferases and cytokinin oxidases/dehydrogenases (CKXs), respectively (Houba-Herin *et al.*, 1999; Morris *et al.*, 1999; Schmulling *et al.*, 2003; Galuszka *et al.*, 2007; Martin *et al.*, 2001; Mok and Mok, 2001; Hou *et al.*, 2004). IPTs, CKXs and LOGs are expressed at specific locations and time points, especially at regions of active growth during plant development (Werner and Schmulling, 2009; Kuroha *et al.*, 2009). However, cytokinin precursors were also identified in phloem and xylem saps, suggesting that the place of cytokinin precursor biosynthesis does not always correspond with the places of cytokinin activation (Corbesier *et al.*, 2003; Hirose *et al.*, 2008).

The LOG proteins seem to be very important to release active cytokinins during plant development. The first *LOG* gene, *OsLOG*, was identified in rice (*Oryza sativa*) in a screen for mutants deficient in the maintenance of shoot meristems (Kurakawa *et al.*, 2007). In the genome of Arabidopsis, 9 *LOG* homologs were subsequently identified, of which 7 had enzymatic activities equivalent to that of *OsLOG* (Kuroha *et al.*, 2009). LOG proteins were generally localized in the cytosol and nuclei (Kurakawa *et al.*, 2007; Kuroha *et al.*, 2009). Functional analysis of the *AtLOG* genes illustrated their importance for cytokinin effects. Ectopic overexpression of *AtLOG* genes resulted in reduced apical dominance, retardation of leaf senescence and elevated rate of cell division in the root vasculature and the embryo (Kuroha *et al.*, 2009). Double and triple mutants showed altered root and shoot morphology and a reduced sensitivity to cy-

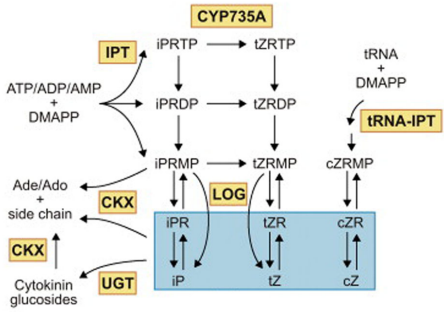


Figure 8.1: Overview of cytokinin metabolism. TransferRNA-isopentenyltransferases (tRNA-IPTs) act during the formation of *cis*-zeatin (cZ), while adenosine phosphate-isopentenyltransferases (IPTs) catalyze the synthesis of ($\triangle^2$–isopentenyl)adenine (iP) and *trans*-zeatin (tZ) precursors. The cytokinin precursors are converted into active cytokinins by a two-step pathway, which converts cytokinin riboside 5'-monophosphates into the corresponding nucleosides and subsequently into active cytokinin nucleobases. The release of iP and tZ can also be performed directly in a one-step reaction which is mediated by a cytokinin riboside 5'-monophosphate phosphoribohydrolase, designated LOG. Inactivation of cytokinins is achieved through either conjugation or degradation, by cytokinin glycosyltransferases (UGT) and cytokinin oxidases/dehydrogenases (CKXs), respectively.

tokinins during lateral root formation (Kuroha *et al.*, 2009). The partially overlapping expression patterns of the *LOG* genes, as well as the absence of phenotype in *AtLOG* single mutants, compared to *AtLOG* multiple mutants, suggested redundant functions for LOG proteins (Kuroha *et al.*, 2009).

Cytokinin signaling involves a phosphorelay mechanism, which is very similar to the two-component systems occurring in prokaryotes (Figure 3.2) (To and Kieber, 2008; Werner and Schmulling, 2009). Three genuine transmembrane cytokinin receptors were identified in Arabidopsis: the ARABIDOPSIS HISTIDINE KINASE2 (AHK2), AHK3 and AHK4, the latter also referred to as WOODENLEG (WOL) or CYTOKININ RESPONSE1 (CRE1) and homologs were identified in several plant species, amongst which *Medicago truncatula* and *Lotus japonicus* (Riefler *et al.*, 2006; To and Kieber, 2008; Inoue *et al.*, 2001; Hwang and Sheen, 2001). Upon cytokinin binding, these receptors autophosphorylate and transfer the phosphate group to ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEINS (AHPs). The AHPs move in and out the nucleus, independently of the phosphorylation state (Punwani *et al.*,

2010), but inside the nucleus, phosphorylated AHPs relay the signal to a group of ARABIDOPSIS RESPONSE REGULATORS (ARRs) which are classified according to their C-terminal domain (To *et al.*, 2007). Type A ARRs repress cytokinin signaling in a negative feedback loop, while type B ARRs are transcription factors regulating the expression of primary cytokinin response genes among which type A ARRs (To *et al.*, 2007; Kim, 2008). In addition, the expression of cytokinin response genes is also regulated by CYTOKININ RESPONSE FACTORS (CRFs), whose accumulation is also mediated by AHPs (Rashotte *et al.*, 2006; Rashotte and Goertzen, 2010).

Cytokinins control cell division at various positions within the plant. They are positive regulators of shoot apical meristem (SAM) activity and are involved in defining the boundaries between the SAM and shoot organ primordia (Tucker and Laux, 2007; Shani *et al.*, 2006; Giulini *et al.*, 2004; Lee *et al.*, 2009). The specification and maintenance of the vascular meristem might also be mediated by cytokinins (Mahonen *et al.*, 2006a,b). In cytokinin deficient mutants, a reduced vascular diameter was indeed observed due to a decrease in vascular cell proliferation in combination with an enhancement of the differentiation rate of pericycle cells towards protoxylem cells (Mahonen *et al.*, 2006a,b). In addition, in Arabidopsis and poplar, cytokinins were shown to have a positive effect on secondary vascular development, via the regulation of cambial activity (Mahonen *et al.*, 2006a,b; Matsumoto-Kitano *et al.*, 2008; Nieminen *et al.*, 2008).

Cytokinins might play opposite roles in the root apical meristem (RAM) and SAM because cytokinins have been shown to enhance the differentiation rate of RAM-located stem cells, suggesting their involvement in the determination of the root meristem size (Dello Ioio *et al.*, 2007; Blilou *et al.*, 2005). Moreover, cytokinins are involved in root stem-cell specification during early embryogenesis and in the negative regulation of lateral root formation (Muller and Sheen, 2008; Fukaki and Tasaka, 2009; Benkova *et al.*, 2003).

Legume plants gained the ability to establish a nitrogen fixing symbiosis with rhizobia. As a result, root nodules are formed in which the bacteria reside to fix atmospheric nitrogen for the plant. Nodule development requires the coordinated establishment of bacterial infection and *de novo* organ formation (Jones *et al.*, 2007; Ferguson *et al.*, 2010). Downstream of the Nod Factors (NF), signal molecules are produced that activate the nodulation process. Cytokinins are essential to activate cortical cell division for nodule primordium formation (Gonzalez-Rizzo *et al.*, 2006; Murray *et al.*, 2007; Tirichine *et al.*, 2007). In several legume species, amongst which alfalfa (*Medicago*

sativa), *M. truncatula*, white clover (*Trifolium repens*), pea (*Pisum sativum*) and *L. japonicus* the importance for cytokinins in nodulation has been demonstrated. Early experiments have shown that a NF-deficient *Sinorhizobium meliloti* strain, which constitutively produced tZ, induced nodule formation (Cooper and Long, 1994). The induction of nodule-like structures was also observed by exogenous application of cytokinins to alfalfa, *Sesbania rostrata* and white clover (Dehio and de Bruijn, 1992; Bauer *et al.*, 1996; Fang and Hirsch, 1998; Mathesius *et al.*, 2000).

Recently it was shown that *M. truncatula* transgenic plants with suppressed expression of *MtCRE1, Mtcre1* mutants and *L. japonicus* knockout mutants for the *MtCRE1* homolog, *LHK1*, were defective in nodule primordia formation (Gonzalez-Rizzo *et al.*, 2006; Murray *et al.*, 2007; Plet *et al.*, 2011). Additionally, a *L. japonicus* gain-of-function mutant for the LHK1 receptor provoked spontaneous nodules, indicating that cytokinin signaling is both necessary and sufficient for nodule formation (Tirichine *et al.*, 2007). In agreement, reduced cytokinin accumulation blocks nodulation, as in *L. japonicus*, ectopic expression of *CKX* genes, involved in cytokinin degradation, inhibits nodulation (Lohar *et al.*, 2004). In addition, the type B *MtRR1* and the type A *MtRR4* are activated by rhizobial inoculation in a manner dependent on several components of the NF signalization pathway (Gonzalez-Rizzo *et al.*, 2006). Attempts have been made to localize the cytokinin response during nodulation. Histochemical localization of type A RR *ARR5* in transgenic *L. japonicus* roots, indicated a rapid induction of ARR5 activity in root hairs and dividing cortical cells after rhizobial inoculation (Lohar *et al.*, 2004). Also in *M. truncatula*, the expression of *ARR4-5* and *ARR10-12* homologs, as well as the cytokinin receptor *MtCRE1*, is induced upon inoculation (Gonzalez-Rizzo *et al.*, 2006; Lohar *et al.*, 2006). *pMtCRE1:GUS* analysis and *in situ* hybridizations revealed expression in the NF response zone of the cortical cells and later on in the developing nodule primordium (Lohar *et al.*, 2006; Plet *et al.*, 2011). Based on these results it was proposed that cytokinin might be the mobile factor, activated/produced in the epidermis downstream of *DMI3* and translocated to the cortex were subsequent cytokinin signaling would induce cell division for primordium formation. In the cortex, cytokinin would be sensed by the cytokinin receptor MtCRE1 and subsequent signaling via type A MtRR4 and type B MtRR1 would result in the activation of the transcription factors ERN1, NSP2 and NIN, involved in the transcription of early nodulation (ENOD) genes to induce cortical cell divisions (Tirichine *et al.*, 2007; Frugier *et al.*, 2008; Plet *et al.*, 2011). MtCRE1 would also regulate nodulation-related auxin signaling by reducing PIN protein expression (Plet *et al.*, 2011). Moreover, histochemical

analysis of *pMtCRE1:GUS* activity revealed expression in the meristematic cells of mature nodules, suggesting cytokinins to be involved in nodule meristem homeostasis of mature *M. truncatula* indeterminate nodules (Plet *et al.*, 2011). Finally, cytokinins might also be involved in mediating root susceptibility to rhizobial infection and nodule organogenesis, as hyperinfection is observed in *hyperinfected1* (*hit1*) a mutant of the cytokinin receptor gene *LHK1* (Murray *et al.*, 2007; Frugier *et al.*, 2008).

Until now, it is not clear whether NF signaling involves *de novo* cytokinin synthesis, cytokinin activation or relocation. We addressed this question by identifying possible *LOG* genes in the *M. truncatula* genome. For two *MtLOG* genes, *MtLOG1* and *MtLOG2*, expression was upregulated during nodulation in a NF-dependent way. The expression of both genes was analyzed in detail during nodule formation. Functional analysis by RNAi and overexpression indicate an important role for *MtLOGs* during nodulation as well as lateral root formation.

8.2 Results

8.2.1 Identification of potential nodulation-related *LOG* genes in the genome of *Medicago truncatula*

Kurakawa *et al.* (2007) identified 9 members of the Arabidopsis LOG protein family. Based on the sequences of these *AtLOG* genes, homologs were identified in the genome of *M. truncatula* by tBLASTx analysis against the *M. truncatula* genomic data (Mt3.0). Seven of the 9 members of the Arabidopsis *LOG* family genes (*AtLOG1* till *AtLOG7*) were highly homologous to AC148995_43.5 and AC150977_28.5, which were renamed *MtLOG1* and *MtLOG2*, respectively. The other two members of the Arabidopsis LOG family genes (*AtLOG8* and *AtLOG9*), which cluster separately in the phylogenetic tree of the Arabidopsis LOG family proteins (Kuroha *et al.*, 2009), were highest homologous to AC152551_11.5, designated *MtLOG3*. No other *M. truncatula* genes were retrieved that had a significant similarity with the *AtLOG* genes. We calculated the similarities between the *MtLOG* and *AtLOG* genes using the supermatcher tool of wEMBOSS. *MtLOG1* and *MtLOG2* share 87.6 % similarity, *MtLOG1* and *MtLOG3* 69.2 % and *MtLOG2* and *MtLOG3* 67.2 %. *MtLOG1* is most homologous with *AtLOG3* (78.7 %) and shares between 60.6 % and 78.0 % similarity with the other Arabidopsis *LOG* genes. *MtLOG2* is also most homologous with *AtLOG3* (78.5 %) and shares between 57.3 % and 77.5 % similarity with the other Arabidopsis *LOG* genes,

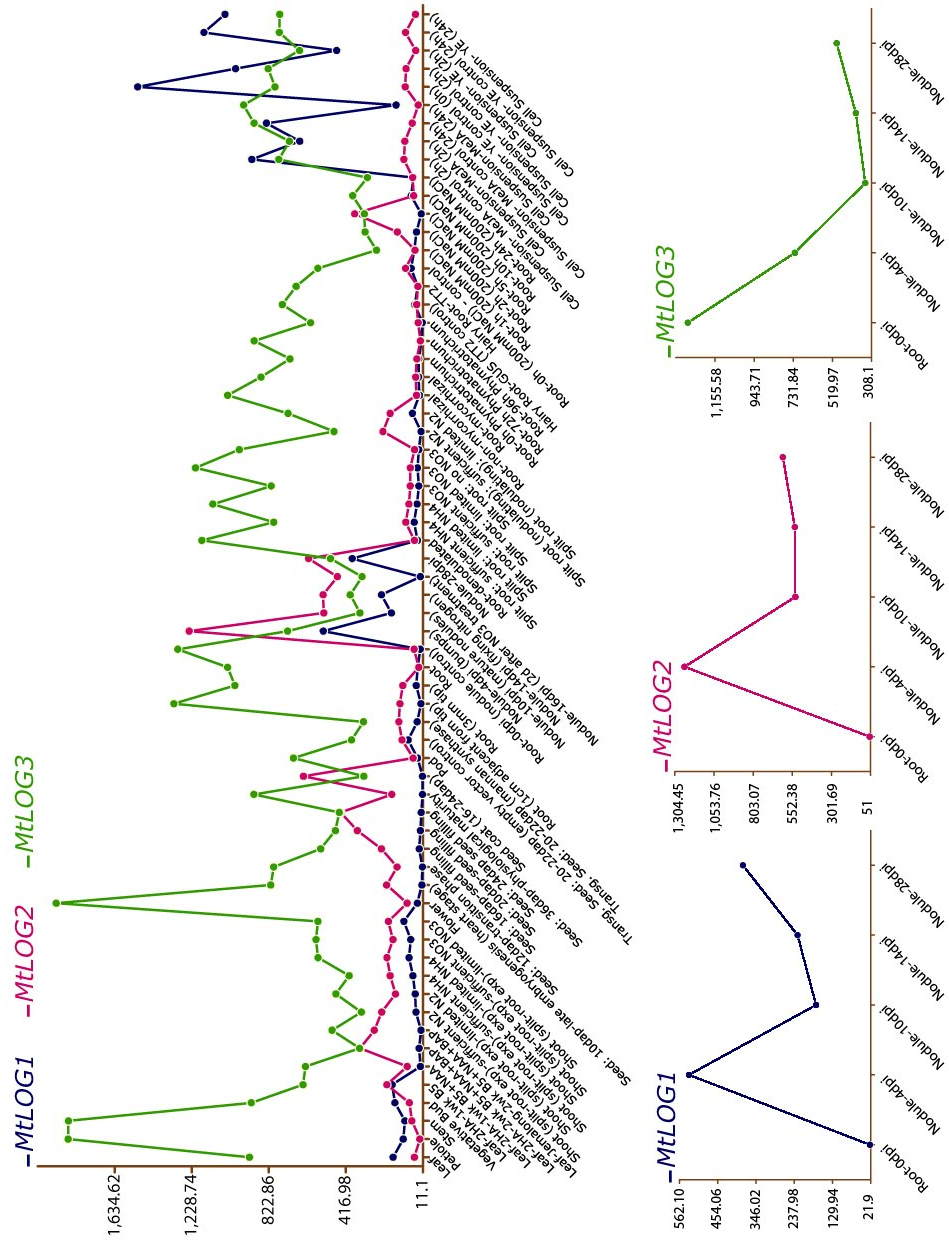
while *MtLOG3* is most homologous to *AtLOG8* (77.5 %) and shares between 55.5 % and 68.9 % similarity with the other Arabidopsis *LOG* genes.

In order to retrieve an *in silico* expression pattern of the three putative *MtLOG* genes, their sequences were blasted (BLASTn) against the Medicago Gene Expression Atlas from the Noble Foundation[26]. For *MtLOG1* (Mtr.39530.1.S1_at), an upregulation was observed in 4-day-old nodules, as compared to the expression in uninoculated control roots (Figure 8.2A). Moreover, according to this data, *MtLOG1* is not expressed in root tissue and a low level of expression is detected in leaves, petioles, buds, stems and cell suspension cultures (Figure 8.2A). Also for *MtLOG2* expression (Mtr.50458.1.S1_at) an upregulation during nodulation is observed in the data presented by the Medicago Gene Expression Atlas, with the highest expression at 4 days post inoculation (dpi) (Figure 8.2B). Compared to *MtLOG1, MtLOG2* seemed to be expressed more broadly in several other plant tissues, amongst which leaves, seeds and roots (Figure 8.2B). Expression of *MtLOG3* (Mtr.24814.1.S1_at) was detected in almost every tissue analyzed, and a reduction in *MtLOG3* transcripts was observed in nodule tissues from 4 dpi onward (Figure 8.2C). The temporal expression patterns during nodulation were confirmed by qRT-PCR analysis (Figure 8.3, A-C).

The transcript levels were analyzed in nodulated root tissues at 4, 6, 8, and 10 dpi and compared to the expression levels in uninoculated control roots. The elongation zone of uninoculated roots, the nodule initiation site, was used as the reference tissue. The *MtLOG1* transcript level steadily increased from 4 dpi on (Figure 8.3A). *MtLOG2* expression was low at 4 dpi, but increased at 6 dpi, and remained high until 10 dpi (Figure 8.3B). The *MtLOG3* transcript level decreased from 4 dpi until 10 dpi (Figure 8.3C). These data confirm the *in silico* data. Further studies only involved the *MtLOG1* and *MtLOG2* genes.

[26] `http://mtgea.noble.org/v2/`

Figure 8.2 ***(facing page)*****:** *In silico* expression profiles of *MtLOG1, MtLOG2* and *MtLOG3*, retrieved from the Medicago Gene Expression Atlas from the Noble foundation. Tissues analyzed are indicated below the diagrams. The three diagrams at the bottom of the page are magnifications of the top diagram and represent the expression profiles of *MtLOG1, MtLOG2* and *MtLOG3* in nodulation-specific tissues.


–MtLOG1
–MtLOG2
–MtLOG3
1,634.62
1,228.74
822.86
416.98
11.1
Root-0dpi
Nodule-4dpi
Nodule-10dpi
Nodule-14dpi
Nodule-28dpi
562.10
454.06
346.02
237.98
129.94
21.9
1,304.45
1,053.76
803.07
552.38
301.69
51
1,155.58
943.71
731.84
519.97
308.1

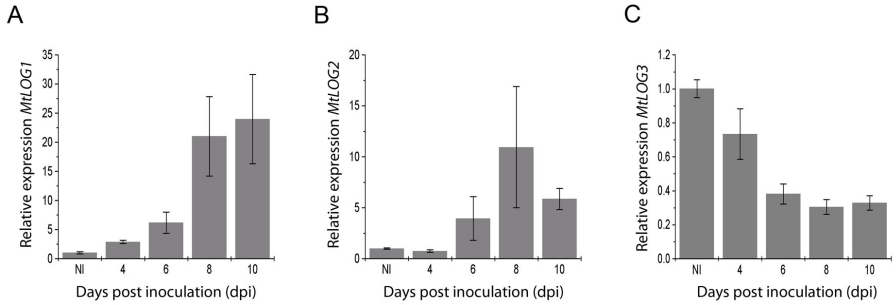


Figure 8.3: Expression analysis of *MtLOG* genes during nodulation. A, Relative expression of *MtLOG1*. B, Relative expression of *MtLOG2*. C, Relative expression of *MtLOG3*. Expression levels were measured by qRT-PCR on cDNA samples of zone I root tissues of uninoculated plants (NI) and at 4, 6, 8, and 10 dpi. Data and error bars represent means ± SD. This experiment was repeated twice with comparable results.

8.2.2 *MtLOG1* expression pattern in developing nodules and in lateral roots

Spatial expression pattern of *MtLOG1* in developing nodules was investigated by *promoter:GUS* analysis. A 2-kb region upstream of *MtLOG1* was isolated based on the available genomic data[27] and cloned 5' to the *uidA* gene. Transcriptional activation of the *uidA* gene was visualized by GUS staining.

In uninoculated roots, no GUS staining was seen in the root zone susceptible for nodulation. Three days after inoculation, when incipient nodule primordia are present, GUS staining was seen in the centre of the nodule primordium and to a much lesser extent at the base of the nodule primordium. In the outer cortex, no GUS staining was observed (Figure 8.4, A and B). At a slightly later stage, GUS staining was detected throughout the nodule primordium (Figure 8.4, C and D). In elongated, mature nodules weak *MtLOG1* expression was seen at the apex of the nodule, more specifically in the meristem and early differentiating nodule cells (Figure 8.4, E and F). No GUS staining was observed in the outer cortical cells and in cells of the mature infection zone (Figure 8.4F). *MtLOG1:GUS* expression was also observed in lateral root primordia (Figure 8.4G) and in roottips (data not shown).

Due to technical problems, cloning of *pMtLOG2:GUS* was not finished in time, but

[27] http://www.ncbi.nlm.nih.gov/

currently being performed.

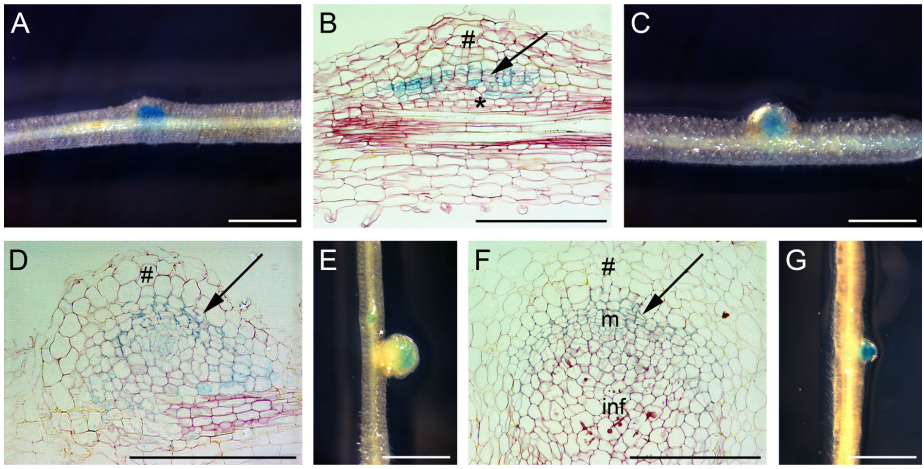


Figure 8.4: *MtLOG1* promoter activity during nodulation. A-B, *pMtLOG1:GUS* activity in nodule primordia. B, Bright-field picture of a longitudinal section through the nodule primordium represented in A. Expression is observed in the upper dividing cells of the primordium (arrow), but not in the dividing cells close to the root vasculature (asterisk) and not in the outer cortical cells (hash). C-D, *Promoter:GUS* activity of *MtLOG1* in young nodules. D, Bright-field picture of a longitudinal section through the nodule represented in C. Expression is observed throughout the nodule cortical tissue (arrow), except in the outer cell layers (hash). E-F, *pMtLOG1:GUS* activity in mature nodules. F, Bright-field picture of a longitudinal section through the nodule represented in E. A weak expression is observed in the meristem (arrow) and early differentiating cells, but not in the infection zone, nor in the outer cortical cells of the nodule (hash). G, *pMtLOG1:GUS* activity in lateral root primordia. All sections were counterstained with 0.05 % ruthenium red. xy, xylem; ph, phloem, p, pericycle, m, meristem; inf, infection zone. Bars = 1 mm (A, C, E and F) and 0.5 mm (B and D).

8.2.3 Influence of auxin and cytokinin on *MtLOG1* and *MtLOG2* expression

A proper auxin/cytokinin balance is a prerequisite for nodule formation (Oldroyd and Downie, 2008; Ding and Oldroyd, 2009). To determine whether auxins and/or cytokinins affect *MtLOG1* and *MtLOG2* expression, 10^{-6} M indole-3-acetic acid (IAA) or 10^{-7} M 6-benzylaminopurine (BAP) was supplemented to the growth medium of

5-day-old seedlings. For each condition, roots of 18 seedlings were harvested after 0, 3, 6, 12, 24, and 96 h. Roots from plates without hormone addition were used as a negative control. The expression of *MtCLE13* was measured as a positive control (Mortier *et al.*, 2010).

No significant differences in *MtLOG1* expression was detected in treated versus control roots. In roots treated with 10^{-7} M BAP, *MtLOG2* transcripts were upregulated after a 96 h treatment (Figure 8.5). The upregulation of *MtLOG2* expression observed after addition of 10^{-6} M IAA was not confirmed in an independent experiment (Figure 8.5).

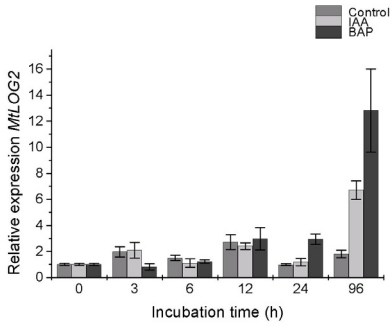


Figure 8.5: Influence of auxin and cytokinin on *MtLOG2* expression. qRT-PCR analysis of *MtLOG2* expression on cDNA samples of roots grown in the presence of 10^{-6} M auxin (IAA) or 10^{-7} M cytokinin (BAP). Growth medium without hormones was used for the control plants. Samples of 5-day-old plants were taken at 0, 3, 6, 12, 24, and 96 h after hormone addition. Data and error bars represent means $\pm$ SD. This experiment was repeated twice with comparable results.

