The transcription factor NIN mediates a dynamic balance between gene activation and repression in *L. japonicus*

Dissertation zur Erlangung des Doktorgrades der Naturwissenschaften an der Fakultät für Biologie der Ludwig-Maximilians-Universität München

vorgelegt von

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München, Dezember 2020

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Dissertation eingereicht am:	03.12.2020
Tag der mündlichen Prüfung:	19.03.2021

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Rosa Elena Andrade Aguirre

Para mi familia

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I. List of Abbreviations

° C	degree Celsius
Δ	Delta
µ	Micro
aa	Amino Acid
AD	Activation Domain
AM	Arbuscular Mycorrhiza
ANOVA	Analysis Of Variance
AON	Autoregulation Of Nodulation
ASL18	ASymetric Leaves 2-like 18
BD	Binding Domain
BF	BrightField
CCaMK	Calcium-and Calmodulin-dependent Kinase
ChIP	Chromatin ImmunoPrecipitation
CLE	CLavata 3/Endosperm surrounding region
CLE-RS	CLE Root Signal
CYC-RE	Cyclops Response Element
CPK	Ca^{2+} - sensor Protein Kinase
Da	Dalton
dpi	Days Post Inoculation
dpt	Days Post Transformation
*Ds*Red	*Discosoma* sp. Red fluorescent protein
EMSA	Electrophoretic Mobility Shift Assay
ENOD11	Early Nodulin 11
EPR3	Exopolysaccharide Receptor kinase 3
ERN1	Ethylene Response Factor required for Nodulation 1

EV	Empty Vector
FL	Full Length
FLT	Fluorescent Lifetime
FLIM	Fluorescence Lifetime Imaging Microscopy
FRET	Förster Resonance Energy Transfer
GFP	Green Fluorescent Protein
GUS	β - glucuronidase
HAR1	Hypernodulation Aberrant Root formation
HR	Hairy Roots
HRP	Horse Radish Peroxidase
IPN2	Interacting Protein of NSP2
IT	Infection Thread
LBD16	Lateral organ Boundaries Domain 16a
LCOs	LipoChitoOligosaccharides
Lj	*Lotus japonicus*
LHK1	Lotus Histidine Kinase 1
LRR-RKs	Leucine-Rich Repeat Receptor Kinases
M	Molar
m	Mili
Mt	*Medicago truncatula*
NBS	NIN Binding Site
NFC	Nitrogen-Fixing Clade
NFR	Nod Factor Receptor
NF-Y	Nuclear Factor Y
NIN	Nodule INception
NIN^{FL}	NIN Full Length (1-878 aa)
NIN^{N}	NIN N-terminal (1-296 aa)

NIN^{C}	NIN C-terminal (546-878 aa)
$NIN^{\Delta RWP}$	NIN (1-545..655-878 aa)
$NIN^{\Delta PB1}$	NIN (1-643 aa)
NLP	NIN-Like Protein
NLS	Nuclear Localization Signal
NNC1	Nodule Number Control 1
NPL	Nodulation Pectate Lyase
NRE	Nitrate Response Element
NRSYM1	Nitrate unResponsive Symbiosis 1
NSP	Nodulation Signaling Pathway
OD_{600}	Optical Density at a wavelength of 600 nm
OE	OverExpression
PB1	Phox and Bem1
PCR	Polymerase Chain Reaction
PNR	Primary Nitrate Response
qPCR	Quantitative Polymerase Chain Reaction
RAM1	Reduced Arbuscular Mycorrhiza 1
RINRK1	Rhizobial Infection Receptor-like Kinase1
RNS	Root Nodule Symbiosis
RPG	Rhizobium-directed Polar Growth
SCAR	Suppressor of cAMP Receptor defect
SIP1	SymRK Interacting Protein 1
SN	Spontaneous Nodule
snf	Spontaneous Nodule Formation
SymRK	Symbiosis Receptor Kinase
TA	Transactivation Assay
TF	Transcription Factor

TML	Too Much Love
UAS	Upstream Activated Sequence
µL	Microliter
wpi	Week Post Inoculation
WT	Wild Type
Y2H	Yeast-2-Hybrid assay

II. List of Publications

Rosa Elena Andrade Aguirre, the author of this thesis contributed to the following manuscripts as follows:

Manuscript 1: The transcription factor NIN mediates a dynamic balance between gene activation and repression in *L. japonicus* (in preparation).

Reference: **Andrade, R.E.**, Lambert, J., Chiasson, D, Parniske, M.

Manuscript 2: A novel *cis*-regulatory element enabled the emergence of the root nodule symbiosis (in preparation).

Reference: Cathebras, C., Gong, X., **Andrade, R.E.**, Vondenhoff K., Keller J., Delaux P.M., Griesmann, M., Parniske, M.

List of presentations at scientific conferences

- Poster presentation at the ICNF 2017 in Granada, "The inhibitory role of NIN in nodulation".
- Poster and short presentation at the ENFC 2016 in Budapest, "Do you want to join the complex? Towards the identification of new CCaMK/Cyclops interactors"

III. Summary

The Nodule inception (*NIN*) gene encodes a transcription factor positioned at the top level of the transcriptional regulatory cascade indispensable for the establishment of root nodule symbiosis (RNS), an intimate relationship between legumes and nitrogen-fixing bacteria. Gaining insights into regulation and activity of this transcription factor, is pivotal for the mechanistic understanding of RNS development. A previous model suggested that NIN regulates its own expression via a negative feedback loop by binding to the *NIN* promoter and through protein-protein interaction between NIN and Cyclops (Doctoral thesis, Lambert, 2017). In this study, I confirmed that NIN is involved in the regulation of its own expression by comparing the *NIN* expression between wild type and the *nin-2* mutant. In a transient transactivation assay in *Nicotiana* leaves, NIN inhibited the transactivation of all known CCaMK/Cyclops targets, namely *NIN*, *ERN1* and *RAM1*. To elucidate the mechanism by which NIN interferes with the CCaMK/Cyclops activity, I tested whether NIN recognizes a specific sequence in the promoter of *NIN*. Such sequence was not detected; moreover, NIN was able to inhibit Cyclops transactivation activity independently of the Cyclops DNA binding sequence. These results suggest that the inhibition of Cyclops by NIN is not mediated at the level of DNA binding (for example by competition). The interaction between NIN and Cyclops was confirmed and the interaction domain was delimited to their N-terminal regions. However, the delimited interaction domain of Cyclops was dispensable for the NIN-mediated inhibition. In addition, the presence of autoactive CCaMK versions disrupted the Cyclops/NIN interaction *in vivo*, although NIN was able to inhibit *NIN* expression mediated by this complex. My data suggest that the Cyclops/NIN interaction and the NIN-mediated inhibition of the CCaMK/Cyclops complex are two independent processes. I tested the impact of amino and carboxy-terminal domains of NIN on the local inhibition of nodulation and in the transcriptional repression of *NIN*, and observed that both processes require the carboxyterminal domain, specifically the PB1 protein-protein interaction domain. The following model is suggested: NIN is present at basal levels and interacts with Cyclops; thus, repressing it. When calcium spiking occurs, CCaMK phosphorylates Cyclops and the Cyclops/NIN interaction is disrupted. Cyclops binds to the *NIN* promoter and transactivates its expression (Singh et al., 2014). Once the NIN protein concentration increases, NIN inhibits the transactivation of Cyclops by possibly recruiting other PB1-containing protein. This could be a mechanism that allows a rapid response against further infection by rhizobia in an autoregulation of nodulation (AON)-independent manner.

IV. Zusammenfassung

Nodule inception (*NIN*) kodiert für einen Transkriptionsfaktor an der Spitze einer regulatorischen Signalkette, welche zur Ausbildung der Knöllchensymbiose (RNS), einer engen Partnerschaft zwischen Leguminosen und stickstofffixierenden Bakterien, unerlässlich ist. Zu Verstehen wie dieser Transkriptionsfaktor reguliert und aktiviert wird, ist zentral für das mechanistische Verständnis der RNS-Entwicklung. In früheren Modellen wurde angenommen, dass NIN seine Genexpression über eine negative Rückkopplungsschleife reguliert, indem es an den eigenen Promoter bindet und mit dem Transkriptionsfaktor Cyclops interagiert (Doctoral thesis, Lambert, 2017). In dieser Arbeit konnte ich bestätigen, dass NIN zur Regulation seiner eigenen Genexpression erforderlich ist, indem ich die Transkriptmenge an *NIN* zwischen Wildtyp-Pflanzen und Pflanzen, die im *NIN*-Gen mutiert sind (*nin-2*), verglichen habe. In transienten Transaktivierungsversuchen in *Nicotiana* Blättern inhibierte NIN die Transaktivierung aller bisher bekannten Zielgene von CCaMK/Cyclops, nämlich *NIN*, *ERN1* und *RAM1*. Um den Mechanismus aufzuklären durch welchen NIN die Aktivität von CCaMK/Cyclops beeinflusst, untersuchte ich, ob NIN spezifische Sequenzen in seinem Promoter erkennt. Es konnte allerdings keine solcher Sequenzen identifiziert werden. Des Weiteren war NIN dazu in der Lage, unabhängig vom Vorhandensein einer Cyclops-DNA-Bindesequenz die Aktivität von selbigem zu reprimieren. Diese Ergebnisse ließen darauf hindeuten, dass die Inhibierung von Cyclops durch NIN nicht auf DNA-Ebene (z.B. durch gegenseitigen Wettbewerb um Bindestellen) stattfindet. Ich konnte bestätigen, dass NIN und Cyclops einen Komplex bilden und die Interaktionsdomäne auf die N-terminalen Regionen eingrenzen. Allerdings war der gefundene Interaktionsbereich von Cyclops für die NIN-vermittelte Inhibierung nicht notwendig. Außerdem waren autoaktive CCaMK-Versionen *in vivo* in der Lage, die Interaktion zwischen Cyclops und NIN zu unterbrechen, obwohl NIN die von diesem Komplex ausgelöste *NIN*-Expression inhibieren konnte. Meine Ergebnisse deuten darauf hin, dass die Cyclops/NIN-Interaktion und die NIN-vermittelte Inhibierung des CCaMK/Cyclops-Komplexes zwei unabhängige Prozesse sind. Ich untersuchte den Einfluss von N- und C-terminalen NIN-Domänen auf die lokale Inhibierung von Nodulierung und die transkriptionelle Reprimierung von *NIN* und konnte feststellen, dass beide Prozesse die C-terminale Domäne, insbesondere die PB1-Protein-Protein-Interaktionsdomäne, benötigen.

Folgendes Modell wird vorgeschlagen: NIN ist in basalen Mengen in der Zelle vorhanden, interagiert mit Cyclops und reprimiert diesen. Sobald Calcium-Oszillationen ausgelöst werden, phosphoryliert CCaMK Cyclops und löst die Cyclops/NIN-Interaktion auf. Daraufhin kann

Cyclops an den *NIN* Promoter binden und *NIN*-Expression transaktivieren (Singh et al., 2014). Sobald die Menge an gebildetem NIN ansteigt, inhibiert NIN die Transaktivierung von Cyclops, vermutlich durch Rekrutierung anderer Proteine mit PB1-Domänen. Durch diesen Mechanismus wäre es möglich zeitnah weitere Infektionen durch Rhizobien unabhängig von der längerfristigen Autoregulierung von Nodulierung (AON) entgegenzuwirken.

V. Introduction

1. The nitrogen-fixing root nodule symbiosis

Nitrogen is an indispensable inorganic nutrient that is essential for plants throughout their lives as a component of amino acids, nucleic acids, chlorophyll, coenzymes, and numerous plant secondary products (Frink et al., 1999). It is predominantly present as atmospheric di-nitrogen gas (N_2) which is not directly accessible for plants. Instead, plants rely on reduced nitrogen forms, such as ammonium (NH_4^+) or nitrate (NO_3^-) that they generally absorb from the soil. Thus, agricultural yields are often limited by nitrogen availability (Peoples et al., 1995).

During evolution, some plants acquired and maintained the ability to engage in a mutualistic metabolic symbiosis with nitrogen-fixing bacteria to form the root nodule symbiosis (RNS, Doyle, 2011; Werner et al., 2014). RNS is built upon the trading of reduced carbon from the plant for reduced nitrogen from the bacteria which is hosted within the plant cells, in a specialized organ called the nodule (Roy et al., 2020). On the whole plant level, root nodules represent nitrogen sources and carbon sinks. Assimilated products of nitrogen fixation are exported via the xylem, while the nodule is supplied with the products of photosynthesis, sugars, via the phloem. (Pawlowski and Demchenko, 2012).

Two types of RNS exist between higher plants and nitrogen-fixing soil bacteria, namely legume-rhizobia and actinorhizal symbioses. The classification of the nodules is based on differences in ontogeny and histology. Legumes (Fabales, Fabaceae) and the non-legume *Parasponia* (Rosales, Cannabaceae) host a polyphyletic group of diazotrophic α- and β-proteobacteria collectively referred to as rhizobia. Plants from eight other dicotyledonous families, mostly woody shrubs, also engage into root nodule symbiosis (RNS), and are known as actinorhizal plants because they host diazotrophic filamentous *Actinobacteria* of the genus *Frankia* (van Velzen et al., 2019). Some features of tissue and cell invasion show similarities between actinorhizal and rhizobial symbioses. As for the legume/rhizobia symbioses, bacteria can enter the plant root either intracellularly through an infection thread formed in a curled root hair, or intercellularly. The entry mechanism is determined by the host plant species (Pawlowski and Demchenko, 2012). A striking difference between actinorhizal nodules and legume nodules is reflected in their tissue organization. Nodules produced by legumes are formed in the root cortex, have a stem-like structure with peripheral vascular system and infected cells in the inner tissue. In contrast, actinorhizal nodules, as well as nodules induced by rhizobia on the non-

legume *Parasponia*, are coralloid organs composed of multiple lobes, each of which represent a modified lateral root. Like lateral root primordia they are formed in the root pericycle, and have a central vascular cylinder, without root cap, with a superficial periderm and infected cells in the expanded cortex (Pawlowski and Demchenko, 2012). Recently, it was reported that the ontogeny between legume-type nodules (Fabales) is much more similar to that of actinorhizal-type nodules (*Parasponia andersonni* and *Alnus glutinosa*) than generally assumed (Shen et al., 2020). The authors proposed that both types of nodules share a common evolutionary origin and that legume-type nodules evolved from an actinorhizal-type nodule.

Legumes mainly develop two types of root nodules depending on whether or not the meristem remains active for the life of the nodule. Indeterminate nodules such as the ones in *Medicago truncatula*, *Medicago sativa* (alfalfa), *Pisum sativum* (pea), *Vicia* species (vetches), and *Trifolium* species (clovers) have a persistent meristem at their apex, are continuously infected, and have an elongated shape. The newly infected cells in this type of nodules, and the bacteria inside them, develop further and form new nodule tissue that actively fixes nitrogen. Indeterminate nodules are formed by a gradient of developmental stages, from the young meristem at the nodule tip to the older senescent tissue near the root (Hirsch, 1992; Gage, 2004). In contrast, determinate nodules have a meristem at the periphery that is only active at early stages, and have usually a round shape (Hirsch, 1992; Sprent, 2001). Legumes that form determinate nodules are typically tropical in origin and include *Glycine max* (soybean), *Vicia faba* (bean), and *Lotus japonicus* (Gage, 2004). Both *M. truncatula* and *L. japonicus* have genetic characteristics that make them particularly suitable as model species for the formation of indeterminate and determinate nodules, respectively (Handberg and Stougaard, 1992; Cook, 1999).

The development of the nitrogen-fixing rhizobia-legume symbiosis is the result of a culmination of a complex series of chemical and physical interactions between a compatible pair of bacteria and a plant host. These interactions include signaling processes that trigger changes in gene expression in both partners, shape partner selection, and suppress plant defenses (reviewed in Fliegmann and Bono, 2015; Poole et al., 2018; Roy et al., 2020). Following successful recognition, the establishment of the RNS requires two concomitant events: infection and nodule organogenesis.

For infection, the most common and advanced way is when bacteria attach to the root hair tip and stimulate the root hair to curl (Oldroyd et al., 2011). The bacteria are entrapped within root hairs in a so-called infection chamber formed of plant-derived membrane and cell wall material

(Brewin, 2004). Bacteria multiplies within this chamber and penetrate from the epidermis towards the cortex through a plant-derived tubular infection structure, named infection thread (IT), functioning as a loading route that isolates the rhizobia from the host cell cytoplasm (Rae et al., 1992; Gage, 2004; Jones et al., 2007; Murray, 2011; Fournier et al., 2015). Alternatively, the bacteria can enter the roots through intercellular infection or crack entry (González-Sama et al., 2004; Sprent, 2007).

In parallel, a specialized root organ, the nodule, emerges from cell divisions in the cortex and gets colonized by bacteria released into nodule cells via endocytosis. Inside these infected cells, bacteria differentiate into nitrogen-fixing bacteroids within a unique plant organelle called the symbiosome. The bacteria remain contained within plant membranes from the initial invagination of the root hair membrane to the symbiosome. The symbiosome consists of two membranes: one derived from the plant and one from the bacteria/bacteroids. As such, nutrient exchange between plant and bacteroid requires transport across these two membranes (Oldroyd et al., 2011; Terpolilli et al., 2012). Inside these symbiosomes, bacteria find appropriate physiological conditions to catalyze the conversion of atmospheric N_2 to NH_4^+ by the bacterial enzyme complex nitrogenase (Hoffman et al., 2014).

2. Evolution of the RNS

RNS is a functionally and genetically complex trait with a high developmental and physiological complexity (van Velzen et al., 2019). Plants that are able to engage in RNS belong to the so-called nitrogen-fixing clade (NFC), a monophyletic group comprising the orders *Fabales*, *Fagales*, *Cucurbitales*, and *Rosales* (Soltis et al., 1995; Doyle, 2011). Within this clade, the nodulation trait is restricted to 10 paraphyletic lineages, of which eight nodulate with *Frankia* and two with rhizobia (Geurts et al., 2012). RNS is widespread in the *Leguminosae* family of the Fabales (although not in all legume species), while it is scattered among species of the other three orders. This has led to two hypotheses on RNS evolution. The first one suggests that nodulation evolved from a single origin in the root of the nitrogen-fixation clade, followed by a massively parallel loss of this trait. The second implies parallel evolution of nodulation in some descendants and fewer losses based on phylogenetic ancestral state reconstruction and strong differences in nodules of different lineages (Soltis et al., 1995).

Nodule types and modes of infection within the NFC are diverse. Nevertheless, recent studies indicated that the nodulation trait has a shared evolutionary origin (Griesmann et al., 2018; van Velzen et al., 2018; Shen et al., 2020). Later during evolution, many species within the NFC

lost the ability to establish RNS. In a genome-wide comparative analysis, Griesmann et al. (2018) could not detect any common ancestor gene maintained in all nodulating species. Strikingly, they discovered signatures of multiple independent loss-of-function events for *NIN* (NODULE INCEPTION, Schauser et al., 1999) and *RPG* (RHIZOBIUM-directed POLAR GROWTH, Arrighi et al., 2008), which are exclusively associated with nodulation. van Velzen et al. (2018) conducted comparative studies on gene expression between *Parasponia andersonii* and *M. truncatula* and described that nodules in these different lineages may share a single origin. By comparing the genome of *Parasponia* with its closest non-nodulating relative, *Trema*, they found that *NFP/NRF5*, *RPG* and *NIN* are consistently pseudogenized or lost in *Trema* and other nonnodulating species of the Rosales order. Both studies suggest that RNS is subject to an underestimated negative selection pressure.

As possible factors that could drive the loss of nodulation, Griesmann et al. (2018) suggested either an increased availability of fixed nitrogen in the soils, or that plants avoid this symbiosis due to the emergence of "cheating" bacterial partners that imbalance the trade-off between the costs and benefits of this symbiosis. In contrast, van Velzen et al. (2019) proposed that a factor acting globally and at geological times, such as CO_2 decrease, could explain this widespread parallel loss.

Complex traits, such as the RNS, may evolve by deploying homologous developmental programs. This is likely achieved by modifications of gene-expression networks through the evolution of transcription factors (TFs) (Shubin et al., 2009). Soyano and Hayashi (2014) suggested that RNS evolved from recruiting pre-existing transcriptional networks and modes of gene regulation from the arbuscular mycorrhizal symbiosis, nitrate responses and aspects of lateral root development. The most prevalent cause of phenotypic divergence is thought to result from mutations affecting the activity of *cis*-regulatory sequences rather than the necessity for gene duplication or protein evolution (Carroll, 2008; Wittkopp and Kalay, 2011). Griesmann et al. (2018) suggested that rewiring of existing gene networks via gains or losses of *cis*-regulatory elements could have contributed to the emergence of the RNS trait. The same idea, that the evolution of RNS was a gain of regulatory elements rather than protein coding sequences, was suggested by Doyle (2016).

Arbuscular mycorrhiza symbiosis (AM) is the most ancient plant root endosymbiosis (around 450 million years ago) that most land plants (80-90%) form with fungi of the subphylum Glomeromycotina within the Mucoromycota family (Parniske, 2008; Spatafora et al., 2016; Brundrett and Tedersoo, 2018). The hypothesis that RNS evolved from AM is supported by the

observation that both symbioses share a set of genes, called “common symbiosis genes”, that are required for the successful establishment of both symbioses (Kistner et al., 2005; Parniske, 2008; Delaux et al., 2013b). In addition, it was proposed that the same exocytosis pathway is used for the intracellular hosting of rhizobia as well as AM fungi (Ivanov et al., 2012). The arbuscular mycorrhiza symbiosis promotes the uptake of phosphate and other nutrients by the plant; in return, plants provide up to 20% of their fixed-carbon in the form of hexoses and lipids to the symbiont (Pimprikar and Gutjahr, 2018). The nutrients are collected by a fungal extraradical hyphal network, transported into the root and exchanged in highly branched tree-shaped structures, called arbuscules, within root cortical cells (Choi et al., 2018). Arbuscules are the workhorse of this symbiosis as they are the primary site of mineral nutrient exchange between both partners (Luginbuehl and Oldroyd, 2017). Besides this nutritional benefit, AM also increases the overall fitness of the host plant by an elevated resistance to pathogens and to abiotic stresses (drought, salinity or heavy metals) (Gianinazzi et al., 2010).

3. The root nodule symbiosis signaling pathway

Two model legumes, *Lotus japonicus* and *Medicago truncatula* and two crop species, *Phaseolus vulgaris* (common bean) and *Glycine max* (soybean) have led, through forward and reverse genetic approaches, to the discovery of nearly 200 genes required for RNS in legumes. The host mechanisms that control root nodulation include three distinct processes that can be uncoupled genetically: mutual recognition, infection and nodule organogenesis (Roy et al., 2020).

3.1 Signal perception

Mutual recognition is the first step for the initiation of the intimate symbiosis between rhizobium and host plant and involves a reciprocal exchange of diffusible chemical signals that activate a signaling pathway leading to gene activation (Fig. 1). When nitrogen becomes limiting for growth, legumes secrete specific secondary metabolites of the flavonoid type in the rhizosphere to attract the bacteria. These signals are taken up by rhizobia, bind the transcriptional regulator NodD, and activate a suite of bacterial nodulation genes (Liu and Murray, 2016). Induction of the rhizobial *nod* genes leads to the synthesis of lipo-chitooligosaccharides (LCOs) called Nod factors (NFs), composed of a N-acetylglucosamine (GlcNAc) backbone with a fatty acid attached to the nonreducing terminal glucosamine (Fliegmann and Bono, 2015). NFs from different rhizobia have the same backbone, but they

differ in the length of their backbone, size and saturation of the fatty acid chain, and have additional modifications at either end, such as glycosylation and sulfation. Such decorations within the NF structure contribute to host-rhizobium compatibility and recognition specificity (Denarie et al., 1996; Long, 1996). These key signaling molecules are recognized by the plant and induce a series of specific responses in the root, including root hair deformation, alkalization of the cytosol, host cell membrane depolarizations, calcium influx, and the induction of calcium spiking around the nucleus of epidermal root cells (Oldroyd and Downie, 2004). The latter is a common feature of nodulating species within the nitrogen-fixing clade including legumes, actinorhiza plants and *Parasponia* (Granqvist et al., 2015).

The symbiotic signaling network expands from plasma membrane-localized LysM-type and LRR-type receptor kinases down to a nuclear-localized transcriptional network (reviewed in Kelly et al., 2017; Zipfel and Oldroyd, 2017). In legumes, recognition of the rhizobial NFs involves LysM receptor kinases, namely NOD FACTOR RECEPTOR 1 (*Lj*NFR1) and *Lj*NFR5 *in L. japonicus* and LYK3/NFP in *M. truncatula*, which function in a heterodimeric complex that mediates downstream signaling (Madsen et al., 2003; Radutoiu et al., 2003; Arrighi et al., 2006; Madsen et al., 2011; Broghammer et al., 2012; Moling et al., 2014). In addition, some lipid raft proteins, such as flotillins (FLOT2/4) and symbiosis-specific remorins (SYMREM), interact with and immobilize the receptors in membrane nanodomains to help mediate rhizobial infection (reviewed in Ott, 2017).

The receptors *Lj*NFR1/*Lj*NFR5 interact with the receptor-like kinase *Lj*SymRK (SYMBIOSIS RECEPTOR KINASE) (Stracke et al., 2002; Ried et al., 2014). *SymRK*, which was the first gene identified as a part of the common symbiosis genes, is essential for induction of calcium oscillations by both NFs and chitin-derived molecules for AM symbiosis, named Myc factors (Endre et al., 2002; Stracke et al., 2002; Genre et al., 2013; Sun et al., 2015). Ried et al. (2014) reported that ectopic expression of *LjNFR1*, *LjNFR5* and *LjSymRK* in the absence of rhizobia is sufficient for the activation of signaling cascades that lead to spontaneous nodule (SN) formation in the absence of symbiont and induced the expression of AM and nodulation-related genes. The formation of SN by overexpression of *SymRK* was confirmed also by Saha et al. (2014). Moreover, the intracellular kinase domain of SymRK lead to excessive formation of SN in *M. truncatula* in the absence of *Sinorhizobium meliloti*. Interestingly, in the presence of the symbiont, epidermal infection was restored by the kinase domain but colonization was still compromised (Saha et al., 2014). The authors concluded that ligand-independent deregulated activation of the intracellular kinase domain of SymRK is capable of inducing nodule

organogenesis in the absence of bacteria, but the ectodomain of the receptor is important for the proper colonization of *S. meliloti*.

3.2 The common symbiotic genes

Following NFs perception, several nuclear proteins have been identified in the symbiosis pathway that are shared between RNS and AM symbiosis (Fig. 1). These are the *Lj*SymRK/*M. truncatula* DOES NOT MAKE INFECTIONS 2 (*Mt*DMI2) (Endre et al., 2002; Stracke et al., 2002); nuclear pore complexes including *Lj*NUP85, *Lj*NUP133 and *Lj*NENA (Kanamori et al., 2006; Saito et al., 2007; Groth et al., 2010); two potassium channels *Lj*POLLUX/*Mt*DMI1, *Lj*CASTOR (recently described to act also as a calcium channel) (Ané et al., 2004; Imaizumi-Anraku et al., 2005; Charpentier et al., 2008; Kim et al., 2019), a cyclic nucleotide–gated calcium channel (MtCNGC15, Charpentier et al., 2016); *Mt*MCA8, a calcium pump (Capoen et al., 2011), a calcium calmodulin-dependent protein kinase *Lj*CCaMK/*Mt*DMI3 (Levy et al., 2004; Tirichine et al., 2006), a nuclear coiled-coil *Lj*Cyclops/*Mt*IPD3 (Messinese et al., 2007; Yano et al., 2008), *Mt*VAPYRIN and *Mt*NSP2 (Pumplin et al., 2010; Maillet et al., 2011; Murray et al., 2011).

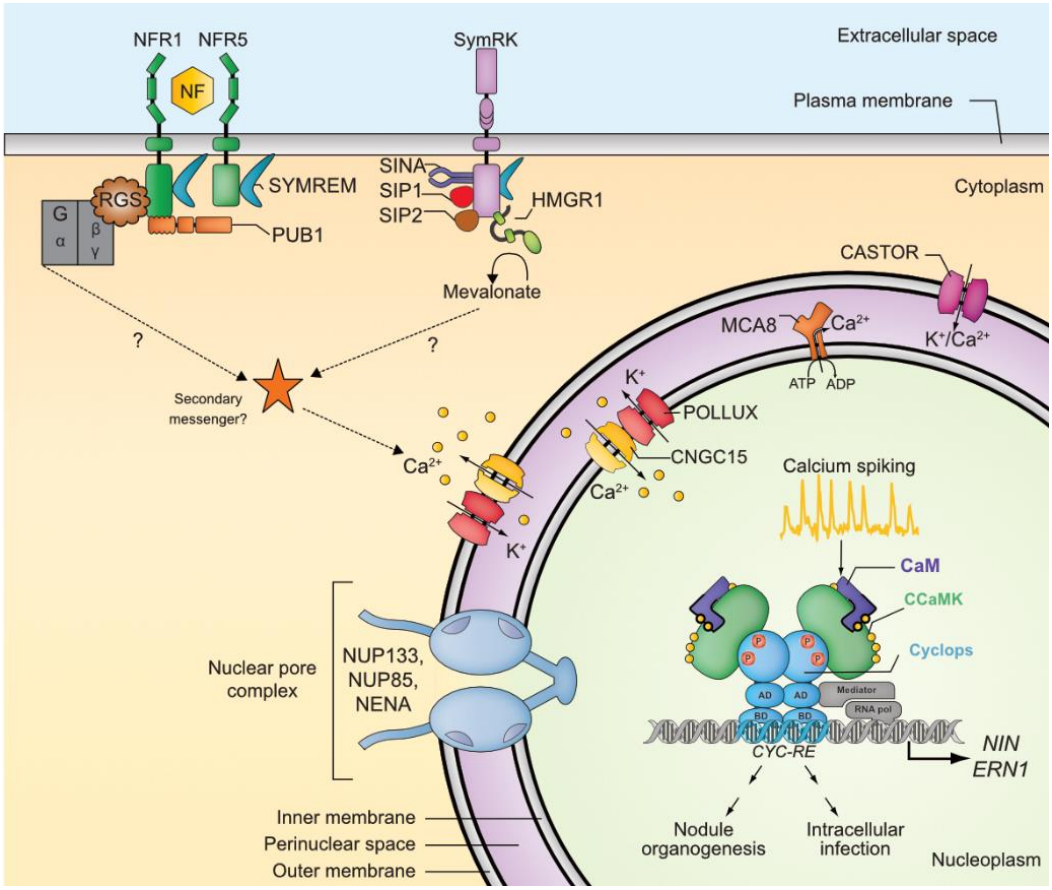


Fig. 1 Overview of symbiotic signal transduction upon perception of Nod Factors in root nodule symbiosis

Rhizobium secretes Nod factors (NFs) which are perceived by the lysine motif receptor-like kinases, named NFR1/NFR5 in *L. japonicus* and LYK3/NFP in *M. truncatula* (Amor et al., 2003; Limpens et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003; Arrighi et al., 2006; Broghammer et al., 2012), which may be recruited to membrane micro-domains by remorins (SYMREM1) and flotillins (Haney and Long, 2010; Lefebvre et al., 2010; Toth et al., 2012). PUB1: is an E3 ubiquitin ligase interacting with the kinase domain of LYK3, and exerts a negative regulatory role on nodulation signaling (Mbengue et al., 2010). The receptors associate with the putative co-receptor-like kinase in *L. japonicus* SymRK and DMI2 in *M. truncatula* (Endre et al., 2002; Stracke et al., 2002; Ried et al., 2014). SINA, SIP1, SIP2 and HGMR1 interact with SymRK (Kevei et al., 2007; Chen et al., 2012; Den Herder et al., 2012). The last one produces mevalonate, a possible activator of nuclear Ca^{2+} oscillations (Kevei et al., 2007; Venkateshwaran et al., 2015). NFR1 interacts with and phosphorylates RGS proteins, which positively regulate nodulation (Choudhury and Pandey, 2015). Mevalonate and the G-protein signaling pathway might be required to stimulate the production of a so far unidentified secondary messenger (Charpentier, 2018). Three components of the nuclear pore complex NUP133, NUP85 and NENA (Kanamori et al., 2006; Saito et al., 2007; Groth et al., 2010) ion-channels such as CASTOR, POLLUX/DMI1 and CNGCs (Ané et al., 2004; Imaizumi-Anraku et al., 2005; Charpentier et al., 2008; Charpentier et al., 2016; Kim et al., 2019) as well as Ca^{2+} transporters (MCA8; Capoen et al., 2011) are required for the generation of Ca^{2+} spikes in the nucleus. A decoder of the calcium spiking signature in the nucleus comprises calmodulin (CaM), CCaMK/DMI3 and its phosphorylation target Cyclops/IPD3 (Levy et al., 2004; Tirichine et al., 2006; Messinese et al., 2007; Yano et al., 2008). Cyclops is displayed as a dimer with two key phosphorylation sites S50 and S154. Cyclops phosphorylation induces a conformational change releasing the DNA-binding (BD) domain from autoinhibition exerted by the N-terminal regulatory domain (Singh et al., 2014). RNA-Pol.: RNA Polymerase II. So far, it has been reported that Cyclops transactivate the expression of *NIN* and *ERN1* via binding to *CYC-RE*s in their promoters (Singh et al., 2014; Cerri et al., 2017). Figure was initially created by Dr. Andreas Binder and is modified based on Singh and Parniske (2012), Singh et al. (2014), and Charpentier (2018).

