MOLECULAR GENETICS OF NODULE NUMBER REGULATION: CLONING, CHARACTERIZATION AND FUNCTIONAL STUDIES OF THE ROOT DETERMINED NODULATOR1 (RDN1) GENE IN Medicago truncatula

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MOLECULAR GENETICS OF NODULE NUMBER REGULATION: CLONING, CHARACTERIZATION AND FUNCTIONAL STUDIES OF THE ROOT DETERMINED NODULATOR1 (RDN1) GENE IN Medicago truncatula

A Thesis
Presented to
the Graduate School of
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In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Genetics

by
Tessema Kebede Kassaw
December 2012

Accepted by
Dr. Julia Frugoli, Committee Chair
Dr. William Marcotte
Dr. Hong Luo
Dr. Harry Kurt
ABSTRACT

Nitrogen is critical to life. However, the majority of nitrogen on earth (in the atmosphere) is inert and unavailable to nearly all organisms. Metabolically diverse prokaryotes are the only organisms capable of fixing atmospheric nitrogen; rhizobia set up a symbiosis with legume plants allowing the plants to benefit from this ability. Since nodulation and the subsequent nitrogen fixation processes are energy intensive, the host plant must balance hosting of the rhizobia by limiting the number of nodules it forms through a mechanism called Autoregulation of Nodulation (AON). My study of mutants in the model legume *Medicago truncatula* defective in AON allowed identification of loss-of-function alleles of the ROOT DETERMINED NODULATION1 (*RDN1*) gene (Medtr5g08952). I identified *RDN1* by genetic mapping, transcript profiling, and rescue of the mutant phenotype. *RDN1* is predicted to encode a 357-amino acid protein and is a member of an uncharacterized, highly conserved gene family unique to green plants. The promoter drives expression in the vascular cylinder and subcellular localization places *RDN1* in the secretory pathway, consistent with a role for *RDN1* in intracellular and long distance signaling in plants. I used grafted plants to show that *RDN1* regulatory function occurs in the root before the shoot-derived suppression signal regulated by SUNN, another AON gene. Using a combination of gene expression assays, analysis of *sunn/rdn1* double mutants and shoot-to-root reciprocal grafting I showed SUNN and *RDN1* act in the same signaling pathway. *RDN* genes from poplar, rice and Arabidopsis can rescue the *Mtrdn1* mutant suggesting *RDN1* protein function is retained in non-legumes. I report multiple root defects in Arabidopsis and Medicago mutants with defects in *RDN* genes. Together
These findings help establish RDN as a family of proteins with previously uncharacterized regulatory functions involved not only AON but also root growth and lateral root development in land plants. Building on RDN’s AON role, I also developed a split root inoculation system to understand the timing of autoregulation of nodulation in *M. truncatula* and discovered evidence for a previously unknown secondary AON signal.
DEDICATION

I dedicate this dissertation to my wonderful family and friends. A special feeling of gratitude goes to my loving, understanding and patient wife, Kidist Molla Teshome, who accompanies me through the ups and downs of these many years of research. This work would not have been possible without her constant love and care. To my precious daughter Joanna Tessema, you are the joy of my life. I must also thank my loving and faithful father (Kebede Kassaw) and mother (Guzguz Asfaw) for instilling the importance of hard work and education. I also dedicate this work to my brother Neguse Kebede, my sisters Workinesh Kebede and Almaze Kebede, you are my inspiration. Finally, I dedicate this dissertation to all my friends for their encouragement and constant support. I am lucky to have you all. Thank you!
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CHAPTER 1

LITERATURE REVIEW

Portions of the material in this chapter will appear in the book Progress in Symbiotic Endophytes, 2013, R. Aroca, Ed, Springer as Chapter 1 “Journey to Nodule Formation: From Molecular Dialogue to Nitrogen Fixation” Tessema Kassaw and Julia Frugoli and appears here with permission from the editor.

INTRODUCTION

Nitrogen Fixation and Legume

The use of legumes in agricultural rotations was documented by Pliny the Elder in 147 BC (Crawford et al. 2000). The leguminosae family is taxonomically categorized into three subfamilies which encompass nodulating plants, the Caesalpinioideae, Mimosoideae, and Papilionoideae (Doyle and Luckow 2003), although most nodulation occurs in the Papilionoideae. They are the third largest family of angiosperms consisting of more than 650 genera and over 18,000 species (Lewis et al. 2005) and are second only to Graminiae in their importance as human food accounting for 27% of the world’s crop production and contributing 33% of the dietary protein nitrogen needs of humans (Graham and Vance, 2003). Legumes include a large number of domesticated species harvested as crops for human and animal consumption as well as for oils, fiber, fuel, fertilizers, timber, medicinals, chemicals, and horticultural varieties (Lewis et al. 2005). One of the reasons legumes are so popular is their ability to satisfy their nutritional needs
by establishing symbiotic relationships with microbes in the rhizosphere. Like 80% of the terrestrial plant species, legumes form arbuscular mycorrhizal (AM) associations, where the fungus colonizes the cortical cells to access carbon supplied by the plant and the fungus helps the plant in the transfer of mineral nutrients, particularly phosphorus, from the soil (Smith and Read, 2008). AM is a very ancient symbiosis more than 400 million years old. In contrast, the endosymbiosis of plants with nitrogen-fixing bacteria is limited to only a few plant families and is more recent evolutionarily, approximately 60 million years old (Godfroy et al. 2006). Around 88% of legumes examined to date form nodules in association with rhizobia and important agricultural legumes alone contribute about 40-60 million metric tons of fixed N₂ annually while another 3-5 million metric tons are fixed by legumes in natural ecosystems (de Faria et al. 1989; Smil, 1999).

The legume-rhizobia symbiosis has been investigated using Pisum sativum (pea), Vicia sativa (vetch), Medicago sativa (alfalfa), Medicago truncatula (barrel medic), Glycine max (soybean), Phaseolus vulgaris (bean), Sesbania rostrata (sesbania) and Lotus japonicus (lotus). The large genome size and low efficiency of transformation of many crop legumes combined with the advent of genomic research resulted in a concentration on two symbiotic models, M. truncatula and L. japonicus (Oldroyd and Geurts, 2001; Udvardi et al. 2005). These systems provide an opportunity for researchers to study both bacterial and fungal symbioses not supported by the well-studied model plant, Arabidopsis thaliana at the molecular level. Both species have all the tools for a model system such as a small diploid genome, self-fertility, ease of transformation, short life
cycle, high level of natural diversity and a wealth of genomic resources (Handberg and Stougaard, 1992; Cook, 1999). Both belong to the Papilionoideae subfamily and based on the nature of the nodule they develop they are classified as determinate (L. japonicus) and indeterminate (M. truncatula) nodulators. Determinate nodules are characterized by a non-persistent meristem which ceases development at early stage. This results in round nodules with a homogeneous central fixation zone composed of infected rhizobia-filled cells interspersed with some uninfected cells. In contrast, indeterminate nodules are cylindrical and consist of a gradient of developmental zones with a persistent apical meristem that supports indeterminate growth, an infection zone, a fixation zone, and zone of senescence (Figure 1). The two model plants represent the two nodule development strategies and most findings discussed in this chapter come from these systems.

Early Steps in Legume Rhizobia Symbiosis

Overview

Soil bacteria belonging to the genera Rhizobium, Bradyrhizobium, Sinorhizobium, Allorhizobium, and Mesorhizobium establish a unique beneficial interaction with most legumes and a few non-leguminous plants in the family of Ulmaceae (Parasponia sp.). The interaction between rhizobia and the host plant results in the formation of N₂-fixing nodules. Within these nodules bacteria are provided with a carefully regulated oxygen and carbon supply which makes it possible for the bacteria to reduce nitrogen efficiently for the plant. The early steps of the symbiosis begin with the exchange of discrete signals, a molecular dialogue, between the bacteria and the plants (Shaw and Long, 2003). Plants
produce and release chemicals, mainly flavonoids and isoflavonoids, into the rhizosphere. These molecular signals initiate root nodulation by the induction of nod genes in rhizobia, promoting bacterial movement towards the plant, and enhancing the growth of the bacterial cells (Phillips and Tsai, 1992). The plant factors are recognized by rhizobial NodD proteins, transcriptional regulators that bind directly to a signaling molecule, and are able to activate downstream nod genes (Mulligan and Long, 1985). Rhizobial nod genes are responsible for the production and secretion of species-specific Nod factors, lipochito-oligosaccharidic signaling molecules (Zhu et al. 2006). Upon exposure to Nod factors, the plant root hair cells growth is altered, a periodic calcium spiking is induced, a preinfection thread structure is formed, gene expression is altered, and inner cortical cells in the root are mitotically activated, which together leads to the formation of nodule primordia (Ane et al. 2004; Kuppusamy et al. 2004; Mitra et al. 2004; Geurts et al. 2005; Middleton et al. 2007). The infection thread housing the bacteria advances through this actively dividing zone of cells to the nodule primordia. The subsequent release of the bacteria into individual cortical cells by endocytosis results in the enclosure of the bacteria within a plant membrane called the peribacteroid or symbiosome membrane. The peribacteroid membrane effectively isolates the bacteria from the host cell cytoplasm while controlling transport of selected metabolites in both directions (Puppo et al. 2005). The bacteria inside the symbiosome membrane differentiate into bacteroids that produce nitrogenase for nitrogen fixation (Lodwig et al. 2003). However, for effective nitrogen fixation, nitrogenase needs a low oxygen environment, while at the same time rapid transport of oxygen to the sites of respiration must be ensured. These conflicting demands
are met by the presence of millimolar concentrations of the oxygen-binding protein leghemoglobin within the cytoplasm of nodule cells (Ott et al. 2005). Recent work using leghemoglobin RNA-interference lines in *L. japonicus* showed altered bacterial and plant cell differentiations, decreased amino acid levels in nodules and a defect in nitrogen fixation (Ott et al. 2009). The resulting physiological and morphological changes in the host plant lead to the formation of nodules, a suitable environment for bacterial nitrogen fixation (Figure 1.1). The fixed nitrogen obtained by the plant is not without cost, as the plants in return must contribute a significant amount of energy in the form of carbon skeletons to the bacteria.

![Figure 1.1](image.png)

*Figure 1.1 Initial Phases in the Legume-rhizobium Symbiosis.* The interaction between rhizobia and legume microsymbionts is determined by two specific steps in the mutual signal exchange. First, bacterial nodulation (nod) genes are activated in response to plant-secreted signal molecules (Plant factors), especially flavonoids, resulting in biosynthesis and secretion of Nod factors by rhizobia bacteria. In the second step, Nod factors elicit two simultaneous processes in the host plant roots; triggering the infection process and nodule formation (cortical cell division). The infection process includes curling of the root hair around the attached bacteria, infection thread formation, and release of rhizobia from the infection thread into the dividing cortical cells while nodule formation includes mitotic activation of the inner cortical cells,
division and establishment of a meristem zone (I), and infection zone (II) a nitrogen fixation zone (III) and, in a senescence zone (IV).

**Plant derived signals**

The plant starts the molecular dialogue by releasing flavonoid and isoflavonoid compounds to the rhizosphere (Redmond *et al*. 1986; Kossak *et al*. 1987). Flavonoids have multiple roles in rhizobia-legume symbiosis. These compounds serve as chemoattractants for the rhizobial symbiont and trigger the biosynthesis and release of Nod factors from the bacteria. They do so by acting as a signaling molecule, binding to the bacterial transcription factor *NodD*. *NodD* in turn activates the expression of rhizobial *nod* genes, which are responsible for the production of Nod factors (lipochitin oligosaccharides). The perception of Nod factors by a receptor in the legume host triggers a sequence of events, including curling of root hairs around the invading rhizobia, the entry of the rhizobia into the plant through infection threads, and the induction of cell division in the root cortex that marks formation of the nodule primordium. The recognition of specific flavonoids secreted by the root by compatible rhizobia is the earliest step in determining host specificity.

Flavonoids are also involved in the initiation of the nodule through their action on the plant hormone auxin and could thus play a developmental role in addition to their action as *nod* gene inducers (Hirsch, 1992). RNA interference (RNAi) in *M. truncatula* used to silence the enzyme that catalyzes the first committed step of the flavonoid pathway, chalcone synthase (CHS), reduced the level of flavonoids and the silenced roots were
unable to initiate nodules, even though normal root hair curling was observed (Wasson et al. 2006). In addition, Wasson et al. (2006) rescued nodule formation and flavonoid accumulation by supplementing plants with the precursor flavonoids naringenin and liquiritigenin. Subramanian et al. (2006) used a similar RNAi mediated approach to silence isoflavone synthase (the entry point enzyme for isoflavone biosynthesis in soybean). Isoflavonoids levels in these plants were below detection and a major decrease in nodulation was observed suggesting that endogenous isoflavones are essential for the establishment of symbiosis between soybean and Bradyrhizobium japonicum. In M. truncatula, RNA interference-mediated suppression of two flavone synthase II (MtFNSII) genes, the key enzymes responsible for flavone biosynthesis, resulted in flavone depleted roots and significantly reduced nodulation providing genetic evidence that flavonones are important for nodulation in M. truncatula as well (Zhang et al. 2007). Combined, this genetic evidence reinforces the importance of flavonoids in nodule initiation and establishment.

Even though flavonoids are the most potent nod gene inducers, other non-flavonoid compounds such as jasmonates, aldonic acid, betaines and xanthones can also induce the expression of nod genes in rhizobia but only at high concentrations (Mabood and Smith, 2005; Gagnon and Ibrahim, 1998; Phillips et al. 1992). In addition to their role in defense response against pathogens, both jasmonic acid and methyl jasmonate strongly induced the expression of nod genes in Bradyrhizobium japonicum (Mabood and Smith, 2005). B. japonicum inoculants pre-incubated with jasmonic acid and methyl jasmonate can
accelerate nodulation, nitrogen fixation, and plant growth of soybean under controlled environment conditions (Mabood and Smith, 2005). While *Lupinus albus* secretes diverse compounds into the rhizosphere, the majority are other non-flavonoid compounds, aldonic acids. The family of aldonic acids, erythronic and tetronic acids (4-C sugar acids) induced the expression of *nod* genes in several bacteria (such as *Rhizobium lupini*, *Mesorhizobium loti*, and *Sinorhizobium meliloti*) and led to low but significant increases in β-galactosidase activities (Gagnon and Ibrahim, 1998). In addition to the flavonoids, alfalfa (*Medicago sativa*) releases two betaines, trigonelline and stachydrine, that induce *nod* genes in *Rhizobium meliloti* (Phillips *et al.* 1994). These compounds are secreted in large quantities by germinating alfalfa seeds. Another plant-derived signal important for bacterial attachment to the plant root, are plant lectins. Lectins are glycoproteins secreted from the tip of root hairs which mediate specific recognition of the bacterial surface carbohydrate molecules. Several experiments have shown the host specificity of plant lectin mediated bacterial attachment by expressing plant lectin genes from one legume species in another and cross-inoculated with non-compatible rhizobia (Diaz *et al.* 1989; van Rhijn *et al.* 1998). Diaz *et al.* (1995) also reported the sugar binding activity of pea lectin in white clover and the localization on the external surface of elongating epidermal cells and tips of emerging root hairs, similar to the result observed in pea.

**Bacterial derived signals**

Rhizobia establish the nodulation symbiosis in different legume plants by exchanging chemical signals with their legume partners. The molecular communication begins on the
bacterial side with the recognition of the flavonoids by rhizobial NodD proteins (NodD1, D2 and D3). These proteins are transcriptional regulators which bind directly to a signaling molecule and activate downstream nod genes (Oldroyd and Downie, 2004; Mandal et al. 2010). Upon activation of the nod genes by the plant signal, the bacteria release species-specific Nod factors to the rhizosphere. Nod factor molecules are lipochito-oligosaccharides consisting of three to five β(1-4) linked N-acetyl-glucosamine residues that are acylated with a fatty acid of 16–20 C-atoms in length on the amino group of the non-reducing glucosamine (Price and Carlson, 1995). The common nod genes nodABC are structural nod genes important for the biosynthesis of the core backbone of all the Nod factors and have a pivotal role in infection and nodulation process (Spaink et al. 1993). The nodABC operons are structurally conserved and functionally interchangeable among different rhizobia without altering the host range (Martinez et al. 1990). This common core structure may, however, be modified by a number of species-specific substituents on the distal or reducing terminal residues which make each bacterial factor unique for each host plant. The substituents include acetate, sulfate, carbamoyl, glycosyl, methyl, arabinose, fucose and mannose groups. Therefore, the host specific nod genes (nodHPQGEFL) are important to specify the different substitution present on the backbone of Nod factors, allowing nodulation of a specific host plant (Brelles-Marino and Ane, 2008). Mutation of these particular genes leads to an extended or altered host range (Djordjevic et al. 1985). In general a correct chemical structure is required for induction of a particular plant response and Nod factor-induced signal transduction cascade.
Nod factors act as signal molecules to simultaneously initiate the nodule formation process programmed in the host plant as well as to trigger the infection process (Kouchi et al. 2010). But several other bacterial molecules are important in the legume-rhizobial interaction. For example, rhizobial extracellular polysaccharides (EPS) are host plant-specific molecules involved in signaling or in root hair attachment. Extracellular polysaccharides are species- or strain-specific polysaccharide molecules with a large diversity in structure and are secreted into the environment or retained at the bacterial surface as a capsular polysaccharide (Laus et al. 2005). EPS-deficient mutants are impaired in efficient induction of root hair curling and especially in infection-thread formation which finally leads to the formation of ineffective nodules (Pellock et al. 2000; van Workum et al. 1998). K-antigen polysaccharides are among the most studied acidic polysaccharides involved in nodulation (Becker et al. 2005). The mutation on both rkpJ and rkpU genes of S. fredii HH103 which are vital for production of K-antigen polysaccharides led to reduced nodulation and starvation for nitrogen; their expression was unaffected by flavonoids (Hidalgo et al. 2010). Hence, other bacterial molecules besides Nod factors play a critical role in the progression of the rhizobia-legume interaction.

**NOD FACTOR SIGNAL TRANSDUCTION PATHWAY**

Genetic studies in the model legumes *M. truncatula* and *L. japonicus* led to the identification of plant genes involved in the early steps in nodulation (Limpens and
Bisseling, 2003; Levy et al. 2004). A series of mutant screens identified a number of key regulators essential for Nod factor (NF) signaling. Similar set of genes have been found for the two model systems and are described below in spatial/temporal order from the surface of the root hair, based on their mutant phenotypes.

At the cell surface are the LysM-RK receptor kinases (LYK3 and NFP in *M. truncatula* and their counterparts NFR1 and NFR5 in *L. japonicus*) which perceive Nod factors and trigger the signal transduction cascade essential for all early symbiotic events (Limpens et al. 2005; Smit et al. 2007; Broghammer et al. 2012). MtNFP is orthologous to LjNFR5 and a knockout mutation in this gene causes complete loss of Nod factor-inducible responses (Amor et al. 2003). *M. truncatula* LYK3, a putative high-stringency receptor that mediates bacterial infection, has been localized in a punctate distribution at the cell periphery, consistent with plasma membrane localization and upon inoculation co-localizes with FLOTILLIN4 (FLOT4) tagged with mCherry, another punctate plasma membrane–associated protein required for infection (Haney et al. 2011). Catoira et al. (2001) reported that the hair curling (*hcl*) mutants in *M. truncatula* altered the formation of signaling centers that normally provide positional information for the reorganisation of the microtubular cytoskeleton in epidermal and cortical cells. Genetic analysis of calcium spiking in *hcl* mutants showed wild type calcium spiking in response to NF suggesting *HCL* acting downstream of earlier NF signaling events (Wais et al. 2000). Using a weak *hcl* allele, *hcl*-4, Smit et al (2007) found that *hcl* mutants were defective in *LYK3* (LysM receptor kinase) and act as Nod factor entry receptor important for both root hair curling
and infection thread formation. Since both MtNFP and MtLYK3 encode trans-membrane receptors containing LysM domains, they were proposed to be good candidates for binding the chitin backbone of NF (Limpens et al. 2003; Arrighi et al. 2006). Recently, two groups reported that the Lotus orthologs of NFP (NFR5) and LYK3(NFR1) make a heterodimer and perceive the rhizobial lipochitin oligosaccharide signal molecules by direct binding (Madsen et al. 2011; Broghammer et al. 2012). A Rho-like small GTPase (LtROP6) from Lotus japonicus was also identified as an NFR5-interacting protein both in vitro and in planta but did not interact with NFR1 (Ke et al. 2012).

Subsequently, Does not Make Infections genes (DMI1, DMI2, and DMI3), and the GRAS type transcription regulators Nodulation Signaling Pathway genes (NSP1 and NSP2) in M. truncatula (Geurts et al. 2005) as well as the SYMRK, CASTOR, POLLUX, Nup85, Nup133 and CCaMK genes of L. japonicus are involved (Ane et al. 2004; Paszkowski, 2006; Zhu et al. 2006; Murakami, 2007). Plants carrying a single mutation in one of these genes are defective for most of the early responses of Nod factor signaling as well as mycorrhization (Paszkowski, 2006; Zhu et al. 2006) with the exception of NSP1 which is nodulation specific (Maillet et al. 2011), indicating that both mycorrhizal and rhizobial symbiosis rely on partially overlapping genetic programs that regulate both signaling pathways. Since the arbuscular mycorrhizal symbiosis is a very ancient association while the legume/rhizobia symbiosis is relatively more recent (Godfroy et al. 2006), the existence of common genes led to a hypothesis that ancestral legumes may have co-opted
part of the signaling machinery of this ancient symbiosis to facilitate the more recent symbiosis with nitrogen fixing rhizobia (Udvardi and Scheible, 2005; Zhu et al. 2006).

As reported by Geurts et al. (2005) and Udvardi and Schieble (2005), among the seven key genes identified in *M. truncatula*, the *DMI1*, *DMI2* and *DMI3* downstream genes are shared between both rhizobial and fungal symbiosis in the *DMI* dependent signaling pathways (Figure 1.2). The *dmi1*, *dmi2* and *dmi3* mutants do not show root hair deformation, gene expression, or mitotic induction of cortical cells but do show swelling at the tip of the root hairs in response to Nod factor and are blocked early in the establishment of mycorrhizal association (Wais et al. 2000; Oldroyd and Long, 2003; Mitra et al. 2004).
Figure 1.2. Nod factor signaling cascade in legume-rhizobia symbiosis. The Nod factor receptors NFR5 and NFR1, consisting of extracellular LysM domains and intracellular kinase domain, are positioned at the surface of the root cells to perceive the Nod factor signal from the bacteria and trigger the downstream events. Downstream genes common to both mycorrhizal and bacterial symbiosis (DMI1, DMI2, DMI3) and the mostly rhizobial symbiosis-specific downstream transcription factors (GRAS protein, NSP1 and NSP2, and NIN) are activated upon Nod factor perception. The two processes simultaneously triggered by Nod factor are the infection process in epidermal cells and nodule organogenesis in cortical cells opposite to the xylem poles.

The DMI1 and DMI3 proteins are highly conserved in most land plants, in contrast to the less conserved DMI2 protein. The DMI2 gene encodes a receptor like kinase with extracellular leucine rich repeats, a transmembrane domain and intracellular kinase domain (Endre et al. 2002; Levy et al. 2004). DMI2 is called NORK for Nodule Receptor Kinase in M. sativa and the corresponding ortholog in L. japonicus is called SYMRK (symbiosis receptor kinase) (Endre et al. 2002; Stracke et al. 2002). The only interacting partner for DMI2 reported thus far is 3-hydroxy-3-methyl-glutaryl-CoA reductase (MtHMGR1) (Kevei et al. 2007). Mutagenesis and deletion analysis showed that the interaction requires the cytosolic active kinase domain of DMI2 and the cytosolic catalytic domain of MtHMGR1 (Kevei et al. 2007). Several interacting partners for SYMRK have been reported. These include SymRK-interacting proteins SIP1 and SIP2, which have essential roles in the early symbiosis signaling and nodule organogenesis (Zhu et al. 2008; Chen et al. 2012) and a SymRK-interacting E3 ubiquitin ligase (SIE3) shown to bind and ubiquitinate SymRK in vitro and in planta (Yuan et al. 2012). The DMI1 gene encodes an ion channel like protein which mediates the early ion fluxes observed in root hairs responding to Nod factors (Ane et al. 2004; Zhu et al. 2006). DMI1 and its orthologs are important either to trigger the opening of calcium release channels or compensate for the charge release during the calcium efflux as counter ion
channels. DMI1 is required for the generation of Nod factor-induced, nucleus-associated Ca$^{2+}$ spikes that are critical for nodule initiation and protein localization to the nuclear envelope of *M. truncatula* root hair cells correlates with the nuclear association of Ca$^{2+}$ spiking (Peiter et al. 2007). Both DMI1 and DMI2 are upstream of calcium spiking and plants with mutations in these genes are blocked for calcium spiking and downstream nodulation events (Shaw and Long, 2003). The DMI1 orthologs CASTOR and POLLUX were initially reported to localize in the plastids of pea root cells and onion epidermal cells (Imaizumi-Anraku et al. 2005). However, a functional DMI1::GFP fusion protein localized to the nuclear envelope in *M. truncatula* roots when expressed both under a constitutive 35S promoter and a native *DMI1* promoter (Riely et al. 2007). Recently, immunogold labeling localized the endogenous CASTOR protein to the nuclear envelope of *L. japonicus* root cells, consistent with a role of CASTOR and POLLUX in modulating the nuclear envelope membrane potential (Charpentier et al. 2008).