8.2.4 *MtLOG1* and *MtLOG2* expression in nodulation mutants

To investigate whether NF signaling is required for the induction of *MtLOG1* and *MtLOG2* expression, we analyzed the transcript levels by qRT-PCR, before and after inoculation of *nin-1, nsp1, nsp2, dmi1, dmi2, dmi3*, or *Mtcre1-1* mutants (Figure 8.6). The mutant lines did not develop nodules, except to a very low extent for *Mtcre1-1*, which formed arrested primordia and infection foci (Andriankaja *et al.*, 2007; Middleton *et al.*, 2007; Plet *et al.*, 2011). For *MtLOG1* and *MtLOG2*, gene expression

was upregulated upon inoculation in wild type *M. truncatula* roots compared to control roots (Figure 8.6, A-D). In the roots of the nodulation mutants this effect was not observed anymore (Figure 8.6, A-D). These results suggest that *MtLOG1* and *MtLOG2* expression during nodulation is dependent on the components of the early NF signaling pathway.

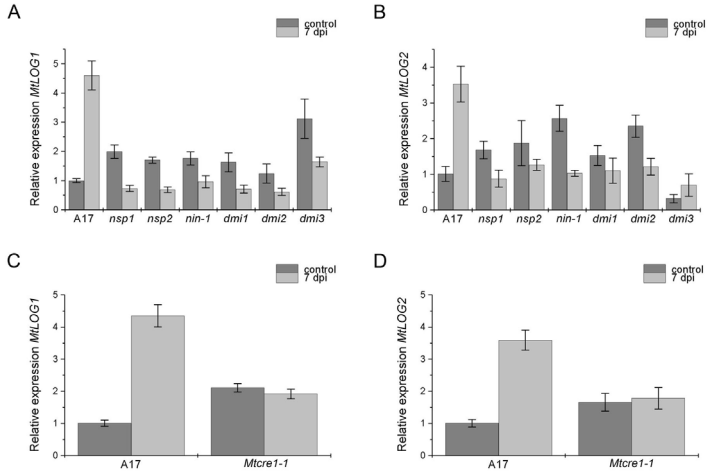


Figure 8.6: *MtLOG* expression in nodulation mutants. A and C, Relative expression of *MtLOG1*. B and D, Relative expression of *MtLOG2*. A-B, Expression analysis by qRT-PCR on cDNA samples of zone I root tissues of wild type plants (A17) and *nsp1, nsp2, nin-1, dmi1, dmi2* and *dmi3* mutants before inoculation (control) and at 7 dpi. The upregulation in *dmi3* for *MtLOG2* is not statistically relevant, as evaluated with ANOVA by means of the GenStat software ($p > 0.05$). C-D, Expression analysis by qRT-PCR on cDNA samples of zone I root tissues of wild type plants (A17) and *Mtcre1-1* mutants before inoculation (control) and at 7 dpi. Data and error bars represent means $\pm$ SD. These experiments were repeated twice with comparable results.

To confirm the results of the qRT-PCR analysis, *pMtLOG1:GUS* transgenic roots were generated in a *Mtcre1-1* and *nin-1* mutant background and analyzed 5 days after inoculation with *Sm2011* pBHR-mRFP. For wild type (A17) and the *Mtcre1-1* mutant, root zones harboring infection threads were isolated based on mRFP fluorescence of the *Sm2011* pBHR-mRFP bacteria. As no infection threads are observed on *nin-1* plants, root zones, susceptible to rhizobial infection, were harvested. *pMtLOG1:GUS* expression at the level of nodule primordia was only observed in wild type plants and not in *Mtcre1-1* and *nin-1* mutants (Figure 8.7). The *pMtLOG1:GUS* expression pattern

in lateral root primordia was also observed in the *Mtcre1-1* and *nin-1* mutants (Figure 8.7). These results confirm that *MtLOG1* is dependent on *MtCRE1* and *NIN* for its nodulation related expression, but not for its expression in lateral root primordia.

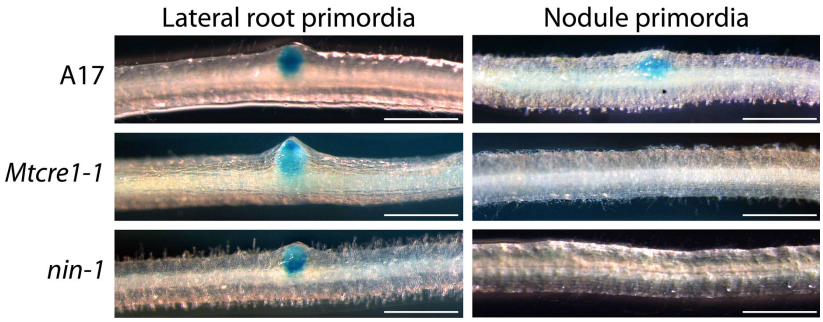


Figure 8.7: *pMtLOG1:GUS* activity in lateral root and nodule primordia in a *Mtcre1-1* and *nin-1* mutant background, as compared to wild type plants (A17) (n = between 15 and 20). Expression in the lateral root primordia is observed in every plant analyzed, while expression in nodule primordia was only detected in wild type plants and not in *Mtcre1-1* and *nin-1* mutant plants. Bars = 1 mm.

8.2.5 Effect of ectopic expression of *MtLOG1* on nodulation

To analyze the function of MtLOG1, composite plants with *35S:MtLOG1* expressing roots were made by *A. rhizogenes* transformation. QRT-PCR analysis confirmed the ectopic overexpression of the *35S:MtLOG1* constructs (Figure 8.8A). Preliminary cytokinin measurements indicated that *35S:MtLOG1* roots contain less cytokinin nucleotides (iPMP) than *35:GUS* roots ($p < 0.001$, t-test) (Figure 8.8B), while the level of active cytokinins (free base iP) was not affected by *MtLOG1* overexpression (Figure 8.8C) (Experiment executed by P. Tarkowski).

Nodulation of these transgenic roots was assessed at 7 dpi. Control roots (*35S:GUS*) contained on average 8.91 ± 0.50 nodules per root (Figure 8.8D), while on *35S:MtLOG1* transgenic roots, an average of only 0.57 ± 0.14 nodules were counted (Figure 8.8D). This difference in nodule number is statistically relevant ($p < 0.001$, Regression analysis).

To analyze whether the few nodules that are formed are functional, composite plants were inoculated with *Sinorhizobium meliloti 2011* pBHR-mRFP to be able to follow

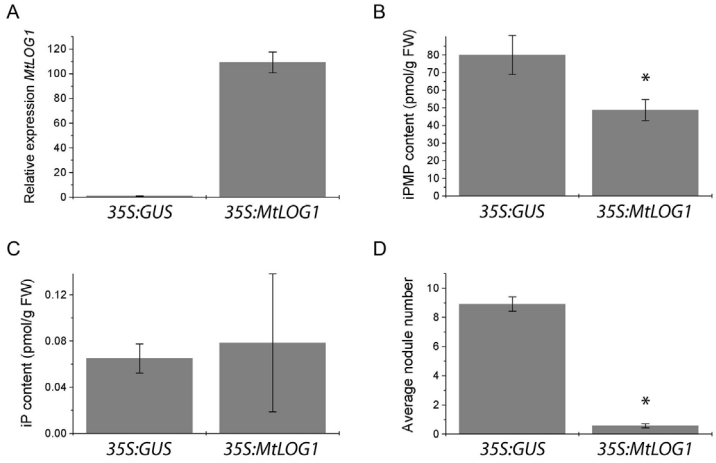


Figure 8.8: Effects of the ectopic expression of *MtLOG1* on nodulation and cytokinin levels. A, Relative expression of *MtLOG1* in *35S:MtLOG1* roots compared to *35S:GUS* roots. Expression levels were measured by qRT-PCR on cDNA samples of uninoculated root tissues made by *Agrobacterium rhizogenes* transformation with either *35S:GUS* or *35S:MtLOG1*, 45 days after germination. The experiment was repeated twice with comparable results. B, Quantification of cytokinin nucleotides (iPMP) (pmol/g FW) in *35S:MtLOG1* roots compared to *35S:GUS* roots (n = 3) ($p < 0.001$, Student t-test). Data and error bars represent means $\pm$ SD. C, Quantification of active cytokinins (free base iP) (pmol/g FW) in *35S:MtLOG1* roots compared to *35S:GUS* roots (n = 3). Data and error bars represent means $\pm$ SD. D, Average nodule number. Measurements were done 7 dpi on *35S:GUS* or *35S:MtLOG1* transgenic roots of composite plants (n = 29-35). The total mean of two biological repeats is represented in the graph. Data and error bars represent means $\pm$ SE. Asterisks indicates a statistically significant difference in comparison to *35S:GUS* transgenic roots ($p < 0.001$, Regression analysis).

infection via mRFP fluorescence. The bright red color observed throughout the central tissue of young *35S:MtLOG1* transformed nodules reveals that the nodules are totally filled with bacteria (Figure 8.9D). GFP fluorescence was used to visualize co-transformed roots (Figure 8.9, A and C).

Next, we investigated whether the infection progressed normally in *35S:MtLOG1* plants by performing microscopic analysis on 3-μm tick Technovit sections of 14 day-old nodules. Infection threads are clearly distinguishable on sections of *35S:GUS* and *35S:MtLOG1* transgenic nodules (Figure 8.9, F and I), indicative of normal rhizobial infection. However, the meristematic tissue of *35S:MtLOG1* transformed nodules

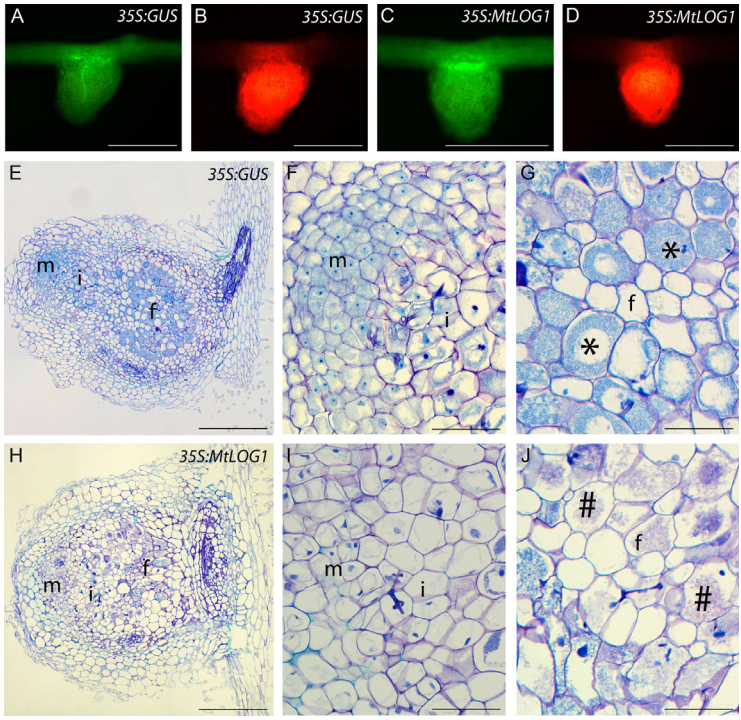


Figure 8.9: Nodulated roots of plants transformed with either *35S:GUS* or *35S:MtLOG1*. A and C, *35S:GUS* and *35S:MtLOG1* co-transformed roots were visualized by GFP fluorescence, respectively. B and D, *Sinorhizobium meliloti 2011* pBHR-mRFP were depicted based on mRFP fluorescence imaging in *35S:GUS* and *35S:MtLOG1* transformed nodules, respectively. E-J, Microscopic analysis of nodules ectopically expressing *GUS* (E-G) or *MtLOG1* (H-J) at 14 dpi. E and H, Longitudinal section through a nodule ectopically expressing *35S:GUS* (control) and *35S:MtLOG1*, respectively. F, Detail of the meristem and infection zone of the nodule represented in E. The meristematic tissue and infection zone are distinguishable. G, Detail of the fixation zone of the nodule represented in E. Large infected cells that are totally filled with symbiosomes are observed (asterisks). I, Detail of the meristematic tissue and fixation zone of the nodule represented in H. No dense cells, indicative of meristematic tissue, are observed anymore. J, Detail of fixation zone of the nodule represented in H. Senescence is observed in most infected cells (hashes). The sections were stained with toluidine blue. m = meristem, i = infection zone, f = fixation zone. Bars = 5 mm (A to D), 0,5 mm (E and H) and 50 μm (F-G and I-J).

seemed to disappear (Figure 8.9I). In addition, a high level of nodule senescence was observed on the sections of *35S:MtLOG1* transformed nodules (Figure 8.9, E and G), while on sections of control nodules (*35S:GUS*) of the same age, almost no senescent tissue was present (Figure 8.9, H and J).

As a *L. japonicus* gain-of-function mutant for the LHK1 receptor provoked spontaneous nodules, we wondered whether the ectopic expression of *MtLOG1* would result in a similar phenotype (Tirichine *et al.*, 2007). Therefore, *35S:MtLOG1* chimeric plants were grown under uninoculated and nitrogen-poor conditions and screened for nodule formation at several time points. On two-month-old plants, neither nodules nor bumps were observed, indicating that ectopic expression of *MtLOG1* is insufficient to provoke spontaneous nodule formation.

8.2.6 Interaction of *MtLOG1* with autoregulation of nodulation

Because *35S:MtLOG1* resulted in fewer nodules, we wondered whether *MtLOG1* might be involved in autoregulation of nodulation (AON), a long distance mechanism that controls nodule number. Previously it was shown that the CLE peptide gene, *MtCLE13*, putatively activating the AON pathway, was induced by cytokinin (Mortier *et al.*, 2010). Therefore, the expression of *MtCLE13* was investigated by qRT-PCR analysis on cDNA samples of uninoculated root tissues transformed with *35S:MtLOG1* by use of *A. rhizogenes*. In addition, two other *MtCLE* genes (*MtCLE4 and MtCLE12*) were tested. *MtCLE12* and *MtCLE13* expression was higher in *35S:MtLOG1* roots compared to the level seen in control roots (Figure 8.10, A-B). For *MtCLE4* no differential expression was measured (data not shown). We next investigated whether *MtCLE12* and/or *MtCLE13* overexpression has an effect on *MtLOG1* and *MtLOG2* expression. QRT-PCR analysis on cDNA of chimeric plants ectopically expressing either *MtCLE12* or *MtCLE13* revealed no consistent differential expression of *MtLOG1* and *MtLOG2* between control and *35S:MtCLE12* or *35S:MtCLE13* roots. Hence, *MtLOG1* expression might contribute to *MtCLE12* and *MtCLE13* expression during nodulation.

We previously have shown that nodulation is totally inhibited on *35S:MtCLE12* and *35S:MtCLE13* roots and that this effect involves long-distance mechanisms (Mortier *et al.*, 2010). To test whether ectopic expression of *MtLOG1* might also result in long distance effects, composite plants were generated with small *35S:GUS* or *35S:MtLOG1* transgenic roots in addition to the wild type main root. At 7 dpi with *Sm2011* pBHR-mRFP, nodule number was assessed. On average 17.36 $\pm$ 1.72 nodules were counted

on the primary wild type root of *35S:GUS* plants, while 14.62 $\pm$ 1.59 nodules were counted on the primary wild type root of *35S:MtLOG1* plants (Figure 8.10C). Regression analysis (Poisson distribution, logarithmic link) by means of the GenStat software revealed that these differences were not statistically relevant ($p > 0.05$). This result indicates that the effect of the ectopic overexpression of *MtLOG1* on nodulation acts locally and not systemically.

The negative effect of *35S:MtCLE12* and *35S:MtCLE13* was abolished in *sunn-4* mutants which are affected in the AON pathway due to a mutation in a leucine-rich-repeat receptor-like-kinase (LRR-RLK) possibly perceiving CLE peptides (chapter 5). In a next step, the effect of ectopic overexpression of *MtLOG1* was tested in a *sunn-4* mutant background. Nodulation was assessed 7 days after inoculation with *Sm2011* pBHR-mRFP and resulted in 41.50 $\pm$ 4.41 nodules on *35S:GUS* roots (control), and in 8.67 $\pm$ 1.75 nodules on *35S:MtLOG1* roots (Figure 8.10D). Regression analysis (Poisson distribution, logarithmic link) by means of the GenStat software revealed that these differences are statistically relevant ($p < 0.001$, Regression analysis).

Together, these results indicate that the effect of ectopic expression of *MtLOG1* might involve MtCLE12 and MtCLE13 peptide signaling but that the inhibition acts locally and is independent of *SUNN*.

8.2.7 Effect of knock-down of *MtLOG1* and *MtLOG2* expression on nodulation

To further unravel the role of *MtLOG* genes during nodulation, an RNAi hairpin construct was created that simultaneously down-regulates the expression of *MtLOG1* and *MtLOG2* (experiments executed by Ulrike Mathesius, Australia). Due to the homology between *MtLOG1* and *MtLOG2* it was impossible to create hairpin constructs which discriminated between both genes. Composite plants were inoculated with *S. meliloti* strain E65 and nodule number was assessed at 14 dpi. In wild type A17 plants, an average of 3.52 $\pm$ 0.30 nodules were counted on control roots transformed with an empty vector, while RNAi *MtLOG1/MtLOG2* plants had on average 2.64 $\pm$ 0.31 nodules ($p <$, ANOVA) 0.05) (Figure 8.11A). QRT-PCR analysis confirmed the simultaneous knock-down of *MtLOG1* and *MtLOG2* in A17 plants (Figure 8.11B).

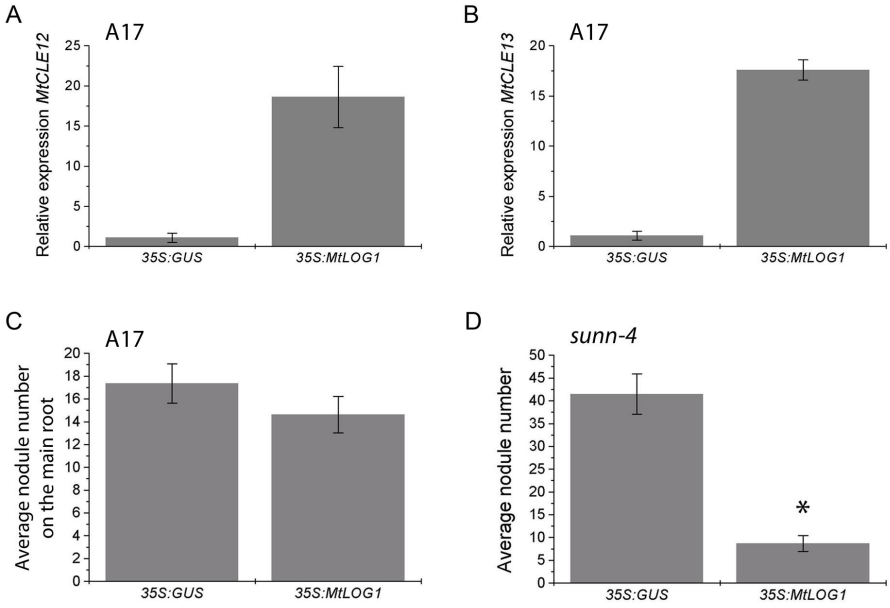


Figure 8.10: Interaction of *MtLOG1* with autoregulation of nodulation. A-B, Expression analysis of genes that are differentially regulated in *35S:MtLOG1* roots compared to *35S:GUS* roots. A, Relative expression of *MtCLE12*. B, Relative expression of *MtCLE13*. Expression levels were measured by qRT-PCR on cDNA samples of uninoculated root tissues transformed by *A. rhizogenes* transformation with either *35S:GUS* or *35S:MtLOG1* 45 days after germination. Data and error bars represent means $\pm$ SD. The experiment was repeated twice with comparable results. C, Long-distance effect of roots expressing *MtLOG1* on wild type root nodulation of composite plants. Average nodule number at 7 dpi on the wild type main root of composite plants bearing additional transgenic *35S:GUS* (control) or *35S:MtLOG1* roots (n = 28-31). Data and error bars represent means $\pm$ SE. Regression analysis by means of the GenStat software revealed that there are no statistical differences ($p > 0.05$). The total mean of two biological repeats is represented in the graph. D, Effect of ectopic overexpression of *MtLOG1* on nodulation in a *sunn-4* mutant background. Average nodule number at 7 dpi on composite plants bearing transgenic *35S:GUS* (control) or *35S:MtLOG1* roots (n = between 26 and 28). Data and error bars represent means $\pm$ SE. Statistical differences were evaluated with a regression analysis by means of the GenStat software. Asterisk indicates a statistically relevant difference in comparison to *35S:GUS* transgenic roots ($p < 0.001$, Regression analysis). The total mean of two biological repeats is represented in the graph.

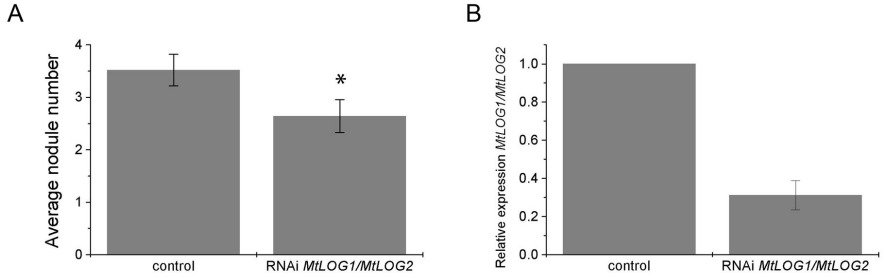


Figure 8.11: Effect of knock-down of *MtLOG1* and *MtLOG2* on nodulation. A, Nodule numbers in wild type hairy roots 14 days after inoculation with *S. meliloti* strain E65. Wild type (A17) plants were transformed with an empty vector (control) or an RNAi construct targeting *MtLOG1* and *MtLOG2* (RNAi *MtLOG1/MtLOG2*) and inoculated 3 weeks post transformation (n = between 70 and 99). Data and error bars represent means ± SE. Asterisk indicates statistically significant differences in comparison to control roots ($p < 0.05$, ANOVA). B, Expression of *MtLOG1/MtLOG2* in RNAi-transformed hairy roots (RNAi *MtLOG1/MtLOG2*) of wild type (A17) plants compared to empty vector controls determined by qRT-PCR. Values show means and standard deviations of three biological replicates and three technical replicates. Each biological replicate contains 8 to 10 roots and was harvested 14 dpi (at the time of counting nodules).

8.2.8 Effect of ectopic expression of *MtLOG1* and knock-down of *MtLOG1* and *MtLOG2* on root development

To analyze the function of *MtLOG1*, composite plants with *35S:MtLOG1* expressing roots were made by *A. rhizogenes* transformation. A difference in root length was measured at 40 days post germination, since the control roots were on average 40.52 ± 3.55 cm long, while the *35S:MtLOG1* roots measured 22.88 ± 3.55 cm (Figure 8.12A). In accordance, the lateral root number was on average 40.07 ± 3.31 for control transgenic roots (*35S:GUS*) and 18.23 ± 2.45 for *35S:MtLOG1* transgenic roots (Figure 8.12B). Finally, the lateral root density was calculated, based on the lateral root numbers and the root length of the respective plants, and resulted in 1.36 ± 0.13 lateral roots per cm for control roots and in 1.38 ± 0.14 lateral roots per cm for *35S:MtLOG1* roots (Figure 8.12C). Except for the lateral root density, all differences in measurements were statistically relevant ($p < 0.001$, Regression analysis).

In addition to the root phenotypes described above, *35S:MtLOG1* expressing roots had a more robust appearance than *35S:GUS* roots. To analyze this observation more in detail, transversal sections of root tissue located 1 to 2 cm above the roottip of 1-

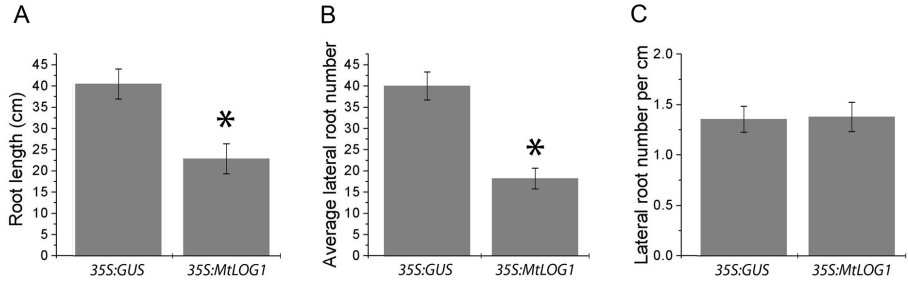


Figure 8.12: Effects of the ectopic expression of *MtLOG1* on root formation. Measurements were done 40 days after germination on *35S:GUS* or *35S:MtLOG1* transgenic roots of composite plants (n = 29-35). A, Average lateral root number. B, Average root length. C, Average lateral root density. Asterisks indicate statistically significant differences in comparison to *35S:GUS* transgenic roots ($p < 0.001$, Regression analysis). Data and error bars represent means $\pm$ SE. The total mean of two biological repeats is represented in the graphs.

month-old plants were made and analyzed. As shown in Figure 8.13, the vascular tissue of *35S:MtLOG1* transgenic roots was more expanded than that of control roots. The diameter of the vascular bundle, comprising xylem tissue, phloem tissue, pericycle and endodermal cell layer was measured and an average diameter of 276.2 $\pm$ 6.0 μm was obtained for *35S:MtLOG1* roots, while this was only 182.0 $\pm$ 5.0 μm for control roots ($p < 0.001$, Student t-test) (Figure 8.13C). Careful analysis of the sections indicated larger but also more cells in *35S:MtLOG1* vascular tissues (Figure 8.13B). The same measurements were performed for the cortical tissue of these roots. No change in size of the cortical zone, neither in number of cortical cells was seen in radial sections of *35S:MtLOG1* roots compared to control roots. These data suggest that the thickening of *35S:MtLOG1* transformed roots is the result of expansion of the vascular tissue.