3.3 Decoding of the calcium signal

Downstream of SymRK, sustained calcium oscillations are activated in the nuclei of epidermal root hair cells, which is referred to as calcium spiking. It is believed that calcium spiking is the core of the symbiosis signaling pathway, as it is required for signal transduction (Ehrhardt et al., 1996; Oldroyd and Downie, 2006; Capoen et al., 2011). It is so far not clear how the signals are transduced from the components on the plasma membrane to those residing in the nucleus. Two possible pathways have been suggested for connecting the receptor like-kinases and nuclear calcium spiking (Charpentier, 2018). The first one involves heterotrimeric G-protein complexes that interact with *Mt*RGS (regulator of G-protein signaling) (Choudhury and Pandey, 2015), and the second one requires the 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (*Mt*HGMR1) which produces mevalonate related metabolites and is sufficient to activate nuclear calcium oscillations (Kevei et al., 2007; Venkateshwaran et al., 2015).

After calcium spiking several transcription factors are activated including *Lj*Cyclops/*Mt*IPD3L, NIN, ERF Required for Nodulation 1/2 (ERN1/ERN2), *Mt*NF-YA1, the GRAS proteins such as Nodulation Signaling Pathway 1/2 (NSP1, NSP2) and DELLAs which will ultimately drive changes in gene expression associated with infection and nodule development (Schauser et al., 1999; Oldroyd and Long, 2003; Kaló et al., 2005; Smit et al., 2005; Heckmann et al., 2006; Andriankaja et al., 2007; Middleton et al., 2007; Yano et al., 2008; Laloum et al., 2014; Fonouni-Farde et al., 2016; Jin et al., 2016; Cerri et al., 2017; Kawaharada et al., 2017a; Jin et al., 2018).

3.4 The CCaMK/Cyclops complex

It is believed that *Lj*CCaMK (*Mt*DMI3) is the prime decoder of symbiotic factor-induced nuclear calcium oscillations as autoactive CCaMK versions were found to suppress loss-of-function mutations of common symbiosis genes required for the generation of Ca^{2+} spiking (Hayashi et al., 2010; Madsen et al., 2010; Singh and Parniske, 2012). Additionally, autoactive forms of CCaMK are sufficient to induce spontaneous nodulation and the formation of the arbuscular mycorrhizal pre-penetration apparatus (Levy et al., 2004; Mitra et al., 2004; Gleason et al., 2006; Tirichine et al., 2006; Hayashi et al., 2010; Takeda et al., 2012; Miller et al., 2013). Using an *in vitro* biochemical approach, Miller et al. (2013) described that calcium concentration is a trigger for the conformational change of *Lj*CCaMK. *Lj*CCaMK can bind Ca^{2+} through two different mechanisms, either directly via EF-hand domains or indirectly through a CaM (calmodulin) binding domain. Ca^{2+} binding affinities of the EF-hands and CaM allow

CCaMK to discriminate between basal and higher Ca^{2+} concentrations that reflect absence or presence of calcium spiking, respectively, which together influence its autophosphorylation state. EF-hands bind calcium at basal concentrations and this ensures the default autophosphorylated state of inactive CCaMK. Mutation of the autophosphorylation residue T265, $CCaMK^{T265I}$, leads to spontaneous nodulation in *L. japonicus* (*snf1-1* mutant) (Tirichine et al., 2006). Hayashi et al. (2010) also reported that $CCaMK^{T265D}$ can complement nodule organogenesis and infection in mutants upstream of CCaMK; however, infection was not restored in *nfr* mutants. Both autoactive versions could only activate nodulation-specific gene expression patterns but not AM-specific genes (Takeda et al., 2011; Takeda et al., 2012). The importance of the autoinhibitory domain of CCaMK was also confirmed in *M. truncatula*, as its deletion leads to the activation of the nodulation signaling pathway with spontaneous nodule formation and nodulation gene expression in the absence of bacterial elicitation (Gleason et al., 2006). These studies highlight that the release of autoinhibition from CCaMK after calmodulin binding is a central switch that is sufficient to activate nodule organogenesis. Moreover, Takeda et al. (2012) reported that the kinase domain alone of CCaMK ($CCaMK^{1-314}$) is sufficient for the induction of both RNS and AM specific genes, *NIN* and *SbtM1*, in *L. japonicus* roots. This suggests that the autoregulatory domain of CCaMK is required for maintaining downstream signaling specificity (Singh and Parniske, 2012). Importantly, the kinase domain alone was also able to restore AM symbiosis and nodule organogenesis but not rhizobial infection in the *ccamk-3* mutant (Takeda et al., 2012). In summary, nuclear calcium spiking induces association of Ca^{2+}-calmodulin with CCaMK, promoting a conformational change in the kinase domain that is then able to phosphorylate its targets (Horvath et al., 2011; Miller et al., 2013).

CCaMK forms a preassembled complex with its phosphorylation target *Lj*Cyclops (*Mt*IPD3) (Messinese et al., 2007; Yano et al., 2008). The *L. japonicus cyclops* mutant phenotype is less penetrant than the one of other common symbiosis genes. In *cyclops-3* mutant roots, curled root hairs are colonized by rhizobia and nodule primordia are also induced. However, the mutant is impaired in IT formation and full colonization by AM. Moreover, $CCaMK^{T265D}$ is able to induce spontaneous nodules in *cyclops* (Yano et al., 2008). These findings suggest that infection is Cyclops-dependent whereas nodule organogenesis is Cyclops-independent, at least in *L. japonicus*. Consistent with this idea, Liu et al. (2019c) reported that the induction of *NIN* by cytokinin is equal for both *cyclops-3* and wildtype. Ovchinnikova et al. (2011) reported that in *M. truncatula* the severity of the mutant phenotype depends on the ecotype, implying possible redundancy. Jin et al. (2018) identified an *IPD3-like* gene in *M. truncatula*. The double mutant *ipd3l/ipd3-2* had a stronger phenotype than each of the single mutants as it was completely

unable to initiate IT formation and nodule organogenesis, resembling the *dmi3-1* mutant (CCaMK), which can only entrap the bacteria. So far, the presence of such homolog has not been reported in *L. japonicus*.

Cyclops is a modular DNA-binding transcriptional activator (Singh et al., 2014). Both the DNA-binding (BD) and activation domains (AD) of Cyclops are located at the C-terminal part of the protein. A minimal version of Cyclops (Cyclops^{min}, aa 255 to 518), which contains only the AD and BD of Cyclops, is sufficient to mediate DNA-binding and transcriptional activation. Singh et al. (2014) suggested that a negative regulatory domain of Cyclops exists within the N-terminal part of the protein.

It has been proposed that the activated CCaMK/Cyclops complex switches the endosymbiotic program on by regulating two nodulation-specific promoters (*NIN* and *ERN1*; Singh et al., 2014; Cerri et al., 2017), and an arbuscular mycorrhiza (AM) related promoter (*RAM1*, Pimprikar et al., 2016). Singh et al. (2014) reported that Cyclops is a phosphorylation substrate of CCaMK. The authors detected five phosphorylated serines by mass spectrometry, of which two were essential for RNS and AM. The phosphomimetic version Cyclops^{DD} (Cyclops $^{S50D\ S154D}$) is able to induce spontaneous nodules, bind and transactivate the expression of the *NIN* promoter. Jin et al. (2018) explored higher phosphorylated versions of Cyclops and reported that Cyclops^{8D} has a lower transcriptional activity than Cyclops^{DD}. This strongly indicates that the phosphorylation level of Cyclops plays an important role for its transcriptional activity in RNS.

The establishment of the CCaMK/Cyclops complex is the last step shared for both RNS and AM symbioses, which leads to the question of how specificity is achieved for one pathway or the other. The specificity upstream of CCaMK can be conceptualized by perception of the according symbiont signals via its receptors; however, the mechanisms conferring transcriptional specificity downstream of the complex remain elusive (Singh and Parniske, 2012). The CCaMK/Cyclops complex is a hub for recruiting a variety of interaction partners, which probably mediate signaling specificity that leads to the activation of the appropriate targets and therefore the correct cell developmental decision. Indeed, a growing number of proteins have been recently described to be directly or indirectly associated with the CCaMK/Cyclops complex (Kudla et al., 2018). It remains to be demonstrated if any of them confer the specificity between the pathways.

4. Nodule inception (NIN) and NIN-like proteins (NLPs)

4.1 Molecular structure of NLPs and NIN

NIN is the founding member of NIN-like proteins (NLPs), a protein family present in all land plants that has even homologs in algae (Schauser et al., 2005; Chardin et al., 2014). Historically, NLPs were described to have six blocks of conservation (Schauser et al., 2005). Blocks I to IV are unique to this protein family, and the latter two (RWP-RK and PB1) are more common protein domains. The function of blocks I-III was unknown and IV was described to be a potential transmembrane domain (TM). However, evidence suggests that NLPs and NIN are localized in the nucleus (Yokota et al., 2010; Soyano et al., 2013; Lambert, 2017; Nishida et al., 2018). It is currently accepted that NLPs contain three major domains, the nitrate responsive domain (NRD), RWP-RK and PB1 (Fig. 2a). Previously, a GAF-like domain was described to be present in the N-terminal part of NLPs (Camargo et al., 2007; Chardin et al., 2014). Nevertheless, the existence of the GAF-like domain in the NLPs is disputed as the folded GAF-related structure has not been confirmed (Mu and Luo, 2019). This N-terminal conserved domain of NLPs is currently referred as the NRD, as it mediates activation of NLP transcription factors in response to nitrate (Konishi and Yanagisawa, 2013; Konishi and Yanagisawa, 2019).

The main difference between NIN and NLPs is their N-terminal region with a deletion of around 210 bp between block I and II, which is attributed to the lack of nitrate responsiveness for NIN (Schauser et al., 2005; Konishi and Yanagisawa, 2013; Suzuki et al., 2013). Suzuki et al. (2013) reported that the N-terminal part of NIN activates transcription regardless of the presence of nitrate, while the N-terminus of NLP6 only does it when nitrate is present. One hypothesis is that NIN evolved from an NLP protein that lost nitrate responsiveness in an ancestral leguminous plant, and has been recruited to regulate nodule development (Konishi and Yanagisawa, 2014). In this work, the predicted TM separated the N-terminal and C-terminal truncated versions of NIN, as depicted in Fig. 2b.

The C-terminal part is highly conserved and contains the DNA-binding and a protein-protein interaction domain, RWP-RK and PB1 (Phox and Bem1, Ponting et al., 2002), respectively (Schauser et al., 2005) (Fig. 2a). The RWP-RK domain contains two α-helices and an amphipathic leucine zipper with the conserved sequence Arg–Trp–Pro–X–Arg–Lys (where X indicates any amino acid, Schauser et al., 1999; Schauser et al., 2005; Liu et al., 2018). NLPs are able to bind via their RWP-RK domain to conserved nitrate-responsive *cis*-regulatory elements (*NREs*) and activate the transcription of several nitrate-induced genes responsible for

nitrate assimilation defined as primary nitrate response (PNR) (Konishi and Yanagisawa, 2013; Marchive et al., 2013; Suzuki et al., 2013; Soyano et al., 2014a; Nishida et al., 2018).

The PB1 domain is a protein-protein interaction domain and consist of about 80 amino acid residues, containing either one or both type I and type II motifs, and are highly conserved among animals, fungi, amoebas, and plants (Korasick et al., 2015). The type I motif contains three glutamate or aspartate residues between β3 and β4 found on the rear surface of the PB1 domain. The type II motif is located on the front surface and it contains an invariant lysine residue in the β1 region (Fig. 2a). The interaction between two PB1 domains thus occurs in a front-to-back manner, with electrostatic interactions between the basic lysine residue in one PB1 domain and the acidic glutamate/aspartate residues in the other (Sumimoto et al., 2007; Korasick et al., 2015). NLPs contain both type I and type II PB1 domains, which can interact with type I, type II, type I/II PB1 domains or even mediate interactions with proteins lacking the PB1 domain (Konishi and Yanagisawa, 2019). The PB1 domain is required for homo and heterodimerizations such as the interaction between AtNLP6/7 with TCP20, which constitutes the molecular link between nitrate signaling and root meristem growth (Guan et al., 2017). Recently, it was described that in the presence of nitrate the interaction between *Mt*NIN and *Mt*NLPs mediated via its PB1 domains inhibits rhizobial infection and nodule formation in a nitrate-dependent manner (Lin et al., 2018).

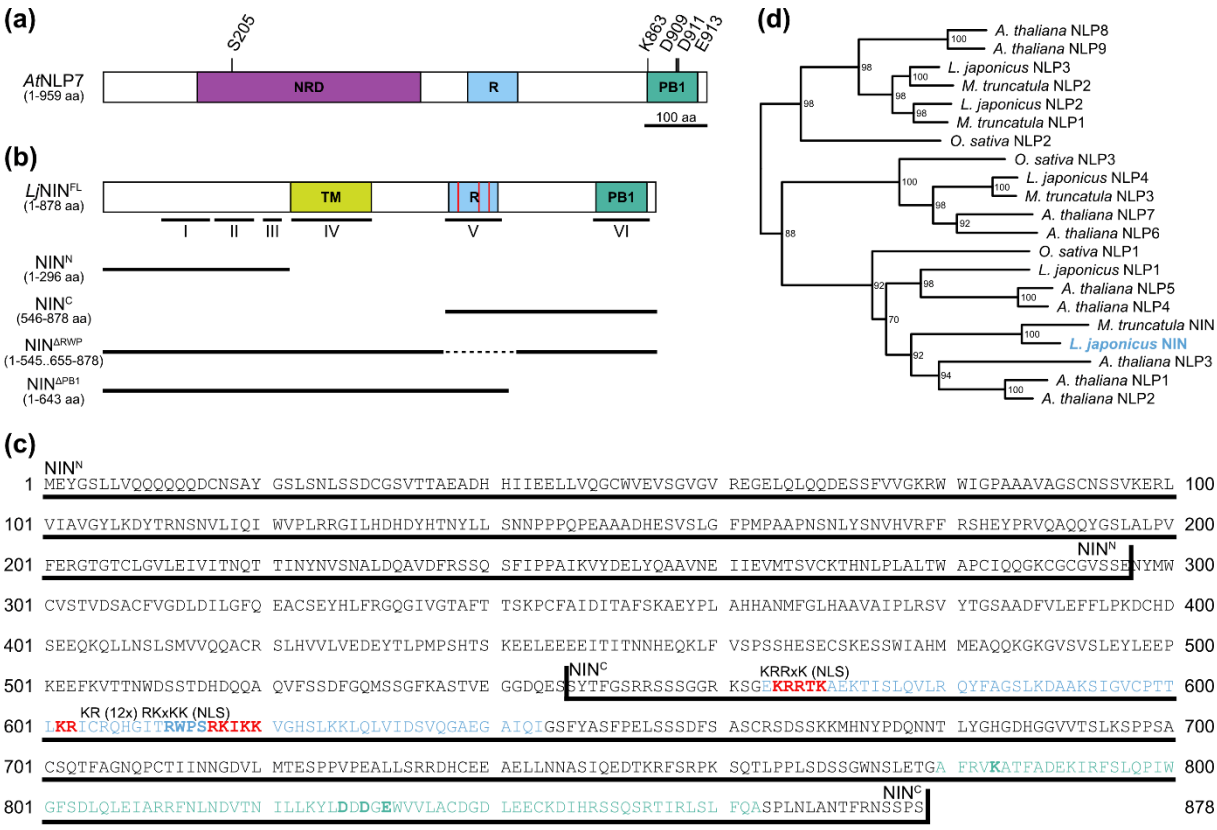


Fig. 2 Domain structure of NIN and NLPs

(a) Protein domain structure of *A. thaliana* NLP7. NLPs contain three domains: nitrate-responsive domain (NRD), the RWP-RK DNA binding domain (R) and the Phox and Bem1 interaction domain (PB1) which are highlighted in purple, blue and green, respectively. The evolutionary conserved S205 within the NRD is an essential phosphorylation site for the nuclear retention of NLPs (Liu et al., 2017). The PB1 domain contains both type I and type II motifs that facilitates protein-protein interactions (Konishi and Yanagisawa, 2019). Type I comprises the three glutamate and aspartate residues (D909, D911, E913), whereas the type II motif contains an invariant lysine residue (K863). (b) Protein domain structure of *L. japonicus* NIN and the truncated versions used in this thesis. The predicted nuclear localization signals in the RWP-RK domain are marked as red lines. Six blocks of conservation (blocks I to VI), which were previously proposed by (Schauser et al., 2005), are indicated by black underlines. (c) Detailed amino acid sequence of *L. japonicus* NIN. The N-terminal and C-terminal parts of NIN are indicated. Within the RWP-RK domain the two NLS are depicted in red. The amino acids corresponding to the type I and type II motifs within the PB1 domain are highlighted in bold. (d) An un-rooted maximum likelihood phylogenetic tree of NIN and NLPs from *L. japonicus*, *M. truncatula*, *A. thaliana* and *O. sativa*. Numbers on each node represent the respective bootstrap values.

4.2 Role of NLPs

NLPs play essential roles in many aspects of plant growth and development via the finely tuned nitrate signaling pathway (reviewed in Mu and Luo, 2019). In *A. thaliana* it has been demonstrated that they play roles in nitrate sensing, uptake and assimilation (Castaings et al., 2009). The molecular mechanism leading to PNR has been characterized in Arabidopsis. Nitrate is perceived by the NITRATE TRANSPORTER 1.1 (NRT1.1) which induces a transient increase in Ca^{2+} concentration (O'Brien et al., 2016). Liu et al. (2017) described that this signal

is perceived by Ca^{2+}-sensor protein kinases (CPKs). CPKs are then able to phosphorylate an evolutionary conserved site (serine 205) in NLP7, a signal that is essential for the retention of this protein in the nucleus to activate genes involved in the PNR. Besides the role of NLPs as master regulators in nitrate sensing, the roles of NLPs for several other processes such as nitrogen/phosphate interactions, root cap cell release, and nitrate signaling during germination have been clarified these last years (Karve et al., 2016; Yan et al., 2016; Maeda et al., 2018).

NLPs in *L. japonicus*

L. japonicus has four NLPs, NLP1 being the closest homolog to NIN and NLP4 within the same clade of the nitrate sensor NLP7 from *A. thaliana* (Suzuki et al., 2013) (Fig. 2d). *Lj*NLP4 and *Mt*NLP1 have been recently identified as key regulators of the nitrate-induced control of nodulation (Lin et al., 2018; Nishida et al., 2018). When nitrate is present, NRSYM1 (*Lj*NLP4) is retained in the nucleus and regulates the production of CLE-RS2, a root-derived mobile peptide that inhibits nodule formation via the systemic Autoregulation of Nodulation pathway (AON, more details in Regulation of RNS). The *nrsym1* mutant is not able to reduce its nodule number at high nitrate concentrations (Nishida et al., 2018). Lin et al. (2018) reported that mutations in NLP genes prevent nitrate inhibition of infection, nodule formation and nitrogen fixation. The mechanism suggested implies interaction between NIN and NLPs through their PB1 domains and/or possibly competition for their similar binding sequences, which ultimately leads to inhibition of NIN targets expression.

Because the C-terminal parts of NIN and NLPs are highly similar, it was hypothesized that both proteins can bind to the same promoters (Schauser et al., 2005). Suzuki et al. (2013) reported already that *Lj*NIN binds to the *NRE in vitro*, although with a weaker affinity than its closest homolog *Lj*NLP1, and activating transcription from an *NRE*-containing promoter *in vivo*. A NIN consensus binding nucleotide sequence (NBS) was determined by *in vitro* experiments, using NIN^{C} in a selected amplification binding assay (SAAB) (Soyano et al., 2014a). Interestingly, the derived sequence was very similar to the *NRE*, which confirmed the hypothesis that NIN might target nitrate-inducible genes with *NREs* in their promoters. Indeed, NIN targets promoters such as *LjNIR1*, *LjNRT2.1* and *LjNIR2.2* as revealed by chromatin immunoprecipitation (ChIP)–PCR analyses (Soyano et al., 2014a). Surprisingly, the expression of these nitrate-inducible genes was neither associated with NIN transcriptional activity nor with rhizobial infection, even if NIN bound to these promoters. Likewise, nitrate also did not up-regulate the expression of two NIN targets, *LjNF-YA1* and *LjNF-YB1* (Role of NIN in nodule

organogenesis, page 27). These results indicate that NIN and nitrate selectively activated symbiotic NIN-transcriptional targets and nitrate-inducible genes whose promoters possess *NBS/NREs*, respectively. Apart from this key discovery, the authors also reported that NIN and nitrate antagonize their action in gene expression. Ectopic expression of *NIN* interfered with the activation of nitrate-dependent genes and nitrate treatment followed by NIN activation down-regulated expression of symbiotic NIN target genes. The ability to repress and activate gene expression might be a consequence of differential regulation of both NIN and NLPs. NIN transcriptional activity is regulated primarily at the transcriptional level, while NLPs are regulated post-transcriptionally in response to nitrate (Soyano et al., 2014a)

4.3 The NIN transcription factor and its transcriptional targets

NIN has been shown to be essential for the establishment of the RNS. It is required for the formation of different types of nodules. In detail, for determinate [e.g., *L. japonicus* (Schauser et al., 1999)] and indeterminate [e.g., *M. truncatula* and *P. sativum* (Borisov et al., 2003; Marsh et al., 2007)] nodules, as well as actinorhizal (-like) [(e.g., *Casuarina glauca* and *P. andersonii*) (Clavijo et al., 2015; Bu et al., 2020)] nodules. *NIN* encodes a nodulation specific transcription factor that plays a key role in both infection and nodule organogenesis processes. In several legumes *nin* mutants display an excessive root hair curling across a widened susceptible zone, but ITs and cortical cell divisions fail to initiate (Schauser et al., 1999; Borisov et al., 2003; Marsh et al., 2007). NIN most likely also plays a role in infection of actinorhizal plants, as shown to be required for *Frankia*-induced root hair deformation in *Casuarina glauca* (Clavijo et al., 2015). This suggests that the role of NIN in infection thread formation is conserved in both legumes and actinorhizal plants.

Transcriptome data in both *L. japonicus* and *M. truncatula* suggest that NIN acts as a central regulatory hub in rhizobial infection, with possibly more roles than the currently described ones (Soyano et al., 2014b; Liu et al., 2019a). New potential targets include genes involved in regulation of rhizobial infection, cell wall modification, nutrient uptake and assimilation, hormonal responses, and in general with cell growth-related processes (Liu et al., 2019a). The experimental evidence of NIN as a transcriptional regulator of putative target genes varies greatly. A compilation of our current knowledge is summarized, highlighting the evidence provided for each of the targets.

Role of NIN in the infection process

NIN plays a key role in rhizobial infection by regulating genes such as *ENOD11*, *NPL, SCARN*, *EPR3, RINRK1* and RPG (Xie et al., 2012; Qiu et al., 2015; Vernie et al., 2015; Kawaharada et al., 2017b; Li et al., 2019; Liu et al., 2019a). *ENOD11* (Early Nodulin 11) encodes a putative repetitive proline-rich cell wall protein that is widely used as marker for early signaling and symbiotic infection responses in *M. truncatula* (Journet et al., 2001). Andriankaja et al. (2007) reported a NFs responsive *cis* element within the *ENOD11* promoter, called *NF-box*, that is regulated by ERN1/2 (ETHYLENE RESPONSE FACTOR REQUIRED FOR NODULATION) upon NF treatment. Vernie et al. (2015) reported that NIN competes with ERN1 binding to the *NF-box* and thus suppresses the transactivation of *ENOD11* by ERN1 in *N. benthamiana* transactivation assays. NIN contributes also to cell wall remodeling, a process required for initiation and elongation of ITs. NIN binds to the *NODULATION PECTATE LYASE* (*NPL*) promoter and regulates the synthesis of an enzyme secreted to the infection chamber and the lumen of the IT, which is hypothetically involved in pectate degradation of the cell wall and therefore potentially implicated in IT initiation (Xie et al., 2012; Liu et al., 2019a). Qiu et al. (2015) reported that NIN could also bind to the *SCAR-NODULATION* (*SCARN*) promoter and transactivate its expression, contributing to the rearrangement of the actin cytoskeleton required for the development of infection threads.

EPR3 encodes for a LysM-type exopolysaccharide receptor kinase that distinguishes compatible and incompatible rhizobial exopolysaccharides at the epidermis (Kawaharada et al., 2015). EPR3 is involved in the intracellular rhizobial infection from epidermal passage to endocytotic release into symbiosomes. Within the *EPR3* promoter potential ERN1 and NIN binding sites were identified, which conferred epidermal and possibly a cortical expression, respectively. Both proteins were able to transactivate the expression of *EPR3* in *N. benthamiana*, although at very low levels (Kawaharada et al., 2017b). Another leucine-rich receptor-like kinase (LRR-RLK) was recently described to be regulated by NIN. RHIZOBIAL INFECTION RECEPTOR-LIKE KINASE1 (RINRK1) was found to be an atypical kinase localized in the plasma membrane with a role on IT formation. Full induction of early infection genes, including *NIN*, required RINRK. In addition, *RINRK1* expression was strongly reduced in *nin-2*, and the activation of its promoter by rhizobia was impaired in this mutant. NIN was also shown to bind to the *RINRK1* promoter and regulate its expression. Because RINRK1 is required for full *NIN* induction and NIN is required for *RINRK1* induction, the authors suggest a positive feedback involving RINRK1 and NIN which could result in amplification of NF

signaling associated with rhizobial infection (Li et al., 2019). Other genes that are important for successful rhizobial infection in *M. truncatula* and depend on NIN include: *RPG* and *CBS1* (Arrighi et al., 2008; Sinharoy et al., 2016; Liu et al., 2019a). The phenotypes caused by mutations in these genes show that they are required for infection, but their precise biological function and the exact mechanism by which NIN potentially regulates them is not clear yet. In *L. japonicus*, it was detected via ChIP-seq that NIN binds to the *RPG* promoter (Soyano et al., 2014b; Liu et al., 2019a); however, evidence of transactivation is still not reported. RPG is a long coiled-coil protein that is nuclear-localized. In *M. truncatula* seems to be required for root hair curling and infection thread formation (Arrighi et al., 2008). RPG is specifically expressed in nodules of *M. truncatula*, *L. japonicus* and *P. andersonii* (Arrighi et al., 2008; Mun et al., 2016; van Velzen et al., 2018), which is consistent with the loss of a functional *RPG* gene in several non-nodulating NFC species (Griesmann et al., 2018; van Velzen et al., 2018). This suggest a symbiotic function in the NFC.

Role of NIN in nodule organogenesis

Nodule organogenesis is mediated by NIN, which promotes cell division in the cortex via targeting the promoters from Nuclear Factor-Y subunit genes (*NF-YA1* and *NF-YB1*), *ASL18/LBD16*, and the cytokinin receptor (*CRE1*) (Soyano et al., 2013; Vernie et al., 2015; Soyano et al., 2019). NF-YA and NF-YB together with NF-YC belong to a CCAAT-box heterotrimeric Nuclear Factor-Y (NF-Y) complex, present in all higher eukaryotes. Of the three subunits, NF-YA determines the DNA-binding specificity (Mantovani, 1999; Myers and Holt, 2018). Mutant analyses in legumes (*L. japonicus* and *M. truncatula*) and in *P. andersonii* have demonstrated that the connection between NIN and NF-YA1 is part of a core genetic network required for rhizobium symbiosis (Liu and Bisseling, 2020). In legumes and *Parasponia*, *NF-YA1* mutations lead to the formation of small nodules and/or reduced nodule numbers (Combier et al., 2006; Soyano et al., 2013; Bu et al., 2020). Apart from defects in nodule organogenesis, aberrant IT formation and block of intracellular infection was also reported in *M. truncatula* and in *Parasponia*, respectively (Laloum et al., 2014; Laporte et al., 2014; Bu et al., 2020). Furthermore, the expression of *NF-YA1* is correlated with the one of *NIN* (Liu et al., 2019b; Bu et al., 2020). This dual role of NF-Ys for infection and nodule organogenesis might be explained through the activation of cell cycle genes (Liu and Bisseling, 2020).

Soyano et al. (2013) provided the first evidence that NIN can act as a transcription factor with both activation and DNA binding properties. They identified both *NF-Y* subunit genes, *LjNF-*

YA1 and *LjNF-YB1*, as transcriptional targets of NIN in *L. japonicus*. Both genes were expressed in the dividing cortical cells of early root nodule primordia and in developing root nodules, and the knockdown of *LjNF-YA1* inhibited root nodule organogenesis but did not have an effect on infection. NIN could bind to the promoter regions of both *NF-Ys in vivo* and *in vitro*. Furthermore, *NIN* overexpression induced in 17% of the plant's root nodule primordium-like structures, without peripheral vascular system, that originated from cortical cells in the absence of bacterial symbionts. Ectopic expression of either *NIN* or both *NF-Y* subunit genes caused lateral roots with malformed tips (Soyano et al., 2013). These results were already pointing towards a common regulatory mechanism that regulates cell proliferation during root nodule and lateral root organogenesis. Moreover, the nodule primordium and the persistent meristem of indeterminate *M. truncatula* nodules have features reminiscent of the formation of lateral roots and the root apical meristem (Mergaert et al., 2020). Indeed, lateral roots and nodules share an extensive genetic and hormonal overlap that converges on the formation and interpretation of auxin maximum in *M. truncatula* (Schiessl et al., 2019). The authors indicated LBD16 as the point of convergence between both programs. Soyano et al. (2019), elegantly demonstrated that NIN can recruit the developmental program of lateral root for root nodule organogenesis through ASL18/LBD16a. NIN was able to bind to the introns of *ASL18a/b*, and this was sufficient for its transcriptional activation and for conferring expression in nodule primordia. A complex between NF-Y and ASL18a formed, and overexpression of *NF-YA1/NF-YB1* together with *LBD16* increased six-fold lateral root densities and induced bump formation in wild type and in *nin-9*. This stimulation of cell division was also observed even in a non-legume, as thicker roots were obtained in tomato. Moreover, co-expression of *ASL18a* and *NF-Y* subunits, allowed some infected nodule primordia in the *daphne* mutant. Altogether, these data strongly suggest that LBD16 stimulates cell division in collaboration with NF-Y subunits.

Activation of cytokinin signaling is a pivotal event in nodule initiation in legumes. External application of cytokinin or autoactivated cytokinin receptors are sufficient to activate cortical cell divisions that lead to the formation of structures resembling nodules (Gonzalez-Rizzo et al., 2006; Murray et al., 2007; Tirichine et al., 2007; Heckmann et al., 2011; Plet et al., 2011). Notably, NFs application results in the accumulation of cytokinin (van Zeijl et al., 2015). In *L. japonicus* the cytokinin receptor is called LOTUS HISTIDINE KINASE 1 (LHK1) and its *M. truncatula* ortholog is CYTOKININ RESPONSE 1 histidine kinase (CRE1). Their mutants are hyperinfected and fail to initiate timely cortical cell divisions in response to rhizobial signals (Gonzalez-Rizzo et al., 2006; Murray et al., 2007; Held et al., 2014). Cytokinin is not only necessary but also sufficient for the induction of cortical cell divisions, as demonstrated in the

lhk1 gain-of-function mutant (*snf2*) in *L. japonicus*, which has a cytokinin hypersensitivity and forms spontaneous nodules in the absence of rhizobia (Tirichine et al., 2007). Three transcription factors act downstream of cytokinin to initiate the nodulation process: NSP2, ERN1 and NIN (Heckmann et al., 2011; Plet et al., 2011). Vernie et al. (2015) reported that NIN in the root epidermis is required to promote cytokinin signaling and nodule organogenesis in the inner root cortex. Moreover, NIN was sufficient to promote the expression of *CRE1* in the root cortex. NIN could bind to the *CRE1* promoter and transactivate its expression. Recently, Lin et al. (2018) reported that the interaction between NIN and NLP1 in *M. truncatula* inhibits the transactivation of *CRE1* by NIN in the presence of nitrate, and therefore suppresses nodulation.

