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Lucas Gontijo Silva Maia

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**INVOLVEMENT OF TWO NOVEL GRF-TYPE ZINC FINGER NODULINS DURING
RHIZOBIAL SYMBIOTIC ESTABLISHMENT IN *MEDICAGO TRUNCATULA*
NODULES**

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**Dissertation submitted to the Davis College of Agriculture,
Natural Resources and Design
at West Virginia University**

in partial fulfillment of the requirement of the degree of

**Doctor of Philosophy
in
Genetics & Developmental Biology**

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**Morgantown, West Virginia
2017**

KEYWORDS: Symbiotic Nitrogen Fixation, Nodulation, Bacteroid Differentiation, Genetic Network, Transcription Factors, Legumes

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ABSTRACT

INVOLVEMENT OF TWO NOVEL GRF-TYPE ZINC FINGER NODULINS DURING RHIZOBIAL SYMBIOTIC ESTABLISHMENT IN *MEDICAGO* *TRUNCATULA* NODULES

Lucas Gontijo Silva Maia

In legumes, symbiotic nitrogen fixation (SNF) occurs through a cooperative relationship established with rhizobial diazotrophic bacteria. This association triggers the de novo development of a highly specialized organ from root, called nodule. In this dissertation, we explore the molecular functional genetics of nodulation in the model legume *Medicago truncatula* in the scope of knowledge. Followed by a linearization of the current knowledge about the roles of Transcription Factors in legume nodule symbiosis. In an attempt of understanding the orchestration dynamics of nodulation and processes that generate SNF. Legumes evolved novel genes related with nodule development, symbiosis establishment and nitrogen fixation, which are exclusively expressed at different stages of nodule development, and during nitrogen reduction per se. In the model legume *Medicago truncatula*, two genes encoding GRF-type zinc finger proteins present high and distinct expression pattern during symbiotic nitrogen fixation in root nodules. Both genes encode for very similar protein, part on a non-characterized protein family, GRF-type zinc finger, with putative function as transcriptional factor. The gene expression data present high and distinct transcription for both genes in specific zones of the indeterminate nodule: *MtGRF1/MtN20* (Medtr7g086040.1) is highly expressed in an area below the nodule meristem (zone II, where endosymbiosis occurs), while *MtGRF2* (Medtr1g064350.1) is expressed in the interzone, where bacteroid differentiation occurs. *MtN20* is exclusively present in the distal-zone II of nodules, in tissue where the plant cells become colonized and bacteroids start to differentiate in SNF “organelles”. And a Tnt1-insertional line for *MtN20* was genotyped and phenotype. Nodules of *mtn20* present normal nodule formation but disruption in nitrogen fixation, showing early senescence, and loss of the rhizobial maturation, required in this process. *MtGRF2* present expression pattern in the interzone of nodules, in a tissue where the and bacteroids pass through a singular differentiation stage to become a in SNF “organelles”. A genetic network for each gene of interest highlight high correlated genes that possible play essential roles on distinct stages of symbiotic nitrogen fixation and bacteroid differentiation, await to be functional characterized. This information contributes to understanding of symbiotic nitrogen fixation and emphasize important questions to be investigated and make possible to explore root nodulation in non-legumes.

DEDICATION

In dedication to my family, in special to my parents, for making me who I am, and my wife for supporting me all the way!

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CHAPTER 1

OF LEGUMES AND SYMBIOTIC NITROGEN FIXATION: EXPLORING THE MOLECULAR GENETICS OF NODULATION IN THE MODEL LEGUME *MEDICAGO TRUNCATULA*

ABSTRACT

In legumes, symbiotic nitrogen fixation (SNF) occurs through a cooperative relationship established with rhizobial diazotrophic bacteria. This association triggers the *de novo* development of a highly specialized organ from the root, called nodule. Legumes evolved novel genes related to nodule development, symbiosis establishment and nitrogen fixation, which are exclusively expressed at different stages of nodule development, and during nitrogen reduction *per se*. In the model legume *Medicago truncatula*, we identified two GRF-type zinc finger genes as expressed specifically in nodules and were chosen for characterization of their molecular roles during symbiotic nitrogen fixation. Gene expression levels are high for both genes in specific zones of the indeterminate nodule: *MtGRF1/MtN20* (Medtr7g086040) is highly expressed in an area below the nodule meristem (zone II, where cell infection or endosymbiosis occurs), whereas *MtGRF2* (Medtr1g064350) is expressed in the interzone, where bacteroid differentiation occurs. Understanding their functions is of paramount importance to enhance our basic knowledge about how nodules develop and establish symbiotic nitrogen fixation. Therefore, in order to functionally characterize the role of these genes, this research aims to: 1) Further detail their expression patterns during nodule development and in specific zones of the mature indeterminate nodule of *M. truncatula*; 2) Assess the essentiality of these genes for symbiotic nitrogen fixation; 3) Generate regulatory gene networks centered on these nodulins. In this chapter, we review modern concepts of legume biology and symbiotic nitrogen fixation in the light of genetics and genomics studies using model species, and especially the recent advances in the body of scientific knowledge based on studies with the model legume *Medicago truncatula*.

OF LEGUMES AND SYMBIOTIC NITROGEN FIXATION: EXPLORING THE MOLECULAR GENETICS OF NODULATION IN THE MODEL LEGUME *MEDICAGO* *TRUNCATULA*

INTRODUCTION

Legume (Fabaceae) species occupy an indisputable place in modern agriculture, not only as food and feed crops but also as key players of natural systems and essential components of sustainable agriculture due to its capability of naturally injecting fixed nitrogen into the soil through root symbiosis with rhizobia. Nutritionally, pulses are a substantial source of protein, vitamins, fibers and minerals as food and feed. Legume crops contribute also to enrich and restore nitrogen fertility in farming fields (Graham, 2003; Herridge et al., 2008; Peoples et al., 2009). In natural ecosystems, legume species are vital to nitrogen (N) cycling and soil fertility. Most legumes can associate with diazotrophic bacteria, *Rhizobium* spp. and make indirect use of atmospheric N₂ for their metabolism (Sprent and James, 2007; Den Herder and Parniske, 2009; Oldroyd et al., 2011). Diazotrophic bacteria have an enzyme (nitrogenase) that under hypoxic conditions captures dinitrogen and reduces it into ammonia. This trait, which evolved independently at least six times in the Rosids I clade, and most remarkably in the legume family (Leguminosae or Fabaceae), making this family capable of establishing in nitrogen-poor environments. Ultimately, legumes radiated globally and became one of the most successful botanical families, currently encompassing 689 genera and over 19,000 species (Doyle, 2016). Besides legumes, Parasponia (Cannabaceae) can also associate with *Rhizobium* and develop nodules for nitrogen fixation (A. D. L. Akkermans, 1978). Moreover, eight more families of the Rosid I clade contain species that are capable of symbiotically fix nitrogen with *Frankia* bacteria through actinorrhizal association (Rosaceae, Casuarinaceae, Myricaceae, Rhamnaceae, Elaeagnaceae, Datisceae, Coriariaceae), including alder (*Alnus* spp., Betulaceae) and *Trema* spp. (Ulmaceae) (Soltis et al., 1995). Both, rhizobial and actinorrhizal intracellular associations occur in an organ that is developed *de novo* from root tissues, called nodule (Oldroyd and Downie, 2008; Oldroyd et al., 2011).

The legume-*Rhizobium* symbiosis (i.e., nodulation and symbiotic nitrogen fixation, SNF) has been co-evolving for circa 58 million years (Sprent, 2008). In order to be viable and sustainable, SNF requires balanced physiological conditions and chemical exchanges between the plant and rhizobia. For example, a source of direct N for the plant or lack of a finely-tuned genetic coordination between the symbionts can inhibit this mutualistic relationship (Oldroyd and Downie, 2008; Valentine et al., 2010).

Although large datasets have been amounting in the last decade on the molecular genetics of SNF in legumes, we still do not have a complete understanding of how genes are regulated in the root and nodule

cells in order to develop the nodule and sustain SNF. Transcription factors are proteins that bind to specific DNA sequences to control the rate of transcription, thus directly controlling growth, development, and metabolism in living organisms (Udvardi et al., 2007; Yamasaki et al., 2013). By exploring the gene expression atlas of the legume model *Medicago truncatula* (Benedito et al., 2008), we identified two novel putative transcription factors that are highly and specifically expressed in nodules, each with a unique expression pattern within nodule zones. They belong to the largely uncharacterized GRF-type zinc finger family of (putative) transcription factors (Brown, 2005; Gamsjaeger et al., 2007).

LITERATURE REVIEW

Nitrogen on Earth: Nitrogen (N) is a macroelement essential for all living organisms. It constitutes a key element necessary to sustain life, participating in the composition of amino acids, nucleic acids, coenzymes, signals, and secondary metabolites (e.g., alkaloids). In agriculture, N is often the most limiting nutrient to crop yields and one of the most costly inputs (Smil, 1999; Vitousek et al., 2002). On the other hand, N is very abundant in the Earth's atmosphere, composing approximately 78% as a very stable gas, dinitrogen (N_2). This molecule has a triple covalent bond between the two N atoms that requires $200 \text{ kJ} \cdot \text{mol}^{-1}$ of energy to split in order to generate an accessible form to plants, such as ammonia (NH_3) (Peoples et al., 2009; Valentine et al., 2010). N_2 can be converted into NH_3 naturally by lightning, industrially by the Haber-Bosch process, or enzymatically via the nitrogenase present in diazotrophic prokaryotes (Smil, 2001; Herridge et al., 2008).

Diazotrophs and symbiotic nitrogen fixation: Diazotrophs can be “free-living” or “symbiotic” prokaryotic organisms that have the nitrogenase, an enzymatic multi-unit complex that catalyzes this highly energetically reaction to reduce N_2 into NH_3 (Graham, 2003). Nitrogenase has iron (Fe) and iron/molybdenum (MoFe) protein cofactors. While the Fe-protein complex hydrolyzes ATP, the MoFe-complex carries out the N_2 reduction into NH_3 (Kim and Rees, 1992; Anderson et al., 2013). Importantly, the nitrogenase active site can also interact with dioxygen (O_2), which generates an oxidation damage to the MoFe-protein site and irreversibly inactivates the enzyme (Robson and Postgate, 1980; Ott et al., 2005). Therefore, nitrogenase must work in an anoxic or hypoxic cellular environment. The mechanisms that evolved to deal with N_2 fixation in the presence of our oxygenic atmosphere by lowering the O_2 pressure through an extracellular polysaccharide protection layer that hinders O_2 diffusion and allocating nitrogenase into a heterocyst with low O_2 pressure and anaerobic metabolism as seen in cyanobacteria, or confining SNF in nodule cells that contain leghemoglobin, which is a protein with high affinity to oxygen and allows for an aerobic metabolism and SNF to happen concomitantly, as seen in legumes (Robson and Postgate, 1980). Symbiotic diazotrophs, such as *Rhizobium* and *Sinorhizobium* bacteria, are capable of interacting

and associating endosymbiotically with legume roots, develop the nodule anew and create ideal conditions for SNF to happen (Lodwig et al., 2003; Udvardi et al., 2007; Den Herder and Parniske, 2009; Libault et al., 2009; Oldroyd et al., 2011).

Symbiotic nitrogen fixation and legume crops: Legumes are recognized as essential to agricultural sustainability (Graham, 2003; Herridge et al., 2008; Peoples et al., 2009). Legumes are also an important component of human and animal diets and the main source of primary proteins. Pulses, which are grain legumes used for human consumption and comprise of dry beans, peas, lentils, chickpeas, cowpeas, lupins and vetches. Pulse reached a global production of 4.5 million tons in 2014 (FAOSTAT, 2017). N corresponds to 3-4% of organic matter in legumes and SNF performed by pulses and other legume crops were estimated to fix annually over 20 tons of N, 10.7-23 kg N₂ per metric ton of biomass produced, depending on the species (Unkovich et al., 2010). Thus, legumes are a major factor of N input in agricultural systems and significantly contributing to low-cost and sustainable N fertilization (Herridge et al., 2008; Peoples et al., 2009). During the evolution of N₂-fixing nodules, *Rhizobium* root colonization has been presented by at least three different ways, intercellular infection, crack entry and the most complex root hair infection thread (Madsen et al., 2010). Beside those difference, the nodule growth also diverged in two types, such as determinate growth present in Phaseoloid legumes clade (such as soybean, peanut, *Phaseolus* and *Lotus japonicus*), and indeterminate growth, present in *Medicago truncatula* (barrel medic, henceforth called just Medicago) (Sprent and James, 2007; Sprent, 2008). The indeterminate nodule, even when fully mature, maintains all developmental tissue stages from meristem in the apex to a senescence zone proximal to the root. *M. truncatula* and *Lotus japonicus* are models for legume nodulation along with other important crop legumes, such as soybean (*Glycine max*), bean (*Phaseolus vulgaris*), pea (*Pisum sativum*) and lentil (*Lens culinaris*), which present inconveniences for adoption as models, due to large genomes and low transformation rates.

Evolution of symbiotic nitrogen fixation in legumes: SNF rose circa 58 million years ago (Lavin et al., 2005; Sprent and James, 2007; Sprent, 2008), just about two million years after the Fabaceae family emerged (Doyle and Luckow, 2003; Sprent, 2007). The appearance of SNF provided a distinguished evolutionary advantage during the evolution of legumes, and this trait is thought to have provided the opportunity to conquer otherwise inhospitable, N-poor habitats and radiate globally (Sprent and James, 2007; Yokota and Hayashi, 2011; Geurts et al., 2016; Mus et al., 2016).

Unsurprisingly, legumes coopted existing as well as evolved novel genes to enable the establishment of the symbiotic relationship, which encompasses a continued “molecular communication” and exchange of metabolites between legumes and rhizobia (Udvardi and Day, 1997; Prell and Poole, 2006; Benedito et al., 2010; Udvardi and Poole, 2013). This association triggers the development of a new specialized organ from root tissues, the nodule, to intracellularly host the rhizobia and capacitate SNF

(Figure 1) (Lodwig et al., 2003; Oldroyd and Downie, 2008; Den Herder and Parniske, 2009; Oldroyd et al., 2011).

Figure 1. Nodule organogenesis in *Medicago truncatula*. Roots of 10-day plants were inoculated with *Sinorhizobium meliloti* strain *Sm-1021* constitutively expressing the *beta-galactosidase (lacZ)* gene. Roots were stained with X-gal and the blue color reveals the bacteria. At 2 dpi, *S. meliloti* interact with root epidermis, 3 dpi *S. meliloti* form the infection thread inducing cortex cells to divide, 4 dpi *S. meliloti* start to colonize the nodule primordia, 10 dpi nodule presents a bump in the root, establishes the fixation zone, 14 dpi nodule is completed development of a nodule and at 21 dpi nodules keep growing indeterminately. Scale bars in all images represent 100µm.

Rhizobium recognition and nodule development initiation: Before nodulation starts, a nitrogen-starving legume plant begins a chemical communication with free-living *Rhizobium* in the rhizosphere by exuding flavonoids from its root system via unknown efflux transporters at the plasma membrane of root cells. Flavonoid transporters in plant species so far identified include MULTIDRUG AND TOXIC COMPOUND EXTRUSION (MATE) efflux in *Arabidopsis thaliana*, ATP-BINDING CASSETTE (ABC) transporter in soybean and RESISTANCE-NODULATION-CELL DIVISION (RND)-type efflux in *Lotus japonicus* (Wasson et al., 2006; Sugiyama et al., 2008; Thompson et al., 2010). Firstly, flavonoids/isoflavonoids exuded by low nitrogen root, are recognized by free-living *Rhizobium* in the rhizosphere and induces synthesis and exudation of lipo-chitoooligosaccharides (LCOs), called Nod-factors (NFs), with the expression of common *nodABC* and species-specific *nodD* *Rhizobium* *ssp.* genes (Peters et al., 1986; Hartwig et al., 1990; Limpens, 2003; Limpens and Bisseling, 2003; Radutoiu et al., 2003).

In legumes, NFs are recognized species-specifically by constitutive membrane receptors localized in the root hairs, such as *M. truncatula* NOD-FACTOR PROTEIN (MtNFP) and *Lotus japonicus* NOD-FACTOR RECEPTOR (LjNFR1) and LjNFR5 in *Lotus japonicus*. These receptors are dimeric LYSIN

MOTIF (LysM) receptor-like kinases that act upstream of the "Common Symbiotic Signaling (SYM) Pathway" that is shared between arbuscular mycorrhizal fungi (AMF) and root nodule symbiosis (RNS). Nodulation, therefore, occurs by the association between a legume root and species-specific rhizobial bacteria, in which the *Rhizobium* commonly enters through the root epidermis (root hairs), where it forms an infection thread (IT) to colonize the root cortex (Vernié et al., 2015).

In an attempt to better illustrate the nodulation process, we present the characterized genes involved in each developmental event of the *M. truncatula* indeterminate nodules (**Table 1**).

Modulation and symbiosis nitrogen fixation event:	Genes:	Medicago gene ID:	References:
Nitrogen Starving Plant	Flavonoids transmembrane efflux		Peter et al., 1986
Rhizobium spp. NodFactor synthesis genes	NFs (<i>nodABC</i> and <i>nodD</i>)		Hartwig et al., 1990
Pre-infection genes - Rhizobium ssp.	<i>MtNFP</i> (<i>LysM-RLK</i>), <i>LjNFR1</i> and <i>LjNFR5</i> (<i>MtNFP</i>)	Medtr5g019040	Amor et al., 2003; Radutoiu S, et al., 2003
ITs	<i>MTLYK3</i> (entry receptor-later signaling)	Medtr5g086130	Smit P, et al., 2007
A) Common Pathway AMS and RNS			
Activation of Ca ²⁺ spiking signaling (root hair) and ITs	<i>MtDMI2</i> and <i>LjSYMRK</i> (<i>NORK</i>)	Medtr5g030920	Limpens et al., 2005
Maintenance of Ca ²⁺ spiking signaling	<i>MtDMI1</i> (<i>Lj. CASTOR</i> and <i>POLLUX</i>)	Medtr2g005870	Riely et al., 2007
Phospholipase C (PLC) transduction of responses to NFs	G-protein (MAPK)	Medtr4g005830	Miles, G. P., et al., 2004.
Perception of Ca ²⁺ spiking signaling (nuclei)	<i>MtDMI3</i> (CCaMK)	Medtr8g043970	Lévy J, et al., 2004
CCaMK-CYCLOPS	<i>MtIPD3</i> , <i>CYCLOPS</i> (<i>Lotus japonicus</i>)	Medtr5g026850	Messinese et al., 2007
early stages of the nodulation programme - activation of gene expression	<i>MtNSP1</i>; <i>CERBERUS</i>	Medtr8g020840	Smit P, et al., 2005
MtNSP1-(MtENOD11, MtERN1, MtNIN, MtCRE1(root cortex))	<i>MtNSP2</i>, <i>LjNSP2</i>	Medtr3g072710	Kaló P, et al., 2005
Infection thread propagation	<i>MtNIN</i>, <i>LjNIN</i> (epidermis)	Medtr5g099060	Vernié, et al., 2015
LjNSP2 induces	<i>MtCBS1</i> ; <i>MtCBS2</i>	Medtr6g052300	Sinharoy, et al., 2016
Cytokinin	<i>LjENOD40</i> - <i>LjNIN</i>		Murakami, Y., et al., 2006
Auxins	<i>MtRR4</i> and <i>MtNSP2</i>	Medtr5g036480	Murakami, Y., et al., 2006
<i>MtNSP2</i> , <i>MtERN1</i> and <i>MtNIN</i> and <i>PIN</i> activates	MtPIN (auxin efflux) MtLAXs (MtPINs and MtPAT)	Medtr6g011400	Sanko-Sawcenko, et al., 2016
	MtCLE13	Medtr4g079610	Mortier et al., 2010
	ERF1 (ERNS-ERFs)	Medtr4g008860	Vernié et al., 2008
Ethylene	<i>MtNIN</i> in cortex	Medtr5g099060	Vernié, et al., 2015
B) Nodule Meristema (ZI)	<i>WUS</i>/<i>WOX-CLV</i> (<i>WOX5</i>)	Medtr5g081990	Osipova, et al., 2012
ENODs (<i>bona fide</i> marker following mechanisms)	MtCRE1 (cytokinin receptor 1)	Medtr8g106150	Gonzalez-Rizzo, et al., 2006; Laffont et al., 2015
	MtN13	Medtr4g120950	Gamas et al., 1998
	MtCLE12	Medtr4g079630	Popp and Ott, 2011
	MtROP2	Medtr6g087980	Limpens et al., 2013
	CYCA2;4	Medtr2g102520	Roudier et al., 2003
	MtLYK3	Medtr5g086130	Limpens et al., 2005
	NOOT/COCH	Medtr7g090020	Couzigou, 2012
	MtSPK1	Medtr4g129010	Andrio et al., 2013
Activates ERN1 and ENOD11	MtNF-YA1 (<i>HAP2.1</i>)	Medtr1g056530	Combie et al., 2006; Laporte et a., 2014
	MtNF-YA2	Medtr7g106450	Laloum et al., 2014
	MtRR4	Medtr5g036480	Plet et al., 2011
C) Rhizobium Infection Specific (ZII-d)	<i>MtAnn1</i>	Medtr8g038210	de Carvalho Niebel F, et al., 1998
	ENOD11	Medtr3g415670	Journet E-P, et al., 2001
	MtERN1 (NFs activator)	Medtr7g085810	Cerri, 2012
	MtN6	Medtr1g062710	Mathis et al., 1999
	MtENOD12	Medtr3g415650	Limpens et al., 2013
	MtN1	Mtr.37500.1.S1_at	Gamas et al., 1998
	MtERN2 (NFs activator)	Medtr6g029180	Cerri et al., 2012
	MtERN3 (putative NFs repressor-ENOD11)	Medtr8g085960	Adriankaja, 2007
	NCR-α (NFS1)	Medtr8g073380	Yang et al., 2017
	NCR-β (NFS2)	Medtr8g465280	Wang et al., 2017
	LjAPN1		Yamaya-ito et al., 2017
D) Nodule maturation - Early Bacteroid Differentiation (ZII-p)	<i>MtENOD20</i> (ZII-d-IZ)	Medtr4g130800	Vernoud et al., 1999
CLE peptides	MtMMPL1/MtN9	Medtr5g036087;Me	Combie et a., 2007
NCRs	MtNCR247	Medtr5g056815;Me	Farkas et al., 2014
	MtCP1 (Trx s1)	Medtr2g079400	Ribeiro et al., 2017
	MtIRE	Medtr5g069000	Pislaru and Dickstein 2007
	NAC1 (ZII-d-ZIII)	Medtr2g064470	D'haeseleer, 2011
	MtENOD16 (ZII-d-ZIII)	Medtr4g130780	Catalano et al., 2004
	MtSYMREM1 (ZII-d-ZIII)	Medtr8g097320	Lefebvre et al., 2010
	MtDNF1 (ZII-d-ZIII)	Medtr3g027890	Wang et al., 2010; Van de Velde et al., 2010
	MtDNF2 (ZII-d-ZIII)	Medtr4g085800	Bourcy et al., 2013
	MtCKX1 (ZII-d-ZIII)	Medtr1g015410	Moreau et al., 2011
	MtKNOX3 (downregulate <i>MtIPT3</i> and <i>MtLOG2</i>)	Medtr1g012960	Azarakhsh, 2015
E) Nodule maturation - Bacteroid Late Differentiation (IZ)	MtPUB1	Medtr5g083030	Mbengue et al., 2010
	NCR035 (IZ-ZIII)	Mtr.10684.1.S1_at	Van de Velder et al., 2010
	NCR001 (IZ-ZIII)	Medtr6g463200 Me	Van de Velder et al., 2010
	HSP70 (Symbiosome)	Medtr4g130540	Catalano et al., 2004
	MtNip1 (Symbiosome) (IZ-ZIII)	Medtr8g087710	Catalano et al., 2004
	RSD (C2H2)	Medtr7g063220	Sinharoy, et al., 2013
	MtNAC074	Medtr7g085220.1	Vidyadhar Karmarkar, 2014
	MtCCS52a	Medtr3g067940	Vindardell et al., 2003
F) Nitrogen Fixation (ZIII)	bHLH1	Medtr3g150170	Godiard, 2011
leghemoglobin	MtIP3 (interacts with DMI3)	Medtr5g026850	Messinese et al., 2007
Anapargine Synthetases (4)	MtENOD8.1	Medtr1g030275	Coque et al., 2008
	MtRBOHA	Medtr1g083290	Marino et al., 2011
	MtENOD8.2	Medtr1g030268	Dickstein et al., 2002; Limpens et al., 2013
	Krüppel-like zinc finger (<i>Mszpt2-1</i>)		Frugier, 2000
	GmNAT2		Laursen, 1994
G) Nodule Senescence (IV)	MtATB2 (bZIP)	Medtr4g070860	D'haeseleer et al., 2010
	MtNAC969	Medtr4g081870	de Zelicourt et al., 2012
	MtNAC920	Medtr3g096920.2	Vidyadhar Karmarkar, 2014

Table 1. Characterized genes involved in nodulation of *Medicago truncatula*. Genes noted in bold genes are transcription factors.