As *MtCRE1* is an important cytokinin receptor in *M. truncatula*, we controlled whether the root phenotypes observed on *35S:MtLOG1* hairy roots are *MtCRE1*-dependent. Therefore, composite plants with *35S:MtLOG1* expressing roots were made by *A. rhizogenes* transformation in a *Mtcre1-1* mutant background. The dry weight of 20 roots of each line was measured at one month post germination. Control roots weighted on average 9.54 $\pm$ 0.99 mg, while *35S:MtLOG1* roots weighted on average 9.57 $\pm$ 0.99 mg (Figure 8.13D). These differences are statistically not relevant (Regression analysis, $p > 0.05$). The diameter of the vascular tissue was also measured, and no statistically relevant differences were observed. The diameter of the vascular tissue of *35S:GUS*

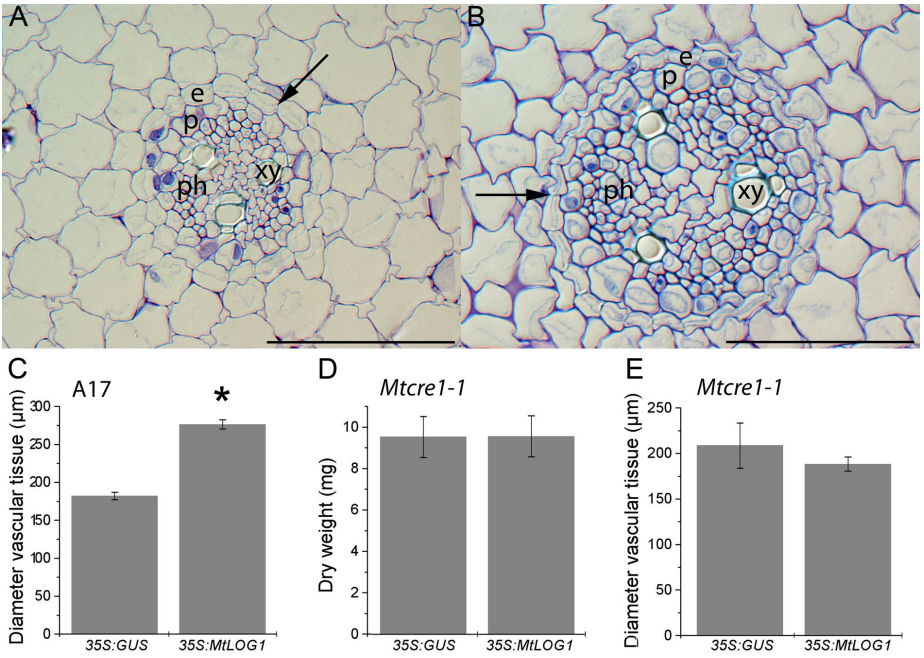


Figure 8.13: Enlargement of the root vascular tissue due to cell expansion and increase in cell number caused by the ectopic expression of *MtLOG1* is dependent on *MtCRE1*. A and B, Toluidine blue stained sections through young root segments ectopically expressing *GUS* and *MtLOG1*, respectively. C, Average diameter of the root vascular tissue of *35S:GUS* and *35S:MtLOG1* chimeric wild type (A17) plants (n = 3). Asterisk indicates a statistically significant difference in comparison to *35S:GUS* transgenic roots ($p < 0.001$, Student t-test). D-E, Effects of the ectopic expression of *MtLOG1* on root formation in a *Mtcre1-1* mutant background. D, Dry weigth of the total root material. Measurements were done 1 month post germination on *35S:GUS* and *35S:MtLOG1* transgenic roots of composite *Mtcre1-1* mutant plants (n = 42). No statistical differences were measured ($p > 0.05$, ANOVA). E, Average diameter of the root vascular tissue of *35S:GUS* and *35S:MtLOG1* chimeric plants (n = 3-7). Statistical differences were evaluated with a two-tailed, unpaired Student t-test. Measurements represented in C and E were made on root material harvested 1 to 2 cm above the roottip of 1 month old plants. Measurements of the diameter of the vascular tissue included the endodermal cell layer, identified by the presence of Casparian strips (arrows). Data and error bars represent means ± SE. e, endodermis; p, pericyclus; xy, xylem; ph, phloem. Bars = 0.25 mm.

roots was on average 208.67 $\pm$ 24.86 μm long, while the diameter of the vascular tissue of *35S:MtLOG1* roots was on average 188.29 $\pm$ 7.75 μm long (Figure 8.13E). These results suggest that the root phenotypes observed on *35S:MtLOG1* plants is *MtCRE1* dependent.

Next, the number of lateral root was analyzed in the RNAi *MtLOG1/MtLOG2* plants (Experiments executed by U. Mathesius and A. Wasson). In wild type A17 plants, an average of 2.70 $\pm$ 0.30 lateral roots were counted on control roots transformed with an empty vector, while RNAi *MtLOG1/LOG2* plants had on average 3.72 $\pm$ 0.36 lateral roots ($p < 0.05$, ANOVA) (Figure 8.14, A). Also in *Mtcre1-1* mutants a statistically significant difference in lateral root number was counted, as an average of 4.20 $\pm$ 0.47 lateral roots were counted on control roots transformed with an empty vector, while RNAi *MtLOG1/LOG2* plants bared on average 6.17 $\pm$ 0.66 lateral roots ($p < 0.05$, ANOVA) (Figure 8.14B). QRT-PCR analysis confirmed the simultaneous knock-down of *MtLOG1* and *MtLOG2* in A17 and *Mtcre1-1* mutants, respectively (Figure 8.14, C-D).

In agreement with a function in releasing active cytokinins, ectopic overexpression resulted in smaller root systems with reduced lateral root numbers, while RNAi resulted in more lateral roots. MtCRE1 seems to be the most important cytokinin receptor, but the RNAi experiments suggest that other receptors mediate the cytokinin effect on lateral root number.

8.3 Discussion

We identified 3 *LOG* genes (*MtLOG1, MtLOG2* and *MtLOG3*) in the genome of *M. truncatula* (470 Mb) by tBLASTx analysis. *LOG* genes encode cytokinin riboside 5'-monophosphate phosphoribohydrolases involved in the direct conversion of cytokinin riboside 5'-monophosphates to active cytokinins (Kurakawa *et al.*, 2007; Kuroha *et al.*, 2009). In comparison to the 11 *LOG* genes identified in the genome of rice (430 Mb) and the 9 *LOG* genes identified in the genome of Arabidopsis (157 Mb), more *MtLOG* genes were expected to be retrieved. Possibly the *M. truncatula* and Arabidopsis *LOG* genes are too divergent to be identified via tBLASTx analysis. Alternatively, the 16 % of the genome of *M. truncatula* that still needs to be sequenced might contain several more *LOG* genes.

The 3 *MtLOG* genes share a high degree of similarity with the 7 *AtLOG* genes (*AtLOG1* to *AtLOG5*, *AtLOG7*, and *AtLOG8*), which were shown to be involved in

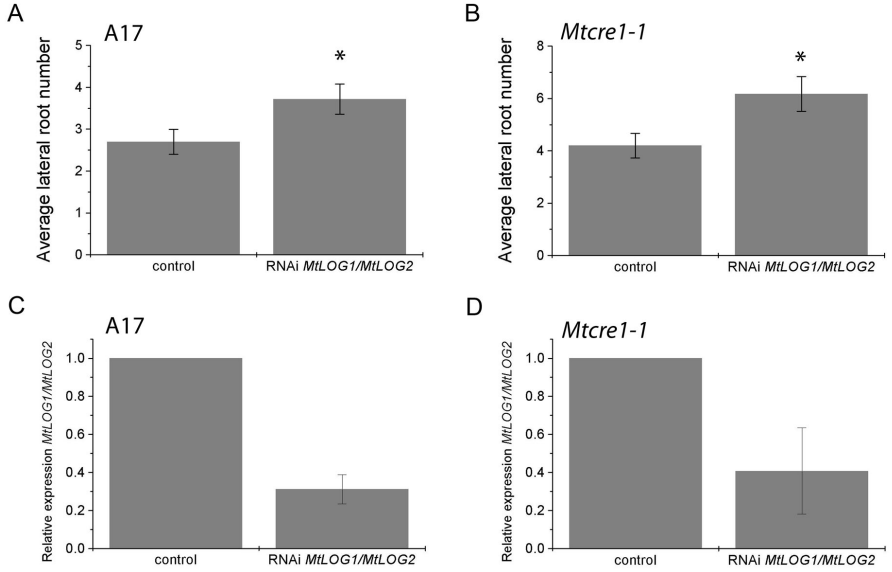


Figure 8.14: Effect of knock-down of *MtLOG1* expression on lateral root formation in wild type (A17) and *Mtcre1-1* mutant plants. A-B, Lateral root numbers in hairy roots 14 dpi with *S. meliloti* strain E65. Wild type (A17) (A) or *Mtcre1-1* mutants (B) were transformed with an empty vector (control) or an RNAi construct targeting both *MtLOG1* and *MtLOG2* (RNAi *MtLOG1/MtLOG2*) and inoculated 3 weeks post transformation. (n = between 70 and 99). Data and error bars represent means ± SE. Asterisks indicate statistically significant differences in comparison to control roots ($p < 0.05$, ANOVA). C-D, Expression of *MtLOG1/MtLOG2* in RNAi-transformed hairy roots (RNAi *MtLOG1/MtLOG2*) of wild type (A17) (C) or *Mtcre1-1* mutants (D) compared to empty vector controls determined by qRT-PCR. Values show means and standard deviations of three biological replicates and three technical replicates. Each biological replicate contains 8 to 10 roots and was harvested 14 dpi.

the release of the active cytokinin nucleobases from the cytokinin riboside 5'-monophosphates similar to what was reported for *OsLOG* (Kurakawa *et al.*, 2007; Kuroha *et al.*, 2009). Preliminary results indeed have shown that *35S:MtLOG1* roots contain less cytokinin nucleotides compared to *35S:GUS* roots, indicating that MtLOG1 is a true 5'monophosphate phophoribohydrolase. However, no more free cytokinins were measured in these roots. This is in contrast to the results of Kuroha *et al.* (2009) and Kurakawa *et al.* (2007). The difference between their experiments and the experiments

described here is that they used DEX inducible constructs allowing the analysis of short-term effects, while here the constitutive *35S* promoter was used, enabling the analysis of long term effects only. Possibly, the plants have adapted their physiology by activating cytokinin degrading or conjugating mechanisms to counteract the effect of *LOG* overexpression. New experiments should involve more sensitive measurement on more plant material, as well as CKX analysis (P. Tarkowski, personal communication).

Transcripts of two of the three *MtLOG* genes were upregulated during nodulation. *MtLOG1*, expression is restricted to lateral root primordia and nodules, while *MtLOG2* is more broadly expressed in different plant tissues, suggesting its participation in several development processes. The nodulation-related expression was shown to be dependent on the components of the early NF signaling pathway, *DMI1, DMI2, DMI3, NSP1, NSP2, NIN* and *MtCRE1*, indicating that the genes are only induced after the activation of the MtCRE1 receptor. In addition, the earliest stage at which the *MtLOG1* expression was seen, was in cells of developing nodule primordia. Hence, it is unlikely that the cytokinins released via *MtLOG1* and possibly *MtLOG2* act as a primary cytokinin pool that is sensed by MtCRE1 to initiate nodule formation. Rather, *MtLOG1* and *MtLOG2* might be involved in a positive feedback loop to maintain the cytokinin levels to allow continued cell division. Other triggers such as translocation or activation of cytokinins, or changes in cytokinin susceptibility might be needed to allow the initiation of cortical cell divisions.

How *MtLOG1* and *MtLOG2* expression is triggered is currently unknown. Although the nodulation-related expression is dependent on *MtCRE1*, addition of cytokinin did not activate *MtLOG1* expression. Also exogenous addition of auxin could not trigger the expression of both genes. Because, the expression of *MtLOG1* was only observed after the first cell divisions, a developmental factor related to cell division might induce both genes. A combination of cytokinin and auxin as well as the stele factor, uridine, isolated years ago as a factor that controls cortical cell division (Smit *et al.*, 1995b; Crespi and Galvez, 2000), are interesting possibilities to test.

In mature nodules, *pMtLOG1:GUS* staining was seen in the meristematic zone and early differentiating nodule cells suggesting that *MtLOG1* might be involved in cell division and differentiation of mature nodule meristems. In accordance, *MtCRE1* and the cytokinin response regulator 4 (*MtRR4*) are expressed in the same zone of mature nodules (Plet *et al.*, 2011). Hence at this stage, *MtLOG1* and possibly *MtLOG2* might account for cytokinins that are perceived by MtCRE1 to trigger cell division. Also in other plant developmental processes, *LOG* genes control meristematic activities.

For instance, the *OsLOG* gene was shown to be essential to maintain SAM activity (Kurakawa *et al.*, 2007; Kuroha *et al.*, 2009).

Functional analysis revealed an important function for *MtLOG1* and *MtLOG2* during nodulation. Simultaneous knock-down of *MtLOG1* and *MtLOG2* expression resulted in a reduction in nodule number. These data further support the positive effect of cytokinins on nodulation as was previously suggested by numerous experiments (Gonzalez-Rizzo *et al.*, 2006; Murray *et al.*, 2007; Plet *et al.*, 2011; Tirichine *et al.*, 2007; Lohar *et al.*, 2004). For instance, in *L. japonicus*, a cytokinin degrading enzyme of Arabidopsis or rice resulted in a reduction of nodulation (Lohar *et al.*, 2004). Also the knock-down and knock-out of the gene encoding the cytokinin receptor MtCRE1 resulted in plants with a strongly reduced nodule number (Gonzalez-Rizzo *et al.*, 2006; Plet *et al.*, 2011). Finally, a loss-of-function mutation in the gene encoding the cytokinin receptor LHK1 of *L. japonicus* induced a reduction of nodulation (Murray *et al.*, 2007). Hence, the cytokinins produced by the MtLOG proteins might account at least partly for the cytokinin pool needed to support nodule formation. The *lhk1* loss-of-function mutation also exhibited hyperinfection (Murray *et al.*, 2007). It might be interesting to analyze whether hyperinfection is also observed on the RNAi *MtLOG1/MtLOG2* transformed roots.

A gain-of-function mutation in the *L. japonicus LHK1* gene resulted in spontaneous nodulation indicating that activation of the cytokinin receptor is sufficient to induce nodule development (Murray *et al.*, 2007). In agreement, exogenous application of cytokinin resulted in spontaneous nodule formation in alfalfa, Sesbania and white clover and a rhizobial mutant ectopically producing cytokinin could induce nodule formation (Cooper and Long, 1994; Dehio and de Bruijn, 1992; Bauer *et al.*, 1996; Fang and Hirsch, 1998; Mathesius *et al.*, 2000). Ectopic expression of *MtLOG1* did however not result in spontaneous nodule formation. This discrepancy might be explained by the absence or by low levels of cytokinin precursors in the root cortex, the place where the first cell divisions for primordium formation occur. In Arabidopsis, high concentrations of cytokinin precursors were measured in both the xylem and phloem tissue (Corbesier *et al.*, 2003; Hirose *et al.*, 2008). These precursors might not reach the cortex until nodulation is initiated. Interestingly symplasmic continuity between the phloem and nodule initials is installed early after inoculation (Complainville *et al.*, 2003). Hence, the cytokinin precursors might only reach the cortical cells once this continuity is made.

In agreement with the restricted availability of precursors in the vascular system,

only an enlargement of the root vascular tissue was observed in *35S:MtLOG1* transgenic roots. The ectopic expression of *AtLOG* genes also resulted in an enlargement of the root vascular tissue (Kuroha *et al.*, 2009). Cytokinins are known positive regulators of cambial activity (Ye, 2002; Matsumoto-Kitano *et al.*, 2008; Nieminen *et al.*, 2008). Hence it would be interesting to investigate whether the enlarged vascular tissues observed here are consequences of induced activity of the cambium.

Because many experiments have shown that cytokinins are positive regulators of nodulation it came as a surprise that inoculation of *35S:MtLOG1* roots resulted in a reduced number of nodules. Analysis of the few nodules that were still formed on those roots indicated that bacterial infection threads were normal in size and shape indicating that rather the root susceptibility towards nodulation might be decreased in *35S:MtLOG1* roots. Such a reduction in root susceptibility was not observed after exogenous application of cytokinins (Gronlund *et al.*, 2005; Radutoiu *et al.*, 2003). However, Lorteau *et al.* (2001) indicated that high concentrations of exogenous cytokinins inhibit nodulation. Possibly, the ectopic expression of *MtLOG1* might result in the release of too high levels of cytokinins inducing a negative effect on nodulation. Alternatively, the ectopic expression of *MtLOG1* might activate additional factors, as illustrated by the profound changes in vascular development that affect nodule formation.

The MtCLE12 and MtCLE13 peptides might act as a downstream factor of *MtLOG1* because the expression of both corresponding genes was enhanced in *35S:MtLOG1* transgenic roots compared to the levels observed in control roots. These CLE peptides negatively regulate nodulation and interact with the AON pathway that systemically controls nodule number and is activated at the onset of primordium formation (Okamoto *et al.*, 2009; Mortier *et al.*, 2010; Reid *et al.*, 2011; Li *et al.*, 2009). As a result, ectopic expression of *MtCLE12* or *MtCLE13* ensues a systemic and *SUNN*-dependent inhibition of nodulation (Mortier *et al.*, 2010). Hence, the activation of both *MtCLE* genes might explain the observed inhibition of nodulation. Although this hypothesis is tempting, differences were observed between the nodulation phenotype of *MtLOG1* and *MtCLE12* or *MtCLE13* overexpressing roots. The effect of *35:MtLOG1* was milder compared to the effect of overexpression of either CLE peptide gene because, instead of a total inhibition, only a reduction in nodule number was observed in the case of *MtLOG1*. In addition, in contrast to what was observed for *MtCLE12* and *MtCLE13*, the effect of *35S:MtLOG1* was local and independent of *SUNN*. It is currently difficult to interpret these data, but it cannot be excluded that *MtCLE12* and/or

MtCLE13 exert *SUNN*-independent effects on nodulation, which might be invisible because they are overruled by *SUNN*-dependent effects.

In accordance with the upregulation of *MtCLE13* expression by the ectopic expression of *MtLOG1*, exogenous addition of cytokinins could induce *MtCLE13* (Mortier *et al.*, 2010). In contrast however, *MtCLE12* was not detected after cytokinin addition (Mortier *et al.*, 2010). Currently, no hypothesis can explain this observation, but it might have to do with the observation that *MtCLE12* is only expressed from a late stage of nodule primordium formation on, while *MtCLE13* is expressed very early on, at a stage at or even before the first cell divisions are observed (Mortier *et al.*, 2010). Together, although the upregulation of *MtCLE12* and *MtCLE13* might explain the inhibitory effect seen on nodulation of *MtLOG1* plants, many aspects should still be resolved. It will be interesting to control the localized expression of both *MtCLE* genes in the *35S:MtLOG1* plants. Moreover, the analysis of the *MtCLE12/MtCLE13* RNAi in *35S:MtLOG1* roots might reveal whether the CLE peptides play an active role in the *35S:MtLOG1* induced reduction of nodulation.

Analysis of the few nodules that did form on *35S:MtLOG1* transgenic roots revealed no or only a small meristem and a high level of senescence. Premature nodule senescence might be a consequence of an imbalance between cell division and differentiation leading to aberrant differentiation of infected cells and subsequently senescence. Also in *ENOD40* RNAi roots as well as in *MtCLE12/MtCLE13* RNAi lines a similar observation was made (Kumagai *et al.*, 2006). The link between meristem activity and senescence or the need for a coordinated differentiation between bacterial and plant cells might be an interesting subject to further study. Alternatively, *35S:MtLOG1* might have a direct effect on nodule senescence. In agreement, Coba de la Pena *et al.* (2008) reported the upregulation of a cytokinin receptor homolog in *Lupinus albus* during nodule senescence. The induction of nodule senescence by cytokinin activation is in contrast to the positive effect cytokinins have on leaf senescence (Wingler *et al.*, 1998; Van de Velde *et al.*, 2006). Because *MtLOG1* is expressed in the nodule apical zone, it is however more appealing that the senescence observed is a consequence of a modulation of the meristematic activities. It will be interesting to analyze whether *MtLOG1* might work together with the CLE peptides at the level of meristem activities rather than in the control of nodule numbers.

The *MtLOG* genes also contribute to the cytokinin effect on root development. In Arabidopsis, *LOG* genes were shown to negatively control lateral root emergence (Kuroha *et al.*, 2009). This is in agreement with observations that demonstrated the

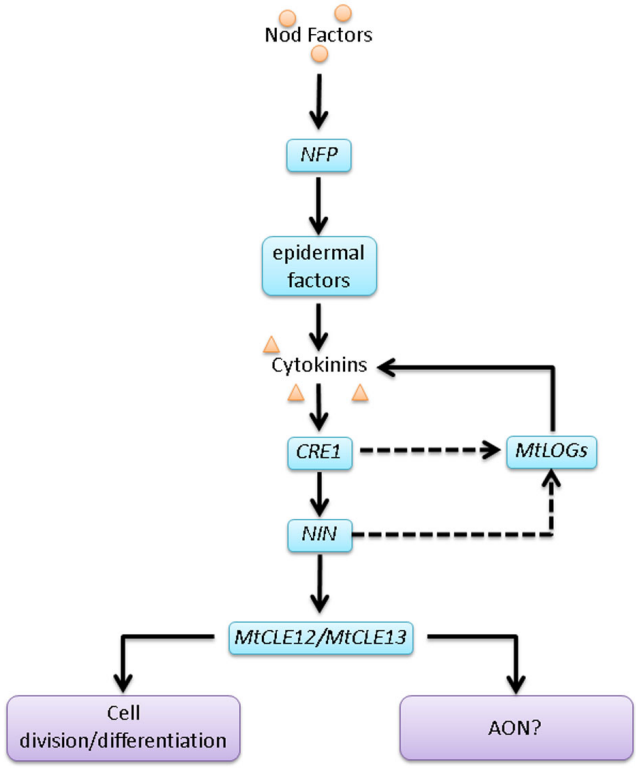


Figure 8.15: Overview of the involvement of *MtLOG* genes during early nodulation. Expression of *MtLOG* genes was shown to be located downstream of the component of the early NF signaling pathway, including epidermal factors and *MtCRE1*. In addition, histochemical analysis indicated initiation of *MtLOG1* expression after the first cell divisions for primordium development did take place. Hence, it is unlikely that the cytokinins released via *MtLOG* genes act as a primary cytokinin pool that is sensed by MtCRE1 to initiate nodule formation, but rather are involved in a positive feedback loop to maintain the cytokinin levels. Functional analysis of *MtLOG* genes by RNAi and ectopic overexpression analysis suggested a role during cell division and differentiation and possibly during AON. This might happen via CLE peptides, as ectopic expression of *MtLOG1* resulted in the ectopic expression of *MtCLE12* and *MtCLE13*.

negative effect of cytokinins on root growth and lateral root formation in many plant species, including *M. truncatula* (Gonzalez-Rizzo *et al.*, 2006; Bishopp *et al.*, 2009). *MtLOG1* might indeed account for the production of cytokinins that negatively regu-

late lateral root formation because *pMtLOG1:GUS* activity was detected in lateral root primordia and an elevated number of lateral roots was observed on *MtLOG1/MtLOG2* RNAi plants. Moreover, on *35S:MtLOG1* transgenic roots, a reduction in root length and lateral root number was observed indicative for a possible enhanced production of cytokinins that inhibit root growth. Yet, the length of the primary root in plants ectopically expressing a *AtLOG* gene was similar to the root length of control plants (Kuroha *et al.*, 2009), while here a reduction in root length was measured. Possibly, morphological or molecular differences between *A. thaliana* and *M. truncatula* but also between the activities of the LOG proteins might account for this discrepancy.

Previous experiments with the cytokinin receptor MtCRE1 suggested that signaling via this receptor might account for the negative role of cytokinin on root growth (Nishimura *et al.*, 2004; Lohar *et al.*, 2004; Gonzalez-Rizzo *et al.*, 2006). We confirm this as indeed more lateral roots were observed on *Mtcre1-1* mutants compared to wild type. However, the *MtLOG1/MtLOG2* RNAi constructs also resulted in an enhanced number of lateral roots on *Mtcre1-1* mutant plants indicating that other cytokinin receptors, apart from MtCRE1, might play a role. Also in Arabidopsis, multiple cytokinin receptors control the effect on lateral root growth (Nishimura *et al.*, 2004; Li *et al.*, 2006). Two other cytokinin receptors have been found in *M. truncatula*, it will be interesting to find the other receptors that contribute to the influence of cytokinins on lateral root growth.

Together, these results suggest that *de novo* synthesis of cytokinins via MtLOGs accounts for proper nodule primordium development and nodule meristem function (Figure 8.15). In addition, the same *MtLOG* genes might negatively regulate lateral root formation. Further studies will reveal the individual contributions of both MtLOG proteins studied here, but stable mutants will be needed for additional analyses. In addition, *MtLOG1* might be involved in the activation of the autoregulation pathway via the activation of two *MtCLE* genes that control nodule number. It is well known that the AON mechanism is activated when the first cell divisions appear in the cortex (Li *et al.*, 2009). However, further experiments are required to clearly demonstrate the potential link between cytokinin production and the AON mechanism.