Calcium spiking is a central component of the common symbiotic pathway. Recent work using calcium chameleon reporters in *M. truncatula* roots suggests that tightly regulated Ca$^{2+}$-mediated signal transduction is key to reprogramming root cell development at the critical stage of commitment to endosymbiotic infection (Sieberer et al. 2012). Two nucleoporin genes (*NUP133* and *NUP85*), have been identified at an equivalent position in the Nod factor signaling pathway with the DMI1 protein, and are required for the common symbiotic pathways and for calcium spiking responses (Kanamori et al. 2006, Saito et al. 2007). *NUP133* encodes a protein that has sequence similarity to human
nucleoporin Nup133 and localizes in the nuclear envelope, indicating that both NUP133 and NUP85 are member of the nuclear pore complex in legumes (Kanamori et al. 2006). Both genes are required for the calcium spiking that is induced in response to Nod factors but further research is required to clarify the roles of NUP133 and NUP85 in leguminous plants.

DMI3 acts immediately downstream of calcium spiking in the nodulation signaling pathway and is required for both nodulation and mycorrhizal infection (Levy et al. 2004; Figure 2). In contrast to the other mutants mentioned, calcium spiking and root hair swelling in response to Nod factor are wild type in a dmi3 mutant background whereas symbiotic gene expression or cell divisions for nodule formation are defective (Mitra et al. 2004). The DMI3 gene encodes a Ca$^{2+}$ and calmodulin-dependent protein kinase (CCaMK) that responds to the Ca$^{2+}$ signal (Mitra et al. 2004; Geurts et al. 2005). These protein families are multifunctional, with a kinase domain, a calmodulin (CaM) binding domain and a Ca$^{2+}$ binding domain with three EF hands (Oldroyd and Downie, 2004). The CCaMKs have the capacity to bind calcium in two ways, either by direct binding to the three EF hands or by forming a complex with calmodulin to regulate the kinase activity. The interaction of the Ca$^{2+}$ with the C-terminal EF hands results in autophosphorylation of the CCaMK and allows CaM binding, which leads to substrate phosphorylation (Cook, 2004; Levy et al. 2004). In general CCaMK perceives the calcium spiking signature and transduces this to induction of the downstream genes involved in mycorrhizal or rhizobial symbiosis. Split yellow fluorescent protein
complementation and yeast-2-hybrid systems demonstrated that the highly conserved nuclear protein IPD3 is an interacting partner of DMI3 and that the interaction is through a C-terminal coiled-coil domain (Messinese et al. 2007). In a separate report, characterization of three independent retrotransposon Tos17 insertion lines of rice OsIPD3 upon AM fungus *Glomus intraradices* inoculation revealed that the Osipd3 mutants were unable to establish a symbiotic association with *Glomus intraradices* confirming the role of this CCaMK in root symbiosis with AM fungi (Chen et al. 2008).

Beyond this point in the common symbiotic pathway transcriptional regulators of the *NIN, GRAS (NSP1, NSP2) and ERF* families are required for upregulation of nodulation expressed genes and initiation of nodulation (Madsen et al. 2010). Both *NSP1* and *NSP2* are found to encode putative transcriptional regulators of the GRAS protein family (Smit et al. 2005; Kalo et al. 2005). The NSP1 protein has been localized in the nucleus similar to the upstream gene DMI3 (Smit et al. 2005). NSP2, however, migrates from its original location in the nuclear envelope and endoplasmic reticulum into the nucleus where it regulates the transcription of early nodulin genes after Nod factor elicitation (Kalo et al. 2005). Since both NSP1 and NSP2 form a complex (Hirsch et al. 2009) and are genetically downstream of DMI3, at least one of these genes is the target of DMI3 action. Cross species complementation studies also showed *NSP1* and *NSP2* functions are conserved in non-legumes. *OsNSP1* and *OsNSP2* from rice were able to fully rescue the root nodule symbiosis-defective phenotypes of the mutants of corresponding genes in the model legume, *L. japonicus* (Yokota et al. 2010). Recently, Liu et al. (2011) reported that
NSP1 and NSP2 are also a vital component of strigolactone biosynthesis in *M. truncatula* and rice. Mutations on both genes reduced expression of DWARF27, a gene essential for strigolactone biosynthesis (Liu *et al.* 2011). Downstream of NSP1 and NSP2, another putative transcription factor, NIN, first identified in *L. japonicus* is essential for coordinating nodule organogenesis and bacterial entry (Marsh *et al.* 2007). NIN encodes a transmembrane transcriptional regulator with homology to Notch of Drosophila (Schauser *et al.* 1999). Early NF-induced gene expression using an *ENOD11: GUS* reporter fusion in the *Mtnin-1* mutant showed that *MtNIN* is not essential for early nod factor signaling but may function downstream of the early NF signaling pathway to coordinate and regulate temporal and spatial formation of root nodules (Marsh *et al.* 2007). The perception of Nod factors also leads to the activation of another transcription factor with DNA binding capability, ERN1, an AP2-like transcription factor in the ERF subfamily, which is necessary for nodulation and functions in early Nod factor signaling (Middleton *et al.* 2007). Mutations in *ERN* block the initiation and development of infection threads and thus block nodule invasion by the bacteria. *ERN1* is induced rapidly after *S. meliloti* inoculation and is necessary for Nod factor–induced gene expression. Unlike wild type plants, *ern1* mutants do not form spontaneous nodules when transformed with activated calcium- and calmodulin-dependent protein kinase, and Nod factor application does not induce *ENOD11:GUS* expression (Middleton *et al.* 2007). A second ERF transcription factor, EFD (for ethylene response factor required for nodule differentiation) is required for the differentiation of functional Fix*+* nodules and may participate in an ethylene-independent feedback inhibition of the nodulation process as
well as regulating the expression of the primary cytokinin response regulator MtRR4 (Vernie et al. 2008).

The formation of nodule primordia involves dedifferentiation and reactivation of cortical root cells to establish the nodule primordium, a mass of rapidly proliferating undifferentiated cells, opposite to the protoxylem poles (Timmers et al. 1999; Penmetsa and Cook, 1997). Both gain-of-function and loss-of-function mutants have shown that cytokinin signaling through the cytokinin receptor kinase (LHK1) is important for reactivation of cortical cells (Tirichine et al. 2007; Murray et al. 2007). Plet et al. (2011) also reported cytokinin signaling in M. truncatula integrates bacterial and plant cues to coordinate symbiotic nodule organogenesis in an MtCRE1 dependent manner. Simultaneous with the formation of the primordia are the endocytic-like entry of the bacteria to the plant root cells, associated with the plant driven infection thread formation, bacterial cell division within the infection thread, progression of the infection threads towards the dividing nodule primordia and finally the invasion of the developing nodule. Several genes important to Nod factor recognition have been reported to affect infection thread initiation and growth. The M. truncatula Hair Curling (HCL) gene encodes the LYK3 receptor-like kinase a specific function of which is to initiate the infection thread on Nod factor recognition (Limpens et al. 2003; Smit et al. 2007). Besides its role in nodule initiation, the receptor kinase DMI2 plays a key role in symbiosome formation and is expressed both on the host cell plasma membrane and the membrane surrounding the infection thread (Limpens et al. 2005). Also required for both
infection thread growth in root hair cells and the further development of nodule primordia is the orthologous \textit{LIN/CERBERUS} genes in \textit{M. truncatula} and \textit{L. japonicus}, which encode predicted E3 ubiquitin ligases containing a highly conserved U-box and WD40 repeat domains functions and function at an early stage of the rhizobial symbiotic process (Kiss \textit{et al.} 2009 Yano \textit{et al.} 2009). The Vapyrin (VPY) gene is essential for the establishment of the arbuscular mycorrhizal symbiosis and is also important for rhizobial colonization and nodulation acts downstream of the common signaling pathway (Murray \textit{et al.} 2011). In addition, flotillin (FLOT2 and FLOT4) and remorin (MtSYMREM1) proteins, which promote trafficking and aggregation of membrane proteins, are required for infection by rhizobia, possibly by acting as scaffolds for recruitment of membrane proteins involved in nodulation signaling (For review see Oldroyd \textit{et al.} 2011).

**NODULE AUTOREGULATION**

The symbiosis between leguminous plants and rhizobia under conditions of nitrogen limitation, leads to the development of new plant organs, the N$_2$-fixing nodules that are usually formed on roots but also on stems in a few plants. The bacteria require energy and a suitable environment for nitrogenase, the enzyme important for nitrogen fixation (Crawford \textit{et al.} 2000). Hence, the nodulation process is energy intensive and the plants need to maintain a balance between cost and benefit by limiting the number of nodules that form. Plants use local and long-distance or systemic signaling to coordinate and regulate the number of nodulation events (Kosslak and Bohlool, 1984; Caetano-Anolles and Gresshoff, 1991). This feedback inhibition, in which the earlier nodulation events
suppress the subsequent development of nodules in young tissues, is called Autoregulation of Nodulation (AON) (Pierce and Bauer, 1983; Searle et al. 2003; Oka-kira and Kawaguchi, 2006). AON employs root-derived and shoot-derived long-distance signals. The root-derived signal is generated in roots in response to rhizobial infection and then translocated to the shoot, while the shoot-derived signal is generated in shoot and then translocated back to the root to restrict further nodulation. AON is activated upon the perception of Nod factor in the elongation zone of the root with emerging root hairs where rhizobial infection occurs being most affected (Bhuvaneswari et al. 1981).

AON is under both environmental and developmental controls and appears to be universally used by legumes to control the extent of nodulation. Mutations affecting AON lead to supernodulation or hypernodulation, associated with root developmental defects. Genetic analysis of AON began with the isolation of supernodulating mutants, which have lost their ability to autoregulate nodule number. AON mutants are characterized by the formation of an excessive number of nodules and a short root compared to their wild type counterparts. For instance, Glycine max nts (Carroll et al. 1985); Lotus japonicus har1 (Krusell et al. 2002), tml (Magori et al. 2009) and klavier (Oka-Kira et al. 2005); Pisum sativum sym29 (Krusell et al. 2002) and nod3 (Sidorova and Shumnyi, 2003); Medicago truncatula sunn (Penmetsa et al. 2003; Schnabel et al. 2005), lss (Schnabel et al. 2010) and rdn1 (Schnabel et al. 2011) mutants are defective in autoregulation and thus form an excessive number of nodules. The NTS/NARK, HAR1, SYM29 and SUNN genes encode a leucine-rich repeat receptor kinase with homology to
Arabidopsis CLAVATA1 (Searle et al. 2003; Krusell et al. 2002; Schnabel et al. 2005). KLAVIER also encodes a different LRR receptor kinase (Miyazawa et al. 2010) while NOD3 and RDN1 encode proteins of unknown function (Schnabel et al. 2011). Since autoregulation is mediated through long distance signaling involving shoot and root, shoot to root reciprocal grafting studies using wild type and autoregulation defective mutants revealed that there are both shoot-controlled as well as root-controlled supernodulators (Carroll et al. 1985; Penmetsa et al. 2003; Magori et al. 2009). It is believed that the shoot-controlled supernodulators are impaired either in the perception of the root-derived infection signal or in the transmission of the shoot-derived autoregulation signal. On the other hand, the root-controlled mutants are thought to be impaired in either the transmission of the root-derived infection signal or in the perception of the shoot-derived autoregulatory signal.

Nodule initiation and development are also determined by physiological conditions and phytohormones. Successful nodule formation and subsequent nitrogen fixation occur normally only under nitrogen limiting conditions (Schultze and Kondorosi, 1998). However, the mutants defective in AON are partially nitrate tolerant (Caba et al. 1998; Carroll et al. 1985). This suggests that at some stage the autoregulation signal and the nitrate signal talk to each other to inhibit nodule formation. In addition, the gaseous phytohormone, ethylene, is a negative regulator of nodule organogenesis. The M. truncatula mutant skl, encoding an EIN2 ortholog (Pentmetsa et al. 2008), is insensitive to ethylene and shows a 10-fold increases in nodule number relative to the wild type
Rhizobial inoculation and exogenous ACC induce ethylene synthesis and thereby lead to suppressed nodule and root development in *sunn* mutants (Penmetsa *et al.* 2003). Similarly, the addition of the ethylene inhibitors like L-α-aminoethoxyvinyl-glucine enhanced nodule development in common bean and pea (Guinel and Sloetjes, 2000; Tamimi and Timko, 2003). Another phytohormone, auxin, is mostly produced in younger plant shoots and moves long distance to the root tip following an auxin concentration gradient to trigger root, nodules and other plant organ development (Pacios-Bras *et al.* 2003; Schnabel and Frugoli, 2004). In fact, IAA produced by the rhizobia is reported to increase nodule formation (Pii *et al.* 2007). In uninoculated roots of *sunn* mutant plants, auxin transport from shoot to root is approximately 3 times higher than in wild type plants (van Noorden *et al.* 2006) and auxin transport inhibitors such as NPA significantly reduce nodulation in wild type plants and *sunn* mutants but not *skl* mutants (van Noorden *et al.* 2006; Prayitno *et al.* 2006), suggesting a role for auxin in regulating nodule number as well.

The plant hormone, cytokinin is also implicated in nodulation. Exogenous application of cytokinins to legume roots induced responses similar to rhizobial Nod factors, including cortical cell division, amyloplast deposition, and induction of early nodulin gene expression (Bauer *et al.* 1996). Gonzalez-Rizzo *et al.* (2006) identified an *M. truncatula* homolog of Arabidopsis *HISTIDINE KINASE 4 (CRE1)*, a histidine kinase cytokinin receptor. Using RNA interference to downregulate *MtCRE1* they demonstrated that *MtCRE1* acts as a negative regulator of lateral root formation and as a positive regulator
of nodulation. Expression analysis of genes downstream in cytokinin signaling, \textit{MtRR1} and \textit{MtRR4}, and the early nodulin \textit{MtNIN1} in \textit{M. truncatula} suggests that these three genes are involved in crosstalk between Nod factor and cytokinin signaling pathways depending on \textit{MtCRE1} (Gonzalez-Rizzo \textit{et al.} 2006). Cytokinin activation of \textit{MtCLE13}, a short peptide involved in nodulation (Mortier \textit{et al.} 2010) depends on \textit{MtCRE1} and NIN but not on NSP2 and ERN1, suggesting two parallel pathways triggered by cytokinin in the root cortex; activation of cortical cell division and activation of \textit{MtCLE13} to inhibit further nodulation (Mortier \textit{et al.} 2012).

In addition, CLE genes (12-13 amino acid long secreted peptides) which are involved in both shoot and root meristem homeostasis, vascular differentiation and nodulation, comprise a gene family of up to 40 members and play a role in either activation of the root derived AON signal or have the potential to interact with leucine-rich repeat receptor kinases such as SUNN (Mortier \textit{et al.} 2010). Identification of these AON genes in combination with phytohormones and other growth regulators, and the intensive study of nodulation signaling cascade will aid understanding of the fascinating and complex events leading to legume nodule formation and regulation and plant development in general.

**NODULE SENESCEENCE**

Functional nodules are not maintained throughout the life cycle of the host plant. The peak nitrogen fixation period in both determinate and indeterminate nodules is restricted
to between 3 to 5 weeks after infection (Lawn and Burn 1974; Puppo et al. 2005). During the vegetative growth stage and flowering, nodules are the major carbon sink in legumes. In the course of pod filling, however, the seed is the strongest sink for photosynthate and nodules start to gradually senesce. The first symptoms of senescence are the deterioration of leghemoglobin, resulting in a pink to green color change in the nodule and loss of turgidity in old nodules (Perez Guerra et al. 2010). In mature indeterminate nodules the senescence zone starts in zone IV (Figure 1). Upon aging, this senescence zone gradually moves in a proximal-distal direction until it reaches the apical part and leads to nodule degeneration (Puppo et al. 2005; Van de Velde et al. 2006). On the other hand, in determinate nodules it expands radially from the center to the periphery. The primary targets for nodule senescence are symbiosomes, in the same manner as chloroplasts in leaf senescence, with several common senescence associated genes both up and down regulated in leaf and nodule senescence suggesting a shared pathway (Van de Velde et al. 2006). Since symbiosomes are derived from the uptake of prokaryotic cells that fix nitrogen and chloroplasts are postulated to have originated from the uptake of cells that fix carbon, this common pathway is not surprising. Using transmission electron microscopy, two consecutive stages were distinguished during nodule senescence: a first stage, characterized by bacteroid degradation with a few dying plant cells, and a more advanced stage of nodule senescence, during which cells had completely resorbed their symbiosomes and started to decay and collapse (Van de Velde et al. 2006). Hence, the final fate of the bacteria and the plant cells that form the nitrogen fixing organelle is death. Plant cysteine proteinases are important in controlling nodule senescence. An
Asnodf32 protein which encodes a nodule-specific cysteine proteinase in *Astragalus sinicus* was reported to play an important role in the regulation of root nodule senescence. In Asnodf32-silenced hairy roots, the period of bacteroid active nitrogen fixation was significantly extended and enlarged nodules were also observed (Li et al. 2008). Recently a *M. truncatula* transcription factor, MtNAC969, was also reported to participate in nodule senescence. MtNAC969 is induced by nitrate treatment, similar to senescence markers was antagonistically affected by salt in roots and nodules; MtNAC969 RNAi silenced nodules accumulated amyloplasts in the nitrogen-fixing zone, and were prematurely senescent (de Zelicourt et al. 2012). Nodule senescence is an active process programmed in development, thus reactive oxygen species, antioxidants, hormones and proteinases also play a key role (Puppo et al. 2005).

**PLANT ENDOMEMBRANE SYSTEM**

Vesicle trafficking is a phenomenon essential for many processes in multicellular organisms including symbiotic interactions. Organelles in the endomembrane system (vesicle trafficking) are part of the dynamic network in which materials are shuttled in (endocytosis) and out (exocytosis) of the cell. By following the endocytosis pathway, materials move from the outer cell surface to different compartments of the cytoplasm of the cell. In animal cells, the cargo molecules upon internalization are transported to early endosomes, distributed to late endosomes and lysosomes, and molecules may be recycled back to the plasma membrane from early endosomes (Mellman, 1996). In comparison to animal cells, endocytosis in plants is ill-defined and has recently undergone a paradigm
shift, with strong evidence for a different route of trafficking in plants. Clathrin-mediated endocytosis is reported as the predominant endocytic mechanism in plant cells (Chen et al. 2011). Several studies support that endocytosis begins by internalizing plasma membrane (PM) proteins and cell wall pectins to the early endosomes where the cargo is further sorted, either for recycling back to the PM, or to the vacuole for degradation (Geldner et al. 2001; Shah et al. 2002; Baluska et al. 2002; McMahon and Boucrot, 2011). The relocalization of polarly localized PIN auxin efflux carriers (PIN1 and PIN2) from Arabidopsis are good examples of clathrin-mediated endocytosis in plants (Dhonukshe et al. 2007). Among many roles, plant endocytosis is important for polar tip growth, auxin-mediated cell–cell communication, gravitropic responses and defensive responses against pathogenic attack (Voigt et al. 2005; Jaillais et al. 2006; Dhonukshe et al. 2007; Leborgne-Castel et al. 2008).

As eukaryotic cells have the ability to internalize a variety of macromolecules by endocytosis, they also have a secretory pathway composed of functionally similar distinct membrane compartments but opposite in flow to endocytosis. Secretion of proteins and other molecules is the primary means by which a cell interacts with its surroundings. The secretory pathway consists of intracellular membrane structures: the endoplasmic reticulum (ER), the Golgi apparatus, and vesicles. Proteins travel from the ER through the Golgi apparatus to arrive at the cell surface or at vacuoles. ER is the site of protein synthesis, folding and assembly, cleavage of signal peptides from nascent proteins, N-linked glycosylation, quality control (a possible site of degradation), and final export of
cargo proteins to Golgi apparatus (Vitale and Denecke, 1999). In plants, the Golgi maintains a close physical relationship with the ER for shuttling molecules back and forth (Boevink et al. 1998). The Golgi apparatus is the central organelle mediating protein and lipid transport within the eukaryotic cell. Polysaccharides and secreted proteins reach the cell wall after their production or modification in the Golgi apparatus (Driouich et al. 1993). Numerous secreted proteins are constantly trafficking through the Golgi from cis-to-trans Golgi in the direction of secretion. Both endocytosis and exocytosis interact at trans-Golgi network (TGN) (Voitti et al. 2010). Recent results suggest the trans-Golgi network in plants may be considered either as an integral part of the endocytic network or as an independent organelle—the early endosome for plants (Dettmer et al. 2006; Lam et al. 2007; Voitti et al. 2010). After further processing and sorting the cargo molecules, vesicles bud from Golgi and transported either to the vacuole for storage and degradation or to the plasma membrane for secretion. Secretory vesicles either tether at the plasma membrane (Steyer et al. 1997) or coalesce with the plasma membrane and form fusion pores, resulting in the release of vesicle contents into the extracellular matrix (Thiel and Battey, 1998). Proteins in the secretory pathways have several roles in plant development. Examples include the Keep on Going (KEG) protein in Arabidopsis that regulates signaling during stress responses (Gu and Innes, 2011), the CLE peptides that control nodule formation in legumes and the pattern of root growth and lateral root development in Arabidopsis (Mortier et al. 2010; Okamoto et al. 2009; Meng et al. 2012) and the Root Meristem Growth Factor (RGF) secreted peptide signals required for maintenance of root stem cell niche in Arabidopsis (Matsuzaki et al. 2010). In general,
secretory proteins are important for intercellular signaling that coordinate and specify cellular functions in plants.

**SCOPE OF THE STUDY**

As world population grows alarmingly (~7 billion according to US census records), massive pressure is being placed on arable lands, water, energy, and biological resources to provide an adequate supply of food while preserving the integrity of the natural ecosystem. Since the advent of the green revolution most agricultural production has been reliant on industrial fertilizers to periodically replenish the nutrient reserve of agricultural soils. The production and application of these chemical fertilizers is a major source of pollution as well as the major use of energy in agricultural systems. Hence, maintaining sound and productive agricultural system using alternative biological resources is critical to survival. Researchers have been looking for a natural nitrogen fertilizer replacement giving special emphasis for Biological Nitrogen Fixation (BNF). BNF is of great importance in agriculture contributing a large share of usable nitrogen to the biosphere. N\textsubscript{2}, which makes up 80% of the atmosphere, contains a triple bond, rendering it so tightly bonded that it does not participate in most chemical reactions and is not available to most biological organisms. Rhizobia in association with legumes overcome this limitation by breaking the triple bond and converting the molecular nitrogen to available forms like ammonia. Expansion of this association to other plants would ease the long-term pressure for expanded industrial production.
Tremendous progress has been made in cloning the genes that are essential for legume-rhizobial symbioses from the two model legumes, *M. truncatula* and *L. japonicus*. However, most of the legume-rhizobia interaction studies to date concentrated on the initial stages involved in nodulation. In the past few years, several shoot controlled hypernodulation mutants have been characterized and published contributing to our understanding of the opposite of nodule initiation, nodule regulation. To understand the molecular mechanism involved in nodule regulation, establish a model for the signal transduction pathway, and in long run to utilize the findings for plant production improvements, we have explored both shoot- and root-controlled hypernodulation mutants. This study deals mainly with *rdn1*, a novel root controlled hypernodulator in the autoregulatory pathway of *M. truncatula*. Here, I report the cloning of *MtRDN1* and the investigation of the function and molecular mechanisms of RDN1 action in nodule number regulation, including the subcellular level localization of *MtRDN1*. I report the establishment of a split root system and inverted-y grafting approach in *M. truncatula* to determine the timing of Autoregulation of Nodulation (AON) and investigate where and when RDN1 acts in the AON pathway. I employed cross-species complementation to determine the role of RDN genes beyond nodule number regulation and characterized Arabidopsis T-DNA insertion lines to investigate the non-symbiotic role of RDNs.
REFERENCES


legumes requires NSP2, a member of the GRAS family of transcriptional regulators. Science 308: 1786-1789.


CHAPTER 2

Note for doctoral dissertation: Tessema Kassaw, in addition to manuscript writing & editing for these two papers, contributed part of the data for Figure 2.1, 2.2 and 2.10 and all of the data for Figures 2.3, 2.4, 2.8, and S2.1. Material in this chapter has been published in Plant Physiology (2011) 157: 328-340 and Plant Signaling and Behavior (2012) 7:1-3 and is reproduced here with permission of the publishers

Plant Physiology: The ROOT DETERMINED NODULATION 1 gene regulates nodule number in roots of Medicago truncatula and defines a highly conserved, uncharacterized plant gene family.