Role of NIN in the autoregulation of nodulation

In addition to its role as a transcription activator of nodulation genes, NIN appears to have a role in the onset of the autoregulation of nodulation (AON), a signaling pathway that dynamically controls nodule number (more details in

Autoregulation of nodulation, page 33). Soyano et al. (2014b) reported that NIN directly targets the promoters of *CLE-RS1* and *CLE-RS2* and activates their transcription, leading to activation of AON. Both *CLE-RS1/2* promoters are expressed in nodule primordia generated beneath infected root hairs as well as in developing nodules, an expression pattern similar to the one of *NIN*. Besides, the induction of both *CLEs* depends on NIN and is mediated by cytokinin signaling. The authors reported that overexpression of *NIN* systemically represses activation of endogenous *NIN* expression in untransformed roots of the same plant in a HAR1-dependent manner, leading to systemic suppression of nodulation and down-regulation of *CLE* expression. Recent evidence suggest that the regulation of AON mediated by NIN is conserved in NFC, as the expression of *CLE9* is also induced in *Parasponia* nodules (van Velzen et al., 2018).

4.4 Regulation of the *NIN* promoter

The spatiotemporal regulation of *NIN* has been studied in detail in *M. truncatula* and *L. japonicus,* and it is highly complex. *NIN* expression is induced exclusively after NFs treatment or rhizobial inoculation, with a characteristic two-phase response, correlated with both the epidermal and cortex response (Schauser et al., 1999; Borisov et al., 2003; Marsh et al., 2007). The epidermal expression of the *NIN* promoter occurs shortly after inoculation and the expression in the root cortex contributes to cell division at a later stage (Heckmann et al., 2011; Kosuta et al., 2011; Popp and Ott, 2011). In the epidermis, *NIN* expression is required for infection. This was demonstrated by *in situ* hybridization, promoter GUS reporter constructs and root hair transcriptome analyses (Breakspear et al., 2014; Yoro et al., 2014; Vernie et al., 2015; Liu et al., 2019b). It has also been reported that *NIN* expression can also be slightly induced by flg22, a peptide derived from bacterial flagellin that triggers innate immune responses (Lopez-Gomez et al., 2012). Likewise, ectopic cortical cell division and *NIN* expression can be induced by exogenous cytokinin application without rhizobial infection (Gonzalez-Rizzo et al., 2006; Murray et al., 2007; Heckmann et al., 2011).

Complementation of *nin* mutants has been a long-standing enigma. Several attempts in different species to fully complement this mutant were not successful. IT formation in the epidermis could be restored, but not nodule organogenesis (Yokota et al., 2010; Soyano et al., 2013; Yoro et al., 2014). Very recently, the first successful complementation of a *M. truncatula nin* mutant was reported (Liu et al., 2019b). The key for complementation relied on the identification of remote cytokinin-responsive elements, which are essential for the cell division initiation in the root pericycle that leads to nodule primordia formation. These elements are located in about 18 kb and 41 kb upstream of the transcriptional start site in *M. truncatula* and *L. japonicus*, respectively. In addition, the authors further dissected the *NIN* promoter and revealed that it is highly modular. *NIN* expression driven by up to 2.2 kb of the promoter could rescue excessive rhizobia-responsive root hair curling and infection-pocket formation, while 5 kb restored IT formation. This modularity of the *NIN* promoter was already reported for the *L. japonicus daphne* mutant, a weak *nin* allele, whose coding sequence is intact but its promoter is altered by a chromosomal translocation 7 kb upstream of *NIN* exhibiting no nodule organogenesis but hyperinfection (an excessive number of ITs (Yoro et al., 2014). This, together with the expression pattern of *NIN*, strongly indicates that the regulation of the *NIN* promoter involves close but also very distant upstream regions that modulate its proper spatio-temporal expression.

The experimental evidence for regulators of the *NIN* promoter is highly variable. Hirsch et al. (2009) were the first to report that two GRAS domains proteins, NODULATION SIGNALING PATHWAY 1 (NSP1) and NSP2, could potentially modulate the expression of the *NIN* promoter. NSP1 and NSP2 formed a complex that bound the promoters of *ENOD11*, *ERN1* and *NIN* though an AATTT *cis*-element. The binding site of NSP1 was delimited to -892bp to -13bp upstream of the transcriptional start site within the *NIN* promoter of *M. truncatula*. Notably, the induction of *NIN* and *ERN1* by rhizobia required both NSP1 and NSP2 as assessed by RT-PCR. There is however, so far, no evidence that NSPs are able to transactivate the expression of the *NIN* promoter directly. NSP1 is constitutively expressed with no marked change upon rhizobia inoculation and localizes to the nuclei of all epidermal and cortical cells (Smit et al., 2005). In contrast, *NSP2* expression is induced by *S. meliloti* and NF treatments, and NSP2 fully localizes to the nucleus upon NF treatment (Kaló et al., 2005). Still, subcellular localization should be taken with caution, as in both studies protein localization of either NSP1 or NSP2 was investigated upon expression from constitutive promoters. *nsp* mutants are impaired in the induction of cortical cell divisions and blocked on IT formation. Genetically, NSPs were positioned downstream of DMI3 (CCaMK) (Kaló et al., 2005; Smit et al., 2005) and LHK1, as their mutants crossed with *snf1/snf2* failed to form spontaneous nodules (Gleason et al., 2006; Hayashi et al., 2010; Madsen et al., 2010). It was recently hypothesized that the GRAS domain protein DELLA bridges the interaction between NSP1/NSP2 to the CCaMK/Cyclops complex, which may act in concert to regulate the expression of the *NIN* promoter (Jin et al., 2016). Taken together, NSPs have been considered to be key regulators for RNS. Interestingly, *NIN* expression during symbiotic interaction with rhizobia was induced in the *nsp1* mutant (Jin et al., 2016), indicating that other proteins might be involved.

IPN2 (Interacting Protein of NSP2), a MYB coiled-coil type transcription factor belonging to the GARP protein family, is another protein that was shown to bind to the *NIN* promoter via *in vitro* EMSA. Downregulation of *IPN2* caused a decrease in nodule numbers and infection. Additionally, *NIN* and *ENOD40-1* transcripts were reduced in *IPN2-RNAi* roots. Indicating that IPN2 plays a positive role in the regulation of infection and nodule formation (Kang et al., 2014). Evidence of direct regulation of the *NIN* promoter by IPN2 has been very recently presented by Xiao et al. (2020). The authors described the phenotype of *ipn2-1*, a *LORE1* mutant, which displayed defective phloem cells in the vascular tissue and was severely impaired in establishing rhizobia-induced symbiotic responses. The expression of genes such as *NIN*, *NF-YA1*, *NPL* and *ERN1* was also strongly reduced in the *ipn2-1* mutant. IPN2 was able to specifically bind and transactivate the expression of the *NIN*, *NPL* and *ERN1* promoters. Xiao

et al. (2020) identified a *cis* element within the *NIN* promoter, *IPN2-RE* located between -192 bp to -162 bp, where IPN2 could directly bind and mediate the expression of the *NIN* promoter. Interestingly, NSP1 also bound to the *IPN2-RE*. In *N. benthamiana* transactivation assays, the addition of NSP1 significantly decreased the IPN2-mediated transactivation of either the *NIN* promoter or *2xIPN2-RE*. In contrast, the addition of both NSP1 and NSP2 enhanced the transactivation mediated by IPN2. The authors proposed a new model where IPN2, NSP1, and NSP2 form a trimeric complex that targets the *NIN* promoter and modulates its expression.

It was first reported in *L. japonicus* that Cyclops and CCaMK^{T265D} mediate the transactivation of the *NIN* promoter. Furthermore, Cyclops^{DD} (Cyclops $^{S50D\ S154D}$), an autoactive and phosphomimetic version of Cyclops, is able to induce spontaneous nodules, bind to a *cis* responsive element (*CYC-RE*) within the *NIN* promoter and activate its expression (Singh et al., 2014). The *CYC-RE* resides within a 30bp region containing a palindromic sequence, referred to as *CYC-box* (TGCCATGTGGCA), located between -717 bp and -683 bp upstream of the transcriptional start site. The presence of this palindromic sequence suggests that Cyclops might bind to the *NIN* promoter as a dimer. Interestingly, Cyclops binding site is conserved in legume *NIN* promoters and in *P. andersonii* (Liu and Bisseling, 2020).

There are reports of other proteins that can bind to the *NIN* promoter, but the regulatory mechanisms are not yet understood. A SymRK interacting protein (SIP1) bound to the *NIN* promoter in yeast one-hybrid (Y1H) and electrophoretic mobility shift assays (EMSA). The binding was delimited to an AT-rich region located at -69 to -59 upstream of the transcriptional start site (Zhu et al., 2008), contained within the minimal *NIN* promoter (Singh et al., 2014). The role of SIP1 in RNS was not clear. *SIP1* is constitutively expressed but was weakly upregulated 5h after *M. loti* treatment. Notably, SIP1 can bind to DNA via its N-terminal part, but it does not contain an AD. The authors hypothesized that SIP1 functions as a DNA sequence-specific binding protein that forms hetero-oligomers with another unidentified protein that contains an AD or repressor domain (Zhu et al., 2008). Wang et al. (2013) reported the existence of a longer splice variant of SIP1, whose transcript is more abundant than the short version. However, only the truncated version interacted with SymRK. Downregulation of *SIP1* by an RNAi approach impaired nodule organogenesis (although not the infection process) and AM development (fungal hyphae penetrated, but failed to enter cortical cells). Moreover, overexpression of either *SIP1L* or *SIP1S* increased the number of nodules, indicating a positive role of each variant in nodulation. Remarkably, even though SIP1 appeared to bind the *NIN* promoter, the *NIN* transcript was not affected in the *SIP1-RNAi* roots (Wang et al., 2013). The mechanism by which SIP1 regulates *NIN* expression is still unknown.

5. Regulation of RNS

Plants that establish RNS thrive on nitrogen-poor soils where other plant species generally struggle to survive. However, excessive root nodule formation can be detrimental to the growth of the host plant by over-consumption of plant-derived energy resources. RNS carries a high metabolic cost for the host plants as forming and maintaining nodules is resource intensive. It is estimated that legumes dedicate 10-20% of their carbon to nodules. Energy is not only consumed to drive rhizobial nitrogen fixation, but also to transport the fixed nitrogen to the other tissues (Phillips, 1980; Yoshida et al., 2010; Ferguson et al., 2019). To keep an optimal balance of gains and costs, plants have developed mechanisms that allow them to control each step of nodulation in concert with endogenous and environmental inputs (Oka-Kira and Kawaguchi, 2006; Reid et al., 2011; Nishida and Suzaki, 2018b).

5.1 Autoregulation of nodulation

One of the negative regulation systems that plants possess is a systemic negative feedback regulation called autoregulation of nodulation (AON), where earlier nodulation suppresses the development of new nodules (Caetano-Anolles and Gresshoff, 1991; Oka-Kira and Kawaguchi, 2006; Suzaki et al., 2015).

Rhizobial infection induces the production of root-derived nodulation-specific peptides belonging to the CLAVATA3/EMBRYO SURROUNDING REGION-related (CLE) family, named in *L. japonicus*, CLE-ROOT SIGNAL 1 (CLE-RS1), -RS2 and -RS3, in *M. truncatula* CLE12/13 and in *G. max* RHIZOBIA INDUCED CLE1 (RIC1) and RIC2 (reviewed in Roy et al., 2020). Mature and functional CLE peptide ligands are around 12–13 amino acids that have undergone post-translational modifications before being secreted to the xylem and transported to the shoot (Okamoto et al., 2013). In the shoot, these CLE peptides are thought to be recognized by phloem-expressed leucine-rich repeat receptor-like protein kinases (LRR-RLK), specifically HYPERNODULATION AND ABERRANT ROOT FORMATION 1 (HAR1) that is proposed to form a receptor complex with another LRR-RLK, KLAVIER (KLV), and an LRR-RL protein, *Lj*CLAVATA2. HAR1 orthologs have been identified as SUPER NUMERIC NODULES (SUNN) in *M. truncatula* and NODULE AUTOREGULATION RECEPTOR KINASE (NARK) in *G. max*. The perception of the nodulation-suppressing CLE peptides by the receptor(s) activates the generation of shoot-derived inhibitors (SDIs), which travel to the roots via the phloem to suppress further nodule development (Nishida and Suzaki, 2018b; Ferguson et al., 2019). The identity of this molecule is still debatable, although some evidence has revealed that

an increase in shoot cytokinin production mediated by a ISOPENTENYL TRANSFERASE (IPT3) may have a role in the AON pathway (Sasaki et al., 2014).

The inhibitory effect of AON requires TOO MUCH LOVE (TML), a root- active Kelch-repeat F-box protein which forms part of an E3 ubiquitin ligase complex. TML is possibly involved in proteasomal degradation of target proteins with positive roles in nodulation, considered the late step in AON (Magori and Kawaguchi, 2009; Takahara et al., 2013; Suzaki et al., 2015). Deleterious mutations in either *HAR1* or *TML* result in an excessive nodule formation (hypernodulation) phenotype as well as a significant increase in epidermal infection threads (Wopereis et al., 2000; Magori et al., 2009). These observations already implied that HAR1 and TML and, by inference, AON *per se*, regulate not only nodule formation but also root susceptibility to infection (Miri et al., 2019). Indeed, a mechanism involving a microRNA, miR2111, and TML was described for ensuring susceptibility to infection. miR2111 acts as a shoot-derived activator of nodulation that targets the root-localized *TML* mRNA, which is regulated by *HAR1* and *LHK1* (Tsikou et al., 2018).

In soybean, a mechanism involving other miRNA, referred as the NMN regulatory module, has been recently described to control nodule number by orchestrating dynamic cross talk between nodulation and long-distance feedback signaling in soybean (on-off switch for AON) (Wang et al., 2020). The module consists of two positive regulators, NIN (GmNINa) and MicroRNA172c (miR172c), and as suppressor NODULE NUMBER CONTROL 1 (NNC1). This module starts with the activation of *miR172c* by GmNINa, which targets *NNC1* for degradation and leads to nodulation (Wang et al., 2014). NNC1 acts as a repressor of the early nodulin *ENOD40* and of *miR172c*, forming a negative feedback loop. Wang et al. (2019) described that NIN and NNC1 interact with each other and antagonistically regulate *GmRIC1* and *GmRIC2* expression by competing for *cis*-element binding, which leads to AON activation or attenuation. Conversely, activation of AON inhibits *GmNINa* and *miR172c* expression, thereby reducing their inhibitory effects on NNC1 to attenuate both nodulation signaling and AON.

5.2 Nitrogen regulation of nodulation

The fact that legumes are able to reprogram nodulation in response to changing environments underpins its adaptation capacity. RNS is negatively regulated when sufficient nitrogen is available in the soil, thereby enabling plants to save the cost associated with nodulation. Upon increasing nitrogen concentrations, nodule number is gradually reduced and even completely abolished when nitrate is sufficient (Nishida and Suzaki, 2018a; Ferguson et al., 2019).

Although the sites of nitrate action are different among leguminous species, high nitrate generally inhibits pleiotropic phases of RNS, including production of flavonoids, rhizobial infection, nodule initiation, nodule growth, nitrogen fixation activity. These responses are quickly reversible when nitrate is removed (Nishida and Suzaki, 2018a). To explain the pleiotropic effects of nitrate on RNS, two possible hypotheses have been proposed. The first one may be related to a relocation of carbon from the plant, implying less carbon in the nodules and more in lateral root growth. The second might involve a nitrate-induced inhibition of the nitrogenase activity, probably manipulating the bacteroid metabolism through NODULE-SPECIFIC CYSTEINE-RICH peptides (NCRs) (Nishida and Suzaki, 2018a).

Barbulova et al. (2007) reported that AON requires a few days for its mechanism to be active, while nitrogen regulation of nodulation acts faster. The induction of *NIN* was prevented within 24 hours after nitrate or ammonia treatments. In split-root experiments, systemic AON signaling takes about three days to inhibit nodule formation (Suzuki et al., 2008). Conversely, sufficient inhibition of nodule growth and nitrogen fixation activity is observed within one day of nitrate treatment. On the basis of this quick response, the latter two processes may be regulated locally in the root (Nishida and Suzaki, 2018b).

It has been proposed that the molecular mechanism underlying the control of nodulation by nitrate may involve systemic components of the AON, as both nitrate and rhizobia promote the expression of *LjCLE-RS2* connecting nodulation, CLEs, and nitrate signaling. However, AON alone is insufficient to fully explain the pleiotropic regulation induced by nitrate (Okamoto et al., 2009; Nishida and Suzaki, 2018a). For instance, high nitrate application reduced the number of ITs and inhibited nodule development in a *HAR1* independent manner, which differs from nodule suppression (Soyano et al., 2014a; Okamoto and Kawaguchi, 2015; Nishida et al., 2018). Another layer of complexity arises by analyzing inhibition of nodulation by nitrate in other species. For instance, the nitrogen regulation pathway acts locally in the roots as demonstrated by split-root experiments in soybean (Ferguson et al., 2019). It is however possible that some components of AON might also be used for the inhibition of nodulation by nitrate. The separation for both mechanisms has been challenging since AON-related hypernodulating mutants such as *har1*, *klv*, *tml* retain nodule formation even in presence of a high nitrate concentration (Krusell et al., 2002; Oka-Kira et al., 2005; Magori et al., 2009).

Nishida et al. (2018) described *nrsym1* (*nitrate unresponsive symbiosis 1*), a mutant with normal nodule number but unable to inhibit nodulation upon high nitrate concentrations. NRSYM1 (NLP4) is a key regulator for inhibition of nodulation by nitrate by regulating the expression of

NIR1 and *CLE-RS2* promoters in a nitrate-dependent manner. Both genes are also NIN targets (Soyano et al., 2014b; Soyano et al., 2014a). Interestingly, NRSYM1 has a higher affinity to bind to the *CLE-RS2* promoter than NIN (Nishida et al., 2018). Given that NRSYM1 and NIN bind to the same *cis*-element there might some competition between these two proteins for the control of their common target genes expression (Nishida and Suzaki, 2018a). This was already observed in transient expression assays where the transcriptional activation of *NRE* by *L. japonicus* NLP1 is competitively down-regulated when NIN is included (Suzuki et al., 2013). Nishida et al. (2018) conclude that the signaling pathway NRSYM1>CLE-RS2>HAR1 plays a pivotal role for the control of nodule number by nitrate, while other processes such as rhizobial infection, nodule cell-size, and nitrogen fixation activity are controlled through an AON-independent mechanism, where NRSYM1 likely uses different downstream targets.

5.3 Ethylene regulation of nodulation

The involvement of the plant hormone ethylene in nodulation was initially proposed from pharmacological studies showing that the application of exogenous ethylene or its biosynthetic precursor ACC (1-amino-cyclopropane-carboxylic acid) severely inhibits calcium spiking, symbiotic gene expression, IT development, and nodule organogenesis (Penmetsa and Cook, 1997; Oldroyd et al., 2001). Conversely, application of the ethylene biosynthesis inhibitor, AVG (amino ethoxyvinyl glycine) increases nodule numbers and IT numbers (Nukui et al., 2000; Heckmann et al., 2011).

The gaseous hormone ethylene plays both positive and negative roles in the regulation of nodulation. Under favorable growing conditions, ethylene is involved in calcium spiking initiation, IT formation and nodule primordium maintenance. It is also thought to act in positioning the nodule around the root and interacts with other phytohormones to regulate nodule organogenesis. In contrast, under environmental stress high levels of ethylene have a strong inhibitory effect on RNS (reviewed in Guinel, 2015; Ferguson et al., 2019). Ethylene can also influence the type of rhizobial infection according to the environment, as observed with the semi-aquatic tropical legume *Sesbania rostrata.* In non-flooded conditions ethylene promotes the NF dependent initiation of infection pockets and cell divisions; however, when the roots are flooded, it promotes intercellular infection via crack entry at lateral root bases (D'Haeze et al., 2003; Goormachtig et al., 2004).

Ethylene-insensitive mutants are hypernodulating and hyperinfected as reported in both *M. truncatula* (*ethylene insensitive 2*, *ein2*, formerly called *sickle*) and *L. japonicus* (Penmetsa and

Cook, 1997; Miyata et al., 2013; Reid et al., 2018). The *Mtein2* mutant is also unable to control infection by mycorrhizal fungi, as well as infection by fungal and oomycete root pathogens (Penmetsa et al., 2008). The ethylene-dependent inhibition of nodulation acts in a distinct genetic pathway from that of AON, as *sunn* is ethylene sensitive and the *sunn/sickle* double mutant has an enhanced hypernodulation phenotype (Penmetsa et al., 2003).

Reid et al. (2018) developed a sensitive laser-based assay for determining ethylene emission from *L. japonicus* that allowed detection of ethylene production as early as 6 hours after inoculation with *M. loti* with a peak at 24 hours. The ethylene response depends on the nodulation pathway as well as NF production by compatible rhizobia. Upon *M. loti* inoculation, mutants in the receptors and CCaMK failed to increase ethylene production, while transcription factors acting downstream of calcium spiking, such as NSPs, Cyclops, NIN and ERN1, showed moderate to no effect in the ethylene response. Notably, ethylene production was elevated in spontaneously nodulating mutants (both *snf1* and *snf2*). The high ethylene production in *snf2* is consistent with the well-established cytokinin-ethylene crosstalk for the local regulation of infection (Miri et al., 2019).

6. Aim of the Thesis

The establishment of RNS requires three processes: intracellular infection, nodule organogenesis, and a negative feedback mechanism to ensure an optimal number of nodules. The transcription factor NODULE INCEPTION (NIN) plays an indispensable role in all these processes. NIN has a conserved key position for RNS, as suggested from both functional and phylogenomic analysis. Thus, it has been postulated that the recruitment of NIN in the nodulation process seems to be the key step in the birth of this symbiosis (Liu and Bisseling, 2020). As a result, extensive research about new potential targets of NIN as well as regulation of its promoter have been carried out.

Recent studies have shown that a gene involved in lateral root development has been co-opted for nodule organogenesis downstream of NIN and that there is an extensive overlap between both processes (Schiessl et al., 2019; Soyano et al., 2019). This discovery has drawn attention to the importance of studying in detail how NIN works, which could bring us closer to the vision of transferring this beneficial symbiosis to non-leguminous crop plants.

Despite all the previous research performed on NIN, knowledge about the regulation of its own expression remained limited. It was previously hypothesized that NIN is able to regulate its own expression (Doctoral thesis, Lambert, 2017). However, the evidence was mainly obtained with overexpression experiments and the mechanism for inhibition was not clear.

Therefore, the aims of this doctoral thesis were to I) investigate whether NIN indeed plays a role in the regulation of its own promoter in a non-overexpression context, II) dissect the activating and repressing potential of NIN and III) describe by which possible mechanism NIN inhibits the Cyclops-mediated transcriptional activation of its targets. To do so, I analyzed the specificity of this inhibition, tested if the NIN-mediated inhibition requires a specific sequence in the *NIN* promoter, confirmed the Cyclops/NIN interaction, delimited its domains, analyzed whether the addition of CCaMK influences it and assessed the effect of this physical interaction on the inhibition, and delimited the specific NIN sequence required for the local inhibition of nodulation by *NIN* overexpression.

VI. Results

1. NIN is required for the regulation of its own promoter in *Lotus japonicus* roots

During nodule development the spatio-temporal expression pattern of the *NIN* gene is dynamic (Tirichine et al., 2006; Heckmann et al., 2011; Kosuta et al., 2011; Yoro et al., 2014). We investigated whether NIN plays a role in the regulation of its own expression. Therefore, I compared the activity of the *NIN* promoter between WT and *nin-2* upon inoculation with *M. loti* in HRs of the model legume *L. japonicus*. The *nin-2* mutant contains a premature stop codon caused by a frame shift due to a transposon footprint and is considered to be a null mutant (Schauser et al., 1999).

Seven days post inoculation, the activity of the *NIN* promoter was restricted to nodule primordia in WT plants. In contrast, the *NIN* promoter activity in the *nin-2* mutant was deregulated with a strong patchy induction in the epidermis possibly correlated to infected root hairs (Fig. 3a). *NIN* transcript accumulation in both WT and *nin-2* under mock and *M. loti* treatment was quantified by qPCR. A low *NIN* expression for both WT and *nin-2* mutant under mock conditions was detected. At 7 dpi, in *nin-2*, a ten-fold increased *NIN* transcript abundance relative to the wild-type upon inoculation with *M. loti* (34 vs 346-fold) was observed, which is partially explained by a lower *NIN* expression under mock conditions in the mutant (Fig. 3b). A higher *NIN* transcript accumulation in *nin-2* compared to WT was also detected at 3 dpi. These results suggested that in the presence of *M. loti*, NIN inhibits its own expression.

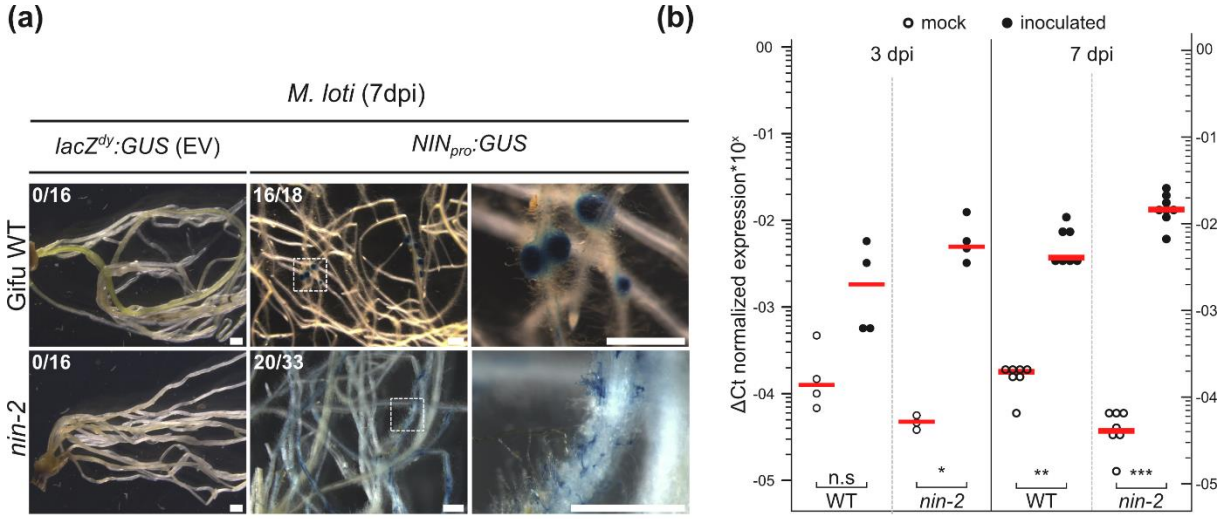


Fig. 3 *NIN* positively and negatively influences its own expression.

The expression profile of *NIN* was analyzed in roots of *L. japonicus* WT and *nin-2* at 7 days post-inoculation (dpi) with *M. loti Ds*Red by (a) the localization of a NIN_{pro}*:GUS* reporter fusion and (b) by quantification of *NIN* transcript abundance by qRT-PCR. (a) Hairy roots of *L. japonicus* WT and *nin-2* transformed with the empty vector (EV, $lacZ^{dy}$*:GUS*) or with the 3 kb *L. japonicus NIN* promoter driving the expression of the *β-glucuronidase* (*GUS*) reporter gene (NIN_{pro}*:GUS*) were generated. Reporter activation was visualized by blue GUS staining of transformed roots. Transformed plants were identified by expression of the *GFP* transformation marker. Dotted squares mark the area magnified in the images on the right. Leading numbers indicate roots with blue staining either of nodules (for WT) or deregulated *NIN* expression in the epidermis (*nin-2*), and are compared to the total number of transgenic root systems analyzed for that genotype and condition. Bars: 1mm. (b) *NIN* transcript abundance in mock-treated samples (50 ml FP medium) or in samples inoculated with *M. loti* was determined at 3 and 7 dpi in four and seven biological replicates for each genotype, respectively. For each genotype and condition 3-4 root systems (~100 mg) were pooled and treated as one biological sample. Transcript levels for every plant genotype and each treatment were determined with technical duplicates. Circles indicate expression relative to the housekeeping gene *EF1alpha*. Open circle, mock-treated; black circle, inoculated with *M. loti*. Statistical analysis was performed based on a Welch t test (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; n.s non-significant).

2. NIN negatively interferes with CCaMK/Cyclops-mediated transcriptional activation of *NIN*, *ERN1* and *RAM1* targets

A transcriptional regulator of *NIN* expression is the CCaMK/Cyclops complex (Singh et al., 2014). It was previously reported that NIN inhibits $Cyclops^{DD}$-mediated transactivation of the *NIN* promoter in both *N. benthamiana* leaves and *L. japonicus* roots (Doctoral thesis Lambert, 2017). Here, I tested if NIN could inhibit the transactivation of its own promoter by the CCaMK/Cyclops complex. The combination of Cyclops and the gain-of-function variant of CCaMK ($CCaMK^{1-314}$) transactivated the expression of NIN_{pro}*:GUS*. Interestingly, in the presence of NIN, a repression in the transactivation of the complex was observed (Fig. 4). In order to evaluate the specificity of this inhibition, the repressing capability of NIN on Cyclops-mediated transactivation of two other known Cyclops targets: *ERN1* and *RAM1* (Pimprikar et

al., 2016; Cerri et al., 2017) were tested. I observed that the transactivation of these two promoters by the CCaMK/Cyclops complex was also repressed in the presence of NIN (Fig. 4).

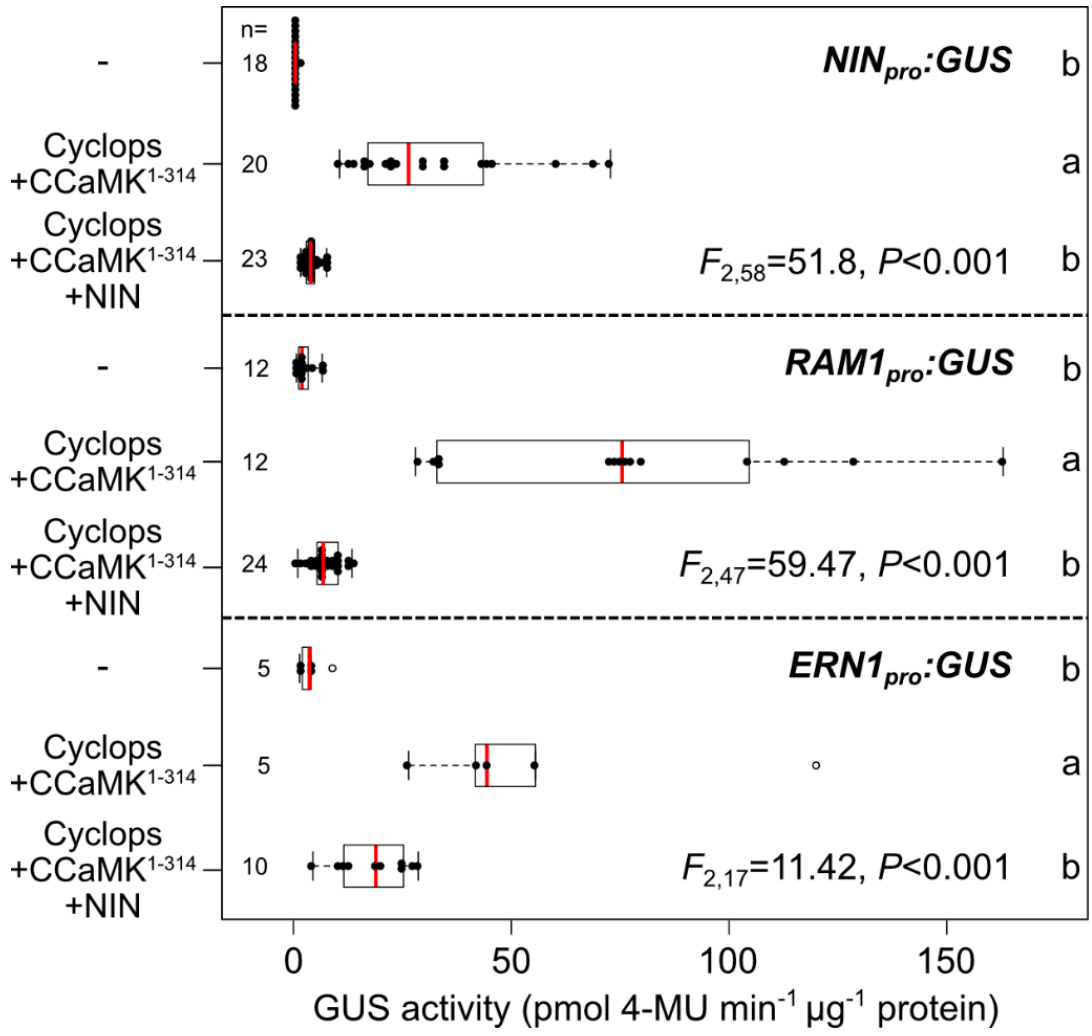


Fig. 4 The Cyclops mediated activation of *NIN*, *ERN1* and *RAM1* promoters is inhibited in the presence of NIN.