8.4 Materials and Methods

Biological material

M. truncatula Gaertn. cv. Jemalong A17 and J5 as well as *nin-1, nsp1, nsp2, dmi1, dmi2, dmi3, Mtcre1-1* and *sunn-4* mutants (Catoira *et al.*, 2000; Oldroyd and Long, 2003; Marsh *et al.*, 2007; Middleton *et al.*, 2007; Schnabel *et al.*, 2005; Gonzalez-Rizzo *et al.*, 2006) were grown and inoculated as described (Mergaert *et al.*, 2003). *Sinorhizobium meliloti* 1021, *Sm1021* pHC60-GFP (Cheng and Walker, 1998), and *Sm2011* pBHR-mRFP (Smit *et al.*, 2005) were grown at 28°C in yeast extract broth medium (Vervliet *et al.*, 1975), supplemented with 10 mg $L-1$ tetracycline for the *Sm1021* pHC60-GFP and *Sm2011* pBHR-mRFP strains.

For RNAi analysis, the *S. meliloti* strain E65 was maintained on Bergensen's Modified Medium (BMM) (Rolfe *et al.*, 1980) containing 50 μM tetracycline, while the *Agrobacterium rhizogenes* ARqua1 strain (Boisson-Dernier *et al.*, 2001) was maintained on Tryptone Yeast (TY) medium containing 100 μg/ml streptomycin.

For *promoter:GUS* analysis, a 2-kb region upstream of *MtLOG1* was isolated from genomic DNA based on the available genomic data[28]. The promoters were fused to the *uidA* gene in pKm43GWRolDC1 (Karimi *et al.*, 2002). Primers used for amplification are presented in Table 8.1.

For the qRT-PCR analysis, *M. truncatula* J5 plants were grown *in vitro* in square Petri dishes (12 x 12 cm) on nitrogen-poor SOLi agar (Blondon, 1964). For the analysis of temporal expression during nodulation, nodules were harvested 4 to 10 dpi from plants grown in pouches, watered with nitrogen-poor SOLi medium, and inoculated with *Sm1021* pHC60-GFP. Infection threads were visible from 4 dpi on, nodule primordia at 6 dpi, and small nodules at 8 dpi. Two days later, at 10 dpi, slightly bigger nodules were observed. Tissue was collected by visualizing the green fluorescent bacteria under a stereomicroscope MZFLII (Leica Microsystems, Wetzlar, Germany) equipped with a blue-light source and a Leica GFP Plus filter set (λ_{ex} = 480/40; λ_{em} = 510 nm LP barrier filter). The zones I of uninoculated roots were isolated at the same developmental stage as the 4 dpi stage. For the analysis of *MtLOG1* expression in nodulation mutants, plants were grown in an aeroponic system. Half of the plants were harvested at the age of 6 days, while the other half of the plants was inoculated with *Sm2011* pBHR-mRFP and was harvested 7 days later. Chimeric plants bearing one

[28] `http://www.ncbi.nlm.nih.gov/`

of the following constructs, *35S:GUS, 35S:MtLOG1, 35S:MtCLE4, 35S:MtCLE12* or *35S:MtCLE13*, were placed in an aeroponic system at the age of 1 month, were grown under nitrogen-rich conditions and their roots were harvested 2 weeks later.

Table 8.1: Primers used in the analyses.

Gene	Sense primer	Anti-sense primer
Gateway cloning		
ORF *MtLOG1*	ATGGACTCACGCAATGGT	CTACCTTGAGATGTCATAATC
promoter *MtLOG1*	TCAGAAAGAGACGCCAAACA	CATTTGTTAGTTGGAATAAAAAGGTAA
RNAi *MtLOG1/MtLOG2*	TGGAGGAGGAAGCATT	GAGTTCCATATCCCCT
qRT-PCR amplification		
MtLOG1	AACAAATGGACTCACGCAATG	GGCAGCATCTTGGTAGGTAG
MtLOG2	CTGATTTCCCTTCTAAGTTGTAAG	TGAACGTAACCGCATGTATTG
MtCLE4	AATTTCACAAGTTCTGCTTCATCGC	TGGCACACCTCTCTTGTCTTCC
MtCLE12	CAACGTCTCTTGCATGAGTTAATGG	ACCTGGTGAAAGCCTATCTCCTG
MtCLE13	CCGAAGCCTTCTACAGAAACTACG	TCTTGGTGGTGATCTTCCATTATGC
40S	GCCATTGTCCAAGTTTGATGCTG	TTTTCCTACCAACTTCAAAACACCG
RNAi *MtLOG1/MtLOG2*	ACCAGGGGGATATGGAACTC	CTCTTTTGCAGTTGGTGCTG
GAPDH	TGCCTACCTCGATGTTTCAGT	TTGCCCTCTGATTCCTCCTTG

Generation of *LOG12* RNAi vector and transformation of *A. rhizogenes*

We used the pHellsgate8 RNAi vector. A 220 bp fragment of the *MtLOG1/MtLOG2* gene of *M. truncatula* was amplified with the primers listed in Table 8.1, which also inserted attb sites for cloning into the pDONOR vector. The inserts were verified by restriction digests. The verified construct was then transformed into the *A. rhizogenes* strain ARqua1 using the freeze-thaw transformation method of (Hofgen and Willmitzer, 1988). *M. truncatula* cv. Jemalong A17 seeds were scarified on sandpaper, sterilized in 6 % (w/v) sodium hypochlorite for 10 min and thoroughly rinsed in sterile water. Seeds were vernalized at 4°C overnight and germinated at 25°C in the dark. The transformation of *M. truncatula* with *A. rhizogenes* followed the technique of (Boisson-Dernier *et al.*, 2001) Transformation with empty pHellsgate8 vector was used as a negative control. Composite plantlets were grown on 15 cm diameter Petri dishes containing sloped modified Fahraeus (F) medium with Kanamycin (25 mg/l) (Boisson-Dernier *et al.*, 2001). Plantlets were grown at 20°C for 7 days, before being transferred to 25°C. A 16 h light period at 150 μE light intensity was used throughout. Plants were then transferred to plates containing F medium without Kanamycin and

incubated for one more week before inoculation with a fresh culture of *S. meliloti* E65 (OD600 = 0.2). Individual roots were marked on the plate and inoculated with 10 μL of bacterial culture. Plates were incubated for two weeks at 25°C with a 16 h light period at 150 μE light intensity. After two weeks, each of the marked roots was analyzed for nodule and lateral root numbers. Immediately afterwards, three lots of 8-10 roots for each treatment were excised, frozen in liquid nitrogen and used for RT-PCR.

RNA extraction, cDNA synthesis, and qRT-PCR analysis

Total RNA was isolated with the RNeasy Plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After a DNAse treatment, the samples were purified through NH_4Ac (5 M) precipitation, quality controlled, and quantified with a Nanodrop spectrophotometer (Isogen, Hackensack, NJ). RNA (2 μg) was used for cDNA synthesis with the Superscript Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA). The samples were diluted 50 times and stored at -20°C until further use. The qRT-PCR experiments were done on a LightCycler 480 (Roche Diagnostics, Brussels, Belgium) and SYBR Green was used for detection. All reactions were done in triplicate and averaged. The total reaction volume was 5 μl (2.5 μl master mix, 0.25 μl of each primer [5 μM] and 2 μl cDNA). Cycle threshold (C_T) values were obtained with the accompanying software and data were analyzed with the $2^{-\triangle\triangle C_t}$ (Livak and Schmittgen, 2001). The relative expression was normalized against the constitutively expressed 40S ribosomal S8 protein (TC100533, MGI). Primers used (Table 8.1) were unique in the MGI version 9.0 and the Medicago EST Navigation System database (Journet *et al.*, 2002). For RNAi analysis, data was analyzed in excel using a relative quantification method based on Pfaffl (2001) with GAPDH as the reference gene. Except for the RNAi analysis, each experiment was repeated at least two times with independent biological tissue.

Statistical analysis

The following generalized linear model (GLM) $Y_{ijk} = \ + genotype_j + experiment_k + error_{ijk}$ (Regression analysis) was fitted to the nodule number, lateral root number and lateral root density data, partitioning phenotypic variation into fixed genotype and experiment effects and random error effects. Y_{ijk} is the phenotype of the i-th plant from the genotype j analyzed in experiment k; μ is the overall mean of the phenotypes obtained for all lines considered. Because the data has a Poisson distribution, a logarithm

base e link function was incorporated. In the case of root length, an analysis of variance (ANOVA) was performed. All analyses were done by means of the GenStat software[29].

In vitro application of auxins, and cytokinins

Auxins (10^{-6} M IAA) and cytokinins (10^{-7} M BAP) were diluted in dimethylsulfoxide and supplemented to the medium of 5-day-old, *in vitro*-grown plants. As a control, plants were grown without supplemented hormones. The plants were cultured at 25°C, 16-h photoperiod, and 70 μE $m^{-2}m^{-1}$ light intensity per day. The roots were covered with aluminum foil for light protection. After 0, 3, 6, 12, and 24 h of incubation, the roots of 18 plants under each condition were harvested and analyzed by qRT-PCR. The experiment was repeated twice with comparable results.

A. rhizogenes-mediated transgenic root transformation

The protocol was adapted from Boisson-Dernier *et al.* (2001). Approximately 48 h after germination, the radicle was sectioned at 5 mm from the roottip with a sterile scalpel. Sectioned seedlings were infected by coating the freshly cut surface with the binary vector-containing *A. rhizogenes* Arqua1 strains. The *A. rhizogenes* strain was grown at 28°C for 2 days on solid yeast extract broth medium with the appropriate antibiotics (Quandt *et al.*, 1993). The infected seedlings were placed on agar (Kalys) containing the SOLi medium, supplemented with 1 mM NH_4NO_3, in square Petri dishes (12 $\times$ 12 cm) placed vertically for 5 days at 20°C, 16-h photoperiod, and 70 μE $m^{-2}m^{-1}$. Subsequently, plants were placed on the same medium between brown paper at 25°C and under identical light conditions. One and 2 weeks later, plants were screened for transgenic roots, characterized by GFP fluorescence with a stereomicroscope MZFLII (Leica Microsystems) equipped with a blue-light source and a Leica GFP plus filter set. One main transgenic root was retained per composite plant. Four weeks after infection, plants were transferred to an aeroponic system, pouches, or perlite-containing pots and incubated with SOLi medium. Three to 7 days after planting, composite plants were inoculated.

To check the long distance effect of *MtLOG1*, the main root was kept on the juvenile plant and infected by stabbing the hypocotyls with a fine needle containing an *A. rhizogenes* culture and cotransformed as described above, after which the plants were

[29] http://www.vsni.co.uk/software/genstat/

grown for 2 weeks at 25°C with a 16-h photoperiod and light at 70 μE m 2 s 1. After the plants had been transferred to an aeroponic system for 7 days, nodulation was analyzed on the main, untransformed root of plants bearing GFP-positive hairy roots.

Histochemical localization of GUS activity

GUS activity in cotransformed roots and nodules was analyzed using 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid as substrate (Van den Eede *et al.*, 1992). Roots and nodules were vacuum infiltrated during 20 min and subsequently incubated in GUS buffer at 37°C. Incubation lasted 4 h for *pMtLOG1-GUS*. After staining, roots and root nodules were fixed, dehydrated, embedded with the Technovit 7100 kit (Heraeus Kulzer, Wehrheim, Germany), according to the manufacturer's instructions, and sectioned with a microtome (Reichert-Jung, Nussloch, Germany). The 3-μm thick sections were mounted on coated slides (Sigma-Aldrich, St. Louis, MO). For tissue-specific staining, sections were submerged in a 0.05 % (w/v) ruthenium red solution (Sigma-Aldrich, St. Louis, MO), washed in distilled water, and dried. Finally, sections were mounted with Depex (BDH Chemicals, Poole, England). Photographs were taken with a Diaplan microscope equipped with bright- and dark-field optics (Leitz, Wetzlar, Germany).

Cytokinin measurements

Samples were devided into two technical replicates (100 mg). Cytokinins were extracted and separated, essentially as outlined by Novak *et al.* (2003). Frozen plant tissue (100 mg) was homogenized by vibration mill and extracted overnight at -20°C in 1 ml Bielski buffer enriched with stable isotope internal standards. Passing the extract, in sequence, through a cation (SCX-cartridge) and an anion [DEAE-Sephadex combined with an SPE(C18)cartridge] exchanger afforded fraction 1 containing the cytokinin free bases, ribosides, and glucosides, and fraction 2 containing the nucleotides. Both fractions were purified further by immunoaffinity extraction based on generic monoclonal anticytokinin antibodies, but fraction 2 was first treated with alkaline phosphatase. Quantification was done by isotope dilution method (UPLC-MS/MS) according to Novak *et al.* (2008).

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Authors contributions

The main part of this work was performed by V. Mortier. The RNAi analyses were done by U. Mathesius, while P. tarkowski performed the cytokinin measurements. S. Goormachtig and M. Holsters were involved in designing the research and revising the manuscript.

9

Search for nodulation related *CLE* genes in the genome of *Glycine max*

Virginie Mortier, Berhanu Amsalu Fenta, Cindy Martens, Stéphane Rombauts, Marcelle Holsters, Karl Kunert and Sofie Goormachtig

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Abstract
CLE peptides are potentially involved in nodule organ development and in autoregulation of nodulation (AON), a systemic process that restricts nodule number. A genome-wide survey of *CLE* peptide genes in the soybean genome resulted in the identification of 39 *GmCLE* genes, the majority of which were not annotated yet. qRT-PCR analysis indicated two different nodulation-related CLE expression patterns, one linked with nodule primordium development and a new one linked with nodule maturation. Moreover, two *GmCLE* gene pairs, encoding group-III CLE peptides which were previously shown to be involved in AON, had a transient expression pattern during nodule development, were induced by the essential nodulation hormone cytokinin and one pair was also slightly induced by addition of nitrate. Hence, our data support the hypothesis that group-III CLE peptides produced in the nodules are involved in primordium homeostasis and intertwined in activating AON, but not in sustaining it.

9.1 Introduction

Legumes can grow on nitrogen-poor soils by establishing a symbiosis with soil-borne bacteria called rhizobia. This symbiosis results in the formation of new root organs, the nodules, in which the bacteria fix nitrogen for the plant. In return, the microsymbiont receives carbon sources and a protective niche. The rhizobia-legume interaction is initiated by mutual recognition of molecular signals. Upon sensing flavonoids exuded by the roots of a compatible host, the rhizobia produce decorated lipochitooligosaccharides, the nodulation (Nod) factors (NFs) that are recognized by LysM receptor-like kinases (RLKs) (D'Haeze and Holsters, 2002). NF recognition activates two coordinated plant developmental programs: initiation of an infection process by which bacteria enter the host and simultaneously elicitation of cortical and pericycle cell division, resulting in the nodule organ. When infection threads reach the cells of the nodule primordium, the bacteria are released into the symbiosomes to fix nitrogen. Two main nodule types are observed. Determinate nodules, with *Lotus japonicus* as the model legume, are initiated in the outer cortex. Early in development, the primordium cells cease to divide and nodule enlargement is mainly due to cell expansion, resulting in spherical, mature nodules. Indeterminate nodules, for which *Medicago truncatula* (barrel medic) is the model, arise from inner cortical cell division. Some cells of the

primordium will become meristematic and will form a persistent apical meristem (Patriarca *et al.*, 2004; Crespi and Frugier, 2008). Downstream from the NFs, nodule primordium formation depends on cytokinin signaling (Frugier *et al.*, 2008; Oldroyd and Downie, 2008), as demonstrated by knockout mutants for the cytokinin receptor gene *LHK1* in *L. japonicus* or by transgenic *M. truncatula* plants with reduced expression of the ortholog *CRE1* that were defective in nodule primordia formation (Gonzalez-Rizzo *et al.*, 2006; Murray *et al.*, 2007). Additionally, the *L. japonicus snf2* gain-of-function mutant for the LHK1 receptor provoked spontaneous nodules, indicating that cytokinin signaling is both necessary and sufficient for nodule formation (Tirichine *et al.*, 2007). Also auxin flow and signaling are important factors for primordium formation (Mathesius *et al.*, 1998; Boot *et al.*, 1999; Pacios-Bras *et al.*, 2003; van Noorden *et al.*, 2006; Wasson *et al.*, 2006). Recently, a group of CLAVATA3 (CLV3)/ESR-RELATED (CLE) peptides has been investigated for their role in nodulation (Okamoto *et al.*, 2009; Hirakawa *et al.*, 2010a; Mortier *et al.*, 2010). CLE peptides are small (12-13 amino acids) secreted peptides derived from the C-terminal region of pre-proproteins (Mitchum *et al.*, 2008; Oelkers *et al.*, 2008). The *Arabidopsis thaliana* genome contains 32 family members that are involved in balancing proliferation and differentiation during plant development. For instance, in Arabidopsis, CLV3 signaling via the RLK CLAVATA1 (CLV1) is essential to maintain stem cell homeostasis at the shoot apical meristem (SAM). Ectopic expression of *CLV3* resulted in the disappearance of the SAM, while *clv3* mutants enhanced SAM proliferation (Clark *et al.*, 1997; Fletcher *et al.*, 1999; Brand *et al.*, 2000; Ogawa *et al.*, 2008). Another well-known example is the CLE41-PHLOEM INTERCALATED WITH XYLEM (PXY) ligand receptor pair that regulates xylem differentiation, and the rate and orientation of vascular cell division (Ito *et al.*, 2006; Hirakawa *et al.*, 2008; Whitford *et al.*, 2008; Etchells and Turner, 2010). CLE peptides with related sequences exhibit functional redundancy (Strabala *et al.*, 2006; Jun *et al.*, 2008). Gain-of-function analysis divided the Arabidopsis CLE peptides in at least three groups. Group-I peptides, exemplified by CLV3, arrest premature root and shoot meristem growth when exogenously applied or ectopically produced, indicating that they promote cellular differentiation. Group-II members, exemplified by CLE41, prevent cellular differentiation. No clear function has been described yet for the group-III peptides, comprising the CLE1 to CLE7 peptides (Ito *et al.*, 2006; Strabala *et al.*, 2006; Etchells and Turner, 2010). In *L. japonicus* and *M. truncatula*, three (*LjCLE-RS1*, *LjCLE-RS2* and *LjCLE3*) and two (*MtCLE12* and *MtCLE13*) *CLE* genes, respectively, are upregulated specifically dur-

ing nodulation. The CLE domain of LjCLE-RS1 and LjCLE-RS2 and MtCLE12 and MtCLE13 is highly similar, indicating that these group-III peptides might exert comparable functions (Okamoto *et al.*, 2009; Mortier *et al.*, 2010). In *M. truncatula*, the *MtCLE12* and *MtCLE13* expression patterns suggest a role in primordium and apical meristem homeostasis (Mortier *et al.*, 2010), while functional analysis revealed that LjCLE-RS1, LjCLE-RS2, MtCLE12, MtCLE13 derived CLE peptides might be implicated in auto-regulation of nodulation (AON) (Okamoto *et al.*, 2009; Mortier *et al.*, 2010). Long-distance AON signaling controls the nodule number to avoid excess of nitrogen sources that would be deleterious for plant growth (Nutman, 1952). Insight into the process of AON was gained by the isolation of mutants affected in a leucine-rich repeat (LRR)-RLK, designated 'nodule autoregulation receptor kinase' (NARK) or 'nitrogen-tolerant symbiosis 1' (NTS1) in soybean, 'hypernodulation aberrant root formation' (HAR1) in *L. japonicus*,'symbiosis 29' (SYM29) in pea, and 'super numeric nodules' (SUNN) in *M. truncatula*. They all have a super-nodulation phenotype and exhibit a nitrate-tolerant nodulation, suggesting that the negative control exerted by nitrate on nodulation might happen via the same process (Pierce and Bauer, 1983; Carroll *et al.*, 1985a,b; Wopereis *et al.*, 2000; Oka-Kira *et al.*, 2005; Barbulova *et al.*, 2007; Magori and Kawaguchi, 2009; Kosslak and Bohlool, 1984; Duc and Messager, 1989; Krusell *et al.*, 2002; Nishimura *et al.*, 2002a; Searle *et al.*, 2003; Schnabel *et al.*, 2005, 2010). Grafting experiments have shown that this AON LRR-RLK is active in the shoot (Nishimura *et al.*, 2002a; Searle *et al.*, 2003) and leads to a return signal that is translocated to the roots, to inhibit further nodulation (Nishimura *et al.*, 2002a; Lin *et al.*, 2010). The nature of the AON signaling molecules is still elusive. AON is activated upon NF signaling at the onset of or during cortical cell division (Mathews *et al.*, 1989b; Caetano-Anolles and Gresshoff, 1991; Sagan and Gresshoff, 1996; Suzuki *et al.*, 2008; Li *et al.*, 2009) and, in pea and *L. japonicus* become stronger as the nodules matured (Suzuki *et al.*, 2008; Li *et al.*, 2009). SUNN and its orthologs might perceive CLE peptides to provoke AON, because they are phylogenetically related to many known and putative CLE receptors (Shiu and Bleecker, 2001; Okamoto *et al.*, 2009). The LjCLE-RS1, LjCLE-RS2, MtCLE12, and MtCLE13 peptides are good candidates for triggering AON. Indeed, ectopic expression of the corresponding genes strongly reduced or abolished nodulation locally and systemically in a *HAR1* and *SUNN* dependent way, in *L. japonicus* and *M. truncatula*, respectively (Okamoto *et al.*, 2009; Mortier *et al.*, 2010). Importantly, inhibition of nodulation was specific for overexpression of *LjCLE-RS1*, *LjCLE-RS2*, *MtCLE12*, and *MtCLE13* and ectopic

expression of *CLE* genes with a structurally unrelated CLE domain did not induce this effect (Okamoto *et al.*, 2009; Mortier *et al.*, 2010). So far, however, it is not proven that the peptides derived from these genes act as long-distance signals travelling from the developing nodules to the shoot, where SUNN and its orthologs are active.

To gain more insight into the function of CLE peptides in nodulation, the *CLE* gene expression was analyzed during the development of determinate soybean nodules. Specialized searches predicted 24 peptide genes in the genome of soybean on top of the 15 previously identified (Oelkers *et al.*, 2008). Expression was assayed in various tissues, including developing and mature nodules, after application of various concentrations of nitrate and of cytokinin and auxin. Several *GmCLE* genes were found of which the expression was upregulated during nodulation. For six *GmCLE* genes, encoding group-I CLE peptides and divided in three gene pairs, the expression increased steadily during nodulation. Two pairs of CLE genes encoded group-III GmCLE peptides. These genes, as well as one group-I *GmCLE* gene, were expressed at high levels in developing, but not in mature, nodules. The group-III soybean genes were induced by cytokinin. These data support the hypothesis that group-III CLE peptides produced in the nodules are involved in primordium homeostasis. These peptides might also activate AON, but not sustain it because genes encoding this group of peptides were absent in mature nodules.

9.2 Results

9.2.1 *In silico* identification of *GmCLE* genes

Because CLE pre-proproteins are short and the conserved CLE domain is only 12 amino acids (AAs) long, neither BLAST nor phylogeny could be reliably applied to identify the genes. For that reason, to search for *GmCLE* genes, a pipeline (S. Rombauts and Y Van de Peer, unpublished data) was used based on the HMMer software, which is more sensitive and specific than BLAST or PSI-BLAST (Eddy, 2009). As a first step, Hidden Markov Models (HMMs) were constructed that were derived from a multiple alignment, made with MUSCLE (Edgar, 2004), of all known *M. truncatula* CLE proteins. All conserved regions in the alignment (domains) from 6 AAs onwards were taken into consideration. Subsequently, an orfeome was constructed from the whole soybean genome and combined with the known *M. truncatula* CLE peptides was screened with the obtained HMMs. The scores for each HMM, received from the HMMer software, were normalized in function of the length of each individual domain

allowing domains to contribute equally. The final scores were ordered in vectors per gene and stored in a matrix. By applying hierarchical clustering (Cluster 3.0) on the matrix, genes with highly correlated vectors were grouped together. The *M. truncatula* CLE peptides included in the analysis pointed to the clusters of interest. The soybean genes that clustered together with known *M. truncatula CLE* genes were taken as primary candidates. In total, 39 GmCLE proteins were identified, of which 15 had been described previously, but none of them had been annotated in the soybean genome (Oelkers *et al.*, 2008) (Table 9.1). All hits were annotated and made available at NCBI (Table 9.1). Due to the duplicated genome of soybean, 17 pairs of highly (at least 85 %) homologous sequences were found.

The corresponding GmCLE pre-proproteins varied in length between 49 AAs and 131 AAs and show a high level of sequence divergence outside the CLE motif (Table 9.1). Except for GmCLE13, all proteins had an N-terminal signal peptide or signal anchor as predicted by HMM signalP and neural networks (Bendtsen et al., 2004) (Table 9.1). Moreover, *GmCLE13* was the only gene bearing an intron (Table 9.1). The *GmCLE* genes were scattered throughout the chromosomes, except for chromosome 4 and 16 on which no *GmCLE* genes were identified (Table 9.1). A tree-based alignment was done with the CLE domain encoded by all soybean and Arabidopsis *CLE* genes as well as the nodulation-specific *CLE* genes of *M. truncatula* and *L. japonicus*. Twenty-nine *GmCLE* genes encoded CLE peptides designated as group-I and exemplified by *CLV3* (Figure 9.1). Six *GmCLE* genes encoded peptides identical to TDIF/CLE41/CLE44 and were designated as group-II. Four *GmCLE* genes (*GmCLE14, GmCLE35, GmCLE37*, and *GmCLE39*), encoded peptides that are most homologous to the peptides of the nodulation-specific genes *MtCLE12, MtCLE13, LjCLE-RS1*, and *LjCLE-RS2* and to Arabidopsis *CLE1* to *CLE7*. This group was named group-III.