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Plant Signaling & Behavior: The *M. truncatula* SUNN gene is expressed in vascular tissue, similarly to *RDN1*, consistent with the role of these nodulation regulation genes in long distance signaling

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ABSTRACT

(Paper 1)

The formation of nitrogen-fixing nodules in legumes is tightly controlled by a long-distance signaling system in which nodulating roots signal to shoot tissues to suppress further nodulation. A screen for supernodulating *Medicago truncatula* mutants defective in this regulatory behavior yielded loss-of-function alleles of a gene designated *ROOT DETERMINED NODULATION 1* (*RDN1*). Grafting experiments demonstrated that *RDN1* regulatory function occurs in the roots, not the shoots, and is essential for normal nodule number regulation. The *RDN1* gene, *Medtr5g089520*, was identified by genetic mapping, transcript profiling, and phenotypic rescue by expression of the wild type gene in *rdn1* mutants. A mutation in a putative *RDN1* ortholog was also identified in the supernodulating *nod3* mutant of pea. *RDN1* is predicted to encode a 357 aa protein of unknown function. The *RDN1* promoter drives expression in the vascular cylinder, suggesting RDN1 may be involved in initiating, responding to, or transporting vascular signals. *RDN1* is a member of a small, uncharacterized, highly conserved gene family unique to green plants, including algae, that we have named the *RDN* family.
INTRODUCTION

Legume plants benefit from their symbiosis with rhizobial bacteria because the bacteria are able to fix molecular nitrogen and share it with the plant, allowing legumes to grow under nitrogen limiting conditions. In exchange, the plant provides the rhizobia residing in root nodules with fixed carbon from photosynthesis. The interaction is complex and involves multiple layers of regulation by both partners. Genetic analysis of nodulation, initially begun because of the potential for agricultural improvement offered by understanding nitrogen fixing symbioses, has revealed regulators relevant both to nodule formation and to non-leguminous plants (Kouchi et al. 2010).

The establishment of the symbiosis follows a similar pattern in most legumes. Legume roots secrete flavonoid signals into the rhizosphere. Rhizobia respond to flavonoids by producing a lipochitin oligosaccharide termed Nod factor. Perception of species-specific Nod factor by the compatible species of legume triggers Ca\(^{2+}\) spiking in root hair cells and induces changes in gene expression. Perception also results in a physical response; the plant-root hair cell curls to sequester the bacteria. In indeterminate nodulators such as pea (Pisum sativum) and alfalfa (Medicago sativa) the inner cortical cells leave the G0 stage of the cell cycle and begin to divide. At the same time, the plant forms a structure called an infection thread, which allows the trapped, dividing bacteria to pass through the root hair and epidermal and outer cortical cells to be released in symbiosomes within the dividing inner cortical cells. The resulting structure, called a
nodule, establishes the physical and biochemical environment to support nitrogen fixation (reviewed in Oldroyd and Downie, 2008).

Because the maintenance of active nodules has an energy cost to the plant estimated at 12-17 grams of carbon per gram of nitrogen obtained (Crawford et al. 2000), regulation of nodule number by the plant is presumed important to balance the need for fixed nitrogen with the cost of supporting the rhizobia. In addition to regulating nodule initiation based on available nitrogen status, the plant regulates spatial location of the nodules and the number of nodules that form in a given symbiotic interaction (reviewed in Ferguson et al. 2010). In wild type plants, early nodules suppress the development of later nodules (autoregulation of nodulation-AON (Caetano-Anollés and Gresshoff, 1991). Grafting experiments demonstrated that AON involves whole plant signal transduction as well as local signaling events (Delves et al. 1986). Genetic analysis of AON has identified several mutants with an increased number of nodules, often accompanied by an inability to regulate nodule number based on nitrogen status and by abnormalities in root length and lateral root formation. The nodules formed on these mutants have normal morphology and are able to fix nitrogen.

Genes corresponding to these mutants can be divided into those with disruptions that regulate nodule number from the shoot (shoot-controlled supernodulators) and those with a point of action in the root (root-controlled supernodulators). For some of these supernodulators the corresponding gene has been cloned, while others are presently...
identified only by phenotype. Additional genes are likely to be involved in the pathway, as evidenced by nodulation phenotypes that result from gene overexpression, but are not yet represented by mutations in the genes themselves.

The first AON gene cloned, *HAR1* in *Lotus japonicus* (ortholog *Sym29* in pea), encodes a leucine-rich repeat receptor-like kinase (LRR-RLK) with homology to the Arabidopsis (*Arabidopsis thaliana*) meristematic regulator *CLV1*. *HAR1* functions in the shoots to regulate nodulation (Krusell *et al.* 2002; Nishimura *et al.* 2002c). Orthologs in soybean (*Glycine max*; *NARK*) (Searle *et al.* 2003) and *M. truncatula* (*SUNN*) (Schnabel *et al.* 2005) have also been identified. Plants with mutations in these genes display shortened roots, excessive nodules (5- to 10-fold more than wild type plants), nodulation in the presence of nitrate levels that prevent nodulation in wild type plants, and in some cases excessive lateral roots (Carroll *et al.* 1985; Sagan and Duc, 1996; Wopereis *et al.* 2000; Schnabel *et al.* 2005). Identified as an independent genetic lesion, the *lss* shoot-controlled supernodulator in *M. truncatula* displays reduced *SUNN* expression (Schnabel *et al.* 2010). Another gene encoding an LRR-RLK involved in shoot regulation of nodulation, *KLAVIER (KLV)* in *L. japonicus*, has recently been identified (Miyazawa *et al.* 2010). The *klv* mutant, like *har1* mutants, supernodulates and is able to nodulate in the presence of abundant nitrate. Additionally, the *klv* mutant has dwarf shoots and roots, altered vascular and floral development, and delayed flowering (Oka-Kira *et al.* 2005). Shoot-controlled supernodulators with similar
nodulation phenotypes but for which the molecular identity is unknown include *ntsn* in bean (Park and Buttery, 1989) and *sym28* in pea (Sagan and Duc, 1996).

A number of root-controlled AON loci have been identified by mutational analysis, but only one, the *M. truncatula* EIN2 ortholog *SICKLE*, has been cloned (Penmetsa et al. 2008). The supernodulation phenotype of *sickle* mutants, which have disrupted ethylene signaling, demonstrates the role of ethylene in controlling nodulation. The mutants *rdh1*, *tml* and *plenty* of *L. japonicus* and *nod3* of pea-like *har1/sym29/nark/sunn*, form short roots with excessive nodules and nodulate in the presence of nitrate, but the nodulation phenotype of a grafted plant depends on the genotype of the root, not the shoot (Postma et al. 1988; Ishikawa et al. 2008; Magori et al. 2009; Yoshida et al. 2010). None of these mutants appear to have a defect in ethylene signaling.

The *astray* mutant of *L. japonicus* has approximately twice the nodules of wild type plants (Nishimura et al. 2002a) which is termed “enhanced” rather than supernodulation. Also in contrast, nodulation in this mutant is sensitive to nitrate in the same degree as wild type. *ASTRAY* encodes a basic leucine zipper protein with a RING-finger motif, but whether it acts in the shoot or root has not been reported (Nishimura et al. 2002b).

Overexpression of nodulation-induced CLE peptides (Mortier et al. 2010; Okamoto et al. 2010) has been shown to reduce nodule number. In *L. japonicus*, overexpression of
LjCLE-RS1 or LjCLE-RS2 systemically reduces nodule number in a HAR1-dependent manner (Okamoto et al. 2009), while in soybean overexpression of the CLE peptides RIC1, RIC2 or NIC1 systemically reduce nodulation in a NARK-dependent manner (Reid et al. 2011a). Similar effects of MtCLE12 or MtCLE13 overexpression are seen in M. truncatula (Mortier et al. 2010). Additionally overexpression of MtCLE12 and MtCLE13 in roots impacts shoot growth, allowing speculation that the CLE peptides act as long distance signaling molecules. However, long distance transport of CLE peptides in any system has not been demonstrated.

Plant hormones have also been shown to be involved in nodule number regulation. The sunn-1 mutant has a defect in long distance auxin transport that may affect nodule number (van Noorden et al. 2006); cytokinin receptor mutations can suppress the nodule number defect of har1 (Murray et al. 2007) and sunn-1 (E. Schnabel and J. Frugoli, unpublished data); and inducing abscisic acid insensitivity by expression of a dominant negative allele of Arabidopsis ABSCISIC ACID INSENSITIVE 1 results in hypernodulation (Ding et al. 2008). Methyl jasmonate and brassinosteroid have also been implicated in nodule number regulation (Nakagawa and Kawaguchi, 2006; Terakado et al. 2006).

Here we report the cloning of a gene from M. truncatula and its ortholog in pea with an essential root-localized function in AON. The ROOT DETERMINED NODULATION 1 (MtRDN1) gene and PsNOD3 are members of a previously uncharacterized gene family
conserved across the plant kingdom from green algae to higher plants. *RDN1* encodes a protein of unknown function that appears to be expressed at low levels in the vasculature of *M. truncatula*. Although *RDN1* is involved in the legume AON signal transduction pathway, the high level of conservation of *RDN* family genes throughout the green plant lineage suggests a role for RDN family proteins in basic plant function.
RESULTS

Identification and mapping of a root controlled supernodulation locus in *M. truncatula*
Supernodulating mutants of *M. truncatula* were identified by a visual screen of fast neutron bombardment M2 seedlings for nodulation phenotypes. Grafting experiments demonstrated that for four mutants (GY15-2E3, D39-13F-V1, D39-1H-T2, and D39-9X-V2) the supernodulation phenotype was conferred by the root tissue (Table 1.1). None displayed the delayed petal senescence phenotype seen in plants with lesions in the *SICKLE* gene (Penmetsa and Cook, 1997), the only root-determined supernodulation locus reported thus far in *M.*

Table 2.1. Grafting experiments to determine site of action of RDN1. Number of nodules formed on grafted seedlings 7 to 10 days after inoculation with *Sinorhizobium medicae* ABS7. Shown is average nodule number per seedling ± standard error (number of seedlings).

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Allele</th>
<th>Mutant shoot/Wildtype root</th>
<th>Wildtype shoot/mutant root</th>
</tr>
</thead>
<tbody>
<tr>
<td>GY15-2E3</td>
<td><em>rdn1</em>-1</td>
<td>11 ± 1.7 (7)</td>
<td>34 ± 7.4 (8)</td>
</tr>
<tr>
<td>D39-13F-V1</td>
<td><em>rdn1</em>-2</td>
<td>13 ± 1.0 (15)</td>
<td>49 ± 14.0 (3)</td>
</tr>
<tr>
<td>D39-1H-T2</td>
<td><em>rdn1</em>-3</td>
<td>9 ± 1.0 (11)</td>
<td>63 ± 2.5 (7)</td>
</tr>
<tr>
<td>D39-9X-V2</td>
<td><em>rdn1</em>-4</td>
<td>12 ± 2.1 (8)</td>
<td>56 ± 4.9 (5)</td>
</tr>
</tbody>
</table>
truncatula, and therefore these new mutants were presumed to define at least one new supernodulation locus in M. truncatula and have been designated root determined nodulation 1 (rdn1) mutants. Genetic mapping was performed for three of the mutants. In each case the supernodulation phenotype cosegregated with markers on the bottom of chromosome 5 to a region syntenic with the Psnod3 supernodulation locus region (Figure 2.1) (Temnykh et al. 1995; Gualtieri et al. 2002). A 1291 kb region centered on marker h2_7n20d was delineated for mutant D39-1H-T2 (rdn1-3), an approximately 2900 kb region for mutant D39-13F-V1 (rdn1-2), and much larger region for GY15-2E3 (rdn1-1).

Figure 2.1 Positional cloning of rdn1 alleles. The rdn1 locus was mapped to the distal end of the long arm of chromosome 5 using publically available markers. The alignment of the rdn1 region to the syntenic region of the nod3 supernodulation locus of pea is shown. Fine mapping defined a 1291 kb region for the rdn1 locus flanked by markers TK20M5-1 and TK103O8-1. Multigene deletions found in three of the rdn1 mutants (rdn1-1, rdn1-3, and rdn1-4) are indicated with grey boxes. The 103 kb region missing in common between the three alleles (indicated with black vertical lines) spans predicted genes
Medtr5g089510 through Medtr5g089720 (IMGAG v3.5). Medtr5g089520 was found to be altered in rdn1-2 by an indel. cM (centimorgan).

Transcript profiling of the *rdn1-1* mutant versus A17 on the GeneChip® Medicago Genome Array (Mitra *et al.* 2004) identified three genes in the 1291 kb mapped region with significantly reduced signals in the mutant. PCR analysis revealed that *rdn1-1*, *rdn1-3*, and *rdn1-4* have large nested deletions of 103 kb, 209 kb, and approximately 240 kb, respectively, spanning two of these genes (Figure 2.1). In the genomic segment corresponding to the shortest deletion, seventeen genes have been annotated by the International Medicago Genome Annotation Group (IMGAG v3.5). In the *rdn1-2* mutant one of these seventeen candidate genes (*Medtr5g089520*) is altered by a 1 kb indel. These data are consistent with the *rdn1* mutants representing four alleles of a gene we designate *RDN1*.

**rdn1 mutants have increased nodulation and reduced root growth**

The extent of nodulation of the *rdn1-1* mutant was compared to that of wild type using an aeroponic growth chamber. Seedlings were grown under nitrogen-limiting conditions, which favor the development of nodules, and assessed ten days after inoculation with the compatible rhizobia *Sinorhizobium medicae* strain ABS7 (Bekki *et al.* 1987). An average of five times more nodules formed on *rdn1-1* than on wild type (Figure 2.2A).
Figure 2.2. Supernodulation phenotype of \textit{rdn1-1}. Seedlings of the two genotypes were grown together in an aeroponic chamber containing nutrient solution lacking nitrogen (A) or supplemented with 10 mM \(\text{NH}_4\text{NO}_3\) (B) and were assayed after 14 d of growth (10 d post inoculation with \textit{S. medicae}). Nodules per plant (means ± se) are shown (A, 40 to 45 plants of each genotype from three combined experimental replicates; B, 17 to 20 plants per genotype from two combined experimental replicates). Student’s t-tests were used to determine significance of differences from wild type (* \(P < 0.0001\)).

Because previously isolated supernodulation mutants, such as the \textit{sun}n mutants, have been reported to have nitrate tolerant nodulation, nodulation of \textit{rdn1-1} in the presence of supplemental nitrogen was evaluated (Figure 2.2B). Aeroponic growth chambers supplemented with 10 mM \(\text{NH}_4\text{NO}_3\) were run side by side with the nitrogen-limited
growth chambers. Under these conditions, wild type roots had very limited nodulation, with the majority of seedlings producing no nodules. In contrast, \textit{rdn1-1} produced abundant numbers of nodules, although the numbers formed were less than what was seen in plants grown without \textit{NH}_4\textit{NO}_3. We conclude that supplemental nitrogen has a moderate suppressive effect on nodulation in \textit{rdn1-1} similar to what has been seen previously in \textit{sunn} mutants.

Root length in \textit{sunn} mutants is shorter than in wild type plants even in the absence of rhizobia (Schnabel \textit{et al.} 2005; Schnabel \textit{et al.} 2010) presumably due to shorter cells (van Noorden \textit{et al.} 2006). Root growth in \textit{rdn1} mutants was evaluated to see if disruption of the \textit{RDN1} locus also impacts root length. The growth rate of \textit{rdn1-1} and \textit{rdn1-2} roots was less than that of wild type roots and resembled that of \textit{sunn-4} roots (Figure 2.3). No other obvious gross morphological differences were noted between \textit{rdn1} mutants and wild type plants. Phenotypes similar to those of \textit{rdn1-1} were observed for the other \textit{rdn1} alleles (data not shown).
Figure 2.3. Root growth in rdn1 mutants compared to wild type (A17) and the sunn-4 supernodulation mutant. Root lengths of seedlings were measured after fourteen days of growth in an aeroponic chamber containing nutrient solution supplemented with 5 mM NH₄NO₃ as a nitrogen source. Means ± SE are shown. n = 8 plants per genotype. Student’s t-tests were used to determine the significance of differences from wild type (*P < 0.001).

Rescue of the rdn1 phenotype
Based on mapping, transcript profiling, and PCR analysis we identified a candidate gene, Medtr5g089520, for RDN1. To verify that Medtr5g089520 was RDN1, the full-length coding sequence driven by the CaMV35S promoter (35Spro::RDN1) was introduced into rdn1-1 and rdn1-2 mutant roots by Agrobacterium rhizogenes mediated transformation using a T-DNA vector also carrying a UBQ10pro::DsRed1 reporter (see Materials & Methods). The same vector carrying only the reporter was used as a control. Plants transformed by this method can produce both transgenic and non-transgenic roots. All non-transgenic roots, i.e. those lacking DsRed1 reporter fluorescence, were removed prior to inoculation with S. medicae for nodulation
assessment. The *rdn1-1* and *rdn1-2* mutants transformed with control T-DNA produced, respectively, 82 ± 10 and 75 ± 5 nodules per plant whereas transformed with the *RDN1* candidate gene T-DNA they produced only 31 ± 6 and 23 ± 3 nodules per plant, respectively (Figure 2.4; Appendix A). This level of nodulation in the presence of the *Medtr5g089520* transgene was similar to the nodulation of wild type roots (21 ± 5). Because restored expression of *Medtr5g089520* was sufficient to restore nodule number regulation in *rdn1* mutants, we conclude that it includes the segment corresponding to the *rdn1* mutant locus.

![Image of bar chart](image.png)

**Figure 2.4. Rescue of the *rdn1* phenotype with Medtr5g089520 (RDN1) cDNA.** Mean nodule number per plant ± SE on seedlings with transformed hairy roots. Non-transformed roots (DSRed negative) were trimmed off prior to inoculation. A17 with EV (empty vector), n=18; rdn1-1 with EV, n=25; rdn1-1 with 35Spro:RDN1, n=10; rdn1-2 with EV n=25; rdn1-2 with 35Spro:RDN1, n=38.
Analysis of the *RDN1* gene sequence

The sequence of *RDN1* cDNA amplified from root tissue shows an open reading frame (ORF) of 1071 bases. The cDNA included several hundred bases of sequence preceding this ORF suggesting a long 5’ UTR. Further PCR analysis localized the transcription start site to a region between positions -872 and -689 relative to the predicted translation start site and identified a 157 bp intron at positions -419 to -263. The predicted 5’ UTR includes several potential start codons, one with an ORF of 177 bases and the rest with ORFs of 51 bases or less. Alignment of the cDNA sequence with the genomic sequence shows a 7.4 kb gene consisting of nine exons and eight introns predicted to encode a 357 amino acid protein (Figure 2.5).

The *RDN1* coding sequence has an ATG sequence at the seventh codon position in our annotation that is the predicted start site in the IMGAG annotation of Medtr5g089520. We predict the more 5’ start because of the high level of conservation of the six amino acids predicted by these codons among *RDN1-like* sequences we have identified in other organisms. Three of the *rdn1* alleles are null alleles with multigene deletions, and the remaining allele, *rdn1-2*, harbors an indel within intron 2. Sequence analysis of the indel region revealed that a 9 bp segment in the middle of the 1148 bp intron 2 had been replaced with a 1071 bp sequence of unknown origin (Figure 2.5A).
Figure 2.5. RDN1 gene structure and predicted RDN1 protein sequence. A, A schematic representation of the exon and intron structure of the MtRDN1 gene. Grey boxes indicate coding sequence, white boxes indicate untranslated sequences, and intervening lines indicate introns. The rdn1-2 allele has a 9 bp deletion replaced with a 1017 bp sequence in the second intron. The Psnod3 mutant has a G-to-A transition altering a 3’ intron splice site in the pea ortholog of RDN1. B, The deduced amino acid sequence of MtRDN1. The conflicting SignalP 3.0 predicted signal peptide cleavage site (arrow) and TMHMM 2.0 predicted transmembrane domain (double underline) are indicated. The two predicted globular domains of MtRDN1 are underlined in blue. The strength of sequence conservation in RDN family members is shown for 43 aligned sequences from 12 species of land plants as colored bars above each amino acid (percent identity: red, 100%; orange, 80 to 99%; green, 60 to 79%; light blue, 40 to 59%; dark blue, 20 to 39%). Sequence conservation between MtRDN1 and predicted algal sequences is shown below each amino acid as colored dots for identity between MtRDN1 and sequences from both clusters of algal RDN family members (100% identity with 13 aligned sequences from four species of algae, red dots) and between MtRDN1 and the algal RDN family members in the more closely related cluster (five aligned sequences from three species of algae, orange dots). For description of and relationships between the sequences used to analyze conservation refer to Figure 2.7 and Appendix D.
Because it was not clear that this alteration of intron 2 would impact gene function, we first determined if *rdn1-2* produced *RDN1* mRNA. Root cDNA from *rdn1-2* was analyzed by PCR and *RDN1* transcript was detected (Appendix B). To determine if *RDN1* mRNA levels were altered in the mutant, cDNAs from *rdn1-2* and wild type roots were analyzed using reverse transcription quantitative PCR (qRT-PCR) by amplifying a fragment from within exon 2 to measure overall transcript levels. The level of *RDN1* cDNA was ~75% lower in *rdn1-2* than in wild type (Figure 2.6).

![Figure 2.6. Reduced RDN1 expression in rdn1-2.](image)

**Figure 2.6. Reduced RDN1 expression in rdn1-2.** A, A schematic representation of the MtRDN1 gene showing locations of primers used in RT-qPCR analysis. Indicated are coding regions (black boxes), untranslated regions (white boxes), introns (black lines), and primers a through d (arrowheads). Due to the large size of the second intron (2.9 kb in wild type and 3.9 kb in rdn1-2), the PCR product from primers c and d is only amplified from cDNA if the intron has been spliced out. B, The abundance of RDN1 mRNA in rdn1-2 compared to wild type as estimated by qRT-PCR using the primers indicated in (A). Overall abundance (left panel) and splicing of the second intron (right panel) are shown. The level of RDN1 transcript was normalized to the expression level of the reference gene Secret Agent for each cDNA sample using the Pfaffl method (Pfaffl, 2001). The normalized levels of PCR products from A17 were defined as 1. The values represent the average of three independent biological replicates (for rdn1-2: means ± SE).
We speculated that the insertion within intron 2 could have an impact on the splicing dynamics of *RDN1* mRNA such that a significant portion of the *RDN1* mRNA in *rdn1-2* mutants would be incompletely spliced. Therefore, to assess splicing of *RDN1* mRNA, the levels of *RDN1* cDNA from which intron 2 had been spliced were measured by qRT-PCR using primers spanning from exon 2 to exon 3. Splicing of *RDN1* mRNA was severely reduced in *rdn1-2* compared to wild type, with over 500-fold less spliced transcript detected in the mutant than in wild type.

The similarity between *rdn1* mutants and the pea *nod3* mutant, as well as the location of the mutated loci in syntenic regions, led us to speculate that *RDN1* and *NOD3* were orthologs. We identified a homolog of *MtRDN1* in the *Psnod3* mutant and its parental cultivar Rondo by PCR. The sequence of the predicted protein is 90% identical to the *MtRDN1* sequence. The *Psnod3* allele has a point mutation at the 3’ end of the first coding region intron that results in the production of an mRNA with an altered splice site (Figure 2.5A; Appendix C). The predicted protein from *Psnod3* includes the first 126aa of the wt protein followed by two novel amino acids and a premature stop codon.

**RDN1 is predicted to encode a protein of the endosomal system with unknown function**

Analysis of the predicted 357aa RDN1 protein using TargetP 1.0 and SignalP 3.0 (Bendtsen et al., 2004; Emanuelsson et al., 2007) suggests that RDN1 enters the secretory pathway and has a 24 aa cleaved signal peptide. In contrast, the topology prediction software TMHMM 2.0 (Krogh et al. 2001) and the transmembrane topology
and signal peptide predictor Phobius (Käll et al. 2004) instead find the N-terminal sequence to be a transmembrane domain, not a cleaved signal peptide. No potential glycosylphosphatidylinositol lipid anchoring sites were detected by the big-PI Plant Predictor (Eisenhaber et al. 2003). No endoplasmic reticulum retention signal was detected as determined by ScanProsite analysis (http://www.expasy.ch/tools/scanprosite/). The predicted protein contains no conserved characterized domains and does not resemble any previously characterized protein. GlobPlot 2.3 (http://globplot.embl.de; (Linding et al. 2003)), which identifies regions of globularity and disorder within protein sequences, predicts two globular domains in RDN1 (Figure 2.5B). The C-terminal region of RDN1 contains a proline rich segment (PPXP_5PPPXXP).

**RDN1 is a member of a small uncharacterized plant gene family**

BLAST searches revealed that **RDN1** is a member of a small gene family conserved throughout the land plants and green algae. No members of the gene family were detected outside of the Viridiplantae. Three **RDN** family genes were found in the *M. truncatula* genome and EST databases including **MtRDN1** and two other closely related genes designated **MtRDN2** (*Medtr8g039290*) and **MtRDN3**, encoding predicted proteins of 361 and 360 aa, respectively, which are 63% and 74% identical to MtRDN1.