Notwithstanding, in a distinguished way, rhizobium recognition elicits membrane depolarization and causes root hair curling (Amor et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003). In *Lotus japonicus*, at least seven genes (SYMRK, CASTOR, POLLUX, SYM3, SYM6, SYM15, and SYM24) were found essential for both, RNS and AMF (Kistner et al., 2005). Another membrane receptor with a LysM domain, *M. truncatula* DOES NOT MAKE INFECTIONS (*MtDMI2*), is present on the plasma membrane of cells surrounding the ITs and share the common SYM pathway that is needed for the formation of symbiosomes (Bersoult et al., 2005). In Lotus, this recognition elicits Ca^{2+} spiking at the perinuclear boundary via SYMRK receptor kinases homologous to *MtDMI2*, downstream of NFP/NFR under recognition of *Rhizobium* NFs (Limpens et al., 2005).

This signaling cascade is marked by calcium spiking ripple through the cell cytoplasm to reach the nucleus in order to induce transcriptional changes to drive nodule development via a dramatic rewiring of the genetic networks (Kistner et al., 2005; Czaja et al., 2012; Moreau et al., 2014). In the nuclear envelope, a similar signaling characterized by Ca^{2+} spiking is induced by *MtDMI1* in Medicago or POLLUX in Lotus, which are membrane-spanning proteins that participate in the formation of a symbiotic receptor complex (Ané et al., 2004; Riely et al., 2007). In the nucleus, Ca^{2+} signaling is produced by a Ca^{2+} /calmodulin-dependent protein kinase (CCaMK), *MtDMI3* in Medicago and *CYCLOPS* in Lotus, which ultimately activates the gene expression of transcription factors involved in the nodulation signaling pathway, such as *NODULE INCEPTION (NIN)*, *NODULATION SIGNALING PATHWAY (NSP1)*, *NSP2*, ETHYLENE RESPONSIVE FACTOR (*ERF*) REQUIRED FOR NODULATION (*ERN1*) and *EARLY NODULIN (ENOD11)*.

As primary transcriptional regulator induced by NF signaling is the GRAS TF NSP1 (MtNSP1) in Medicago and CERBERUS (CER) in Lotus, which share RNS and AMF pathway and essential for the symbiosis transcriptional change (Smit, 2005; Nagae et al., 2014). A second GRAS-type transcriptional regulator, MtNSP2, cannot bind directly to DNA, but it acting by forming a heterodimer with MtNSP1, which has a DNA-binding activity to TA-rich *cis* elements in many promoters of nodulation genes, such as *MtERN1* (Kalo, 2005; Cerri et al., 2012a). Indeed, *ENOD11* expression is a *bona fide* marker for early nodule organogenesis (Lévy et al., 2004; Boisson-dernier et al., 2005; Kalo, 2005; Smit, 2005; Marsh et al., 2007; Cerri et al., 2012; Soyano et al., 2013; Vernié et al., 2015). Interestingly, nodulation that is independent of *Rhizobium* interaction can occur by CCaMK auto-inhibition, proving that DMI3 in Medicago (and CYCLOPS in Lotus) is a central regulator of nodulation (Lévy et al., 2004; Gleason et al., 2006; Yano et al., 2008).

The nodulation signal cascade travels from the root epidermis to the inner cortex and stimulates the nodule primordia formation while the “shepherd’s crook” forms at the tip of the root hair (Oldroyd et al., 2011). Induced and maintained by NIN and high cytokinin levels, nodule primordia formation starts at the

root cortex via cell division (Vernié et al., 2015). Besides its symbiotic role in initiative nodule primordia development, the *M. truncatula* CYTOKININ RESPONSE (MtCRE1) also has positive effects on root architecture and resistance to different stress conditions, such as salinity and some pathogens (Laffont et al., 2015). Cytokinin signaling also regulates transcription via *M. truncatula* RESPONSE REGULATOR (MtRR1), a response regulator that interacts with RR binding sites found in RR4 and NSP2 promoters (Kalo, 2005; Vernié et al., 2008; Ariel et al., 2012).

***Rhizobium* infection and endocytosis:** After the symbiotic recognition occurs and the concurrent nodule primordia formation starts, the micro-symbiont is entrapped at the root hair, which tip curls under bacterial recognition and forms the “shepherd’s crook” (Stougaard et al., 1999; Veereshlingam, 2004; Marsh et al., 2007; Pislariu and Dickstein, 2007; Laporte et al., 2014). This recognition enable the bacterial entrance via endocytosis and trigger nodule primordia formation in the inner root cortex, where cells start to multiply to form the nodule meristem, while *Rhizobium* colonization initiates at the root hair epidermis via the IT formation towards the developing nodule meristem (Limpens, 2003; Oldroyd and Downie, 2008; Farkas et al., 2014; Laloum et al., 2014).

During rhizobial infection, the plant defense apparatus against pathogens is locally “placed on hold” and a symbiotic status takes place, providing conditions for the *Rhizobium* to colonize the root cortical cells (Domonkos et al., 2013; Sinharoy et al., 2013). During this process, the plant cell wall is locally digested, the cytoskeleton reorganizes in response to NF perception, directing root hair curling and formation of an IT, culminating with rhizobium endocytosis by the plant plasma membrane, and creating a new organelle, the symbiosome, which interface (the symbiosome or peribacteroid membrane) forms both a structural as well as functional boundary between both symbionts (Veereshlingam, 2004; Prell and Poole, 2006; Zhao et al., 2012).

Driven by a Ca^{2+} gradient and guided by the plant nucleus, the IT forms by aligning the plant cell cytoskeleton and creating cytoplasmic bridges that invaginate towards the nodule meristem (van Brussel et al., 1992; Timmers et al., 1999). In some instances, the nodule meristem and active bacterial infection zone are maintained in the mature nodule, which allows the nodule to grow indeterminately and contain four spatially distinct zones: the meristem (zone I), cell infection (zone II), bacteroid differentiation (zone II-III or interzone, IZ), nitrogen fixation (zone III), and senescence (zone IV) (Franssen et al., 1992; Mergaert et al., 2006). On the other hand, in determinate nodules, the cortical cells of the developing nodule rather go through these phases synchronously. In temperate legumes, such as pea and alfalfa, the primordium is formed from cells of the root inner cortex and develop indeterminate nodules (Timmers et al., 1999). Meanwhile, tropical legumes, such as soybean and common beans, develop the nodule primordium from the root outer cortex and present nodules with determinate growth (Popp and Ott, 2011; Suzaki et al., 2012).

Transition from infection to SNF: Some nodule cells become colonized by *Rhizobium*, which becomes specialized in SNF and nutritionally dependent on the plant metabolism (Wienkoop and Saalbach, 2003; Oldroyd et al., 2011). During cell infection, the *Rhizobium* is engulfed by the plant cell through an endocytosis process (Timmers et al., 2006) and receives a membrane derived from the plasma membrane (PM), called symbiosome (or peribacteroid) membrane (SM). This membrane encompasses a physical barrier between the bacteria and the plant cytosol and is specialized in signaling and transport of substances in and out of the symbiosome to maintain and regulate the symbiosis (Udvardi and Poole, 2013). After endocytosis, the bacteria will redefine its transcriptional program to become able to fix (i.e., reduce) N_2 into NH_3 . During bacteroid differentiation, the *Rhizobium* passes through five well-defined differentiation stages (cell division, cell elongation, maximum bacteroid elongation with invaded cells with starch accumulation in plastids, bacteroid cytoplasmic heterogeneity with DNA endoreduplication, and synthesis and organization of the SNF apparatus) (Vasse et al., 1990; Udvardi and Day, 1997). Meanwhile, the *Rhizobium* undergoes a differentiation process, such as DNA endoreduplication up to 64C (Cebolla, 1999), cell elongation, repression of some essential biosynthesis pathways, which thereon needs to be supplied by the host (Hosie et al., 2002; Prell et al., 2009), and synthesis of the SNF apparatus, thus becoming mature bacteroids capable of fixing N_2 (Oldroyd and Downie, 2008; Domonkos et al., 2013; Guan et al., 2013; Sinharoy et al., 2013). A good example of this auxotrophic dependency is the halting of the branched-chain amino acids (Leu, Ile, Val) biosynthesis pathways, making the bacteria completely dependent on the host supply and requiring metabolite transporters at both, the symbiosome membrane as well as bacteroid membrane (Prell et al., 2009).

Symbiotic N_2 fixation: Energetically, SNF is a highly demanding reaction. Therefore, the establishment of this symbiosis depends on the host plant providing photosynthates (a reduced C source, such as the dicarboxylates malate and succinate) and, in return, receiving reduced (fixed) N as ammonia (NH_3) from the bacteroid (White et al., 2007; Kaschuk et al., 2009). In order to reduce one molecule of N_2 into two NH_3 , nitrogenase requires 16 ATPs (Patriarca et al., 2002). SNF can consume up to 14% of the photosynthesized C from the plant (Kaschuk et al., 2009). Once the endosymbiont fixes N, NH_3 moves out the bacteroid to the acidic peribacteroid space and is instantly protonated into NH_4^+ (Udvardi and Day, 1997). The NH_4^+ is sent to the plant cytosol via a channel and rapidly assimilated into glutamine via the GS/GOGAT cycle (Tyerman et al., 1995; Castillo et al., 2000; Hosie et al., 2002; Lodwig and Poole, 2003; Mulley et al., 2011). In plants, NH_4^+ transporter families were identified and partially characterized based on their affinity to the substrate, high-affinity AMMONIUM TRANSPORTER (AMT1) and low-affinity AMT2 channels. Some NH_4^+ transporters were characterized in legume nodules, such as MtAMT1;1 and MtAMT2;1, LjAMT2;1 and GmSAT1/GmbHLHm1 (Simon-Rosin et al., 2003; Chiasson et al., 2014; Straub et al., 2014).

During SNF, the infected nodule cell must provide ideal conditions to the bacteroid, such as energy and other organic compounds (TCA metabolites, amino acids), and minerals. The conundrum of high energy demands (which require aerobic respiration) and the nitrogenase sensitivity to O₂, which binds to the active site and permanently inhibits the enzyme, has been resolved in legume nodules with the use of leghemoglobins (Lb) to create a hypoxic environment (low O₂ pressure) in the cytosol of the infected cell for a sustained but slow release of O₂ for respiration (Witty et al., 1983; Ott et al., 2005). Leghemoglobins are not required for plant growth and development when external nitrogen source is available, however, are of paramount importance to support SNF into the nodule (Ott et al., 2005). By and large, Lb is the most prominent nodulin expressed in nodules and provides the characteristic pink color of functional nodules (Govers et al., 1985; Ott et al., 2005; Appleby, 1984; Ott et al., 2005).

Along with the complexity of legume-*Rhizobium* interaction, the N and C status of the plant exert autoregulation of nodulation (AON), which is activated by CLAVATA3 (CLV3)/ENDOSPERM SURROUNDING REGION (ESR) (CLE) peptides (Reid et al., 2011; Osipova et al., 2012) to control SNF (Murray et al., 2017). For example, 0.5 mM KNO₃ supply in an inorganic medium did not inhibit nodulation in soybean, but a 7.5 mM KNO₃ level inhibited 50% of nodulation (Day et al., 1989). In spite of the physiological control of nodulation and SNF, genetic mechanisms encompass another level of regulating nodule development and SNF. The CCaMK DMI3 protein contains an auto-inhibition domain that coordinates the expression of nodulins via its kinase activity through Ca²⁺ spiking (Gleason et al., 2006).

Plant hormones also play a fundamental role during nodulation and SNF. In general terms, auxin triggers nodule development (Bensmihen, 2015; Hiltenbrand et al., 2016); cytokinin is important for NF signaling and nodulation via cytokinin response regulators (RRs); gibberellic acid, brassinosteroid and strigolactones also seem to have a positive impact in nodulation at low concentrations. On the other hand, abscisic acid and jasmonic acid negatively regulate NF signaling and nodule development, along with high levels of strigolactones and gibberellic acid (Bensmihen, 2015).

Overall, the most basic mechanisms of legume-*Rhizobium* interaction have been resolved at the anatomic, cellular, physiological, biochemical, and genetic levels. However, a more refined picture is needed to reveal the molecular mechanisms that coordinate and link each step altogether to define the cell infection, nodule development and establishment as well as maintenance of SNF. In focusing in one or a few model species, and delving into modern techniques, involving -omics and functional genetics approaches, we will be able to dissect each element and eventually reveal the complete picture that makes legumes with such unique features of developing nodules *de novo* from root tissues and establish SNF.

***M. truncatula* as a model legume system:** While mycorrhizal associations encompass broad groups of plants and fungi to establish symbiosis, the legume-*Rhizobia* associations are highly species-specific. As a case in point, *M. truncatula* associates specifically with *Sinorhizobium meliloti* (*Sm*) to

develop nodules and fix N₂. The Fabaceae family is the third largest plant family (after Orchidaceae and Asteraceae) and encompasses over 19,000 species with around 700 of them able to fix N₂ through rhizobial symbiosis (Doyle, 2016). Legumes are also the second most important crop family, after the Poaceae (cereals) (Young and Bharti, 2012). Most legume crops are in the Faboideae (Papilionoideae) subfamily. Even though the foundation of genetics were established from pea studies carried out by Mendel, the genetics of many important crop legumes are complex often regarding polyploidy (e.g., tetraploids like alfalfa, soybean), allogamy (alfalfa), and large genomes (soybean: 1.1 Gb; peanut: 2.7 Gb; pea: 4.3 Gb) (Soltis et al., 2015).

The three major models in the Papilionoideae subfamily are *M. truncatula*, *Lotus japonicus* and soybean (Oono et al., 2010). Additionally, molecular genetic resources, such as genome sequences, are available for common bean, chickpea, lupin, pigeon pea, and a few others (<https://legumeinfo.org/genomes>).

M. truncatula (barrel medic, henceforth called just Medicago) emerged as an ideal genetic model to study nodulation and SNF due to its small, sequenced genome (~450 Mb), diploid genome distributed in a small number of chromosomes (n=8), autogamous pollination, natural diversity availability, short lifecycle (<100 days from seed to seed), not to mention being relatively easy to grow, having genetic transformation techniques well established (for both, transformation of whole plants with *Agrobacterium tumefaciens* as well as hairy root transformation with *Agrobacterium rhizogenes* to produce composite plants) (Young et al., 2011). Medicago nodules are indeterminate (**Figure 2**), and each symbiosome contains a single bacteroid (Oono et al., 2010).

Figure 2. The indeterminate nodule of *Medicago truncatula*. Notice the five distinct zones of symbiosis development in the nodule internal tissues. A 28-dpi nodule was harvested and sectioned with vibratome with 70 μ m thickness. Scale bar represents 100 μ m.

Systems biology of legume symbiosis: Understanding the evolution of root symbioses and how the nodule emerged as a new trait in legumes is fundamental to guide us tailor breeding efforts in legume crops and perhaps extend this trait to non-legumes. Genome sequences and annotation are available for many legume species, making genome-wide association studies (GWAS) feasible and allowing RNA-Seq analyses for construction of gene networks involving traits of particular relevance. Gene expression atlases are available for model legumes, such as *Medicago* (Benedito et al., 2008; He et al., 2009), *Lotus japonicus* (Verdier et al., 2013) and soybean (Severin et al., 2010). Furthermore, a detailed mapping of gene expression in *Medicago* nodule tissues through laser capture microdissection (LCM) samples can be publicly assessed (Roux et al., 2014). During nodule development, a massive transcriptional reprogramming takes place in both symbionts, in which transcription factors forge essential rules in the molecular orchestration of cell differentiation and *de novo* organogenesis (Moreau et al., 2011). The characterization of the DNA methylation landscape revealed 1,425 epigenetically regulated genes during the development and SNF in *Medicago* nodules (Satgé et al., 2016). A proteome atlas of all *Medicago* organs and three nodule developmental stages revealed more than 23,000 proteins, 20,000 phosphorylation and 700 lysine acetylation sites of potential post-translational modifications (Marx et al., 2016). A large collection of *M. truncatula* insertional mutants encompassing 190,000 stable *Tnt1* retrotransposon

insertions in 7,600 independent lines is available for functional characterization of individual genes (Tadege et al., 2008).

The development of the legume nodule is orchestrated molecularly by a precise genetic network program that is ultimately coordinated by transcription factors (Moreau et al., 2011; Oldroyd et al., 2011). Transcription factors (TFs) control the genetic expression of the cellular machinery, which fundamentally coordinate plant growth and development. In this regard, nodule organogenesis shows an intricate genetic regulatory network that still needs to be better characterized (Udvardi et al., 2007; Moreau et al., 2011; Oldroyd et al., 2011; Cerri et al., 2012a; Limpens et al., 2013; Oldroyd, 2013; Laloum et al., 2014; Soyano and Hayashi, 2014; Vernié et al., 2015; Geurts et al., 2016; Zipfel and Oldroyd, 2017). In the *M. truncatula* genome, 1,513 TF genes were previously identified (Young et al., 2011), with 1,111 pseudomolecule genes expressed in nodules, 92 TF of which showing a nodule-specific expression pattern (Young et al., 2011). A more detailed analysis of TFs in *M. truncatula* is provided in **Chapter 2** of this dissertation.

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CHAPTER 2

THE MAKING OF THE LEGUME NODULE: TRANSCRIPTION FACTORS, REGULATORY GENE NETWORKS AND THE NOVEL GRF-TYPE ZINC FINGER FAMILY

ABSTRACT

The genome sequencing of the nitrogen-fixing model legume *Medicago truncatula* provided tools to better understand the nodulation and nitrogen-fixation processes by genetic characterization and molecular approaches. Transcription factors (TFs) have fundamental functions throughout bacterial recognition, symbiosis establishment, nodule development, maintenance of nitrogen-fixation, to nodule senescence. Some of them have been characterized in diverse legume species, although most potential TFs still await functional studies. Understanding the orchestration dynamics of TFs during nodule symbiosis will open new avenues to explore nodulation in non-legumes. In this chapter, we delineate the current knowledge about the roles TFs play in legume nodule symbiosis. We also introduce and analyse a novel family of GRF-type zinc finger family, of which some members present genes that are highly expressed in the indeterminate nodule of *Medicago truncatula*.

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INTRODUCTION

Transcription factors (TFs) control the genetic expression of the cellular machinery, fundamentally coordinating every aspect of metabolism, growth and development of an organism. Essentially, the entire phenome and the phenotypical plasticity of an organism (i.e., the entire set of phenotypes expressed by an individual) are dictated by the controlled its genome genes expression. The environment can only influence the phenotype inasmuch as the appropriate genes are present, and if so while they are induced (or repressed), whereas it cannot modulate traits without the coordination of a gene network that makes the phenotype. TFs are proteins with functional domains that interact with the DNA at promoter regions and directly regulate gene expression. Many TFs are hubs of regulatory gene networks that orchestrate, directly or indirectly, often a large number of genes. In plants, around 5-10% of the genes in the genome encode for TFs (Udvardi et al., 2007; Libault et al., 2009; Jin et al., 2017). As hubs on which important gene networks are centered, TFs usually possess highly conserved domains that impart them with biochemistry properties to recognize distinct *cis*-elements of a gene promoter and either repress or call the basic transcriptional machinery. Therefore, understanding the essential roles that transcription factors play on biological systems allows us to delve more purposefully into the molecular genetics of particular attributes of an organism.