To facilitate the comparison of the AAs between CLE domains, they were numbered as described by Oelkers *et al.* (2008) with the zero position assigned to the conserved glycine (G) residue located at the center of the CLE motif and the positions of the other AAs numbered relative to this G. The peptide sequence derived from the pair GmCLE35-GmCLE37 was very similar to that of the nodulation-related CLE peptides, LjCLE-RS1/LjCLE-RS2 and MtCLE13 (Figure 9.1). The sequence only differed at AA positions -3 (A $\leftrightarrow$ S) with LjCLE-RS1/LjCLE-RS2 and -1 (G $\leftrightarrow$ A) and -3 (A $\leftrightarrow$ S) with MtCLE13 (Okamoto *et al.*, 2009; Mortier *et al.*, 2010). In contrast, the CLE domain of *GmCLE14-GmCLE39*, which belongs to the same group-III peptides, differed at least at three AA positions with the other group members. The conserved

Table 9.1: Overview of the GmCLE peptide genes, the derived CLE domain sequences, identification number, homologous partner, protein length, chromosome number, intron presence and signal peptide/anchor probability as predicted by HMM signalP and neural networks. *G. max* nomenclature as defined in this article. General nomenclature is according to Oelkers *et al.* (2008), who numbered each CLE member independently of the species origin and prefixed the numbers with *CLE*.

G. max nomenclature	General nomenclature	CLE domain sequence	Identification number	Homologous partner	Length (AA)	Chromosome number	Intron	Signal peptide probability	Signal anchor probability
GmCLE01	*CLE23*	RRVPTGSNPLHN	HM585099	*GmCLE22*	71	14	No	1	0
GmCLE02	*CLE34*	RRVPNGPDPIHN	HM585100	*GmCLE27*	131	1	No	0.864	0.135
GmCLE03	*CLE51*	HEVPSGPNPISN	HM585101	*GmCLE31*	113	8	No	0.99	0.009
GmCLE04	*CLE52*	RRVPTGPNPLHH	HM585102	*GmCLE24*	111	20	No	0.104	0.89
GmCLE05	*CLE53*	HEVPSGPNPISN	HM585103	*GmCLE26*	123	18	No	0.021	0.968
GmCLE06	*CLE54*	RKVYTGPNPLHN	HM585104	*GmCLE38*	94	19	No	0.998	0.002
GmCLE07	*CLE55*	RRVPSCPDPLHN	HM585105	*GmCLE30*	97	20	No	0.968	0.031
GmCLE08	*CLE56*	RIIHTGPNPLHN	HM585106	*GmCLE28*	114	20	No	0.001	0.999
GmCLE09	*CLE57*	TATPGGPNPLHN	HM585107		86	9	No	0.968	0.032
GmCLE10	*CLE58*	RLVPSGPNPLHN	HM585108		86	10	No	0.985	0.014
GmCLE11	*CLE59*	RKVPNASDPLHN	HM585109	*GmCLE34*	82	14	No	0.265	0.71
GmCLE12	*CLE60*	HEVPSGPNPISN	HM585110	*GmCLE29*	127	15	No	0.999	0.001
GmCLE13	*CLE61*	REVPTGPDPLHH	HM585111		49	12	Yes	0	0
GmCLE14	*CLE62*	RLAPEGPDPHHN	HM585112	*GmCLE39*	95	13	No	0.974	0.025
GmCLE15		RDVPGGPNPLHN	HM585113	*GmCLE36*	87	5	No	0.995	0.005
GmCLE16		RGVPSGANPLHN	HM585114	*GmCLE33*	67	11	No	0.968	0.032
GmCLE17		REVPSSPDPLHN	HM585115	*GmCLE32*	76	20	No	0.997	0.003
GmCLE18		RIIYTGPNPLHN	HM585116	*GmCLE23*	90	2	No	0.745	0.254
GmCLE19		RLVPSGPNPLHN	HM585117		107	1	No	0.992	0.008
GmCLE20		RLVPTGPNPLHH	HM585118	*GmCLE21*	114	17	No	0.268	0.732
GmCLE21		RLVPTGPNPLHH	HM585119	*GmCLE20*	118	5	No	0.859	0.141
GmCLE22		RRVPTGSNPLHN	HM585120	*GmCLE01*	73	2	No	0.999	0
GmCLE23		RIIYTGPNPLHN	HM585121	*GmCLE18*	89	14	No	1	0
GmCLE24		RRVPTGPNPLHH	HM585122	*GmCLE04*	110	10	No	0.168	0.817
GmCLE25		RRVPTGPNPLHN	HM585123		99	20	No	0.917	0.079
GmCLE26		HEVPSGPNPISN	HM585124	*GmCLE05*	123	8	No	0.018	0.969
GmCLE27		RRVPNGPDPIHN	HM585125	*GmCLE02*	115	2	No	0.959	0.041
GmCLE28		RIIHTGPNPLHN	HM585126	*GmCLE08*	119	7	No	0.001	0.999
GmCLE29		HEVPSGPNPISN	HM585127	*GmCLE12*	125	9	No	0.995	0.004
GmCLE30		RRVPSCPDPLHN	HM585128	*GmCLE07*	96	10	No	0.954	0.045
GmCLE31		HEVPSGPNPISN	HM585129	*GmCLE03*	113	5	No	0.993	0.006
GmCLE32		REVPSSPDPLHN	HM585130	*GmCLE17*	74	10	No	0.999	0.001
GmCLE33		RGVPSGANPLHN	HM585131	*GmCLE16*	67	1	No	0.983	0.016
GmCLE34		RKVPNASDPLHN	HM585132	*GmCLE11*	84	17	No	0.267	0.708
GmCLE35		RLAPGGPDPQHN	HM585133	*GmCLE37*	93	6	No	0.977	0.018
GmCLE36	*CLE63*	RDVPGGPNPLHN	HM585134	*GmCLE15*	87	8	No	0.971	0.027
GmCLE37		RLAPGGPDPQHN	HM585135	*GmCLE35*	94	12	No	0.981	0.018
GmCLE38		RKVYTGPNPLHN	HM585136	*GmCLE06*	100	3	No	0.981	0.019
GmCLE39		RLTPEGPDPHHN	HM585137	*GmCLE14*	96	12	No	0.957	0.041

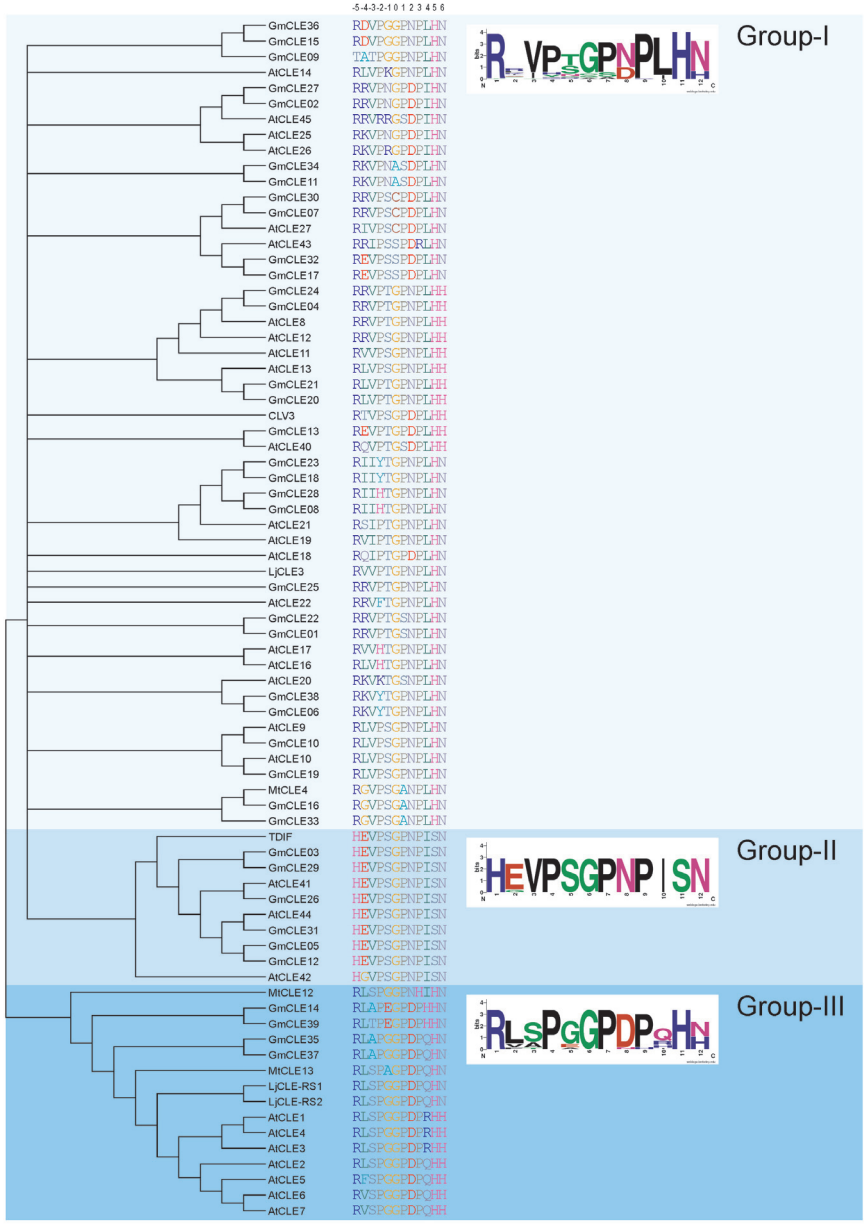
pattern of the residues in each group is shown in a WebLogo representation (Figure 9.1).

9.2.2 Identification of two stages in soybean nodule development with different division and differentiation activities

To identify nodule-related *CLE* genes with expression patterns that are linked with cell division and differentiation, two soybean nodulation stages were analyzed of which one was linked with dividing and differentiating cells and the other corresponded to mature, fully differentiated nodules. At 2 wpi, many small dividing cells were observed at the periphery of a central section through the nodule (Figure 9.2, A and B). More to the centre, cells differentiated in fixing cells with many infection threads in-between the small cells (Figure 9.2, A and C). Enlargement showed that many cells in that region were partially filled with symbiosomes, indicating that differentiation is in progress. At 4 wpi, a typical nitrogen fixation tissue was observed consisting of large, infected cells that were completely filled with blue-stained symbiosomes (Figure 9.2, E and F), and interspersed by vacuolated, uninfected cells (Figure 9.2, E and F). Cell division or differentiation was no longer observed.

To confirm the difference in cell division activity between the two nodulation stages, the expression of the cell division marker, the B-type cyclin *CycB2;1*, was analyzed (Umeda *et al.*, 1999; Lee *et al.*, 2003). With BLAST searches, a soybean homolog of the Arabidopsis *AtCycB2;1* was found and was designated *GmCycB2;1*. qRT-PCR was carried out with cDNA derived from both nodulation stages and from uninoculated roots used as a reference tissue. The relative expression of *GmCycB2;1* was higher in the 2-wpi nodule sample than in uninoculated roots (Figure 9.3). At 4 wpi, expression was strongly reduced and was even much lower than in uninoculated roots. These results indicate that cell division was high in nodules at 2 wpi and that no cell divisions occurred in mature determinate soybean nodules.

Figure 9.1 ***(facing page)*****:** Tree-based alignment of the CLE domain encoded by all *GmCLE* genes and of the CLE domain of all Arabidopsis *CLE* genes as well as the nodulation-specific *CLE* genes.


-5 -4 -3 -2 -1 0 1 2 3 4 5 6
GmCLE36 RDVPGGPNPLHN
GmCLE15 RDVPGGPNPLHN
GmCLE09 TATPGGPNPLHN
AtCLE14 RLVPKGPNPLHN
GmCLE27 RRVPNGPDPIHN
GmCLE02 RRVPNGPDPIHN
AtCLE45 RRVRRGSDPIHN
AtCLE25 RKVPNGPDPIHN
AtCLE26 RKVPRGPDPIHN
GmCLE34 RKVPNASDPLHN
GmCLE11 RKVPNASDPLHN
GmCLE30 RRVPSCPDPLHN
GmCLE07 RRVPSCPDPLHN
AtCLE27 RIVPSCPDPLHN
AtCLE43 RRIPSSPDRLHN
GmCLE32 REVPSSPDPLHN
GmCLE17 REVPSSPDPLHN
GmCLE24 RRVPTGPNPLHH
GmCLE04 RRVPTGPNPLHH
AtCLE8 RRVPTGPNPLHH
AtCLE12 RRVPSGPNPLHH
AtCLE11 RVVPSGPNPLHH
AtCLE13 RLVPSGPNPLHH
GmCLE21 RLVPTGPNPLHH
GmCLE20 RLVPTGPNPLHH
CLV3 RIVPSGPDPLHH
GmCLE13 REVPTGPDPLHH
AtCLE40 RQVPTGSDPLHH
GmCLE23 RIIYTGPNPLHN
GmCLE18 RIIYTGPNPLHN
GmCLE28 RIIHTGPNPLHN
GmCLE08 RIIHTGPNPLHN
AtCLE21 RSIPTGPNPLHN
AtCLE19 RVIPTGPNPLHN
AtCLE18 RQIPTGPDPLHN
LjCLE3 RVVPTGPNPLHN
GmCLE25 RRVPTGPNPLHN
AtCLE22 RRVFTGPNPLHN
GmCLE22 RRVPTGSNPLHN
GmCLE01 RRVPTGSNPLHN
AtCLE17 RVVHTGPNPLHN
AtCLE16 RLVHTGPNPLHN
AtCLE20 RKVKTGSNPLHN
GmCLE38 RKVYTGPNPLHN
GmCLE06 RKVYTGPNPLHN
AtCLE9 RLVPSGPNPLHN
GmCLE10 RLVPSGPNPLHN
AtCLE10 RLVPSGPNPLHN
GmCLE19 RLVPSGPNPLHN
MtCLE4 RGVPSGANPLHN
GmCLE16 RGVPSGANPLHN
GmCLE33 RGVPSGANPLHN
TDIF HEVPSGPNPISN
GmCLE03 HEVPSGPNPISN
GmCLE29 HEVPSGPNPISN
AtCLE41 HEVPSGPNPISN
GmCLE26 HEVPSGPNPISN
AtCLE44 HEVPSGPNPISN
GmCLE31 HEVPSGPNPISN
GmCLE05 HEVPSGPNPISN
GmCLE12 HEVPSGPNPISN
AtCLE42 HGVPSGPNPISN
MtCLE12 RLSPGGPNHIHN
GmCLE14 RLAPEGPDPMHN
GmCLE39 RLTPEGPDPMHN
GmCLE35 RLAPGGPDPQHN
GmCLE37 RLAPGGPDPQHN
MtCLE13 RLSPAGPDPQHN
LjCLE-RS1 RLSPGGPDPQHN
LjCLE-RS2 RLSPGGPDPQHN
AtCLE1 RLSPGGPDPRHH
AtCLE4 RLSPGGPDPRHH
AtCLE3 RLSPGGPDPRHH
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AtCLE7 RVSPGGPDPQHH
Group-I
Group-II
Group-III

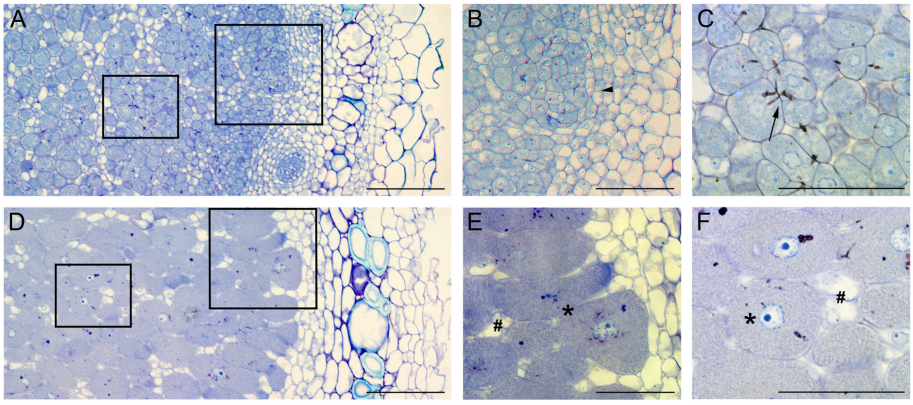

Figure 9.2: Microscopic analysis of nodules at 2 and 4 wpi. A, Section through a nodule of 2 wpi. B, Detail of large square indicated in (A). Small dividing cells are indicated by an arrowhead. C, Detail of small square indicated in (A). Differentiation into nitrogen-fixing cells is observed by the presence of many infection threads (arrow). D, Section through a nodule of 4 wpi. E and F, Details of (D). Nitrogen fixation zone consisting of large infected cells that are totally filled with symbiosomes (asterisks), interspersed by vacuolated uninfected cells (hashes). No signs of cell division or differentiation are visible anymore. Sections were stained with toluidine blue. Bars = 100 μm (A and D) and 50 μm (B, C, E, and F).

9.2.3 Search for *GmCLE* genes that are differentially expressed during nodulation

To determine the temporal expression during nodulation, qRT-PCR was carried out with cDNA derived from the nodulation-susceptible zone of non-inoculated roots and from nodules at 2 and 4 wpi. The non-inoculated roots were used as reference tissue. With the primer 'Beacon designer 7' program, no primers could be designed that discriminated between the two highly homologous genes of the different *GmCLE* gene pairs. Therefore, primer combinations were used that recognized transcripts of both genes of a single pair. Except for *GmCLE25*, all other 38 *GmCLE* genes were expressed. For 8 primer combinations, no differential expression was observed upon nodulation (see Supplementary Table 9.4). Compared to the expression in uninfected roots, the transcript level of *GmCLE06-GmCLE38, GmCLE14-GmCLE39* and *GmCLE35-GmCLE37* increased in nodules at 2 wpi, but decreased again in nodules at 4 wpi (Figure 9.4, A-C). The transcript level of *GmCLE11-GmCLE34, GmCLE13* and

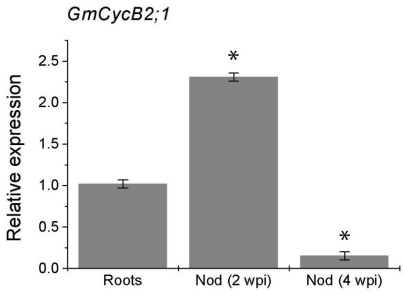

Figure 9.3: Expression analysis of *GmCycB2;1* during nodulation. qRT-PCR on cDNA samples of uninoculated roots (Roots) and of nodules (Nod) at 2 and 4 wpi. Error bars represent standard errors (n = 2). Asterisks indicate statistically significant differences in comparison to uninoculated roots (Roots) ($p < 0.001$, Anova).

GmCLE17-GmCLE32 steadily increased as nodulation progressed and was the highest in mature 4-wpi nodules (Figure 9.4, D-F). Expression of *GmCLE04-GmCLE24, GmCLE09, GmCLE12-GmCLE29, GmCLE15-GmCLE36, GmCLE16-GmCLE33, GmCLE18-GmCLE23* and *GmCLE20-GmCLE21* was much lower in nodules at 2 wpi and 4 wpi than in uninoculated roots (see Supplementary Table 9.4).

9.2.4 Tissue- or organ-specific expression of nodulation-related *CLE* peptide genes

GmCLE genes, for which the expression was upregulated upon nodulation, were also investigated for expression in other tissues or organs. qRT-PCR analysis was carried out with cDNA derived from roots, roottips, stems, SAMs, cotyledons, mature leaves, and first leaves. Expression measured in roots grown without nitrogen was taken as a reference. A basal expression was observed for every *GmCLE* gene in most of the cDNA samples (Figure 9.5; Supplementary Table 9.5). The expression of *GmCLE06-GmCLE38* and *GmCLE13* was higher in the different shoot tissues than in nitrogen-starved roots. Expression of *GmCLE14-GmCLE39* and *GmCLE35-GmCLE37*, both transiently expressed upon nodulation, was higher in roots than in the different shoot tissues (Figure 9.5; Supplementary Table 9.5).

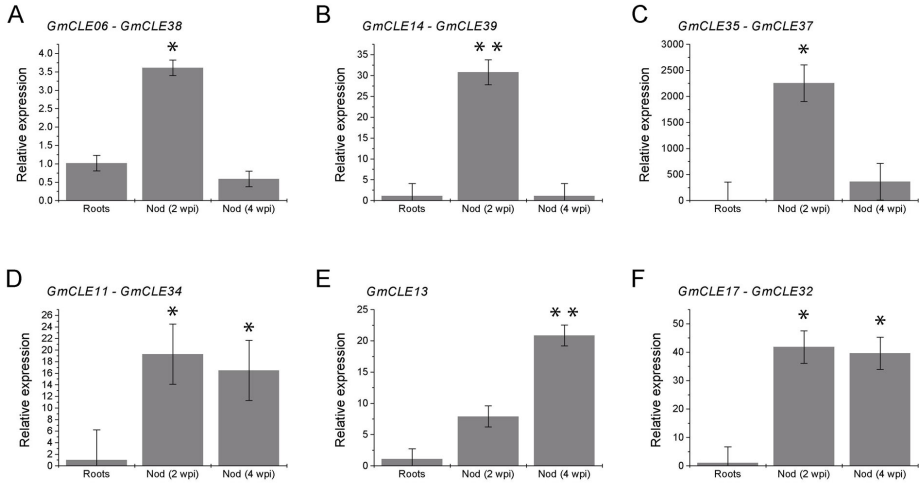

Figure 9.4: Expression analysis of *GmCLE* genes during nodulation. qRT-PCR on cDNA samples of uninoculated roots (Roots) and of nodules (Nod) at 2 and 4 wpi. Error bars represent standard errors (n = 2). Statistically significant differences in comparison to uninoculated roots (Roots) are indicated with * ($p < 0.05$, Anova) or ** ($p < 0.01$, Anova).

9.2.5 Induction of nodulation-related *GmCLE* genes by the addition of auxin or cytokinin

To see whether the expression of nodulation-related *GmCLE* genes was linked with primordium formation, their expression was analyzed after addition of either auxins or cytokinins, two hormones that control nodule organ formation (Oldroyd and Downie, 2008; Ding and Oldroyd, 2009). Expression of the nodulation-related *GmCLE* genes was assayed in roots of 5-day-old soybean seedlings grown in the presence of 10^{-6} M IAA or 10^{-7} M BAP and roots were harvested at 0, 4, 8, and 24 h after treatment. Addition of auxin had no influence on any of the tested genes (see Supplementary Table 9.6; Figure 9.6). In samples supplemented with 10^{-7} M BAP, *GmCLE14-GmCLE39* and *GmCLE35-GmCLE37* transcripts were upregulated 24 h after treatment (Figure 9.6, A and B). The expression of the other nodulation-related *GmCLE* genes did not change after BAP treatment (see Supplementary Table 9.6).

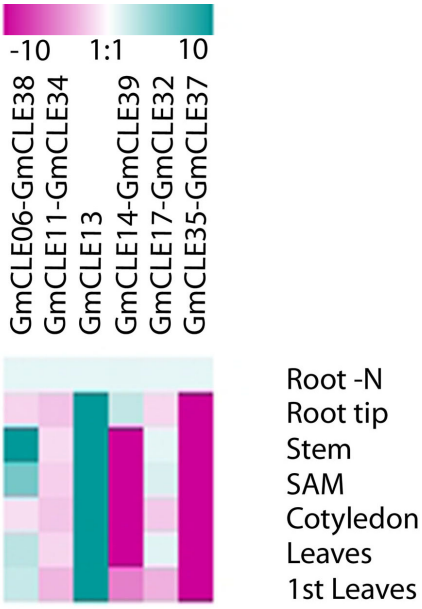

Figure 9.5: Tissue- or organ-specific expression analysis of nodulation-related *GmCLE* genes. Heat map of *GmCLE* expression in different tissues as measured by qRT-PCR. Samples are cDNA from roots grown in the absence of NH_4NO_3 (Root -N), roottips, stems, SAMs, cotyledons, mature leaves (leaves) and first leaves (1st leaves).

9.2.6 Influence of nitrate on the expression of the nodulation-related *GmCLE* genes

Nitrogen starvation is a prerequisite for nodulation and high nitrate availability negatively regulates nodulation (Streeter and Wong, 1988; Barbulova *et al.*, 2007). The influence of nitrate on nodulation has been proposed to happen via the AON mechanism because nodulation of mutants affected in AON is nitrate tolerant (Pierce and Bauer, 1983; Carroll *et al.*, 1985a,b; Wopereis *et al.*, 2000; Oka-Kira *et al.*, 2005; Barbulova *et al.*, 2007; Magori and Kawaguchi, 2009). To analyze whether nitrate has an influence on the expression of nodulation-related *GmCLE* genes, soybean seedlings were grown for 6 days in the presence of 0, 1, 5 or 10 mM KNO_3. In both biological repeats, the expression of *GmCLE14-GmCLE39* increased after addition of 10 mM KNO_3 (see

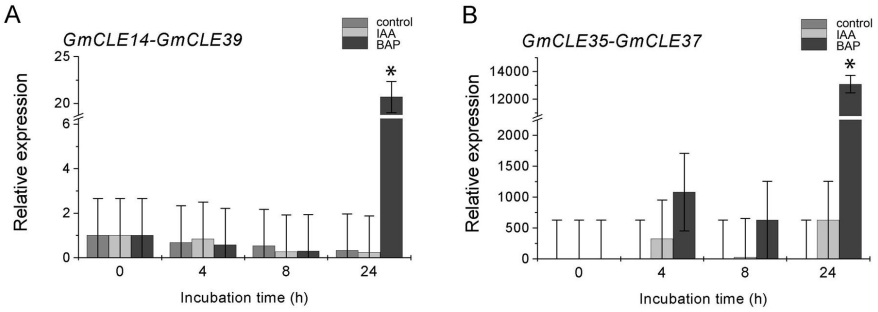

Figure 9.6: Influence of auxin and cytokinin on *GmCLE14-GmCLE39* and *GmCLE35-GmCLE37* expression. qRT-PCR analysis of *GmCLE14-GmCLE39* and *GmCLE35-GmCLE37* expression on cDNA samples of roots grown in the presence of 10^{-6} M auxin (IAA) or 10^{-7} M cytokinin (BAP). Growth medium without hormones was used for the control plants. Samples of 5-day-old plants were taken at 0, 4, 8, and 24 h after hormone addition. Error bars represent standard errors (n = 2). Asterisks indicate statistically significant differences in comparison to control plants, grown without hormone addition ($p < 0.001$, Anova).