Predicted **RDN**-related sequences were analyzed from the sequenced genomes of eleven other land plant species including the dicots Arabidopsis, *L. japonicus*, soybean, cucumber (*Cucumis sativus*), poplar (*Populus trichocarpa*), and grape (*Vitis vinifera*);
the monocots rice (Oryza sativa), maize (Zea mays), and sorghum (Sorghum bicolor); the moss Physcomitrella patens; and the lycophyte Selaginella moellendorfii. A total of 43 predicted RDN family genes were identified (Appendix G). The deduced protein sequences were aligned (Figure S4) and subjected to phylogenetic analysis (Figure 2.7A). Predicted RDN family proteins ranged in size from 344 to 380aa, with most of the variability in size and much of the sequence variation occurring in the amino terminal signal peptide region. The predicted proteins from dicots clustered into three groups, designated the RDN1, RDN2, and RDN3 groups. As was seen for M. truncatula, in L. japonicus, cucumber, and grape a single member of each group was found. In poplar and soybean, which have undergone major genome duplication events, pairs of genes were found for each group; the soybean genome has an additional apparent duplication of the RDN1 group genes with two pairs of RDN1 group sequences (genes GmRDN1A, GmRDN1B, and GmRDN1.2A, and the pseudogene GmRDN1.2B). Arabidopsis was the only analyzed dicot species for which an RDN1 group gene was not found. Three RDN family genes were found in each of the monocots rice, maize, and sorghum. The inferred protein sequences clustered into two groups distinct from the dicot RDN groups. The lycophyte Selaginella moellendorfii and moss Physcomitrella patens have RDN family members that cluster outside of the dicot and monocot groups.
Figure 2.7. Phylogeny of the RDN protein family. Phylogenetic relationships between predicted RDN proteins in land plants (A) and in algae (B) derived using the neighbor-joining method. Branches supported by at least 50% of the bootstrap replicates (n=1000) are shown. Genes were identified by BLAST analysis of the sequenced genomes of the higher plants Medicago truncatula (Mt), Glycine max (Gm), Lotus japonicus (Lj), Cucumis sativus (Cs), Populus trichocarpa (Pt), Vitis vinifera (Vv), Arabidopsis thaliana (At), Oryza sativa (Os), Sorghum bicolor (Sb), and Zea mays (Zm); the moss Physcomitrella patens (Pp); the lycophyte Selaginella moellendorfii (Sm); and the green algae Chlamydomonas reinhardtii, Micromonas sp. RCC299, Ostreococcus tauri, and Ostreococcus lucimarinus. The gene names and sequence sources are given in Appendix D.

The degree of sequence conservation between the predicted RDN family proteins of land plants is striking. Among the 43 sequences analyzed, amino acid identity ranged from 55 to 98%. Within the RDN1 group, the proteins were 74 to 95% identical. Similar levels of conservation were found within the RDN2 group. Sequence conservation among the RDN3 group members was somewhat higher (83 to 98%). Of the 357 amino acids of the MtRDN1 sequence, 104 were identical in the other 42 RDN sequences (Figure 2.5B). An additional 87 amino acids were identical in at least 80% of the RDN family sequences. This corresponds to ~60% of predicted mature RDN family protein residues matching in over 80% of RDN family proteins.
Thirteen predicted RDN1-related genes were identified in the sequenced genomes of the green algae Chlamydomonas reinhardtii, Micromonas sp. RCC299, Ostreococcus tauri, and Ostreococcus lucimarinus (Table S2.1). The predicted algal RDN family proteins ranged in size from 409 to 697 amino acids, which was larger than for any of the identified land plant sequences. Longer predicted N-terminal regions in the algal sequences accounted for much of the difference. An alignment of predicted RDN family proteins from algae, M. truncatula, and S. moellendorffii beginning at amino acid 60 of MtRDN1 was used for analysis and generation of a phylogenetic tree (Figure 2.7B; Appendix E). Among the algal sequences divergence was greater than for the land plant sequences. For example, over this aligned region the three predicted proteins from C. reinhardtii were only 26 to 50% identical to each other compared to over 73% identity among the MtRDN proteins. The predicted algal proteins were separated into two main clusters composed of sequences ~50% and ~35% identical to MtRDN1. Amino acids highly conserved between the algal sequences and MtRDN1 are shown in Figure 2.5B.

RDN1 expression

We determined where RDN1 was expressed in vivo, to gain preliminary clues about function. RDN1 expression was detected in both roots and shoots of seedlings by PCR from cDNA (Appendix B). RDN1 is represented on the Affymetrix Gene Chip® Medicago Genome Array by probe set Mtr.42387.1.S1_at. Examination of microarray data using the Medicago truncatula Gene Expression Atlas revealed widespread low level expression of RDN1 with roots appearing to have higher levels of RDN1 mRNA
than shoots (Benedito et al. 2008). *RDN1* expression in roots did not vary during the course of nodulation in this dataset.

The other two *MtRDN* genes are also represented on the microarray. Each exhibited expression throughout the plant at higher levels than for *RDN1*. Compared to *RDN1*, signal intensities were generally 2 to 5 fold higher for *RDN2* (Mtr.40743.1.S1_at) and over 10 fold higher for *RDN3* (Mtr.45545.1.S1_at and Mtr.13077.1.S1_at). Expression of these two genes also appeared to be higher in the roots than in the shoots.

Localization of *RDN1* expression within *M. truncatula* roots was evaluated by use of a reporter construct introduced by *Agrobacterium rhizogenes*-mediated transformation. The upstream region of the *MtRDN1* gene was used to drive expression of the β-glucuronidase (GUS) gene using 2.1 kb of promoter sequence (*RDN1-2.1_pro;GUS*). GUS activity was detected in the vascular cylinder throughout the root (Figure 2.8A, C, D, E, and F). The staining was specific to the presence of the *RDN1* promoter construct as evidenced by a lack of staining in roots transformed with a construct lacking the *GUS* gene (Figure 2.8B). The GUS staining appeared most intense in the area of the xylem, although other regions of the vascular cylinder also often showed staining. No activity was detected in mature nodules besides the expression in the nodule vasculature, which is located on the periphery of the nodule (Figures 2.8G and 2.8H).
Figure 2.8. Localization of RDN1 promoter activity using a promoter: GUS construct. Roots transformed with T-DNA carrying RDN1-2.1pro: GUS (A and C-H) or lacking the GUS gene (B and E inset) were incubated with GUS detection reagent. A, GUS activity was detected in the central cylinder throughout RDN1-2.1pro:GUS transformed roots. B, Without the GUS gene, roots show no staining. C and D, Close up of root tissue (C) and root tip (D). E, Transverse root cross section with staining apparent in the vasculature. Negative control shows no background staining (inset). F, Transverse root cross section (3 µM) of fixed and counterstained tissue. G and H, Intact nodule (G) and nodule cross section (H) showing GUS activity only in the vasculature around the nodule following extended incubation (36 h) with GUS detection reagent. Scale bars where shown are 50 µm.
DISCUSSION

**rdn1 mutants**

Four mutant alleles of a previously uncharacterized *M. truncatula* nodulation regulation locus, designated *ROOT DETERMINED NODULATION 1* (*RDN1*), were identified. The *RDN1* gene was cloned and confirmed by mapping, transcript profiling, and rescue of mutant phenotype by the candidate gene in *rdn1* mutant roots. Three of the alleles have deletions of greater than 100 kb encompassing the *RDN1* gene and represent null alleles; the other allele has an indel within an intron which dramatically reduces production of mature *RDN1* (Figures 1 and 6). The effect of insertions in introns has been observed in other systems. In *A. thaliana* mutants with T-DNA insertions located within introns, transcript levels were shown to be impacted in nearly all cases (Wang, 2008).

*RDN1* lies on chromosome 5 in a region syntenic with the region of the pea *nod3* root-controlled supernodulation locus. We found a corresponding pea gene with over 90% sequence identity to *MtRDN1* and that is mutated in the *nod3* mutant, indicating that this apparently orthologous gene is the *NOD3* gene (Figures 2.1 and 2.5A).

Autoregulation of nodulation (AON) shows similarities in distinct groups of legumes although these show differences in nodule development and structure. For example, the orthologous LRR-RLKs SUNN, SYM29, HAR1, and NARK control AON in the shoots
of *M. truncatula* and pea, which form indeterminate nodules, and in *L. japonicus* and soybean, which form determinate nodules. It might be expected that other proteins involved in AON, such as RDN1, would be similar between legumes that form indeterminate and determinate nodules. We have identified a putative *RDNI* ortholog in the *L. japonicus* genome database located on chromosome 2 in a region with synteny to the *RDNI* region of *M. truncatula* (Cannon et al. 2006). *LjRDNI* lies within the 1.2 cM region of chromosome 2 defined for the *L. japonicus* PLENTY root-controlled supernodulation locus (Yoshida et al. 2010), suggesting that the plenty mutant phenotype could be caused by a lesion in *LjRDNI*.

Disruption of the *RDNI* locus causes an approximately five fold increase in root nodulation of seedlings. The *rdn1* mutants behave similarly to *sunn* mutants in nodulation and root growth behaviors: nodulation under nitrogen limiting conditions is abundant while the presence of available nitrogen has a moderately suppressive effect on nodule formation; the roots are shorter than wild type roots (Figures 2.2 and 2.3). In contrast to *SUNN* which exerts its effect in the shoots, the role of *RDNI* in regulating nodule number is in the roots (Table 2.1).

AON requires transmission of a signal from nodulating roots to the shoot and then the relay of information to the whole root system. Such long distance communication presumably involves the movement of signaling molecules through the vascular cylinder. The promoter of the *SUNN* gene is active in the vasculature throughout the
plant (Karve and Frugoli, unpublished results), as has also been found for its orthologs 
*LjHAR1* and *GmNARK*, reported as active predominantly in the phloem (Nontachaiyapoom *et al.* 2007). Reporter gene analysis in roots shows that like the *SUNN* promoter, the *RDN1* promoter appears to be active in cells of the vasculature although its activity appears to be more widespread than the *SUNN* promoter (Figure 2.8). *RDN1* message is also detected in shoots and although our promoter activity assay was limited to root tissues, we expect *RDN1* promoter activity in the shoots to be located in the vasculature as well.

Evidence that RDN1 may act in the production or transmission of the AON signal and not in responding to the shoot derived inhibitor comes from experiments using a pea supernodulating line with the recessive *RisfixC* mutation, which by mapping and phenotype represents an allele of *nod3* (Novak, 2010). Grafted plants possessing a large *RisfixC* main root system and smaller wild type adventitious roots produced high numbers of nodules on both the mutant roots and wild type roots. This observation suggested to the authors that the root system, composed mainly of mutant roots, was unable to send a signal sufficient to trigger the AON response in the shoot, thereby allowing the wild type roots to produce excessive numbers of nodules.

**RDN1 protein and related proteins**
The *RDN1* gene is predicted to encode a protein of 357aa composed of a secretory signal sequence, two uncharacterized globular domains, and a proline-rich segment (Figure 2.5B). Its cellular function is unknown. We obtained two conflicting structural
predictions: one that RDN1 is released as a soluble protein into the ER lumen following cleavage of its signal sequence and the other that RDN1 is an integral membrane protein with a single transmembrane domain. However, the predicted localization of RDN proteins to membranes is supported by the identification of the Arabidopsis RDN3 family protein At5g25265 in proteomics analyses in plasma membrane (Marmagne et al. 2007; Mitra et al. 2009) and vacuolar fractions (Carter et al. 2004; Jaquinod et al. 2007). Determining the subcellular location of RDN1 experimentally would provide an important clue as to the function of the protein, such as whether RDN1 is secreted, located in the plasma membrane, or targeted elsewhere in the cell.

*RDN1* related sequences were found in the genomes of all plants examined, including those of green algae, and represent a gene family we have designated the *RDN* family. All of the putative RDN family proteins identified are predicted by TargetP to have signal sequences, although, as was found for MtRDN1, there are conflicting predictions for whether or not the signal sequences are cleaved. Three *RDN* family genes were found in most land plant genomes. For example, *M. truncatula* has two genes similar to *MtRDN1*, named *MtRDN2* and *MtRDN3*, which are both predicted to encode proteins ~70% identical and over 80% similar to MtRDN1. Predicted dicot RDN family proteins clustered into three groups represented by the three MtRDN proteins (Figure 2.7). Predicted monocot RDN family proteins clustered separately into two groups. The structure of the RDN family phylogenetic tree suggests the existence of an *RDN* gene
family prior to the divergence of monocots and dicots, followed by duplications forming the RDN1 and RDN2 groups of the dicots and forming one of the monocot clusters.

Arabidopsis does not have an \textit{RDN1} ortholog but does have an additional \textit{RDN} family gene, At2g25260, divergent from other dicot \textit{RDN} family genes. Analysis of database sequences from other plants of the order Brassicaceae revealed no \textit{RDN1} orthologs in close relatives of Arabidopsis in the family Brassicaceae, but a gene similar to At2g25260 was found, while \textit{Carica papaya}, a member of the family Caricaceae, possesses \textit{RDN1}, \textit{RDN2}, and \textit{RDN3} homologs like the other dicot lineages. This suggests that loss of \textit{RDN1} and appearance of the At2g25260 type gene occurred in the Brassicaceae lineage.

The predicted RDN family proteins from land plants are highly conserved. Over 60\% of the amino acids of the predicted mature proteins are highly conserved among family members with over 80\% of the family members identical at those positions. Many residues (10\%) are also invariant in the algal RDN family sequences.

The conservation of \textit{RDN} family genes across the Viridiplantae, including unicellular algae, suggests a basic cellular function for RDN family proteins. Of the nearly 7000 protein families identified in the \textit{Chlamydomonas} genome, 172 families appear to be unique to the green plants, with almost two-thirds of these proteins predicted to be
chloroplast-localized (Merchant et al. 2007). The RDN family is one of only 61 identified green plant-specific protein families whose members are not predicted to be localized to chloroplasts and one of only ten whose members are predicted to have secretory signal sequences.

**Expression of RDN family genes**

A survey of the major organ systems of *M. truncatula* (Benedito et al. 2008; He et al. 2009) using the Medicago Gene Expression Array revealed that the three *RDN* genes are expressed in all organs examined, including leaves, stems, flowers, vegetative buds, roots, nodules, pods and seeds (Appendix F). The abundance of the message appears to vary between the genes with *RDN1* detected only at low levels, *RDN2* and *RDN3* at higher levels.

Similarly, using the Arabidopsis Electronic Fluorescent Pictograph browser for whole genome tiling array data (Winter et al. 2007; Laubinger et al. 2008), the Arabidopsis *RDN2* and *RDN3* family genes At5g13500 and At5g25265 were found to be expressed in most tissues examined (roots, leaves, shoot apex, cotyledons, hypocotyls, seeds, flowers, young siliques) with the *RDN3* family gene being expressed at higher levels (Appendix F). In contrast, At2g25260, the Arabidopsis *RDN* family gene without an ortholog in the other dicots, was detected primarily in roots and shoot apex inflorescences at high and moderate levels, respectively, and only weakly in other tissues.
In microarray analyses of fluorescently sorted cells from the roots of a series of GFP-marked lines, the Arabidopsis *RDN2* family gene was most strongly expressed in the root hair cell lineage with strong expression also throughout the elongation zone (Brady *et al*. 2007; Dinneny *et al*. 2008). Strong elongation zone expression was also observed in another study (Dinneny *et al*. 2008). Thus, it appears that RDN family proteins are expressed in most plant organs, with perhaps higher levels of expression in certain cell types such as vascular cells for *MtRDN1* and root hair cells for the Arabidopsis *RDN2* family gene.

Consistent with the demonstrated role of *MtRDN1* in AON and its expression in vascular tissue, we propose that MtRDN1 is involved in initiating, responding to, or transporting vascular signals and that this vascular signaling function of RDN1-related proteins may be present in all dicots. Furthermore, the wide conservation of *RDN* family genes across the green plants, including unicellular algae, suggests other conserved molecular functions for the RDN family proteins in the plant cell.
MATERIALS AND METHODS

Plant Materials
Preparation of *Medicago truncatula* seeds and growth of plants in aeroponic chambers was performed as described in Schnabel *et al.* (2010). For all other assays, scarified and imbibed seeds were vernalized in the dark at 4°C for 2 d on HMF agar (Huo *et al.* 2006) covered with Whatman filter paper. Following overnight germination at room temperature, seedlings were transferred to plates half covered with filter paper with the radicals on the paper and the cotyledons on the uncovered medium, placed vertically in a growth chamber at 25°C with a 16 h photoperiod for 5 days, and then used for experiments. For all nodulations experiments the strain *Sinorhizobium medicae* strain ABS7 was used (Bekki *et al.* 1987).

The *rdn1* mutants were identified from independent M2 seed pools collected from fast neutron bombarded M1 seeds of *M. truncatula* cv. Jemalong. M3 plants were rescreened in an aeroponic chamber for nodulation phenotype and in the greenhouse for petal senescence. Lines used for detailed analyses were backcrossed to A17 wild type three times (*rdn1-1*) or once (*rdn1-2*). A near-isogenic line of the *Pisum sativum* cv. Rondo mutant *nod3* backcrossed into *P. sativum* cv. Juneau (*nod3I*, PI 598367) and *P. sativum* cv. Rondo were obtained through the National Plant Germplasm System (http://www.ars-grin.gov/).
Mapping of \textit{rdn} and Sequence Analysis

The F2 self-pollinated progeny from crosses of \textit{rdn1} mutants GY15-2E, D39-1H-T2, and D39-13F-V1 to \textit{M. truncatula} ecotype A20 were used for genetic mapping of the \textit{rdn1} locus. DNA from F2 individuals with high nodule numbers was evaluated for the segregation of CAPS and other markers as previously described (Schnabel et al., 2010; Appendix H). For each mutant, 80 to 195 supernodulating F2 plants were tested. For D39-1H-T2 and D39-13F-V1, supernodulating plants represented ~25% of the F2 progeny as expected for a single recessive locus. In the F2 progeny of the GY15-2E mapping cross, fewer than expected supernodulating plants were observed (~10%); recombination around the \textit{rdn1} locus was suppressed at least five-fold; and skewed marker segregation was observed, with cosegregation of markers from the long arms of chromosomes 5 and 8. These data suggest a genomic rearrangement within the GY15-2E mutant.

The Gene Chip ® Medicago Genome Array (Affymetrix, Santa Clara, CA) was used for oligonucleotide hybridization experiments comparing transcript profiles of buffer-inoculated wild type A17 roots with buffer-inoculated \textit{rdn1-1} mutant roots. Methods were as described by Mitra et al. (2004). The search for RDN family members used BLAST algorithms blastp and tblastn (Altschul et al. 1990). Sequences from selected organisms with sequenced genomes were used for analysis. No sequences with a probability relationship to \textit{MtRDN1} of e<3 were found outside of the Viridiplantae.
**Phylogenetic Analysis**
RDN family predicted protein sequences were aligned using the ClustalW algorithm of MegAlign in Lasergene 7.1.0 (DNAStar, Madison, WI). Phylogenetic trees were constructed with *MEGA* version 4 (*Tamura et al.* 2007) using the Neighbor-Joining method with 1000 bootstrap replicates. Branches supported by at least 500 of the 1000 bootstrap replicates are shown in Figure 2.7.

**RNA Isolation and qRT-PCR**
RNA was isolated from plant tissues using the Qiagen RNeasy Mini Kit (http://www.qiagen.com), treated with RQ1 RNase-Free DNase (http://www.promega.com) followed by phenol and chloroform extractions and ethanol precipitation, and quantified spectrophotometrically. cDNA was synthesized in 20 ml reactions from 0.5 to 2 mg RNA using random hexanucleotide primers and Superscript Reverse Transcriptase II (http://www.invitrogen.com) following the manufacturer’s recommendations. The absence of genomic DNA in cDNAs was verified by PCR with primers JF1330 and JF1331, specific for a non-coding region near Medtr5g089720. The expression of *RDN1* and the specificity of *RDN1* primers were evaluated by visualizing PCR products from genomic DNA and cDNA on 1% Tris-Borate-EDTA gels following PCR amplification (Appendix B).

Expression levels were quantified in an iQ5 Thermocycler (http://www.bio-rad.com) using PerfeCTa SYBR Green Supermix (Quanta Biosciences). Expression levels and splicing of *RDN1* transcripts were assessed using primers for amplifying within exon 2
(primers qPCR-a and qPCR-b) and from exon 2 to exon 3 (primers qPCR-c and qPCR-d). Levels of cDNA were normalized using the Secret Agent gene as a reference (Kuppusamy et al. 2004) and ratios were calculated using the Pfaffl method (Pfaffl, 2001). Three independent biological replicates were evaluated. For each cDNA three technical replicates were performed and the values averaged. The efficiency of each primer pair was assessed by use of a dilution series. In all runs, the primer pairs for exon 2, exon 2 to exon 3, and Secret Agent had measured efficiencies of 2.0, 2.0, and 1.8, respectively. Across the biological replicates, threshold cycles for all products from the cDNAs fell within the valid range of the standard curves with the exception of spliced products from rdn1-2 which had high Ct values.

**Generation of transgenic hairy roots and histochemical analysis**

For expression of RDN1 in transgenic plants, a fragment of RDN1 cDNA was PCR amplified from *M. truncatula* cDNA using primers RDN1cDNA-A and RDN1cDNA-B and cloned downstream of the cauliflower mosaic virus 35S promoter in pC-DsRED2 using KpnI and XhoI. The vector pC-DsRed2 was constructed from pCAMBIA0390 by replacing a portion of the polylinker with the polylinker region of pCAMBIA3201 (EcoRI to PstI), adding the Ascl/HindIII UBQ10pro:DsRed1 fragment of pRedRoot (Limpens et al. 2004), and adding additional restriction sites to the polylinker by ligating an EcoRI/MluI/XhoI adaptor into the EcoRI site. For promoter activity analysis, 3.3 and 2.1 kb fragments from upstream of the predicted RDN1 translation start site were amplified by PCR from *M. truncatula* genomic DNA using primers P2.1-F and P2.1-R and primers P3.3-F and P3.3-R, respectively, and cloned using NcoI and
EcoRI into pC2381ES (Huo et al. 2006). The coding and promoter sequences in the binary vector were confirmed by sequencing.

Prepared seedlings were transformed as previously described (Limpens et al. 2004) using *Agrobacterium rhizogenes* strain ARqua1 (Quandt et al. 1993) containing the appropriate binary vector. Seedlings were maintained on plates until sufficient root tissue had grown. For nodulation experiments, non-transgenic roots (those lacking DsRed fluorescence) were trimmed off prior to transfer of plants to pots of perlite mixed with HMF medium without nitrate. After five days of nitrogen starvation, plants were flood inoculated with *S. medicae* (OD600=0.1) and nodules were counted 21 days later.

For promoter experiments, transformed tissue was washed twice in 0.1 M Na2HPO4/NaH2PO4, pH 7.2 for 15 minutes and GUS activity was localized based on a protocol by Jefferson and others (Jefferson et al. 1987). Samples were infiltrated with substrate under vacuum for 30 min and incubated at 37°C for 18 h, unless otherwise indicated. Where indicated, roots were fixed in 5% glutaraldehyde/0.1 M sodium phosphate buffer, pH 7.2 for 2 hr under vacuum. Serial ethanol dehydration was then performed by increasing the concentration (10, 30, 50, 70, 90, and 100%) at room temperature for 10 minutes each. Samples were embedded in Technovit 7100 resin (Heraeus Kulzer) using the manufacturer’s instruction. Sections were prepared using a RM2165 microtome (Leica Microsystems), dried onto glass slides at 42 °C, and
counterstained for 1 min in 1% aqueous saffronin-O solution. Slides were washed briefly with water, dried, and mounted in permount (Fisher Scientific). Tissue was photographed using a Zeiss Axiostar plus microscope and a Nikon E600 microscope with a Retiga EXi FAST monochrome CCD 12-bit camera.