This chapter reviews what is known about TFs during nodulation and symbiotic nitrogen fixation (SNF) in legumes, and provides genomic and transcriptomic perspectives of nodule TFs, with a particular focus on the genetic model *M. truncatula*. Furthermore, I provide the first insights on nodule-specific putative TFs belonging to a largely uncharacterized family, the GRF-type zinc-finger.

The making of the nodule at the molecular level

The nodule is a new organ that develops *de novo* from the cortical tissues of the root. Determinate nodules rise from the outer cortex while indeterminate nodules grow from the inner cortex (Crespi and Gálvez, 2000). The developmental program of the nodule starts in response to the symbiotic colonization of diazotrophs bacteria. In legumes, this interaction is highly species-specific for both symbionts (Liu and Murray, 2016; Wang et al., 2017; Yang et al., 2017). The morphological and physiological changes observed during nodule development are orchestrated by a precise genetic network program triggered by signaling molecules and signal transduction pathways but that is ultimately coordinated by TFs (Moreau et al., 2011; Oldroyd et al., 2011). In this regard, nodule organogenesis shows an intricate genetic regulatory

network that still needs to be better understood (Udvardi et al., 2007; Moreau et al., 2011; Oldroyd et al., 2011; Cerri et al., 2012; Limpens et al., 2013; Oldroyd, 2013; Laloum et al., 2014; Soyano and Hayashi, 2014; Vernié et al., 2015; Geurts et al., 2016; Zipfel and Oldroyd, 2017).

Initially, legume roots under low nitrogen status exude flavonoids, such as luteolin, to the rhizosphere (Liu and Murray, 2016), which are perceived by soil-borne *Rhizobium*. In response, *Rhizobium* synthesizes membrane lipo-chitoooligosaccharides (LCOs) called Nod factors (NF) and release them to the soil (Peters et al., 1986; Oldroyd, 2001; Limpens and Bisseling, 2003). These molecules are then recognized on the plasma membrane of root hair cells via sugar-binding receptor-like kinases containing lysin motifs (LysM) and a leucine-rich repeat (LRR) encoded by NFP and LYK3 in *M. truncatula* and NFR1 and NFR5 in *L. japonicus* (Madsen et al., 2003; Ané et al., 2004; Oldroyd and Downie, 2008). Early symbiotic events are dependent of LysM receptor, which elicit Ca^{2+} spiking signal-transduction cascade at the perimembrane of root hairs via the leucine-rich-repeat-containing receptor kinase *M. truncatula* DOES NOT MAKE INFECTIONS (MtDMI2), or SYMBIOSIS RECEPTOR LIKE KINASES (LjSYMRK) and (MtLYK) (Endre et al., 2002; Stracke et al., 2002; Limpens et al., 2005). Another gene, *DMI1*, is localized in the nuclear membrane of *M. truncatula* root hairs, while CASTOR and POLLUX are DMI1 homologs in *Lotus japonicus* (Ané et al., 2004; Imaizumi-Anraku et al., 2005; Hogg et al., 2006; Riely et al., 2007). DMI1 was localized in Endoplasmatic Reticulum (ER) of yeast presenting function of releasing Ca^{2+} of ER storage (Peiter et al., 2007). *DMI1* is involved in Ca^{2+} spiking, of the common symbiotic pathway, (*DMI1*) modulate the perinuclear cytosolic Ca^{2+} spiking signaling (Ané et al., 2004; Peiter et al., 2007). The Ca^{2+} spiking signal transduction cascade continues until it achieves the interior of the nucleus where, modulated by MtDMI3 or LjCYCLOPS, the signal induces nodule primordia formation in the root cortical cells (Lévy et al., 2004; Mitra et al., 2004). Another NF receptor, MtLYK3, acts as *Rhizobium* ssp. entry receptor controlling infection. MtLYK3 is not required for *M. truncatula* NODULE INCEPTION (MtNIN) induction, but it seems to play a function in the polar growth of ITs and regulation of a subset of NF-induced genes (Limpens, 2003; Smit et al., 2007; Arrighi et al., 2008). In summary, NFP functions as a signaling reception that elicits Ca^{2+} spiking, which induces MtNIN for nodule primordial initiation, while LYK3 coordinates the bacterial entry by developing ITs.

NF stimulation elicits the early responses required for root hair deformation (“curling”), like Ca^{2+} oscillations, cytoskeletal changes, root hair deformation, while also inducing the expression of early nodulins, like NIN and EARLY NODULINS (ENODs) during the early development of the nodule (Timmers et al., 1999). By means of the NF signaling cascade, cortical cells are elicited to start dividing in order to trigger nodule organogenesis. **Figure 1** illustrates the most relevant legume TFs described during the process of nodulation and symbiotic nitrogen fixation.

Figure 1. Characterized transcription factors involved in the common symbiotic pathway as well as the *Rhizobium*-specific nitrogen fixation symbiosis in legumes.

During early NF signaling event, NOD-FACTOR PROTEIN (NFP) and DMI3 are responsible for rhizobial perception and ENOD11 induction present in the epidermis and root cortex, followed by infection thread formation and nodule organogenesis. A plant-specific Ca²⁺/calmodulin-dependent protein kinase (CCaMK) encodes by DMI3 in *Medicago* and SYM15 in *Lotus* is fundamental for symbiotic gene expression, localized in the nucleus of epidermis and cortex cell of the root, which recognize and triggers the signal transduction cascade of the Ca²⁺ spiking (Lévy et al., 2004; Mitra et al., 2004; Tirichine et al., 2006; Rival et al., 2012). The first recognized TFs that play a role during this stage are the GRAS-domain regulators NODULATION SIGNALING PATHWAY 1 and 2 (NSP1 and NSP2) (Kalo, 2005; Smit, 2005; Zipfel and Oldroyd, 2017). This family has important roles in diverse biological processes, such as root development, phytohormone signal transduction, as well as meristem development and maintenance. In legumes, NSP1 and NSP2 are essential for nodule development and involved at early stages of organogenesis, but not for mycorrhizal associations. The *nsp1* and *nsp2* mutants in *M. truncatula* and *Lotus japonicus* show a *nod*/*myc*⁺ phenotype (Kaló et al. 2005, Smith et al. 2005, Heckmann et al. 2006, Murakami et al 2006). Moreover, NSP1 plays an additional role beyond early signaling, as it is required for maintenance of infection or nodule development (Heckmann et al., 2006; Zipfel and Oldroyd, 2017). This

protein binds to the *ENOD11* promoter as well as ETHYLENE RESPONSIVE FACTOR (ERF) REQUIRED FOR NODULATION 1 (ERN1) and NIN, two other TFs acting downstream of CCaMK/DMI3 (Hirsch et al. 2009). Both, MtNSP1 and MtSNP2 form homo- as well as heterodimers at the *MtENOD11* promoter and their expression levels are enhanced by NF treatment (Hirsch et al., 2009; Cerri et al., 2012). Both genes are induced by CCaMK/DMI3, possibly via phosphorylation, in which the kinase site of CCaMK phosphorylates DMI3 (Kalo, 2005; Smit, 2005; Heckmann et al., 2006; Messinese et al., 2007; Yano et al., 2008).

Downstream, DMI3 activates the gene expression of *ERN1*, a necessary for NF-induced TF involved at the early signaling pathway (Andriankaja et al., 2007). Via DMI3 activation, ERN1 is required for Nod-factor gene expression signaling, and necessary to induce spontaneous nodulation via DMI3 activation (Middleton et al., 2007). ERN1 encodes an APETALA2 (AP2)-domain protein that binds specifically to the NF box, which is a conserved NF-responsive element present in the promoters of *MtENOD11*, *MtENOD12* and *MtENOD9* (Andriankaja et al., 2007). The highly conserved AP2 DNA-binding domain of ERN is necessary to initiate NF signaling in presymbiotic cells. Therefore, loss of this function blocks the initiation and development of rhizobial invasion structures, termed infection threads, and thus block nodule invasion by the bacteria (Middleton et al., 2007). Indeed, *ern* knockout mutants have defective in infection and those that develop, form small primordia. (Middleton et al., 2007).

Subsequently, NIN is an early signaling component of the NF signaling pathway (Marsh et al., 2007). Although NIN is not required for NF signaling *per se*, it regulates negatively the spatial expression pattern of *ENOD11* and bacteria entry. NIN expression occurs in nodule primordium cells, in the nodule parenchyma and vascular bundles, as well as infected and non-infected cells of mature determinate nodules (Borisov et al. 2003). Data also suggest that NIN may be a positive regulator of both, bacterial entry as well as nodule primordium development, because it abolishes nodulation when ectopically expressed. In conclusion, the TF NIN may be a key integrator that controls NF perception, nodule organogenesis, bacterial invasion and nodule number (Marsh et al., 2007).

Beyond TF regulation, the hyper-nodulating mutants *sickle (skl)* and *supernumerary nodules (sunn)* exhibit 10-fold number of nodules at the primary nodulation zone. Penmetsa et al. (2003) described that *MtSUNN* and *MtSKL* define distinct genetic pathways, in which SKL encodes for an ethylene sensitive that regulates nodule number and distribution while *MtSUNN* for a leucine-rich repeat receptor-like kinase that regulates the nodule number via autoregulation of nodulation (AON) (Schnabel et al., 2005).

The WUSCHEL (WUS)-RELATED HOMEBOX (WOX) family transcription factors, WOX5 transcription factor upon nodulation in *M. truncatula* and pea (*Pisum sativum*) form indeterminate nodules. Mature nodules can be of the indeterminate or determinate type depending on whether an apical meristem is sustained through development or not. Which type grows relies on the host: typical models for

indeterminate nodule development are *M. truncatula* and pea. The expression of WOX5 in nodules is suppressed by the CLAVATA (CLV) signaling complex that controls the AON. AON is activated early after the perception of the Nod factors, at the onset of cell division for primordium formation, and its strength increases as nodule development proceeds (Pierce and Bauer, 1983; Caetano-Anolle's and Gresshoff, 1991; Li et al., 2009). So WOX5 is another component of the AON mechanism in addition to the CLV signaling mechanism in the shoot and the CLAVATA3/EMBRYO-SURROUNDING REGION (CLE) peptides in the root. Hence, a conserved WUS/WOX-CLV regulatory system might regulate cell proliferation and differentiation not only in regular plant apical meristems (RAM) and shoot apical meristem (SAM) but also in nodule meristems. The identified AON component, NOD3, might act downstream or alongside the CLE peptides during AON. Thus, although the exact interaction between the different AON components remains unsolved, the AON puzzle that can be used in the future to gain an integrated insight into how AON works. In summary, a conserved WUS/WOX-CLV regulatory system might control cell proliferation and differentiation not only in the root and shoot apical meristems but also in nodule meristems. And the component AON, NODULATION3 of pea, might act downstream from or beside the CLE peptides during AON (Osipova, et al., 2012)

The development stage is characterized by the nodule growth and zones differentiation, a stage between the early stages and the nodule operation, in nitrogen fixation. During the development of root nodules a basic helix-loop-helix (bHLH) transcription factor gene family from *M. truncatula* has its expression regulated produced upon infection by rhizobia bacteria in *M. truncatula*. The *MtbHLH1* is expressed in nodule primordia cells derived from pericycle divisions, in nodule vascular bundles and in uninfected cells of the nitrogen fixation zone. *MtbHLH1* is also expressed in root tip, lateral root primordia cells and root vascular bundles and induced upon auxin treatment. The *MtbHLH1* gene is involved in the control of nodule vasculature patterning and nutrient exchanges between nodules and roots, Godiard et al., 2011).

Plant microRNAs (miRNAs) have an impact on the regulation of several biological processes such as development, growth and metabolism by negatively controlling gene expression at the post-transcriptional level. Branscheid et al. (2011) identified 243 novel Medicago microRNAs and 118 mRNA cleavage targets of miRNA mature and star sequences. Several AM symbiosis-relevant genes were identified as miRNA targets. The transcript of *MtNSP2* encodes for a GRAS transcription factor involved in the nodule and mycorrhizal signaling pathway. *MtNSP2* mRNA can be downregulated via miR171h microRNA inducing cleavage (Lauressergues et al., 2012). This is an example of own primary transcript showing the ability it miRNA to self regulate.

A transcription factor of CCAAT-binding family identified by Combier et al. 2006, *MtHAP2-1*, for which RNA interference (RNAi) and in situ hybridization experiments indicate a key role during nodule

development, possibly by controlling nodule meristem function. This TF is regulated by a microRNA169, whose overexpression leads to the same nodule developmental block as *MtHAP2-1* RNAi. In addition, the restriction of *MtHAP2-1* expression to the nodule meristematic zone is essential for the differentiation of nodule cells, by the miR169 (Combier et al., 2006). An small peptide from an alternative splicing of *MtHAP2-1* first exon Open Reading Frame, uORF1p, binds to MtHAP2-1 5' leader sequence mRNA and downregulates *MtHAP2.1* during nodule development (Combier et al., 2008).

In Arabidopsis, the (NAC1) transcription factor is involved in lateral root formation, and is regulated post-transcriptionally by miRNA164 and by SEVEN IN ABSENTIA HOMOLOG OF *A. thaliana* (SINAT)5-dependent ubiquitination. MtNAC1 shows a different expression pattern in response to auxin than its Arabidopsis homolog and no changes in lateral root number or nodulation were observed in plants affected in MtNAC1 expression. In addition, no interaction was found with SINA E3 ligases, suggesting that post-translational regulation of MtNAC1 does not occur in *M. truncatula*. The miR164 and *MtNAC1* show an overlapping expression pattern in symbiotic nodules, and overexpression of this miRNA led to a reduction in nodule number, D'haeseleer et al., 2011.

Several transcription factors including genes encoding NAC (NAM/ATAF/CUC) identify by Zélicourt et al. (2012) have been linked to the *M. truncatula* response to salt stress in roots. The expression of MtNAC969 in central symbiotic nodule tissues was induced by nitrate treatment, and antagonistically affected by salt in roots and nodules, similarly to senescence markers. Accordingly, expression of salt stress markers was decreased in MtNAC969 over-expressing and induced in RNAi roots, suggesting a repressive function for this transcription factor in the salt-stress response. The MtNAC969 RNAi nodules accumulated amyloplasts in the nitrogen-fixing zone, and were prematurely senescent. This way, the MtNAC969 transcription factor is differentially affected by environmental cues in root and nodules and participates in several pathways controlling adaptation of the *M. truncatula* root system to the environment.

The ethylene response factor required for nodule differentiation transcription factor (EFD) belongs to the Ethylene Response Factor (ERF) group V, which contains ERN1, 2, and 3. These three ERFs involved in Nod factor signaling. The EFD is a negative regulator of root nodulation and infection by *Rhizobium* and that EFD is required for the formation of functional nitrogen-fixing nodules. As expected this gene is up-regulated during nodulation in *M. truncatula*. EFD appears to be involved in the plant and bacteroid differentiation processes taking place beneath the nodule meristem. Vernié et al. (2008) showed that the EFD activated *MtRR4*, a cytokinin primary response gene that encodes a type-A response regulator. At this work, the EFD induction of *MtRR4* leads to the inhibition of cytokinin signaling, with two consequences: the suppression of new nodule initiation and the activation of differentiation as cells leave the nodule meristem. Thus reveals a key regulator linking early and late stages of nodulation and suggests that the

regulation of the cytokinin pathway is important both for nodule initiation and development (Vernie et al., 2008).

KNOTTED1-LIKE HOMEODOMAIN (KNOX) genes have been characterized active during morphogenetic processes delimitating undifferentiated cells and organ outgrowth, such as during the formation of leaf primordia, leaf serrations and compound leaves (Di Giacomo et al., 2013; Bar and Ori, 2015). KNAT3/4/5-like class 2 KNOX transcription factors decreased the expression of the *MtEFD*, an ethylene response factor required for nodule differentiation, TF and its direct target *MtRR4*, a cytokinin response gene, therefore regulate legume nodule development, as it has been presented in leaf development (Di Giacomo et al., 2016).

A *M. truncatula* member of the Cysteine-2/Histidine-2 (C₂H₂) family of plant TFs, REGULATOR OF SYMBIOSOME DIFFERENTIATION (RSD), is required for normal symbiosome differentiation during nodule development has maximal transcript levels in the bacterial invasion zone. Transcriptome analysis of an *rsd* mutant identified 11 genes as potential targets of RSD repression. RSD interacted physically with the promoter of one of these genes, VAMP721a, which encodes vesicle-associated membrane protein 721a. Thus, RSD may influence symbiosome development in part by repressing transcription of VAMP721a and modifying vesicle trafficking in nodule cells. This establishes RSD as a TF implicated directly in symbiosome and bacteroid differentiation and a transcriptional regulator of secretory pathway genes in plants. So the RSD represses the transcription of the secretory pathway gene VAMP721a and promotes the symbiosome development in *M. truncatula* (Sinharoy et al., 2013).

Plant phytohormones also have a fundamental role in nodulation and SNF. In summary, auxin allows indeterminate nodules to grow, cytokinin is important for NF signaling and nodulation linked with cytokinin response regulator (RRs), gibberellic acid, brassinosteroid and strigolactones also seem to have a positive impact in nodulation at low concentrations. In another hand, abscisic acid and jasmonic acid negative regulates NF signaling and nodule development and high doses of strigolactones and gibberellic acid (Bensmihen, 2015).

The nodule senescence in *M. truncatula* is a genetically programmed process that is accompanied by degradation of the bacteroids and then of the plant structures. And this process is associated with nutrient recycling and remobilization of resources (Van de Velde, 2006). Fixation zone cells have a limited functional lifespan, old cells senesce and are continuously replenished by meristem descendants. During the lifespan of the indeterminate nodules, the senescence zone keeps expanding, but remains surrounded by a functional peripheral zone.

Throughout the lifespan of the nodules, the exchange of C sources and N compounds is maintained balance between the host plant and the bacteria. Sucrose provides C skeletons and energy to the bacteroids in the form of C₄ carboxylic acids such as malate, and ammonia is assimilated into GS/GOGAT pathway.

Nodule senescence of nodule cells apoptosis are established when C/N ratio is out of balance or free nitrogen source is available.

The transcription of *M. truncatula A. thaliana bZIP (MtATB2)*, encoding a bZIP transcription factor, down-regulated by sucrose and it is enhanced during nodule senescence, expressed in the vascular tissue of nodules, nodule apex and roots. *MtATB2* transcription is influenced by light and sucrose, regulation of downstream functions related to linking C metabolism and metabolite partitioning and seems to reduce nodule formation and affected root growth in ectopic expression (D'Haeseleer et al., 2010).

Hubs of nodule development: identification of nodule-specific transcription factors

Based on the *Affymetrix* gene expression data and a last genome *M. truncatula* annotation version, we identified 1,298 TFs, with 1,169 of which falling into 45 known families, and 129 considered “putative novel TF” (Benedito et al., 2008). Later, using RNA-seq dataset from six different mature organs of the *M. truncatula* plant (leaf, seed/pod, bud, flower, root and nodule), 3,692 potential TFs and transcriptional regulators (TRs) were identified (5.9% of all predicted gene in the genome) allocated into 74 families, including 47 *bona fide* TF families, 11 chromatin remodeling components and 16 potentially novel families. *M. truncatula* present 1,513 nodule specific TFs, in which approximately 142 genes were derived from whole-genome duplication, that occurred 58-Myr-ago, and 92 are high expressed in nodules (Young et al., 2011). Herein, we offer an update on the TF inventory in the Medicago genome and an overview of transcriptional activity regarding available RNA-Seq datasets. Furthermore, we explore nodule-specific TF genes, and in special those that are expressed in particular developmental stages or zones of the mature nodule.

Transcriptional regulomics of the *M. truncatula* nodule and symbiotic nitrogen fixation

As an important aspect of systems biology, the regulome refers to the study of the whole set of regulators present in an organism (Ramalingam et al., 2015). The term encompasses TFs as well as other regulators, including miRNA and signaling molecules and proteins.

Here, we are present a deep investigation of TF and Transcriptional Regulators (TRs) using the proteome of the important model plants for nodulation and symbiosis nitrogen fixation, *M. truncatula*, *Lotus japonicus*, *Glycine max* and *Phaseolus vulgaris* and the most genetically explored model plant *Arabidopsis thaliana*. *Arabidopsis thaliana*, on PlantTFcat website (<http://plantgrn.noble.org/PlantTFcat/>). The PlantTFcat a transcription factor and transcriptional regulator categorization analysis tool, that possess a list of 108 published TF/TR/CR families in a systematic analysis in the InterProScan domains by common conserved domains families (Dai et al., 2013). PlantTFBD is considered as a benchmark tool with 58 families, and PlantTFcat came to complement and develop a deeper prediction, providing a better output including “Family-type”, InterPro protein signature databases, in spite of “Family” (Dai et al., 2013; Jin et

al., 2017). The result of this analysis is presented in **Table 1**. Each species assessed owns a categorized number of TFs and TRs identified family, followed by the respective abundance of proteins and Proteome Version used.

Table 1. Transcription factors and transcriptional regulators identified in the proteome of species of interest.

Species	TFs+TRs	Families	Proteins	Proteome Version
<i>Arabidopsis thaliana</i>	5,004	99	35,386	Athaliana_167_TAIR v.10.protein
<i>Glycine max</i>	13,462	98	88,647	Gmax_275_Wm82.a2.v1.protein
<i>Lotus japonicus</i>	4,741	99	48,105	Lj3.0_pep
<i>Medicago truncatula</i>	5,486	98	57,585	Mt4.0v2_GenesProteinSeq_20140818_1100-1
<i>Phaseolus vulgaris</i>	5,460	98	36,995	Pvulgaris_442_v2.1.protein-7 MB-PDT 2017

TFs (Transcription Factors), TRs (Transcription Regulators)

In which the GRF-type zinc finger is one of the recognized TFs or TRs families recognized by PlantTFcat tool. With that we generate the **Table 2**, which show the representation of members on the five species analyzed. The abundance of proteins that each species owns the domain IPR010666, GRF-type zinc finger family, is shown in column IPR010666, followed by the Family that they were categorized and Associate domains, also present in some members, **Table 2**. And some of the proteins present other recognized domains, such as CCHC-ZF, LIM and topoisomerase beyond GRF-type zinc finger, as can be seen on **Figure 3A**.