Supplementary Table 9.7). However, due to the variation in the level of upregulation between both repeats, these data were not proven to be statistically different (Figure 9.7; see Supplementary Table 9.7). Also for the other genes no statistical difference in gene expression level was seen between control roots and roots treated with the various concentrations of nitrate (see Supplementary Table 9.7).

9.3 Discussion

The previously identified *L. japonicus LjCLE-RS1/LjCLE-RS2* and the *M. truncatula MtCLE12* and *MtCLE13* genes encode structurally related CLE peptides that are involved in nodulation (Okamoto *et al.*, 2009; Mortier *et al.*, 2010). While tissue specific expression patterns hinted at a role in nodule primordium and meristem homeostasis, functional analysis revealed a role during AON (Okamoto *et al.*, 2009; Mortier *et al.*, 2010). To get a better insight into the role of CLE peptides in nodulation, the expression of CLE peptide genes was examined during determinate nodule development in soybean, so far, the only legume for which the complete genome sequence is available (Schmutz *et al.*, 2010). By specialized searches, 39 *CLE* genes were identified, 34 of which form homologous pairs, as a result of the duplicated genome (Schmutz *et al.*,

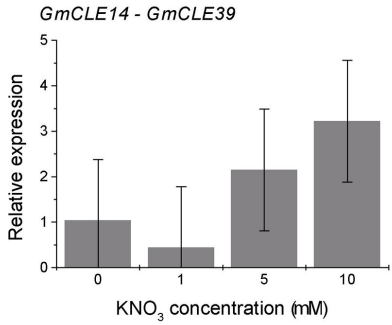

Figure 9.7: Influence of KNO_3 on *GmCLE14-GmCLE39* expression. qRT-PCR analysis of *GmCLE14-GmCLE39* expression in cDNA samples of roots grown for 6 days in the presence of 0, 1, 5 and 10 mM KNO_3. No statistical differences were measured as compared to the plants grown in the absence of KNO_3 ($p > 0.05$, Anova). Error bars represent standard errors (n = 2).

2010). Although 15 of these had been previously identified (Oelkers *et al.*, 2008), none of them had been annotated in the genome (Table 9.1).

By comparing the GmCLE peptide sequences with those of Arabidopsis, 29 GmCLE peptides were found to belong to group-I, putative promoters of cellular differentiation exemplified by CLV3 (Ito *et al.*, 2006) (Figure 9.1); six GmCLE peptides are related to TDIF and AtCLE41/AtCLE44, which are group-II members, known to prevent cellular differentiation and to control the rate and orientation of vascular cell division (Ito *et al.*, 2006; Etchells and Turner, 2010) (Figure 9.1). Two GmCLE pairs, GmCLE14-GmCLE39 and GmCLE35-GmCLE37, belong to group-III and are similar to the Arabidopsis CLE1 to CLE7 peptides. In Arabidopsis, the functional analysis of group-III peptides resulted in conflicting data. Ectopic addition caused root meristem consumption only at high peptide concentrations and the peptides did not inhibit xylem differentiation in a zinnia (*Zinnia elegans*) cell culture system (Ito *et al.*, 2006). Overexpression of the corresponding genes did not affect the root meristem, while the shoot meristem disappeared (Meng *et al.*, 2010). Also the nodulation-related LjCLE-RS1/LjCLE-RS2, MtCLE12 and MtCLE13 peptides belong to group-III (Okamoto *et al.*, 2009; Mortier *et al.*, 2010). Hence, GmCLE14-GmCLE39 and GmCLE35-GmCLE37 might exert a similar function as LjCLE-RS1/LjCLE-RS2, MtCLE12 and MtCLE13.

A nodulation-related function of GmCLE14-GmCLE39 and GmCLE35-GmCLE37 is also suggested by the expression patterns analyzed at two different stages of soybean nodule development. Microscopic analysis, confirmed by the expression of a soybean homolog of the G2-M phase-related marker CycB2;1 (Umeda *et al.*, 1999; Lee *et al.*, 2003) revealed that under our experimental conditions, the central tissue of 2-wpi nodules contains dividing and differentiating cells, while 4-wpi nodules are terminally differentiated. Expression analysis in these two nodulation stages revealed two different nodulation-related *CLE* patterns. The group-III genes *GmCLE14-GmCLE39* and *GmCLE35-GmCLE37* as well as the group-I *GmCLE06-GmCLE38* had a transient expression pattern with the highest expression in 2-wpi nodules, after which the expression disappeared again in mature differentiated nodules. The expression of *GmCLE11-GmCLE34, GmCLE13* and *GmCLE17-GmCLE32* steadily increased as nodulation progressed and was the highest in 4-wpi nodules. Thus, although the expression of six *GmCLE* genes, from which five had a second copy in the genome, are induced during nodulation, only the expression of *GmCLE06-GmCLE38* and the group-III peptide genes *GmCLE14-GmCLE39* and *GmCLE35-GmCLE37* was linked with stages of nodule cell division and differentiation in the nodule primordium (Table 9.2). Compared to *L. japonicus* and *M. truncatula*, more *GmCLE* genes were upregulated during nodulation of soybean, and different nodulation-related expression patterns were seen. Of course completion of the genome sequences might reveal more CLE peptides involved in nodulation in *M. truncatula* and *L. japonicus*.

Similar to the previously identified *M. truncatula* group-III gene *MtCLE13* (Mortier *et al.*, 2010), the *GmCLE14-GmCLE39* and *GmCLE35-GmCLE37* genes of soybean were expressed in the developing nodule primordium and expression of both pairs is induced in roots by the addition of cytokinins, but not of auxins. *GmCLE14-GmCLE39* and *GmCLE35-GmCLE37* might therefore be not only the structural but also the functional equivalents of *MtCLE13* (Table 9.2).

The third transiently expressed gene pair, *GmCLE06-GmCLE38*, encodes group-I type peptides that are very similar to LjCLE3 (Okamoto *et al.*, 2009). So far, no localized expression pattern is known for any of the nodulation-related *LjCLE* genes. Our analysis indicates a role for *GmCLE06-GmCLE38* during primordium homeostasis. In contrast to *GmCLE14-GmCLE39* and *GmCLE35-GmCLE37*, no induction of *GmCLE06-GmCLE38* gene expression was found upon cytokinin or auxin addition to roots.

GmCLE11-GmCLE34, GmCLE13 and *GmCLE17-GmCLE32*, for which the ex-

Table 9.2: Integrated overview of knowledge about nodulation-related CLE peptides. Summary of all data presented here and those obtained by Mortier *et al.* (2010) and Okamoto *et al.* (2009). In black, supported by expression and functional data; grey, still hypothetical functions, because of lack of functional data; NA, not analyzed.

	Summary						Possible function in nodulation		
Gene	CLE peptide group	Expression in developing nodules	Expression in mature nodules	Cytokinin induction	Auxin induction	KNO_3 induction	AON	Nodule development	Nitrate regulation
GmCLE11-GmCLE34	I	+	+	-	-	-	-	+	-
GmCLE13	I	+	+	-	-	-	-	+	-
GmCLE17-GmCLE31	I	+	+	-	-	-	-	+	-
GmCLE06-GmCLE38	I	+	-	-	-	-	-	+	-
GmCLE14-GmCLE39	III	+	-	+	-	+/-	+	+	+/-
GmCLE35-GmCLE37	III	+	-	+	-	-	+	+	-
MtCLE12	III	+	+	-	-	-	+	+	-
MtCLE13	III	+	+	+	-	-	+	+	-
LjCLE-RS1	III	+	NA	NA	NA	-	+	+	-
LjCLE-RS2	III	+	NA	NA	NA	+	+	+	+

pression steadily increased during nodulation, encode group-I type peptides from which members are known to cause consumption of the root meristem upon ectopic addition or overexpression. The deduced peptide sequence of GmCLE13 differed only at one position with AtCLE40. The latter peptide is involved in the organization of the root apical meristem by repressing the WUSCHEL homolog WOX5 (Stahl *et al.*, 2009). The expression of *GmCLE11-GmCLE34, GmCLE13* and *GmCLE17-GmCLE32* was not modulated by auxin or cytokinin. What the function would be of these CLE peptides in mature, terminally differentiated nodules, without stages of division nor differentiation, is not known, but very intriguing because, so far, in all studied cases, no function other than one linked to cell division and differentiation has been found.

The group-III LjCLE-RS1/LjCLE-RS2, MtCLE12 and MtCLE13 peptides might be involved in AON as over expression of the corresponding genes reduced or abolished nodulation locally as well as systemically in a *SUNN/HAR1*-dependent manner (Okamoto *et al.*, 2009; Mortier *et al.*, 2010). This effect was specific for group-III pep-

tides because ectopic expression of *CLE* genes encoding peptides with a typical group-I signature was ineffective (Okamoto *et al.*, 2009; Mortier *et al.*, 2010). The LjCLE-RS1, LjCLE-RS2, MtCLE12 and MtCLE13 peptides could possibly act as long-distance signals and travel to the shoot to be perceived by SUNN/HAR1 (Okamoto *et al.*, 2009; Mortier *et al.*, 2010). Alternatively, in nodules, the group-III peptides might be perceived by local receptors and provoke an upward long-distance signal that activates, in the shoot, the binding of an as yet unidentified CLE peptide with SUNN/HAR1 for AON.

The soybean group-III peptides GmCLE14-GmCLE39 and GmCLE35-GmCLE37 might, based on the sequence similarity and the expression analysis, be the functional equivalents of LjCLE-RS1, LjCLE-RS2, MtCLE12 and/or MtCLE13 (Table 9.2) and activate AON in soybean. Interestingly, because these soybean group-III *CLE* genes are not expressed in mature nodules, which exert a high AON activity, our data open the possibility that group-III CLE peptides might activate AON, while other mechanisms take over the control of nodule numbers at later stages during nodulation, possibly involving nitrogen fixation efficiency (Nutman, 1952; Magori and Kawaguchi, 2009).

Mutants defective in AON, exhibit a nitrate-tolerant nodulation, suggesting that nitrate inhibition of nodulation act via the AON pathway (Pierce and Bauer, 1983; Carroll *et al.*, 1985a,b; Wopereis *et al.*, 2000; Oka-Kira *et al.*, 2005; Barbulova *et al.*, 2007; Magori and Kawaguchi, 2009). In *L. japonicus*, LjCLE-RS2 transcription was upregulated by nitrate (Okamoto *et al.*, 2009). Under our experimental conditions, the expression of one group-III GmCLE gene pair was moderately induced by the addition of nitrate (Table 9.2). Hence, the link between AON, CLE peptides and the influence of nitrate on nodule formation needs further investigation.

In conclusion, in soybean several *CLE* genes are upregulated during nodulation. Two distinct nodulation-related expression patterns were observed, one linked with nodule primordium formation and differentiation and another linked with nodule maturation. It would be interesting to follow the localized expression of these *CLE* genes and to study the effects of knockout or overexpression. Such studies might be hampered by redundancy because in the soybean genome, CLE peptides are mostly encoded by gene pairs. Expression of group-III gene pairs was linked with developing and not with mature nodules, suggesting that signaling by group-III CLE peptides might initiate AON and that other mechanisms might take over at later stages in nodulation. Our data provide a framework for biochemical and genetic analysis to explore potential interaction with the SUNN/HAR1/NARK receptor and role in nodule primordium and

meristem homeostasis.

9.4 Materials and Methods

Plant material, bacterial strains and growth conditions

Glycine max (L.) Merr. 'Prima 2000' seeds were germinated in the dark for 2 days and grown in pots containing vermiculite (Mandoval, Alberton, South Africa). The greenhouse conditions were 27°C/17°C day/night temperature, 60% relative humidity, 13 h photoperiod, 600 mmol m^{-2} s^{-1} photosynthetically active radiation. The plants were watered every 2 days with Hoagland's solution (Hewitt, 1966). For the plants grown in nitrogen-rich conditions, NH_4NO_3 (1 mM final concentration) was added to the Hoagland's solution. *Bradyrhizobium japonicum* WB74-1 x 109 CFU g^{-1} (Soygro bio-fertilizer Limited, Potchefstroom, South Africa) was inoculated just before sowing by adding 0.5 g of the powder to each pot.

Nodules were harvested 2 and 4 weeks post inoculation (wpi) for microcopy and expression analysis. For the quantitative reverse-transcription (qRT)-PCR experiments, roots from non-inoculated plants grown under nitrogen-poor conditions (Hoagland's solution) were harvested two weeks after sowing. First leaves, cotyledons and SAMs were harvested 1 week after growing under nitrogen-rich conditions (Hoagland's solution + 1 mM NH_4NO_3) and stems, roottips and leaves 1 week later.

RNA extraction, cDNA synthesis, and qRT-PCR analysis

RNA extraction, cDNA synthesis and qRT-PCR analysis were done as described (Mortier *et al.*, 2010). The relative expression was normalized against the constitutively expressed genes encoding the 40S ribosomal protein S8 (AK285894) or ELF1B protein (TC203623) (Jian *et al.*, 2008). For the five single *GmCLE* genes, specific primer pairs could be predicted by comparison to the whole soybean genome. For the remaining *GmCLE* genes, no specific regions could be found because of the highly homologous pairs, in which case, the selected primer pairs amplified both homologous genes. The primers used, (see Supplementary Table 9.3) were purchased at Inqaba Biotechnical Industries (Pretoria, South Africa). Each experiment was repeated twice with independent biological tissues. Statistical differences were evaluated with ANOVA by means

of the GenStat software[30].

In vitro application of auxins, cytokinins and nitrogen

Auxins (10^{-6} M indole-3-acetic acid [IAA]) or cytokinins (10^{-7} M 6-benzylaminopurine [BAP]) were diluted in dimethylsulfoxide and supplemented to the medium of 5-day-old, *in vitro*-grown seedlings. As a control, plants were grown without supplemented hormones. The seedlings were grown in Magenta boxes ($6 \times 6 \times 10$ cm) on Gelrite agar (Sigma-Aldrich, St. Louis, MO, USA) containing Hoagland's solution (Hewitt, 1966) supplemented with 1 mM NH_4NO_3. The plants were cultured in a room at a temperature of 26°C, 16 h photoperiod and light intensity of 70 μE $s^{-1}m^{-2}$ light per day. After 0, 4, 8, and 24 h of incubation, the roots of 4 plants were harvested and analyzed by qRT-PCR. For the *in vitro* application of nitrogen, 0, 1, 5 or 10 mM KNO_3 were supplemented to the medium of 2-day-old seedlings. The roots of 6 plants of each condition were harvested 6 days later and analyzed by qRT-PCR. All experiments were repeated twice with comparable results.

Microscopy

Root nodules were fixed, dehydrated and embedded with the Technovit 7100 kit (Heraeus Kulzer, Wehrheim, Germany), according to the manufacturer's instructions, and sectioned with a microtome (Reichert-Jung, Nussloch, Germany). The 3-μm-thick sections were mounted on coated slides (Sigma-Aldrich). For tissue-specific staining, sections were submerged in a 0.5 % (w/v) toluidine blue solution, washed in distilled water, and dried. Finally, sections were mounted with Depex (BDH Chemicals, Poole, England). Photographs were taken with a Diaplan microscope equipped with bright-field optics (Leitz, Wetzlar, Germany).

9.5 Supplemental data

[30] http://www.vsni.co.uk/software/genstat/

Table 9.3: Primers used in the analysis

Gene	Sense primer	Anti-sense primer
GmCLE01/GmCLE22	ATGAAGCACTTCCACTTGTTCC	CTCTTGTTGTGTAAAGGGTTG
GmCLE02/GmCLE27	TAGTAGTGGTAGTAGTTCTTT	AGAAGACAAACAAGCCCCACCACA
GmCLE03/GmCLE31	GAAGAAAGTGAACAAAATTCCAAG	ACTTCATGTGCACTAGCATTGAAGAT
GmCLE04/GmCLE24	ATTTGACTTCTCTCCTTTCTTCAAG	GTCTGAAGGATCATGGTG
GmCLE05/GmCLE26	CTTTTCTTCACTTCCTCTCAC	GGCTGATTCTGAAGATTTGGT
GmCLE06/GmCLE38	AACAAAAAAATGAGTTGTGTTATCCTCTT	CAAATCTGGTTTTTCTTGCTGCT
GmCLE07/GmCLE30	ATGTTGGTTGCCGGCGGCAAGA	CAAGATGAGTCCTTCCAC
GmCLE08/GmCLE28	CAGAGTCGCAATTTTCTTCTTGT	GAATGGCTGGTTCTGGTT
GmCLE09	AAACCCACTTCATAACTGAATGAAA	AGGAAAATGAAATCAAACTATGTCTTGC
GmCLE10	ATGAGAATTCCAAAATTCTCAACC	TATTTATATGCATGCCATCTAGAGT
GmCLE11/GmCLE34	CAGTGCTCTCTTGTTGGGGGGT	GTGGCTGGTAGTGCTGATC
GmCLE12/GmCLE29	TGATGACCCTCTTCTTCTTTG	GTGTGCTTCCTGCTTCTCTAG
GmCLE13	AGGTTGCAAGATTTGGGAATGA	AGGAAATTCAATGGCCTATAGGGT
GmCLE14/GmCLE39	TAATCATCCTCTCCACCTTCTTC	GCAAAATTATGATGAGGATCTGG
GmCLE15/GmCLE36	ATGGCAGTTCGATATTCAAGGCTCTCA	GAACATCTCGTAGAGAAACTCCATAA
GmCLE16/GmCLE33	ATGCTAAGGAGTGACATGGGTGCTCTTT	CACCATGTCCTCTCTACTCTACCGATT
GmCLE17/GmCLE32	GCAGTTCTTGTTTAAACCATGGAGCATT	GGGTCAGGAGAGGAAGGTAC
GmCLE18/GmCLE23	CTACTGTTGATTCTGTTCTTGTTC	CTTCACCACCACCTTCACC
GmCLE19	CACCATCATCACCACCACCTT	GTTAATAGTGGTGAAATCGGTATAAT
GmCLE20/GmCLE21	CATCAGATTCCAACATCCACTAAG	GGCGTTTCTCAACACCATAAC
GmCLE25	CACTCACCACCACCATCATC	TGGTTGTGAAGTGTTGAAGAAG
GmCLE35/GmCLE37	TCATGTTCTCTACATTCTTCATGACT	CTCCTGGTGCAAGTCTATCTG
GmELF1B	GTTGAAAAGCCAGGGGACA	TCTTACCCCTTGAGCGTGG
Gm40S	GCCAGCCTGCTAACACTAAG	AAGAGTCTGAGTACGCACAAG
GmCYCB	TGTAATCTTGTACCGTCCTTCTC	TCCTCAGAGTAGCCTGTGTAG

Table 9.4: Expression analysis of all *GmCLE* genes during nodulation. qRT-PCR on cDNA samples of uninoculated roots (Roots) and of nodules (Nod) at 2 and 4 wpi. The qRT-PCR analysis was done in duplicate. The average of the relative expression levels of each gene (AV) and the standard error (SE) were calculated. The relative expression levels were defined by the $2^{-\triangle\triangle C_t}$ (Livak and Schmittgen, 2001). Statistically significant differences in comparison to uninoculated roots (Roots) are indicated with * ($p < 0.05$, Anova), ** ($p < 0.01$, Anova) or *** ($p < 0.001$, Anova).

	Roots		Nod (2 wpi)			Nod 4 wpi		
Gene	AV	SE	AV	SE	Anova	AV	SE	Anova
GmCLE01/GmCLE22	1.02	0.16	0.99	0.16		1.69	0.16	
GmCLE02/GmCLE27	1.03	0.17	0.35	0.17		0.36	0.17	
GmCLE03/GmCLE31	1.01	0.25	1.14	0.25		0.28	0.25	
GmCLE04/GmCLE24	1.03	0.06	0.19	0.06	*	0.07	0.06	**
GmCLE05/GmCLE26	1.05	1.00	1.58	1.00		2.81	1.00	
GmCLE06/GmCLE38	1.02	0.21	3.61	0.21	*	0.59	0.21	
GmCLE07/GmCLE30	1.02	0.24	0.85	0.24		0.94	0.24	
GmCLE08/GmCLE28	1.07	0.65	2.12	0.65		0.75	0.65	
GmCLE09	1.02	0.04	0.08	0.04	***	0.02	0.04	***
GmCLE10	1.10	2.22	3.70	2.22		3.20	2.22	
GmCLE11/GmCLE34	1.00	5.20	19.30	5.20	*	16.50	5.20	*
GmCLE12/GmCLE29	1.02	0.05	0.75	0.05	*	0.37	0.05	**
GmCLE13	1.07	3.00	30.80	3.00	**	1.10	3.00	
GmCLE14/GmCLE39	1.10	3.00	30.80	3.00	**	1.10	3.00	
GmCLE15/GmCLE36	1.01	0.02	0.12	0.02	***	0.21	0.02	***
GmCLE16/GmCLE33	1.00	0.03	0.27	0.03	***	0.08	0.03	***
GmCLE17/GmCLE32	1.00	5.71	41.80	5.71	*	39.60	5.71	*
GmCLE18/GmCLE23	1.02	0.14	0.59	0.14		0.08	0.14	*
GmCLE19	1.17	0.41	1.27	0.41		2.34	0.41	
GmCLE20/GmCLE21	1.01	0.01	0.18	0.01	***	0.14	0.01	***
GmCLE35/GmCLE37	1.00	352.60	2254.00	352.60	*	362.00	352.60	

Table 9.5: Tissue- or organ-specific expression analysis of nodulation-related *GmCLE* genes. qRT-PCR analysis on cDNA samples of different tissues. Samples are cDNA from roots grown in the absence of NH_4NO_3 (Root N), roottips, stems, SAMs, cotyledons, mature leaves (leaves) and first leaves (1st leaves). The relative expression levels of two biological repeats (1 and 2) are represented. The relative expression levels were defined by the $2^{-\triangle\triangle C_t}$ method (Livak and Schmittgen, 2001).

Repeat1 Gene	Root -N	Roottip	Stem	SAM	Cotyledon	Leaves	1st Leaves
GmCLE06/GmCLE38	1.00	0.59	19.86	5.40	0.79	2.69	2.22
GmCLE11/GmCLE34	1.01	0.43	0.69	0.54	0.45	0.62	0.35
GmCLE13	1.00	1581	7400	7582	74960	508100	73532
GmCLE14/GmCLE39	1.05	2.40	0.00	0.00	0.00	0.00	0.20
GmCLE17/GmCLE32	1.01	0.64	1.05	1.48	0.46	1.18	0.32
GmCLE35/GmCLE37	1.00	0.00	0.00	0.00	0.00	0.00	0.00
Repeat2 Gene	Root -N	Roottip	Stem	SAM	Cotyledon	Leaves	1st Leaves
GmCLE06/GmCLE38	1.00	0.02	3.59	1.34	0.13	1.06	1.61
GmCLE11/GmCLE34	1.06	0.84	1.40	1.34	1.24	0.12	0.88
GmCLE13	1.03	1.11	5.62	3.80	11.26	73.11	36.68
GmCLE14/GmCLE39	1.00	8.82	0.21	0.18	0.18	0.50	0.48
GmCLE17/GmCLE32	1.00	0.21	1.14	0.61	0.26	0.08	0.12
GmCLE35/GmCLE37	1.00	0.00	0.00	0.00	0.00	0.00	0.00

Table 9.6: Influence of auxin and cytokinin on the expression of *GmCLE* genes. qRT-PCR analysis on cDNA samples of roots grown in the presence of 10^{-6} M auxin (IAA) or 10^{-7} M cytokinin (BAP). Growth medium without hormones was used for the control plants. Samples of 5-day-old plants were taken at 0, 4, 8, and 24 h after hormone addition. The qRT-PCR analysis was done in duplicate. The average of the relative expression levels of each gene (AV) and the standard error (SE) were calculated. The relative expression levels were defined by the $2^{-\triangle\triangle C_t}$ (Livak and Schmittgen, 2001). Asterisk indicates statistically significant differences in comparison to control plants grown without hormone addition ($p < 0.001$, Anova).

	0 h		4 h		8 h		24 h	
Gene	AV	SE	AV	SE	AV	SE	AV	SE
Control treatment								
GmCLE06/GmCLE38	1.01	0.14	0.56	0.14	0.49	0.14	0.56	0.14
GmCLE11/GmCLE34	1.01	0.20	0.78	0.20	0.98	0.20	0.61	0.20
GmCLE13	1.04	0.07	0.69	0.07	0.66	0.07	0.51	0.07
GmCLE14/GmCLE39	1.01	1.65	0.68	1.65	0.53	1.65	0.32	1.65
GmCLE17/GmCLE32	1.00	0.42	0.95	0.42	1.49	0.42	0.67	0.42
GmCLE35/GmCLE37	1	627	0	627	1	627	0	627
IAA treatment								
GmCLE06/GmCLE38	1.01	0.14	0.48	0.14	0.37	0.14	0.37	0.14
GmCLE11/GmCLE34	1.01	0.20	1.19	0.20	0.71	0.20	0.60	0.20
GmCLE13	1.04	0.07	0.42	0.07	0.64	0.07	0.30	0.07
GmCLE14/GmCLE39	1.01	1.65	0.84	1.65	0.27	1.65	0.23	1.65
GmCLE17/GmCLE32	1.00	0.42	2.05	0.42	0.48	0.42	0.76	0.42
GmCLE35/GmCLE37	1	627	326	627	27	627	0	627
BAP treatment								
GmCLE06/GmCLE38	1.01	0.14	0.50	1.14	0.65	0.14	0.62	0.14
GmCLE11/GmCLE34	1.01	0.20	0.51	0.20	0.66	0.20	1.40	0.20
GmCLE13	1.04	0.007	0.95	0.07	1.11	0.07	0.43	0.07
GmCLE14/GmCLE39	1.01	1.65	0.57	1.65	0.29	1.65	20.68	1.65*
GmCLE17/GmCLE32	1.00	0.42	1.29	0.42	1.51	0.42	2.93	0.42
GmCLE35/GmCLE37	1	627	1080	627	1	627	13088	626*

Table 9.7: Influence of KNO_3 on the expression of *GmCLE* genes. qRT-PCR analysis was done on cDNA samples of roots grown for 6 days in the presence of 0, 1, 5, and 10 mM KNO_3. The qRT-PCR analysis was done in duplicate. The average of the relative expression levels of each gene (AV) and the standard error (SE) were calculated. The relative expression levels were defined by the $2^{-\triangle\triangle C_t}$ (Livak and Schmittgen, 2001). No statistical differences were measured as compared to the plants grown in the absence of KNO_3 ($p > 0.05$, Anova).