N. benthamiana leaves were transiently co-transformed with constructs containing the *Lotus NIN*, *ERN1* and *RAM1* promoters driving the expression of the *GUS* reporter gene (*NIN$_{pro}$:GUS*, *ERN1$_{pro}$:GUS* and *RAM1$_{pro}$:GUS*, respectively) together or in absence (-) of other constructs containing the genomic sequence of the proteins indicated at the y axis driven by constitutive promoters. GUS activity was measured at 48 hours post-infiltration and quantified with a fluorometric assay. Box-plots (red line, median; box, interquartile range; whiskers, lowest and highest data point within 1.5 interquartile range; black dots, individual leaf discs; white dots, individual leaf discs outside of the 1.5 interquartile range) and statistical analyses (ANOVA followed by Tukey honest significant difference test) were performed in R. Letters indicate different statistical groups. n indicates the number of individual leaf discs analyzed in two to four independent experiments.

To confirm that the inhibitory effect of NIN is Cyclops dependent, I analyzed the effect of NIN and Cyclops on the transactivation of the *5xUAS$_{pro}$:eGFP:GUS* (*upstream activating sequence*, *UAS*) reporter by GAL4 (BD$_{GAL4}$-AD$_{VP16}$). Both NIN and Cyclops did not significantly affect the transactivation of the reporter by GAL4 (Fig. 5). Taken together, these results indicate that NIN interferes with the transcriptional activity of the CCaMK/Cyclops complex, in a Cyclops-dependent manner.

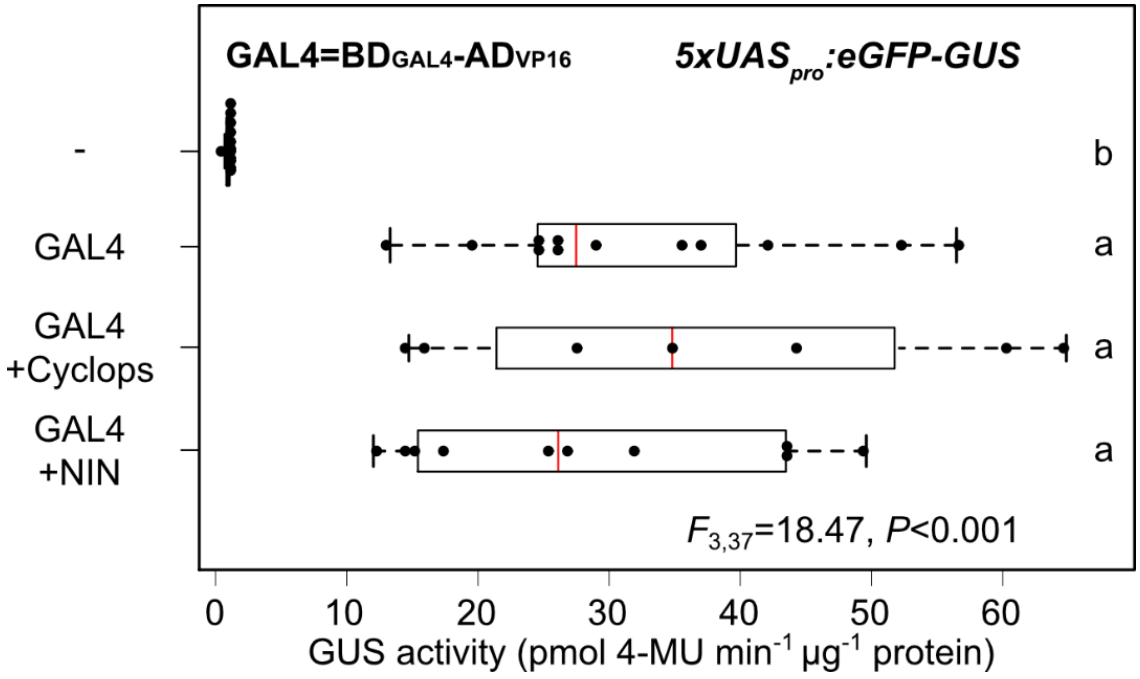


Fig. 5 The GAL4-mediated transactivation of *5xUAS$_{pro}$:GUS* is not inhibited by NIN

N. benthamiana leaves were transiently co-transformed with constructs expressing the *5xUAS* promoter driving the expression of the *GUS* reporter gene together or in absence (-) of other constructs containing the genomic sequence of the proteins indicated at the y axis driven by constitutive promoters. GUS activity was measured at 48 hours post-infiltration from two to four independent experiments and quantified with a fluorometric assay. Box-plots (red line, median; box, interquartile range; whiskers, lowest and highest data point within 1.5 interquartile range; black dots, individual leaf disc) and statistical analyses (ANOVA followed by Tukey honest significant difference test) were performed in R. Letters indicate different statistical groups.

3. The inhibition of the *NIN* promoter by NIN is not mediated via specific binding to the *CYC-RE* or surrounding elements.

We observed that NIN is required for the regulation of its own expression *in planta* upon *M. loti* inoculation (Fig. 3) and that in a *N. benthamiana* transient system NIN inhibits Cyclops-mediated transactivation of all of its so far known targets (Fig. 4). To assess if this inhibitory mechanism requires a specific sequence on the *NIN* promoter, I performed further transactivation assays with selected *NIN* promoter fragments. Cyclops^{DD} was expressed in the presence of shorter versions of the *NIN* promoter containing the *CYC-RE* response element, being the minimal region sufficient for Cyclops-mediated transactivation of the *NIN* promoter (Singh et al., 2014). Previously, Lambert (2017) identified a putative 37 bp NIN binding site (*pNBS*) at the 5' end of the *CYC-RE*, which displayed sequence similarity to a described NIN binding site (Soyano et al., 2013). To assess whether this *pNBS* is required for NIN mediated inhibition, I generated different fragments of the *NIN* promoter either spanning the *pNBS* and the *CYC-RE* (*B3*), containing both elements exclusively (*pNBSCYC-RE*) and only the *CYC-RE* (Fig. 6a). These elements were fused to the minimal *NIN* promoter (Singh et al., 2014), added to the *GUS* reporter gene and tested in transactivation assays.

The transactivation by Cyclops^{DD} of an element containing *CYC-RE* and *pNBS* (*pNBSCYC-RE*) was almost four times stronger compared to the *CYC-RE* alone. Moreover, NIN strongly inhibited the transactivation by Cyclops^{DD} of this shorter *NIN* promoter region (Fig. 6b). To evaluate if this *pNBS* is necessary for the inhibition of Cyclops transactivation mediated by NIN, I mutagenized this element in the context of the *NIN* promoter and performed transactivation assays. The CCaMK/Cyclops complex transactivated the expression of the NIN_{pro}*:GUS* and the NIN_{pro}*::mpNBS:GUS* reporters at the same level, and for both reporters NIN was able to significantly inhibited the Cyclops-mediated transactivation (Fig. 6c). This indicates that this *pNBS* is not or only partly required for the transcriptional inhibition through NIN.

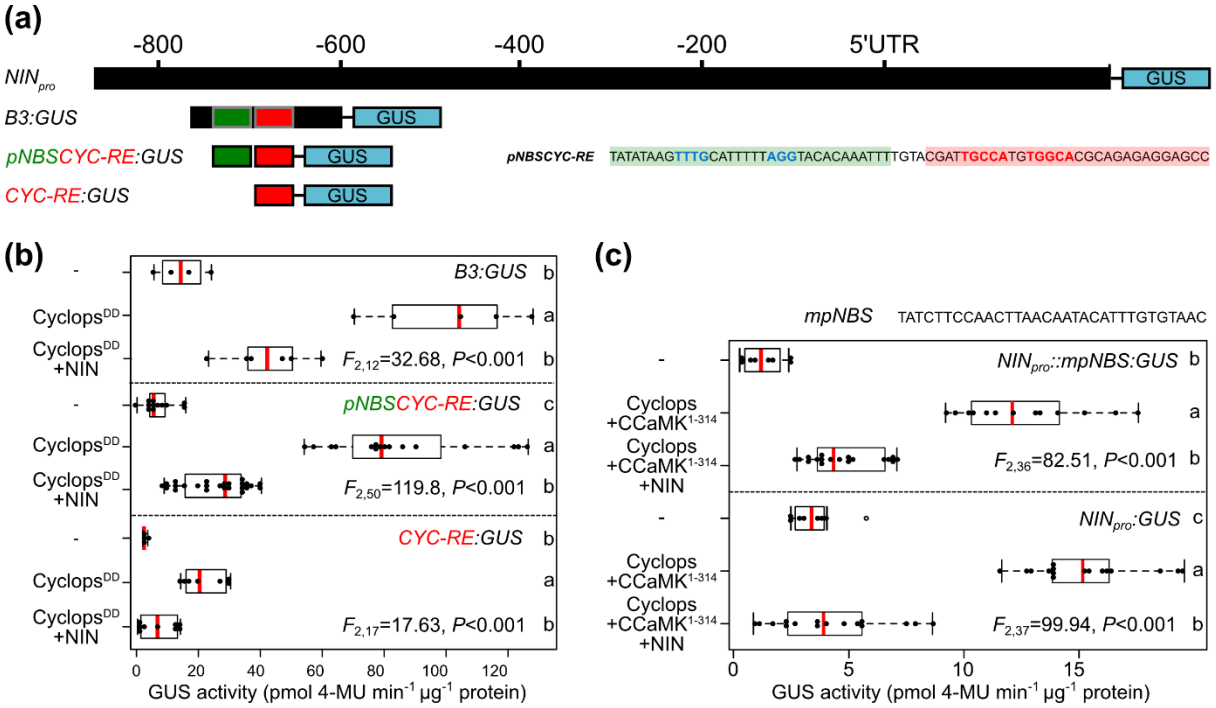


Fig. 6 *NIN* inhibition is not mediated by sequence specific DNA binding of NIN to the previous identified *NIN* binding site.

(a) Schematic representation of the *NIN* promoter and the deletion series fused to *35S* minimal promoter tested in (b). The *NIN* promoter length is annotated from the transcriptional start site. The putative NIN binding site (*pNBS*) and *CYC-RE* are depicted as green and red squares, respectively. The blue letters within *pNBSCYC-RE* represent the sequence similarity to the described *NIN* binding site and the red letters depict the palindromic sequence where Cyclops binds (*CYC-box*). (b-c) *N. benthamiana* leaves were transiently co-transformed with the indicated *NIN* promoter fragments (b) and the mutated *pNBS* within the *NIN* promoter (c) driving the expression of the *GUS* reporter gene together or in absence (-) of other constructs containing the genomic sequence of the proteins indicated at the y axis driven by constitutive promoters. (b) Mapping of the minimal region where NIN can inhibit Cyclops^{DD} mediated transactivation of the *NIN* promoter. (c) Effect of *mpNBS* within *NIN$_{pro}$:GUS* (870 bp) for the inhibition of NIN in the activation of the *NIN* promoter by the CCaMK/Cyclops complex. GUS activity was measured at 48 hours post-infiltration from three independent experiments and quantified with a fluorometric assay. Box-plots (red line, median; box, interquartile range; whiskers, lowest and highest data point within 1.5 interquartile range; black dots, individual leaf disc) and statistical analyses (ANOVA followed by Tukey honest significant difference test) were performed in R. Letters indicate different statistical groups.

To determine whether NIN could bind to the *pNBSCYC-RE* in a sequence specific manner, I designed chimeric transcription factors containing the C-terminal part of NIN or NLP4, which contained the conserved RWP-RK DNA binding domain (R) and fused them to the transcriptional activation domain of the herpes virus protein VP16. Both chimeric constructs, NIN^{C}-VP16 and NLP4^{C}-VP16, transactivated the expression of the nitrate response element *NRE:GUS* but not *pNBSCYC-RE:GUS* (Fig. 7a). To ensure that a possible sequence that might exist outside of the restricted region around the *CYC-RE* was not missed, I extended the search of a possible NIN binding site to a 3 kb *NIN* promoter length. We could not detect transactivation by the chimeric transcription factor NIN^{C}-VP16 even when the full 3 kb *NIN* promoter was present (Fig. 7b). This indicates the absence of any NIN specific DNA binding sequence within a 3 kb *NIN* promoter length.

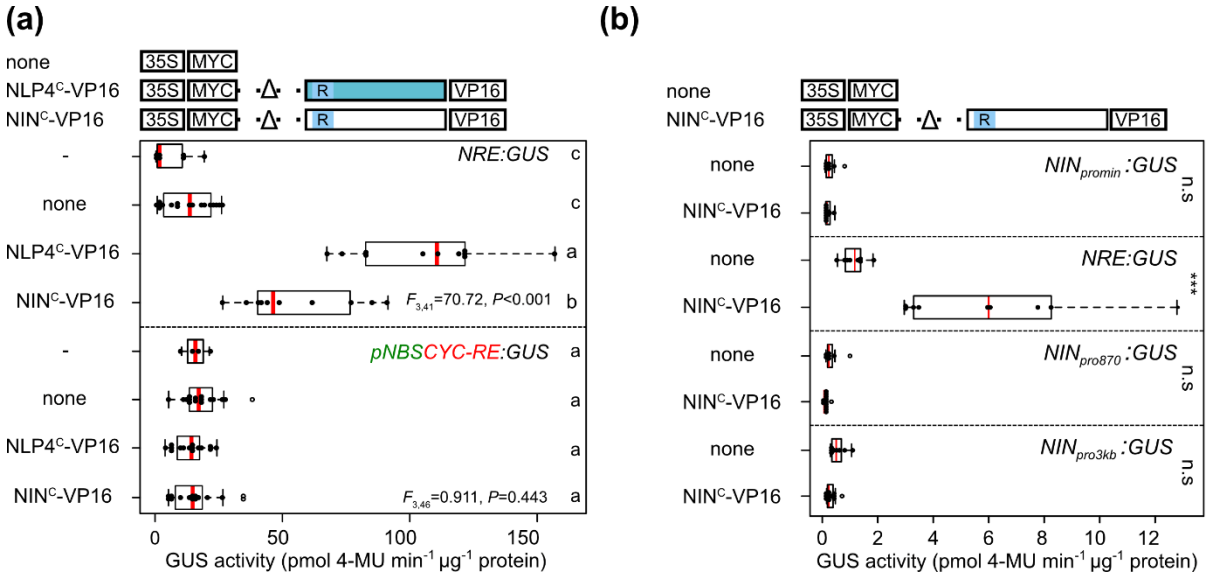


Fig. 7 NIN^{C}-VP16 and NLP4^{C}-VP16 transactivate the expression of the *NRE:GUS* but not the one of *pNBSCYC-RE:GUS*

(a-b) *N. benthamiana* leaves were transiently co-transformed with constructs expressing the indicated *cis* elements or promoters driving the expression of the *GUS* reporter gene together or in absence (-) of other constructs containing the genomic sequence of the proteins indicated at the y axis driven by constitutive promoters. Transactivation of *pNBSCYC-RE* and *NRE* (a), minimal *NIN* promoter (*NINpromin*), *NRE* and *NIN* promoter (870 and 3000 bp, respectively) (b) by the chimeric proteins. GUS activity was measured at 48 hours post-infiltration from two independent experiments and quantified with a fluorometric assay. Box-plots (red line, median; box, interquartile range; whiskers, lowest and highest data point within 1.5 interquartile range; black dots, individual leaf discs; white dots, individual leaf discs outside of the 1.5 interquartile range) and statistical analyses (a) ANOVA followed by Tukey honest significant difference test and (b) Welch t test were performed in R. (a) Letters indicate different statistical groups. (b) ***p ≤ 0.001; n.s = not significant.

4. NIN inhibits Cyclops transactivation activity regardless of its DNA binding sequence

We tested if NIN could inhibit Cyclops transactivation of the *NIN* promoter regardless of its DNA binding sequence. For this, the palindromic Cyclops binding sequence (*CYC-box*) within the 3 kb *NIN* promoter was replaced with the *UAS* of *GAL4* ($NIN_{pro}3kb::GAL4_{UAS}$) (Fig. 8a) and Cyclops was fused to the GAL4 binding domain ($GAL4^{BD}$-Cyclops). First, the inhibitory effect of NIN in the transactivation of the 3 kb *NIN* promoter by the CCaMK/Cyclops complex was confirmed. We observed that $GAL4^{BD}$-Cyclops transactivated the expression of the $NIN_{pro}3kb::GAL4_{UAS}:GUS$ reporter in the presence of $CCaMK^{1-314}$ and that this transactivation was repressed by NIN (Fig. 8b). This observation confirms that NIN can repress the transactivation of the *NIN* promoter by the CCaMK/Cyclops complex independently of the Cyclops DNA binding sequence. All together our results suggest that the inhibition of Cyclops by NIN is not happening via DNA interaction but might be mediated by a different mechanism.

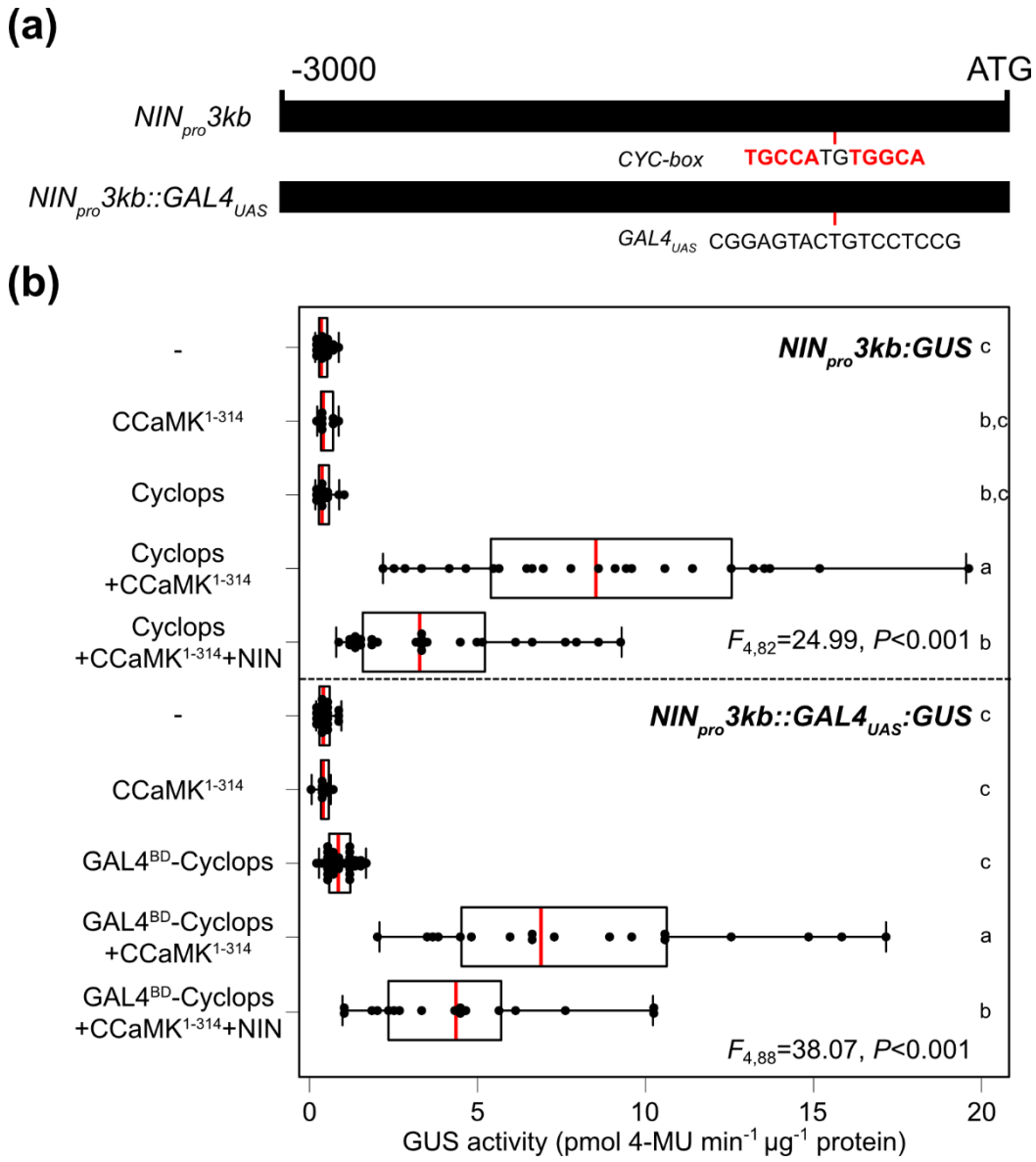


Fig. 8 NIN represses Cyclops transactivation activity regardless of its DNA binding sequence.

(a) Schematic representation of the 3 kb *NIN* promoter with replacement of *CYC-box* by $GAL4_{UAS}$. The palindromic sequence of *CYC-box* is highlighted in red. (b) Transactivation assay was done in *N. benthamiana* leaves using the 3 kb *NIN* promoter wild type (top) and the *NIN* promoter with *1xUAS* instead of the *CYC-box* (bottom) driving the expression of the *GUS* reporter gene. *N. benthamiana* leaves were transiently co-transformed with constructs expressing the 3 kb *NIN* promoter wild type and the *NIN* promoter with $GAL4_{UAS}$ instead of the *CYC-box* driving the expression of the *GUS* reporter gene (NIN_{pro}*3kb:GUS* and NIN_{pro}*3kb::*$GAL4_{UAS}$*:GUS*, respectively) together or in absence (-) of other constructs containing the genomic sequence of the proteins indicated at the y axis driven by constitutive promoters. GUS activity was measured at 48 hours post-infiltration from two independent experiments and quantified with a fluorometric assay. Box-plots (red line, median; box, interquartile range; whiskers, lowest and highest data point within 1.5 interquartile range; black dots, individual leaf discs) and statistical analyses (ANOVA followed by Tukey honest significant difference test) were performed in R. Letters indicate different statistical groups.

5. The N-terminal part of NIN interacts with the N-terminal part of Cyclops

NIN was able to inhibit Cyclops mediated transactivation of *NIN*, *ERN1* and *RAM1* (Fig. 4). In addition, the inhibition by NIN was not dependent on a particular Cyclops DNA binding sequence (Fig. 8). To elucidate the underlying mechanism of this inhibition, previous results indicating that NIN and Cyclops interact (Doctoral thesis Lambert, 2017) were confirmed. I performed FLIM-FRET (Fluorescence Lifetime Imaging - Förster Resonance Energy Transfer) experiments with the FRET pair GFP-mCherry in *N. benthamiana* leaf cells. The fluorescent lifetime (FLT) of the donor (GFP-Cyclops) was significantly reduced in the presence of both mCherry-NIN and CCaMK-mCherry in comparison to free NLS-mCherry, taken as a negative control. This confirmed that NIN and Cyclops interact *in planta*. As a positive control the already reported interaction between Cyclops and CCaMK was used (Yano et al., 2008; Singh et al., 2014).

To identify the protein domain of NIN that interacts with Cyclops, NIN was split, according to the prediction of a transmembrane domain (Fig. 2b, Schauser et al., 1999), into N and C-terminal parts, NIN^{N} (1-296 aa) and NIN^{C} (546-878 aa) (Fig. 9a). A very strong reduction in the FLT of the GFP donor in the presence of NIN^{N} but not NIN^{C} was observed, indicating that Cyclops interacts with the N-terminal part of NIN (Fig. 9b). An increase of nucleus size was noticed when NIN^{N} was expressed in *N. benthamiana*, which was not observed for any of the other combinations (data not shown). To confirm this interaction, a yeast two-hybrid assay (Y2H) was performed (D. Chiasson, unpublished data). Cyclops was fused to the GAL4 activation domain (AD) and NIN to the GAL4 binding domain (BD). Interaction between Cyclops and CCaMK was observed as previously reported (Yano et al., 2008). The interaction between NIN^{FL} and NIN^{C} was also observed, consistent with the observation that the PB1 domain (present in NIN^{C}) is required for homo and heterodimerizations between NLPs (Lin et al., 2018). Surprisingly, a NIN^{N}/NIN^{N} self-interaction was also observed. The interaction between the N-terminal part of NIN and Cyclops was confirmed with Y2H (D. Chiasson, unpublished data). The Cyclops domain required for the interaction with NIN^{N} was further delimited. Interaction between NIN^{N} and these truncated N-terminal versions of Cyclops was detected: $Cyclops^{1-366}$ (lacking the BD), $Cyclops^{84-366}$, $Cyclops^{84-310}$ and even $Cyclops^{84-245}$ (lacking the AD). The interaction was disrupted with $Cyclops^{150-366}$, suggesting that the amino acids 84-150 are required for the interaction with NIN^{N} (D. Chiasson, unpublished data).

Overall, these results suggest that NIN interacts via its N-terminal part with the N-terminal part of Cyclops.

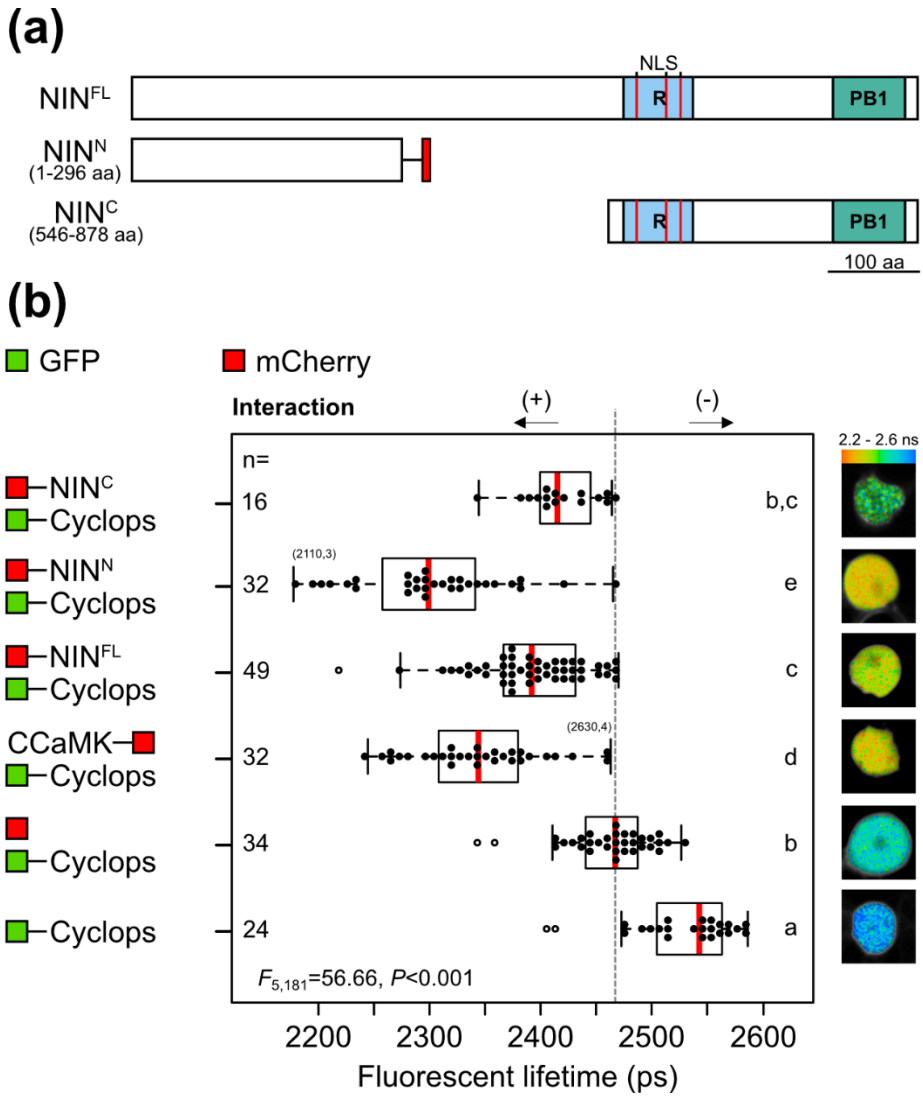


Fig. 9 Cyclops/NIN interaction detected via FLIM-FRET analysis

(a) Schematic representation of the NIN full length (NIN^{FL}) and the truncated NIN proteins used in (b). The RWP-RK (R), DNA-binding domain and PB1 protein-protein interaction domain are depicted in blue and green, respectively. The red lines and box represent the location of the bipartite NLS in NIN^{FL} and the artificial NLS added to NIN^{N}, respectively. (b) *In vivo* FLIM-FRET analysis of the Cyclops/NIN interaction using GFP as FRET donor and mCherry as FRET acceptor, in nuclei of *N. benthamiana* leaf cells 48-60 hours post infiltration. On the images on the right, the fluorescent lifetime (FLT) of the donor is depicted in a color code from red (2.2 ns) to blue (2.6 ns) for one representative nucleus per construct combination. Boxplots represent the FLT of the donor in ps (red line, median; box, interquartile range; whiskers, lowest and highest data point within 1.5 interquartile range; black dots, individual nuclei analyzed; white dots, individual nuclei outside of the 1.5 interquartile range). Data were subjected to ANOVA and *post-hoc* Tukey analyses. Letters indicate different statistical groups. n represents the total number of nucleus analyzed. Grey dotted line represents the median reference FLT of the GFP-Cyclops and free mCherry combination.

I assessed the effect of CCaMK in the Cyclops/NIN interaction. Lifetime measurements of the donor GFP fused to Cyclops were performed in the presence of mCherry-NIN as well as non-tagged CCaMK versions (CCaMK^{WT}, CCaMK$^{1-314}$, CCaMK^{T265D} and CCaMK^{NFG}). In the presence of both autoactive versions, either CCaMK$^{1-314}$ or CCaMK^{T265D}, the FLT of GFP was significantly increased. By contrast, no significant changes were observed in the presence of the kinase inactive CCaMK^{NFG} or CCaMK^{WT} versions (Fig. 10). This suggests that phosphorylation of Cyclops by CCaMK disrupts the Cyclops/NIN interaction.