Table 2. Abundance of and GRF type-ZF proteins in the proteome of species of interest, holds or not additional domains.

Species	IPR010666	Associated domains
<i>Arabidopsis thaliana</i>	7	4-CCHC(ZF); 3-only GRF-ZF
<i>Glycine max</i>	5	2-CCHC(ZF); 3-only GRF-ZF
<i>Lotus japonicus</i>	26	26-only GRF-ZF
<i>Medicago truncatula</i>	52	1-LIM; 51-C2H2
<i>Phaseolus vulgaris</i>	2	1-CCHC(ZF); 1-only GRF-ZF

The IPR010666 domain is spread in the Zinc Finger proteins, and in which of Zinc Finger protein subfamily such as CCHC(Zn), Growth Regulate Factor, LIM and C2H2 (**Figure 3A**). CCHC(Zn)-type zinc finger protein recognizes and interacts with the specific sequence single-stranded DNA or RNA oligonucleotides (Espinosa et al., 2003; Brown, 2005). Growth Regulator Factors transcription regulators are involved in young tissue and present high expression in rice (*Oriza sativa*) when gibberellic acid (GA₃) is applied (Choi et al., 2004). Those genes for two highly conserved regions indicative of transcription factors domains, the QLQ (Gln, Leu, Gln) and WRC (Trp, Arg, Cys) (Kim et al., 2003; Choi et al., 2004).

We identify one nodulin, *MtN20* (Medtr7g086040.1) that presents high and specific expression in nodules, with a peak of expression at 4 days post inoculation (dpi) and persisting in the mature indeterminate nodule with *Sinorhizobium meliloti* (*Sm1021*). The *M. truncatula* gene atlas, which is based on Affymetrix Medicago GeneChip dataset from 274 experiments and 739 microarrays, underscores the nodule-specific expression profile of *MtN20*/*MtGRF1* (probeset Mtr.14503.1.S1_at; **Figure 2**).

Figure 2. Affymetrix expression profile of *MtN20* (gene locus Medtr7g086040; probset Mtr.14503.1.S1_at) in *Medicago truncatula* Gene Expression Atlas (<http://mtgea.noble.org/v3>). (A) Expression across all Affymetrix samples. (B) Expression in nodule specific samples highlighted in A.

A *Tnt1* insertional mutant line presented white and round nodules. This nodulin was predicted as GRF-type Zinc Finger and some papers gave the idea that this protein was a GRF Zinc Finger, which is improper. *MtN20* or (*MtN20*) have been classified as GRF-Zinc Finger (GRF-ZF), which the first member was characterized in *A. thaliana* *Growth Regulator Factor1* (*AtGRF1*). This family was characterized presenting essential roles in stem elongation with the domains Glutamine-Leucine-Glutamine, QLQ:IPR014978 and WRC:IPR014977, which is not the GRF-type Zinc Finger (IPR010666) (Kim et al., 2003; Choi et al., 2004). In table 1 of (Udvardi et al., 2007), 8 GRF-type Zn-finger putative TFs, with domains described as IPR010666, were untimely allocated in the “GRF” TF family.. This gene of interest presents only the GRF-type Zinc Finger domain (IPR010666), which is different from GRF in *Arabidopsis thaliana*, and no protein has been characterized.

Figure 3. Phylogenetic reconstruction of GRF-type zinc finger proteins in select legume species and Arabidopsis and expression profile of the *M. truncatula* genes, in which the bold names are the proteins of interest in this research. (A) The original phylogenetic tree based on the Maximum Likelihood method was built with 10,000 bootstraps for all GRF-type Zinc Finger proteins found in the genomes of *Arabidopsis thaliana*, *Phaseolus vulgaris*, *Lotus japonicus*, *Glycine max* and *Medicago truncatula* generated on MEGA7 software (Kumar et al., 2016). (B) Maximum likelihood phylogenetic tree with 10,000 bootstraps the *M. truncatula* GRF-type zinc finger proteins was generated on MEGA7 (Kumar et al., 2016) and displayed along with their renormalized RNA-Seq data for gene expression profiles on six major tissues (Young et al., 2011) and nodule laser capture microdissection (Roux et al., 2014).

Zinc finger (ZF) proteins present a vast diversity of structures and functions. Generally, a ZF protein is defined as a functional protein with an independently folded domain stabilized by the coordination of one or more zinc ions (Laity et al., 2001). ZF proteins can interact with diverse types of molecules, such as peptides and other proteins, as well as RNAs, DNAs and lipids. In addition, they play several functions, including DNA recognition and signaling, RNA packaging, protein ubiquitination, assembly of transcriptional machinery, and lipid binding (Klug, 1999; Matthews and Sunde, 2002a; Matthews and

Sunde, 2002; Brown, 2005; Gamsjaeger et al., 2007). In the InterPro database (<https://www.ebi.ac.uk/interpro/entry/IPR010666>), ZF proteins are allocated to 95 different families with 182 different domains, with the GRF-type domain being a well-defined group (Mitchell et al., 2015). GRF-type ZF proteins show a signature conserved GRF (Glycine, Arginine and Phenylalanine) residue interspaced domain encompassed within the larger ZF domain (Whitehead et al., 1997). Notwithstanding, very little is known about the functional roles GRF-type TFs play in the cell (Chen et al., 2015).

A special notice is required here in that this domain are different from GRF-type Zinc Finger IPR010666, which owns a GRF conserved residues into Zinc Finger domain presented by spaced Cysteines as seen in the protein alignment (**Figure 4**).

Figure 4. Protein GRF-type zinc finger alignments. (A) Pfam GRF-type zinc finger (PF06839) HMM logo from (<http://pfam.xfam.org/>). (B) Consensus domain for all 52 *Medicago truncatula* GRF-type zinc finger proteins using the “Sequence Logo” generator using fasta Clustal alignment (<http://weblogo.berkeley.edu/>) on (Galaxy.org). (C) Multiple alignment with BLOSUM62 matrix *MtN20*, GRF2 (Medtr1g063450.1) and two closest Arabidopsis homologous proteins. Alignment was generated on Geneious software version R11 (<https://www.geneious.com/>).

These results show that GRF-type Zinc finger family is still not very well defined by the family category, which will be better described after the posterior characterization of the gene and protein of interest (*MtN20*).

The present research highlights our knowledge towards to the molecular role of GRF-type TFs legume nodulation. In **Figure 3A**, we present a phylogenetic tree of the proteins holding the IPR010666 domain from the proteomes shown in **Table 1**. **Figure 3B** shows only the *M. truncatula* proteins phylogenetic analysis linked with the RNA-Seq expression data.

The LIM sequence motif is contained twice on Cysteine-rich proteins coordinating zinc binding protein (Michelsen et al., 1993). A C₂H₂-type zinc finger is a widespread DNA binding motif in TF, coding for two or three β -layers and one α -helix on a zinc finger formed by two cysteine and two histidine residues. In general, C₂H₂-type zinc finger proteins are present as transcriptional regulators involved in important cellular processes, such as development, differentiation, and suppression of malicious cell transformation (Razin et al., 2012). In *M. truncatula*, a C₂H₂ were characterized as transcription factor regulator of symbiosis differentiation, previously described (Sinharoy et al., 2013).

The C₂H₂-type zinc finger domain alone is shown in some highly expressed proteins of *M. truncatula* root nodules, therefore apparently adding unknown but potentially important function in nodulation and nitrogen fixation.

Extensive transcriptome datasets of *M. truncatula* using the *Affymetrix* Medicago GeneChip platform and RNA-Seq data were previously published by Benedito et al. (2008), Young et al., (2011), and Roux et al. (2014). Benedito et al. (2008) presented a transcriptome compendium for all major organs and time-course analysis of nodules and seeds. Roux et al. (2014) studied the whole genome expression in nodule tissues through laser capture microdissection (LCM). Analyzing both datasets, our curiosity caught two putatively novel zinc-finger transcription factors through their nodule specificity and intensity of expression (**Figures 3B & 5 and Table 2**). In this study, both genes are emphasized as novel nodule specific genes potentially involved with transcriptional regulation.

Figure 5. Expression profile of GRF-type zinc finger family members (IPR010666) in nodules of *Medicago truncatula*. **(A)** RNA-seq data was retrieved from Young et al. (2011). **(B)** Data retrieved from LCM-RNA-Seq root nodule (Roux et al., 2014).

Crossing the *M. truncatula* TFs and TRs, from Table 1, with LCM-RNA-Seq of nodule section from (Roux et al., 2014) we can identify all TFs and TRs that are expressed in nodules of *M. truncatula*. Roux et al., (2014) present 36495 genes, in which 2996 genes match with genes in the *M. truncatula* TFs and TRs list, from which 1955 and 2930 present expression more than 100 and 0 respectively. The majority of them, 2757 genes, are unknown and 239 genes present annotation name. Very interestingly the *MtN20* was the fifth position of the most TFs and TRs expressed in nodules of *M. truncatula*, followed by Medtr1g064350.1 (another GRF-type Zinc Finger) (Supplemental material table 1S).

The phylogenetic tree with only *M. truncatula* members present a high similar protein with N20 that is also encoded by a nodule specific gene with high and distinct expression from *MtN20* (**Figure 3B**). This same (**Figure 3B**) give us another very interesting idea, that those genes are the unique genes of the GRF-type zinc finger family that are been high expressed and they are seems to be nodules specific, with distinct expression pattern. This second gene (Medtr1g064350.1) of the GRF-type zinc finger is the second gene of interest on our research, called *MtGRF2*.

The Medicago Gene Expression Atlas (Benedito et al., 2008) shows *MtGRF2*/Medtr1g064350 (probeset Mtr.37013.1.S1_at) with an expression onset at around 6 days post-inoculation (dpi), and the strongest level in the adjacencies of zone 2 of nodule samples (**Figure 6**).

Figure 6. Affymetrix expression profile of *MtGRF2* (gene locus Medtr1g064350; probeset Mtr.37013.1.S1_at) in the *Medicago truncatula* Gene Expression Atlas (<http://mtgea.noble.org/v3>; Benedito et al., 2008). (A) Expression across all Affymetrix samples. (B) Expression in nodule samples as indicated in A.

Since those genes of interest present differential expression we retrieve the data of *Medicago* root nodule LCM RNA-Seq to take it closed (Roux et al., 2014) (**Table 3**).

Table 3. Gene expression of the GRF-type zinc finger nodulins Medtr7g086040 and Medtr1g064350 in nodule zones by RNA-Seq of laser capture microdissection (LCM) samples (Roux et al., 2014). Distribution of expression in percentage of total reads in all samples of the nodule are shown in parentheses.

SAMPLES	Medtr7g086040 (<i>MtN20/MtGRF1</i>)	Medtr1g064350 (<i>MtGRF2</i>)
FI	752690 (10.2%)	3988 (00.1%)
FIID	4662776 (63.0%)	83201 (01.2%)
FIIP	1967835 (26.6%)	828990 (11.6%)
IZ	19999 (00.3%)	4618594 (64.8%)
ZIII	3506 (00.0%)	1594961 (22.4%)

The consistency of both *Affymetrix* (**Figure 2 and 6**) and LCM RNA-Seq of *Medicago* data (**Table 3**), give the level of gene expression indicates that both genes seems to play important functions in different stages of root nodulation in *M. truncatula*.

Taken all transcriptome data, two putative novel zinc-finger transcription factors highlight our curiosity through their nodule specificity and intensity of expression (**Figure 2, 3, 5 and 6, Table 3**). In these studies, both genes are emphasized as novel genes specific to nodules, potentially involved with transcriptional regulation. Having this knowledge it is important now to elucidate the TFs related with each biological activity, such as metabolism, development and function. We hypothesize their importance as

putative TFs for normal nodulation and symbiosis nitrogen fixation linked with the predicted biological process in which they should be involved.

METHODS

Identification of GRF-type Zinc Finger proteins: For each species of interest we download the update latest version of their proteomes and we run them in PlantTFDB to identify what domains each protein owns, then we selected all genes with identify protein domain IPR010666, which is cataloged as GRF-type Zinc Finger. In which *Arabidopsis thaliana*, (Athaliana_167_TAIR10.protein), *Lotus japonicus* (Lj3.0_pep), *Medicago truncatula* (Mt4.0v2_GenesProteinSeq_20140818_1100-1), *Phaseolus vulgaris* (Pvulgaris_442_v2.1.protein-7 MB-PDT 2017) and *Glycine max* (Gmax_275_Wm82.a2.v1.protein).

RNA-Seq data analysis: The SRAs data were analyzed in the Galaxy platform. The fastq-dump tool were used to upload the SRAs files into Galaxy (Leinonen et al., 2011). The quality of read reports were processed using the FastQC tool (Andrews, S.. FastQC A Quality Control tool for High Throughput Sequence Data.). And the expression data were denenerated in Transcription per Million (TPM) using Salmon (Patro et al., 2015). In which the reference transcriptome used was the last version of *M. truncatula* (Mt4.0v2), kmerLen size = 31, single- or paired-end depending of each SRA source, quasi type of index, also no sequence bias correction were applied.

Phylogenetic analyses: The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al., 1992). The tree with the highest log likelihood () is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying the Maximum Parsimony method. A discrete Gamma distribution was used to model evolutionary rate differences among sites (categories (+G, parameter =)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], % sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 92 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 23 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

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CHAPTER 3

MtN20 IS AN ESSENTIAL GRF-TYPE ZINC FINGER NODULIN FROM *MEDICAGO TRUNCATULA* INVOLVED IN THE FIRST STAGE OF BACTEROID DIFFERENTIATION

ABSTRACT

Legume root nodule proteins, called nodulins, have been explored in different ways in order to understand root nodulation, symbiosis nitrogen fixation and evolution of this relationship. Some nodulins have been functionally characterized and allocated in which the mechanism is involved, but many of them remain intangible regarding their molecular function. In this study, we aimed at studying the role of nodulin 20 (MtN20) during nodule development and symbiotic nitrogen fixation (SNF) in *Medicago truncatula*. This protein, MtGRF1/MtN20, has been predicted as a putative transcription factor highly and specifically expressed in root nodules 4 days post inoculation with *Rhizobium*, which refer to the zone II of indeterminate *Medicago truncatula* nodules. Also, this protein belongs to a largely uncharacterized family of putative transcription factors. We hypothesize that MtN20 is a transcription factor playing an essential role during nodule development by controlling expression of key symbiotic genes in early stages of bacterial infection of root nodules cells. Our findings demonstrate that *MtN20* requires bacterial infection, but not bacteroid differentiation to express. We can conclude that *MtN20/MtGRF1* is a novel gene potentially involved during early events of rhizobial infection of nodule cells. MtN20 belongs to a regulatory gene network participating with bona fide symbiotic genes, such as EFD, the EFD target Nod19, NIN and several nodule-specific cysteine-rich (NCR) peptides. A gene expression analysis using mutant bacteria defective to specific symbiotic milestones can be used to help further elucidate which process must occur before a nodulin is triggered to express.

MtN20 IS AN ESSENTIAL GRF-TYPE ZINC FINGER NODULIN FROM *MEDICAGO TRUNCATULA* INVOLVED IN THE FIRST STAGE OF BACTEROID DIFFERENTIATION

INTRODUCTION

Nodulins encompass a large and diverse class of legume proteins defined by their nodule-specific expression pattern. These are a significant group of legume proteins that evidently evolved to express in an organ that is characteristic of this botanical group, with potential roles in nodule development and symbiotic nitrogen fixation (SNF). Nodulins were first identified in soybean nodules by 2D-gel analysis and described as proteins potentially involved in the development of the *Rhizobium*-legume symbiosis (Legocki and Verma, 1980). They were named according to their approximate weight on gel proteins, in kiloDaltons (17kDa) and classified according to the onset of gene expression during nodule development (Gamas et al., 1996). Interestingly, non-legume nodulins are proteins that which families were first characterized with specific expression in legume nodules and then found to exist in non-legume species. A review on dicot and monocot non-legume nodulins was published previously (Denance et al., 2014).

In *Medicago truncatula*, nodulins were first described by Gamas et al. (1996), which 29 novel distinct families of nodulins were identified by Northern blot and classified into three groups based on their expression during nodule development, of root, 24 and 48 hours post inoculation and 4 and 14 days post inoculation (dpi). Later, among the 50,900 *M. truncatula* probesets represented on the Affymetrix microchip, Mtr.14503.1.S1_at was identified as nodule-specific transcripts (<http://mtgea.noble.org/v3/>; **Figure 1**) (Benedito et al., 2008; He et al., 2009). Using a transcriptomic approach based on RNA-Seq, MtN20/MtGRF1 (Medtr7g086040) gene was identified as specifically expressed in nodules (Young et al., 2011; Roux et al., 2014), therefore being nodulins by definition.

While some nodulins have been functionally characterized, such as leghemoglobins (Appleby, 1984; Andersson et al., 1996). The large majority remain elusive regarding their molecular roles. In this study, we aimed at studying the role of nodulin 20 (MtN20) during nodule development and SNF in *M. truncatula*. Given the high and specific gene expression profile of MtGRF1/MtN20 in the nodule and its conserved domain, we hypothesize that MtN20 is a transcription factor playing an essential role during nodule development by controlling gene expression of key symbiotic genes.

RESULTS AND DISCUSSION

Nodulin 20 (MtGRF1) is an elusive protein recurrently identified in early transcriptomic studies

In *M. truncatula*, nodulin 20 (MtN20) was classified Gamas et al. (1996) as a group 1 nodulin, with expression starting at 4 days post-inoculation based on hybridization signal. Györgyey et al. (2000) identified MtN20 using an expression sequence tag (EST) sequencing approach (clone 74), even though it presented a relatively low expression level. The genome sequencing and annotation of *M. truncatula* (Young et al, 2011) and transcriptomic studies allowed to identify MtN20 as encoded by the locus Medtr7g086040, which belongs to a largely unexplored family of putative transcription factors (GRF-type zinc finger, IPR010666). Therefore, we renamed MtN20 as the founder member of this protein family (*MtN20*). It is important to underscore that, given the nodulin name designation via protein mass on gel electrophoresis, other N20 potentially identified in other legume species may be completely unrelated to MtN20.

The Medicago gene expression atlas (Benedito et al., 2008) shows that *MtN20* was down-regulated in 6-dpi *ethylene response factor required for nodule differentiation (efd-1)* mutant nodules (**Figure 2 in Chapter 2**) (Vernie et al., 2008). Furthermore, transcriptional analyses indicated *MtN20* as a potential “Class A3” activator of nodule development (Moreau et al., 2011) due to its expression profile between the early steps of infection thread formation and *EFD* expression, which controls nodule numbers (Vernie et al., 2008). Both data highlight the notion that *MtN20* is implicated in the mechanism of cell infection. The symbiotic ERF transcription factor *MtEFD* (*ETHYLENE RESPONSE FACTOR REQUIRED FOR NODULE DIFFERENTIATION*) was characterized as playing an important role controlling *Sinorhizobium meliloti* infection in nodule cells (Vernie et al., 2008).

MtN20 has been recurrently identified as a nodulin in transcriptome studies. Notwithstanding, very little has been explored about its function in SNF. Using micro- and macroarrays, El Yahyaoui et al. (2004) identified 756 genes differentially expressed during nodule development. Among these, *MtN20* appeared up-regulated at 4-dpi nodules against non-inoculated roots. Using a similar approach, Manthey et al. (2004) also identified *MtN20* as differently expressed into nodules compared to non-inoculated root, but not in arbuscular mycorrhiza. Godiard et al. (2007) grouped *MtN20* as an early nodulin from a study using suppression subtractive hybridization, along with other important early nodulins, such as *MtENOD11*, *MtENOD12*, *MtENOD16*, *MtENOD20*, *MtENOD40*, *RIP1*, *MtANN1*, and *MtN1*.

Fedorova et al. (2002) also identified *MtN20* as a major nodulin significantly expressed in developing and mature nodules, but not in senescent nodules. The extensive Medicago transcriptome studies were published by Benedito et al. (2008), Young et al. (2011), and Roux et al. (2014) based on the

Affymetrix Medicago GeneChip, and RNA-Seq of several whole, mature organs of the plant, and RNA-Seq of nodule laser capture microdissection (LCM) of mature nodule tissues, respectively. All three studies presented *MtN20* expression data as highly and nodule-specifically expressed (**Figure 2 in Chapter 2, Table 1**).

MtN20 is potentially a novel nuclear protein

The GRF-type zinc finger proteins encompass a family (IPR010666) that although it is present in green algae, it has highly expanded in *M. truncatula*, compared even to other legumes, referred in the phylogenetic analysis **Chapter 2**. This protein family is defined by three, non-consecutive very conserved amino acids (Gly, Arg, Phe) in the center of the protein domain, along with additional zinc-finger domains. So far, no function has been attributed to any GRF protein in the scientific literature. Importantly, GRF proteins have been previously reported in plants, especially in *Arabidopsis* and rice (Kim et al., 2003). However, these proteins are named after the acronym Growth-Regulating Factor and encompass a non-related family (IPR031137). Therefore, we refer to the members of the MtGRF1/N20 family as GRF-type zinc finger proteins. Interestingly, among the 53 GRF-type zinc finger genes present in the *M. truncatula* genome, only two of them are expressed in nodules (**Figure 2 in Chapter 2**), and hardly anywhere else in the mature plant. The second member of this family (Medtr1g064350) is even more highly expressed in nodules. We named it MtGRF2 and explored its properties and potential function in **Chapter 4**. The phylogeny of this protein family is presented in **Chapter 2** of this dissertation.