**Accession Numbers**
MtRDN1 mRNA (GU580937), PsNOD3 gene (GU580938), PsNOD3 mRNA (GU580939).

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ABSTRACT
(Paper 2)

Encoding a conserved protein of unknown function, the *M. truncatula* *RDN1* gene is involved in autoregulation of nodulation through signaling in the root. In contrast, the SUNN kinase in *M. truncatula* has been shown by grafting of mutant scions to control nodule number in the root by communication of a signal from the shoot to the root. GUS staining patterns resulting from expression of the *SUNN* promoter fused to *uidA* showed expression of *SUNN* in most parts of plant including the root, but confined to the vascular tissue, a pattern that overlaps with that published for *RDN1*. Real Time PCR analysis showed levels of both *SUNN* RNA and *RDN1* RNA did not change significantly during early nodulation signaling (0-72 hours after inoculation). The similarity in expression in cell types strongly suggests vascular signaling for nodule number regulation, while the lack of changes over early nodule development suggest post transcriptional mechanisms such as protein association or phosphorylation transmit the signal.
The symbiosis between legumes and rhizobia involves signaling, response and regulation by both the bacterial and plant partners. The regulation of nodule number by the plant, termed “autoregulation,” includes a long distance signal transduction pathway involving both the shoot and the root. Because supporting active nodules has energy cost to the plant in the range of 12-17 grams of carbon per gram of nitrogen obtained (Crawford et al. 2000), regulation of nodule number by the plant presumably balances the need for fixed nitrogen with the cost of supporting the bacteria. Isolation of plant mutants unable to regulate the number of nodules that form on the roots identified multiple genes encoding proteins that control nodules number from different parts of the plant (for review see Reid et al. (2011b); Mortier et al. 2011). Grafting experiments in which mutant shoots were grafted onto wild type roots and the reciprocal experiments of wild type shoots grafted onto mutant roots revealed that for mutants in some genes, the genotype of the shoot determined the number of nodules that formed on the root, while for other genes, it was the genotype of the root which determined the number of nodules that formed. We recently identified a gene, RDN1 (ROOT DETERMINED NODULATION1) that controls nodule number from the root but is expressed in both shoots and roots (see previous paper in this chapter). A fusion of the RDN1 promoter to the uidA (GUS) gene gave staining in the vasculature of transgenic roots.
The *SUNN* gene in *M. truncatula* encodes a leucine-rich repeat receptor kinase that controls nodule number from the shoot but like *RDN1* is expressed in both shoots and roots (Schnabel *et al.* 2005). To assess tissue-level expression of *SUNN*, we constructed a transgenic *M. truncatula* plant containing 1360 bp of sequence upstream of *SUNN* driving expression of an *mGFP-GUS* gene fusion. This construct includes the entire region of 5’ DNA between the start of the *SUNN* gene transcript up to and including the next genetic landmark, a genomic MITE insertion. The construct was transformed into wild type (A17) *M. truncatula* using the protocol of Zhou *et al.* (2004) and detection of GUS was via the protocol of Jefferson *et al.* (1987).

The expression of this construct in T3 plants gave a very similar pattern of expression to that reported for the *RDN1* reporter construct. The GUS staining pattern indicated expression in the vasculature of many tissues including leaves, petioles, stems and roots (Figure 2.9). The staining was often in cell layers adjacent to phloem cells in tissues examined under higher magnification. For example, staining was seen in the procambium in petioles (Figure 2.9B) and stems (Figure 2.9D, 2.9E), and the cells surrounding the primary phloem in roots (Figure 2.9F). Despite the fact that SUNN regulates nodule formation, nodule expression was limited to the vasculature (Figure 2.9 G & H). No staining was observed in shoot meristematic tissue with faint staining in the vasculature leading to the meristem (Figure 2.9 I). These results were consistent with those of Nontachaiyapoom *et al.* (2007) who examined the orthologous soybean *NARK* and *Lotus HAR1* promoters in a *L. japonicus* background. They reported
sequence conservation between the promoters of the *Lotus*, *Medicago* and *Glycine* orthologs including a sequence element driving vascular specific expression and our results confirm this experimentally in the Medicago background. Because both the RDN1 and SUNN genes were expressed in the vasculature and regulate nodule number, we asked if SUNN or RDN1 transcription had similar expression level patterns in response to rhizobia. We examined SUNN and RDN1 expression in wild type shoots and roots over a time course of early nodulation with Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR), using protocols from our previous work (Schnabel et al. 2010). We saw little change in expression levels in either gene in root and shoot tissue at 24, 48, or 72 hours after addition of rhizobia (Figure 2.10). Values show no statistical difference with p>0.05 for all comparisons by Student’s t test.
Figure 2.9 Expression of a SUNNpro:mtGFP-GUS fusion in wild type plants. A Nikon E600 microscope with a Retiga EXi-FAST monochrome CCD 12-bit camera was used for visualization and imaging of some stained samples and a Zeiss Lumar.V12 stereoscope equipped with AxioCAM MRC and AxioVision software (Zeiss) for others. Leaf, stem and root transverse sections were made by hand using a razor blade. The sections were routinely ~0.2mm in thickness. (A) Staining in the veins in leaflets; (B)
cross section of petioles; (C) cross section of leaflet with arrows indicating staining in vasculature; (D) cross section of stem; (E) close up of stem showing staining in phloem; (F) cross section of root; (G) mature nodules; (H) cross section of root and nodule and (I) faint staining in the vasculature leading to the meristem from the same plant used for (E), but no meristematic staining.

The presence of SUNN and RDN1 in vascular tissue has mechanistic implications. We speculate that the function of the SUNN kinase and/or RDN1 may affect phloem loading/unloading of auxin or other molecules. Outside of legumes, the SUNN kinase is most closely related by sequence to the Arabidopsis CLV1 kinase and related proteins in monocots involved in meristem maintenance (Schnabel et al. 2005), and AtCLV1, in addition to its well characterized expression in apical meristems, is expressed in the phloem companion cells (Nimchuk et al. 2011). Combined with the observation that the RDN1 protein is completely unknown in function but highly conserved across all green plants including moss, the functions of RDN1 and SUNN cannot be limited to signal transduction in the autoregulation of nodulation. The fact that they are expressed in the vasculature with little change in expression in response to rhizobia could suggest the two proteins are involved in the same signal transduction event and we are investigating this further. The observation that SUNN regulates from the shoot while RDN1 regulates from root, yet both are expressed in both shoot and root tissues, most likely indicates there are other as yet undiscovered genes involved in long distance nodulation signaling events.
Figure 2.10. Quantification of gene expression. Results of reverse transcription quantitative PCR of SUNN and RDN1 gene expression in shoots and roots of wild type plants during early nodulation signaling. Expression is shown relative to expression at time 0 (no rhizobia) for SUNN in shoots (A) and roots (B) as well as for RDN1 in shoots (C) and roots (D). Mean of three independent biological replicates of 3-5 plants per time point and three technical replicates. Error bars are standard error of the mean.
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CHAPTER 3

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SIMPLE AND EFFICIENT METHODS TO GENERATE SPLIT ROOTS AND GRAFTED PLANTS USEFUL FOR LONG-DISTANCE SIGNALING STUDIES IN *Medicago truncatula* AND OTHER SMALL PLANTS

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ABSTRACT

*Background*

Long-distance signaling is a common phenomenon in animal and plant development. In plants, lateral organs such as nodules and lateral roots are developmentally regulated by root-to-shoot and shoot-to-root long-distance signaling. Grafting and split root experiments have been used in the past to study the systemic long-distance effect of endogenous and environmental factors, however the potential of these techniques has not been fully realized because data replicates are often limited due to cumbersome and difficult approaches and many plant species with soft tissue are difficult to work with.
Hence, developing simple and efficient methods for grafting and split root inoculation in these plants is of great importance.

**Results**

We report a split root inoculation system for the small legume *M. truncatula* as well as robust and reliable techniques of inverted-Y grafting and reciprocal grafting. Although the split root technique has been historically used for a variety of experimental purposes, we made it simple, efficient and reproducible for *M. truncatula*. Using our split root experiments, we showed the systemic long distance suppression of nodulation on a second wild type root inoculated after a delay, as well as the lack of this suppression in mutants defective in autoregulation. We demonstrated inverted-Y grafting as a method to generate plants having two different root genotypes. We confirmed that our grafting method does not affect the normal growth and development of the inserted root; the composite plants maintained normal root morphology and anatomy. Shoot-to-root reciprocal grafts were efficiently made with a modification of this technique and, like standard grafts, demonstrate that the regulatory signal defective in *rdn1* mutants acts in the root.

**Conclusions**

Our split root inoculation protocol shows marked improvement over existing methods in the number and quality of the roots produced. The dual functions of the inverted-Y grafting approach are demonstrated: it is a useful system to produce a plant having roots of two different genotypes and is also more efficient than published shoot-to-root
reciprocal grafting techniques. Both techniques together allow dissection of long distance plant developmental regulation with very simple, efficient and reproducible approaches.

**Key Words:** Split Root, Inverted-Y, Grafting, Nodulation, Long-Distance Signaling, Systemic Signaling, *Medicago truncatula*, RDN1
INTRODUCTION

Signaling mechanisms are vital for all living organisms. Plants and animals use long-distance signals to coordinate and adjust their growth in response to endogenous and environmental cues. These signals transmit messages throughout the whole organism to achieve biological homeostasis. Plants use hormones, RNA, proteins, short peptides and lipids for long distance signaling in defense against pathogens (Heil and Ton, 2008), in response to numerous abiotic and biotic stresses (Pant et al. 2008) and in developmental processes such as flowering (Corbesier et al. 2008) branching (Foo et al. 2001) and nodulation (Kossalk and Bohlool, 1984; van Noorden et al. 2006). Our lab uses *Medicago truncatula* to study long distance regulation; specifically the root-to-shoot and shoot-to-root signals that control nodule number and to understand the regulatory network involved in this process.

*M. truncatula* is an excellent model to study legume biology due to its small diploid genome (500Mb), self-fertility, ease of transformation, short life cycle, high level of natural diversity and a wealth of genomic resources (Cook, 1999). In addition, the bacterial and fungal symbionts of *M. truncatula* that lead to the fixation of nitrogen and the increased uptake of phosphorus are well-characterized (Galibert et al. 2001; Martin et al. 2008). In both rhizobial and arbuscular mycorrhizal symbioses, the establishment and maintenance of symbiosis requires expensive plant resources, specifically energy (Crawford et al. 2000). To balance this expense with other plant needs, legumes have a
negative feedback inhibition system called autoregulation of nodulation (AON) (Pierce and bauer, 1983; Searle et al. 2003). Through AON, the early symbiotic events occurring in a root and leading to nodule organogenesis or to arbuscule formation systematically affect later symbiotic interactions through transportable signals. Efforts have been focused on locating time and space-specific root and shoot events including sensor, integrator and effector molecules. Grafting and split root inoculation systems can be very informative when combined with current molecular genetic tools to decipher the signaling. However, very little grafting and split root work has been done in model legumes, with historical work in less genetically tractable plants such as bean, pea, soybean, clover and vetch, some of which have stems much larger than those in model systems.

Establishment of symbiosis in one part of a legume root affects further symbiotic events in other parts of the root inoculated later, and this phenomenon was initially elucidated using split root experiments. In these split root systems, two roots in one plant are partitioned in time and space allowing prior inoculation of one root system (Root A) to systematically regulate nodulation from the separate inoculation of the second root (Root B). Around thirty years ago, it was reported that the suppression of nodule development in the Root B side of the split root system in soybean is associated with prior inoculation of the Root A side (Kossalk and Bohlool, 1984). Five years later, Olsson et al. (1989) reported the lack of systemic suppression of nodulation in supernodulating soybean mutants. Using a split root experiment, Tang, Robson and
Dilworth (1990) showed that iron is required for nodule initiation in lupine, emphasizing the direct and indirect impact of mineral nutrient deficiency on symbiosis. Application of either rhizobia or nod factors to the Root A side of a split root system inhibits nodulation in the B root system, suggesting that nod factors are enough to elicit the autoregulatory responses in vetch (van Brussel et al. 2002). In clover, a non-nodulating strain of *Rhizobium trifolii* inoculated on Root A was unable to inhibit infection by the wild type strain on Root B, suggesting a minimum requirement of Nod factor to initiate the plant inhibitory response (Sargent et al. 1987). In work by Laguerre et al. (2012), one root system was nodulated with a nitrogen-fixing bacterial partner while the other root system was nodulated with non-fixing partner resulting in a plant that compensated for the local nitrogen limitation in the root with non-fixing bacteria. The same group had shown that in split root plants when one root is in a nitrogen-limited condition and the other receives nitrogen, both nitrogen fixation activity and net nitrogen uptake by the root system in the nitrogen-limited condition was higher in the *M. truncatula sunn-2* mutant versus wild type plants (Jeudy et al. 2010). The authors suggested a secondary response of growth stimulation of pre-existing nodules in the wild type and *sunn-2* mutant. Autoregulation signals initiated by either nodulation or mycorrhization on Root A in alfalfa systemically influence both rhizobial and arbuscular mycorrhizal colonization of Root B in a split root system without preferential selection (Catford et al. 2003). Also in alfalfa inoculated with mycorrhizae, isoflavonoid levels are systematically regulated in the uninoculated Root B upon prior
inoculation of Root A, suggesting the involvement of isoflavonoids in the long distance autoregulation of arbuscular mycorrhizal symbiosis (Catford et al. 2006).

Developing a model of signal transduction by comparison across these experiments is difficult due not only to the use of many less tractable and less well developed molecular genetic systems with both determinate and indeterminate nodule development, but also to the use of a broad range of compartmentalization techniques to separate the split roots in various growth systems. For instance, PVC piping elbows have been used in soybean (Kossalk and Bohlool, 1984) and split root tubes have been used in soybean and vetch (Kossalk and Bohlool, 1984; Olsson et al. 1989; van Brussel et al 2002). Split root plate assays were done using Trifolium subterraneum and Lotus japonicus by separating the roots with plastic dividers supported with 0.6% water agar (Sargent et al. 1987) or by removing the center of the agar to create separate root environments (Suzuki et al. 2008). The limitations of these techniques include inability to consistently control various factors known to affect nodule regulation, such as ethylene concentration in plate systems (Penmetsa and Cook, 1997; Tamimi and Timko, 2003) and rhizobial cross contamination. Moreover, the effect on nodulation of root exposure to light (Webb, 1982), balancing the size of the root systems before treatment, the types of containers and the composition of the growth media including the amount of water and the concentration of various root exudates that affect nodulation were not consistently controlled. The above approaches were also targeted for very small laboratory scale applications, often with only 3-5 replicates (plants) per experiment.
In addition to the unintentionally introduced variables in the above experiments, many key factors in autoregulation remain unexplored in a single system/species. These factors include the time the AON signal takes to suppress nodulation and the stages of nodule initiation targeted by the AON signal. Except for the nitrogen experiments described above, the split root technique has not been used in *M. truncatula*. We were unable to find efficient examples of the use of the technique in model plants with growth parameters similar to *M. truncatula* (small stems approximately 0.1cm in diameter). Hence improving the existing split root protocol to consistently generate large numbers of grafted plants was imperative for our AON investigations.

Another technique, reciprocal grafting, is also a valuable tool to study the remote and local interactions of various genotypes and systemic signals. For shoot-to-root reciprocal grafting in Arabidopsis, wedge grafting has been commonly employed, and adapted for other model plants with slight modifications (Turnbull *et al.* 2002; Nishimura *et al.* 2002; Penmetsa *et al.* 2003; Magori *et al.* 2009; Schnabel *et al.* 2010; Schnabel *et al.* 2011). Shoot-to-root reciprocal grafting allows researchers to examine the systemic signals and separate gene functions in above and below ground parts of the plant. The major limitations of the technique, especially in small plants, is the need for agar, parafilm, medical tubing or other physical support materials which interfere with inspection of the graft union and slow the healing of the union, sometimes influencing later plant development (Yin *et al.* 2012). The success rate for *M. truncatula* reciprocal
grafts is reported to be as low as 8% (Lohal and VandenBosch, 2005) and we have observed a rate of 50% depending on genotype in our previous work (Penmetsa et al. 2003; Schnabel et al. 2010; Schnabel et al. 2011; Lucinda Smith personal communication).

Despite the low success rate, reciprocal grafting is quite informative. Reciprocal grafting between a Zn hyperaccumulator, *Thlaspi caerulescens*, and a Zn nonaccumulator, *Thlaspi perfoliatum*, showed the relative importance of roots and shoots in Zn hyperaccumulation and hypertolerance (Guimaraes et al. 2009). The discovery of Flowering Locus T (FT) protein as a long distance signal moving from the leaf to the apex through phloem to induce flowering in *Arabidopsis* was done with grafting (Corbesier et al. 2007). Grafting analysis provided evidence that the shoot genotype controls the supernodulating phenotype in the autoregulation defective mutants *har1* and *klv* in *Lotus japonicus* (Krussel et al. 2002; Miyazawa et al. 2010), *sym29* in *Pisum sativum* (Nishimura et al. 2002), *nark* in *Glycine max* (Delves et al. 1986), *sunn* and *lss* in *M. truncatula* (Penmetsa et al. 2003; Schnabel et al. 2010). For example, grafting *sunn* and *lss* scions on wild type (A17) rootstock produced a hypernodulation phenotype whereas the reciprocal grafting of A17 scion on either *sunn* or *lss* rootstock gave wild type nodulation phenotype (Penmetsa et al. 2003; Schnabel et al. 2010). Grafting also revealed the action of the root determined nodulation 1 mutant *rdn1* in which, unlike the examples above, the root genotype controls the hypernodulation phenotype (Schnabel et al. 2011). In cases of root-determined
hypernodulation, the cause could be a defect in either the synthesis or transmission of the root-derived factor or the transport and/or perception of the shoot-derived descending inhibitory signal. Distinguishing between these possibilities requires a plant with roots of two different genotypes. Working in pea, researchers used approach grafting between wild type pea and lines with mutations affecting AON to reveal that early nodulation events prior to root hair curling cannot induce the AON signal, demonstrating that AON starts after root hair curling but before visible cortical and pericycle cell division (Li et al. 2009). However approach grafting, in which two complete plants are joined in the stem region, adds the complication of having two shoots of different genotypes that may vary in their vascular connections to the roots and their production of the unknown shoot-derived inhibitory signal compared to a single shoot, making the findings from these experiments difficult to interpret definitively. Therefore we developed an inverted-Y grafting technique to provide an extra dimension to the split root experiment by partitioning the two roots of the same plant not only in time and space but also in genotype. This experimental approach allows for the dissection of function of the gene products involved in the regulatory circuit without the complications created by approach grafting.

This methodology report describes these highly efficient split root and inverted-Y grafting protocols in *M. truncatula*. Our techniques provide simple ways of generating many root systems to dissect long distance signaling. These can be either split root experiments where the effect of a treatment on one root is detected on the second root or
inverted-Y graft experiments where plants with two different root genotypes are used to study the root-to-shoot and shoot-to-root signals and individual gene actions. We also report a modification of the inverted-Y technique that allows rapid generation of a large number of reciprocally grafted plants with a single shoot and single root.
RESULTS

A split root technique demonstrates systemic AON

Split-root experiments are valuable for the investigation of the autoregulation of nodulation, i.e. systemic suppression of subsequent root colonization by rhizobia through signaling from an already colonized part of the root system. We developed a split root inoculation protocol for *M. truncatula* by modifying an existing Agrobacterium mediated hairy root transformation method for *M. truncatula* (Limpens et al 2004) diagrammed in Figure 3.1 and detailed in Figure 3.2.

![Generalized diagram of split root inoculation in M. truncatula.](image)

**Figure 3.1. Generalized diagram of split root inoculation in *M. truncatula*.** A plant with two main roots is created via our protocols and each root is placed in Perlite in a separate pot. One root is inoculated first (Root A) and the second root (Root B) is inoculated at a later time point. The plants continue to grow for 21 days after the second inoculation, at which point they are removed from the Perlite system and nodules on each root are counted.
Figure 3.2. Generating split roots in *M. truncatula*. (A) Plants on Petri dishes before cutting roots, (B) after cutting roots and (C) after placing filter paper over the roots. (D) Lateral root initiation 5 days after cutting the root. (E) Example of 2 plants trimmed to two balanced root systems ready for transfer to Perlite. (F) Plants with two root systems, one growing in each pot (G) Experimental design of pots in a standard greenhouse tray that maximizes plants per unit space. Each row is a replicate, and each column contains the following (a) Root A of plant 1 (b) Root B of plant 1 and Root B of plant 2 (c) Root A of plant 2 and Root A of plant 3 (d) Root B of plant 3 and Root B of plant 4 (e) Root A of plant 4 (h) A plant 21 days after Root B inoculation ready for counting.
Briefly, the transformation protocol was followed to the point of removing the root system (Figure 3.2 A & B) and placing on HMF media sandwiched between two half round Whatman filter papers (Figure 3.2 C), but no *Agrobacterium* was applied. Lateral roots were allowed to grow out from the cut (Figure 3.2 D). Two lateral roots of the same size were selected (Figure 3.2 E), while the rest of the roots were removed and the two remaining roots grown in separate root environments by placing them in side-by-side plastic pots in a Perlite system with free drainage (Figure 3.2 F). By combining roots from alternate plants in a single pot (Figure 3.2 G); four split root systems could be accommodated in five pots. One side of the split root system (Root A) was inoculated with *Sinorhizobium medicae* strain ABS7 (Bekki *et al.* 1987) and the other side of the split root system (Root B) was inoculated four days after the first root inoculation with the same rhizobium strain at the same concentration. In agreement with experiments in soybean (kossak and Bohlool, 1984), wild type (A17) roots inoculated second (Root B) had significantly fewer nodules than the root inoculated first (Root A) (Figure 3.3). As might be expected, in *rdn1* mutants known to make too many nodules (Schnabel *et al.* 2011), inoculation of the first root had no effect on nodule number on the second root, confirming a defect in AON (Figure 3.3).
Figure 3.3. Split root plants demonstrate that the suppression of nodulation is lost in an AON mutant. Number of nodules per root on the root inoculated first (Root A) and the root inoculated 4 days later (Root B) counted 21 days after second inoculation. The wild type is ecotype A17 and the mutant rdn1-2. Data represent means (N = 6 for A17, N = 23 for rdn1-2). Error bars are standard error of the mean. Letters (a, b and c) indicate significant differences between Root A and B, and between the two genotypes (p < 0.05).

**Inverted-Y graft plants respond normally to the transmissible signal**
The main objective of inverted-Y grafting is to obtain a graft of one shoot to two different rootstock genotypes in the same plant in order to understand the role and timing of action in long distance regulation of a mutant gene. Since graft union development is a delicate process that involves cell-to-cell communication, there are many factors that lead to poor healing and connection of the graft, such as differences in
morphology between the stock and the scion and the water content of the media (Yin et al. 2012). Yin et al. (2012) used an oblique medium surface to alleviate these problems. Based on this idea, we modified an inverted-Y grafting approach similar to that recently used by Magori (Magori et al. 2009) in Lotus japonicas for M. truncatula and noted its applicability for other model plants.

We grew M. truncatula wild type plants on buffered nodulation media (BNM—see Materials & Methods) for four to five days and performed wild type to wild type inverted-Y grafts to demonstrate the protocol. Two approximately equal sized stock and insert roots were selected for grafting. Plants with rootstocks originating with two different plants were obtained by diagonally cutting completely through the insert root (Root B) at root-shoot junction, while making small incision on the same spot of the stock root (Root A) with same angle as the insert and grafting the insert upwards into the silt of the stock so that the cut surface of the insert faces the path of the stock (Figure 3.4A). Unlike other protocols in the literature (Foo et al. 2001; Li et al. 2009; Li et al. 2012), this technique does not require parafilm, medical tubing or other support materials. Instead the two roots support each other until the graft is healed (Figure 3.4B). Our experience in developing the M. truncatula inverted-Y grafting approach agrees with the comments of Yin, et al. on water and morphology differences (Yin et al. 2012); we too have incidentally observed that the healing process appears to be negatively affected by the water content of the media. We overcame this by using thick pre-wetted brown seed germination paper on BNM media to create a slightly dryer
environment than agar alone and facilitated the healing process by incubating the plates horizontally for five days (Figure 3.4B). Successful grafting was determined by new root growth 2-3 weeks after grafting (Figure 3.4C). Plants with healed roots of equal size (Figure 3.4D) were then transferred to Perlite and grown/inoculated as in the split root protocol (Figure 3.2 F). Because the only way to visually distinguish roots of different genotypes on plates is labeling, we cannot emphasize enough the importance of attention to detail in the labeling of the plates and pots.

**Figure 3.4. Creating an inverted-Y graft between two different rootstock genotypes.** (A) Schematic representation of inverted-Y grafting. Genotype A is the main stock which contributes both the shoot genotype and one of the rootstocks and genotype B is acting as the insert contributing only one of the
rootstocks. (B) Close up of graft immediately after insertion. (C) Four day old plants immediately after grafting on BNM media covered with brown seed germination paper. (D and E) Successful grafts immediately before transferring to a Perlite system. Red arrows in D indicate the new root growth in the successful grafts while blue arrows indicate failed grafts that lack new growth. (F) Representative plant 21 days after the second root inoculation; washed and ready for nodule counting.