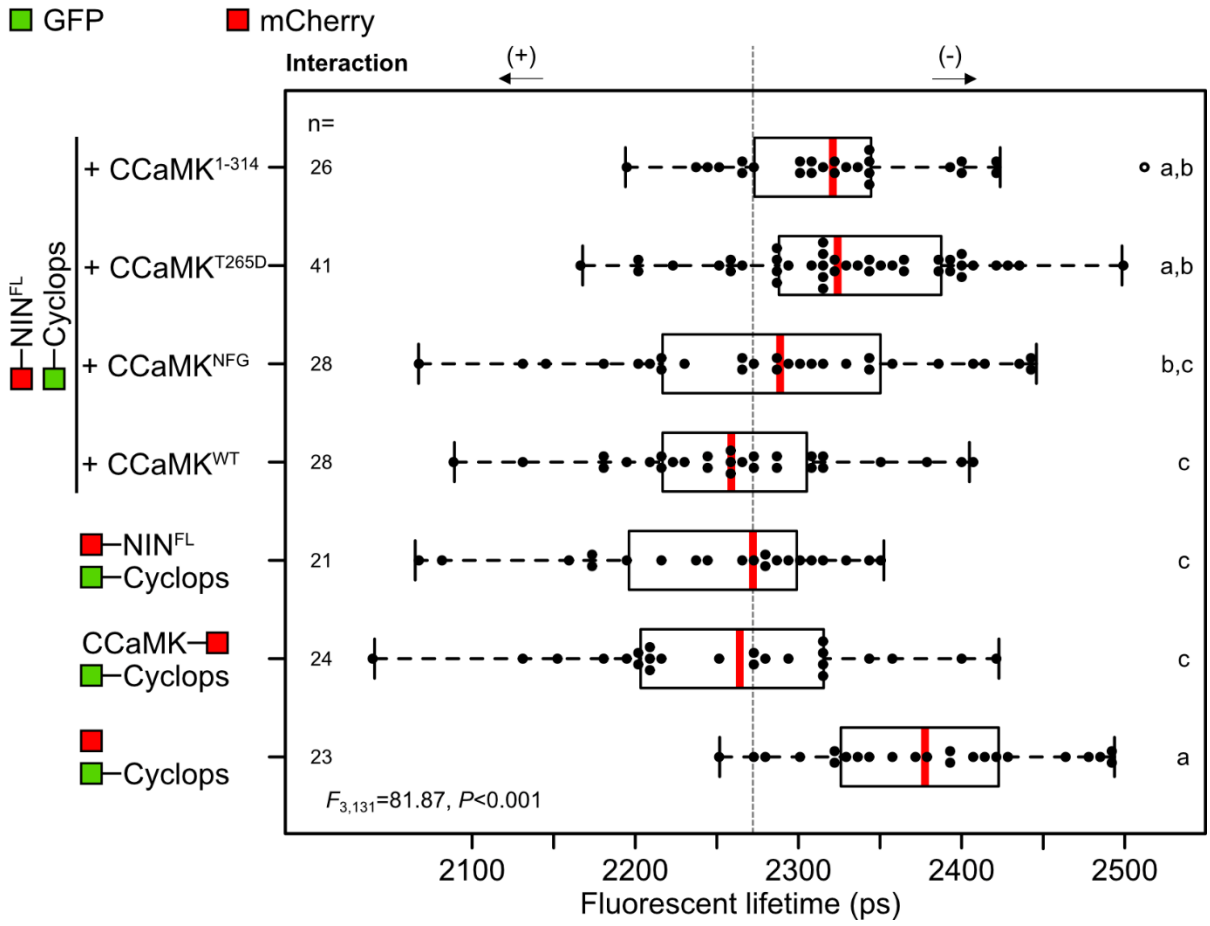


Fig. 10 The Cyclops/NIN interaction is perturbed by CCaMK autoactive versions, CCaMK$^{1-314}$ and CCaMK^{T265D} but not in the presence of CCaMK^{WT} or CCaMK^{NFG}

In vivo FLIM-FRET analysis of the Cyclops/NIN interaction in the presence of non-tagged versions of CCaMK (kinase-only myc-CCaMK$^{1-314}$-NLS, gain-of-function myc-CCaMK^{T265D}, kinase-inactive myc-CCaMK^{NFG} and myc-CCaMK^{WT}), in nuclei of *N. benthamiana* leaf cells 48-60 hours post infiltration. Boxplots represent the FLT of the donor in ps (red line, median; box, interquartile range; whiskers, lowest and highest data point within 1.5 interquartile range; black dots, individual nuclei analyzed). Data were subjected to ANOVA and *post-hoc* Tukey analyses. Letters indicate different statistical groups. n represents the total number of nucleus analyzed. Grey dotted line represents the median reference FLT of the GFP-CYLOPS and mCherry-NIN^{FL} combination.

Because NIN^{N} interacted with an N-terminal part of Cyclops (D. Chiasson, unpublished data), I tested if the inhibition would still occur if a Cyclops truncated protein that lacks this interacting region is used. The effect of NIN on the transactivation of the *2xCYC-RE* with the minimal Cyclops version (Cyclops^{min}, 255-518 aa), which does not contain the delimited interacting region by Y2H, was tested. As previously reported, Cyclops^{min} was able to transactivate the expression of this *cis* regulatory element (Singh et al., 2014). When NIN was included, the transactivation of *2xCYC-RE* mediated by Cyclops^{min} was repressed, suggesting that the delimited interaction region between both N-terminal regions of NIN and Cyclops is not necessary for the inhibition (Fig. 11). I tested whether CCaMK^{T265D} could influence this repression. Significant changes were neither observed in the activation by Cyclops^{min} nor for NIN-mediated repression in the presence of CCaMK^{T265D}.

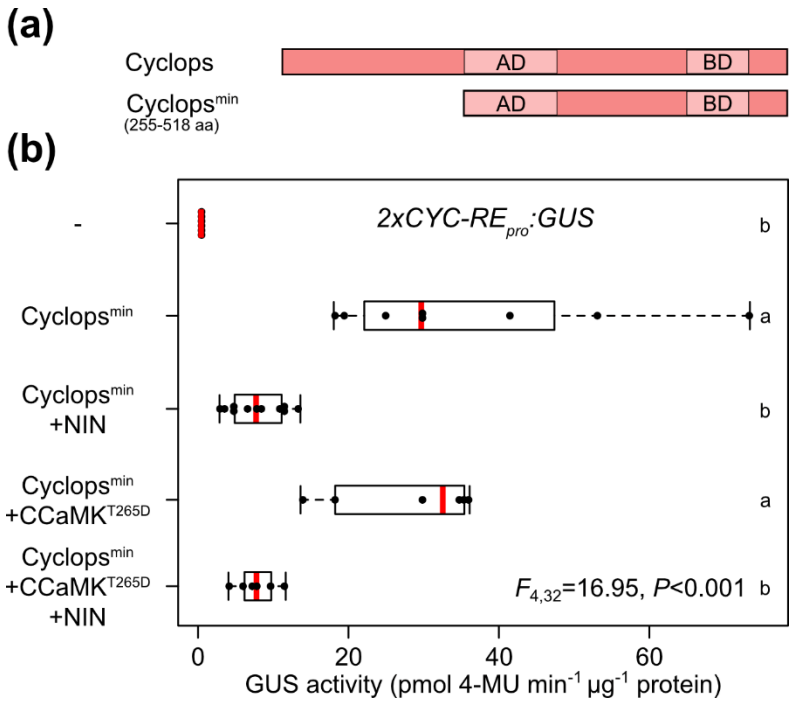


Fig. 11 The delimited Cyclops/NIN interaction domain is not required for the NIN-mediated inhibition of the Cyclops^{min} transactivation of the *2xCYC-RE*.

(a) Schematic representation of Cyclops full length (Cyclops) and the truncated Cyclops protein (Cyclops^{min} 255-518 aa). *N. benthamiana* leaves were transiently co-transformed with constructs expressing the *2xCYC-RE* of the *NIN* promoter driving the expression of the *GUS* reporter gene (*2xCYC-RE$_{pro}$:GUS*) together or in absence (-) of other constructs containing the genomic sequence of the proteins indicated at the y axis driven by constitutive promoters. GUS activity was measured at 48 hours post-infiltration from two independent experiments and quantified with a fluorometric assay. Box-plots (black line, median; box, interquartile range; whiskers, lowest and highest data point within 1.5 interquartile range; black dots, individual leaf discs) and statistical analyses (ANOVA followed by Tukey honest significant difference test) were performed in R. Letters indicate different statistical groups.

6. Ectopic expression of *NIN* and its C-terminal part inhibits nodulation locally

We demonstrated that NIN inhibits its own expression at the level of Cyclops *in planta*. We asked whether *NIN* deregulation would have an effect on nodulation, during both infection and nodule organogenesis processes. To address this question, *NIN* was ectopically expressed under the control of the *L. japonicus Ubiquitin* promoter (Maekawa et al., 2008) and the effect on infection thread formation and nodule organogenesis was investigated. When ectopically expressed in Gifu WT plants, both infection and nodule organogenesis processes were exclusively suppressed in transformed roots, whereas non-transformed roots had similar nodule numbers compared to the EV control (Fig. 12a, b). These results are in accordance with the ones reported in Lambert (2017). To assess which domain is responsible for this suppression of nodulation, I overexpressed NIN^N and NIN^C separately (Fig. 2). A significant reduction in the number of nodules by overexpression of NIN^C but not NIN^N was observed, indicating that the C-terminal part of NIN contains a domain involved in the inhibition of nodulation (Fig. 12b).

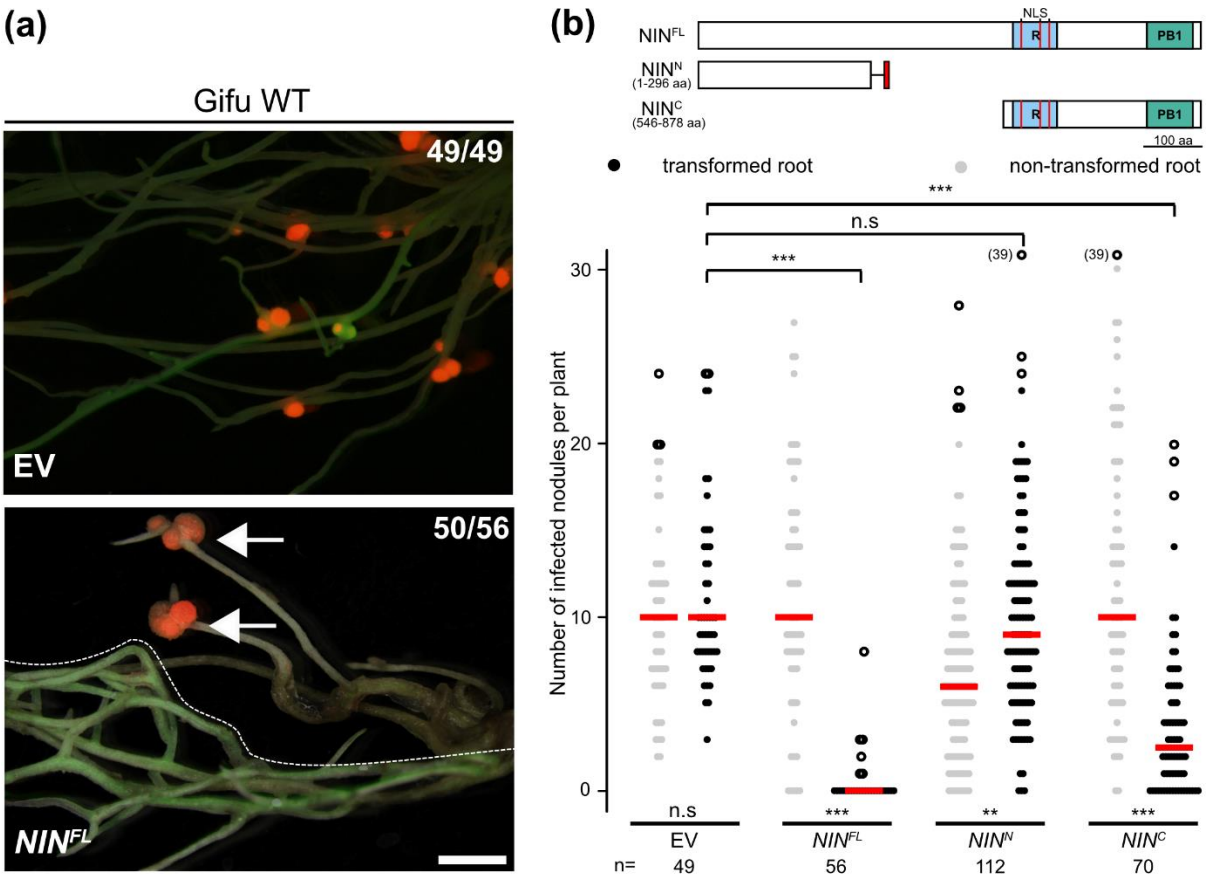


Fig. 12 *NIN* overexpression impairs both infection and nodule organogenesis locally.

Hairy roots of *L. japonicus* WT transformed with the empty vector (EV, *ubq10:NLS-2xGFP*), *ubq:myc-NIN* (*NIN*^{FL}), *ubq:myc-NIN*^{N} (*NIN*^{N}) and *ubq:myc-NIN*^{C} (*NIN*^{C}) were generated. (a) Representative pictures of local inhibition of nodules by overexpression of *NIN*^{FL} in *L. japonicus* WT hairy roots. The nodulation phenotype is presented as overlay images recorded with GFP (indicates transformed roots) and *Ds*Red (*M. loti* MAFF *Ds*Red) filters. Nodulation is inhibited in the transformed roots overexpressing *NIN*^{FL} (GFP positive), but nodules were formed on non-transformed roots of the same root system (no GFP expression), as marked by the white arrows. Numbers represent plants with the displayed phenotype versus total number of plants analyzed. Bar: 2 mm. (b) Plot represents the number of nodules in both transformed and non-transformed in roots overexpressing *NIN*^{FL}, *NIN*^{N} and *NIN*^{C} quantified 3 weeks post inoculation with *M. loti*. Red line, median; grey dots, number of infected nodules in non-transformed roots; black dots, number of infected nodules in transformed roots; white dots, individual infected nodules outside of the 1.5 interquartile range. n indicates the number of individual root systems analyzed. Pairwise Welch t-tests were performed between transformed and non-transformed nodule number per construct and between number of nodules in transformed roots in the EV vs the ones in *NIN*^{FL}, *NIN*^{N} and *NIN*^{C}. *p<0.05, **p<0.01, ***p<0.001.

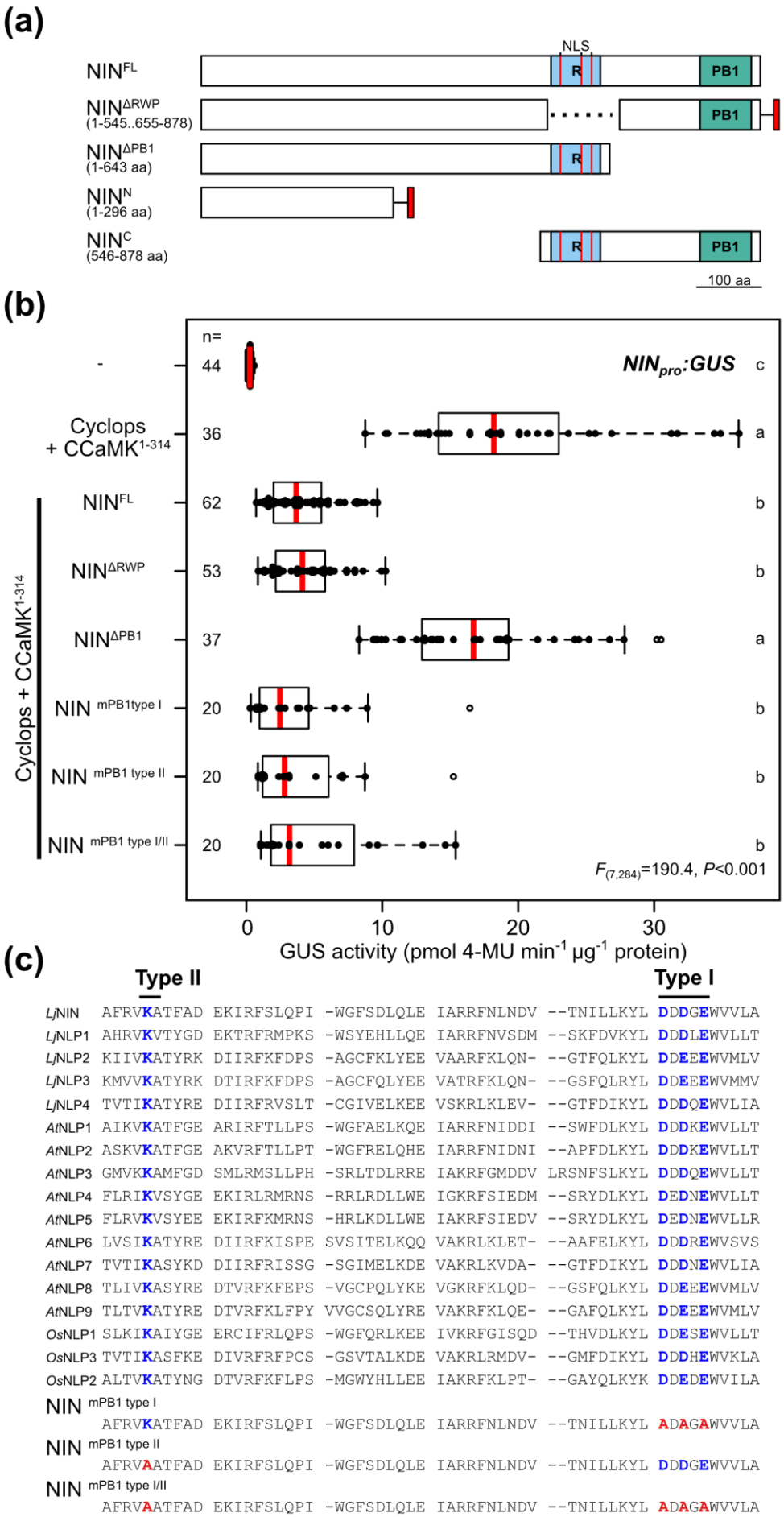
7. The PB1 domain of NIN is required for the NIN mediated inhibition of the CCaMK/Cyclops complex

Because a strong reduction in nodule number was observed when NIN^{C} was overexpressed, I further analyzed the role of two conserved domains present in the C-terminal part of NIN (Fig. 13a). These are the RWP-RK and the PB1 domains, described to be involved in DNA binding and for protein-protein interactions, respectively. In a *N. benthamiana* transactivation assay, the PB1 domain was required for NIN mediated inhibition of Cyclops activity. Upon deletion of the PB1 domain ($NIN^{\Delta PB1}$, 1-643 aa) NIN was not able to inhibit Cyclops mediated transactivation of the *NIN* promoter anymore, while inhibition still occurred with the protein lacking the DNA-binding domain ($NIN^{\Delta RWP}$, 1-545..655-878 aa). This indicates that the PB1 domain but not the RWP-RK domain is required for the NIN mediated inhibition of the CCaMK/Cyclops complex transactivation (Fig. 13b).

NLP transcription factors possess type I/II PB1 domains (Konishi and Yanagisawa, 2019). NIN contains both conserved motifs, type I and type II. Type I motif consists of D827/D829/E831 and type II motif of K784 conserved amino acids (Fig. 13c). Surprisingly, mutagenesis of either Type I ($NIN^{mPB1\ type\ I}$) or Type II ($NIN^{mPB1\ type\ II}$), or even both motifs ($NIN^{mPB1\ type\ I/II}$) did not impair the ability of NIN for inhibiting Cyclops transactivation of the *NIN* promoter in *N. benthamiana* (Fig. 13b).

Fig. 13 The PB1 protein-protein interaction domain is required for the NIN mediated inhibition of the CCaMK/Cyclops complex

(a) Schematic representation of NIN and the truncated NIN proteins used here for the fluorometric assay and for the nodulation experiment in Fig. 14. As the RWP domain contains the predicted nuclear localized signals (NLS), an artificial NLS was added to the $NIN^{\Delta RWP}$ truncated protein. As reference NIN^{N} and NIN^{C} are added (b) Delimitation of the NIN domain required for the inhibition of Cyclops transactivation in the *NIN* promoter. *N. benthamiana* leaves were transiently co-transformed with constructs expressing the *NIN* promoter driving the expression of the *GUS* reporter gene (NIN_{pro}*:GUS*) together or in absence (-) of other constructs containing the genomic sequence of the proteins indicated at the y axis driven by constitutive promoters. GUS activity was measured at 48 hours post-infiltration and quantified with a fluorometric assay. Box-plots (red line, median; box, interquartile range; whiskers, lowest and highest data point within 1.5 interquartile range; black dots, individual leaf discs; white dot outliers, individual leaf discs outside of the 1.5 interquartile range) and statistical analyses (ANOVA followed by Tukey honest significant difference test) were performed in R. Letters indicate different statistical groups. n indicates the number of individual leaf discs analyzed in three to four independent experiments. (c) Alignment of amino acid sequences of PB1 domains present in NLPs from *L. japonicus* (*Lj*), *A. thaliana* (*At*) and rice (*Os*). Amino acids in blue represent core residues in Type I and Type II motifs. Amino acids in blue represent the WT sequence and the red ones the mutation to alanine (A).


(a)
NLS
NIN^FL
NIN^ΔRWP (1-545..655-878)
NIN^ΔPB1 (1-643 aa)
NIN^N (1-296 aa)
NIN^C (546-878 aa)
R
PB1
100 aa
(b)
n=
-
Cyclops + CCaMK^1-314
NIN^FL
NIN^ΔRWP
NIN^ΔPB1
NIN mPB1type I
NIN mPB1 type II
NIN mPB1 type I/II
Cyclops + CCaMK^1-314
44
36
62
53
37
20
20
20
NIN_pro:GUS
c
a
b
b
a
b
b
b
F(7,284)=190.4, P<0.001
0
10
20
30
GUS activity (pmol 4-MU min-1 µg-1 protein)
(c)
Type II
Type I
LjNIN AFRVKATFAD EKIRFSLQPI -WGFSDLQLE IARRFNLNDV --TNILLKYL DDDGEWVVLA
LjNLP1 AHRVKVTYGD EKTRFRMPKS -WSYEHLLQE IARRFNVSDM --SKFDVKYL DDDLEWVLLT
LjNLP2 KIIVKATYRK DIIRFKFDPS -AGCFKLYEE VAARFKLQN- --GTFQLKYL DDEEEWVMLV
LjNLP3 KMVVKATYRK DTIRFKFDPS -AGCFQLYEE VATRFKLQN- --GSFQLRYL DDEEEWVMMV
LjNLP4 TVTIKATYRE DIIRFRVSLT -CGIVELKEE VSKRLKLEV- --GTFDIKYL DDDQEWVLIA
AtNLP1 AIKVKATFGE ARIRFTLLPS -WGFAELKQE IARRFNIDDI --SWFDLKYL DDDKEWVLLT
AtNLP2 ASKVKATFGE AKVRFTLLPT -WGFRELQHE IARRFNIDNI --APFDLKYL DDDKEWVLLT
AtNLP3 GMVKKAMFGD SMLRMSLLPH -SRLTDLRRE IAKRFGMDDV LRSNFSLKYL DDDQEWVLLT
AtNLP4 FLRIKVSYGE EKIRLRMRNS -RRLRDLLWE IGKRFSIEDM --SRYDLKYL DEDNEWVLLT
AtNLP5 FLRVKVSYEE EKIRFKMRNS -HRLKDLLWE IAKRFSIEDV --SRYDLKYL DEDNEWVLLR
AtNLP6 LVSIKATYRE DIIRFKISPE SVSITELKQQ VAKRLKLET- --AAFELKYL DDDREWVSVS
AtNLP7 TVTIKASYKD DIIRFRISSG -SGIMELKDE VAKRLKVDA- --GTFDIKYL DDDNEWVLIA
AtNLP8 TLIVKASYRE DTVRFKFEPS -VGCPQLYKE VGKRFKLQD- --GSFQLKYL DDEEEWVMLV
AtNLP9 TLTVKATYRE DTVRFKLFPY VVGCSQLYRE VAKRFKLQE- --GAFQLKYL DDEEEWVMLV
OsNLP1 SLKIKAIYGE ERCIFRLQPS -WGFQRLKEE IVKRFGISQD --THVDLKYL DDESEWVLLT
OsNLP3 TVTIKASFKE DIVRFRFPCS -GSVTALKDE VAKRLRMDV- --GMFDIKYL DDDHEWVKLA
OsNLP2 ALTVKATYNG DTVRFKFLPS -MGWYHLLEE IAKRFKLPT- --GAYQLKYK DDEDEWVILA
NIN mPB1 type I
AFRVKATFAD EKIRFSLQPI -WGFSDLQLE IARRFNLNDV --TNILLKYL ADAGAWVVLA
NIN mPB1 type II
AFRVAATFAD EKIRFSLQPI -WGFSDLQLE IARRFNLNDV --TNILLKYL DDDGEWVVLA
NIN mPB1 type I/II
AFRVAATFAD EKIRFSLQPI -WGFSDLQLE IARRFNLNDV --TNILLKYL ADAGAWVVLA

I asked whether the PB1 domain was also required for inhibition of nodulation. *NIN*$^{\Delta PB1}$ and *NIN*$^{\Delta RWP}$ were overexpressed in Gifu WT plants and the number of nodules formed in both transformed and non-transformed roots was quantified. For both constructs, a significant reduction in the number of nodules formed was observed on roots overexpressing *NIN*$^{\Delta PB1}$ and *NIN*$^{\Delta RWP}$ compared to the empty vector control. However, only overexpression of *NIN*$^{\Delta RWP}$ but not *NIN*$^{\Delta PB1}$ inhibited nodulation almost to the same levels of *NIN*^{FL} (Fig. 14). This suggests that the PB1 domain is important for the inhibition of NIN in roots as well, while the DNA binding domain of NIN does not seem to be necessary for the observed inhibition.

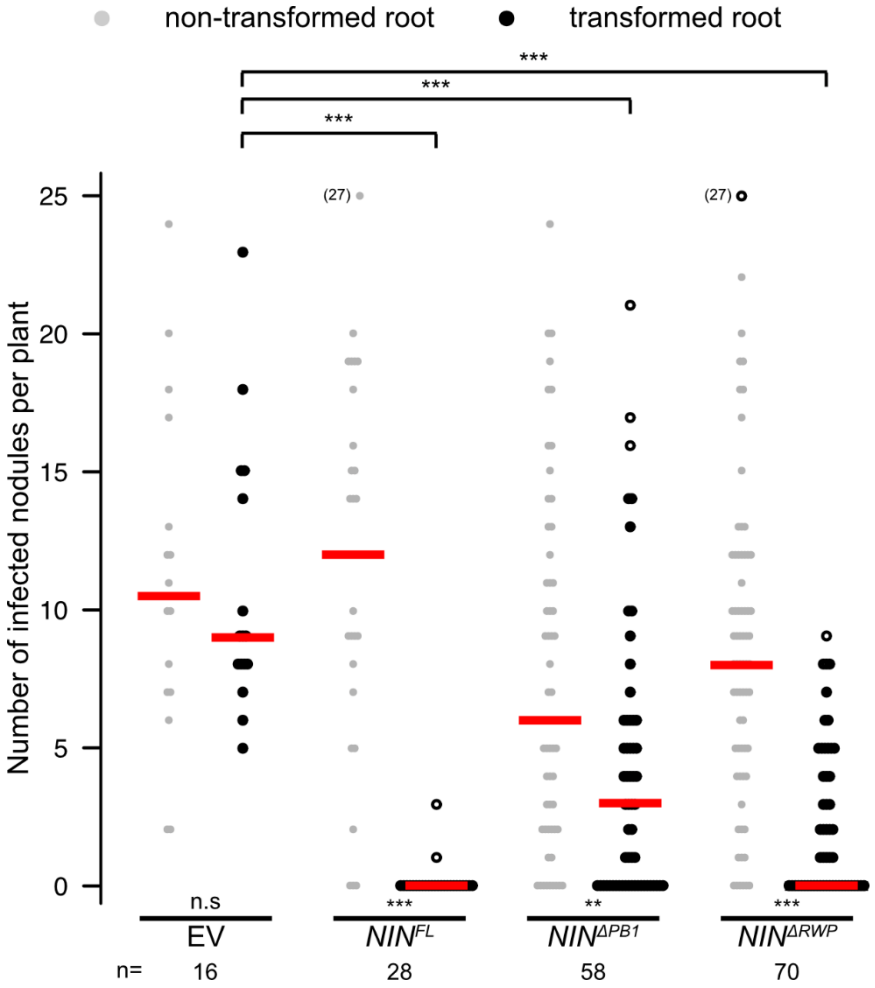


Fig. 14 The PB1 protein-protein interaction domain is required for inhibition of nodulation in *L. japonicus* roots

Hairy roots of *L. japonicus* WT transformed with the empty vector (EV, *ubq10:NLS-2xGFP*), *ubq:myc-NIN* (*NIN*^{FL}), *ubq:myc-NIN*$^{\Delta PB1}$ (*NIN*$^{\Delta PB1}$) and *ubq:myc-NIN*$^{\Delta RWP}$ (*NIN*$^{\Delta RWP}$) were generated. Plot represents the number of nodules in both transformed and non-transformed roots overexpressing *NIN*^{FL}, *NIN*$^{\Delta PB1}$ and *NIN*$^{\Delta RWP}$ quantified 3 weeks post inoculation with *M. loti*. The distinction between transformed and non-transformed roots was possible with the visualization of the GFP transformation marker. Red line, median; grey dots, number of infected nodules in non-transformed roots; black dots, number of infected nodules in transformed roots; white dots, individual infected nodules outside of the 1.5 interquartile range. n indicates the number of individual roots analyzed. Pairwise Welch t-tests were performed between transformed and non-transformed nodule number per construct and between number of nodules in transformed roots in the EV vs the ones in *NIN*^{FL}, *NIN*$^{\Delta PB1}$ and *NIN*$^{\Delta RWP}$. *p<0.05, **p<0.01, ***p<0.001.

The N-terminal part of NIN is different from the NLPs, contributing to different nitrate responses, while the C-terminal part is highly conserved (Schauser et al., 2005; Suzuki et al., 2013). Because the N-terminal part of NIN was interacting with Cyclops (Fig. 9) but the PB1 domain was necessary for the NIN-mediated inhibition of the *NIN* promoter (Fig.13), I constructed a plasmid encoding a chimeric protein with the N-terminal part of NIN and the C-terminal part of NLP4 (NIN-NLP4, Fig. 15a). This chimeric protein was able to also inhibit the transactivation of the *NIN* promoter by Cyclops at the same level that NIN does it in *N. benthamiana* (Fig. 15b).

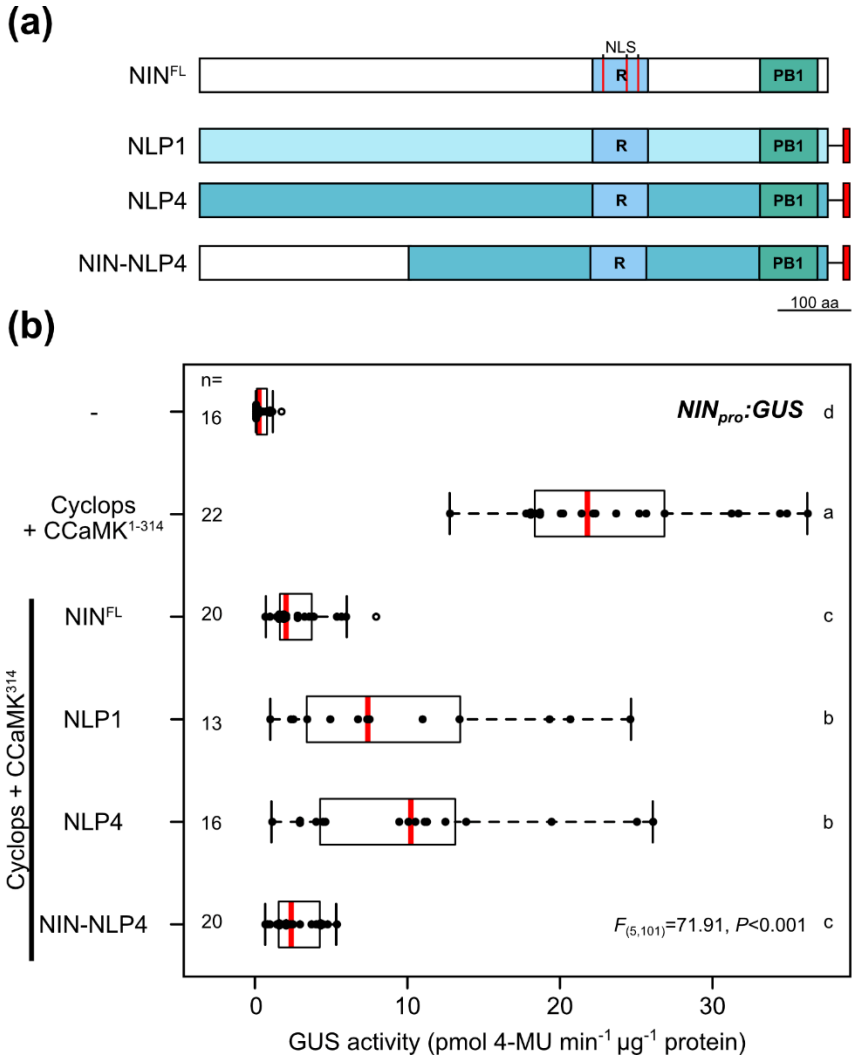


Fig. 15 NIN, NLP1, NLP4 and the chimeric protein NIN-NLP4 inhibit the transactivation of the *NIN* promoter by the CCaMK/Cyclops complex in *N. benthamiana*

(a) Schematic representation of NIN, NLP1, NLP4 and the chimeric protein NIN-NLP4 used here for the fluorometric assay and for the nodulation experiment in Fig. 17. To ensure that the NLPs and the chimeric protein localizes in the nucleus, an artificial NLS was added at the C-terminus, as depicted with the red boxes. (b) *N. benthamiana* leaves were transiently co-transformed with constructs expressing the *NIN* promoter driving the expression of the *GUS* reporter gene (*NIN*$_{pro}$*:GUS*) together or in absence (-) of other constructs containing the genomic sequence of the proteins indicated at the y axis driven by constitutive promoters. GUS activity was measured at 48 hours post-infiltration and quantified with a fluorometric assay. Box-plots (red line, median; box, interquartile range; whiskers, lowest and highest data point within 1.5 interquartile range; black dots, individual leaf discs; white dots, individual leaf discs outside of the 1.5 interquartile range) and statistical analyses (ANOVA followed by Tukey honest significant difference test) were performed in R. Letters indicate different statistical groups. n indicates the number of individual leaf discs analyzed in two to four independent experiments.