MtN20 codes for a 146-amino acid residue protein with a molecular weight of 17 kDa and an isoelectric point of 7.59. Its small protein size (<60 kDa for diffusion through the nuclear pore) and isoelectric point (the pI value of the plant nucleoplasm is ~7.2) are compatible for nuclear import and activity (Wang and Brattain, 2007; Shen et al., 2013). Based on its deduced amino acid sequence, Fedorova et al. (2002) suggested that the nodulin 20 gene product is potentially a peroxisomal protein or localized in the mitochondrial matrix space. However, a nuclear localization signal (NLS) was predicted in the C-terminus of the protein. The *M. truncatula* proteome compendium (Marx et al., 2016) identified MtN20 as a protein highly and specifically expressed in nodules, and it also determined the existence of a phosphorylation site at S138, just before the estimated NLS in the C-terminus. Subcellular localization studies are still necessary to confirm GRF-type zinc fingers are directed to the nucleus of the cell (**Figure 1**).

MTSRVSAAPTCDCEAKCVIFISRTKKNPDRPFRCPYYGOIRKTHCDYFMWEDDFIASQAAMV
ELQETTIERKMEAMIIQMNMKDMKAMKIQINKDMEAKINELKRDTEYKFNILEREIEAMTFQISA
DVVAFDIKHLSLGGLKGSK

Figure 1. MtN20 protein sequence retrieved data from *Medicago truncatula* Proteome compendium on six major organs and three time-points of nodules development from (Marx et al., 2016). Showing one serine phosphorylated

on position 138 (red tag), the protein domain red underline and nuclear localization signal (NLS) in green box (estimated via SBC-NucPred (Brameier et al., 2007))

In the *M. truncatula* Compendium proteome, MtN20/MtGRF1 was identified in 10, 14 and 28 dpi nodules of *Medicago* (**Figure 2**).

Figure 2. MtN20/MtGRF1 quantification retrieved from the *Medicago truncatula* Compendium proteome (Marx et al., 2016). MtN20 is present in nodules samples. The x-axis is presented as log2 of TMT (relative protein abundance). In which positive values indicate more while negative values indicate less amount in of protein against the average of all samples. It is important to highlight that the quantification is based on protein fragments, peptides, which can be common between different proteins.

Tnt1 insertional mutant lines for *MtN20/MtGRF1* were identified in the Noble Foundation collection and are illustrated in the gene structure presented below (**Figure 3**). A disadvantage of these mutants is that they can present more than one insertion in each line, which often generates confounding phenotypes. Therefore, a more reliable approach is to knock-out the gene using a gene editing method, such as the CRISPR-Cas9 system. We had tried to produce *ex-vitro* hairy root transformation knock-out via CRISPR-Cas9 using *Agrobacterium rhizogenes*, but we could not screen the transgenic roots, we had a problem with our selective fluorescence mark, DsRed. And then, we used antibiotic selective media with 25mg/L of Kanamycin, and the plants did not produce healthy roots and plants to conduct the experiment.

A segregating population of *MtN20 Tnt1* mutant lines, NF1858, NF2990, NF3070 and NF17000, had been used in this study, *Tnt1*-insertional position and direction are present in (**Figure 3**). In which we had identified homozygous plants for the insertion line NF1858 in the gene of interest.

Interestingly, although most commonly conserved domains are coded within the confines of an exon (Sibley et al., 2016), the GRF-type zinc finger domain of both genes of interest are split in different exons (**Figures 3**).

Figure 3. Position and gene structure of *Medtr7g086040* (*MtGRF1/MtN20*) in *Medicago truncatula* chromosome 7. Four *Tnt1* *Medicago truncatula* insertion lines (NF-1858, NF-2990, NF-3070 and NF17000) are represented with their respective positions and directions (arrows). The centromere is represented with a black circle on chromosome 7. In the lower representation, exons are indicated by boxes, with the sequence coding for the GRF-type zinc finger domain (IPR010666) represented as black boxes. Introns and UTRs are represented as lines.

Five homozygous recessive plants from the NF1858 line were genotyped via PCR in which the dominant PCR generate an amplicon of 1508bp and recessive of 862bp (653bp-*Tnt1*+209bp-*MtN20*), the recessive PCR band was sequenced in order to verify the position and direction of the *Tnt1* (**Figure 4**).

Figure 4. Genotyping of NF1858 Tnt1 line. **A)** *MtGRF1/MtN20* gene architecture with primers in green arrows. **B)** Gel images of dominant and recessive PCR, in which the dominant and recessive PCR represent amplicons of 1953bp and 866 pb respectively. **C)** PCR-direct sequence with alignment with Tnt1 (blue box) and *MtGRF1/MtN20* (light-green box), in which RC is reverse complement, primers are represented in green.

MtN20 function may be essential for symbiotic nitrogen fixation but not for nodule development

Five Tnt1 insertional lines were identified in the *MtN20* (Tadege et al., 2008; Pislariu et al., 2012). However, all lines presented insertions in the intron between the only two exons (**Figure 3**). Notwithstanding, the line NF1858 developed normally shaped but smaller, white nodules (**Figures 5** and **6A**). Cell infection was defective, with a very narrow infection zone and numerous infection droplets unable to fully carry infection out (**Figure 5**).

Figure 5. Phenotype of NF1858 *Tnt1* insertion line of *Medtr7g086040.1* (*MtN20*). **A-C** is R108 ecotype and **E-G** is the mutant line. In which **A** and **D** are fresh nodule picture in 5X amplification, **B**, **C**, **E** and **F** is 7µm sectioning of nodule after fixation and X-gal stained *lac-Z Rhizobium*, **B** and **E** 5X amplification, **C** and **F** 10 X amplification.

Indeed, the symbiotic phenotype of line NF1858 was already noticed by Pislariu et al. (2012). The nodule phenotype of line NF1858 is indicative that *MtN20* loss-of-function does not affect nodule development, but it is essential for symbiotic nitrogen fixation (SNF), possibly due to a defective cell infection. It is important to note, however, that at least 86 *Tnt1* insertions are scattered in the genome of this line, among which 27 were identified in gene spaces (defined herein as the DNA between the start and stop codons). Of those genes, with only two expressed exclusively in nodules, *MtN20* and another nodule-specific gene is *SUNN* (*Medtr4g070970.1*) (Pislariu et al., 2012). However, *SUNN* is a negative regulator of nodulation and its loss-of-function leads to a striking supernumerary nodulation with functional nodules (Schnabel et al., 2005). Therefore, the symbiotic effect observed in NF1858 nodules is unlikely to be due to *SUNN* loss-of-function. Unfortunately, we were unable to identify homozygous recessive plants in the segregant (T_2) population for insertions in the *MtN20* in the *Tnt1* lines NF2990, NF3070 and NF17000. A

clear approach to establish the essentiality of MtN20 for rhizobial symbiosis is still needed. The use of CRISPR/Cas9 system for loss-of-function is a viable approach to fully tackle this question.

***MtN20* expression occurs specifically in the very early stages of bacterial infection**

The early onset of *MtN20* gene expression (3 dpi - **Figure 6I**) indicates a role during the initial phases of nodule development. Transcriptomic data presented by Roux et al. (2014) showed that most of the *MtN20* expression occurs in the distal portion of zone II, where the early stages of infection occur. In the same study, similar expression patterns were noted for the pre-infection marker *MtN6* (Mathis et al., 1999), the early nodulin *MtENOD12* (Limpens et al., 2013), and EFD (Vernie et al., 2008). Interestingly, the expression of *MtENOD11*, which is an early marker for pre-infection of *Rhizobium* as well as mycorrhizae, is significantly high also in the meristem. This may indicate that MtN20 acts downstream of MtENOD11. Our 2-kb promoter activity (p*MtN20*::GUS) analysis on hairy roots (**Figure 6C**) confirmed the very restrict realm of *MtN20* gene expression in mature nodules, congruent to data published by Roux et al. (2014) (**Figure 6J**).

A gene expression analysis using mutant bacteria defective to specific symbiotic milestones can be used to help further elucidate which process must occur before a nodulin is triggered to express. Our analysis demonstrates that *MtN20* requires bacterial infection, but not bacteroid differentiation to express (**Figure 6H**). This further indicates that *MtN20* is involved in the very early stages of bacterial infection.

Figure 6. *MtN20* (Medtr7g086040) function and gene expression of roots inoculated with *Sinorhizobium meliloti* strain ABS7. (A-B) *Tnt1* insertion in *MtN20* leads to impaired nodule development: (A) 21-day old nodule of *Tnt1* mutant line NF1858 of *Medicago truncatula*. (B) 21-day old nodule of cv. R108, as a control (wild-type). (C-D) Expression profile of the *MtN20* is specifically expressed in zone II of the mature nodule sectioned in 70µm. Nodules were stained with X-Gluc: (C) 28-day old nodule expressing the *pMtN20::GUS* cassette. (D) 28-day old nodule section (non-transgenic control). (E) Location of *Tnt1* insertions in the *MtN20* gene available at the Noble Foundation mutant population. (F-G) *MtN20* expression is specific to nodules: (F) Affymetrix data from *Medicago* Gene Atlas (Benedito et al., 2008); (G) RNA-Seq data from Young et al., 2011. (H-I) *MtN20* qRT-PCR expression analysis: (H) symbiotic mutants of *Sinorhizobium meliloti* in mature nodules, *MtN20* expression requires infection, but not bacteroid differentiation or symbiotic nitrogen fixation. (I) Time course of *MtN20* gene expression in roots inoculated with *lacZ* *Sinorhizobium meliloti* strain ABS7 (qRT-PCR, own data). (J) *MtN20* is expressed in higher in distal side of zone II (RNA-Seq data from Roux et al., 2014).

The *MtN20* transcriptional network correlates with the development of the early infection zone of the nodule

If *MtN20* functions as a transcription factor during early infection, it is relevant to understand the transcriptional module to which it is related in order to better understand the mechanisms it may be involved with. A transcriptional network centered on *MtN20* was developed using 72 sequence reads archive (SRA) files from *M. truncatula* available at the NCBI database. The last annotation version of the *M. truncatula* genome was used to map the public RNA-Seq data and generate a normalized (transcript per million, TPM) expression matrix for all identified 57,585 gene models. Following, we gathered the first-degree correlation

with *MtN20* produced by Pearson Coefficient Correlation (PCC, $\rho \geq 0.85$) and then built a second-degree correlation matrix ($\rho \geq 0.90$) to produce a genetic network (Figure 7).

Figure 7. Co-expression genetic network of *MtN20*. In this network, we had our gene of interest *MtN20* in green node in the center and other genes in blue node, the node size corresponds to the level of each gene expression in *Medicago truncatula* root nodule. The correlation of each node is represented by the line between them, edge, in which the thickness is correspondent to the level of Pearson correlation between the genes.

In this network, genes directly connected to *MtN20* include: *MtDAS25* (Medtr5g081900.1), *MtN7* (Medtr3g067645.1), *MtSYMREM* (Medtr8g097320.1), the syntaxin *MtSYP132* (Medtr2g088700.1), *MtENOD20* (Medtr4g130800), *Cysteine-rich receptor-like protein kinase* (Medtr3g415610.1), *MtN21*

(Medtr3g012420.1), *Ankyrin repeat family protein* (Medtr3g016210.1), *MtGRPs* (Medtr5g084160.1), *MtEFD-1* (Medtr4g008860.1), *MtEFD-target* (Medtr2g030470.1).

MtSYMREM1 characterized as a protein of the Remorin presents interaction with at least three essentials root nodule symbiosis (RNS) receptor-like kinases (RLKs). *MtSYMREM1* has a lotus homologs (*LjSYMREM1*), that increases the nodulation when overexpressed. It is important to highlight that this gene presents changes in mapping annotation, such as Medtr8g097320 for Mt4.0v2.1, Medtr8g098650 for Mt3.5v1 and Mtr.13003.1.S1_at for *Affymetrix* probeset. *MtSYMREM1* was found related to infection process and with symbiosomes [56], In the microarray analysis of transcription reprogramming of root nodule development, (Moreau et al., 2011).

The syntaxin *MtSYP132* (Medtr2g088700.1), which is localized to the symbiosome membrane contributing to the specialization involved in infection thread development and endocytosis (Catalano et al., 2004).

The early nodulin *ENOD20* (Medtr4g130800), was induced in inoculated *lumpy infections* (*lin*) roots and present similar expression pattern (Kuppusamy et al., 2004). *ENOD20* was predicted to participate in the reorganization of the cell wall during infection thread growth and/or in the differentiation of the infected cells of the root nodules (Greene et al., 1998).

ERF transcription factor *ETHYLENE RESPONSE FACTOR REQUIRED FOR NODULE DIFFERENTIATION* (*MtEFD-1*, Medtr4g008860.1), which is a *bona fide* marker for infection zone at early steps of nodulation, been expressed between zones I and II border (Vernie et al., 2008; Limpens et al., 2013). Which seems to be involved with the *MtEFD-target* (Medtr2g030470.1).

Second-order correlations with the *MtN20* hub revealed additional genes involved in cell infection processes: *MtRR19 type A*, *LOG-like*, *MtC00553*, *MtDAS12*, *MtDAS18*, *MtDNF1* (*SPC22*), *ENOD12*, *ENOD16*, *MtIPD3*, *MtIRE*, *MtN24*, *MtN24*, *MtN25*, *MtN26*, *MtNF-YA1*(*HAP2.1*), *MtN-FYB16*, *MtNIN*, *MtRR4 type A*, *MtSPK1*, *MtTubb1*, *nodule signal peptide peptidase*, *Subtilisin-like protease SDD1* and the putative transcriptional factor *WRKY72* , *NCR140-TC40943* (Medtr4g059900.1), *NCR193-TC34601* (Medtr3g084910.1) and 5 the nodule-specific cysteine-rich peptides Medtr4g035705.1-NCR, Medtr4g088265.1-NCR, Medtr5g056710.1-NCR, Medtr5g068585.1-NCR, Medtr6g445080.1-NCR, Medtr7g028550.1-NCR, Medtr8g442760.1-NCR.

Nodule-specific cysteine-rich (NCR) peptide are large family of bacteroid-targeted plant-driven antimicrobial peptides that place essential rules on terminal bacteroid differentiation process, such as host-bacteria discrimination (Van de Velde et al., 2010; Wang et al., 2017b; Yang et al., 2017). NCR seems to be the most abundant type of symbiosis target molecules that coordinates symbiosis nitrogen fixation via plan secretory pathway. In which many other proteins are important for bacteroid differentiation such as VAMPs, DNF1, DNF2, Trx s1 and many others (Alunni and Gourion, 2016).

Overall, this transcriptional module reflects a potential function during the very early stages of rhizobial infection of the nodule cell. Interestingly, novel genes are revealed in this network, which should be functionally characterized for a full understanding of the rhizobial infection process in the developing nodule. From those we can highlight some that are highly expressed (>200TPM) in root nodules, such as Medtr1g051120.1, Medtr3g415590.1, Medtr0117s0080.1, Medtr8g012800.1, Medtr1g025830.1 and Medtr7g096690.1 within the first degree of correlation, and Medtr2g016730.1, Medtr2g014250.1, Medtr8g012800.1, Medtr2g014250.1, Medtr1g051120.1, Medtr5g036083.1, Medtr7g068290.1, Medtr5g036083.1, Medtr4g116060.1, Medtr3g101480.1, Medtr2g014250.1, Medtr7g068290.1, Medtr5g009780.1 and Medtr2g014250.1 for the second degree of correlation (**Table 1**).

GENE1	GENE1	ρ	Module (TPM)	p-value	GENE1	GENE1	ρ	Module (TPM)	p-value
Medtr7g086040.1-MIN20	Medtr2g030470.1-EFD1-target	0.84	22.8456	0	Medtr2g014250.1	Medtr1g05530.1-MIN1-YA1 (HAP2.1)	0.97	12.5099	0
Medtr7g086040.1-MIN20	Medtr7g056660.1	0.94	217.721	0	Medtr3g415590.1	Medtr7g087880.1	0.97	17.9846	0
Medtr7g086040.1-MIN20	Medtr1g058350.1	0.93	5.65266	0	Medtr3g101480.1	Medtr7g105370.1	0.97	3.506	0
Medtr7g086040.1-MIN20	Medtr7g013170.1	0.93	5.32864	0	Medtr3g110430.1	Medtr5g081900.1-MIDAS25	0.97	21.3347	0
Medtr7g086040.1-MIN20	Medtr1g010220.1	0.92	5.88425	0	Medtr5g081900.1-MIDAS25	Medtr3g110430.1	0.97	21.3761	0
Medtr7g086040.1-MIN20	Medtr1g020860.1-MREFD1	0.92	11.9728	0	Medtr7g020870.1	Medtr7g020870.1	0.97	107.749	0
Medtr7g086040.1-MIN20	Medtr5g009780.1	0.92	42.9765	0	Medtr7g020870.1	Medtr5g064980.1	0.97	2.79246	0
Medtr7g086040.1-MIN20	Medtr5g084160.1-MGRPs	0.91	7.79521	0	Medtr7g101370.1	Medtr3g101480.1	0.97	429.483	0
Medtr7g086040.1-MIN20	Medtr7g016730.1	0.9	3.506	0	Medtr8g097230.1-MSYMREM1	Medtr8g07160.1-MIN24	0.97	17.9846	0
Medtr7g086040.1-MIN20	Medtr7g010480.1	0.9	39.5314	0	Medtr1g051120.1	Medtr1g051120.1	0.96	15.9098	0
Medtr7g086040.1-MIN20	Medtr1g062770.1	0.89	92.724	0	Medtr1g051120.1	Medtr4g025743.1	0.96	9.54486	0
Medtr7g086040.1-MIN20	Medtr2g014250.1	0.89	4.51776	0	Medtr1g058350.1	Medtr5g009780.1	0.96	235.405	0
Medtr7g086040.1-MIN20	Medtr3g016230.1-Ankyrin repeat family protein	0.89	13.5991	0	Medtr2g014250.1	Medtr7g092230.1	0.96	5.32864	0
Medtr7g086040.1-MIN20	Medtr7g055370.1	0.89	153.357	0	Medtr3g016730.1	Medtr1g051120.1	0.96	536.099	0
Medtr7g086040.1-MIN20	Medtr5g00850.1	0.89	17.9846	0	Medtr3g012420.1-MIN21	Medtr3g078623.1	0.96	5.8031	0
Medtr7g086040.1-MIN20	Medtr8g012800.1	0.89	429.483	0	Medtr3g072360.1	Medtr7g105370.1	0.96	42.9765	0
Medtr7g086040.1-MIN20	Medtr00040990.1	0.88	5.32864	0	Medtr3g110430.1	Medtr7g068290.1	0.96	14.2506	0
Medtr7g086040.1-MIN20	Medtr1g051120.1	0.88	652.945	0	Medtr3g115610.1-Cysteine-rich receptor-like protein kinase	Medtr3g115620.1	0.96	5.32864	0
Medtr7g086040.1-MIN20	Medtr1g051120.2	0.88	2.79246	0	Medtr4g008860.1-MREFD1	Medtr7g096690.1	0.96	5.32864	0
Medtr7g086040.1-MIN20	Medtr3g012420.1-MIN21	0.88	66.642	0	Medtr5g009780.1	Medtr1g058350.1	0.96	6.46929	0
Medtr7g086040.1-MIN20	Medtr7g02360.1	0.88	61.4137	0	Medtr3g036083.1	Medtr7g031370.1	0.96	11.749	0
Medtr7g086040.1-MIN20	Medtr3g077000.1	0.88	95.0174	0	Medtr5g081900.1-MIDAS25	Medtr7g068290.1	0.96	451.347	0
Medtr7g086040.1-MIN20	Medtr3g415590.1	0.88	536.099	0	Medtr5g464480.1	Medtr4g071400.1	0.96	95.0174	0
Medtr7g086040.1-MIN20	Medtr4g116060.1	0.88	39.5314	0	Medtr7g11170.1	Medtr5g036083.1	0.96	451.347	0
Medtr7g086040.1-MIN20	Medtr7g055980.1	0.88	5.65266	0	Medtr7g068290.1	Medtr3g110430.1	0.96	25.1159	0
Medtr7g086040.1-MIN20	Medtr7g064290.1	0.88	89.8674	0	Medtr7g068290.1	Medtr5g081900.1-MIDAS25	0.96	2.79246	0
Medtr7g086040.1-MIN20	Medtr0117s0080.1	0.87	451.347	0	Medtr7g092230.1	Medtr2g014250.1	0.96	429.483	0
Medtr7g086040.1-MIN20	Medtr1g042900.1	0.87	21.7761	0	Medtr7g096690.1	Medtr4g008860.1-MREFD1	0.96	17.9846	0
Medtr7g086040.1-MIN20	Medtr7g02360.1	0.87	22.7041	0	Medtr7g105370.1	Medtr7g072360.1	0.96	9.2374	0
Medtr7g086040.1-MIN20	Medtr7g089815.1	0.87	15.9598	0	Medtr00030590.1	Medtr2g014250.1	0.95	235.405	0
Medtr7g086040.1-MIN20	Medtr3g415610.1-Cysteine-rich receptor-like protein kinase	0.87	107.749	0	Medtr00030590.1	Medtr3g101480.1	0.95	6.3476	0
Medtr7g086040.1-MIN20	Medtr5g464480.1	0.87	63.4768	0	Medtr00030590.1	Medtr7g105370.1	0.95	91.2789	0
Medtr7g086040.1-MIN20	Medtr3g020240.2	0.86	4.75499	0	Medtr1g010220.1	Medtr2g016730.1	0.95	6000.15	0
Medtr7g086040.1-MIN20	Medtr3g101480.1	0.86	16.1319	0	Medtr1g051120.1	Medtr3g115620.1	0.95	39.5314	0
Medtr7g086040.1-MIN20	Medtr3g110430.1	0.86	3.61438	0	Medtr1g058350.1	Medtr2g030470.1-EFD1-target	0.95	61.4137	0
Medtr7g086040.1-MIN20	Medtr4g130900.1-MENOC20	0.86	6.46929	0	Medtr2g014250.1	Medtr00040990.1	0.95	6.46929	0
Medtr7g086040.1-MIN20	Medtr1g010480.1	0.86	6.46929	0	Medtr2g014250.1	Medtr2g016730.1	0.95	6.26871	0
Medtr7g086040.1-MIN20	Medtr1g010480.1	0.86	6.46929	0	Medtr2g014250.1	Medtr7g105370.1	0.95	39.5314	0
Medtr7g086040.1-MIN20	Medtr7g092230.1	0.86	36.0218	0	Medtr2g016730.1	Medtr1g010220.1	0.95	1.88425	0
Medtr7g086040.1-MIN20	Medtr1g010480.1	0.85	235.405	0	Medtr2g016730.1	Medtr2g014250.1	0.95	652.945	0
Medtr7g086040.1-MIN20	Medtr7g079870.1	0.85	10.528	0	Medtr2g020240.2	Medtr2g079760.1	0.95	95.0174	0
Medtr7g086040.1-MIN20	Medtr2g088700.1-MSYM132	0.85	22.2412	0	Medtr2g030470.1-EFD1-target	Medtr1g058350.1	0.95	95.0174	0
Medtr7g086040.1-MIN20	Medtr2g102340.1	0.85	4.81554	0	Medtr2g030470.1-EFD1-target	Medtr7g031370.1	0.95	5.88425	0
Medtr7g086040.1-MIN20	Medtr7g055270.1	0.85	29.0893	0	Medtr2g079870.1	Medtr2g020240.2	0.95	2.65387	0
Medtr7g086040.1-MIN20	Medtr3g057645.1-MIN7	0.85	16.5225	0	Medtr0088700.1-MSYM132	Medtr2g103520.1-MIDAS18	0.95	122.139	0
Medtr7g086040.1-MIN20	Medtr5g081900.1-MIDAS25	0.85	45.4931	0	Medtr3g012420.1-MIN21	Medtr4g130780.1-MENOC20	0.95	38.2819	0
Medtr7g086040.1-MIN20	Medtr7g020870.1	0.85	5.65266	0	Medtr3g012420.1-MIN21	Medtr5g036083.1	0.95	63.4768	0
Medtr7g086040.1-MIN20	Medtr7g055123.1	0.85	9.98254	0	Medtr3g012420.1-MIN21	Medtr7g020870.1	0.95	100.748	0
Medtr7g086040.1-MIN20	Medtr8g012805.1	0.85	5.88425	0	Medtr3g012420.1-MIN21	Medtr7g068290.1	0.95	26.3284	0
Medtr7g086040.1-MIN20	Medtr8g097230.1-MSYMREM1	0.85	13.0974	0	Medtr3g012420.1-MIN21	Medtr7g092230.1	0.95	33.1558	0
					Medtr3g012420.1-MIN21	Medtr7g114880.1-MIN26	0.95	29.144	0
					Medtr3g11210.1-Ankyrin repeat family protein	Medtr7g096690.1	0.95	17.9846	0
					Medtr3g072360.1	Medtr7g078770.1	0.95	4.58628	0
					Medtr3g101480.1	Medtr7g096690.1	0.95	36.3346	0
					Medtr3g110430.1	Medtr00040990.1	0.95	18.2352	0
					Medtr3g110430.1	Medtr2g103520.1-MIDAS18	0.95	2.55613	0
					Medtr3g110430.1	Medtr7g114880.1-MIN26	0.95	3.506	0
					Medtr4g116060.1	Medtr8g012800.1	0.95	652.945	0
					Medtr4g130900.1-MENOC20	Medtr5g009780.1	0.95	61.4137	0
					Medtr4g130900.1-MENOC20	Medtr4g130900.1-MENOC20	0.95	2.79246	0
					Medtr5g036083.1	Medtr3g012420.1-MIN21	0.95	14.206	0
					Medtr5g036083.1	Medtr7g068290.1	0.95	4.51776	0
					Medtr5g08087.1	Medtr8g013660.1	0.95	91.2789	0
					Medtr5g081900.1-MIDAS25	Medtr2g103520.1-MIDAS18	0.95	11.7466	0
					Medtr5g081900.1-MIDAS25	Medtr6g007160.1-MIN24	0.95	107.749	0
					Medtr5g081900.1-MIDAS25	Medtr7g114880.1-MIN26	0.95	9.618	0
					Medtr5g081900.1-MGRPs	Medtr7g013170.1	0.95	5.32864	0
					Medtr7g013170.1	Medtr2g030470.1-EFD1-target	0.95	6.968254	0
					Medtr7g013170.1	Medtr5g084160.1-MGRPs	0.95	13.0974	0
					Medtr7g020870.1	Medtr1g079730.1	0.95	6.46929	0
					Medtr7g020870.1	Medtr2g008860.1	0.95	92.724	0
					Medtr7g020870.1	Medtr2g020870.1	0.95	2.65387	0
					Medtr7g020870.1	Medtr5g099060.1-MIN1	0.95	12.5099	0
					Medtr7g020870.1	Medtr6g007160.1-MIN24	0.95	6.58935	0
					Medtr7g068290.1	Medtr7g020870.1	0.95	901.623	0
					Medtr7g068290.1	Medtr3g012420.1-MIN21	0.95	1.38792	0
					Medtr7g068290.1	Medtr5g036083.1	0.95	536.099	0
					Medtr7g068290.1	Medtr7g068290.1	0.95	2.79246	0
					Medtr7g068290.1	Medtr7g114880.1-MIN26	0.95	6.53166	0
					Medtr7g092230.1	Medtr3g05530.1-MIN1-YA1 (HAP2.1)	0.95	0.509304	0
					Medtr7g092230.1	Medtr3g012420.1-MIN21	0.95	48.103	0
					Medtr7g092230.1	Medtr3g078770.1	0.95	2.65387	0
					Medtr7g092230.1	Medtr7g021230.1	0.95	21.7761	0
					Medtr7g096690.1	Medtr3g01610.1-Ankyrin repeat family protein	0.95	5.32864	0
					Medtr7g096690.1	Medtr3g072360.1	0.95	33.9727	0
					Medtr7g096690.1	Medtr7g105370.1	0.95	61.4768	0
					Medtr7g105370.1	Medtr00030590.1	0.95	11.749	0
					Medtr7g105370.1	Medtr2g014250.1	0.95	652.945	0
					Medtr7g105370.1	Medtr7g096690.1	0.95	1.02312	0
					Medtr8g012800.1	Medtr4g116060.1	0.95	451.347	0
					Medtr8g097230.1-MSYMREM1	Medtr5g064980.1	0.95	89.5314	0
					Medtr8g097230.1-MSYMREM1	Medtr5g064980.1	0.95	5.88425	0