The wild type to wild type grafts resulted in large root systems and the plants nodulated normally upon rhizobial inoculation (Figure 3.4E). Consistent with our split root inoculation experiment, the initial inoculation of Root-A systemically suppressed nodule formation on the Root-B demonstrating the grafted roots behaved as a single system. In order to confirm that there was no effect of the healing process on the graft transmissible signal, we also used the grafted root and the main stock root interchangeably as “Root-A” and inoculated with rhizobia first. As indicated by comparing the two combinations in Figure 3.5A, inoculating either root first did not affect the systemic suppression of nodulation in the respective second root. This suggests that the grafted root is functionally and morphologically identical to the main root and the transmissible signal is not affected by the procedure. As further confirmation of vascular integrity, we performed microscopic analysis of cross sections of the Y graft junctions and noted normal vascular connections (Figure 3.5 B, D, and E).
Figure 3.5. Nodule number on inverted-Y grafted plants. (A) Wild type (A17) self grafts show systemic inhibition of nodulation on the second root. The X axis label indicates whether the first root inoculated (Root A) was the stock root or the inserted root. Root B was inoculated 4 days after Root A and data was collected 21 days after Root B inoculation. The Y axis is average nodule number per root; error bars indicate standard error of the mean. Both comparisons are significant by pair-wise t-test with P<0.05. N= 6 for the first set of bars and 8 for the second. (B) Longitudinal section of vasculature at Y graft junction (C) Reciprocal graft longitudinal section. (D & E) Cross sections of stock root and grafted root respectively of plant in (B).
The inverted-Y grafting approach can also be used for shoot to root reciprocal grafting
As noted, there is a long history of reciprocal grafting, at least in larger species, to demonstrate transmissible signals. However, in *M. truncatula* the root/shoot junction and the roots themselves are soft and have a narrow diameter making them difficult to handle during standard grafting procedures. By using the above inverted-y grafting approach with slight modifications (cutting deeper into the hypocotyl of the main root and then removing the original root after the graft heals), we improved the success rate of reciprocal grafts from 50% in our previous work (Penmetsa et al. 2003; Schnabel et al. 2010; Schnabel et al. 2011) and 8% in that of Lohar and VandenBosch (2005) to 66% (Table 3.1). This deeper cut prevents the stock root from growing and favors the healing of the vasculature in the new root. Successful grafts, indicated by fresh root growth on the inserted root (Figure 3.6A), were selected and the main rootstocks, which usually did not show further development due to deep cutting, were removed by excision with a razor blade before planting (Figure 3.6B). Figure 3.6C shows a comparison of nodule number in reciprocal and self-grafted plants between the wild type (A17) and the autoregulation defective mutant rdn1-2 21 days post inoculation. Hypernodulation resulted when a wild type shoot was grafted onto an rdn1-2 root. In contrast, wild type nodulation resulted when an rdn1-2 shoot was grafted onto a wild type root, in agreement with the conclusion of our previous grafting studies that nodule number in rdn1-2 is determined by the root genotype (Schnabel *et al.* 2011). However, the use of our new technique added statistical power to our observations by allowing us to increase the number of plants used in a given experiment up to 20. Indeed our new
approach also provided healthy grafts morphologically indistinguishable from each other and intact plants except for the number of nodules (Figure 3.5C, Appendix H).

**Table 3.1. Success rate of shoot to root reciprocal grafting using a modification of the inverted-Y technique.** The description of the graft combination refers to Wild type: ecotype Jemalong A17, mutant-1: the shoot controlled hypernodulator *sunn-4* (Schnabel et al 2005) and mutant-2: the root controlled hypernodulator *rdn1-2* (Schnabel et al 2011)

<table>
<thead>
<tr>
<th>Graft Combination</th>
<th>Initiated</th>
<th>Successful Grafts</th>
<th>Success Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type/Wild type</td>
<td>24</td>
<td>18</td>
<td>75</td>
</tr>
<tr>
<td>Mutant-1/Mutant-1</td>
<td>21</td>
<td>11</td>
<td>52.4</td>
</tr>
<tr>
<td>Mutant-2/Mutant-2</td>
<td>23</td>
<td>14</td>
<td>60.9</td>
</tr>
<tr>
<td>Mutant-1/Wild type</td>
<td>12</td>
<td>10</td>
<td>83.3</td>
</tr>
<tr>
<td>Mutant-1/Mutant-2</td>
<td>23</td>
<td>14</td>
<td>60.9</td>
</tr>
<tr>
<td>Mutant-2/Mutant-1</td>
<td>24</td>
<td>15</td>
<td>62.5</td>
</tr>
<tr>
<td><strong>Mean ±SE</strong></td>
<td><strong>21.2 ± 1.9</strong></td>
<td><strong>13.7 ± 1.2</strong></td>
<td><strong>65.8 ± 4.6</strong></td>
</tr>
</tbody>
</table>
Figure 3.6. Shoot to root reciprocal grafting. (A & B) Successfully grafted plants on BNM plates immediately before planting on Perlite. (A) The actively growing roots (indicated by red arrows) are the insert roots; the stock roots have ceased growth. (B) Red arrow marks root to be cut before transfer to Perlite. (C) Average nodule number on reciprocally grafted M. truncatula plants. Error bars indicate standard error of the mean. For the combinations noted as shoot/root, N=27 for A17/A17, 4 for A17/rdn1-2, 4 for rdn1-2/A17 and 20 for rdn1-2/rdn1-2. Letters designate significant difference by Tukey-Kramer test (p<0.05)
DISCUSSION

This study presents two improved and reliable techniques of dissecting long distance autoregulatory mechanisms of nodule regulation in *M. truncatula*; (1) a split root experiment to assay AON inhibition that can be adapted to test long distance signaling in any combination of root environments and plants that can be grown on plates and moved to Perlite and (2) an inverted-Y graft experiment that in addition to the above benefits, can be used to differentiate signaling originating in the root from downstream roots responses to shoot signals and that can also be used to generate reciprocal grafts.

The ease of the described split root protocol increases the number of split root plants most investigators can generate for a particular experiment by growing the split root seedlings on HMF media until they are ready for planting on soil system. In addition, the planting of the separate root systems does not require PVC piping or special split root tubes. Instead, ten days after the cutting of the primary root, the regenerated lateral roots are long enough to plant directly in separate pots filled with washed Perlite. The initiation of lateral root development on HMF media sandwiched between two half round Whatman filter papers provided the option to select two equivalent roots and trim the rest for the split root assay (Figure 3.2). The choice of the Perlite growth system for the nodulation portion of the assay allows ethylene to dissipate, eliminating a significant variable in root response. We demonstrated the inhibitory effect of the autoregulation signal in the delayed inoculated wild type root and showed that the *rdn1*-2 mutant is defective in AON (Figure 3.3). We are now using the techniques to define precisely
when the AON circuit initiates, how long it lasts, and whether there is any second message after the initial signal.

Grafting is a valuable tool for dissection of long distance plant communication between roots and shoots. The advantage of the grafting methods presented here is the elimination of the need to hold the grafts together with extra materials, making the method applicable for plants with very small stem diameters. Instead of two shoots from approach grafting (Li et al. 2009), an inverted-Y graft provides a single shoot that perceives the root derived factor and transduces the shoot derived inhibitory signal. Because our grafting technique does not need materials for physical support (Figure 3.4), it also requires less manual dexterity by the experimenter and should be suitable for growing plants out on soil, plates and in aeroponic chambers after grafting. The success rate is high for a complex technique; in our hands it is so high that we routinely make the less complex reciprocal grafts by the modification described to the inverted-Y experiment rather than the technique used in our previous work (Penmetsa et al. 2003; Schnabel et al. 2010; Schnabel et al. 2011). Using the inverted-Y method, we demonstrated that the shoot derived inhibitory signal initiated by inoculating the one of the roots (Root A) first suppresses nodulation in the delayed inoculated second root (Root B). We also confirmed the success of the graft union and the intact nature of cell-to-cell communication in the plants by showing that the results were not dependent on whether the grafted root or the original root was inoculated first (Figure 3.5). Currently, using the inverted-Y technique we are investigating the role of RDN1 in long distance
nodule regulation. Taken together, these data suggest that the inverted-Y graft will be invaluable in identifying the different components of the AON circuit by grafting different combinations of wild type and the autoregulation defective mutants together.

The modification of the inverted-Y grafting technique we developed for shoot-to-root reciprocal grafting is simple and highly efficient, with a 66% success rate in plants carrying two different mutations in genes of the AON pathway (Table 3.1). It does not require external support as the stock root will act as a support until the graft heals. Most importantly, the grafting method does not affect the normal development of the plant since healthy and completely healed grafts are attained rapidly and the morphology and the nodulation of the grafted seedlings are the same as the intact wild type and mutant plants (Appendix I). Using this approach, we demonstrated that rdn1 mutants are defective in sending the root derived signal initiated by inoculation or perceiving the shoot derived inhibitory signal confirming the rdn1 mutation is appropriately named root determined nodulator (Figure 3.6 and Schnabel et al. 2011).
CONCLUSION

The described methods are useful for the dissection of long distance signaling in plants. Our split root inoculation protocol showed marked improvement over existing methods by eliminating complex apparatuses and allowing selection of root systems balanced for size. Elimination of external supports coupled with defined growth conditions allows even dexterity challenged experimenters to create experimental plants with split root systems. The inverted-Y graft approach has the dual advantage of being more efficient than published shoot-to-root reciprocal grafting techniques and is also a useful system to produce a plant having two different rootstock genotypes. Both techniques together allow dissection of long distance plant developmental regulation using very simple, efficient and reproducible techniques. We suggest the combination of autoregulation defective mutants, grafting, and inoculation of split root systems will complement other molecular genetic and biochemical approaches to unravel the signal transduction involved in AON. We propose the techniques should be broadly applicable to other plants, including those of small size such as Arabidopsis.
MATERIALS AND METHODS

Plant Materials and Growth Conditions
Seeds of *Medicago truncatula* cv Jemalong ecotype A17 and the root determined nodulator *rdn1-2* (Schnabel *et al*. 2011) were utilized for this experiment. Seeds were acid scarified and imbibed as described in Schnabel *et al*. (Schnabel *et al*. 2011). Seeds were vernalized in dark at 4°C for 2 days on Harrison Modified Farhaeus (HMF) media (Huo *et al*. 2006) covered with two half round filter papers (Whatman, catalog # 1001090). The seeds were then germinated in the dark at room temperature for 1 day and used for the following techniques.

Split Root Technique
Using sterile technique under a positive flow hood (for all work described in this report done before transfer to Perlite), one day old seedlings were placed five seedlings per plate on 9 cm Petri dishes containing HMF media covered with sterilized half round filter papers (Whatman) and allowed to grow vertically for five days in a growth chamber (25°C and 16 hr photoperiod). Lateral root initiation was stimulated by removing the roots of these five day old seedlings at the root-shoot junction and transferring them to new HMF plates sandwiched between two half round Whatman filter papers, important to keep the roots moist and in the dark. We used a sterile razor blade to cut the root under aseptic conditions. After growing vertically in the growth chamber for an additional five days, the top filter paper was removed. Approximately one week later, lateral roots of sufficient length had formed and plants having two
adventitious roots of approximately equal size were chosen for planting in split-root pots filled with washed Perlite (Figure 3.2E). Each of these two lateral roots (Root A and Root B in Figure 3.1) was separately planted to an individual pot, and the remaining lateral roots were cut off. To establish the plants in the Perlite system, the plants were watered for 5 days with a 100-fold dilution of water-soluble 20:10:20 Peat-Lite Special fertilizer (Scotts). Fertilization was then withdrawn and the plants were hydrated with water alone an additional five days in order to induce nitrogen deficiency required for nodulation. The plants were then used for split-root inoculation experiments with bacteria. The first root (Root A) was inoculated via flood inoculation (see inoculation below) and second root (Root B) was inoculated in the same manner four days later. Nodules on each root were counted 21 days after the Root B inoculation.

**Inverted-Y grafting**
One day old seedlings were grown on sterile Nunc Bio-Assay dishes (245mm x 245mm x 25mm) each containing 250 ml of BNM [42] covered with 16.5cm x 22.9cm brown seed germination paper (Anchor Paper 76 # heavy weight brown seed germination paper) and sealed with Curasilk™ Hypoallergenic cloth tape (Kendall). We found this particular weight of paper to be important for good results. The seedlings were grown vertically for 5 days in the growth chamber at 25°C with a 16 hr photoperiod. Relatively equal roots from the respective stock and insert genotypes were selected for grafting. Using a sterile razor blade under aseptic conditions in a positive flow hood, the insert root was diagonally sliced at the hypocotyl, while making a small incision (slit) about half way through the hypocotyl of the stock root with same angle as the insert. In our
experience, cutting deeper than halfway into the stem prevents the stock root from growing, presumably because the vasculature is damaged, and the delay in growth affects the healing of the graft favoring the new root over the stock root. The graft was inserted upwards into the slit of the stock so that the cut surface of the insert faced the path of the stock. The plates were sealed again with Curasilk™ Hypoallergenic cloth tape (Kendall). Five days after grafting, the plates were turned vertically and the grafted plants allowed to grow for more than ten days under the same growth chamber conditions. Occasionally (at most twice in an experiment) any extra lateral roots coming out from the stock were trimmed to stimulate the growth of the inserted root. After at least 10 days, the well-grafted seedlings (determined by new growth on both roots) were transferred into the Perlite system and grown and evaluated as in the split root experiment described above.

**Inverted-Y grafting for reciprocal grafts**

For shoot to root reciprocal grafting, the inverted-Y grafting was performed as mentioned above except the incision for inserting the graft was cut diagonally 75-80 percent into the hypocotyl of the stock, with same angle as the insert. As mentioned in the methodology for the inverted-Y graft, this prevents the stock root from growing and favors the graft to preferentially heal the new root. Successful grafts, indicated by fresh root growth on the inserted root, were selected and the main rootstocks, which usually did not show further development due to the deep cutting, were removed by excision with a razor blade before planting.
Inoculation & sectioning

All plants were inoculated with *Sinorhizobium medicae* strain ABS7, carrying a LacZ reporter gene on a plasmid with tetracycline resistance (Bekki *et al.* 1987). The rhizobia were grown in liquid TY media containing 15μg/ml tetracycline at 30°C on a rotary shaker at 250 rpm for approximately 48 hrs. Before inoculation, the rhizobia were diluted to an OD 600nm of 0.2 with water and 6 ml of the bacterial solution was applied to each root compartment at root collar region (flood inoculation). Nodulation was observed and nodules were counted 21 days after the second root inoculation for both the split root and inverted-Y graft experiment and 21 days after the only inoculation for the reciprocal grafting. Nodules were counted using an Olympus SZX12 Dissecting Stereo Microscope after gentle washing of the Perlite away from the roots. Hand sections of roots were made with a sharp razor blade while holding the root down with forceps.

Photography and Data analysis

Roots were photographed with the same Olympus SZX12 Dissecting Stereo Microscope using an Olympus DP11 Digital Camera System or a Zeiss Axiostar plus with a Nikon coolpix5000 digital camera for the cross sections in Figure 3.5C & D. Larger pictures were also obtained obtained with the Nikon coolpix5000 camera. For split root inoculation and inverted-Y graft data paired t-tests were used to identify statistical significance differences between root systems using p<0.05 as the significance level. The Tukey-Kramer test was used for shoot to root reciprocal grafted data (p<0.05).
COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHOR’S CONTRIBUTIONS

TK participated in the design of the experiments, carried out the experiments, and drafted the manuscript. JF also participated in the design and coordination of the experiments and helped write the manuscript. All authors read and approved the final manuscript.

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REFERENCES


CHAPTER 4

FURTHER CHARACTERIZATION OF MTRDNI LOCALIZATION AND FUNCTION, COMPARISON OF RDN1 AND SUNN AUTOREGULATORY MUTANTS AND INVESTIGATION OF THEIR GENETIC INTERACTIONS

ABSTRACT

Rhizobia species fix molecular nitrogen in association with legumes in a process called nodulation. Since nodulation is energetically costly to the host, legume plants balance the nitrogen demand with the energy expense by limiting the number of nodules through long-distance signaling. After cloning the RDN1 gene in Chapter 2, in this chapter we localized RDN1 to a large number of tiny moving organelles in the cytoplasm, and present evidence of co-localization with secretory pathway components, suggesting that RDN1 may engage in vesicle trafficking. We used both split root system and grafting techniques to investigate the timing of the long distance nodule autoregulation signal in Medicago truncatula and the role of RDN1 and SUNN genes in the regulatory circuit. We found that prior nodulation events do not affect later nodulation events for plants carrying mutations in RDN1 and SUNN genes, unlike wild type plants. The full induction of the autoregulation of nodulation took three days in wild type plants. However, we report of evidence for the initiation of a second signal in matured nitrogen fixing nodules ten days after the first root inoculation which
suppressed nodulation on the second root in both mutant and wild type plants. Inverted Y graft combinations of wild type plants and plants carrying mutations in \textit{RDN1} and \textit{SUNN} demonstrated that \textit{RDN1} regulatory function occurs in the root before the shoot-derived suppression signal which involves \textit{SUNN}. The \textit{sunnen/rdn1} double mutation and the shoot to root reciprocal grafting between \textit{sunnen-4} and \textit{rdn1-2} supports \textit{SUNN} and \textit{RDN1} acting in the same pathway. Combined with our data that the \textit{SUNN} message is down regulated in the \textit{rdn1} background whereas \textit{RDN1} message level is normal in the \textit{sunnen} background, we suggest RDN1 acts before SUNN.
INTRODUCTION

Autoregulation of Nodulation (AON) encompasses root-shoot-root long distance signaling. Hence, both shoot and root controlled AON defective mutants have been reported in Medicago truncatula, Lotus japonicus, Glycine max and Pisium sativum providing important insight to dissect plant developmental regulation mechanisms. The shoot controlled AON genes which are cloned so far known to encode a leucine-rich repeat receptor protein kinase like the CLAVATA1 (CLV1) of Arabidopsis thaliana (Krusell et al. 2002; Nishimura et al. 2002; Searle et al. 2003; Schnabel et al. 2005). This protein kinase is abundant in plants and has an extracellular domain of leucine-rich repeats, a membrane spanning domain and an intracellular protein kinase domain (Downie and Parniske 2002). Because they exert their effect from the shoot, they are believed to be the receptors for the root-derived signal and triggers for the biosynthesis and release of the shoot derived inhibitory signal. However, recent genetic and biochemical studies continue to reveal the complex nature of the interaction of different receptor kinases. CLV1 interacts with CLV3, a precursor of a secreted peptide, and they have been shown to function in the same genetic pathway to maintain stem cell proliferation in Arabidopsis shoot apical meristem (Ogawa et al. 2008). The CORYNE (CRN) receptor kinase can readily form heterodimers with CLV2 and then a CLV2/CRN receptor complex binds CLV3 and regulates the stem cell-promoting homeodomain transcription factor WUSCHEL (WUS) in a pathway separate from CLV1, although there is evidence for the formation of a CLV1/CLV2/CRN receptor
complex at the plasma membrane (Muller et al. 2008; Ogawa et al. 2008; Guo et al. 2010; Bleckmann et al. 2010; Zhu et al. 2010). The genomes of both Arabidopsis and M. truncatula encode a large family of CLAVATA3/embryo-surrounding region (CLE) genes, which have varied expression and function. Two genes, MtCLE12 and MtCLE13, are involved in indeterminate nodule development with nodulation related expression patterns that were linked to proliferation and differentiation (Mortier et al. 2010). The expression of both MtCLE12 and MtCLE13 increases upon rhizobia inoculation and remains high 10 days post inoculation (Mortier et al. 2010). Furthermore, both genes were induced by nitrate addition in a concentration dependent manner (Okamoto et al. 2009). Nodule number on transgenic roots expressing an MtCLE12/13 RNAi hairpin construct is significantly higher than the control plants, suggesting a role for MtCLE12/13 in nodule number regulation (Mortier et al. 2012). Ectopic expression of the group III peptide genes MtCLE12 and MtCLE13 was shown to result in systemic and SUNN-dependent inhibition of nodulation, although the effect differed between sunn alleles, being much stronger in the sunn-4 null allele versus the sunn-1 missense allele (Mortier et al. 2010; Saur et al. 2011). Consistent with this, Glycine max GmRIC1 and GmRIC2, and Lotus japonicus LjCLE-RS1 and LjCLE-RS2 regulate nodule formation systemically in GmNARK and HAR1 dependent manner respectively (Lim et al. 2011; Okamoto et al. 2009). Thus, CLE peptides are strong candidates for the root-derived mobile signal induced both by nitrate and rhizobial inoculation. However, the long distance movement of CLE peptides has not been experimentally demonstrated in
any system and their connection with upstream components of the root-derived signal has not been shown.

Long-distance transport of a variety of molecules relies on the plant vascular system, which includes two vital tissues; xylem and phloem. GUS staining patterns resulting from expression of *SUNN* promoter fused to *uidA* showed expression of *SUNN*, one of the shoot receptor kinases, confined to the vascular tissue, a pattern that overlaps with *RDNI* expression (Schnabel *et al.* 2012). The similarity in tissue level expression strongly suggests vascular signaling for nodule number regulation and direct and/or indirect long-distance connection between the two genes. The root-controlled supernodulating mutants are thought to be impaired in either the biosynthesis or transmission of the root-derived signal or in the perception of the shoot-derived inhibitory signal. However, the nature of the root- and the shoot-derived signals are still unknown and the nodule autoregulation pathway is very complex. The shoot-derived inhibitory signal is reported to be a small compound with molecular weight of less than 1kDa and reported to be neither RNA nor protein, since RNase and proteinase treatments did not alter nodule suppression in a vascular feeding experiment in soybean (Lin *et al.* 2010). AON activates early upon the nod factor perception; the elongation zone of the root with emerging root hairs is vulnerable to rhizobial infection and most affected by the autoregulation process (Bhuvaneswari *et al.* 1981). Long distance signaling was initially demonstrated by split root inoculation experiments by first inoculating one side of the split root that systematically suppresses further nodulation in
the other side of the split root. As noted in Chapter 3, the time of the full induction of AON varies from species to species.

Nodule initiation and development are also under various physiological and developmental controls. Successful nodule formation and subsequent nitrogen fixation occur normally under nitrogen limiting conditions. In the presence of high concentrations of biologically available nitrogen, plants shut down nitrogen fixation and hence nodule formation is suppressed (Schultze and Kondorosi 1998). In contrast, mutants lacking AON are reported to be nitrate tolerant and produce 5-10 times more nodules than wild type plants (Caba et al. 1998; Carroll et al. 1985). This suggests that the autoregulation signal and the nitrate signal may interact to inhibit nodule formation.

The nitrogen sources taken up by higher plants are nitrate or ammonium. However, nitrogen assimilation requires the reduction of nitrate to ammonium, followed by ammonium assimilation into amino acids. Using attached nodules and pulse-chase labeling, it was demonstrated that fixed nitrogen is transported in the xylem of nodulators largely as glutamine, asparagine or uriede (Baker and Parsons 1997). The large pool of asparagine that is present in nodules may buffer the transport of nitrogen and thus act to regulate nitrogen fixation via a feedback mechanism (Baker and Parsons 1997). In addition, inhibition of nitrogen fixation can affect the partitioning of carbon and other metabolites from the shoot. Singleton and Kessel (1987) showed that the flux of photosynthate to nitrogen fixing nodules and their associated roots is greater than in non-nitrogen fixing sections of the root. This suggests that nitrogen assimilation, either
from N₂ fixation or inorganic sources, has a localized effect on both nodule and root development. It has been also demonstrated by split root systems that the lack of nitrogen acquisition by half root system nodulated with non-fixing rhizobia triggers a compensatory response enabling the other half root system nodulated with nitrogen fixing partners to compensate the local nitrogen limitation (Laguerre et al. 2012).

In addition, the gaseous plant hormone, ethylene is a negative regulator of nodule formation (Caba et al. 1998). The M. truncatula mutant, skl (sickle), defective in ethylene perception, is insensitive to ethylene and has a 10-fold increase in nodule number relative to the wild type (Penmetsa and Cook 1997). In contrast to skl mutants which are unaffected, the number of nodules produced by the sunn-1 hypernodulation mutant is reduced when inoculated after aminocyclopropane carboxylic acid (ACC) application (Penmetsa et al. 2003). The addition of ethylene inhibitors like L-α-aminoethoxyvinyl-glucine (AVG) enhanced nodule development in pea (Tamimi and Timko 2003). Taken together, ethylene, like AON, is a negative regulator of nodule formation, and hence inhibition of ethylene enhances nodule development. The phytohormone, auxin, which exists predominantly in the form of Indole-3-Acetic Acid (IAA), has long been proposed to play a crucial role in plant development. It is mostly produced in younger plant shoots and moves long distance down to the root tip following an auxin concentration gradient with the help of auxin efflux protein complexes (Pacios-Bras et al. 2003; Schnabel and Frugoli 2004). This triggers the development of roots, nodules and other plant organs. In uninoculated roots of sunn-1
mutant auxin transport from shoot to root is approximately three times higher than the wild type (van Noorden et al. 2006). In addition, auxin transport inhibitors like NPA, when locally applied at shoot-root junction and added directly into the growth media, significantly reduce the nodule number in sunn-1 mutants but not the sickle mutants just as ethylene does (Prayitno et al. 2006; van Noorden et al. 2006). These results and other related experiments suggested that SUNN acts in the auxin signaling pathways and SICKLE in ethylene signaling pathways, and defects in either can lead to supernodulation.