Western blots confirmed that NIN^{FL}, NIN$^{\Delta RWP}$, NIN$^{\Delta PB1}$, NLP1 and the chimeric NIN-NLP4 protein were correctly expressed in *N. benthamiana* (Fig. 16).

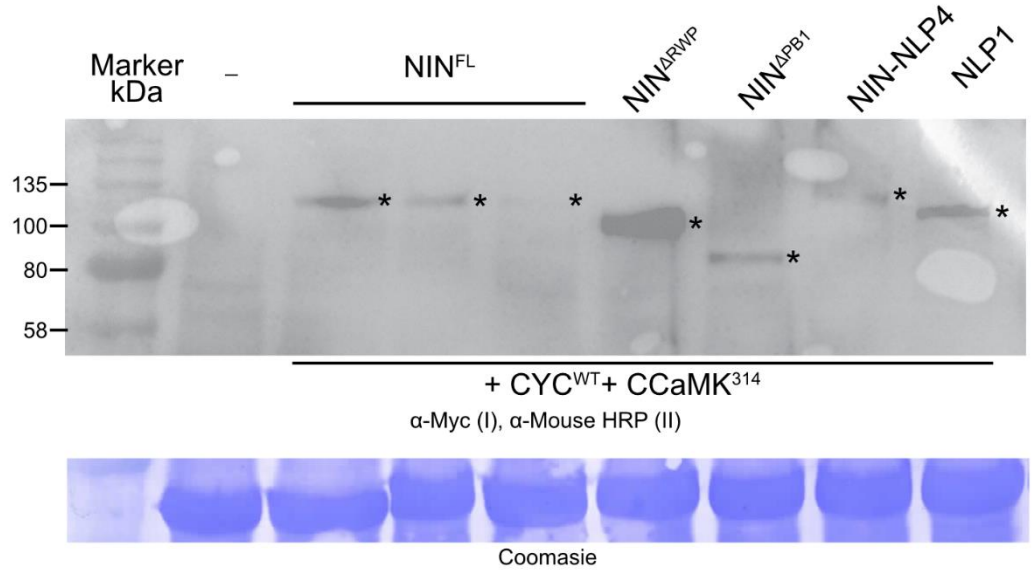


Fig. 16 Confirmation of protein expression in the transactivation assays performed in *N. benthamiana* leaves

Protein accumulation in *N. benthamiana* of NIN^{FL}, NIN$^{\Delta RWP}$, NIN$^{\Delta PB1}$, NLP1 and the chimera NIN-NLP4 used for transactivation assays. Asterisks denote the presence of the proteins NIN^{FL} (99 kDa), NIN$^{\Delta RWP}$ (83 kDa), NIN$^{\Delta PB1}$ (73 kDa), NIN-NLP4 (103 kDa) and NLP1 (100 kDa). The primary (I) and secondary (II) antibodies are indicated. The detection of the secondary antibody was performed with chemi-luminescence.

Because NIN-NLP4 had an inhibitory effect in the transactivation of the *NIN* promoter by the complex similar to NIN (Fig. 15), I asked whether it would have an inhibitory effect in nodule number when being overexpressed. A significant reduction in nodule numbers when *NIN-NLP4* was overexpressed was not observed compared to the reduction caused by *NIN* overexpression (Fig. 17). This highlights that results between *N. benthamiana* and *L. japonicus* roots are not necessarily correlating. We tested the specificity of NIN-mediated inhibition in both *N. benthamiana* and *L. japonicus* systems by using *Lj*NLP1 (the closest ortholog to NIN) and *Lj*NLP4 (Fig. 2d). Both *Lj*NLPs were able to reduce the Cyclops-mediated transactivation of the *NIN* promoter, but not to the same extent as NIN^{FL} or the NIN-NLP4 chimeric protein (Fig. 15b). We evaluated the effect of overexpressing both NLPs for inhibition of nodulation in WT plants. Neither *NLP4* nor *NLP1* overexpression interfered with nodulation (Fig. 17), indicating that the inhibition of nodule formation by NIN is specific.

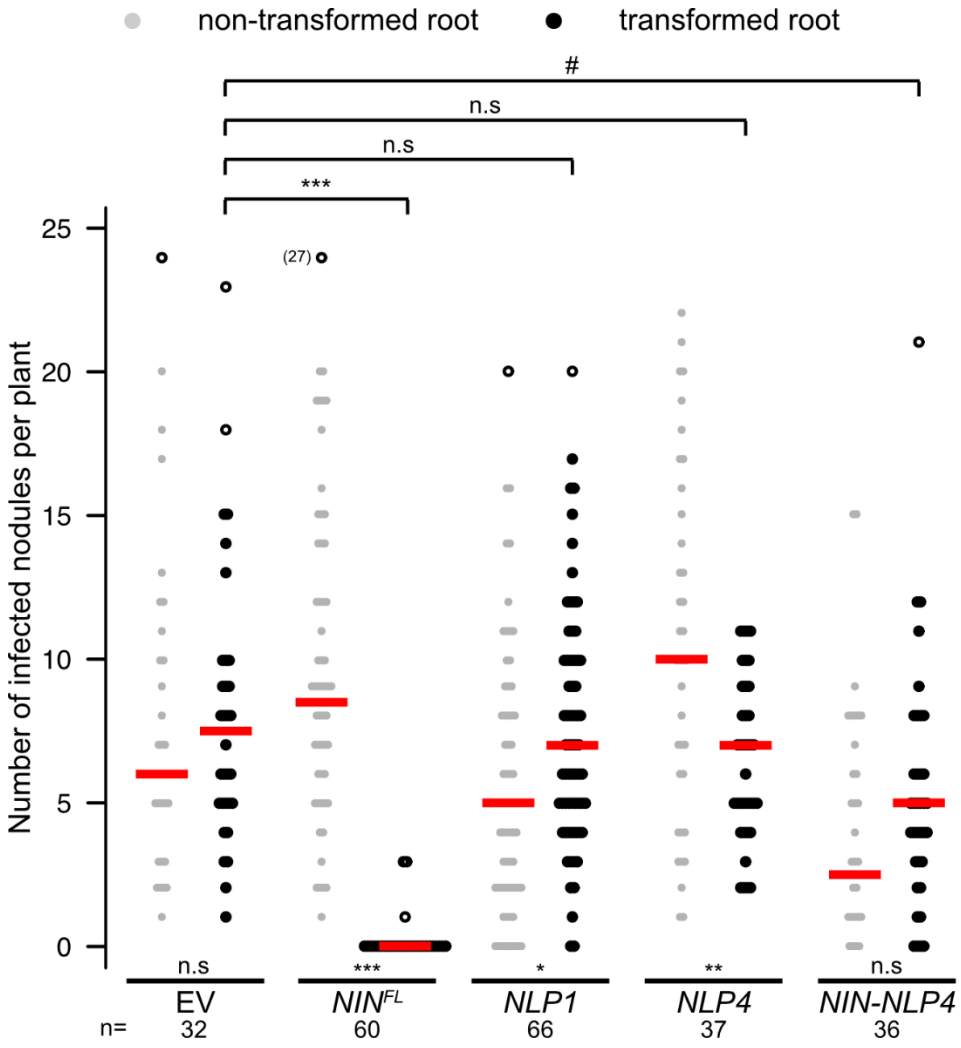


Fig. 17 Overexpression of neither *NLP1*, *NLP4* nor *NIN-NLP4* influence the nodule number in *L. japonicus*.

Hairy roots of *L. japonicus* WT transformed with the empty vector (EV, *ubq10:NLS-2xGFP*), *ubq:myc-NIN* (*NIN*^{FL}), *ubq:myc-NLP1* (*NLP1*), *ubq:myc-NLP4* (*NLP4*) and *ubq:myc-NIN-NLP4* (*NIN-NLP4*) were generated. Plot represents the number of nodules in both transformed and non-transformed in roots overexpressing *NIN*^{FL}, *NLP1*, *NLP4* and *NIN-NLP4* quantified 3 weeks post inoculation with *M. loti*. The distinction between transformed and non-transformed roots was possible with the visualization of the GFP transformation marker. Red line, median; grey dots, number of infected nodules in non-transformed roots; black dots, number of infected nodules in transformed roots. n indicates the number of individual roots analyzed. Pairwise Welch t-tests were performed between transformed and non-transformed nodule number per construct and between number of nodules in transformed roots in the EV vs the ones in *NIN*^{FL}, *NLP1*, *NLP4* and *NIN-NLP4*. *p<0.05, **p<0.01, ***p<0.001.

8. Endogenous *NIN* expression was reduced by *NIN* overexpression in spontaneous nodule mutants

It was previously reported that overexpression of *NIN* inhibits the formation of spontaneous nodules locally, in both *snf1-1* and *snf2-2* mutants (Doctoral thesis Lambert, 2017). The *snf1-1* mutant encodes a CCaMK protein with a mutation in the autophosphorylation residue (T265I) and therefore is a constitutive active kinase (Tirichine et al., 2006). An inhibition of spontaneous nodule formation by *NIN* overexpression in this mutant suggests an inhibitory mechanism downstream of CCaMK. The *snf2-2* mutant contains a single amino acid substitution in a cytokinin receptor (Lotus Histidine Kinase, LHK1) (Tirichine et al., 2007). The formation of spontaneous nodules was exclusively reported in transformed roots, implying that this mechanism is independent of the *LHK1* regulation and cytokinin perception (Doctoral thesis Lambert, 2017). Similar results were obtained here by *NIN* overexpression with a local inhibition of spontaneous nodule formation in both mutants (Fig. 18a)

In these two mutants, I evaluated whether the endogenous *NIN* expression was reduced by *NIN* overexpression. Moreover, I asked whether a different response between both mutants could be observed. The overexpression construct (*ubq:myc-NIN*, NIN^{FL}) does not contain the 3'UTR of the *NIN* gene, enabling us to discriminate the expression of the endogenous *NIN* gene from the one of the transgene. A slight reduction of the endogenous *NIN* expression was detected in both mutants (Fig. 18b). This suggests that the endogenous *NIN* expression is indeed down-regulated upon *NIN* overexpression.

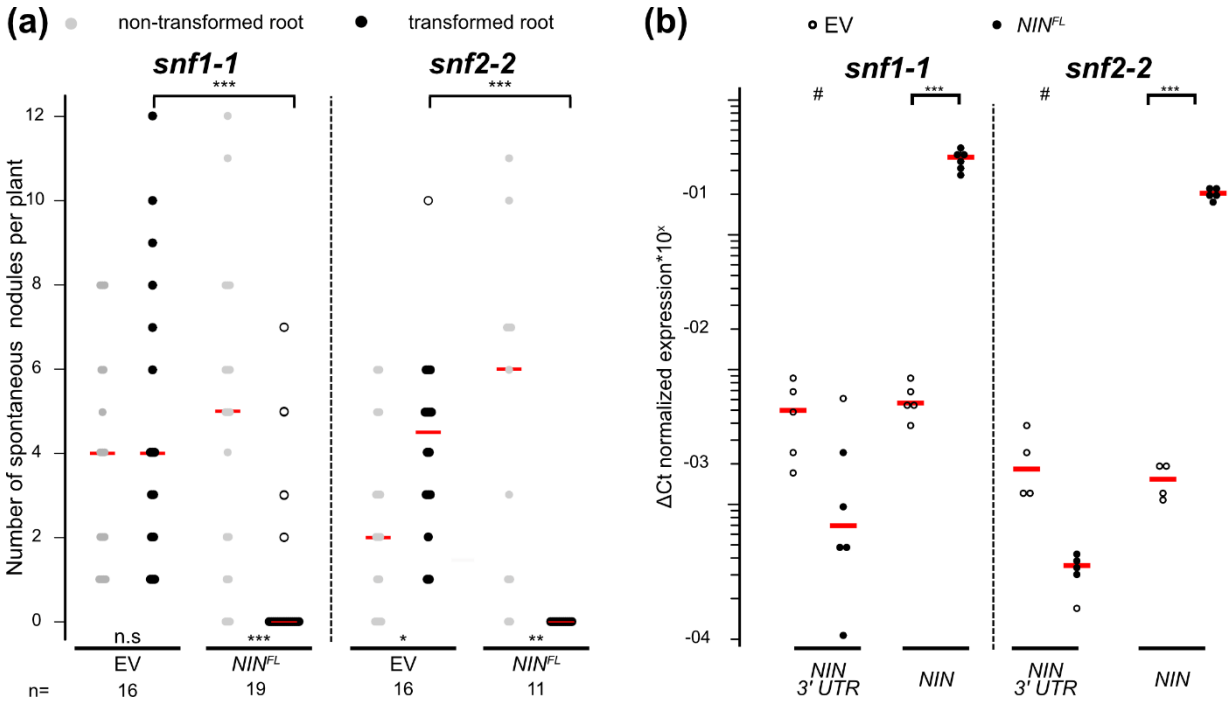


Fig. 18 Effect of *NIN* overexpression on spontaneous nodule formation and on the endogenous *NIN* expression in the *snf1-1* and *snf2-2* mutants.

Hairy roots of *L. japonicus snf1-1* and *snf2-2* transformed with the empty vector (EV, *ubq10:NLS-2xGFP*) or *ubq:myc-NIN* (NIN^{FL}) were generated. (a) Plot represents the number of nodules in both transformed and non-transformed in roots overexpressing NIN^{FL} formed in the absence of rhizobia at 60 dpt. The distinction between transformed and non-transformed roots was possible with the visualization of the GFP transformation marker. Red line, median; grey dots, number of spontaneous nodules in non-transformed roots; black dots, number of spontaneous nodules in transformed roots. n represents the number of individual roots analyzed. Pairwise Welch t-tests were performed between transformed and non-transformed spontaneous nodule number per construct and between number of spontaneous nodules in transformed roots in the EV vs the ones in NIN^{FL}. *p<0.05, **p<0.01, ***p<0.001. (b) Transcript accumulation of endogenous (*NIN 3'UTR*) and transgene (*NIN*) in transformed roots from (a) was determined by qRT-PCR. Transcripts levels for EV and NIN^{FL} in each genotype were determined with technical duplicates. Circles indicate expression relative to the housekeeping gene *EF1alpha*. Open circle, EV; black circle, NIN^{FL}. Statistical analysis used a Welch t test ($^{\#}$p<0.1, ***p<0.001).

VII. Discussion

1. Cyclops/NIN interaction and its dynamics

In this study, we confirmed the interaction between NIN and Cyclops, further delimited the domains involved, tested the requirement of the interaction for the inhibition and interrogated the effect of CCaMK in the Cyclops/NIN interaction.

Apart from NIN, several interactors have been described for Cyclops. Yano et al. (2008) reported that Cyclops interacts via its N-terminal part with CCaMK (81-366 aa), a region also required for Cyclops self-interaction. Other interactors of Cyclops include DELLA proteins (Jin et al., 2016; Pimprikar et al., 2016). Pimprikar et al. (2016) revealed that the CCaMK/Cyclops/DELLA complex constitutes a major regulatory hub interconnecting symbiosis and GA signaling during arbuscule development, while Jin et al. (2016) reported that the presence of DELLAs enhances the intensity of phosphorylation of IPD3 by CCaMK. A model was suggested where *Mt*DELLA bridges the interaction between IPD3 and NSP2 (Jin et al., 2016). Making use of truncated NIN and Cyclops protein variants, we delimited the interaction domain to the N-terminal part of both proteins. Notably, the delimited interaction region of Cyclops (84-245 aa) with NIN neither contained the AD nor the BD, present in the C-terminal part (D. Chiasson, unpublished data). Singh et al. (2014) previously proposed that the N-terminal part of Cyclops acts as a negative regulatory domain since its removal lead to an active transcription factor. As NIN interacts with the N-terminal part of Cyclops, it is tempting to hypothesize that NIN might have an influence in the conformation of this negative regulatory domain, which could ultimately impact in Cyclops activity.

Several interactors have also been described for the NLP protein family, but so far, a unique domain for interaction has not been delimited. It has been reported that the PB1 domain, present at the C-terminus of the protein, is required for the formation of homo and heterodimers between NLP6, NLP7 and TCP20 in Arabidopsis and for the interaction between NIN and all the NLPs in *M. truncatula* (Guan et al., 2017; Lin et al., 2018). In contrast, Wang et al. (2019) reported that NNC1 mainly interacts with NINa in soybean through the N-terminus of GmNINa but not with the RWP-RK nor the PB1 domain. Here, we also report that the N-terminal part of NIN (NIN^N, 1-296 aa) is required for the Cyclops/NIN interaction (Fig. 9). This indicates that NIN has at least two surfaces for interaction, one in the N-terminus and one at the C-terminus via the PB1 domain.

In the Y2H experimental setup, the interaction between Cyclops and NIN^{FL} could not be directly tested due to strong autoactivation of the system. Therefore, as previously reported, Cyclops was fused to the activation domain (AD) of the yeast GAL4 protein (Yano et al., 2008; Singh et al., 2014), and two truncated pieces of NIN (NIN^{N} and NIN^{C}) were fused to the $GAL4^{BD}$. Both NIN truncated pieces did not autoactivate the system, suggesting that the AD of NIN resides in the middle part of NIN (297-545 aa).

NIN and NLPs share a conserved C-terminal part, but the N-terminal region of NIN is distinct from the ones of NLPs (Schauser et al., 2005; Suzuki et al., 2013), and might have acquired the ability to interact with Cyclops and possibly other symbiotic and non-symbiotic proteins. To confirm this hypothesis, we would need to test if also the N-terminal part of NLPs could interact with Cyclops. We tried to assess this hypothesis; however, the N-terminal part of the four NLPs present in *L. japonicus* when fused to the $GAL4^{BD}$ caused strong autoactivation of the system (D. Chiasson, unpublished data). In the future, a version of Cyclops lacking its intrinsic autoactivation domain is required, so that Cyclops could be fused to the BD and the NLPs to the AD in the GAL4 yeast two hybrid system.

Previously, Lambert (2017) reported that the Cyclops/NIN interaction occurs regardless of Cyclops phosphorylation status, as $Cyclops^{WT}$, $Cyclops^{DD}$ and $Cyclops^{AA}$ interacted with NIN. My results indicated that the presence of autoactive versions of CCaMK negatively influence the Cyclops/NIN interaction (Fig. 10). This suggests that higher phosphorylated versions of Cyclops (other than $Cyclops^{DD}$) might play a role in fine-tuning the Cyclops/NIN interaction. According to this hypothesis, Jin et al. (2018) reported that in *M. truncatula* $IPD3^{2D}$ (S50 and S155, equivalent to S50 and S154 in $Cyclops^{DD}$ in *L. japonicus*) plays a positive role, while $IPD3^{8D}$ has a negative role in RNS. The authors described the presence of a homolog of IPD3 in *M. truncatula*, IPD3L. The double mutant *ipd3l ipd3-2* had a stronger phenotype than *ipd3* as it was completely unable to initiate IT formation and nodule organogenesis. The complementation of the double mutant with $IPD3^{8D}$ yielded a lower nodule number compared with $IDP3^{2D}$. Moreover, the expression levels of genes such as *NIN*, *ENOD11* and *FLOT4* were lower in the $IPD3^{8D}$ transformed roots compared with $IPD3^{2D}$. So apart from regulating interaction, as suggested here, extra phosphorylation sites modulate Cyclops transcriptional activity.

In this study, the dynamics of protein-protein interactions between NIN and Cyclops was studied in heterologous systems, where the biological symbiotic context is not taken into consideration. In the future, studies of Cyclops/NIN interaction need to be performed in *L.*

japonicus roots and ideally *in situ*. This will contribute to a better understanding of this interaction, especially in terms of when and where this interaction occurs.

2. A local inhibition of nodulation mediated by the PB1 domain of NIN

Soyano et al. (2014b) proposed that NIN is involved in AON and that roots constitutively expressing *NIN* repress nodulation systemically. We observed a local, not systemic, inhibition response due to *NIN* overexpression (Fig. 12, this work and Lambert, 2017). There are several possible explanations for this different outcome including (1) *NIN* driven by different promoters, (2) the addition of an inducible system and (3) different techniques for the induction of hairy roots. Soyano et al. (2014b) reported that dexamethasone treatment of roots expressing *NIN* fused with a glucocorticoid receptor (NIN-GR) under the *35S* promoter induced two morphological phenotypes in the root architecture: malformed and also normal roots. The systemic response was exclusively occurring in roots forming malformed structures. We could not observe these malformed structures in our overexpression analysis using the *L. japonicus Ubiquitin* promoter, that could be specifically correlated to *NIN* overexpression. In the work of Soyano, nodule number was reduced in positively transformed roots with normal architecture, whereas non-transformed roots displayed normal nodulation, which is consistent with our work. This strongly suggests the existence of a local inhibitory mechanism.

Results from Yoro et al. (2014) also supports our hypothesis of local inhibition. The authors described that NIN has a negative role for rhizobial infection, specifically on IT formation. They overexpressed *NIN* in the *daphne* mutant, a hyperinfective *nin* allele unable to develop nodules, and observed that non transformed roots maintained the hyperinfective phenotype whereas transformed roots had a drastic reduction of IT numbers. This observation suggests the existence of a NIN-mediated local inhibitory mechanism, at least for the infection process. Consistent with this hypothesis, is that *NIN* expression upon inoculation is lower than WT in another hyperinfected mutant, *lkh1* (Murray et al., 2007). These hyper-IT mutant phenotypes prompt exploration of the mechanism by which newly divided cortical cells repress IT formation during early nodule development. This could be part of a crosstalk mechanism between infection and nodulation.

Recently, using the *daphne har1-8* double mutant (the hyperinfected *daphne* mutant crossed with a mutant with impairment in AON regulation), the existence of a CLE-HAR1 systemic signaling and a new HAR1-independent local control of infection mediated by NIN was

described (Yoro et al., 2019). The *daphne* hyperinfective phenotype was suppressed with overexpression of *CLERS1/2* in a systemic manner. Notably, this effect was lost in the *daphne har1-8* double mutant, which indicates that CLE peptides do play a role in the suppression of increased formation of ITs via the HAR1 signaling pathway, as reported for nodule suppression (Okamoto et al., 2009). This scenario was different upon *NIN* overexpression in the *daphne har1-8* double mutant where 8/18 transformed roots maintained the hyper-IT phenotype, while 10/18 had a strong reduction in IT numbers despite the presence of the *har1-8* mutation. Interestingly, the strong reduction of ITs was exclusively observed in transformed roots (Yoro et al., 2019). These results confirm that NIN can act as a local negative regulator for IT formation, as what we observed for nodule organogenesis.

We investigated whether infection plays a role in this inhibitory effect. In both spontaneous nodule formation mutants, *NIN* overexpression inhibited the appearance of spontaneous nodules exclusively on transformed roots (Fig. 18). This indicates that this local inhibition is a) rhizobia independent b) downstream of CCaMK and c) independent of LHK1 and cytokinin perception. Taken together, our data reveal that NIN is also able to repress nodule organogenesis in a local manner.

In this work, I aimed to delimit the domain of NIN responsible for the local inhibition of nodulation to better understand the underlying mechanism. The C-terminal part of NIN (NIN^{C}, 546-878 aa) seems to be involved in the negative regulation, as a reduced number of nodules, compared to empty vector, was observed upon its overexpression. In contrast, the N-terminal part of NIN which is interacting with Cyclops, did not cause any effect in the nodule number (Fig. 12b). This is consistent with our results that the delimited Cyclops/NIN interaction domains do not play a role for inhibition *in planta*, at least under overexpression.

The role of the two conserved domains present at the C-terminal part, namely the RWP-RK and the PB1 domain, was further assessed. A strong inhibition for nodule number in transformed roots was observed upon overexpression of $NIN^{\Delta RWP}$ (a truncated version of NIN without the DNA binding domain), comparable to the overexpression of NIN^{FL} (Fig. 14). This is consistent with our previous results that DNA-binding is not required for the NIN inhibition. Notably, overexpression of $NIN^{\Delta PB1}$ also reduced the nodule number in transformed roots; however, not at the level of NIN^{FL}. This observation suggests that the inhibition is possibly mediated via the PB1 domain. This supports our transactivation assay results, which revealed that the PB1 domain is required for the inhibition of NIN on its own promoter (Fig. 13).

Because PB1 is a conserved domain within the NLP family, we assessed whether two other *L. japonicus* NLPs had the same inhibitory effect as the one observed for *NIN* overexpression. Overexpression of neither *NLP1* nor *NLP4* interfered with nodulation, which indicates that the negative regulation observed is a unique feature of *NIN* overexpression (Fig. 17). Lin et al. (2018) also reported that in *M. truncatula* no difference was observed in nodule number between empty vector and overexpression of *NLP1*. According to preliminary results, NLP4 is not required for the local inhibition of nodule numbers caused by *NIN* overexpression (data not shown). However, the phenotype of the mutant used for this experiment differed significantly from the one reported by Nishida et al. (2018). Using 10 mM nitrate, we observed a reduction in the nodule number in the here characterized *nlp4* mutant, comparable to the one of WT plants (data not shown). In contrast, the *nrsym1* mutant was unable to inhibit nodule number upon high nitrate concentration. Although, the strongest phenotype of *nrsym1* was observed with 50 mM nitrate concentration (Nishida et al., 2018). The *nrsym1* mutant was obtained through an ethylmethane sulfonate (EMS) screening in the MG-20 ecotype, while the one used for this experiment came from a *LORE1* insertion in the Gifu ecotype. The ecotype of the mutant might have played a role in the lack of phenotype, as MG-20 has higher sensitivity to nitrate for inhibition of nodule number compared with Gifu (data not shown). Another possibility for the lack of response by overexpressing *NIN* in the *nlp4* mutant is redundancy, as suggested by Lin et al. (2018). The authors reported a stronger phenotype when they expressed *NLP4-RNAi* in the *nlp1* mutant in *M. truncatula*, indicating redundancy of NLP1 and NLP4 in nitrate inhibition of nodulation. Further experimental studies are needed for the identification of the other PB1 containing protein that mediates together with NIN the inhibition.

The results presented in this work were obtained by overexpression using the strong *L. japonicus Ubiquitin* promoter. It would be interesting to analyze the effect of *NIN* expression under a weaker but still constitutive promoter, such as the *actin* promoter. In addition, to better understand the effect of NIN on nodule inhibition, the use of tissue specific promoters is advisable. Preliminary data suggested that at least expression of *NIN* under the epidermal promoter *EXPA7* (Hayashi et al., 2014), did not inhibit nodule formation (data not shown). The effect of *NIN* expression in the cortex could be assessed using the root cortex-specific promoter *AtCortex* (Gavrilovic et al., 2016) as reported by Miri et al. (2019).

Because our inhibition of nodulation by *NIN* overexpression is local, it would be valuable to analyze the expression of AON components in the transformed roots. An RNA-seq approach in those roots could also reveal a list of differentially expressed genes that could be involved in this local inhibition. It was recently described that nodules and lateral roots share an extensive

overlap in organogenesis and transcription. The point of convergence for both developmental programs is the activation of *LBD16*, a NIN target (Schiessl et al., 2019; Soyano et al., 2019). The authors reported that overexpression of *NIN* was sufficient to activate *LBD16* expression and that co-expression of *LBD16* with both *NF-Y* subunits increased lateral root densities six-fold over the controls (Soyano et al., 2019). This is an important result for future research. The sole effect of *NIN* overexpression for lateral root emergence should be analyzed, especially in the roots that do not develop nodules, to see if the lateral root program overrides the one for nodule organogenesis.

3. Role of NIN in the regulation of its promoter

The expression pattern of the *NIN* promoter is highly dynamic through the nodulation process. We compared the *NIN* promoter expression in both WT and *nin-2* upon inoculation with *M. loti*. The *nin-2* mutant is described as a null mutant, characterized by an excessive root hair response with over-curling, but unable to form ITs and nodule primordia (Schauser et al., 1999). Our comparison revealed that (1) the expression of the *NIN* promoter is deregulated in the epidermis, (2) there are at least some detectable *NIN* transcripts under mock conditions and (3) the mutant displays a high concentration of *NIN* transcripts upon *M. loti* treatment (Fig. 3). These results suggest that in the presence of *M. loti*, NIN is required for inhibition of its own expression. The reduction of endogenous *NIN* by *NIN* overexpression in *snf2* also supports our hypothesis that NIN represses its own expression, even in a rhizobia independent manner (Fig. 18).

Similar results to ours were also observed in another *nin* mutant allele, *daphne*, where *NIN* expression in the inner cortex is abolished and displays a hyperinfected phenotype. In *daphne*, the expression of the *NIN* promoter is in a larger zone of the root compared to WT, almost covering the whole root area (Yoro et al., 2014). The authors suggested that the loss of NIN caused this increase in rhizobial infection as overexpression and cortical expression of *NIN* suppressed the hyperinfection phenotype. A model has been suggested where epidermal *NIN* expression at early stages, positively regulates IT formation, whereas cortical *NIN* expressed at later stages negatively regulates IT formation (Yoro et al., 2014; Liu et al., 2019b). The deregulated expression in the epidermis that we observed is consistent with this model, as the *nin-2* mutant is unable to form nodule primordia, and according to the model unable to establish the negative regulation in the epidermis. Importantly, our observations could be either due to a lack of regulation of the promoter in the epidermis or caused by the lack of nodules formed. However,

the same pattern of expression and transcript accumulation as *nin-2* was observed in *nin-15*, a mutant that is impaired in infection but not in nodule organogenesis (data not shown). This suggests that the observed deregulated expression of the *NIN* promoter in the epidermis is partially independent of the cortical program absence. In the future, it would be interesting to confirm these results in a full *nin* knock-out mutant.

Cytokinin signaling is also crucial for regulation of *M. loti* infection, as *lhk1* mutants are hyperinfected (Murray et al., 2007). Miri et al. (2019) recently reported that expression of the cytokinin receptor *LHK1* in the cortex, which functions upstream of *NIN*, apart from being essential for nodule development, is required for the negative regulation of epidermal infection. Importantly, this LHK1-dependent signaling that restricts subsequent infection is initiated before nodule primordium formation. This suggests a possible mechanism of regulation of infection that does not necessarily require the activation of the cortex program, which could explain our observation of deregulated *NIN* expression in the epidermis in the *nin-2* mutant.

A possible role of NIN acting as a repressor was much earlier already suggested by Marsh et al. (2007). They described that the *M. truncatula nin* mutant displays a greatly expanded and contiguous expression of the *ENOD11* promoter. A dual role of NIN was more recently proposed according to the tissue implicated (Vernie et al., 2015). In the epidermis, NIN restricts the extent of *ENOD11* transactivation via competitive inhibition with ERN1. In contrast, NIN promotes the expression of *CRE1* in the root cortex. Therefore, in this case NIN works not only as an activator in the cortex, but also as a suppressor in the root epidermis. This indicates that a higher level of complexity arises in terms of crosstalk among specific tissues and time points which are necessary for the establishment of RNS. A case example of gene expression interactions in the control of developmental transition comes from flower development. Flower development requires a very precise spatial and temporal regulation of gene expression initiated by so-called floral meristem identity genes. In *A. thaliana* LEAFY (LFY), APETALA1 (AP1) and the AP1-paralog CAULIFLOWER (CAL) are key transcription factors which regulate the expression of floral meristem identity genes. They constitute hubs in the flowering gene regulatory network and control floral meristem specification in a partially overlapping manner (reviewed in O'Maoileidigh et al., 2014; Goslin et al., 2017).

4. NIN interferes with Cyclops transcriptional activity

Previously, Lambert (2017) reported that NIN inhibited the transactivation of its promoter by Cyclops^{DD} (a phosphomimetic version of Cyclops) in *N. benthamiana* transactivation assays and in *L. japonicus* roots. Despite the fact that this phosphomimetic version of Cyclops can transactivate the expression of the *NIN* promoter and induce spontaneous nodules, it is still not sufficient for full restoration of RNS in *ccamk* mutants (Singh et al., 2014). This suggests the existence of either additional phosphorylation sites on Cyclops by CCaMK and/or missing additional interaction partners of the complex. Therefore, we wanted to investigate if NIN could also inhibit the transactivation of its promoter in presence of Cyclops and CCaMK$^{1-314}$, or if the inhibition only occurs at the level of Cyclops^{DD}. NIN also inhibited the transactivation of its own promoter in the presence of both CCaMK1-314 and Cyclops, indicating that the inhibition is not only Cyclops^{DD} dependent and that it also occurs in presence of a WT version of Cyclops together with its phosphorylation partner, that could potentially phosphorylate Cyclops at different sites, not just S50 and S154. This confirms that NIN acts as a repressor of its own transcriptional activation influencing the activity of the CCaMK/Cyclops complex. This supports the previous hypothesis that the regulation of the *NIN* promoter is mediated by a negative feedback loop. In addition, we demonstrated that this transcriptional inhibition by NIN could be extended to two other symbiotic promoters, namely *ERN1* and *RAM1* (Fig. 4), that also happen to be targets of the CCaMK/Cyclops complex (Pimprikar et al., 2016; Cerri et al., 2017). This overall suggests a more general inhibitory mechanism of NIN at the level of Cyclops.