Table 1. First and second degree of correlated expression genes with *MtN20/MtGRF1*. The first degree of correlated genes with $\rho > 0.95$ and the second degree of correlation the $\rho > 0.95$ correlated genes, follows by their expression level in root nodule (TPM), in which we ordered the table by *MtN20/MtGRF1* in the analysis. In which the gene annotation are separated with “-“ following the gene mapping.

Identifying *cis*-elements in the *MtN20* promoter

A reasonable question raised by the very specific transcriptional domain of *MtN20* in the nodule is which *cis*-elements in the promoter (and which transcription factors) are responsible for this transcriptional activity during rhizobial infection. In order to start looking into this question, we identified common plant *cis*-elements using the PLACE tool (<http://www.dna.affrc.go.jp/htdocs/PLACE>; Higo et al., 1999) in the 2011bp regions upstream the *MtN20* start codon (**Figure 8**).

For the *MtN20*, several *cis*-elements are present in multiple copies (Higo et al., 1999): DOFCOREZM (18 times), GATABOX (17), CACTFTPPCA1 (15), ROOTMOTIFTAPOX1 (12), CAATBOX (12), IBOXCORE (10), GT1CONSENSUS (8), ARRIAT (8), OSE2ROOTNODULE (6), NODCON2GM (6), MYCCONSUSAT (6), EBOXNNAPA (6), and several others with lower representation (**Figure 7**). DOFCOREZM was identified as a binding site of Dof transcription factors in maize (Wang et al., 2017a). GATABOX is highly conserved *cis*-element for GATA transcription factors. The conserved sequence CACTFTPPCA1 was found in the promoter of PEP carboxylase gene of the C4 plant, *Flaveria trinervia* (Gowik et al., 2004), that also had been characterized in Arabidopsis male meiocytes (Li et al., 2014). Interestingly, ROOTMOTIFTAPOX1 is found in *rolD* promoters of *Agrobacterium rhizogenes*. CAATBOX relates to the CAAT box, which is a heat shock element (HSE) characterized as a synergistic element to induce expression during stresses (Rieping and Schöffl, 1992). Unexpectedly, IBOXCORE refers to the highly conserved domain (I-box) previously described as induced by light (Terzaghi and Cashmore, 1995). The potential contribution of each of these elements to the tissue specificity of *MtN20* should be assessed to better characterize the molecular mechanisms involved with the transcriptional activity of this gene during nodule development.

CONCLUSIONS AND PERSPECTIVES

MtN20 is a novel gene potentially involved in rhizobial infection of nodule cells. It belongs to a largely uncharacterized family of putative transcription factors. *MtN20* belongs to a regulatory gene network encompassing bona fide symbiotic genes, such as EFD, the EFD target Nod19, NIN and several nodule-specific cysteine-rich (NCR) peptides. Several overrepresented *cis*-elements were identified in the *MtN20* gene promoter, with potential implications to understand its tissue specificity.

Figure 9. *Cis*-regulatory elements identified in promoter region of *MtN20* (Medtr7g086040.1). A 2008bp region upstream the transcriptional start site (TSS) was analyzed in PLACE (Higo et al., 1999). *Cis*-elements are highlighted and names are provided underneath. For a complete list of elements found in this promoter region.

The second gene of interest, *MtGRF2*, described in the chapter 4 of this dissertation, and detailed explored in the next chapter 4, *MtN20* and *MtGRF2* present distinguished expression pattern conferred by each promoter element. A comparative table was generated, in which we pointed out the number of copies of differential promoter element between both genes of interest in a 2008bp upstream region (**Chapter 4 - Table 2**).

MATERIAL AND METHODS

Plant growth conditions: The plants were grown in 2:1 turface:vermiculite medium in 4" Azalea pots under 16:8-h (light:dark) photoperiod and 24°C:22°C (day:night) temperature conditions in a Percival growth chamber. Plants received 50 mL 1X B&D (Broughton and Dilworth, 1971) fertilizer solution with low nitrogen (0.5mM ammonium nitrate) every other day except at day of inoculation with rhizobium.

Cloning of *MtN20* promoter: A 1968bp genomic region upstream the MtGRF1/MtN20 (Medtr7g086040.1) start codon (coordinates chr7:33359964..33361035 of the *M. truncatula* genome assembly v.4.0) was cloned into a binary vector to express the *GUS* reporter gene. Medicago genomic DNA (ecotype ‘A17’) was extracted from young leafs using the IDT Plant DNA Extraction Protocol (www.idtdna.com/pages/docs/educational-resources/plant-dna-extraction-protocol.pdf?sfvrsn=5). PCR was carried out with the high-fidelity KOD Extreme Hot-Start DNA polymerase (Toyobo, catalog #71086) in an Mastercycler ep Gradient S Thermal Cycler (Eppendorf). The amplicon was run on 1% agarose gel, purified and further cloned into the directional pENTR-D/ TOPO vector (ThermoFisher), followed by Gateway LR reaction, using Gateway LR Clonase II Enzyme Mix (ThermoFisher) to recombine it into the the destination vector (pKGWFS7). and finally incorporate into the All final cassettes were confirmed by Sanger sequencing at the WVU Genomics Core Facility. Plasmids were used to transform *Agrobacterium rhizogenes* strain ‘ARqual’ for *ex-vitro* hairy-root transformation. All primer sequences used in this dissertation are provided in **Annex I**.

***Ex-vitro* hairy-root transformation:** A slightly modified version of the freeze-thaw method for *Agrobacterium* transformation protocol (openwetware.org/wiki/Maloof_Lab:Agro_Transformation) was used for *Agrobacterium rhizogenes* strain ‘ARqual’. *Ex-vitro* hairy root transformation of *M. truncatula* ecotype “A17” was performed as described by Boisson-Dernier et al. (2006). In brief, roots of four-day old seedlings were diagonally cut out and the wound was inoculated with a three day-old plate cultivated with ARqual transformed with the final destination on solid LB medium containing the respective selective antibiotic (100mg/L spectinomycin plus 100mg/L streptomycin). The seedlings were then grown on modified Fahraeus medium (pH 7.5) with 15g/L agar in tissue culture tubes for 2 weeks under 16:8-h light:dark photoperiod regimen at 24°C:22°C day:night temperature before transplanting to a turface:vermiculite (2:1 ratio) under the same growth conditions. 0.5mM were provided ½-strength B&D fertilizer solution (50mL) every other day 7 days before rhizobial inoculation. When applicable, transgenic roots and nodules were visualized under fluorecence stereoscope via expression of the fluorescent marker DsRed (excitation/emission ~580/605 nm).

Rhizobial inoculation: Unless specified otherwise, roots of one week-old plants were inoculated with 50 mL suspension ($OD_{600}=0.05$) of *Sinorhizobium meliloti* rhizobial strain *Sm1021* (*Sm1021-lacZ*) harboring the *hemA::LacZ* cassette in 1X B&D solution without nitrogen to induce nodulation. One inoculation loop of *Sinorhizobium meliloti* rhizobial strain *Sm1021* from glycerol stock was grown for three days in 3 mL YM medium supplemented with 6mM $CaCl_2$ and proper antibiotic, according to the destination vector (e.g., 10 mg/L Tetracycline or 100 mg/L Streptomycin). Sixteen hours before rhizobial inoculation, the 1-mL

inoculum was regrown in 200mL flask until OD₆₀₀~1.0. The suspension was centrifuged at 10,000g for 10 minutes, the pellet washed by resuspension and centrifugation, and the clean pellet was then resuspended and diluted with 1X no-N B&D solution without nitrogen to an OD₆₀₀ of 0.05. Fifty milliliters of the rhizobial suspension were used to inoculate each plant.

Histochemical assay (tissue fixation and GUS staining): Sections were cut by hand from unfixed stems of plants grown in vitro, essentially as described (Jefferson et al., 1987) and fixed in 0.3% formaldehyde in 10 mM MES, pH 5.6, 0.3 M D-mannitol for 45 min at room temperature, followed by several washes in 50 mM NaH₂PO₄, pH 7.0. All fixatives and substrate solution were introduced into sections with a brief (1 min) vacuum infiltration. Histochemical reactions with X-Gluc were performed with 1 mM substrate in 50 mM NaH₂PO₄, pH 7.0 at 37°C for 3 hours and 16 hours. After staining, sections were rinsed in 70% ethanol for 5 min, and then mounted for microscopy (Jefferson et al., 1987). For anatomical analyses, transformed nodules at 28 dpi were embedded in 5% low-melting agarose and sectioned on a vibratome at 70µm thickness. Those sections were extracted from agarose and kept in 1.5mL tube with NaH₂PO₄, pH 7.0, to be observed under light microscopy (Nikon Eclipse E600 microscope coupled with a Nikon DS-Ri1 camera).

Gene expression profile: Gene expression profile analyses of *MtN20/MtGRF1* in nodulating roots inoculated with rhizobial mutants were performed in ecotype “A17” plants inoculated with *fixJ*, *bacA*, *exoA*, or *hemA::LacZ* strains of *Sinorhizorium meliloti* in the *Sm1021* background. For gene expression analyses via qRT-PCR, root samples not inoculated with rhizobia provided full nitrogen fertilization (6 mM N provided as KNO₃ and NH₄NO₃) were used as control. Whole root systems were harvested at 0, 3, 14, 21, and 28 dpi. RNA were extracted with mirVana mRNA Isolation kit (Life Technologies) followed by DNA removal with Turbo DNA-free kit (Applied Biosystems). First strand cDNA was synthesized with oligo-dT priming using the SuperScript III First-Strand Synthesis Super mix (ThermoFisher), following the manual instructions. Primers are described in cf. **Suppl. Table 1**.

Three biological and three technical replicates were run for qRT-PCR analyses. SYBR green master mix (Applied Biosystems) we used for 5-µL reactions on ABI7500 Real Time qPCR System (Applied Biosystems). In which was made two MasterMix one with SyBR green (2.5µL) and cDNA (1µL 1:10) and another between with primers (2x0.5µL 20mM) and water (0.5µL). All templates were amplified using the same cycling conditions: 50°C for 2 minutes; 95°C for 10 minute; 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Melt curves were produced following the same protocol in 10-fold serial dilutions of cDNA (1:10 to 1:100,000). Ct (threshold cycle) values were exported to Excel to organize data and comparative analysis of gene expression.

The symbiotic marker genes used: *NCR2A* (Medtr2g042480, marker of bacteroid differentiation: Moreau et al., 2011), *LEG* (Medtr5g081000, marker of nitrogen fixation: Vinardell, 2003), *Sennex2* (Medtr7g104360, an uncharacterized gene expressed specifically in the senescence zone: cf. Roux et al., 2014), *LYK3* (Medtr5g086130, marker of root hair curling: Smit et al., 2007), the early nodulin *ENOD11* (Medtr3g415670, marker of pre-infection and infection: Svistoonoff et al., 2010), and *HAP2.1* (Medtr1g05653, which expresses exclusively in the nodule meristem: Combier et al., 2006; Combier et al., 2008). The housekeeping genes used for RT-qPCR were: *EF1α* (Medtr6g021800) and *Tub1* (Medtr7g089120). LinRegPCR was used to extract PCR efficiencies for each primer pair (REF). The software NormFinder (Andersen, 2004) was used to identify stably-expressed housekeeping genes for the experiment. Relative expression was calculated according to the Pfaffl method (Pfaffl, 2001). All primers are described in **Annex 1**.

Light microscopy: At 28 days post-inoculation with *Sm1021-LacZ*, nodules were harvested and fixed under vacuum with 2.5% glutaraldehyde in 0.1 M PIPES (pH 7.2) for one hour at room temperature, followed by two rinses with 0.1 M PIPES (pH 7.2). When applicable, samples were stained in fresh staining solution containing 50mM potassium ferricyanide, 50mM potassium ferrocyanide, 0.08% X-Gal (0.08mg/mL) or 0.08% Red-Gal (0.08mg/mL) and 0.1M PIPES (pH 7.2) for 16 hours at room temperature (Boivin et al., 2016), followed by two rinses with 0.1M PIPES (pH 7.2), and stored at 70% ethanol until processing. After fixation (and staining), tissues were cleared with 2.4% sodium hypochlorite for 5 min and dehydrated with an ethanol series (50%, 70%, 90%, 95%, and 100% twice for 45 minutes each). Samples were then transferred to 100% xylene for 15 minutes or until translucent. The completely dehydrated samples were embedded in fresh 60°C xylene:paraplast (1:1) mix for two hours at 60°C and transferred to melted (60°C) 100% paraplast and incubated at 60°C for at least three hours. Each sample were blocked and cut in 7µm section with a conventional microtome. The slices were placed on glass slides and incubated overnight at 37°C. On the next day, the slides were washed twice with 100% xylene for 3 minutes and twice with 100% ethanol, followed by the hydration process using submersion in 95% ethanol and then water for 5 minutes each. The samples were then ready to accept water-based dyes. The slides were observed under bright field microscope (Nikon Eclipse E600 microscope coupled with a Nikon DS-Ri1 camera). Often, X-gal staining was used to visualize *lacZ* bacteria and the tissue was counterstained with chloramphenicol to visualize the lignin in the plant cell wall.

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CHAPTER 4

MtGRF2 IS A GRF-TYPE ZINC FINGER NODULIN FROM *MEDICAGO TRUNCATULA* POTENTIALLY INVOLVED IN BACTEROID DIFFERENTIATION

ABSTRACT

Nodule specific proteins, called nodulins, are involved in the coordination of nodule formation and symbiotic nitrogen fixation in legumes. In addition to MtN20/MtGRF1, a second GRF-type zinc finger nodulin (MtGRF2, Medtr1g064350.1) is highly and specifically expressed in interzone of *Medicago truncatula* nodules. During nodule development, nodule cells are colonized by *Rhizobium* bacteria, which differentiate into a symbiotic form specialized to fix nitrogen: the bacteroid. Bacteroids go through five distinct steps of differentiation (types 1-5) and each stage is present sequentially in the interzone of *Medicago truncatula* indeterminate nodules. Based on its very discrete gene expression pattern in the interzone and regulatory gene network, MtGRF2 is potentially involved in late bacteroid differentiation. Three Ser residues were experimentally verified as phosphorylated in the amino terminus of the protein, for which the biological relevance and interacting kinase need to be determined. We also identified a putative nuclear localization signal at the carboxyl terminus of the protein, suggesting a potential function as a transcription factor. A comparative analysis of *cis*-elements presents in *MtN20/MtGRF1* and *MtGRF2* gene promoters revealed overrepresented shared and unique sequences between that may account for the unique spatial-temporal patterns of expression of each GRF-type zinc finger nodulin.

MTGRF2 IS A GRF-TYPE ZINC FINGER NODULIN FROM *MEDICAGO TRUNCATULA* POTENTIALLY INVOLVED IN BACTEROID DIFFERENTIATION

INTRODUCTION

Distinctly from the processes of legume-*Rhizobium* recognition and cell infection, the plant mechanisms and genes involved in controlling bacteroid maturation largely remain a “black box”. In legumes, many species belonging to the inverted repeat-lacking clade (IRLC), such as *Medicago truncatula*, impose terminal bacteroid differentiation (Czernic et al., 2015). Nodule-specific cysteine-rich (NCR) peptides have been implicated in this process (Mortier et al., 2010; Van de Velde et al., 2010; Farkas et al., 2014; Pan and Wang, 2017).