	0 mM		1 mM		5 mM		10 mM	
Gene	AV	SE	AV	SE	AV	SE	AV	SE
GmCLE06/GmCLE38	1.01	0.55	0.87	0.55	1.22	0.55	2.21	0.55
GmCLE11/GmCLE34	1.00	0.18	0.72	0.18	1.08	0.18	0.73	0.18
GmCLE13	1.03	0.98	0.51	0.98	0.58	0.98	0.41	0.98
GmCLE14/GmCLE39	1.04	1.34	0.44	1.34	2.15	1.34	3.22	1.34
GmCLE17/GmCLE32	1.00	0.46	0.60	0.46	0.69	0.46	0.67	0.46
GmCLE35/GmCLE37	1.10	4.65	1.20	4.65	0.30	4.65	0.10	4.65

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Authors contributions

The main part of the work was done by V. Mortier. B. Fenta was involved in growing plant material for the assays in Pretoria (South-Africa). C. Martens and S. Rombauts performed the phylogenetic and bioinformatic analysis, respectively. K. Kunert, S. Goormachtig and M. Holsters were involved in designing the research and revising the manuscript.

10

Identification of putative CLE peptide receptors involved in determinate nodulation on soybean

Virginie Mortier, Berhanu Amsalu Fenta, Karl Kunert, Marcelle Holsters and Sofie Goormachtig

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Abstract
CLAVATA3/EMBRYO SURROUNDING REGION (CLE) peptides tightly control the balance between stem cell proliferation and differentiation in several plant developmental processes. Transmission of the CLE peptide signal has been shown to be rather complex. Despite their recent identification, little is known about the receptors by which nodulation-specific CLE peptides, which were identified in soybean (*Glycine max*), are perceived. Genetic analysis has indicated that the leucine-rich repeat receptor-like kinase NARK of soybean and its orthologs in other legumes are possible candidates. However, more receptors need to be identified because CLE peptides are often detected by heteromultimeric complexes. Here, we identified two additional putative CLE peptide receptor pairs in the soybean genome with a nodulation-related expression pattern, *GmRLK1-GmRLK2* and *GmRLK3-GmRLK4*, and discuss their role in CLE peptide perception during nodulation.

Due to their sessile nature, plants rely on meristem-located stem cells for growth. Divisions of stem cells need to be tightly controlled to preserve a balance between proliferation and differentiation towards appropriate tissues and organs (Stahl and Simon, 2010). CLAVATA3/EMBRYO SURROUNDING REGION (CLV3/ESR or CLE) peptides have a main role in regulating this balance (Wang and Fiers, 2010). These intercellular peptides are 12 or 13 amino acids long and act as short-distance signaling molecules in a non-cell-autonomous manner (Wang and Fiers, 2010). CLE peptides are involved in several aspects of plant development, among which homeostasis of shoot apical meristem (SAM) and root apical meristem (RAM), vascular development and nodulation (Okamoto *et al.*, 2009; Hirakawa *et al.*, 2010b; Mortier *et al.*, 2010, 2011; Reid *et al.*, 2011).

Of the recently identified CLE peptide receptors (Wang and Fiers, 2010), many belong to the subclass XI of leucine-rich-repeat receptor-like kinases (LRR-RLKs) (Shiu and Bleecker, 2001) and almost all interact to form either homomultimers or heteromultimers (Guo *et al.*, 2010). Whereas the expression of some CLE peptide receptors is restricted to certain plant tissues, other receptors are expressed throughout the plant and are involved in several developmental processes.

The CLE peptide CLV3 that controls the SAM homeostasis (Clark *et al.*, 1995) is perceived in parallel and with a similar ligand-binding affinity by several receptor complexes, including homomultimers of the subclass XI LRR-RLK CLV1, heteromultimers of CLV1 with their close homologs BARELY ANY MERISTEM1 (BAM1) and

BAM2, heteromultimers of the LRR-containing membrane-anchored protein CLV2 and the membrane anchored kinase CORYNE/SUPPRESSOR OF OVEREXPRESSION OF LLP1-2 (CRN/SOL2), and finally by homodimers of the RECEPTOR-LIKE PROTEIN KINASE2/TOADSTOOL2 (RPK2/TOAD2), an LRR-RLK that is only distantly related to CLV1 (Ogawa *et al.*, 2008; Ohyama *et al.*, 2009; Bleckmann *et al.*, 2010; Guo and Clark, 2010; Zhu *et al.*, 2010a). In the RAM homeostasis of *Arabidopsis thaliana* as well, several receptors are involved in the transmission of CLE peptide signals. The CLE40 ligand is perceived by the LRR-RLK ARABIDOPSIS CRINKLY4 (ACR4), which is unrelated to CLV1, to sustain distal stem cell regulation (Stahl *et al.*, 2009), whereas CLE14 and CLE20 ligands might interact with CLV2 and CRN/SOL2 to inhibit cell division in the root meristem (Meng and Feldman, 2010). Finally, TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR (TDIF), a phloem-expressed CLE peptide, involved in the inhibition of tracheary element differentiation, signals through the procambium-located receptor, TDIF RECEPTOR/PHLOEM INTERCALATED WITH XYLEM (TDR/PXY) that is very similar to CLV1 (Hirakawa *et al.*, 2008).

Recently, CLE peptides have been shown to function during determinate and indeterminate nodulation (Okamoto *et al.*, 2009; Mortier *et al.*, 2010, 2011; Reid *et al.*, 2011). Nodulation is the result of symbiotic interactions between legumes and soilborne bacteria called rhizobia. The interaction is characterized by the development of nodules, root-based, organ-like structures in which the inner cells are colonized by nitrogen-fixing bacteria. A complex signal exchange between rhizobia and their hosts triggers nodulation, followed by the activation of the early Nodulation factor (NF) signaling pathway in the plant epidermis to allow rhizobial infection and the activation of cortical cell division for nodule organogenesis (Ferguson *et al.*, 2010). Many legumes, such as soybean (*G. max*), develop determinate nodules in which all meristematic cells differentiate to build the central tissue of the nodule that consists of infected cells interspersed by some uninfected cells. As a result, these legumes develop round-shaped nodules made up of only fully differentiated cells. However, in many other legumes, such as *Medicago truncatula*, indeterminate nodules are formed that are characterized by a cylindrical shape and a persistent apical meristem. Detailed expression analysis in *M. truncatula* has indicated that two *CLE* genes, encoding structurally related CLE peptides, are expressed in nodule primordium cells and, later on, in the nodule meristematic tissues, indicating that they might control nodule differentiation (Mortier *et al.*, 2010).

As nodulation is an energy-consuming process, plants develop the minimal number of nodules to ensure optimal growth. One of the mechanisms that controls the number of nodules is a long-distance feedback mechanism, called autoregulation of nodulation (AON) (Nutman, 1952; Pierce and Bauer, 1983; Kosslak and Bohlool, 1984; Carroll *et al.*, 1985a,b; Delves *et al.*, 1986).

In shoots, a CLV1-like LRR-RLK of subclass XI plays a crucial role for AON, namely NODULE AUTOREGULATION RECEPTOR KINASE (NARK) in soybean, SUPER NUMERIC NODULES (SUNN) in *M. truncatula*, HYPERNODULATION ABERRANT ROOT FORMATION1 (HAR1) in *Lotus japonicus* and SYMBIOSIS29 (SYM29) in *Pisum sativum* (pea) (Krusell *et al.*, 2002; Nishimura *et al.*, 2002a; Searle *et al.*, 2003; Oka-Kira *et al.*, 2005; Schnabel *et al.*, 2005). Recently, two additional members of this receptor complex have been identified, a CLV2 homolog (PsSYM28/-PsCLV2 and LjCLV2) and an Arabidopsis RPK2/TOAD2 homolog, LjKLAVIER (Lj-KLV) (Kinoshita *et al.*, 2010; Krusell *et al.*, 2011). Interestingly, in contrast to the *nark/sunn/har1/sym29* mutants, mutants in these two genes were also affected in the SAM homeostasis, indicating that, in legumes, a receptor complex similar to that described for Arabidopsis, controls not only the SAM activity, but also the nodule number (Kinoshita *et al.*, 2010; Krusell *et al.*, 2011). In addition to their expression in the shoot, *NARK* and its homologs are also expressed in the root vascular tissue, but their root-specific function is still unknown. The similarity with the Arabidopsis LRR-RLKs of subclass XI (e.g., CLV1, BAM1, BAM2, BAM3 and TDR/PXY) predicts that one or several CLE peptides might be perceived by this complex, but thus far their identity remains elusive. In soybean, expression of several *GmCLE* genes was upregulated during nodulation (Mortier *et al.*, 2011; Reid *et al.*, 2011). Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis revealed that at least three *GmCLE* genes were upregulated in both developing and mature nodules, while the expression of at least three other *GmCLE* genes was transiently induced in developing nodules, but absent in mature nodules (Mortier *et al.*, 2011). Hence, two different nodulation-related *CLE* expression patterns were found: one linked with nodule primordium development and differentiation and one linked with nodule maturation. Functional analysis has shown that for *GmRIC1/GmCLE14-GmCLE39* and *GmRIC2/GmCLE35-GmCLE37*, which are transiently expressed during nodulation, NARK might be a putative receptor, because overexpression of these genes resulted in a decrease in nodule number in a *NARK*-dependent manner (Mortier *et al.*, 2011; Reid *et al.*, 2011).

These observations together with those in *M. truncatula* and *L. japonicus* have raised the hypothesis that these CLE peptides might move toward the shoot to activate the NARK-containing complex for AON (Okamoto *et al.*, 2009; Mortier *et al.*, 2010, 2011; Reid *et al.*, 2011), but these nodulation-related CLE peptides might equally well be perceived locally by still unknown receptors to control nodule number and nodule differentiation (Mortier *et al.*, 2010, 2011). Besides these AON peptides, three additional *GmCLE* genes encoding structurally unrelated CLE peptides accumulate during nodulation and might act locally to control the balance between cell division and differentiation inside the nodules.

We looked for putative nodulation-related CLE peptide receptors in the genome of *G. max*, based on the observation that many known and putative CLE peptide receptors such as CLV1, BAM1, BAM2, BAM3 and TDR belong to group XI of LRR-RLKs (Shiu and Bleecker, 2001). By a tBLASTx algorithm (Altschul *et al.*, 1990), based on *NARK* homology, 13 LRR-RLKs of subclass XI were identified in the soybean genome (Schmutz *et al.*, 2010). The first two hits corresponded to *NARK* and its homolog, whereas the remaining 11 were designated *GmRLK1* to *GmRLK11*. In-depth sequence analysis revealed that *GmRLK1* and *GmRLK2* and *GmRLK3* and *GmRLK4* also form homologous pairs as a result of the soybean genome duplication (Shoemaker *et al.*, 2006). For *GmRLK5* to *GmRLK11*, no homologs were identified. *GmRLK2* and *GmRLK3* corresponded to Glyma14g03770 and Glyma18g14680, respectively, but *GmRLK1* and *GmRLK4* have not been annotated yet. According to the supermatcher tool of wEMBOSS (Rice *et al.*, 2000), *GmRLK2* and *GmRLK3* share 47.3 % and 48.4 % similarity with *NARK*, respectively. qRT-PCR analysis of cDNA samples at two nodulation stages in soybean with the expression of uninoculated roots as a reference revealed that one sample was linked with dividing and differentiating cells (2 weeks post inoculation (wpi)), whereas the other corresponded to mature, fully differentiated nodules (4 wpi). As no differential expression was measured for *GmRLK5, GmRLK6, GmRLK7, GmRLK8, GmRLK9, GmRLK10* and *GmRLK11*, these genes were not retained for further analysis in the context of nodule development. With the 'Beacon designer 7' program[31], no primers could be designed that discriminated between the two highly homologous genes *GmRLK1-GmRLK2* and *GmRLK3-GmRLK4*. Therefore, the primer combinations used recognized transcripts of both genes of a single pair (Table 10.1). Compared to its expression in uninoculated roots, the *GmRLK1-GmRLK2* transcript level increased at the two nodulation stages and was the highest in

[31] www.premierbiosoft.com

mature nodules (Figure 10.1A). Hence, the expression pattern of *GmRLK1-GmRLK2* overlaps with that of the nodulation-related CLE peptides, especially with *GmCLE11-GmCLE34, GmCLE13* and *GmCLE17-GmCLE32* of which the expression also gradually increases during nodulation (Mortier *et al.*, 2011). Functional and *promoter:GUS* analyses will demonstrate whether these genes are expressed in overlapping and neighboring cells and their possible interaction. Tissue-specific analysis carried out with cDNA derived from roots, roottips, stems, SAMs, cotyledons, mature leaves and first leaves (as described in Mortier *et al.* (2011)), revealed that *GmRLK1-GmRLK2* is expressed in every tissue, with the highest expression in roots and stems, although at a level lower than that in nodules (Figure 10.1, A and C), suggesting that *GmRLK1-GmRLK2* might also be involved in nodulation-independent processes. Accordingly, CLV2 and CRN/SOL2 also have a broad expression pattern and are involved in homeostasis of both the SAM and RAM (Jeong *et al.*, 1999; Miwa *et al.*, 2008; Muller *et al.*, 2008). NARK is expressed in roots and shoots as well, but not in nodules (Nontachaiyapoom *et al.*, 2007). Whether the nodulation-independent expression pattern of *GmRLK1-GmRLK2* overlaps with that of *NARK* in the root and shoot vasculature is currently unknown. Furthermore, functional analysis by RNAi and ectopic overexpression might indicate whether *GmRLK1-GmRLK2* is involved in AON and/or in nodule differentiation.

Table 10.1: qRT-PCR primers used in the analyses.

Gene	Sense primer	Anti-sense primer
GmRLK1-GmRLK2	GGAACAATGCCAAATGGAGAG	TGTACCTGTGCCTGATTCTTC
GmRLK3-GmRLK4	CTACTCTTGATGATTGCCAATG	TGTGTTGGAATCTCAACAAAAAAGCAG

In contrast to *GmRLK1-GmRLK2, GmRLK3-GmRLK4* expression is downregulated in nodule primordia and mature nodules, in comparison to its expression in uninoculated roots (Figure 10.1B). The tissue- or organ-specific expression of *GmRLK3-GmRLK4* resembled the expression profile of *GmRLK1-GmRLK2*, because expression was detected in every tissue analyzed (Figure 10.1D). For *GmRLK3-GmRLK4*, the highest expression was measured in stems and SAMs. This tissue-specific expression pattern is broadly similar to that of *NARK* and its homologs in other legumes (Krusell *et al.*, 2002; Schnabel *et al.*, 2005; Nontachaiyapoom *et al.*, 2007).

In addition, in *M. truncatula*, the expression of *SUNN* is also downregulated during nodulation (unpublished results). The expression of *NARK* in the vasculature of the

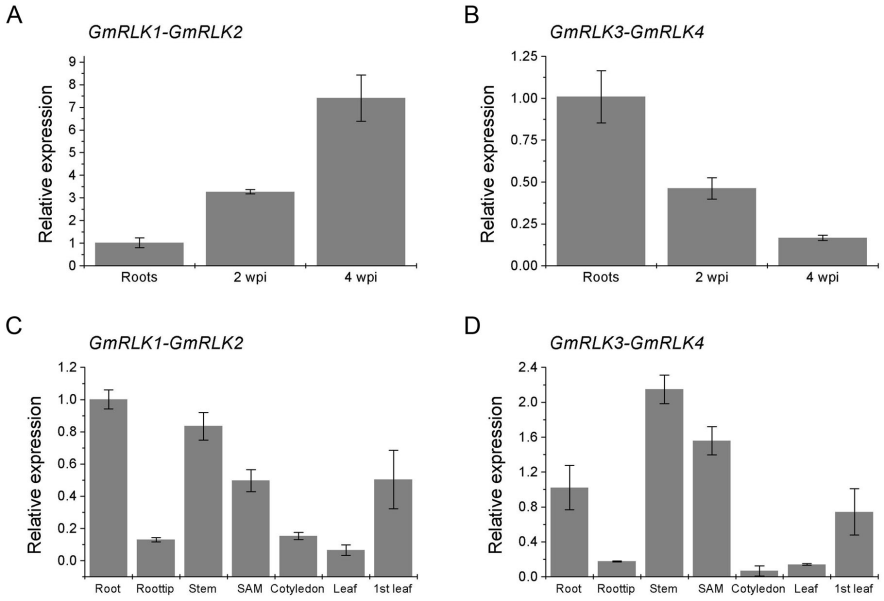

Figure 10.1: Nodulation and tissue-specifc expression analysis of nodulation-related *GmRLK* genes. A and B, qRT-PCR on cDNA samples of uninoculated roots (Roots) and of nodules at 2 and 4 wpi. C and D, qRT-PCR on cDNA samples grown without NH_4NO_3 from roots, roottips, stems, SAMs, cotyledons, mature leaves (leaf) and first leaves (1st leaf). Error bars represent standard deviations (n = 3). The experiments were repeated twice with comparable results. For detailed experimental procedures, see Mortier *et al.* (2011).

root might be the underlying cause for the decreased expression in nodule-enriched material (Nontachaiyapoom *et al.*, 2007).

Together, the temporal expression during nodulation, as well as the tissue-specific expression pattern of *GmRLK3-GmRLK4* suggest that *GmRLK3-GmRLK4* might interact with *NARK*. Functional and biochemical analyses need to confirm this hypothesis. As the expression during nodulation does not coincide with the expression pattern of nodulation-related GmCLE peptides (Mortier *et al.*, 2011), *GmRLK3-GmRLK4* might probably not play a role in short-distance signaling of the nodulation-related GmCLE peptides.

In conclusion, genome-wide analysis combined with expression analysis identified

two additional LRR-RLKs of subclass XI, of which the expression is modulated by nodulation. Hence, besides NARK, similar receptors might control CLE peptide signaling during nodulation, accordingly to the multiple receptor complexes involved in CLE signal transduction in the SAM and RAM. Based on their opposite nodulation-related expression profiles, *GmRLK1-GmRLK2* and *GmRLK3-GmRLK4* are likely to function differently: *GmRLK3-GmRLK4* might together with NARK control nodule numbers and *GmRLK1-GmRLK2* might perceive CLE peptides at short distances to regulate nodule development. Functional analysis of these *GmRLK* genes by ectopic expression or RNAi analysis should confirm their precise role in nodulation, but biochemical analyses are essential to demonstrate interactions between these receptors and CLE peptides. Finally, because several CLE peptide receptors (e.g. CLV2, CRN/SOL2, RPK2/TOAD2 and ACR4) do not belong to subclass XI of LRR-RLKs, receptors with unrelated sequences might be involved in CLE peptide binding during nodulation and should, therefore, be investigated as well.

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Authors contributions

The main part of the work was done by V. Mortier. B. Fenta was involved in growing plant material for the assays in Pretoria (South-Africa). K. Kunert, S. Goormachtig and M. Holsters were involved in designing the research and revising the manuscript.

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Discussion and perspectives

Legumes develop root nodules as a result of a symbiotic interaction with soil borne bacteria, called rhizobia. Inside the nodules the bacteria find the ideal niche to fix atmospheric nitrogen for the plant in return for energy sources. Nodule formation is a complex developmental process that requires the spatio-temporal expression of many plant and bacterial genes. Nodulation can be divided in two developmental pathways, bacterial infection and organ initiation, and the two merge when the bacteria are taken up by the plant cells. Two types of nodules have been characterized, determinate nodules such as originating on soybean (*Glycine max*) and indeterminate nodules such as observed on *Medicago truncatula*. Determinate nodules are round-shaped and are terminally differentiated, while indeterminate nodules have a persistent apical meristem. This work mainly focuses on *M. truncatula* nodule development and therefore, although very often orthologs were studied in other legumes, gene names mentioned in this summary will be from *M. truncatula*.

Nodule primordia develop after re-initiation of cell division in the root cortex upon sensing the bacterial signal molecules, the Nod Factors (NFs). The putative NF receptors, NFP, LYK3, LYK4 and the early NF signaling components, DMI1, DMI2, DMI3, IPD3, NSP1, NSP2, ERN1 and NIN were shown to be crucial for proper nodule

primordium formation (reviewed by Oldroyd and Downie (2008) and Ferguson *et al.* (2010)). Central to primordium initiation is cytokinin signaling as has been demonstrated by studies on the cytokinin receptor MtCRE1, which is expressed in cortical cells but not in the epidermis (Lohar *et al.*, 2006; Gonzalez-Rizzo *et al.*, 2006; Murray *et al.*, 2007; Tirichine *et al.*, 2007; Plet *et al.*, 2011). How the MtCRE1 receptor is activated in the cortical cells after NF perception is currently unknown. To get more insight in this process, two *MtLOG* genes, *MtLOG1* and *MtLOG2*, encoding cytokinin riboside 5'-monophosphate phosphoribohydrolases that release active cytokinins from nucleotide precursors, were studied in detail (**Chapter 7**). The expression of both *MtLOG* genes was rapidly induced upon nodulation (Figure 11.1) and *pMtLOG1:GUS* was expressed in the dividing cortical cells, supporting a role for the encoded proteins in the release of active cytokinins for primordium development. In addition, simultaneous knock-down of both genes resulted in a reduced nodule number indicating that the two MtLOGs contribute to the free cytokinin pool that is needed for the establishment of a nodule. However, it is improbable that the cytokinins released via MtLOG1 and possibly MtLOG2 act as a primary cytokinin pool that is sensed by MtCRE1 to initiate nodule formation because the MtCRE1 receptor itself was needed for their expression (Figure 11.1). In addition, *pMtLOG1:GUS* activity was not detected in the inoculated roots until an early primordium stage was visible, after the first cell divisions had taken place. These data suggest that *MtLOG1* and possibly *MtLOG2* might rather be involved in a positive feedback loop to maintain high cytokinin levels for continued cell division (Figure 11.1). An interesting hypothesis is that after symplastic continuity is made between the cortex and the vasculature (Complainville *et al.*, 2003), cytokinin precursors might reach susceptible cortex cells to be activated by MtLOG and maintain division. This hypothesis is supported by our observations that ectopic expression of *MtLOG1* in uninoculated roots resulted only in more cells in the vasculature and not in the cortex, where no precursors might be available. Careful analysis of other cytokinin biosynthesis genes together with sensitive cytokinin measurements are required to further study the initiation process. How the *MtLOG* genes are activated is currently unknown. Exogenous application of cytokinins or auxins could not induce the genes. However, given the expression of *MtLOG1* in dividing cortical cells, a combination of both hormones or the stele factor uridine (Smit *et al.*, 1995b; Crespi and Galvez, 2000), previously shown to activate cortical cell division, might be the trigger. Although we clearly demonstrate that the two *MtLOG* genes have a positive role for nodule primordium development, how the MtCRE1 receptor is initially activated upon

nodulation remains elusive and needs further study.