Several transcription factors are able to regulate the *ERN1* promoter both in *M. truncatula* and in *L. japonicus*, including Cyclops, NSP1/NSP2 and NY-A1/NF-YB1 (Hirsch et al., 2009; Cerri et al., 2012; Laloum et al., 2014; Cerri et al., 2017). Here I report an additional level of complexity and show a negative effect of NIN on the regulation of the *ERN1* promoter by the CCaMK/Cyclops complex (Fig. 4). Evidence of an interplay between NIN, Cyclops and ERN1 for IT formation has been recently provided (Liu et al., 2019c). Upon *M. loti* treatment, some *NIN* transcripts were detected in the *cyclops-3* mutant, suggesting the existence of another mechanism independent of Cyclops required for *NIN* activation. Moreover, ectopic expression of *NIN* or *ERN1* suppressed the IT-deficient *cyclops* mutant phenotype. However, *ERN1* and *NIN* failed to rescue the IT defect of *nin-2* and *ern1-1*, respectively. The authors reported that in addition to Cyclops, ERN1 also contributes to *NIN* expression. This together with our results, could potentially indicate the existence of another negative feedback loop between NIN and

ERN1. It appears that transcriptional regulatory networks between NIN, Cyclops and ERN1 are partially overlapping, which might be necessary for the coordination of molecular networks in order to integrate different stages during early signaling and subsequent infection pathways.

RAM1 was identified as a direct target of the CCaMK/Cyclops/DELLA complex (Pimprikar et al., 2016). *RAM1* encodes a GRAS protein required for arbuscule branching and induction of marker genes related to arbuscule development (Park et al., 2015; Xue et al., 2015). Here we report that NIN interferes with the transactivation of *RAM1* by the CCaMK/Cyclops complex (Fig. 4), suggesting a possible role of NIN in AM symbiosis. Since the description of the first mutant allele of NIN by Schauser et al. (1999), it has been commonly accepted by the community that NIN is exclusively involved in RNS, as all *nin* mutants analyzed were not impaired in AM symbiosis (Borisov et al., 2003; Marsh et al., 2007; Perry et al., 2009). However, it is believed now that mild phenotypes might have been missed in initial AM phenotyping screens. This has been the case for NSP1, considered for years to be specifically involved in RNS (Catoira et al., 2000; Maillet et al., 2011). Later, it was reported that *nsp1* mutants exhibit reduced AM colonization and/or reduced responsiveness to Myc-LCOs (Delaux et al., 2013a; Takeda et al., 2013). In the case of NIN, two laboratories investigated again and in more details its potential involvement in AM, producing contradictory results. Guillotin et al. (2016) claimed that NIN plays a role in AM. This claim was based on a reduced colonization rate in *M. truncatula nin-1* compared to WT plants and a 2.5-fold increase in *NIN* expression by treating roots with Myc-LCOs. Recently, Kumar et al. (2020) contradicted the results presented by Guillotin et al. (2016). Kumar and colleagues, employed a time course study in two *nin* alleles with different background, namely *nin-1* and *nin-2* from *M. truncatula* cv Jemalong A17 and *M. truncatula* ssp. *tricycla* R108, respectively. They could neither detect any induction of *NIN* by AM nor a consistent difference in AM colonization between those two *nin* mutants and WT plants. The experimental set-up used in Guillotin et al. and Kumar et al. were radically different. The first one employed the spore inoculation system, checked colonization after 9 wpi, and quantified *NIN* induction by Myc-LCOs application (Guillotin et al., 2016). The second study used a mixture of inoculum in form of a chive inoculation system, performed a time course and monitored *NIN* induction after inoculation with the fungi (Kumar et al., 2020). Kumar et al. (2020) argued that the colonization level is often in decline at the time point checked by Guillotin et al. (2016), therefore pinpointing the importance of a time course experiment for drawing conclusions about colonization. The lack of *NIN* induction by AM reported by Kumar et al. (2020) was also consistent with an earlier report from *L. japonicus*

that monitored *NIN* expression using qPCR over a four-point time course spanning from four days to four weeks after inoculation with *R. irregularis* (Takeda et al., 2013).

Recent phylogenetic analyses also argue against NIN being required for AM symbiosis. It was reported that the loss of NIN is correlated with the loss of RNS but not with the ability to form AM symbiosis, as the majority of the plants that lost NIN can still engage in AM symbiosis (Griesmann et al., 2018; van Velzen et al., 2018). Therefore, it seems that the role of NIN for RNS is highly specific and the evidence refutes at least a direct role of NIN in AM symbiosis.

We attempted to rationalize our finding that NIN interferes with Cyclops activation of the *RAM1* promoter. Preliminary data, collected after twelve days with the chive inoculation system, revealed that overexpression of *NIN* in *L. japonicus* roots inhibited all stages of AM colonization, including intracellular hyphae, arbuscule and spore counting. However, the expression of AM markers including *RAM1* were not reduced in these roots (data not shown). These preliminary data suggest that overexpression of *NIN* interferes with AM colonization; however, this does not involve inhibition of *RAM1*, as observed in the *N. benthamiana* transactivation assays. The lack of inhibition of *RAM1* could be due to a transient inhibition that was possibly missed at the stage observed. In our experimental setup after twelve days, we already had the presence of a lot of vesicles which indicates a rather late stage of the interaction; therefore, it might be that we missed early stages where overexpression of *NIN* could have had an impact on some AM markers. Thus, this experiment should be repeated to confirm the trend observed, and more importantly a time course should be included.

It is also important to highlight that our knowledge on the regulation of AM and RNS symbioses has been mainly obtained from studies on RNS and AM separately, even though both symbioses can simultaneously occur in nature. Lace and Ott (2018) reported that rhizobia and AM can be found in close physical proximity to each other on the same root system in either natural soils or in lab conditions. However, the influence on each other and how both symbioses are spatially maintained in the same root is still not clear. So far, a role of NIN in this mechanism has not been studied. It has been observed that co-inoculation has a negative effect on the microsymbionts. In *Vicia faba*, the presence of Rhizobium resulted in a significant decline in AM colonization, and in *Phaseolus vulgaris* co-inoculation decreased colonization success for both symbioses (Jia et al., 2004; Ballesteros-Almanza et al., 2010). To determine possible interconnection between both symbiosis, co-inoculation experiments need to be considered for the future.

One limitation of our work is that our hypothesis, NIN inhibits Cyclops transcriptional activity, is based in *N. benthamiana* transient assays. This is a limitation since we cannot discard the presence of other components present in the *N. benthamiana* leaves that might have had a potential effect in our results. However, there is evidence that this inhibition also occurs in roots, at least for the case of the *NIN* promoter. Previously, Lambert (2007) demonstrated that co-expression of Cyclops^{DD} and NIN led to a reduced induction of the *NIN* promoter in *L. japonicus* roots. Consequently, more evidence needs to be collected in order to confirm that this inhibition also occurs in *L. japonicus* roots for the two others promoters, namely *ERN1* and *RAM1*.

5. By what mechanism does NIN impact on Cyclops activity?

Overall, our results suggest that NIN is a negative regulator of Cyclops transcriptional activity. We aimed to unravel which molecular mechanism could explain this repression mode. We tested if this could be mediated by either NIN binding to these promoters and competing with Cyclops for *cis*-DNA binding elements or interacting with Cyclops at the level of the protein, and thus repressing its activation.

An element located within the *NIN* promoter (*pNBS* - putative NIN binding site) was previously identified as the region where NIN binds *in vitro* via EMSA. It was hypothesized that NIN binds to this element and represses the transactivation of its own promoter by Cyclops^{DD} (Doctoral thesis Lambert, 2017). We first aimed to delimit the minimal region where NIN can inhibit Cyclops^{DD} transactivation of the *NIN* promoter. However, the activation of all the deletions tested by Cyclops^{DD} was inhibited when NIN was present. In addition, repression was still observed when this *pNBS* was mutagenized in the context of the *NIN* promoter (Fig. 6). This strongly suggests that this *pNBS* is not required for inhibition as initially proposed. Furthermore, this element was not activatable by the synthetic transcription activator NIN^{C}-VP16, which could transactivate the expression of *NRE:GUS* (Fig. 7a). Altogether, our results suggest that even though NIN might bind to this region *in vitro*, this element does not play a role for the inhibitory mechanism *in planta*. Further evidence that NIN binding to its promoter does not mediate inhibition came from the observation that the deletion of the RWP-RK domain (DNA binding domain) of NIN did not impair the ability of NIN to repress the transactivation of the *NIN* promoter by the complex (Fig. 13).

Our data suggested that NIN can repress the transactivation of its own promoter by the CCaMK/Cyclops complex independently of the DNA binding sequence bound by Cyclops

(*CYC-box*, Fig. 8). This is consistent with the observation that NIN could repress the transactivation of, so far, all known Cyclops targets (Fig. 4). Notably, the existence of other Cyclops *cis*-elements within the *NIN* promoter tested (3 kb length) needs to be taken into consideration. However, preliminary data from Cathebras et al (personal communication) demonstrated that mutagenesis or deletion of an element containing the *CYC-RE*, within the 3 kb *NIN* promoter impaired the transactivation of this promoter by the CCaMK/Cyclops complex. This suggests that, at least in transactivation assays, there are no other sequences within the 3 kb *NIN* promoter that could be transactivated by Cyclops. It would be advisable to test the effect of NIN in the $GAL4^{BD}$-Cyclops/$CCaMK^{1-314}$ transactivation of the *5xUAS* promoter. If NIN is still able to inhibit this activation, that would confirm that the DNA binding site of Cyclops is not required for the inhibition.

All together our results suggested that the inhibition of Cyclops by NIN is not happening via DNA binding but might rather be mediated by a different mechanism, e.g. protein-protein interaction. We hypothesized that the interaction between NIN and Cyclops was required for the inhibition of Cyclops activity. However, NIN was still able to inhibit the transactivation of *2xCYC-RE* by $Cyclops^{min}$, a Cyclops version lacking the delimited interaction region (Fig. 11). This suggests that the interaction between NIN and Cyclops is not necessary for the inhibition, at least in this transient *N. benthamiana* assay.

My data suggests that actually the PB1 domain of NIN is required for the inhibition of Cyclops transactivation in the *NIN* promoter (Fig. 13). The versatile PB1 interaction domain is required for protein-protein interactions through either dimerization or oligomerization that allows the development of higher-order functionalities (Mutte and Weijers, 2020). PB1 domains are widely conserved and involved in responses to nutrients, growth factors and stress (Schauser et al., 2005; Sumimoto et al., 2007; Guilfoyle, 2015). PB1 domains are critical for the establishment of homo and heterodimers and in a lesser extend interactions with proteins lacking PB1 domains (Sumimoto et al., 2007; Guilfoyle, 2015). The requirement of the PB1 domain for inhibition indicates that NIN probably recruits other PB1 containing protein which ultimately impairs Cyclops activity. It was reported that deletion or even mutagenesis of key amino acids of this domain disrupts the interaction between NLPs (Guan et al., 2017; Konishi and Yanagisawa, 2019). As the PB1 domain was required for the inhibition, I mutagenized conserved amino acids within this domain. Surprisingly, mutagenesis of these conserved residues did not impair the ability of NIN for inhibiting Cyclops transactivation of the *NIN* promoter in *N. benthamiana* (Fig. 13). Konishi and Yanagisawa (2019) described recently that mutagenesis of these key residues did not affect the ability to transactivate the expression of

nitrate responsive promoters in protoplast transient assays. The authors attribute this to possible artifacts created by transient assay systems that do not completely mimic transcription of chromosomal genes. This might also explain the lack of an effect by mutagenesis of these key residues in our transactivation assays.

6. A model for the negative feedback loop caused by NIN

We observed that NIN turns into a repressor according to the partners it interacts with. This interaction is dynamic and regulated by the amount of protein present. According to the data accumulated, I propose the following model: NIN interacts with Cyclops and therefore regulates its own expression interfering with Cyclops transactivation capacities. My data revealed that even in non-inoculated conditions some *NIN* transcripts are present. Under very low NIN concentration, the Cyclops/NIN interaction could occur through the N-terminal part of both proteins. When NIN and Cyclops interact, the area for the CCaMK/Cyclops interaction is not available. Consequently, this interaction ensures that Cyclops does not transactivate the expression of its targets without the proper stimuli. Once calcium spiking occurs, CCaMK phosphorylates Cyclops, which disrupts the interaction with NIN, and allows Cyclops to transactivate the expression of *NIN*. When there is a lot of NIN protein produced, NIN interferers with the transactivation of Cyclops by possibly recruiting other PB1-containing protein which can interact with Cyclops and remove it from the *CYC-RE* or directly bind to the *CYC-RE* with higher affinity and ultimately stop the transactivation of the *NIN* promoter. Our work has led us to propose that NIN establishes a negative feedback loop, which contributes to the regulation of nodule formation in a local manner. This type of regulation has been extensively studied for explaining circadian rhythms. A model named transcription-translation feedback loops (TTFLs) has become the dominant paradigm for conceptualizing circadian oscillations in both plants and animals. The simplified version of this model involves the formation of a heterodimer between CLOCK and BMAL1 that together activate the transcription of *Per*, *Cry* and *Rev-erb* genes through E-box enhancers in mammalian cells. Once the levels of PER increase, a complex is formed with CRY and CKI1/CKId proteins. This complex acts as a negative regulator by directly interacting with the CLOCK-BMAL1 heterodimer and inhibiting the transcription of its targets (Wulund and Reddy, 2015).

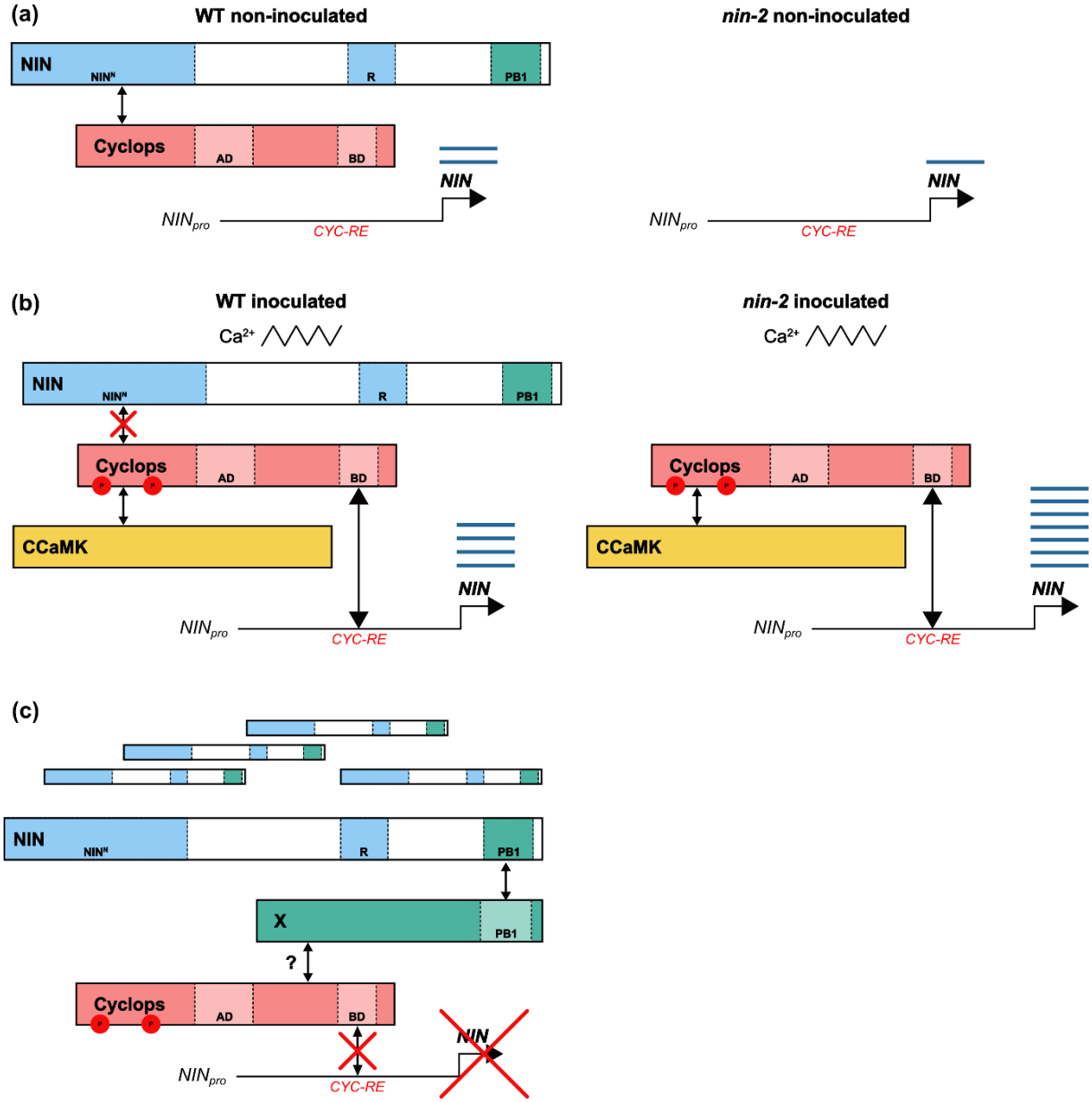


Fig. 19 Model of mechanistic action of NIN for the regulation of Cyclops activity in the *NIN* promoter.

A comparison of the regulation of the *NIN* promoter by NIN between WT and *nin-2*. (a) Under non-inoculated conditions there is a basal *NIN* expression. NIN and Cyclops interact through their N-terminal domains and this interaction blocks Cyclops for transactivation of the *NIN* promoter. (b) Upon calcium spiking, CCaMK phosphorylates Cyclops and the interaction with NIN is lost. Cyclops is then able to bind and transactivate the expression of the *NIN* promoter. In the mutant a high amount of *NIN* transcript is accumulated. (c) After a while the NIN protein interferes with Cyclops activity by recruiting other protein containing a PB1 domain.

Cases where the same transcription factor controls the expression of genes both positively and negatively, according to their interaction partners, have been reported. This interaction enables transcription factors a specific-context dependent gene activation or repression. One example is ZNF750, a transcription factor responsible for epithelial homeostasis. Two types of global gene expression changes are required for progenitor differentiation, namely, induction of epidermal differentiation genes and the repression of epidermal progenitors. ZNF750 can bind to both the progenitor and differentiation genes, but the interaction with different proteins specifies its mode of action. When ZNF750 interacts with another transcription factor, KLF4, the activation of differentiation target genes occurs. In contrast, interaction with a chromatin regulator, KDM1A, facilitates the repression of progenitor genes (Boxer et al., 2014).

The coordinated and massive induction of genes required for nodulation needs certainly the activation of specific TFs, but in addition, other mechanisms such as regulation at the chromatin level might play key important roles. For instance, genes with high tissue-specific expression are often actively silenced throughout much of the plant lifecycle via epigenetic mechanisms (Mergaert et al., 2020). For instance, the soybean protein CPP1, part of a chromatin related repression complex, binds to the promoter of the leghemoglobin gene *Gmlbc3* and represses its expression (Cvitanich et al., 2000). Another level of epigenetic control for the expression of symbiotic nodule cell-specific genes represents DNA methylation. Promoters that are methylated usually lead to gene silencing. Satge et al. (2016) reported that DNA demethylation controls many symbiotic nodule cell specific genes, which are essential for nodule differentiation and the acquisition of organ identity in *M. truncatula*.

Our work has highlighted the level of interconnection between components in the symbiosis signaling, in our case the Cyclops/NIN complex. The Cyclops/NIN interaction might be one of the many possible interactions of NIN, where the interactome of NIN can potentially modulate its activity. Moreover, additional work on deciphering the spatio-temporal relevance of this inhibitory NIN function is required. Studies in these directions might contribute to a better understanding of this key TF.

VIII. Materials and Methods

1. Plant material

Experiments were performed using *L. japonicus* ecotypes Gifu (B-129) (WT, Handberg and Stougaard, 1992), and the symbiotic mutants *nin-2* (Schauser et al., 1999), *snf1-1* (Tirichine et al., 2006) and *snf2-2* (Tirichine et al., 2007).

2. Bacterial strains

Transactivation assays and FLIM-FRET analysis were performed in *N. benthamiana*. Root transformation was performed with *Agrobacterium rhizogenes* strain AR1193 (Stougaard et al., 1987) and nodulation experiments were done with *Mesorhizobium loti* MAFF 303099 expressing *Ds*Red (Markmann et al., 2008). *A. tumefaciens* strains AGL1 and GV3101 were used for transient expression in *N. benthamiana*.

3. DNA constructs and oligonucleotides

Plasmids were generated by Gateway cloning using pENTR-D/TOPO (Invitrogen) as entry vector and LR clonase (Invitrogen) for recombination into the destination vector or using the Golden Gate cloning system described by Binder et al. (2014). A detailed description of the oligonucleotides and constructs used in this study is listed in the following tables.

Table 1. Constructs

Purpose	**Plasmid name**	**Construction/Reference**	**Plasmid ID**
Golden Gate level III plasmids			
GUS expression in HRs/TA	LIIIβ F A-B *NIN_{pro}:GUS/ NIN_{pro}3kb:GUS*	Assembled by Esp3I cut ligation from: LIIIβ F A-B lacZdy:GUS + LI NINpro 2kb + LI NINpro 1kb	pRA53
GUS expression in HRs	LIIIβ F A-B *lacZdy:GUS*	Assembled by BpiI cut ligation from: LIIc F 1-2 ubq10:NLS-2xGFP + LIIβ F2-3 lacZdy:GUS + LII 3-4 dy (BB64) + LIIIβ F A-B (BB53)	pRA48

Purpose	Plasmid name	Construction/Reference	Plasmid ID
TA	LIIIβ F A-B *NIN_{pro870}:GUS*	Assembled by Esp3I cut ligation from: LIIIβ F A-B lacZdy:GUS + LI A-B NINpro (870)	pRA52
TA	LIIIβ F A-B *NIN_{pro}::mpNBS:GUS*	Assembled by Esp3I cut ligation from: LIIIβ F A-B lacZdy:GUS + LI A-B NINpro::mNIN-RE (870)	pRA134
TA	*NIN_{pro}3kb::$GAL4_{UAS}$:GUS*	Gift from Dr. Chiasson	GC1348, DC
OE in HRs	LIIIβ F A-B (EV) *ubq10:NLS-2xGFP*	Assembled by BpiI cut ligation from: LIIc F 1-2 ubq10:NLS-2xGFP + LII 2-6 dy (BB42) + LIIIβ F A-B (BB53)	pRA18
OE in HRs	LIIIβ F A-B *ubq:myc-NIN* (*NIN^{FL}*)	Assembled by BpiI cut ligation from: LIIc F 1-2 ubq10:NLS-2xGFP + LII 2-3 dy (BB39) + LIIβ F3-4 ubq:myc-NINFL + LII 4-6 dy (BB41) + LIIIβ F A-B (BB53)	pRA15
OE in HRs	LIIIβ F A-B *ubq:myc-NIN^{N}* (*NIN^{N}*)	Assembled by BpiI cut ligation from: LIIc F 1-2 ubq10:NLS-2xGFP + LII 2-3 dy (BB39) + LIIβ F3-4 ubq:myc-NINN + LII 4-6 dy (BB41) + LIIIβ F A-B (BB53)	pRA16
OE in HRs	LIIIβ F A-B *ubq:myc-NIN^{C}* (*NIN^{C}*)	Assembled by BpiI cut ligation from: LIIc F 1-2 ubq10:NLS-2xGFP + LII 2-3 dy (BB39) + LIIβ F3-4 ubq:myc-NINC + LII 4-6 dy (BB41) + LIIIβ F A-B (BB53)	pRA17
OE in HRs	LIIIβ F A-B *ubq:myc-$NIN^{\Delta PB1}$* (*$NIN^{\Delta PB1}$*)	Assembled by BpiI cut ligation from: LIIc F 1-2 ubq10:NLS-2xGFP + LII 2-3 dy (BB39) + LIIβ F3-4 ubq:myc-NINΔPB1 + LII 4-6 dy (BB41) + LIIIβ F A-B (BB53)	pRA204
OE in HRs	LIIIβ F A-B *ubq:myc-$NIN^{\Delta RWP}$* (*$NIN^{\Delta RWP}$*)	Assembled by BpiI cut ligation from: LIIc F 1-2 ubq10:NLS-2xGFP + LII 2-3 dy (BB39) + LIIβ F3-4 ubq:myc-NINΔR + LII 4-6 dy (BB41) + LIIIβ F A-B (BB53)	pRA203

Purpose	Plasmid name	Construction/Reference	Plasmid ID
OE in HRs	LIIIβ F A-B *ubq:myc-NLP4* (*NLP4*)	Assembled by BpiI cut ligation from: LIIc F 1-2 ubq10:NLS-2xGFP + LII 2-3 dy (BB39) + LIIβ F3-4 ubq:myc-NLP4-NLS + LII 4-6 dy (BB41) + LIIIβ F A-B (BB53)	pRA238
OE in HRs	LIIIβ F A-B *ubq:myc-NLP1* (*NLP1*)	Assembled by BpiI cut ligation from: LIIc F 1-2 ubq10:NLS-2xGFP + LII 2-3 dy (BB39) + LIIβ F3-4 ubq:myc-NLP1-NLS + LII 4-6 dy (BB41) + LIIIβ F A-B (BB53)	pRA237
OE in HRs	LIIIβ F A-B *ubq:myc-NIN-NLP4* (*NIN-NLP4*)	Assembled by BpiI cut ligation from: LIIc F 1-2 ubq10:NLS-2xGFP + LII 2-3 dy (BB39) + LIIβ F3-4 ubq:myc-NINNLP4-NLS + LII 4-6 dy (BB41) + LIIIβ F A-B (BB53)	pRA240
Golden Gate level II plasmids			
FLIM-FRET	LIIβ F5-6 *ubq:GFP-Cyclops* (GFP-Cyclops)	Gift from C. Cathebras. Assembled by BsaI cut ligation of LI A-B pUbi (G007) + LI sGFP (G28) + LI C-D Cyclops + LI D-E dy (BB08) + LI nos-T (G006) + LI F-G dy (BB09) + LIIβ F5-6 (BB28)	pRA81, CCF4
FLIM-FRET	LIIβ F5-6 *ubq:CCaMK-mCherry* (CCaMK-mCherry)	Gift from C. Cathebras. Assembled by BsaI cut ligation of LI A-B pUbi (G007) + LI B-C dy (BB06) + LI C-D CCaMK + LI D-E mCherry (G25) + LI E-F HSP-T (G045) + LI F-G dy (BB09) + LIIβ F5-6 (BB28)	pRA82, CCF5
FLIM-FRET	LIIβ F3-4 *ubq:NLS-mCherry* (free mCherry)	Gift from C. Cathebras. Assembled by BsaI cut ligation of LI A-B pUbi (G007) + LI B-C NLS (G60) + LI C-D mCherry no ATG (GG57) + LI D-E dy (BB08) + LI E-F nos-T (G006) + LI F-G dy (BB09) + LIIβ F3-4 (BB24)	pRA80, CCF3

Purpose	Plasmid name	Construction/Reference	Plasmid ID
FLIM-FRET	LIIβ F3-4 *ubq:mCherry-NIN* (mCherry-NIN)	Assembled by BsaI cut ligation of LI A-B pUbi (G007) + LI B-C mCherry (G032) + LI C-D gNINFL + LI D-E dy (BB08) + LI nos-T (G006) + LI F-G dy (BB09) + LIIβ F3-4 (BB24)	pRA91
FLIM-FRET	LIIβ F3-4 *ubq:mCherry-NINN-NLS* (mCherry-NIN^{N})	Assembled by BsaI cut ligation of LI A-B pUbi (G007) + LI B-C mCherry (G032) + LI C-D gNINN + LI D-E C-NLS (G035) + LI nos-T (G006) + LI F-G dy (BB09) + LIIβ F3-4 (BB24)	pRA139
FLIM-FRET	LIIβ F3-4 *ubq:mCherry-NINC-NLS* (mCherry-NIN^{C})	Assembled by BsaI cut ligation of LI A-B pUbi (G007) + LI B-C mCherry (G032) + LI C-D gNINC + LI D-E C-NLS (G035) + LI nos-T (G006) + LI F-G dy (BB09) + LIIβ F3-4 (BB24)	pRA140
FLIM-FRET	*ubq:myc-gCCaMK314-NLS-nosT* (CCaMK$^{1-314}$)	Gift from C. Cathebras	pRA108
FLIM-FRET	*ubq:myc-gCCaMKT265D-nosT* (CCaMK^{T265D})	Gift from C. Cathebras	pRA109
FLIM-FRET	*ubq:myc-gCCaMKD186N-nosT* (CCaMK^{NFG})	Gift from C. Cathebras	pRA110
FLIM-FRET	*ubq:myc-gCCaMK-nosT* (CCaMK^{WT})	Gift from C. Cathebras	pRA111
	LIIβ F2-3 *lacZdy:GUS*	Assembled by BsaI cut ligation of LI A-B Esp3I-lacZ dy (G082) + LI B-C dy (BB06) + LI C-D GUS w/o intron (Gift from D. Chiasson) + LI D-E dy (BB08) + LI nos-T (G006) + LI F-G dy (BB09) + LIIβ F2-3 (BB22)	pRA46

Purpose	Plasmid name	Construction/Reference	Plasmid ID
TA	LIIβ F3-4 *Esp3I 35Smin:GUS*	Assembled by BsaI cut ligation of LI A-B Esp3I-lacZ dy (G082) + LI B-C 35Smin (Gift from D. Chiasson), LI C-D GUS w/o intron (Gift from D. Chiasson, DC 720), LI D-E dy (BB08), LI E-F 35S term (G59) and LI F-G dy (BB09) + LIIβ F3-4 (BB24)	pRA106
TA	LIIβ F3-4 *B3:GUS*	Assembled by Esp3I cut ligation from: LIIβ F3-4 Esp3I 35Smin:GUS + LI A-B B3	pRA43
TA	LIIβ F3-4 *pNBSCYC-RE:GUS*	Assembled by Esp3I cut ligation from: LIIβ F3-4 Esp3I 35Smin:GUS + Fragment: RA37 + RA38	pRA195
TA	LIIβ F3-4 *CYC-RE:GUS*	Assembled by Esp3I cut ligation from: LIIβ F3-4 Esp3I 35Smin:GUS + Fragment: RA39 + RA40	pRA196
TA	LIIβ F3-4 *NRE:GUS*	Assembled by Esp3I cut ligation from: LIIβ F3-4 Esp3I 35Smin:GUS + LI A-B 1xNRE	pRA108
TA	LIIβ F3-4 *35S:myc-none* (none)	Assembled by BsaI cut ligation of LI A-B p35S (G005) + LI B-C c-MYC (G069) +LI C-D dy (BB07) + LI D-E dy (BB08) + LI E-F 35S-T (G059) + LI F-G dy (BB09) + LIIβ F3-4 (BB24)	pRA93
TA	LIIβ F3-4 *35S:myc-NLP4$_C$VP16* (NLP4^C-VP16)	Assembled by BsaI cut ligation of LI A-B p35S (G005) + LI B-C c-MYC (G069) +LI C-D cNLP4C + LI D-E dy (BB08) + LI E-F 35S-T (G059) + LI F-G dy (BB09) + LIIβ F3-4 (BB24)	pRA105
TA	LIIβ F3-4 *35S:myc-NIN^C-VP16* (NIN^C-VP16)	Assembled by BsaI cut ligation of LI A-B p35S (G005) + LI B-C c-MYC (G069) +LI C-D cNINC + LI D-E dy (BB08) + LI E-F 35S-T (G059) + LI F-G dy (BB09) + LIIβ F3-4 (BB24)	pRA99