During the rhizobial infection in the nodule cell via endocytosis, they become enclosed within the confines of a semi-organelle called the symbiosome, which is derived from the plant plasma membrane during endocytosis of the infecting bacteria. Each infected nodule cell can hold several thousands of symbiosomes (Kereszt et al., 2011). The rhizobia undergo through an exceptional differentiation process from the free-living, non-nitrogen fixing organism to turn into an auxotrophic endocellular microsymbiont capable of mutualistically fixing nitrogen, called the bacteroid (Jones et al., 2007). Plant peptides coordinate and elicit transcriptional response controlling the bacteroid differentiation (Ferguson et al., 2002; Van de Velde et al., 2010; Bourcy et al., 2013; Berrabah et al., 2015; de Bang et al., 2017). In indeterminate nodules, bacteroid differentiation occurs in the interzone II-III (Emerich and Krishnan, 2014; Maróti and Kondorosi, 2014; Penterman et al., 2014). At first, the bacteria may or may not reproduce to generate a few cells in each symbiosome (Maróti and Kondorosi, 2014). In *M. truncatula*, however, bacteria do not reproduce and each symbiosome holds a single bacteroid. The bacteroid differentiation process starts with NCRs peptides, SymCRK and RSD preventing defense mechanisms, receiving differentiation inducement of plant genes, until the nitrogenase will be synthesized, culminating in a mature, fully functional bacteroid capable to fix nitrogen (Penterman et al., 2014; Berrabah et al., 2015). One important stage of symbiosome differentiation occurs at nodule Interzone (IZ or ZII-ZIII), which is characterized by infected cells showing starch accumulation in plastids, and type-3 bacteroid with cytoplasmic heterogeneity and DNA endoreduplication, as explored in **Chapter 1** (Vasse et al., 1990).

Very few legume genes have been specifically characterized in the process of bacteroid differentiation. The phosphatidylinositol phospholipase C-like (PI-PLC-XD)-containing protein DOES NOT FIX NITROGEN 2 (DNF2) is required for bacteroid persistence (Bourcy et al., 2013; Wang et al., 2017). The C2H2 zinc finger transcription factor REGULATOR OF SYMBIOSOME DIFFERENTIATION (RSD) regulates the early stages of symbiosome development (Sinharoy et al., 2013).

Additionally, SymCRK and DNF2 are required in infected cells to prevent defense-like reactions in nodules after bacterial infection (Berrabah et al., 2015). Recently, the Lotus ASPARTIC PEPTIDASE NODULE-INDUCED 1 (LjAPN1) was characterized as involved in bacteroid persistence (Yamaya-Ito et al., 2017). Bacteroid terminal differentiation requires the interaction between MtTRXS1 with NCR247 and NCR335. This interaction allows MtTRXS1 to activate those NCR247 and NCR335, in order the plant to regulates the bacteroid differentiation (Ribeiro et al., 2017). Given the complexity of the processes occurring in the interzone, it is reasonable to expect that a much larger number of genes are specifically involved and await functional characterization.

Herein, we present a novel putative GRF-type zinc finger transcription factor expressed specifically in the interzone of the indeterminate nodule of *M. truncatula*. Among the 52 genes coding for GRF-type zinc finger proteins in the *M. truncatula* genome, only two were expressed specifically in nodules (**Chapter 2**). The Medicago nodulin 20 (MtN20/MtGRF1) is introduced in **Chapter 3**. We anticipate MtGRF2 is a hub transcription factor controlling the expression of key genes involved in the process of bacteroid differentiation in nodule cells.

RESULTS AND DISCUSSION

Moreover, the Medicago proteome compendium (Marx et al., 2016) of the six major organs of the mature plant and three late time-points during nodules development confirms the presence of the MtGRF2 protein found specifically in nodules (**Figure 1**).

Figure 1. MtGRF2 quantification retrieved from the *Medicago truncatula* Compendium proteome (Marx et al., 2016). The MtGRF2 protein is present in nodules samples. The x-axis is presented as log₂ of the relative protein abundance (TMT), in which the values are normalized against the average of all samples. Note that the quantification is based on protein fragments (small peptides), which sequences may be shared between different proteins in some instances.

Moreover, phosphorylation sites were detected in Ser residues in the positions 10, 14 and 19 (**Figure 2**). The significance of these phosphorylations and the kinase(s) involved in the process are still to be determined. Next, using the NucPred tool (Brameier et al., 2007) we identified a putative nuclear localization signal (NLS) in the very carboxy-terminus of the MtGRF2 protein (**Figure 2**).

MKGVVRFETSP[•]**LL**[•]**SKREASWNGSVSNVD**[•]**TYESAAPTCKCGKKCILYISKTSKNPN****THEYRCPYEK**
QQRPHCN**YFVQKDKFIESQTTMVDLKS****TTMVELLEAKINQLEKDVKIMKSKIEDDIEVKINQLE**
KDMEVKINQFERDIEVIKTQIEMQVKMEQDKNWKRCVRAVG**VVIVVWLYPFV****GSRKRLSK**

Figure 2. The MtGRF2 protein sequence was retrieved from *Medicago truncatula* Proteome Compendium (Marx et al., 2016). Three serine phosphorylated were noticed on positions 10, 14 and 19 (shown with red tags atop). The GRF-type zinc finger protein domain is denoted with red underline. The putative nuclear localization signal (NLS) as estimated by the SBC-NucPred tool is denoted with a green box (Brameier et al., 2007). The actual amino acid residues covered in the proteome sequencing project are shown in bold.

The conserved domains of a protein are usually encoded within the confines of a single exon (Blaker, 1985; Doolittle, 1985; Sibley et al., 2016). Interestingly, the gene architecture of *MtGRF2* is quite unusual in the sense that it is split between its only two exons (**Figure 3**). The same observation was also noticed for *MtGRF1* (**Chapter 3**).

Figure 3. Gene structure of Medtr1g064350 (*MtGRF2*). The centromere position of chromosome 1 of *Medicago truncatula* is represented by a black circle. The transcription direction of genes in the vicinity of *MtGRF2* are indicated by arrows. The exons of *MtGRF2* are represented by boxes, and the intron and extragenic regions, by lines. The sequence coding for the GRF-type zinc finger domain (IPR010666) is represented in black.

Nodules of hairy roots generated with a construct expressing *GUS* expression driven by a 2,062-bp promoter region of *MtGRF2* confirmed the expression domain clearly in the interzone of the mature nodule, although some expression was also noticed at the basis of the nodule, close to the root (**Figure 4A-C**).

MtGRF2 expression is negligible at 3-dpi roots but very high at 10-dpi nodulating root systems (**Figure 4F**). Mutant rhizobial strains impaired in specific milestones of symbiotic establishment showed that bacteroid differentiation, but not nitrogen fixation *per se*, is needed for *MtGRF2* expression, although a leaky expression level was observed in the *bacA* mutant (**Figure 4G**). The RNA-Seq laser capture microdissection dataset of five distinct zones of the mature nodule (Roux et al., 2014) shows *MtGRF2* with maximum expression in the interzone (IZ, a.k.a. zone II-III). The RNA-Seq LCM dataset (Roux et al., 2014) shows *MtGRF2* expressed most strongly in the interzone (**Figure 4H**). Altogether, the level and spatio-temporal transcriptional activity of *MtGRF2* indicates a role during bacteroid differentiation.

Figure 4. *MtGRF2* (Medtr1g064350) gene expression in nodules and roots inoculated with *Sinorhizobium meliloti*. (A-C) *pMtGRF2::GUS* expression profile of root nodules stained with X-Gluc. (A) 28-day old whole, wild-type nodule (non-transgenic control). (B) 28-day old whole nodule expressing the *pGRF2::GUS* cassette. (C) A 70- μ m section of the nodule shown in B. *MtGRF2* expression is localized to the interzone (IZ) or zone II-III of the mature nodule, as well as the basis of the nodule close to the root. (D-E) *MtGRF2* expression is specific to nodules. (D) Affymetrix data from the *Medicago* Gene Atlas (Benedito et al., 2008). (E) RNA-Seq dataset from Young et al. (2011). (F-G) *MtGRF2* qRT-PCR expression analysis of nodulating roots. (F) Time-course of *MtGRF2* gene expression in roots inoculated with *Sinorhizobium meliloti* strain ABS7 expressing *lacZ* (ABS7-*lacZ*). (G) *MtGRF2* gene expression in 28-dpi nodulating roots inoculated with symbiotic mutants of *Sinorhizobium meliloti* (*Sm1021-lacZ*). *MtGRF2* expression requires bacterial differentiation, but not symbiotic nitrogen fixation *per se*. (H) *MtGRF2* is expressed highest in the interzone (IZ or zone II-II) of the mature nodule (data extracted from Roux et al., 2014).

Therefore, *MtGRF2* is a nodule-specific gene expressed after *Rhizobium* infection and before the nitrogen fixation during bacteroid differentiation in the nodule interzone. This indicates that MtGRF2 may play an essential role during the bacteroid differentiation for normal symbiotic nitrogen fixation in root nodules of *M. truncatula*.

Genetic Network Analysis

In order to understand the relationship between MtGRF2 and other genes during nodule development, we developed a correlation genetic network using a selection of 72 SRAs from *M. truncatula* to generate an expression matrix for all 57,585 identified genes. We gathered the significant first- and second-degree Pearson correlations ($\rho \geq 0.90$) to build the correlation genetic network (**Figure 5**). For better perusal of the data, **Table 1** shows the first-degree correlations ($\rho \geq 0.90$) between the expression of MtGRF2 and other genes, along with second-degree correlations ($\rho \geq 0.95$).

Gene1	Gene2	ρ	Nodule(TPM)	p.value
Medtr1g064350.1	Medtr5g063600.1-NCR88-TC38212	0.95	560.411	0
Medtr1g064350.1	Medtr6g090485.1-NCR219-TC3396	0.94	316.068	0
Medtr1g064350.1	Medtr7g056460.1	0.94	10.3232	0
Medtr1g064350.1	Medtr1g068630.1	0.93	12.7759	0
Medtr1g064350.1	Medtr5g043930.1	0.93	1.69487	0
Medtr1g064350.1	Medtr8g069250.1	0.93	23.3169	0
Medtr1g064350.1	Medtr2g048900.1	0.92	43.8192	0
Medtr1g064350.1	Medtr2g090605.1	0.92	14.206	0
Medtr1g064350.1	Medtr1g090820.3	0.91	3359.16	0
Medtr1g064350.1	Medtr2g073170.1-NCR	0.91	540.612	0
Medtr1g064350.1	Medtr3g460850.1	0.91	6.78597	0
Medtr1g064350.1	Medtr4g063735.1-WOX2	0.91	23.0564	0
Medtr1g064350.1	Medtr6g066040.1	0.91	26.186	0
Medtr1g064350.1	Medtr7g078060.2	0.91	9.04986	0
Medtr1g064350.1	Medtr7g088470.1	0.91	9.2261	0
Medtr1g064350.1	Medtr8g012680.1	0.91	95.0776	0
Medtr1g064350.1	Medtr2g073670.1-NCR	0.9	59.2457	0
Medtr1g064350.1	Medtr4g019870.1	0.9	201.425	0
Medtr1g064350.1	Medtr5g084260.1	0.9	1982.68	0
Medtr1g064350.1	Medtr6g445080.1-NCR	0.9	8000.15	0
Medtr1g064350.1	Medtr6g066090.1	0.9	33.8277	0
Medtr1g064350.1	Medtr8g097320.1-MtSYMREM1	0.9	183.723	0
Medtr1g064350.1	Medtr8g103233.1	0.9	26.5023	0
Medtr8g097320.1-MtSYMREM1	Medtr8g097320.2-MtSYMREM1	0.98	19.0302	0
Medtr1g068630.1	Medtr2g090605.1	0.97	14.206	0
Medtr2g090605.1	Medtr1g068630.1	0.97	12.7759	0
Medtr8g097320.1-MtSYMREM1	Medtr6g007160.1-MtN24	0.97	235.405	0
Medtr1g090820.3	Medtr2g103303.1	0.96	631.826	0
Medtr5g084260.1	Medtr2g103307.1	0.96	547.189	0
Medtr5g084260.1	Medtr4g013270.1	0.96	204.653	0
Medtr6g066040.1	Medtr2g007210.1	0.96	73.5612	0
Medtr6g066090.1	Medtr3g465470.1	0.96	22.7632	0
Medtr6g066090.1	Medtr4g060590.1-NCR	0.96	288.245	0
Medtr6g445080.1-NCR	Medtr1g079730.1	0.96	18.7348	0
Medtr6g445080.1-NCR	Medtr5g064980.1	0.96	91.2789	0
Medtr1g090820.3	Medtr4g063735.1-WOX2	0.95	23.0564	0
Medtr1g090820.3	Medtr4g094338.1	0.95	702.329	0
Medtr1g090820.3	Medtr5g084260.1	0.95	1982.68	0
Medtr1g090820.3	Medtr6g464450.1-NCR	0.95	272.111	0
Medtr2g073670.1-NCR	Medtr4g038665.1-NCR	0.95	71.318	0
Medtr4g063735.1-WOX2	Medtr1g090820.3	0.95	3359.16	0

Table 1. First- and second-correlation degree of co-expression genes with *MtGRF2*. Pearson coefficient correlations (PCC) were generated with RNA-Seq normalized as transcripts per million (TPM). Significant correlations were considered $\rho \geq 0.90$ for first-degree associations, and $\rho \geq 0.95$ for second-degree associations in the analysis. The Gene name is shown for each one that has been functionally annotated.

Expression of NCR peptides is highly abundant in indeterminate nodules of *M. truncatula*, including during bacterial infection and bacteroid differentiation. They define the species-specific relationships between legumes and rhizobia (Van de Velde et al., 2010; Farkas et al., 2014; Satgé et al., 2016; Yang et al., 2017; Wang et al., 2017).

The WUSCHEL homeobox-containing (WOX) gene *MtWOX2* (Medtr4g063735.1) is a homolog of the Arabidopsis *AtWOX2*. WOX homeobox proteins are transcription factors involved in embryo apical patterning and cotyledon development (Gamas et al., 1996). *AtWOX2* acts redundantly with STIMPY-LIKE (STPL/WOX8) on regulating the expression of CUC genes to promote the establishment of the cotyledon boundary, without affecting the primary shoot meristem (Lie et al., 2012). This indicates that *MtWOX2* may

be involved in a somewhat similar developmental role in root nodules during differentiation after cell duplication.

MtSYMREM1 (Medtr8g097320 in Mt4.0v2.1, previously named Medtr8g098650 in Mt3.5v1) is a remorin membrane protein that interacts with at least three essential nodule receptor-like kinases (RLKs). *MtSYMREM1* has a Lotus homologs (*LjSYMREM1*) that increase the nodulation when overexpressed. *MtSYMREM1* is involved with infection and symbiosomes formation from the microarray-based analysis of transcription reprogramming of root nodule development (Moreau et al., 2011).

At the second-degree level, i.e. genes associated transcriptionally with those directly associated with *MtGRF2* expression, we highlight the following: the nodulins *MtN22* (Medtr3g055450.1), *MtN24* (Medtr6g007160.1), *MtN25* (Medtr0174s0090.1), the signal peptide peptidase (Medtr1g008280.2), the transcription factor *MtNIN* (Medtr5g099060.1), the leghemoglobins *MtLB29* (Medtr1g049330.1), Medtr1g090810.1 and Medtr5g041610.1, *MtLEC4* (Medtr1g090943.1), *MtIPD3* (Medtr5g026850.1), *MtIRE* (Medtr5g069000.1), *MtNIP1/N26* (Medtr8g087710.1), *MtDAS25* (Medtr5g081900.1), *MtDNF1* (Medtr3g027890.1), *MtDNF2* (Medtr4g085800.3), *MtSYMREM1* (Medtr8g097320.1), *MtSNARPI* (Medtr4g011680.1), *MtNOD25* (Medtr3g055440.5), the organic cation/carnitine transporter *Mt2OS* (Medtr5g082760.1), along with the nodule-specific cysteine-rich peptidases *NCR126-TC355* (Medtr1g071880.1), *NCR193-TC3461* (Medtr3g084910.1), *NCR14-TC4943* (Medtr4g059900.1), *NCR144-TC29276* (Medtr5g068810.1), *NCR164-TC36556* (Medtr7g071720.2), *NCR166-TC3742* (Medtr6g453200.1), *NCR168-TC35186* (Medtr7g071690.1), *NCR194-TC4662* (Medtr5g062510.1), *NCR217-TC41315* (Medtr5g070295.1), *NCR219-TC3396* (Medtr6g090485.1), *NCR235-TC4595* (Medtr4g063690.1), *NCR238-TC29155* (Medtr2g044330.1), *NCR24-TC34386* (Medtr7g114890.1), *NCR26-TC42818* (Medtr5g063580.1), *NCR264-TC42927* (Medtr4g046807.1), *NCR28-TC4257* (Medtr7g022430.1), *NCR88-TC38212* (Medtr5g063600.1) plus 48 other *NCRs*.

The non-characterized nodulins *MtN22*, *MtN24* and *MtN25* (Gamas et al., 1996) show high and specific expression in nodule, with maxima at 45.7% of total expression confined to the IZ for *MtN22*, 39.3% at proximal side of FII (along with 18% to IZ) for *MtN24*, and 70.3% to IZ for *MtN25*, (Roux et al., 2014). Congruently, along with *MtGRF2*, these three nodulins also show maximum expression in the nodule zone 2 sample in the *Medicago* Gene Atlas (Benedito et al., 2008).

Moreover, *MtSNARPI* (Medtr4g011680.1) is small nodulin coding for an acidic RNA-binding peptide that regulates the establishment of symbiosis between *Medicago truncatula*–*Sinorhizobium meliloti*. *MtSNARPI* interacts with the early nodulin *MtENOD40* (Laporte et al., 2010), which is possibly involved in reallocating nuclear proteins to the cytoplasm (Pan and Wang, 2017), although the exact mechanism remains unknown. *MtNIN* (Medtr5g099060.1) is a central important TF acting downstream of

NF signaling pathway that coordinates nodule formation and SNF, also seen in **Chapter 2** (Marsh et al., 2007; Vernié et al., 2015).

Surprisingly, leghemoglobins can be symbiotic or non-symbiotic, and their genes are present not only in legumes, but also non-nodulating species. For example, the Arabidopsis genome codes for two leghemoglobins (Trevaskis et al., 1997). In legumes, nodule leghemoglobins are crucial for sustaining SNF by protecting nitrogenase from O₂ irreversible inactivation (Andersson et al., 1996). In our gene network centered on MtGRF2, three leghemoglobins were associated - *MtLb29* (Medtr1g049330.1), Medtr1g090810.1 and Medtr5g041610.1. Among these, *MtLb29* belongs to a legume-specific phylogenetic clade, while the other two are in clades containing non-legume proteins (Ott et al., 2005). The expression level of leghemoglobin Lb120-1 (Medtr5g066070), which is associated with MtGRF2 via an NCR peptide (Medtr6g445080) is quite remarkable.

The lectin *MtLEC4* is a β ,1-3endoglucanase (MtBGLU1) expressed in nodules starting at one week after inoculation with *Sinorhizobium meliloti*. Medicago mutants (*bit1*, *rit1*, and *mtsym1*) are defective in the initial stages of invasion, whereas the bacterial mutants (*exoA* and *exoH*) were unable to induce *MtLEC4* expression, although nodules infected with the *bacA* mutant do express *MtLEC4* (Mitra and Long, 2004). Nodules of plant mutants with defects in nitrogen fixation (*dnf1* and *dnf5*) also express *MtLEC4* (Mitra and Long, 2004), indicating that *MtLEC4* acts downstream of DNF1 and DNF5. Opposite results were found on the transcription reprogramming analysis of nodule development, where the *MtLEC4* present expression in *exoA* nodules, which are wild-type of *M. truncatula* inoculated with *Rhizobium* capable to colonized plant cells, but impaired to differentiate (Moreau et al., 2011).

MtDNF1 (SPC22, Medtr3g027890) is a subunit of a signal peptidase complex required for protein secretion during symbiosome development (Wang et al., 2010). MtDNF2 (PLC-like phosphodiesterase, Medtr4g085800) is a putative phosphatidylinositol phospholipase C-like enzyme involved with bacteroid differentiation since its down-regulation caused bacteria to not differentiate properly into bacteroids and generated non-functional nodules (Bourcy et al., 2013).

The nuclear protein IPD3 (called *Cyclops* in *Lotus japonicus*), encoded by *MtIPD3*, a phosphorylation substrate of DMI3 (CCaMK), is also essential for rhizobial colonization decoding of the calcium signal and the molecular events downstream required for symbiosis events in both rhizobial and mycorrhizal colonization (Yano et al., 2008; Horváth et al., 2011).

MtIRE an *Arabidopsis thaliana* homologous *INCOMPLETE ROOT HAIR ELONGATION (IRE)* gene encodes to AGC protein kinase expressed in inoculated roots and in a proximal part of the invasion zone (zone II) found in indeterminate nodules (Pislariu and Dickstein, 2007). *MtNIP1*, Nod 26-like, encodes to an aquaporin Nod intrinsic proteins (NIP), which was described as AM-induced and facilitate the cellular uptake of ammonia (Uehlein et al., 2007). *MtDAS25* is been shown to be associated with DNF1 (Wang et

al., 2010), and is been on expression profiles together with, with DNF1 and DAS25 genes in cluster 11 (Moreau et al., 2011; Roux et al., 2014). *MtNDO25* encodes for a signal peptide with high conserved modular nodulin in both N-terminal and C-terminus, that is also been localized in the symbiosome of infected cells. This modular conserved nodulins present one possible mechanism for the delivery of host proteins to the symbiosomes of infected root nodule cells, shown to be sufficient to translocate proteins across the peribacteroid membrane (Hohnjec et al., 2009).

Taken all together, we hypothesize that MtGRF2 is a novel nodulin with a putative transcription factor function apparently involved in the rhizobium differentiation and maturation, the required process for symbiotic nitrogen fixation in root nodule of *M. truncatula*.

Analysis of *cis*-elements in the *MtGRF2* gene promoter

An important question regarding gene expression, is the presence of conserved *cis*-elements that interact with transcription factors and regulators. The very specific expression profile of MtGRF2 in nodule during rhizobial differentiation presents an opportunity to better understand the molecular mechanisms governing this transcriptional pattern. Known *cis*-elements present in the 2,062bp promoter region of the *MtGRF2* gene (**Figure 6**). We identified 63 *cis*-different elements, with 38 of them representing in multiple copies. Those represented at least seven times in this genomic interval are: ARRIAT (20 times), DOFCOREZM (17), CACTFTPPCA1 (14), GTFANTG10 (12), GATABOX (11), CAATBOX1 (10), MYCCONSENSUSAT (9), EBOXNNAPA (9), OSE2ROOTNODULE (7), NODCON2GM (7), and GT1CONSENSUS (7).