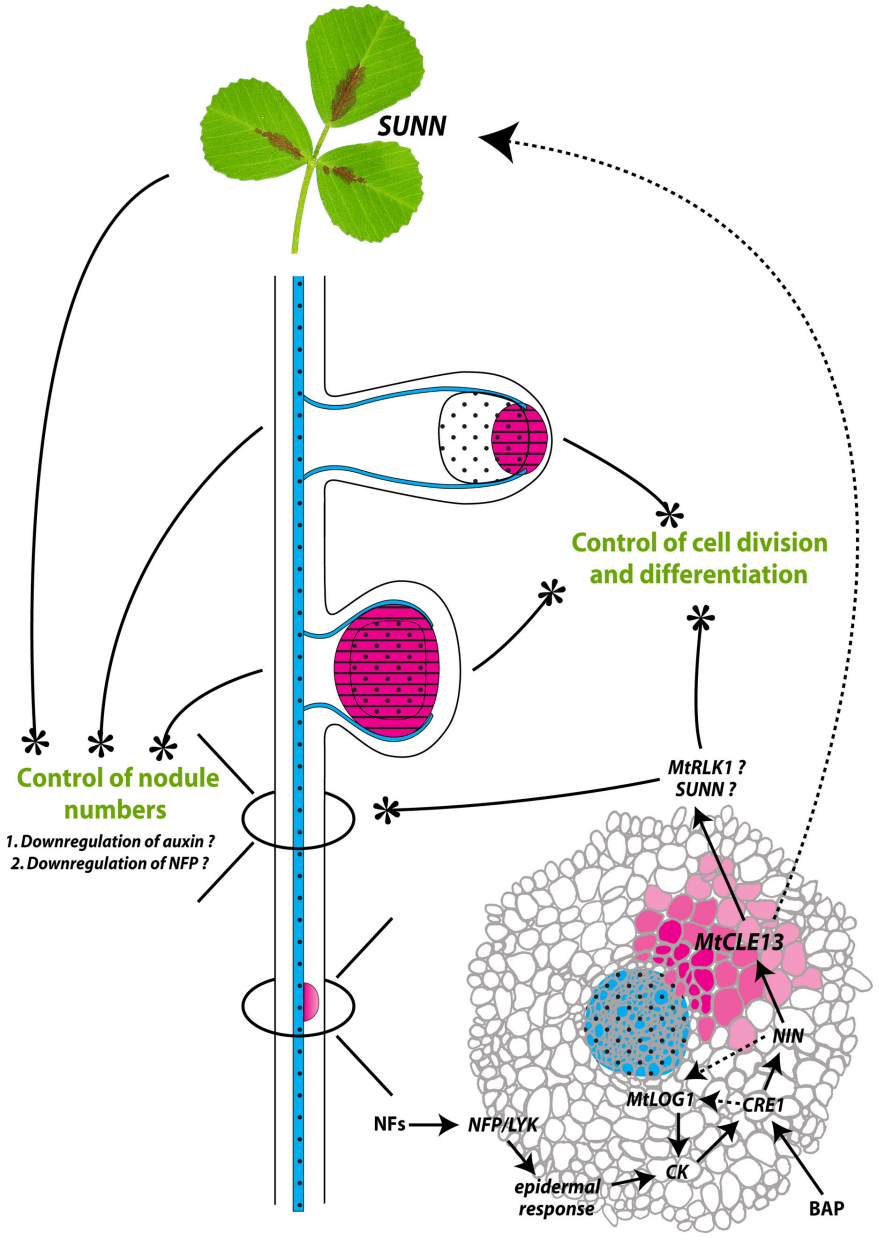
Apart from cytokinin signaling and tightly intertwined with it, many other hormones control the nodulation process (reviewed in **Chapter 1**). Here we demonstrate that a group of peptide hormones, CLV3/ESR (CLE) peptides, plays several roles during determinate and indeterminate nodulation. CLE peptide signaling has been related to the balance between cell proliferation and differentiation during shoot and root apical meristem (SAM and RAM, respectively) homeostasis and during vascular development (Fletcher *et al.*, 1999; Fiers *et al.*, 2005; Hirakawa *et al.*, 2008, 2010b; Ito *et al.*, 2006). By specialized searches, we identified 31 *MtCLE* genes and 39 *GmCLE* genes in the genomes of *M. truncatula* and soybean, respectively (**Chapter 3, 4 and 8**). At least 5 *MtCLE* genes (*MtCLE12, MtCLE13, MtCLE26, MtCLE28* and *MtCLE31*) and 6 *GmCLE* (*GmCLE06-GmCLE38, GmCLE11-GmCLE34, GmCLE13, GmCLE14-GmCLE39, GmCLE17-GmCLE32* and *GmCLE35-GmCLE37*) genes were upregulated in nodulated roots. The CLE domain sequences of many of these genes, including *MtCLE12* and *MtCLE13*, and of the nodulation-related *CLE* genes of *Lotus japonicus* (*LjCLE-RS1* and *LjCLE-RS2*), have a high degree of similarity, indicative of redundant functions (Hobe *et al.*, 2003; Ito *et al.*, 2006; Strabala *et al.*, 2006; Ni and Clark, 2006; Whitford *et al.*, 2008; Okamoto *et al.*, 2009; Reid *et al.*, 2011). This redundancy was reflected, not only by the similarity in gain-of-function phenotypes observed here, but also by the need of a *MtCLE12/MtCLE13* double knock-down mutant to result in any nodulation phenotype (**Chapter 3 and 4**). Moreover, within nodule tissues overlapping expression patterns were observed (Figure 11.1) (**Chapter 3**).

Two of the *M. truncatula CLE* genes (*MtCLE12* and *MtCLE13*) were studied in more detail, and several experiments suggested that one of these, *MtCLE13*, plays a role in the control of nodule initiation downstream of the early NF signaling pathway and cytokinin signaling. Functional *DMI1, DMI2, DMI3, NSP1, NSP2* and *NIN* are necessary for *MtCLE13* transcript accumulation (Figure 11.1) (**Chapter 3**). Moreover, *MtCLE13* transcripts were upregulated after a 3-h cytokinin treatment in a *MtCRE1*- and *NIN*-dependent manner, and levels increased with extended treatment (up to 24 h) (Figure 11.1) (**Chapter 3 and 4**). In agreement, in soybean, the expression of *GmCLE14-GmCLE39* and *GmCLE35-GmCLE37* was upregulated by exogenous cytokinin application, albeit, only from 24 h on (**Chapter 8**). *pMtCLE13:GUS* analysis indicated that, upon cytokinin addition as well as upon inoculation, *MtCLE13* transcripts are localized in the cortex, before visible cell divisions were observed, in a gradient with the highest expression in the inner cortex, and a lower expression in the

outer cortex (Figure 11.1) (**Chapter 3 and 4**). The sites of high *MtCLE13* expression correspond with the places of cortical cell divisions for primordium formation. Because *MtCLE13* expression is dependent on cytokinin, this expression pattern might visualize the cytokinin sensitive root zone. Hence, *MtCLE13* might be involved in the regulation of the balance between cell division and differentiation, similar to what was previously described for other CLE peptides (Simon and Stahl, 2006; Kondo *et al.*, 2006; Ito *et al.*, 2006; Whitford *et al.*, 2008).

After the primordium has been formed, *MtCLE13*, but also *MtCLE12*, might further control cell division and differentiation patterns during the development of the primordium into a mature nodule with an apical meristem. Expression analysis indicated that both genes were expressed in primordia and in the apical zone of mature nodules, comprising the nodule meristem and infection zone (Figure 11.1) (**Chapter 3**). In accordance with an important role in nodule meristem homeostasis, simultaneous knock-down of *MtCLE12* and *MtCLE13* expression resulted in nodules with a smaller nodule meristem and premature nodule senescence (**Chapter 4**). Premature nodule senescence might be a consequence of an imbalance between cell division and differentiation leading to aberrant differentiation of infected cells and subsequently senescence. Consumption of the nodule meristem was also reflected by the reduced expression level of the nodule meristem marker gene, *MtHAP2-1*, in the nodules of these RNAi *MtCLE12/MtCLE13* lines (**Chapter 4**). Hence, the nodulation-related CLE peptides, MtCLE12 and MtCLE13, restrain nodule meristem differentiation, similar to the role described for the CLE peptide TDIF in vascular development (Hirakawa *et al.*, 2008), but opposite to the roles of CLE peptides involved in the maintenance of SAM and RAM homeostasis (Sawa *et al.*, 2006; Ito *et al.*, 2006; Stahl and Simon, 2009). *De novo* synthesis of cytokinins via MtLOG1 might also account for proper nodule meristem functioning, as *pMtLOG1:GUS* staining was observed in nodule primordia

Figure 11.1 ***(facing page)*:** Overview of the role of MtCLE peptides during indeterminate nodulation. CLE peptides play a main role during several aspects of nodulation, more specifically during the control of nodule numbers and in the balance between cell division and differentiation at the level of nodule initiation, nodule maturation and nodule apical meristem homeostasis. Solid arrows, supported by expression and functional data; dashed arrows, still hypothetical, because of lack of functional data. Magenta, *MtCLE13* expression; Stripes, *MtCLE12* expression; Dots, *MtRLK1* expression; Blue, *SUNN* expression; NF, Nod Factor; CK, cytokinin; BAP, benzylaminopurine.


SUNN
Control of cell division
and differentiation
Control of nodule
numbers
1. Downregulation of auxin ?
2. Downregulation of NFP ?
MtRLK1 ?
SUNN ?
MtCLE13
NIN
MtLOG1
CRE1
NFs
NFP/LYK
CK
epidermal
response
BAP

and in the meristematic zone and early differentiating nodule cells of mature nodules (**Chapter 7**). In addition, analysis of the few nodules that formed on *35S:MtLOG1* transgenic roots, revealed no or only a small meristem and a high level of senescence indicating an imbalance between cell division and differentiation (**Chapter 7**). In *35S:MtLOG* roots, *MtCLE12* and *MtCLE13* were shown to be upregulated, indicating that the three genes might act in the same pathway (**Chapter 7**). However, these data are very preliminary, raising many questions and requiring further studies. Careful analysis of the nodules on *MtLOG1/MtLOG2* RNAi roots and analysis of *MtCLE12* and *MtCLE13 promoter:GUS* expression in *35S:MtLOG1* plants certainly would give more information on the relation between *MtLOG1* and *MtCLE12/MtCLE13*.

Apart from a role in the control of cell division and differentiation, *MtCLE12* and *MtCLE13* also control nodule numbers (Figure 11.1). As nodulation is an energy-consuming process, plants develop the minimal number of nodules to ensure optimal growth in specific environmental conditions (reviewed in **Chapter 2**) (Ding and Oldroyd, 2009; Ferguson *et al.*, 2010; Kouchi *et al.*, 2010). One of the mechanisms by which nodule numbers are controlled is called autoregulation of nodulation (AON) and consists of a long-distance feedback mechanism, whereby early nodulation events act to suppress additional nodule formation (Kosslak and Bohlool, 1984; Carroll *et al.*, 1985a,b; Delves *et al.*, 1986; Pierce and Bauer, 1983; Nutman, 1952). AON involves the leucine-rich-repeat receptor-like-kinase (LRR-RLK) SUPER NUMERIC NODULES (SUNN), which shares a high similarity with known CLE peptide receptors, such as CLV1, BAM1-3 and TDR (Schnabel *et al.*, 2005). Here, we have shown that *MtCLE12* and *MtCLE13*, produced upon inoculation, might be involved in AON as ectopic expression abolished nodulation in a systemic and *SUNN*-dependent way (**Chapter 4**). In addition, a combined knock-down of *MtCLE12* and *MtCLE13* resulted in elevated nodule numbers and this effect was not additive to the effect of a mutation in the *SUNN* gene (**Chapter 4**). Similar results were reported for nodulation-related *CLE* genes of *L. japonicus* and soybean, suggesting that a similar control mechanism, involving CLE peptides, regulates determinate and indeterminate nodule numbers (Okamoto *et al.*, 2009; Reid *et al.*, 2011). From these data and the expression data we can propose that after the first nodules are being formed, MtCLE12 and MtCLE13 peptides might signal to the root cortex and epidermis to limit the formation of additional nodules, possibly via the AON mechanism (Figure 11.1). Indeed, several experiments, including the analysis of expression of nodulation-related cortical and epidermal markers, indicated that nodulation on *35S:MtCLE12* and *35S:MtCLE13*

roots was totally abolished at the level of NF perception, before the appearance of any epidermal or cortical responses (**Chapter 3 and 5**). A gene-specific and a micro-array based transcription analysis furthermore revealed that ectopic expression of *MtCLE13* resulted in the differential expression of 17 genes, including a downregulation of the NF receptor *NFP* and two homologous genes, *LYK4* and a close homolog of *LYK5* (**Chapter 5**). The *35S:MtCLE13*-induced downregulation of *NFP* in the root susceptible zone was confirmed by histochemical analysis (**Chapter 5**). Hence, expression of *MtCLE13* and possibly *MtCLE12* during nodulation might repress the expression of NF-receptors in cells susceptible for nodulation and thereby restrain additional nodule formation (Figure 11.1). In addition to the NF receptors, ectopic overexpression of *MtCLE13* also resulted in the differential expression of some more genes among which 2 kelch-repeat containing F-box proteins, which are subunits of E3 ubiquitin ligase complexes, specifying protein substrates for internalization or degradation by the 26S proteasome (**Chapter 5**) (Pickart, 2001; Koepp *et al.*, 2001; Adams *et al.*, 2000; Sun *et al.*, 2007b). Hence, the kelch-repeat containing F-box proteins might reduce root susceptibility for additional nodulation by degrading or internalizing NF receptors or other components of the early NF signaling pathway. The differential expression of *NFP* was shown to be *SUNN*-dependent, suggesting again that the control of nodule numbers by *MtCLE12* and *MtCLE13* might happen via the AON mechanism and that expression of the NF receptor might be a target of the AON process (**Chapter 5**). Interestingly only from part of the differentially expressed genes, the changes were shown to be dependent on *SUNN* (**Chapter 5**). Hence, *MtCLE12* and *MtCLE13* signaling activates both *SUNN*-dependent and *SUNN*-independent processes (Figure 11.1). Further functional analysis as well as a careful expression analysis might reveal more insights into how CLE peptides act on nodule number and interact with the AON pathway.

The expression of *MtCLE12* and *MtCLE13* coincides with the activation and progression of AON which has been shown to be activated at the onset of the establishment of the first nodule primordia (**Chapter 3**) (Li *et al.*, 2009). As SUNN is a LRR-RLK of subclass XI closely related to the CLE peptide receptors, it is generally suggested to bind CLE peptides. Based on our results and recently published data by other groups, it is therefore tempting to speculate that MtCLE12 and MtCLE13 might act as ligands for SUNN in the AON pathway. To do so, the peptides would have to travel to the shoot because grafting experiments have shown that *SUNN*, although also expressed in the phloem of the root and basal part of the nodule vasculature, is active in the shoot for AON (**Chapter 4**) (Delves *et al.*, 1986; Jiang and Gresshoff, 2002;

Krusell *et al.*, 2002; Men *et al.*, 2002; Penmetsa *et al.*, 2003; Francisco and Harper, 1995; Nishimura *et al.*, 2002a). The long-distance root-to-shoot translocation that this hypothesis would entail is in contradiction to the short-distance signaling activities proposed for many CLE peptides (Fukuda *et al.*, 2007; Kurakawa *et al.*, 2007; Whitford *et al.*, 2008; Miwa *et al.*, 2009; Stahl *et al.*, 2009). Alternatively, local perception of MtCLE12 and MtCLE13 in the roots might result in secondary signals that travel to the shoot where they activate other CLE peptides that bind to SUNN to further activate AON. Finally, nodulation-related CLE peptides might control nodule number by acting on the same process as the one on which the *SUNN* pathway acts, without being intertwined with AON. This central process might be the negative regulation of auxin transport in the vasculature, similar to what was reported for CLE peptides in Arabidopsis (Figure 11.1) (Whitford *et al.*, 2008). Indeed, in wild type plants, nodule inhibition is associated with a reduction in polar auxin transport (PAT) from the shoot to the root (van Noorden *et al.*, 2006). In *sunn-1* mutants, no reduction in PAT was seen after inoculation and also a higher auxin flow was measured in *sunn-1* mutants before inoculation (van Noorden *et al.*, 2006). Hence, the absence of any *35S:MtCLE13* phenotypes in *sunn* mutants (**Chapter 4**), might be because the auxin levels are too high and cannot be suppressed to levels low enough to block nodule development. As transcriptome analysis of *35S:MtCLE13* roots did not reflect any changes in expression of hormone-responsive genes (**Chapter 5**), it might be interesting to analyze whether nodulation-related CLE peptides have post-translational effects on proteins involved in hormone signaling or on the level of hormone activation/turnover. The analysis of PIN protein localization in relation to CLE signaling might be of primary interest. Previously it has been shown that the negative effect of exogenous nitrate application on nodulation might be mediated by the AON pathway (Kinkema *et al.*, 2006; Jeudy *et al.*, 2010). In accordance to the inhibition of nodulation by nodulation-related CLE peptides, the partial nitrate tolerant phenotype observed in *sunn-4* mutants, might also be a consequence of the changes in the auxin landscape observed (**Chapter 4**). Indeed, in Arabidopsis, a link between nitrate and auxin transport has recently been resolved (Krouk *et al.*, 2010; Beeckman and Friml, 2010). If nitrate addition would inhibit nodule formation via reducing auxin levels in the root, higher nitrate concentrations would be required in *sunn-4* mutants to reduce the high auxin levels sufficiently to block nodule formation. Whether nitrate inhibition of nodulation involves CLE peptides, is unknown, but several nitrate-induced CLE peptide genes were identified in *L. japonicus* and soybean, which inhibit nodulation in a *HAR1/NARK*-dependent way (Okamoto

et al., 2009; Reid *et al.*, 2011). Currently we favor the hypothesis that many CLE peptides, whether they are expressed in nodules or after the addition of nitrate in the root, or whether they are expressed in the shoot and perceived via shoot-localized SUNN, act on a common component, probably auxin flow and levels, from which a critical concentration is needed to allow nodulation. Many questions still need to be resolved to prove this hypothesis. The interaction between CLE and auxin signaling will reveal more insights. In addition, more CLE receptors active in the root should be identified to support the latter hypothesis, although the root-located SUNN in the vasculature might take part. Reciprocal grafts between *sunn* and wild type in combination with transgenic *35S:MtCLE12* or *35S:MtCLE13* roots might be interesting experiments to perform to further study the relation between *MtCLE12, MtCLE13* and *SUNN*.

In a next step, putative CLE peptide receptors being upregulated during nodulation were searched for based on similarity with SUNN and its orthologs (**Chapter 6**). In *M. truncatula*, qRT-PCR and histochemical analysis of *promoter:GUS* constructs confirmed the nodulation-related expression pattern of *MtRLK1*. *MtRLK1* expression was found in cells adjacent to and partially overlapping with cells in which *MtCLE12* and *MtCLE13* are expressed in the nodule primordium and nodule apex (Figure 11.1). Hence, as CLE peptides are known as short-distance signaling molecules, MtRLK1 might act as a CLE ligand receptor to control stem cell homeostasis in the nodule meristem. In addition, the expression patterns of *MtRLK1* and *SUNN* are overlapping in the root vasculature, suggesting that in these tissues these receptors might interact, possibly to control the auxin landscape to regulate nodule numbers (Figure 11.1) (**Chapter 6**). *MtRLK1* RNAi as well as overexpression might reveal interesting outcomes. Moreover, biochemical analysis should be performed to confirm the interaction between CLE peptides and these putative receptors and might indicate interaction between different receptors. In soybean, two homologous pairs of GmRLKs (GmRLK1-GmRLK2 and GmRLK3-GmRLK4) were found that are differentially regulated during nodulation (**Chapter 9**). One pair of GmRLKs had a similar expression pattern as the *SUNN* ortholog, *NARK*, and might thus control nodule number. The other gene pair was induced upon inoculation and thus might control nodule homeostasis. Functional analysis is needed to further unravel their role. In addition to CLE peptides, *de novo* synthesis of cytokinins by MtLOG1 might also be involved in the control of nodule numbers, as we observed that ectopic overexpression of *MtLOG1* resulted in a reduced nodule number (**Chapter 7**). Because *MtCLE12* and *MtCLE13* were upregulated by *35S:MtLOG1*, we initially taught that both would impinge on the same pathway to in-

hibit nodulation. However, the *35S:MtLOG1* inhibition of nodulation was shown to be local and independent of *SUNN* (**Chapter 7**). Hence, these results suggest that *MtLOG1* might work together with CLE peptides during nodule organogenesis, but not in the control of nodule numbers.

To conclude, in this thesis we have shown that *de novo* cytokinin activation via LOG proteins is needed for normal nodule development. Moreover, our results clearly indicate that CLE peptides play a main role during several aspects of determinate and indeterminate nodulation, more specifically during the control of nodule numbers and in the balance between cell division and differentiation at the level of nodule initiation, nodule maturation and nodule apical meristem. Moreover, we have identified genes that might be involved in the negative feedback mechanism that controls nodule number. Especially the downregulation of the NF receptor, as a way to control nodule number, is interesting and needs further study. Moreover, the interaction of these CLE peptides with the AON pathway is not completely resolved yet but our results opened new possibilities that should be tested in the future.

Summary

Legumes develop root nodules as a result of a symbiotic interaction with soil borne bacteria, called rhizobia. Inside the nodules the bacteria find the ideal niche to fix atmospheric nitrogen for the plant in return for energy sources. Nodule formation is a complex developmental process that requires the spatio-temporal expression of many plant and bacterial genes. Nodulation can be divided in two developmental pathways, bacterial infection and organ initiation, and the two merge when the bacteria are taken up by the plant cells. Two types of nodules have been characterized, determinate nodules such as originating on soybean (*Glycine max*) and indeterminate nodules such as observed on *Medicago truncatula*. Determinate nodules are round-shaped and are terminally differentiated, while indeterminate nodules have a persistent apical meristem. Nodule organogenesis is governed by the bacterially produced Nod factors and downstream of it, cytokinin signaling. As nodulation is an energy-consuming process, legumes developed long distance mechanisms to control nodule number, amongst which autoregulation of nodulation (AON), involving systemic reciprocal signal exchange between the root system and the shoot.

Here, we have shown that structurally related CLE peptides play a main role during several aspects of *M. truncatula* nodulation. During nodulation, CLE peptide genes are activated downstream of the Nod factor and cytokinin signaling cascade and control the balance between cell division and differentiation at the level of nodule initiation, nodule maturation and nodule apical meristem homeostasis. In addition, we could show that CLE peptides control nodule numbers by interacting with the AON pathway. Although the underlying mechanism of this interaction is not yet resolved, our results opened new possibilities to explain the AON pathway. In addition, genes were identified that might be involved in the CLE peptide dependent negative feedback mechanism that controls nodule number. Especially the downregulation of a NF receptor gene, as a way to control nodule number, is an interesting finding. In parallel, we also found nodulation-

related CLE peptide genes that might control determinate nodulation in soybean. In addition, we identified putative receptors of nodulation-related CLE peptides in the genomes of *M. truncatula* and *G. max*. Finally, our results clearly indicate that *de novo* cytokinin activation via a cytokinin riboside 5'-monophosphate phosphoribohydrolase LONELY GUY1 (MtLOG1) of *M. truncatula* is needed for normal nodule development and possibly involves CLE signaling.

Together, this work has shown that apart from the classical phytohormones, CLE peptides greatly contribute to nodulation by controlling cell division and differentiation and restricting nodule number.

Nederlandse samenvatting

De symbiotische interactie tussen rhizobia en leguminosen wordt gekarakteriseerd door de ontwikkeling van nieuwe wortelstructuren, de nodules, waarin bacteriën stikstof fixeren voor de plant. In ruil hiervoor levert de plant energierijke koolstofbronnen en een beschermende omgeving aan de bacteriën. Nodules ontstaan door de initiatie van twee parallele processen: bacteriële invasie en corticale celdeling. Beide processen gaan gepaard met de spatio-temporele expressie van verschillende plantaardige en bacteriële genen en komen samen op het moment dat de bacteriën worden opgenomen door de cellen van het zich ontwikkelende nodule primordium. Twee morfologische types van nodules werden reeds beschreven. Enerzijds gedetermineerde nodules, die onder meer voorkomen op wortels van sojabonen (*Glycine max*) en anderzijds niet-gedetermineerde nodules, zoals waargenomen op *Medicago truncatula*. Gedetermineerde nodules hebben een ronde vorm en worden gekenmerkt door terminale differentiatie, terwijl bij niet-gedetermineerde nodules een persistent apicaal meristeem aanwezig is. Nodule organogenese wordt gecontroleerd door de bacterieel geproduceerde Nod factoren, alsook door de Nod factor geïnduceerde cytokinine signalisatie. Omdat nodulatie een energieconsumerend proces is, hebben planten langeafstandsmechanismen ontwikkeld om het nodule aantal te beperken. Autoregulatie van nodulatie (AON) is één van die mechanismen en bestaat uit een systemische, wederzijdse signaaluitwisseling tussen het wortelsysteem en de scheut.

In deze studie tonen we aan dat structureel gerelateerde CLE peptiden een centrale rol spelen tijdens verschillende aspecten van nodulatie op *M. truncatula*. Tijdens nodulatie, worden deze CLE peptide genen neerwaarts van de Nod factor en cytokinine signalisatieweg opgereguleerd. Daar spelen ze een rol in de balans tussen celdeling en differentiatie, en dit niet enkel tijdens nodule initiatie, maar ook tijdens noduleontwikkeling en in het behoud van het nodule apicaal meristeem. Daarenboven, konden we aantonen dat CLE peptiden bij de controle van nodule aantal betrokken zijn, mogelijks via

interactie met de AON signalisatieweg. Alhoewel de interactie tussen deze nodulatie-gerelateerde CLE peptiden en de AON signalisatieweg nog niet volledig duidelijk is, kunnen uit onze resultaten nieuwe hypothesen afgeleid worden. Daarenboven werden genen geïdentificeerd die mogelijks betrokken zijn bij het negatieve feedbackmechanisme dat het nodule aantal controleert. Voonamelijk de neerregulatie van *NFP* expressie, zou hierbij een mogelijke manier zijn en moet dus verder onderzocht worden. In een tweede luik van deze thesis hebben we CLE peptide genen geïdentificeerd, die betrokken zijn bij gedetermineerde nodulatie op sojaboon. Bovendien zijn we op zoek gegaan naar mogelijke nodulatie-gerelateerde CLE peptidenreceptoren in het genoom van *M. truncatula* en *G. max*. Tenslotte, tonen onze resultaten aan dat *de novo* cytokinine activatie via een cytokinine riboside 5'-monofosfaat fosforibohydrolase LONELY GUY1 (MtLOG1) van *M. truncatula*, noodzakelijk is voor normale nodule ontwikkeling en dat CLE peptidensignalisatie hier mogelijks bij betrokken is.

Kortom, deze doctoraatsthesis toont aan dat naast de klassieke fytohormonen, ook CLE peptidenhormonen een belangrijke rol spelen tijdens nodule organogenese en in de controle van nodule aantal.

Publications

1. **Mortier, V.**, Fenta, B.A., Holsters, M., Kunert, K. and Goormachtig, S. (2011). "Identification of putative CLE peptide receptors involved in determinate nodulation on soybean" *Plant Signaling and behavior*, accepted.

See Chapter 10

2. D'haeseleer, K., Den Herder, G., Laffont, C., Plet, J., **Mortier, V.**, Lelandais-Briere, C., De Bodt, S., De Keyser, A., Crespi, M., Holsters, M., Frugier, F. and Goormachtig, S. (2011). "Transcriptional and posttranscriptional regulation of a NAC1 transcription factor in *Medicago truncatula* roots" *New Phytologist*, accepted.

3. **Mortier, V.**, Fenta, B.A., Martens, C., Rombauts, S., Holsters, M., Kunert, K. and Goormachtig, S. (2011). "Search for nodulation related *CLE* genes in the genome of *Glycine max*" *Journal of Experimental Botany*, 2011 Jan 27. [Epub ahead of print].

See Chapter 9

4. **Mortier, V.**, Den Herder, G., Whitford, R., Van de Velde, W., Rombauts, S., D'haeseleer, K., Holsters, M. and Goormachtig, S. (2010). "CLE peptides control *Medicago truncatula* nodulation locally and systemically" *Plant Physiology*, 153: 222-237.

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