Secretion of proteins and other molecules is the primary means by which a cell communicates with surrounding cells. As mentioned in Chapter 2, the RDN1 protein with an N-terminal signal peptide is predicted to enter the secretory pathway (Schnabel et al. 2011). Proteins destined for the secretory pathway are synthesized and processed in the endoplasmic reticulum (ER) prior to reaching the Golgi for further processing and sorting (Barlowe et al. 1994; Foresti and Denecke 2008). Cargo proteins that pass the quality control of the ER are delivered to the Golgi apparatus which is central for mediating protein and lipid transport within eukaryotic cells. Numerous secreted proteins are constantly trafficking through the Golgi and transported in the direction of secretion, storage or degradation (Handford et al. 2004; Wang et al. 2006). Eukaryotic cells have developed mechanisms to ensure that these proteins are targeted to the correct secretory pathway. The prevacuolar compartment is a post-Golgi protein-sorting organelle, where proteins destined for transport to the lytic vacuole are delivered from
the trans-Golgi network. Many proteins located in the plasma membrane are involved in the transport of cargo molecules in and out of the cell. As eukaryotic cells have a secretary pathway which is composed of several functionally distinct membrane compartments, at the same time they have the ability to internalize a variety of macromolecules by endocytosis. Several markers for both the exocytic and endocytic pathways in plants have been established (Bolte et al. 2004; Nelson et al 2007). These including styryl dyes such as FM4-64 and have been commonly used to study endocytosis in both plant and animal cells. Upon incubation of plant cells with the dye, FM4-64 immediately stains the plasma membrane by becoming inserted and anchored in the outer leaflet of the plasma membrane lipid bilayer; it then is internalized and stains putative endocytic intermediates, prevacuolar compartments, Golgi apparatus and the vacuolar membrane in a time dependent manner (Bolte et al. 2004). Colocalization with FM4-64 alone is insufficient to determine secretory pathway localization since, depending on the time of incubation, cell type, and experimental conditions, the tracer can label almost any compartment of the cell. Hence, in addition to organelle specific markers, researchers have been using brefeldin-A (BFA), which inhibits transport from endoplasmic reticulum to Golgi and from endosomes to plasma membrane and causes aggregate formation in BFA compartments (Kano et al. 2000; Grebe et al. 2003; Jaillais et al. 2006).

In this chapter, we investigate the subcellular localization of RDN1. The role of RDN1 in the long distance root to shoot signaling is addressed, including the timing of AON
and a new finding of a second AON signal correlated with nitrogen fixing nodules and meristem development in *M. truncatula* which has not been previously reported. Our findings support that assertion that the second AON signal is not associated with early nodulation events and meristem activity by comparison of total nitrogen fixation and nodulation phenotypes across different *M. truncatula* genotypes. Using inverted-Y grafting, shoot to root reciprocal grafting, and expression analysis, we also found that nodule number regulation by RDN1 is SUNN dependent and RDN1 acts before SUNN in the cascade of regulation signaling.
RESULTS

MtRDN1 is Associated with Secretory Pathway Components

The MtRDN1 protein has an N-terminal signal peptide which would predict involvement in the secretory pathway in plants (Schnabel et al. 2011). To confirm MtRDN1 localization in the endomembrane system, we used several approaches. Initially, we created a plasmid expressing MtRDN1-GFP under the 35S CaMV promoter (see Materials and Methods). The plasmid was expressed in rdn1-1 and rdn1-2 mutant roots via A. rhizogenes transformation to confirm its functionality by phenotypic rescue. The construct was functional and complements both rdn1-1 (data not shown) and rdn1-2 mutant alleles when compared to the empty vector control roots (Figure 4.1 A). The construct was then transformed into wild type and rdn1 plants for microscopic observation and we noted that MtRDN1-GFP localized in very tiny moving organelles in the cytoplasm of the Medicago root hair cells (Figure 4.1 B), a pattern we also observed in tobacco epidermal cells (Figure 4.1 C & D). To confirm the resulting pattern was not an artifact of the hairy root system and to further pinpoint localization, we examined the localization of the MtRDN1-GFP fusion protein after transient expression in tobacco leaves co-infiltrated with various organelle specific markers developed in Arabidopsis (Nelson and Nebenfuhr 2007). We observed the MtRDN1-GFP fusion protein co-localized with Golgi mCherry-tagged protein marker (Figure 4.2 d-f). In contrast, MtRDN1-GFP did not co-localize with ER-mCherry, Vacuole-mCherry, Peroxisomal-mCherry and mCherry-tagged late endosomal markers (Figure 4.2 a-c and Appendix J).
Figure 4.1. GFP tagged MtRDN1 complements the \textit{rdn1-2} mutation and is localized in a tiny punctuate distribution in the cytoplasm of the root hair cells of \textit{M. truncatula} and epidermal cells of \textit{N. benthamiana} leaves. (A) Average nodule number per transgenic root from plants transformed with empty vector control and MtRDN1-GFP. Error bars indicate standard error of the mean (n=4-9 plants). (B) Root hair cells from uninoculated transgenic root showing punctuate like moving organelles. Axiovert 200 M fluorescence microscope was used to take the picture. (C&D) Subcellular localization of MtRDN1-GFP in tobacco leaf epidermal cell, using FITC filter (C) and overlay of FITC, Rhodamine and DIC images (D). Red dots are chloroplasts. GFP fluorescence was visualized with confocal laser scanning microscope.

The association between the PM mCherry-tagged marker protein and \textit{MtRDN1} was less clear. While not co-localized, we observed \textit{MtRDN1} at the edge of internal side of plasma membrane (Figure 4.2 g-i), but this could be an artifact from compression of the cytosol which occurs in leaf cells. Additionally, we employed the fluorescent dye FM4-64 which is commonly used by many plant researchers as an endocytic tracer (Ueda \textit{et l}.)
2001; Bolte et al. 2004; Qi et al. 2011). Small pieces of tobacco leaves, transiently expressing MtRDN1-GFP, were incubated in 8.2mM FM4-64 dye in dark for the indicated minutes and viewed with confocal microscope. The FM4-64 dye stained the plasma membrane immediately after 10-15 minutes and there was no colocalization of FM4-64–labeled membrane compartments and the fluorescing spots of MtRDN1-GFP (Figure 4.3 a-c, solid yellow arrows) indicating that MtRDN1 may not be associated with the PM. After half an hour, this dye internalized and co-labeled MtRDN1 small punctate structures in some cells (Figure 4.3 d-i). We observed more internalization of the dye after 90 minutes and more colocalization with MtRDN1. Taken together, these results suggest that native MtRDN1 is localized in cellular compartments consistent with trafficking in the secretory pathway.
Figure 4.2. RDN1 localization. Epidermal cells of *N. benthamiana* were co-transformed with MtRDN1-GFP and mCherry tagged organelle specific subcellular markers: endoplasmic reticulum (ER), Golgi apparatus (Golgi) and plasma membrane (PM). Images from mCherry (a,d,g), GFP (b,e,h) and merged images (c,f,i). Using laser scanning confocal microscope. Maximum intensity images from optical sections are shown.

**RDN1 is involved in Synthesis or Sending of the Root-Derived Signal**

Previously we reported that reciprocal grafting demonstrated *rdn1* mutant plants regulated nodule number from the root (Schnabel, et al. 2011a), while *sunn* mutant plants regulate nodule number from the shoot (Pentmetsa *et al.* 2003). While
determining shoot control of nodule number is straightforward with reciprocal grafting, it cannot be used to determine whether RDN1 is involved in sending the root-derived stimulatory signal or perceiving the shoot-derived inhibitory signal. To clarify the role of RDN1, we tested AON using inverted-Y grafts in which roots are separated in time, space and genotype (Kassaw and Frugoli 2012). In this system, a root of one genotype is grafted onto the shoot and root of another and the roots are placed in different pots. The effect of inoculation of one root on the later nodulation of the second root can then be measured. We have previously demonstrated that this method works as expected in wild type plants with a four day delay between inoculation of the first and second root (Kassaw and Frugoli 2012). We created plants carrying wild type shoots and a root each of the wild type and rdn1-2 genotypes, as well as plants carrying rdn1-2 shoots as controls and a root each of the wild type and rdn1-2 genotypes (see Materials & Methods). The first root (Root-A in Figure 4.4) was inoculated with S. medicae strain ABS7 (Bekki et al., 1987) and the second root (Root-B in Figure 4.4) was inoculated with the same strain of Rhizobia 4 days later. Nodules were counted on the two roots 21 days after the second inoculation. We reasoned that if RDN1 is involved in sending a signal to the shoot, in the A17.rdn1-2 graft combination, inoculating the wild type (A17) root first will suppress nodulation in the mutant (rdn1-2) root inoculated later. However, if RDN1 is a receptor of the shoot-derived signal, inoculating the wild type root first should not affect nodulation on the rdn1-2 root inoculated later and hypernodulation would be observed, since the rdn1-2 mutation should not abolish induction of the root-derived signals from the A17 root. The data in Figure 4.4 confirm that whether the
shoot genotype was *rdn1-2* or wild type, inoculating the wild type root first suppressed nodulation in the delayed inoculated *rdn1-2* root, while inoculating the *rdn1-2* root first did not suppress nodulation on wild type roots, implying that *RDN1* is responsible for the synthesis or sending of the root-derived autoregulatory signal.

**Figure 4.3. MtRDN1 is the endomembrane system.** Confocal images of FM4-64–stained tobacco leaf epidermal cells from a region infiltrated with MtRDN1-GFP. Leaves are incubated with FM4-64 dye for 15 minutes (a-c), 30 minutes (d-f), 90 minutes (g-i) in dark before imaging. White arrows indicate co-localization between MtRDN1 and the FM4-64 dye. Yellow arrows indicate no co-localization between the two.
AON in *M. truncatula* occurs within three days and persists at least 15 days
The timing of AON has been reported to vary between species, from as little as 30
hours in *V. sativa* (van Brussel *et al.* 2002) to two, four and five days in *L. japonicus*
(Suzuki *et al.* 2008), *G. max* (Kossak and Bohlool 1984), and *T. subterraneum* (Sargent
*et al.* 1987) respectively. Although we were able to observe autoregulation at 4 days
post inoculation (Kassaw & Frugoli 2012) we determined the precise timing and
duration of autoregulatory signal timing in *M. truncatula* using a split-root system
recently developed in our lab (Kassaw & Frugoli 2012). In this system, plants with two
spatially separated equal roots, a tester (Root A) and a responder (Root B) were
inoculated simultaneously or two to 15 days apart with *S. medicae* as described in the
Materials and Methods. In Figure 4.5A the nodulation of Root-B of the wild type
cultivar A17 is compared to the nodulation of Root A as a percentage of the total
nodules on Root A. When inoculated simultaneously, the responder roots have more
nodules than the initial tester roots (above 100%), but this difference is not statistically
significant.
Figure 4.4. RDN1 involvement in sending a root-derived signal. The genotype before the period is Root-A and the genotype after the period is Root-B. Root-A is inoculated first and Root-B four days after Root-A. (A) Nodule number on inverted-y grafted plants when wild type acts as the main stock contributing the shoot and one of the roots. (B) Nodule number on inverted-y grafted plants when rdn1-2 acts as the main stock contributing the shoot and one of the roots. All experiments are performed in a greenhouse on the perlite system described in Chapter 3.
The percentage of nodules decreases when the roots are inoculated two days apart, and as the interval between inoculations increases from three days to 15 days, the responder root consistently develops close to 50% of the nodules of the first root, and this difference is significant as determined by ANOVA. This indicates that AON occurs between two and three days in *M. truncatula* and the degree of suppression remains constant through at least 15 days between inoculation of the tester and responder.

**Identification of late AON in hypernodulation mutants**

The same analysis was performed using plants carrying either one of two null alleles of the *rdn1* supernodulation mutant, *rdn1*-1 and *rdn1*-2 (Schnabel *et al.* 2011a) or one of two alleles of the *sunn* supernodulation mutant: *sunn*-1 which contains an amino acid change in the kinase domain and *sunn*-4, a null (Schnabel *et al.* 2005). For inoculations separated by zero to eight days, no significant difference in the percent nodulation between Root A and Root B was observed (Figure 4.5 B & C), a result expected in mutants isolated for their defects in AON. However, at ten days between inoculations of roots, significant suppression in Root B in all of the genotypes except *sunn*-4 was observed. Inspection of the nodules on Root A plants from data points separated 10 days or more between inoculations revealed pink nodules, an indication of active nitrogen fixation (Appendix K). Thus, although *rdn1* and *sunn* mutants are defective in AON at the time wild type plants display the phenomenon, by the time the nodules begin to fix nitrogen, three of the four alleles tested display suppression statistically indistinguishable from wild type plants. The discovery of an autoregulatory signal that
two independent AON mutants recognized and responded to led us to speculate on the nature of the signal(s).
Figure 4.5. The timing of autoregulation of nodulation in *M. truncatula* using a split root system. (A) Mean percent nodulation of root-B of the wild type A17. (B) Mean percent nodulation of root-B of the wild type A17 and the rdn1 alleles rdn1-1 and rdn1-2. (C) Mean percent nodulation of root-B of the wild type A17 and the sunn alleles sunn-1 and sunn-4. In all conditions, Root-B was inoculated 0, 2, 3, 4, 6, 8, 10, 11, 12, and 15 days after Root-A and data was collected 21 days after Root-B inoculation. Percentage data is presented as (Mean±SE) of n= 4–47 plants for each time point per genotype.

**Hypernodulation mutants lose the later autoregulation when inoculated with Fix− rhizobia**

One possibility is that wild type plants determine the degree of nodulation initially by the number of meristems initiated in the tester root. Since supernodulation mutants have more nodule meristems than wild type plants, we reasoned that these mutants would display the hypernodulation phenotype because they do not send or respond to the putative meristem signal. However, when nodules begin to supply nitrogen for the plant, a putative second signal based on assimilated nitrogen could be sent, and the hypernodulation mutants could receive this signal. To investigate this possibility, we used the split root assay again but inoculated the tester root with three *S. meliloti* strains; wild type *Rm*1021 (Meade et al. 1982), a Fix−/Nod+ bacterial mutant *Rm*1312 (Ruvkun et al. 1982) and a Fix−/Nod− bacterial mutant SL44 (Fisher et al. 1988). In separate experiments, Root A was inoculated with one of the three strains and the second root (Root B) was inoculated 20 days after Root A with the wild type strain. This late time point was chosen to accommodate the slightly slower pace of nodule development we observed in preliminary experiments when wild type plants were inoculated with *S. meliloti* versus *S. medicae*. As shown in Figure 4.6A, the number of nodules on the second root of both wild type and plants carrying the AON defective mutant alleles were suppressed to wild type levels by inoculating Root A with the wild type *Rm*1021.
strain, indicating that the suppression seen in Figure 4.5 also occurred with this species of rhizobia. Inoculation of Root A with the Fix/Nod$^-$ SL44 strain which does not form nodules does not suppress nodulation on Root B in both wild type and the hypernodulation plant mutants (Figure 4.6A), suggesting that without nodule meristems, AON does not occur. Interestingly, inoculation of the tester root (Root A) with the Fix$^--$/Nod$^+$ bacterial mutant $Rm$1312, resulted in suppression of nodulation in the second root of wild type plants but not the supernodulation mutants (Figure 4.6A), suggesting that the ability of the bacteria to fix nitrogen was not important to the regulation observed in wild type plants at this point in time, but was important to the late regulation phenomenon we observed in the mutants. As further evidence that assimilated nitrogen could be the signal, we measured the rate of nitrogen fixation at 10 and 15 days post inoculation with $S. medicae$ in A17, rdn1-2 and sunn-4 genotypes. The level of nitrogen fixation 10 days after inoculation was higher in both mutants than did the wild type plants but only significantly higher for rdn1-2 mutants (Figure 4.6B). At 15 days post inoculation the total nitrogen fixation was the same for all genotypes (Figure 4.6B).
Figure 4.6. The development of AON in mutants is concurrent with nitrogen fixing nodules. (A) The effect of prior inoculation of Root-A with different strain of *S. meliloti* (*Rm*1021, *Rm*1312, and SL44) on nodulation of Root-B with *Rm*1021, 20 days after Root-A. Error bars are standard error of the mean (n=5-10). (B) The hydrogen production rate measured using a Qubit system as equivalent to the total nitrogen fixed by nodules per plant. Tukey-Kramer minimum significance test with significant level, P<0.05, was used for statistical analysis.

The second signal is not associated with Early Nodulation Events

An alternative explanation for the late regulation phenomenon we observed was that the hypernodulation mutants require a much higher level of the putative meristem signal to respond, and only after this signal has accumulated to high level does AON occur. In this scenario, the increased number of nodules on the tester roots of hypernodulation
mutants in plants in the split root assay eventually sends enough signals to overcome the AON defect. We tested this hypothesis in two ways. First, we compared the total number of nodules and nodule primordia, nitrogen fixing nodules only and nodule fresh weight on Root A of the tester plants inoculated with *S. medicae*. Second, we measured the level of expression of the *CLE12* and *CLE13* genes in both wild type and mutant plants. Since these genes are induced in the root upon nodulation, are expressed in nodule meristems and overexpression reduces nodulation in a SUNN dependent manner (Mortier et al 2010; Saur et al 2011; Lim et al 2011), the CLE peptides, MtCLE12 and MtCLE13, might encode such a root-derived signal and we wondered if CLE expression levels correlated with nodule number. As we reported previously in Chapter 2, both the total and nitrogen fixing nodule numbers were significantly higher in the AON defective mutants (*rdn1-2* and *sunn-4*) than in the wild type plants (Appendix L A&B). The higher number of nodules in the mutant plants may cause them to produce additional regulatory signal related to meristem activities since *Medicago* is indeterminate nodulator; the nodule meristem does not senesce. A qRT-PCR time course analysis showed altered *MtCLE12* and *MtCLE13* expression in hypernodulation mutants. While both genes were up-regulated after rhizobia inoculation and expression level reached a maximum at 10 days post inoculation in wild type and mutants (Figure 4.7), the expression levels of *MtCLE12* and *MtCLE13* were higher in the mutant plants than in the wild type plants for all time points after inoculation for *MtCLE13* and 10 and 15 days after inoculation for *MtCLE12* (Figure 4.7).
Figure 4.7. MtCLE expression in the roots of perlite grown wild type (A17), sunn-4 and rdn1-2 mutants. The plants were inoculated with S. medicae and expression is displayed relative to wild type at day zero. SECRET AGENT (see materials & methods) is used for normalization (A) Relative expression of MtCLE13. (B) Relative expression of MtCLE12. Data is from a single biological replicate and three technical replicates.

Ethylene perception and response is normal in rdn1 mutants
Under some conditions, the inhibitory effect of NO3⁻ on nodulation can be eliminated by growing plants in the presence of the ethylene inhibitor Aminoethoxyvinyl Glycine (AVG), which strongly inhibited ethylene biosynthesis in plants (Ligero et al. 1999). The skl supernodulation mutant of M. truncatula is defective in ethylene response and
displays nitrogen resistant nodulation (Penmetsa & Cook 1997). If nitrogen perception is mediated through the phytohormone ethylene, the postulated second signal perceived by supernodulation mutants might involve ethylene. In order to determine if ethylene was a candidate for the signal, we examined the ethylene production and response in the hypernodulation mutants. In previous work we showed that the sunn-1 and sunn-4 mutants have normal ethylene responses (Penmetsa et al. 2003; Schnabel et al. 2010). For the rdn1 mutants, the same analyses were performed for comparison. The amount of ethylene produced by rdn-1 mutant plants 10 days after inoculation was the same as that produced by wild type plants and significantly lower than that produced by skl plants (Figure 4.8F). Under dark conditions, we compared the sensitivity of wildtype rdn1-1, rdn1-2 and skl seedlings to ACC, the immediate precursor of ethylene synthesis. Just as in wild type plants, ACC induced the triple response in rdn1-1 and rdn1-2 seedlings; including inhibition of root and hypocotyl elongation, radial hypocotyl swelling, and exaggerated curvature of apical hooks, whereas skl seedlings were insensitive to ethylene and did not show this response (Figure 4.8 A-D, Pentmetsa & Cook 1997). Just as in sunn mutants, hypocotyl growth of rdn1-1 and rdn1-2 was compromised in an ACC-dose-dependent manner indistinguishable from the wild type, indicating that ethylene signaling is not impaired in rdn1 mutants (Figure 4.8 E). Taken together, the lack of ethylene response defects in both sunn and rdn1 mutants are consistent with an ability to respond to a second AON signal making ethylene a potential candidate for the second signal.
Figure 4.8. Ethylene response is wild type in rdn1 mutants. One day old wildtype (A17), rdn1-1, rdn1-2 and skl seedlings were sown on agar plates containing the ethylene precursor ACC (0 to 100μM) and grown vertically in darkness for 6 days. (A-D) the triple response of representative (A) A17, (B) rdn1-1, (C) skl and (D) rdn1-2 seedlings. (E) The hypocotyl length with increased concentration of the ethylene precursor, ACC. (F) The amount of ethylene released from nodulating plants of A17, rdn1-1 and skl per gram of fresh weight as measured in Schnabel et al. (2010). Error bars indicates standard error of the mean (n=5 for hypocotyl observations, three biological replicates for ethylene measurements).
**RDN1 and SUNN function in the same genetic pathway**

Using the advantage of having hypernodulation mutants defective in both shoot- and root-based control of nodule number, we generated *sunn: rdn1* double mutants to investigate genetic interactions between SUNN and RDN1 in the AON pathways. The resulting *sunn-1/rdn1-1* double mutant nodulates at the same level as the *sunn-4* null mutant plants and significantly higher than the parental single mutants (Figure 4.9 B). Root length in the *sunn1:rdn1* plants also phenocopies that of *sunn-4* mutant plants (Figure 4.9 A&C). The *sunn-4:rdn1-2* double mutant plants carrying null mutations in both genes did not show enhanced nodulation compared with the parental single mutant *sunn-4*; the absence of both genes does not create additive effects on nodule number when compared to the single parental phenotype (Appendix M), suggesting that RDN1 and SUNN function in the same genetic pathway.
Figure 4.9. **RDN1 and SUNN appear to act in the same signaling pathway.** Data were obtained from aeroponic chamber grown plants 10 days post inoculation. (A) Visual representation of nodulation and root phenotypes on wild type (A17) and AON defective mutants of rdn1 and sunn. (B) Mean nodule number from the same experiment (C) Mean root length from the same experiment. Data are mean±SE (n=20) and different letters indicate significant difference using Tukey test (p<0.05).

**The RDN1 effect on Nodulation is SUNN Dependent**
Given that **SUNN** and **RDN1** appear to be in the same pathway, while exerting their effects from different parts of the plant, we employed shoot to root reciprocal grafting to investigate the connection. We created reciprocal grafts between **sun4-4** and **rdn1-2**, using each genotype as shoot or root respectively. Since **RDN1** signals from the root to the shoot and **SUNN** signals from the shoot to the root, **rdn1-2** shoot and **sun4-4** root reciprocal grafts showed wild type nodulation (Figure 4.10). Self-grafts of **sun4-4** produced a significantly higher number of nodules than self-grafts of **rdn1-2** (Figure
4.10), consistent with the respective mutant phenotypes. However, compared with sunn-4/sunn-4 self-grafts, reciprocal-grafts between sunn-4 shoots and rdn1-2 roots have the same nodule number as sunn-4 self-grafts (Figure 4.10) suggesting that the integration of two contrasting rootstocks does not change the shoot dominance in regulation of nodulation in the root, a result consistent with the phenotype of the double mutant.