Given the unique and distinct expression domains of MtGRF1 and MtGRF2 genes, a comparative analysis can be useful to elucidate their spatial regulation. It has not escaped our notice that two *cis*-elements are frequent, but similarly or uniquely represented in both or either in promoters of the GRF genes of this study. The *MtN20/MtGRF1* promoter has five IBOX, while the *MtGRF2* promoter has six BIHD1OS *cis*-elements, neither of those *cis*-elements are present in the promoter of the other gene (**Table 2**). The biological significance of these elements to the unique expression patterns of each of our genes of interest has yet to be assessed.

Table 2. Comparative analysis of promoter *cis*-elements of *MtN20/MtGRF1* and *MtGRF2* genes. Common and distinct promoter *cis*-elements found in ~2kb upstream of *MtN20/MtGRF1* and *MtGRF2*. #s indicates the number of times that *cis*-elements were founded in the promoter of each gene. Unique *cis*-elements in each gene promoter are shown on the right.

Common <i>Cis</i> -element between pMtGRF1 and pMtGRF2 followed by times that each one appears			Differential <i>Cis</i> -elements between pMtGRF1 and pMtGRF2			
<i>Cis</i> -element	pMtGRF1 #s	pMtGRF2 #s	pMtGRF1		pMtGRF2	
			<i>cis</i> -element	copies #	<i>cis</i> -element	copies #
BOXIINTPATPB	1	1	2SSEEDPROTBANAPA	1	ABRELATERD1	1
CARGCW8GAT	1	1	AMYBOX2	1	ARFAT	1
CIACADIANLELHC	1	1	CANBNNAPA	1	CATATGGMSAUR	1
CPBCSPOR	1	1	IBOXCORENT	1	ELRECOREPCRP1	1
GT1CORE	1	1	MYB26PS	1	MYBPZM	1
MYBCOREATCYCB1	1	1	MYCATRD22	1	SEBFCONSSTPR10A	1
CCAATBOX1	1	2	PRECONSCRHSP70A	1	SEF1MOTIF	1
PYRIMIDINEBOXOSRAMY1A	1	3	PROXBNNAPA	1	TATAPVTRNALEU	1
REALPHALGLHCB21	1	3	SORLIP2AT	1	WUSATAg	1
-300ELEMENT	2	1	TATCCAYMOTIFOSRAMY3D	1	ZDNAFORMINGATCAB1	1
GT1GMSCAM4	2	1	-10PEHVPSBD	2	CARGATCONSENSUS	2
DPBFCOREDCDC3	2	2	AMYBOX1	2	EECCRCAH1	2
SEF4MOTIFGM7S	2	3	CCA1ATLHCB1	2	MYBCORE	2
ACGTATERD1	2	5	GAREAT	2	RHERPATEXPA7	2
CURECORECR	2	5	INRNTPSADB	2	BIHD1OS	6
LECPLEACS2	3	1	MYBGAHV	2		
MYBST1	3	1	RBCSCONSENSUS	2		
RAV1AAT	3	1	TATCCAOSAMY	2		
SREATMSD	3	1	IBOX	5		
ANAERO1CONSENSUS	3	2				
MARTBOX	4	1				
MYB1AT	4	3				
POLLEN1LELAT52	4	4				
POLASIG1	4	6				
POLASIG2	5	3				
GTGANTG10	5	12				
NODCON2GM	6	7				
OSE2ROOTNODULE	6	7				
EBOXBNNAPA	6	9				
MYCCONSENSUSAT	6	9				
GT1CONSENSUS	8	7				
ARR1AT	8	20				
IBOXCORE	10	4				
ROOTMOTIFTAPOX1	12	3				
CAATBOX1	12	10				
CACTFTPPCA1	15	14				
GATABOX	17	11				
DOFCOREZM	18	17				

MATERIAL AND METHODS

Plant growth conditions: *Medicago truncatula* plants (ecotype ‘A17’) were grown in turface:vermiculite (2:1) medium in 4” Azalea pots under 16:8-h (light:dark) photoperiod and 24°C:22°C (day:night) temperature conditions in a Percival growth chamber. Plants received 50 mL 1X B&D (Broughton and Dilworth, 1971) fertilizer solution with low nitrogen (0.5mM ammonium nitrate) every other day, except at day of inoculation with rhizobium.

Cloning of the *MtGRF1* promoter: A 2,062-bp genomic region upstream the *MtGRF2* start codon (coordinates chr1:28315631...28316566 of the *Medicago truncatula* genome assembly v.4.0) was amplified and cloned into a binary vector to express the *GUS* reporter gene. Medicago genomic DNA (ecotype ‘A17’) was extracted from young leafs using the IDT Plant DNA Extraction Protocol (www.idtdna.com/pages/docs/educational-resources/plant-dna-extraction-protocol.pdf?sfvrsn=5). PCR was carried out with the high-fidelity KOD Extreme Hot-Start DNA polymerase (Toyobo, catalog #71086) in an Mastercycler ep Gradient S Thermal Cycler (Eppendorf). The amplicon was run on a 1% agarose gel, column purified and further cloned into the directional pENTR-D/ TOPO vector (ThermoFisher), followed by Gateway LR reaction, using Gateway LR Clonase II Enzyme Mix (ThermoFisher) for recombination it into the destination vector (pKGWFS7). All final cassettes were confirmed by Sanger sequencing at the WVU Genomics Core Facility. Plasmids were used to transform *Agrobacterium rhizogenes* strain ‘ARqual’ for *ex-vitro* hairy-root transformation. All primer sequences used in this dissertation are provided in **Annex 1**.

***Ex-vitro* hairy-root transformation:** A slightly modified version of the freeze-thaw method for *Agrobacterium* transformation protocol (http://openwetware.org/wiki/Maloof_Lab:Agro_Transformation) was used for transformation of *Agrobacterium rhizogenes* strain ‘ARqual’ cells. *Ex-vitro* hairy root transformation of *Medicago truncatula* ecotype “A17” was performed as described by Boisson-Dernier et al. (2006). In brief, roots of four-day-old seedlings were diagonally cut out and the wound was inoculated with a three-day-old plate cultivated with ARqual transformed with the final destination on solid LB medium containing the respective selective antibiotic (100mg/L spectinomycin plus 100mg/L streptomycin). The seedlings were then grown on modified Fahraeus medium (pH 7.5) with 15g/L agar in tissue culture tubes for 2 weeks under 16:8-h light:dark photoperiod regimen at 24°C:22°C day:night temperature before transplanting to a surface:vermiculite (2:1 ratio) under the same growth conditions. 0.5mM were provided ½-strength B&D fertilizer solution (50mL) every other day 7 days before rhizobial inoculation. When applicable, transgenic roots and nodules were visualized under fluorescence stereoscope via expression of the fluorescent marker DsRed (excitation/emission ~580/605 nm).

Rhizobial inoculation: Unless specified otherwise, roots of one week-old plants were inoculated with 50 mL suspension ($OD_{600}=0.05$) of *Sinorhizobium meliloti* rhizobial strain *Sm1021* (*Sm1021-lacZ*) harboring the *hema::LacZ* cassette in 1X B&D solution without nitrogen to induce nodulation. One inoculation loop of *Sinorhizobium meliloti* rhizobial strain *Sm1021* from glycerol stock was grown for three days in 3 mL YM medium supplemented with 6mM $CaCl_2$ and proper antibiotic, according to the destination vector (e.g.,

10 mg/L Tetracycline or 100 mg/L Streptomycin). Sixteen hours before rhizobial inoculation, the 1-mL inoculum was regrown in 200mL flask until OD₆₀₀~1.0. The suspension was centrifuged at 10,000g for 10 minutes, the pellet washed by resuspension and centrifugation, and the clean pellet was then resuspended and diluted with 1X no-N B&D solution without nitrogen to an OD₆₀₀ of 0.05. Fifty milliliters of the rhizobial suspension were used to inoculate each plant.

Histochemical assay (tissue fixation and GUS staining): Sections were cut by hand from unfixed stems of plants grown in vitro, essentially as described (Jefferson et al., 1987) and fixed in 0.3% formaldehyde in 10 mM MES, pH 5.6, 0.3 M D-mannitol for 45 min at room temperature, followed by several washes in 50 mM NaH₂PO₄, pH 7.0. All fixatives and substrate solution were introduced into sections with a brief (1 min) vacuum infiltration. Histochemical reactions with X-Gluc were performed with 1 mM substrate in 50 mM NaH₂PO₄, pH 7.0 at 37°C from 3 hours and 16 hours. After staining, sections were rinsed in 70% ethanol for 5 min, and then mounted for microscopy (Jefferson et al., 1987). For anatomical analyses, transformed nodules at 28 dpi were embedded in 5% low-melting agarose and sectioned on a vibratome with a 70-μm thickness. The sections were extracted from agarose and kept in 1.5mL tube with NaH₂PO₄, pH 7.0 for observation under light microscopy (Nikon Eclipse E600 microscope coupled with a Nikon DS-Ri1 camera).

Gene expression analyses: The gene expression profiling of *MtGRF2* in nodulating roots inoculated with *Sinorhizobium meliloti* strain *Sm1021* in were performed with *M. truncatula* ecotype “A17” plants inoculated with the mutants *fixJ*, *bacA*, *exoA* or the functional strain *hemA::LacZ* strains, all in the same *Sm1021* genetic background. For gene expression analyses via qRT-PCR, non-inoculated root samples provided full nitrogen fertilization (6 mM N from 2 mM KNO₃ and 2 mM NH₄NO₃) were used as a negative control. Whole root systems were harvested at 0, 3, 14, 21, and 28 dpi. RNA was extracted with mirVana mRNA Isolation kit (Life Technologies) followed by DNA removal with Turbo DNA-free kit (Applied Biosystems). First strand cDNA was synthesized with oligo-dT priming using the SuperScript III First-Strand Synthesis Super mix (ThermoFisher), following the manual instructions. All primer sequences used are described cf. **Annex 1**.

SYBR green master mix (Applied Biosystems) we used for 5-μL reactions and ABI7500 Real Time qPCR System (Applied Biosystems) was used to run three biological and three technical replicates. A master mix containing SyBR green (2.5μL) and cDNA (1μL 1:10) was plated with a solution of primer pair mix (0.5μL with 20mM each) and water (0.5μL). All templates were amplified using the same cycling conditions: 50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 15 sec, and 60°C for 1 min.

Melt curves were produced at the end of each run. Ct (threshold cycle) values were exported to Excel to organize data and the comparative analysis of gene expression. The housekeeping genes used as data normalizers for RT-qPCR were *EFlα* (Medtr6g021800) and *Tub1* (Medtr7g089120). LinRegPCR was used to extract PCR efficiencies for each primer pair (<http://linregpcr.nl/>). The software NormFinder (Andersen, 2004) was used to confirm the chosen housekeeping genes as stably expressed. Relative expression was calculated according to the Pfaffl method (Pfaffl, 2001).

Light microscopy: At 28 days post-inoculation with *Sm1021-LacZ*, nodules were harvested and fixed under vacuum with 2.5% glutaraldehyde in 0.1 M PIPES (pH 7.2) for one hour at room temperature, followed by two rinses with 0.1 M PIPES (pH 7.2). When applicable, samples were stained in fresh staining solution containing 50mM potassium ferricyanide, 50mM potassium ferrocyanide, 0.08% X-Gal (0.08mg/mL) or 0.08% Red-Gal (0.08mg/mL) and 0.1M PIPES (pH 7.2) for 16 hours at room temperature (Boivin et al., 2016), followed by two rinses with 0.1M PIPES (pH 7.2), and stored at 70% ethanol until processing. After fixation (and staining), tissues were cleared with 2.4% sodium hypochlorite for 5 min and dehydrated with an ethanol series (50%, 70%, 90%, 95%, and 100% twice for 45 minutes each). Samples were then transferred to 100% xylene for 15 minutes or until translucent. The completely dehydrated samples were embedded in fresh 60°C xylene:paraplast (1:1) mix for two hours at 60°C and transferred to melted (60°C) 100% paraplast and incubated at 60°C for at least three hours. Each sample were blocked and cut in 7-μm section with a conventional microtome. The slices were placed on glass slides and incubated overnight at 37°C. On the next day, the slides were washed twice with 100% xylene for three minutes and twice with 100% ethanol, followed by the hydration process using submersion in 95% ethanol and then water for five minutes each. The samples were then ready to accept water-based dyes. The slides were observed under bright field microscope (Nikon Eclipse E600 microscope coupled with a Nikon DS-Ri1 camera). Often, X-gal staining was used to visualize *lacZ* bacteria and the tissue was counterstained with chloramphenicol to visualize lignin in the plant cell wall.

Regulatory gene network analysis: SRA files of 72 publicly available *Medicago truncatula* RNA-Seq samples were chosen for this study: 33 files with high sequencing depth totaling triplicates of five treatments (Roux et al., 2014: NCBI BioProject Accession PRJNA213402), 15 files for five treatments in triplicates collected at different times points of nodule development (Satgé et al., 2016: PRJNA188524), 36 files of nodules with triplicates of eight treatments plus four root controls (unpublished, PRJNA327225) and six files of major organs of the mature *Medicago truncatula* plant (Young et al., 2011: PRJNA80163). The raw files were submitted to quality check using the FastQC software (www.bioinformatics.babraham.ac.uk/projects/fastqc/), and then mapped onto the *M. truncatula* reference

transcriptome (Mt4.0v2) with the Salmon Transcript Quantification v.0.8.2 software (Patro et al., 2015) in the Galaxy platform (Afgan et al., 2016). All data were normalized as transcript per million (TPM). A gene expression matrix was created in the format a gene-per-row (57,585 genes models) and a sample-per-column (72 samples).

Pearson coefficient correlation (PCC) was performed with MtGRF2 expression values against all *Medicago* genes. A $\rho \geq |0.9|$ threshold was imposed in first-degree correlations with MtGRF2, producing 23 genes with significant correlation. Following, the 23 co-expressed genes were used to generate a second-degree correlation set with a $\rho \geq |0.90|$. The correlation analysis was processed in R in a Linux platform on the WVU Spruce high-performance computer (HPC) cluster using the R-package agricolae in R environment (R Development Core Team, 2016). The correlation datasets for all selected genes were imported to Cytoscape for the genetic network visualization (Smoot et al., 2011).

Identification of cis-elements in promoter region: known *cis*-elements present in the 2-kb promoter region of the *MtGRF2* gene was performed using the PLACE online tool (www.dna.affrc.go.jp/htdocs/PLACE; Higo et al., 1999) using Arabidopsis as the default plant species.

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CHAPTER 5

INVOLVEMENT OF TWO NOVEL GRF-TYPE ZINC FINGER NODULINS DURING RHIZOBIAL SYMBIOTIC ESTABLISHMENT IN *MEDICAGO TRUNCATULA* NODULES

CONCLUSIONS AND PERSPECTIVES

Although a detailed picture has been compiled regarding the first stages of interaction between root cells and free-living rhizobia that triggers nodule development, very little remains known about the molecular players during cell infection, symbiosome formation and bacteroid differentiation that will ultimately produce a fully functional nodule capable of supporting SYMBIOTIC NITROGEN FIXATION (SNF). One major gap in knowledge on SNF, is the involved of plant genes that participate in the regulation of bacteroid differentiation.

In this dissertation, we shed some light on the function of two novel putative transcription factor during the development of root nodules for symbiotic nitrogen fixation in the model legume *Medicago truncatula*. Both genes of interest are highly expressed but in distinct zones of the indeterminate nodule. Both genes encode related proteins that belong to a largely non-characterized protein family of potential transcription factors, the GRF-type zinc finger.

The nodulin *MtN20/MtGRF1* is highly expressed at 4dpi, a stage when the symbiosome starts forming in newly infected cells of the developing nodule. More precisely, *MtN20* is expressed in the distal side of the infection zone. An insertional *Tnt1* mutant line for this gene indicates *MtN20* may be crucial for symbiotic nitrogen fixation, but not for nodule formation. From the experiments with mutant rhizobial, *MtN20* requires infection for transcription induction, but not necessarily bacteroid differentiation *per se*. We conclude that *MtN20* is potentially involved during the early events of rhizobial infection of nodule cells. *MtN20* belongs to a regulatory gene network participating with *bona fide* symbiotic genes, such as EFD, the EFD target Nod19, NIN and several nodule-specific cysteine-rich (NCR) peptides. In order to further our understanding about this gene and reveal its exact molecular function during nodule development, some experiments need to be performed. **1)** Gene knock-out of *MtN20* (e.g., via CRISPR/Cas9 system) will resolve its essentiality for symbiotic nitrogen fixation in *Medicago truncatula*; **2)** Transcriptional profiling (e.g., RNA-Seq) of *MtN20* knock-out and overexpressing nodules will help determine which genes that transcriptionally connected with our gene of interest; **3)** Expression of *MtN20* fused with a fluorescent marker (e.g., GFP) will reveal its subcellular localization. If this protein acts as a transcription factor, it is expected to localize in the nucleus. In this regard, given the putative nuclear localization signal is position at the C-terminus, it is important to test this fusion with fluorescent tag fused at the N-terminus of the protein of interest; **4)** If *MtN20* is a transcription factor, it also needs to interact

with *cis*-elements in the DNA or target genes. This question can be addressed with ChIP-Seq, ChIP-PCR, or motif assay, in which a pull-down approach is used to identify which molecule or DNA sequence the protein is able to interact with. Additional confirmation will require alternate approaches, such as yeast-one-hybrid, and electrophoretic mobility shift assay (EMSA) to help elucidate the exact MtN20 target DNA sites; **5)** A refined bioinformatics analysis to identify and compare *cis*-elements in all co-regulated genes of the MtN20 transcriptional network may render a selection of common *cis*-element responsible for the spatial-temporal resolution of plant genes during cell infection; **6)** the significance of the post-translational modifications experimentally verified in MtN20 also requires some consideration. Expression of MtN20 variants with the phosphorylated Ser residues replaced by a tolerable non-phosphorylated substitute residue (e.g., Ala) (Betts and Russell, 2003) compared to the wild-type allele and a variant with those Ser replaced by phosphomimetic mutations residue (Glu, which works as a mimic of constitutive phosphorylated Ser). In this experiment, it is advisable to use the native promoter of MtN20 to avoid its mis-expression that could lead to pleiotropic effects in nodule development. The potential roles of protein phosphorylation on protein localization or DNA-binding properties are intriguing questions; **7)** finally, the protein-protein interactions involving MtN20 is highly significant in this process. This question can be addressed by yeast-two-hybrid (Y2H) screening of co-expressed proteins, bimolecular fluorescence complementation (BiFC) assay, and fluorescence resonance energy transfer (FRET). Altogether, focusing not only on MtN20, but on other nodule-specific (or nodule zone-specific) genes throughout symbiosis maturation, from cell infection to full bacteroid differentiation, could lead us to a much better dynamic picture of the process.

Furthermore, we also showed that *MtGRF2* is another nodule-specific gene but distinctly expressed specifically in the interzone of the nodule. This result indicates that MtGRF2 may play an essential role during bacteroid differentiation, when many nodule cysteine-rich (NCRs) peptides are essential for normal symbiotic nitrogen fixation in root nodules of *Medicago truncatula*. Indeed, the genetic network generated around MtGRF2 contains several NCRs and other important symbiotic genes, such as *MtNCR88-TC38212* (*Medtr5g063600.1*), *NCR219-TC3396* (*Medtr6g090485.1*). The same follow-up experiments suggested for MtN20 also applies to MtGRF2. Additionally, a comparative approach using the *cis*-elements overrepresented in both genes connected with MtN20 and MtGRF2, or uniquely overrepresented in only one of them, will enable identify relevant regulatory sequences and create testable hypotheses for molecular biology experiments to understand how the genes are sequentially regulated during the process that goes from cell infection into full bacteroid differentiation in a nodule cell that is mature to symbiotically fix nitrogen.

Those two *M. truncatula* GRF-type zinc finger possibly are involved in the processed of bacteroid differentiation. Very few genes have been identified in the process of bacteroid differentiation, e.g. as Regulator of Symbiosome Differentiation (RSD) controlling the expression of vesicle-associated

membrane protein 721a, Does Not Fix (DNF1) and DNF2, Trx s1, Glycine-Rich Protein (GRP), Symbiotic Cysteine-rich Receptor-like Kinase (SymCRK) and many NCRs specific of the Inverted Repeat-Lacking Clade (IRLC) legume group (Alunni and Gourion, 2016). And this dissertation opens up new ideas in that plant genes present fundamental control on the bacteroid initial and terminal differentiation. There are possibilities that those two putative transcription factors GRF-type zinc finger, coordinate the genetic machinery reprogramming, positioning the legume, macrosymbiont, acting as the major regulator of the bacteroid differentiation and sustainment of SNF.

A set of experiments aiming at better understanding the process of mutualistic cell infection and bacteroid differentiation is needed. For that, a batch of knock-out mutant nodule for genes specifically expressed in these stages could be generated, transcriptionally profiled, and phenotypically analyzed by microscopy, for the nodule anatomy by a light or via transmission electron microscopy for bacteroid features. Altogether, these data will determine which (non-redundant) genes are essential and at which stage the process is interrupted by each gene knockout. High-throughput analysis of *cis*-elements overrepresented in nodule-induced genes would also help elucidate the molecular mechanisms by which genes are induced and which transcription factors are responsible for each step of the process.

Overall, this study could provide valuable information about the nodulation and symbiotic nitrogen fixation in terms of reprogramming the genetic machinery required to normal root nodulation and symbiotic nitrogen fixation, as well as important evolutionary events that enabled the occurrence of endosymbiosis. With all the knowledge gathered on molecular genetics and physiology of legumes so far, we are closer than ever, albeit so far yet, of fully understanding nodulation and symbiotic nitrogen fixation in order to introduce this trait in non-legume crops. Only a greater investment in research and education, and substantially more funding to agricultural sciences will enable this ambition to be fulfilled.

REFERENCE

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