Purpose	Plasmid name	Construction/Reference	Plasmid ID
TA	LIIβ F3-4 *ubq:myc-NIN$^{\Delta PB1}$* (NIN$^{\Delta PB1}$)	Assembled by BsaI cut ligation of LI A-B pUbi (G007) + LI B-C c-MYC (G069) +LI C-D gNINΔPB1 + LI D-E dy (BB08) + LI E-F HSP-T (G045) + LI F-G dy (BB09) + LIIβ F3-4 (BB24)	pRA33
TA	LIIβ F3-4 *ubq:myc-NIN$^{\Delta RWP}$* (NIN$^{\Delta RWP}$)	Assembled by BsaI cut ligation of LI A-B pUbi (G007) + LI B-C c-MYC (G069) + LI C-D LI C-D gNINΔR + LI D-E C-NLS (G035) + LI E-F HSP-T (G045) + LI F-G dy (BB09) + LIIβ F3-4 (BB24)	pRA86
	LIIβ F3-4 *ubq:myc-NIN^{N}*	Assembled by BsaI cut ligation of LI A-B pUbi (G007) + LI B-C c-MYC (G069) + LI C-D gNINN + LI D-E dy (BB08) + LI E-F HSP-T (G045) + LI F-G dy (BB09) + LIIβ F3-4 (BB24)	pRA10
	LIIβ F3-4 *ubq:myc-NIN^{C}*	Assembled by BsaI cut ligation of LI A-B pUbi (G007) + LI B-C c-MYC (G069) + LI C-D gNINC + LI D-E dy (BB08) + LI E-F HSP-T (G045) + LI F-G dy (BB09) + LIIβ F3-4 (BB24)	pRA9
TA	LIIβ F3-4 *ubq:myc-NIN^{FL}* (NIN^{FL})	Assembled by BsaI cut ligation of LI A-B pUbi (G007) + LI B-C c-MYC (G069) + LI C-D gNINFL + LI D-E dy (BB08) + LI E-F HSP-T (G045) + LI F-G dy (BB09) + LIIβ F3-4 (BB24)	pRA11
TA	LIIβ F3-4 *ubq:myc-NLP4-NLS* (NLP4)	Assembled by BsaI cut ligation of LI A-B pUbi (G007) + LI B-C c-MYC (G069) + LI C-D cNLP4 + LI D-E dy (BB08) + LI E-F HSP-T (G045) + LI F-G dy (BB09) + LIIβ F3-4 (BB24)	pRA226
TA	LIIβ F3-4 *ubq:myc-NLP1-NLS* (NLP1)	Assembled by BsaI cut ligation of LI A-B pUbi (G007) + LI B-C c-MYC (G069) + LI C-D cNLP1 + LI D-E dy (BB08) + LI E-F HSP-T (G045) + LI F-G dy (BB09) + LIIβ F3-4 (BB24)	pRA225

Purpose	**Plasmid name**	**Construction/Reference**	**Plasmid ID**
TA	LIIβ F3-4 *ubq:myc-NIN-NLP4-NLS* (NIN-NLP4)	Assembled by BsaI cut ligation of LI A-B pUbi (G007) + LI B-C c-MYC (G069) + LI C-D cNIN-NLP4 + LI D-E dy (BB08) + LI E-F HSP-T (G045) + LI F-G dy (BB09) + LIIβ F3-4 (BB24)	pRA228
TA	LIIβ F3-4 *ubq:myc-NIN* $^{mPB1\ 3aa}$ (NIN $^{mPB1\ type\ I}$)	Assembled by BsaI cut ligation of LI A-B pUbi (G007) + LI B-C c-MYC (G069) + LI C-D cNIN mPB1 type I + LI D-E dy (BB08) + LI E-F HSP-T (G045) + LI F-G dy (BB09) + LIIβ F3-4 (BB24)	pRA257
TA	LIIβ F3-4 *ubq:myc-NIN* $^{mPB1\ 1aa}$ (NIN $^{mPB1\ type\ II}$)	Assembled by BsaI cut ligation of LI A-B pUbi (G007) + LI B-C c-MYC (G069) + LI C-D cNIN mPB1 type I/II + LI D-E dy (BB08) + LI E-F HSP-T (G045) + LI F-G dy (BB09) + LIIβ F3-4 (BB24)	pRA258
TA	LIIβ F3-4 *ubq:myc-NIN* $^{mPB1\ 4aa}$ (NIN $^{mPB1\ type\ I/II}$)	Assembled by BsaI cut ligation of LI A-B pUbi (G007) + LI B-C c-MYC (G069) + LI C-D cNIN-NLP4 + LI D-E dy (BB08) + LI E-F HSP-T (G045) + LI F-G dy (BB09) + LIIβ F3-4 (BB24)	pRA259
	LIIc F1-2 *ubq10:NLS-2xGFP*	Transformation marker. Gift from C. Cathebras.	
TA	GAL4^{BD}-Cyclops	Gift from Dr. Chiasson.	GC1329, DC
Golden Gate level I plasmids			
	LI C-D Cyclops	Gift from C. Cathebras.	
	LI C-D CCaMK	Gift from C. Cathebras.	
	LI A-B *NIN$_{pro}$ (870)*	Gift from K. Vondenhoff	
	LI A-B *B3*	Assembled by StuI blunt end cut ligation: pUC57 (BB01) + Fragment: RA28 + RA29	pRA109

Purpose	Plasmid name	Construction/Reference	Plasmid ID
	LI A-B *1xNRE*	Assembled by BpiI cut ligation: LI + Bpi (BB03) + Fragment: RA98 + RA99	pRA107
	LI A-B *NIN_{pro}::mNIN-RE (870)*	Assembled from two PCR amplified fragments from LI A-B NINpro (870). Assembly by BpiI cut ligation into LI-BpiI (BB03). Fragment 1: RA132 and RA133. Fragment 2: RA134 and RA135.	pRA129
	LI C-D $cNLP4^{C}$	PCR amplification from LI C-D cNLP4 with primers RA108 and RA109. Assembly by BpiI cut ligation into LI-BpiI (BB03)	pRA110
	LI C-D $cNIN^{C}$	Assembled from two PCR amplified fragments from LI C-D gNINFL. Assembly by BpiI cut ligation into LI-BpiI (BB03). Fragment 1: RA56 and RA57. Fragment 2: RA58 and RA59.	pRA3
	LI D-E VP16	Gift from Dr. Chiasson	pRA92
	LI C-D $gNIN^{\Delta PB1}$	PCR amplification from LI C-D gNINFL with primers RA60 and RA78. Assembly by BpiI cut ligation into LI-BpiI (BB03)	pRA32
	LI C-D $gNIN^{\Delta RWP}$	Assembled from two PCR amplified fragments from LI C-D gNINFL. Assembly by BpiI cut ligation into LI-BpiI (BB03). Fragment 1: RA60 and RA82. Fragment 2: RA83 and RA84.	pRA36
	LI C-D $gNIN^{N}$	PCR amplification from LI C-D gNINFL with primers RA60 and RA68. Assembly by BpiI cut ligation into LI-BpiI (BB03)	pRA8
	LI C-D $gNIN^{C}$	PCR amplification from LI C-D gNINFL with primers RA56 and RA59. Assembly by BpiI cut ligation into LI-BpiI (BB03)	pRA2

Purpose	Plasmid name	Construction/Reference	Plasmid ID
	LI C-D gNIN^{FL}	Assembled by BpiI cut ligation: L0 NIN_piece 1 + L0 NIN_piece 2 + L0 NIN_piece 3 + LI-BpiI (BB03)	pRA1
	LI C-D cNLP4	Assembled by BpiI cut ligation: L0 NLP4-1 + L0 NLP4-2 + L0 NLP4-3 + LI-BpiI (BB03)	pRA193
	LI C-D cNLP1	Assembled by BpiI cut ligation: L0 NLP1-1 + L0 NLP1-2 + L0 NLP1-3 + LI-BpiI (BB03)	pRA208
	LI C-D cNIN	Assembled from four PCR amplified fragments from LI C-D gNINFL. Assembly by BpiI cut ligation into LI-BpiI (BB03). Fragment 1: RA60 and RA61. Fragment 2: annealed primers RA62 and RA63. Fragment 3: RA64 and RA65. Fragment 4: RA66 and RA67	pRA118
	LI C-D cNIN-NLP4	Assembled by BpiI cut ligation: L0 Chimera NIN + L0 Chimera NLP4 + LI-BpiI (BB03)	pRA222
	LI C-D cNIN $^{mPB1\ type\ I}$	Assembled from two PCR amplified fragments from LI C-D cNIN. Assembly by BpiI cut ligation into LI-BpiI (BB03). Fragment 1: RA60 and RA184. Fragment 2: RA185 and RA67	pRA254
	LI C-D cNIN $^{mPB1\ type\ II}$	Assembled from two PCR amplified fragments from LI C-D cNIN. Assembly by BpiI cut ligation into LI-BpiI (BB03). Fragment 1: RA60 and RA186. Fragment 2: RA187 and RA67	pRA255
	LI C-D cNIN $^{mPB1\ type\ I/II}$	Assembled from two PCR amplified fragments from LI C-D cNIN mPB1 type I. Assembly by BpiI cut ligation into LI-BpiI (BB03). Fragment 1: RA60 and RA186. Fragment 2: RA187 and RA67	pRA256

Purpose	Plasmid name	Construction/Reference	Plasmid ID
Golden Gate level 0 plasmids			
	L0 NIN_piece 1	PCR amplification from pENTR-gNIN with primers JL65 + JL66. Assembly by StuI blunt end cut ligation into LI-Amp (BB02)	
	L0 NIN_piece 2	PCR amplification from pENTR-gNIN with primers JL67 + JL68. Assembly by StuI blunt end cut ligation into LI-Amp (BB02)	
	L0 NIN_piece 3	PCR amplification from pENTR-gNIN with primers JL69 + JL70. Assembly by StuI blunt end cut ligation into LI-Amp (BB02)	
	L0 NLP1-1	PCR amplification from L. japonicus cDNA with primers RA13 + RA2. Assembly by StuI cut ligation into LI-Amp (BB01)	
	L0 NLP1-2	PCR amplification from L. japonicus cDNA with primers RA3 + RA4. Assembly by SmaI cut ligation into LI-Amp (BB01)	
	L0 NLP1-3	PCR amplification from L. japonicus cDNA with primers RA14 + RA15. Assembly by StuI cut ligation into LI-Amp (BB01)	
	L0 NLP4-1	PCR amplification from L. japonicus cDNA with primers RA16 + RA8. Assembly by SmaI cut ligation into LI-Amp (BB01)	
	L0 NLP4-2	PCR amplification from L. japonicus cDNA with primers RA9 + RA10. Assembly by SmaI cut ligation into LI-Amp (BB01)	
	L0 NLP4-3	PCR amplification from L. japonicus cDNA with primers RA11 + RA12. Assembly by SmaI cut ligation into LI-Amp (BB01)	

Purpose	**Plasmid name**	**Construction/Reference**	**Plasmid ID**
	L0 Chimera NIN	PCR amplification from LI C-D cNIN with primers RA60 + RA174. Assembly by SmaI blunt end cut ligation into LI-Amp (BB02)	pRA215
	L0 Chimera NLP4	PCR amplification from LI C-D cNLP4 with primers RA175 + RA176. Assembly by SmaI blunt end cut ligation into LI-Amp (BB02)	pRA216
Gateway plasmids			
TA	Cyclops^{min}	(Singh et al., 2014)	KK172
TA	*35Spro:3xHA-Cyclops* (Cyclops)	(Singh et al., 2014)	pRA7
TA	*35Spro:3xHA-Cyclops^{DD}* (Cyclops^{DD})	(Singh et al., 2014)	pRA8
TA	*35S$_{pro}$:mOrange-CCaMK^{314}* (CCaMK$^{1-314}$)	(Pimprikar et al., 2016)	pRA13
TA	*35S$_{pro}$:3xHA-BD$_{GAL4}$-AD$_{VP16}$* GAL4 (BD$_{GAL4}$-AD$_{VP16}$)	(Singh et al., 2014)	pRA183
TA	*NIN$_{pro}$:GUS*	(Singh et al., 2014)	pRA3
TA	*2xCYCRE$_{pro}$:GUS*	(Singh et al., 2014)	pRA4
TA	*RAM1$_{pro}$:GUS*	(Pimprikar et al., 2016)	pRA6
TA	*ERN1$_{pro}$:GUS*	(Cerri et al., 2017)	pRA5
TA	*5xUAS$_{pro}$:eGFP-GUS*	(Singh et al., 2014)	pRA184

Table 2. Oligonucleotides

Primer Name	5'-3' sequence	Source
Cloning		
RA13	AAGAAGACAATACGGGTCTCACACCATGGGGGATGGTGCTGTGA	This study
RA2	AAGAAGACAAAAGCACGATACCCAAGTTTGA	This study
RA3	AAGAAGACAAGCTTTCAACAAGGCAAAGATGGGTGCC	This study
RA4	AAGAAGACAACTCCTGATAAATTTGGAGAAGCT	This study
RA14	AAGAAGACAACAGAGGTCTCACCTTACT TCTCATACCACTAGAAGCTTGTATGC	This study
RA15	AAGAAGACAAGGAGCCAGCCTGGTATCAGC	This study
RA8	AAGAAGACAAACTAAAGTGTGTGATCCTAGGAGGC	This study
RA9	AAGAAGACAATAGTGAACAAAAGACACTTGTGGGA	This study
RA10	AAGAAGACAAATGATGTCCTCTCTGTATGTCGCCT	This study
RA11	AAGAAGACAATCATAAGGTTTCGTGTCTCCTTGAC	This study
RA12	AAGAAGACAACAGAGGTCTCACCTTCTCCCCTGAG	This study
RA16	AAGAAGACAATACGGGTCTCACACCATGTCAGAATCTGATGAAGAA AAGC	This study
RA28	ATCGCGTCTCAGCGGTTTCGCCGATATCGTAGA	This study
RA29	CGATCGTCTCTCAGAGATTATCATGATGAACTTGACTC	This study
RA30	ATCGCGTCTCAGCGGGTTGTACTGGTAATTATATAGTATTAATTA	This study
RA37	ATCGCGTCTCAGCGGATATAAGTTTGCATTTTTAGGTACACAAATTTT GTACGATTGCCATGTGGCACGCAGAGAGGAGCCTCTGAGAGACGAT CG	This study
RA38	CGATCGTCTCTCAGAGGCTCCTCTCTGCGTGCCACATGGCAATCGTA CAAAATTTGTGTACCTAAAAATGCAAACTTATATCCGCTGAGACGCG AT	This study
RA39	ATCGCGTCTCAGCGGCGATTGCCATGTGGCACGCAGAGAGGAGCCT CTGAGAGACGATCG	This study
RA40	CGATCGTCTCTCAGAGGCTCCTCTCTGCGTGCCACATGGCAATCGCC GCTGAGACGCGAT	This study
RA56c	TACGAAGAAGACAATACGGGTCTCACACCATGTCGTCTTATACCTTT GGAAGCCG	This study

Primer Name	5'-3' sequence	Source
RA57	ATACGAGAAGACAACACCAATGCTCTTTGCTGC	This study
RA58	TACGAAGAAGACAAGGTGTATGTCCCACAACTCTGAAAAGG	This study
RA59	CGTAAAGAAGACAACAGAGGTCTCACCTTAGATGGGCTGCTATTGCG	This study
RA60	TACGAAGACAATACGGGTCTCTCACCATG	This study
RA61	AAGAAGACAACAGCCTGATCAAGAGCATTGGAG	This study
RA62	TCAGAAGACAAGCTGTTGATTTTAGAAGCAGCCAGAGCTTCATTCCACCTGCCATCAAGGTATTTGTCTTCTCA	This study
RA63	TGAGAAGACAAATACCTTGATGGCAGGTGGAATGAAGCTCTGGCTGCTTCTAAAATCAACAGCTTGTCTTCTGA	This study
RA64	AAGAAGACAAGTATATGACGAGTTGTACCAAGC	This study
RA65	AAGAAGACAACATACACCAATGCTCTTTGCT	This study
RA66	AAGAAGACAATATGTCCCACAACTCTGAAAA	This study
RA67	AAGAAGACAACAGAGGTCTCTCCTTAGATGG	This study
RA68	AAGAAGACAACAGAGGTCTCTCCTTCTCAGATGAAACCCCACAA	This study
RA78	AAGAAGACAACAGAGGTCTCTCCTTCTGTATGGCACCCTCAGC	This study
RA82	AGAAGACTTTTCTTGATCCCCACCC	This study
RA83	AAGAAGACAAAGAAAGCTCTTCAGATTTCTCTGC	This study
RA84	AAGAAGACAACAGAGGTCTCTCCTTAGATGGGCTGCTATTGC	This study
RA98	TACGAAGACAATACGATCGCGTCTCAGCGGAGAAACAACTTGACCCTTTACATTGCTCAAGAGCTCATCTCTTTCTGAGAGACGATCGTCTGTTGTCTTCTT	This study
RA99	AAGAAGACAACAGACGATCGTCTCTCAGAAAGAGATGAGCTCTTGAGCAATGTAAAGGGTCAAGTTGTTTCTCCGCTGAGACGCGATCGTATTGTCTTCGTA	This study
RA108	TACGAAGACAATACGATGGTCTCACACCATGATTGATCAAATGTCCTCC	This study
RA109	AAGAAGACAACAGACGATTAGGTCTCTCCTTCTCCCCTGAGCTCTCAC	This study
RA132	ACGAAGACAATACGTACGTCTCAGCGGTTGTACTGGTAATTATA	This study
RA133	AAGAAGACAAGTTAAGTTGGAAGATAAATTTGCGTACATGTCTACG	This study

Primer Name	5'-3' sequence	Source
RA134	TCAGAAGACAATAACAATACATTTGTGTAACTGTACGATTGCCATGTG	This study
RA135	AAGAAGACAACAGATACGTCTCACAGAGCTAGCTGATCCAAT	This study
RA174	AAGAAGACAACCATCTCAGATGAAACCCCACAA	This study
RA175	AAGAAGACAAATGGGGCAAGTTTGCATGT	This study
RA176	AAGAAGACAACAGAGGTCTCACCTTCTCCC	This study
RA184	AAGAAGACTTCATCAGCCAGATACTTGAGAAG	This study
RA185	AAGAAGACAAGATGCTGGAGCGTGGGTAGTTTTAG	This study
RA186	AAGAAGACTTGCCACTCTGAAAGCACCTGTTTCC	This study
RA187	AAGAAGACAATGGCAGCAACTTTTGCAGATGAGAAGATC	This study
KP84	TACGTCTCACAGAGCTAGCTGATCCAATTAAGTACCTG	This study
KP91	ATCGTCTCAGCGGGCTCCGTTTGGTCAACAGAC	This study
KP92	ATCGTCTCTAAGCTAATTTGCAGCGACTTTTTCC	This study
KP93	TACGTCTCAGCTTATATCGCAGCGACCAG	This study
JL65	ATGAAGACTTTACGGGTCTCTCACCATGGAATATGGTTCATTACTAGTGC	This study
JL66	TTGAAGACTTGAACACAGGAAGGGCTAAAGA	This study
JL67	ATGAAGACTTGTTCGAAAGAGGCACCGG	This study
JL68	TTGAAGACTTAGATTCTTGATCCCCACCCTC	This study
JL69	ATGAAGACTTATCTTCTTATACCTTTGGAAGCCGCCGCTCtTCTTCTGGTGGAAGAAAGTCAGGCGAGAAAAGACGAACCAAGGCAGAAAAGACTATCAG	This study
JL70	TTGAAGACTTCAGAGGTCTCTCCTTAGATGGGCTGCTATTGC	This study
F1_CYC	ATGAAGACTTTACGGGTCTCACACCATGGAAGGGAGGGGGTTTTCTG	This study
R1_CYC	TAGAAGACAATTTCAGGAACAATTCTTCACTTGAGTTTC	This study
F2_CYC	ATGAAGACTTGAAAACAGTGATGGAGAG	This study
R2_CYC	TAGAAGACAACTGATTGGAAAATTGAAATC	This study
F3_CYC	ATGAAGACTTTCAGGTAATTGCTCTATTCTTC	This study

Primer Name	5'-3' sequence	Source
R3_CYC	TAGAAGACAACATTTACTGGCGTTTGATTAC	This study
F4_CYC	ATGAAGACTTAATGTTCAAGTAGACTCTAT	This study
R4_CYC	TAGAAGACAAATAGATCCATATCTTTCTAG	This study
F5_CYC	TGAAGACTTCTATAACATCAGCTGTTTCAG	This study
R5_CYC	TAGAAGACAAATCTTCTATCTGCTTTGTTTG	This study
F6_CYC	TAGAAGACAAAGATCTTCAGAAGCAGAATG	This study
R6_CYC	ATGAAGACTTCAGAGGTCTCACCTTCATTTTTTCAGTTTCTGATAG	This study
F1_CC	ATGAAGACTTTACGGGTCTCACACCATGGGATATGATCAAACCAG	This study
R1_CC	TAGAAGACAATGATTAATTGTACTTTTGTATGTTTG	This study
F2_CC	ATGAAGACTTATCATCAAACACTAAGAACAAAG	This study
R2_CC	TAGAAGACAAAGTCTTTTCATAGAAACTGAAATTC	This study
F3_CC	ATGAAGACTTGACTTGGAAGGGCATTACCCAATC	This study
R3_CC	TAGAAGACAATGTTCATGGATATGTTTGAGTAAATAGGTTAACTAAG	This study
F4_CC	TAGAAGACAAAACACATGCACATAGACAAGAATGCACACATATAG	This study
R4_CC	ATGAAGACTTCAGAGGTCTCACCTTTGATGGACGAAGAGAAGAGAG GAGCATG	This study
qRT-PCR		
EF1 alpha_F	GCAGGTCTTTGTGTCAAGTCTT	(Pimprikar et al., 2016)
EF1 alpha_R	CGATCCAGAACCCAGTTCT	
*NIN*_F	AACTCACTGGAAACAGGTGCTTTC	(Soyano et al., 2013)
*NIN*_R	CTATTGCGGAATGTATTAGCTAGA	
3'UTR NIN_F	CCTTTTTCAAGCTTCGCCTCTA	DC548
3'UTR NIN_R	CACAAACAAACACACCTCTCTC	DC549

4. Growth conditions, plant transformation and inoculation

Seeds were scarified and surface-sterilized as described in Ried et al. (2014) prior to germination. For qPCR, plants grew two weeks on 0.8% water agar, were moved to Fahraeus plates for one week, transferred to Weck jars (Weck GmbH, Cat#745-1L Tulip Jar) containing 300 ml dried sand:vermiculite mixture (2:1), mock treated with 50 ml Fahraeus medium (FP) or inoculated with *M. loti* suspension in FP medium set to OD_{600}=0.05 and incubated for 7 days. Hairy root transformation via *A. rhizogenes* was performed as described in Cerri et al. (2017). Transformed roots were detected by visualization of the GFP fluorescent transformation marker in a Leica M165FC stereomicroscope equipped with a DFC 300FX digital camera (Leica Microsystems). After detection of transformed root systems, plants were moved to FP medium plates for one week and then transferred to Weck jars. Nodulation was evaluated three weeks later. Number of spontaneous nodules were scored 60 days post transformation (dpt).

For promoter expression analysis, transformed plants were watered with 35 ml FP medium and then after 3 days mock treated with 15 ml FP or inoculated with *M. loti* suspension in FP medium set to OD_{600}=0.1 and incubated for 7 days. For overexpression of *NIN* in WT, transformed plants were inoculated with 50 ml *M. loti* suspension in FP medium (OD=0.05), and nodulation was scored 3 weeks after inoculation.

N. benthamiana leaves were transiently transformed as described in Cerri et al. (2017), with the addition of K9 plasmid expressing RFP for balancing the amount of *Agrobacterium* strains infiltrated per combination. Leaf discs were collected at 48 hours post-infiltration for transactivation assays and between 48-60 hours for FLIM-FRET experiments.

5. Promoter GUS analysis and transactivation assays

GUS activity from the activation of promoter:GUS reporters in *L. japonicus* was performed as previously described in Singh et al. (2014). For *NIN* expression comparison between WT and *nin-2*, transformed roots were screened and collected under the stereomicroscope 7 days post-inoculation. Enzymatic reaction was incubated for 3 hours at 37° C and subsequently stopped by replacing the GUS solution with two washes of 70% ethanol.

Enzymatic GUS assays in *N. benthamiana* were performed as described in Singh et al. (2014), with the incorporation of a 96-well plate protocol. Briefly, disc leaves were collected in a 96-deep well plate (Thermo Fisher Scientific, Cat#260252) submerged in liquid nitrogen and closed with a silicon lid (Thermo Fisher Scientific, Cat#276000). Frozen samples were

homogenized with beads in a retch mill (2x1min, 30/s). Plate was centrifuged for 5 minutes in a 4° C pre-cooled centrifuge 5810R (Eppendorf AG) for collecting all the material at the bottom of the plate. 350 µL of extraction buffer [50 mM $NaPO_4$, pH 7.0; 10 mM EDTA, pH 8.0; 0.1% Triton X-100; 0.1% N-Lauryl-Sarcosine; 50 mM β-mercaptoethanol; 1x Homemade Plant Protease Inhibitor Cocktail] was added to each well, lid was sealed and homogenizing was done at 4° C. The plate was centrifuged for 30 minutes at 4000 rpm at 4° C. 80 µL of the supernatant was transferred to a 96-well PCR plate kept on ice and used for the fluorometric GUS assay. 10 µL of cell extract was added to a pre-warmed plate containing the same components as the extraction buffer and referred as the GUS buffer plate (supplied with 1 mM 4-Methylumbelliferyl-β-D-glucuronide hydrate, 4-MUG; Biosynth, Cat#EM03216) and the reaction was incubated at 37° C for 10 minutes. 10 µL of the reaction were moved to a plate containing 100 µL of 0.2 M Na_2CO_3, stopping the reaction. The reaction mix was measured using TECAN Infinite 200 PRO (TECAN Group Ltd.) for fluorescence (4-MU) at an excitation wavelength of 360 nm and emission of 465 nm. Protein concentration was estimated with the Bradford test. 1 µL of cell extract was mixed with 100 µL Bradford reagent (Bio-Rad, Cat#5000006) and measured with the TECAN reader at 595 nm.

6. Protein Blot Analysis

Proteins from *N. benthamiana* leaves were extracted with extraction buffer (62.6 mM Tris-HCl, pH 6.8; 2% sodium dodecyl sulphate; 10% glycerol; 735 mM β-mercaptoethanol; 1x Homemade Plant Protease Inhibitor Cocktail) and detected on Western blots using anti-myc mouse monoclonal (Santa Cruz Biotechnology, Cat#9E10 SC-40) and anti-mouse HRP (Biomol, Cat#ARG65350.500) as primary and secondary antibody, respectively. Proteins were detected by chemiluminescence in a Vilber Lourmat Fusion-SL-3500 WL (PeqLab) using SuperSignal™ Maximum Sensitivity Substrate (ThermoFisher Scientific, Cat#34095).

7. Microscopy

Images of *L. japonicus* root systems were taken using a Leica M165FC epifluorescence stereomicroscope equipped with a GFP and RFP filter with an incorporated DFC 450C camera (Leica Microsystems).

8. FLIM-FRET

FLIM-FRET analysis was performed on transformed *N. benthamiana* leaf discs between 48 to 64 hours after inoculation. Images were acquired under Leica TCS SP5 confocal laser-scanning microscope (CLSM), equipped with an HCX PL APO CS 20x/0.7 IMM CORR CS objective lens (Leica). Fluorescent lifetime was measured on the Leica TCS SP5 equipped with a pulsed Ti:Sapphire Mai Tai multiphoton laser (Spectra Physics) and a FLIM PMT detector (Becker & Hickl GmbH). For excitation of sGFP fluorescence, the multiphoton laser (running at 80 mHz with 1.2 ps pulse length) was tuned to 900 nm. Photon counting was recorded for 25 scanning cycles (3s/cycle) at a spatial resolution of 256x256 pixels. Signals were recorded with the TCSPC system using the photon counting software TCSPC 2.80 (Becker & Hickl). Lifetime calculation was performed with the SPCImage software (Becker & Hickl). The nucleus was defined, pixels were binned by a factor two and a double exponential decay model was applied. Scatter and shift were fixed to zero.

9. Gene expression analysis

For analysis of transcript levels by qRT-PCR, 50-100 mg root fresh weight was harvested. RNA was extracted using the Spectrum Plant Total RNA kit (Sigma-Aldrich, Cat#STRN250). DNA was removed with TURBO DNA free (ThermoFisher Scientific, Cat#AM1907) according to manufacture's protocol. RNA integrity was verified on an agarose gel and the absence of genomic DNA was confirmed by PCR. cDNA synthesis was performed with 400 ng RNA in 20 µL reaction volume using the Superscript III kit (ThermoFisher Scientific, Cat#18080044) with oligo (dT) primers. qRT-PCR was performed in 7 µL reactions containing mi-real-time Evagreeen Master mix (Metabion, Planegg, Germany; Provided by Dr. Andreas Brachmann), in 384-well plates with the QuantStudio 5 (ThermoFisher Scientific, Cat#4483285) real-time PCR detection system following manufacturer's instructions. PCR Program: 95° C 10 min, 40 x (95° C for 15 s, 60° C for 30 s and 72° C for 20 s) followed by dissociation curve analysis (95° C for 15 s, melt curve 60° C–95° C: increment 0.075° C per second). Expression values

were normalized to the *Ubiquitin* or *EF1α* reference gene and calculated with the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

10. Statistics and data visualization

All statistical analyses and data plots were generated with R version 3.4.2 (2017-09-28) (R Core Team, 2015) and the packages 'multcompView' (Graves et al., 2015) and 'beeswarm' (Eklund, 2016). Test applied are stated in the figure legend.

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X. List of Figures

XI. List of Tables

XII. Declaration of contribution of other researches

Rosa Andrade, the author of this thesis contributed to the experiments displayed as follows:

Figure 1: designed by Andreas Binder and modified by Rosa Andrade.

Figure 2-19: Experiments were designed, performed and analyzed by Rosa Andrade. Statistical analysis and the preparation of the corresponding figures were done by Rosa Andrade.

XIII. Acknowledgements

I am grateful for all the people who supported me from the beginning to the end of this big project.

I would like to thank my supervisor Prof. Martin Parniske for the time he invested, for always being open for discussion, and invaluable support throughout this process. I am thankful that he gave me the freedom to pursue my own scientific ideas, but as soon as there were some doubts or conflicting results, spent some time to discuss with me about it. Lastly, I would like to thank him for his time to evaluate this thesis.

I would like to thank the remaining committee members for their evaluation of this work.

I would like to also thank the Collaborative Research Center (SFB924) ‘‘Molecular mechanisms regulating yield and yield stability in plants’’ which financially supported my project as part of the “Spatio-temporal dynamics in the composition and function of the CCaMK/Cyclops complex”. Also, for the funding provided for traveling to conferences and for the workshops.

I am grateful for all the people who made being part of “Genetics” a great experience. I am grateful to Dr. David Chiasson for his help with the Y2H experiment and for always being open for discussions. A big thank you goes also to Dr. Marion Cerri. I knew I could always ask for your help and enjoy with you some time and bubble tea. Thank you for motivating me constantly on finishing this project. I would like to thank Dr. Katja Katzer from whom I learned a lot about working with proteins, and also for helping me translating the summary of this thesis. I would also like to thank Dr. Livia Scheunemann; your positive energy was always contagious and kept me motivated. Thanks to the Parniske team of my generation: Chloé, Xiaoyun, Philipp, Isabel, Nimisha, Martin B and Fang-Yu. It was always very helpful to discuss with you about my project. Thanks also to the people in the greenhouse, especially to Anna.

I warmly thank Chloé Cathebras for all the help during and after my time as a doctoral candidate. I will never forget our time together in the lab, especially building correct English sentences between an Ecuadorian and a French. Thank you so much for helping me in each stage of this work.

I want to thank my family in Ecuador, especially my parents, that have always encouraged me to follow and pursue my dreams. The distance between us was never an excuse for being in touch. I am extremely fortunate in my life to have parents like you. A big thanks also goes to my sisters Lu and Eli, who have constantly believed in me.

A big thank you also goes to my German Freyer family. Thank you all for your support and encouragement. You have always made me feel part of your family, and for that thank you so much.

The big thank you is for my multitasking husband/best friend/best dad and project manager, Philipp. I am sure I would have not managed it without you. Thank you so much for trying to give me as much time as possible to work on this thesis. There were so many days that I wanted to drop the towel, but you encouraged me always to continue and finish it. You are my person and you will always be. Finally, thank you Lukas, your sole existence has brought so much light and hope to your family.