![Figure 4.10. Nodule number on shoot to root reciprocal grafted plants.](image)

A second approach used to determine the order of action of RDN1 and SUNN was to examine gene expression of each wild type gene in the other mutant to determine if a mutation in SUNN affected RDN1 expression and vice versa. We used Quantitative Real
Time RT-PCR for this. In previous work, we demonstrated that the expression levels of both \textit{RDN1} and \textit{SUNN} in wild type plants do not change upon rhizobial inoculation, remaining constant for 72 hours post inoculation (Schnabel et al 2012). We therefore tested expression of each gene in root tissue and shoot tissue 24 hours post inoculation from plants grown in an aeroponic chamber. In roots, the expression of \textit{RDN1} in the \textit{sunn}-4 background is the same as the wild type level, while expression in the two null \textit{rdn1} mutants is abolished as expected (Figure 4.11 B). In contrast, \textit{SUNN} transcription was down-regulated in a \textit{sunn}-4 mutant as might be expected as the result of a mutation creates an early stop which most likely leads to RNA degradation (Schnabel \textit{et al.} 2005), but \textit{SUNN} expression was also significantly reduced in both \textit{rdn1} alleles (Figure 4.11 A). On the other hand, \textit{SUNN} expression was wild type in \textit{rdn1}-2 shoots and \textit{RDN1} expression was also wild type in \textit{sunn}-4 shoots (Figure 4.11 C & D).
Figure 4.11. MtSUNN and MtRDN1 expression in roots and shoots of aeroponic chamber grown A17, sunn-4, rdn1-1 and rdn1-2 mutants 24 hrs post inoculation. (A) & (C) SUNN expression. (B) & (D) RDN1 expression. Values are from three biological and three technical replicates for each genotype. Error bars are standard error of the mean.
DISCUSSION

The RDN proteins are well conserved across extremely diverse groups of green plants, including the primitive bryophyte and higher land plants, suggesting an important but unknown role. Since they are proteins with unknown functions, never characterized before and without conserved functional domains (Schnabel et al. 2011) they presented a challenge. The N-terminal signal peptide suggested a secretory pathway role, confirmed with functional GFP tagged version of MtRDN1 (Figure 4.1). The punctate pattern of GFP fluorescence observed in Figure 4.1B streamed along the cytoskeleton in a Brownian fashion (http://youtu.be/ndgyBKFwHFY). These moving organelles approximated the size and pattern of previously reported proteins in endomembrane system (Min et al. 2007; Gu and Innes 2011; Gao et al. 2012). Co-expression of the MtRDN1-GFP fusion protein and organelle specific markers in tobacco epidermal cells identified the Golgi apparatus as a target (Figure 4.2). The Golgi apparatus is an intermediate compartment within the secretory pathway and its most striking property is mobility throughout the cytoplasm (Saint-Jore-Dupas et al. 2004). In addition, MtRDN1-GFP localization did not overlap with the ER marker suggesting no role for RDN in retrieving proteins back and forth between ER and the cis-Golgi. Localization to the Golgi complex raises the possibility that MtRDN1 may be trafficked out of the cell, go to the plasma membrane to interact with downstream receptors like SUNN, or go to the vacuole for storage or degradation. Co-expression of the MtRDN1-GFP fusion with plasma membrane, vacuole, and endosomal markers demonstrated a lack of co-
localization with the vacuole and late endosomal (prevacuole) markers (Supplemental Figure 4.1) which excludes one of the possible routes from Golgi. The plasma membrane has been regarded as the site of receptor mediated signal transduction for both exocytosis and endocytosis. Based on the role of trafficking in the BRI/BIN jasmonate receptor pathway in plants (Bishop and Koncz 2002), one speculation is that MtRDN1 may be trafficked to the membrane to form a ligand-receptor complex and trigger downstream signaling. The co-expression of MtRDN1-GFP with a PM marker in tobacco leaves, however, did not give a conclusive result (Figure 4.2g-i). Although MtRDN1-GFP did not colocalize with PM, it appeared to be associated with it. However, this is inconclusive since labeling of the plasma membrane can sometimes be confused with labeling of the cell wall or cytosol, as the large central vacuole in most leaf pavement cells squeezes the cytosol to the periphery of the cell and therefore causes cytosolic structures to appear adjacent to the plasma membrane. After 30 minutes of staining with the endocytic dye FM4-64, we observed internalization of the dye as small punctuate like structures that partially colocalised with MtRDN1 (Figure 4.3), suggesting trafficking from the PM. However, FM4-64 alone is insufficient as an indication for involvement in endocytosis or exocytosis because the tracer can label almost any compartment of the cell depending on a variety of conditions (Bolte et al. 2004). Nevertheless, our data demonstrate that MtRDN1 can enter the secretory pathway.
Our inverted-Y-graft data in Figure 4.4 suggest that MtRDN1 is responsible for the synthesis or sending of the root derived autoregulatory signal, in agreement with reports on RDN1 ortholog of pea, NOD3 (Li et al. 2009; Novak 2010). Combined with the secretory pathway localization, and the localization of expression in the vasculature (Schnabel et al. 2012) one possibility is that MtRDN1 may be either trafficked itself to the shoot or aid in trafficking a downstream signal to the shoot. In chimeric plants resulting from Agrobacterium mediated transformation of the roots with MtRDN1-GFP expressed under both the CaMV 35S promoter and the native RDN1 promoter we were unable to detect MtRDN1-GFP signal by microscopy in the shoot (data not shown). This suggests that MtRDN1 is not mobile itself and instead may be mobilizing the signal. The suggestion of MtRDN1 localization in association to the PM could be viewed as evidence that MtRDN1 may be aiding an unknown downstream protein at the plasma membrane to move.

Previous studies on Glycine max (soybean) and Lotus japonicus (Lotus) suggested that AON signal is a relatively rapid and time dependent response governing nodulation (Kossak and Bohlool 1984; Suzuki et al. 2008). The full induction of AON in wild type M. truncatula plants was detected three days after the initial inoculation and it persists for at least 15 days (Figure 4.5). Unlike reports by Olsson et al. (1989) in soybean, we did not observe significant suppression under our conditions until day three and nodulation of the root portion receiving the delayed inoculation was not 100% suppressed by prior inoculation of the other root portion even at a 15 day interval. The
three day interval for full induction of AON is shorter than previous reports in soybean of four days (Kossak and Bohlool 1984) and in Lotus of five days (Suzuki et al. 2008) but longer than that of two days in *Trifolium subterraneum* (clover) (Sargent et al. 1987) and 30 hours in *Vicia sativa* (van Brussel et al. 2002). This variation may be due to differences in the split root systems (see discussion in Kassaw & Frugoli 2012), a difference in the speed of translocation of the signal from species to species, morphological and anatomical differences in the species or the different rhizobial partners for each legume species. As expected, prior inoculation did not induce inhibition on nodulation in the delayed inoculated mutant roots (both *sunn* and *rdn1*), confirming that these two genes are critical in long distance nodule autoregulation. Surprisingly, for the first time we observed inhibition of nodulation in the delayed inoculated mutant roots 10 days after the initial inoculation (Figure 4.5). Even though the level of suppression varies between *sunn* and *rdn1*, the result remains consistent for 11, 12 and 15 days intervals. This could be interpreted as the strength of AON signaling increasing as nodule development progresses, allowing mutant roots to eventually respond, or as the existence of a previously undetected second signal associated with nitrogen fixation in mature nodules or with the increased meristem development in hypernodulation mutants of indeterminate nodulators like *M. truncatula*.

*N*₂ fixation creates a strong localized sink for photosynthate that has effects on both nodulation and root development in uninoculated portion of the roots (Singleton and van Kessel 1987), and application of high inorganic nitrogen affects nodulation
(Streeter 1988). Since ethylene biosynthesis inhibitors block the nitrogen suppression effect and increase nodulation (Caba et al. 1998), we examined ethylene response in the AON mutants but found no difference from wild type response in \textit{rdn1} mutants (Figure 4.8) or \textit{sunn} mutants (Penmetsa et al. 2003; Schnabel et al. 2010).

We investigated the systemic effect of nitrogen fixation by rhizobia on root nodule formation with bacterial strains and actual measurements of nitrogen fixation. As indicated in the split root experiments in Figure 4.6A, while suppression of nodulation was observed for the second root (root B) of all genotypes inoculated with wild type bacteria 20 days apart, nodulation on root-B of the AON defective mutants was not suppressed by inoculating root A with a Fix\(^-\) strain of bacteria, while suppression occurred in wild type plants inoculated with the same strain. This is consistent with nitrogen fixed by rhizobia being the second signal detected by the AON mutants-wild type plants detect the unknown first signal and have no need for the second signal, while AON mutants are defective in creating or responding to the first signal, but can still respond to the nitrogen signal when it occurs.

Total nitrogen fixation in \textit{rdn1-2} 10 days post inoculation was significantly higher than the wild type (Figure 4.6B) but both \textit{rdn} and \textit{sunn} mutants responded to the AON signal at 10 days (Figure 4.5) and at 15 days there was no significant difference between AON mutants and wild type in nitrogen fixation (Figure 4.6B). We also counted the nitrogen fixing nodules on each plant and measured the nodule fresh weight per genotype. As
expected, the total number of nitrogen fixing nodules was significantly higher for AON defective mutants than the wild type (Supplemental Figure 4.3 A&B) but there was no difference in nodule fresh weight (Supplemental Figure 4.3C) suggesting that *sunn* and *rdn1* mutants compensate the increased nodule number with reduced nodule size and perhaps reduced nitrogen fixation activity per nodule. In support of this, another group has shown that the systemic regulation of nitrogen fixation activity by the plant is SUNN independent. Jeudy *et al.* (2009) showed that excess capacity in the *sunn*-2 mutant allowed it to up regulate nitrogen fixation activity in response to localized nitrogen starvation in a split root system (Jeudy *et al.* 2009). Taken together, the bacterial experiment suggests nitrogen fixation activity of the nodules might be the second signal, and this activity is normal in AON mutants, but the data are not conclusive.

We also investigated meristem activity. As noted in Supplemental Figure 4.3 A&B AON mutants in indeterminate nodulators have more nodules and therefore more nodule meristems. Since none of the genotypes could suppress nodulation in Root B when Root A was inoculated with bacteria unable to make nodules (Figure 4.6A), we suspected CLE peptides which are associated with nodule meristems could be the second signal. The expression patterns of *MtCLE12* and *MtCLE13* were essentially the same in wild type and AON defective mutants, being induced upon inoculation and remaining high until 15 days post inoculation when measurement ceased (Figure 4.7). The peak of expression occurred at 10 days post inoculation for all genotypes,
correlating with the time we observed nodule suppression in supernodulation mutants. However, the relative expression levels of these two genes were 3-10 fold higher in the mutants versus wild type (Figure 4.7). One possibility therefore is that the level of CLE gene expression may be the initial nodulation suppression signal that AON mutants do not respond to, but the several fold increase by day 10 overwhelms the faulty signal transduction, allowing AON mutants to finally perceive nodulation.

*SUNN* mutants and *RDN1* mutants have more features in common than a late AON suppression response. Both *RDN1* and *SUNN* are expressed in the vascular tissue of both roots and shoots (Schnabel et al. 2011a; Schnabel et al. 2012), neither is induced by rhizobial inoculation (Schnabel et al. 2012), and defects in either gene leads to hypernodulation and short roots in the presence or absence of rhizobia (Schnabel et al. 2005; Schnabel et al. 2011). The most obvious differences are the localization of the effect of the mutation to shoot (*sunn*) or root (*rdn1*) tissue, and unlike RDN1, the SUNN protein is better characterized, a receptor-like kinase similar to Arabidopsis CLAVATA1.

The shoot to root reciprocal grafting between *sunn-4* and *rdn1-2* (Figure 4.10) as well as the *sunn-4*/*rdn1-2* double-mutant analysis (Supplemental Appendix M) showed that the root-determined hypernodulation of *rdn1-2* does not have an additive effect on the shoot determined hypernodulation of *sunn-4*, indicating that RDN1 function and SUNN function impact the same genetic pathway. The *sunn-1*/*rdn1-1* double mutant on the
other hand displays hypernodulation at the level of the sunn-4 null mutant, significantly higher than the two single mutants (Figure 4.9). This is consistent with sunn-1 encoding a weak kinase (Schnabel et al. 2010; Schnabel et al. 2011b) with a weaker hypernodulation phenotype that is compounded by the lack of RDN, leading to the high hypernodulation seen in sunn-4 mutants. The qRT-PCR analysis from root tissue also showed an effect of RDN1 on SUNN expression in root tissue. The expression of RDN1 in a sunn-4 mutant background is at wild type levels in both shoots and roots whereas the SUNN message was down regulated in roots in an rdn1-2 background (Figure 4.11A&B). Since this effect on SUNN was not observed in shoots where SUNN control is localized (Figure 4.11C&D), it may have no effect on long distance signal transduction for nodulation, but it does suggest RDN1 influences SUNN expression in some tissues. It is possible to propose consistent with these data that RDN1 and SUNN are in the same signaling pathway and RDN1 acts before SUNN but further details await exploration.
MATERIALS AND METHODS

Plasmid Construction for Subcellular Localization Studies
The MtRDN1 (MT5G089520) coding sequence was amplified from M. truncatula cv. Jemalong A17 cDNA using forward: GGGGACAAGTTTGTACAAAAAAGCAGGCTatggggagggtaaaatctctact and reverse: GGGGACCACTTTGTACAAGAAAGCTGGGTcgcttctatttaacgaa tccc primers. Bases in upper case are attB sites designed to facilitate use of the PCR product in the Gateway cloning system (Invitrogen™, Carlsbad, USA) and bases in lower cases are derived from the coding sequence. PCR products were gel extract and purified before use to get rid of unspecified products that may cause false positive clones using Zymoclean gel DNA recovery kit (Zymo Research, USA). We used the Gateway BP and LR recombination reactions to clone the MtRDN1 coding sequence without the stop codon per the manufacturer’s instructions. The MtRDN1 PCR product was cloned into pDONR™221vector to generate a Gateway entry clone utilizing kanamycin (50μg/mL) as the selective agent and each of the resulting entry clones were characterized by restriction mapping and sequence analysis. A single correct clone was used for the work.

To generate the expression clone under the CaMV 35S promoter, the LR reaction was performed using the entry clone and pK7FWG2 destination vector (Karimi et al 2002) suitable for C-terminal Egfp fusion to MtRDN1protein. In addition, the destination
vector was reconstructed to have the native MtRDN1 promoter by removing the 35S promoter with restriction digestion (HindIII and SpeI) to express MtRDN1 under its own promoter. Positive bacterial clones were selected by growth in the presence of spectinomycin (50μg/mL). Functionality of the constructs was tested by rescue of the *rdn1* nodulation phenotype using *Agrobacterium rhizogenes* mediated hairy root transformation as in Schnabel *et al.* (2011). The organelle specific markers; Golgi-mCherry, ER-mCherry, PM-mCherry, Vacuole-mCherry, Late endosomal-mCherry and Peroxisomal-mCherry were obtained from the Arabidopsis Biological Resource Center (Nelson and Nebenfuhr 2007). These expression constructs were used for both *Agrobacterium* mediated hairy root transformation (Schnabel *et al.* 2011) and transient tobacco leaf infiltration.

**Plant Materials and Growth Conditions**

Seeds of *Medicago truncatula* cv Jemalong A17 and AON defective mutants *rdn1*-1, *rdn1*-2 (Schnabel *et al.* 2011), *skl* (Penmetsa *et al.* 2008) *sunn*-1, and *sunn*-4 (Schnabel *et al.* 2005) as well as the double mutants *sunn*-1/*rdn1*-1 and *sunn*-4/*rdn1*-2 were acid scarified and imbibed as described in Schnabel *et al.* (2010). Seed was then vernalized in dark at 4°C for 2 days on Harrison Modified Farhaeus (HMF) media (Huo *et al.* 2006) covered with two half round Whatman filter papers (GE Healthcare, USA), followed by germination in the dark at room temperature for 1 day before being used in the experiments described. Plants used for SUND and RDN1 gene expression, phenotypic analysis and comparison of the double mutants were grown in an aeroponic chamber on nodulation medium (Penmetsa and Cook, 1997).
Split Root Development and Inoculation

Lateral root initiation was stimulated by removing the roots of 5 day old seedlings at the root-shoot junction and transferred the trimmed plants to HMF media plates sandwiched between two half round Whatman filter papers and split-root systems were established as described previously (Kassaw and Frugoli 2012). For plant establishment in the perlite system, plants were watered daily with a 100-fold dilution of water-soluble 20:10:20 Peat-Lite Special fertilizer (Scotts Company, OH, USA) for 5 days. After an additional 5 days of nutrient starvation induced by watering with water alone, the plants were used for split-root inoculation experiments with bacteria. The second root (Root B) was inoculated with rhizobia 0, 2, 3, 4, 6, 8, 10, 12, 15, and 20 days after the first root (Root A) inoculation depending on the intended experiment and nodules on both roots were counted 21 days after the second root inoculation. To demonstrate the timing of AON, both Root A and Root B were inoculated with S. medicae strain ABS7 (Bekki et al., 1987). To investigate the possibility of a second nodule inhibitory signal, Root A was inoculated with Rm1021, Rm1312, or SL44 (Meade et al. 1982, Ruvkun et al. 1982 and Fisher et al. 1988) and Root B with the wild type strain Rm1021 alone and nodules on the second root counted after 21 days.

Grafting

The inverted Y and shoot to root reciprocal grafting were performed according to Kassaw and Frugoli (2012). For this particular inverted Y graft experiment, Root B was inoculated four days after Root A inoculation and A17 and rdn1-2 used interchangeably as a stock as well as Root A or Root B. Two genotypes were reciprocal or self-grafted;
shoot to root grafts were denoted as Stock/Insert, and inverted-Y grafts were denoted as Stock.Insert.

**Phytohormone and Nitrogen Fixation Measurements**

To investigate the ethylene sensitivity of *rdn1*, one day old seedlings were sown on HMF media containing increasing concentrations of 1-aminocyclopropane carboxylic acid (ACC) from 0 to 100µM. Seedlings were grown in darkness vertically in a growth chamber at 25 °C with a 16 hrs photoperiod on sterile Nunc Bio-Assay dishes (245 mm x 245 mm x 25 mm) each containing 250 ml of BNM and sealed with Parafilm (BEMIS®, Neenah, USA) and examined for ethylene mediated triple response 6 days after planting. Ethylene measurement after inoculation was performed as in Schnabel *et al.* (2010).

The Q-Box NF1LP package (Qubit Systems Inc, Kingston, Canada) was used for measuring the rate of H₂ production in order to estimate the nitrogen fixation in plants grown on pots filled with washed and autoclaved perlite under the conditions used for the split root experiments. Plants were subjected to the same establishment and nutrient withdrawal regime as the split root plants and then inoculated with *S. medicae*. The flow-through H₂ from N₂-fixing tissues was measured 10 days and 15 days post rhizobial inoculation. The nodulated intact roots were sealed with Qubitac™ sealant to avoid air leakage so that only the shoot portion was exposed to room air. To generate maximum H₂ evolution, roots were flushed with an Ar:O₂ (80:20) gas combination at the rate of 250ml/minute for 5 minutes, with the purpose of ensuring all electrons
measured are utilized for H⁺ reduction. The measurement of H₂ evolution as a means of determining nitrogenase activity used the LabPro interface in conjunction with Logger Pro software provided by Vernier (Portland, Oregon) according to the manufacturer’s instructions.

**Real Time PCR**
Total RNA was isolated from plants grown on aeroponic chamber or perlite with the RNeasy Plant Mini Kit (Qiagen, Maryland, USA) according to the manufacturer's instructions. For aeroponic chamber grown plants, total RNA was extracted from root tissues 24 hrs post inoculation and gathered in three independent biological replicates. For perlite grown plants, RNA was extracted from nodulating roots 10 and 15 days post inoculation and sampled from only one biological replicate. Each RNA sample was digested with RNase free DNase (Promega, Madison, USA) for 40 minutes to remove genomic DNA contamination. The samples were purified by precipitation in 3mM NH₄Ac and 100% ethanol; the RNA quality was checked and quantified with Biophotometer (Eppendorf, Hamburg, Germany). 1µg of total RNA (20µl reaction mixture) was used to synthesize cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, USA). The cDNAs were then diluted to 40 µl before use in qRT-PCR. All the experiments were performed with iQ™ SYBR® Green Supermix and an iCycler iQ5 multicolor detection system (Bio-Rad, Hercules, USA) for amplification and detection. All reactions were done in triplicate and averaged. The total reaction volume was 12.5µL (10µL of master mix including 0.175µL of each primer (25µM) and 2.5µL of diluted cDNA). Cycle threshold (Ct) values were obtained with the accompanying
software and the relative expression value of each gene was analyzed using Pfaffl method (Pfaffl 2001). The relative expression was normalized against the SECRET AGENT gene (Schanbel et al. 2005). Primers used are listed under table 4.1 below. These primers are unique in the Medicago genome based on the NCBI primer BLAST database.

**Table 4.1. List of primers used for qRT-PCR for *M. truncatula* RDN1, SUNN, CLE12 and CLE13 genes.** Secret agent used as housekeeping gene.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Primers (from 5’ to 3’)</th>
</tr>
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<tbody>
<tr>
<td>MtRDN1</td>
<td>TATGCCTGGATCGGCTATGGGAAA</td>
</tr>
<tr>
<td></td>
<td>AACCGCTTTTCTCCAGCCATTGAAC</td>
</tr>
<tr>
<td>MtSUNN</td>
<td>CCATGGCAACGGAACAGACG</td>
</tr>
<tr>
<td></td>
<td>AGCACCACTCCGAAAACATACATCACT</td>
</tr>
<tr>
<td>SA</td>
<td>GGCAGGCTCTGCTATGGTTA</td>
</tr>
<tr>
<td></td>
<td>GGTCAGACGCACAGATTTGA</td>
</tr>
<tr>
<td>MtCLE12</td>
<td>TTTCCAAAATTGGCCTAATCAT</td>
</tr>
<tr>
<td></td>
<td>CCTAGAACGCTTTTGGTATATCC</td>
</tr>
<tr>
<td>MtCLE13</td>
<td>ACAACCGATCAAGTGCAGAGTG</td>
</tr>
<tr>
<td></td>
<td>GGCCTGCTGGGGAGAGGTCA</td>
</tr>
</tbody>
</table>
Transient Protein Expression in Tobacco

The binary vectors described in plasmid constructs were introduced into *Agrobacterium tumefaciens* (EHA105) (Hood *et al.* 1993) by electroporation, and transformed cells were selected on Luria-Bertani (LB) plates supplemented with kanamycin (50 μg/mL) for organelle specific markers and spectinomycin (50 μg/mL) for the Gateway plasmids carrying MtRDN1 at 30°C. An individual colony from each construct was inoculated into 3 ml of liquid TY medium and grown at 30°C in a shaker at 250 rpms for 48 hrs. Cells were harvested by centrifugation and resuspended in 1 ml of infiltration media (10 mM MgCl$_2$ and 100 μM acetosyringone). The cells were further washed two times with 1 ml of infiltration solution by spinning 2 min at 5,500 g in tabletop centrifuge. The resuspended 1 ml *Agrobacterium* solution was incubated at least 2 hrs at room temperature in the infiltration solution without shaking. The bacterial solution was then diluted to 0.4 OD 600 nm and injected into young fully expanded leaves of 4 to 5 week-old tobacco plants grown under greenhouse condition using a 1 mL syringe without a needle by gentle pressure through the small incision made on the lower epidermal surface. Transformed plants then were incubated in laboratory with regular watering with tap water under caisson light conditions. Three to seven days after infiltration leaves were examined for localization experiments using an Axiovert 200 M fluorescence microscope with Apotome (Carl Zeiss, New York, USA) and a laser scanning confocal imaging system. For Axiovert 200 M fluorescence microscope pictures we used Texas red and YFP band pass filters (Chroma).
**Confocal Microscopy Imaging and Analysis**

Transformed leaves and transgenic roots were imaged with an inverted Zeiss LSM 510 laser scanning microscope and plan-apochromat 63 x/1.4 oil DIC objectives (Jena, Germany). For imaging co-expression of the GFP constructs and mCherry tagged organelle specific markers, excitation lines of an argon ion laser of 488 nm for GFP and 543 nm for mCherry were used with a 505/530 nm bandpass filter for GFP and 560 nm long pass filter for mCherry in the single-track facility of the microscope. FM4-64 staining of cells expressing MtRDN1-GFP was performed by submerging pieces of infiltrated leaves in 8.2 mM FM4-64 (stock solution) in water; for 15 to 120 minutes. The confocal filter configurations for FM4-64 stained cells were the same as GFP and mCherry combinations but using the multi-track approach. Post-acquisition image processing was performed with the LSM 5 Image Browser (Carl Zeiss, New York, USA). Images in Figure 4.2 have been adjusted for increased brightness by 25% with Photoshop version 10.0.1.

**Rhizobial Strains and Growth Conditions**

Unless otherwise indicated, plants were inoculated with *Sinorhizobium medicae* strain ABS7 (Bekki *et al*. 1987) carrying a LacZ reporter construct under tetracycline resistance. For split root experiments investigating the nature of the signal, *S. meliloti* strains were used; wild type *Rm*1021 (Meade *et al*. 1982), SL44 Nod-/Fix- carrying a nodD1-nodABC deletion (Fisher *et al*. 1988), and *Rm*1312 Nod+/Fix- carrying nifD::Tn5 (Ruvkun *et al*. 1982). All strains were grown in liquid TY media (5 g tryptone, 3 g yeast extract, 1.4 g CaCl$_2$.2H$_2$O, pH 6.5-7 and 15 g Agar per liter)
containing antibiotics with the following concentrations: tetracycline (15 μg/ml) for *S. medicae* and streptomycin (500 μg/ml) for *S. meliloti* strains. Before inoculation, the rhizobia were diluted to 0.2 OD 600 nm with sterile water and 6 ml of the bacterial solution was applied to each root compartment at root collar region (flood inoculation). For caisson grown plants, a 25 ml rhizobial culture grown for two days was pelleted by centrifugation and resuspended with demineralized water before inoculation. The caisson was inoculated with resuspended bacteria four days after planting. Nodules were counted using an Olympus SZX12 Dissecting Stereo Microscope; for caisson grown plants 10 days after inoculation, for perlite grown shoot to root reciprocal grafted plants 21 days after inoculation, and for the split root 21 days after the second root inoculation after gentle washing of the Perlite from the roots.

**Statistical Analysis**
Analysis of variance was performed on the split root data using JMP-9 software (SAS Institute Inc., Cary, USA). The least square means differences student’s t was performed using percent nodulation of Root B with α <0.05 used as a significant level. For inverted-Y grafted data, paired t-tests were used to identify statistically significant differences between the two root systems. The analysis was done with Excel software (Microsoft, Redmond, WA, USA) with p <0.05 used as a significant level. The Tukey-Kramer test was used for shoot to root reciprocal grafted data and for comparing the nodulation on caisson grown plants (p < 0.05).
REFERENCES


Schnabel EL, Karve A, Kassaw TK, Mukherjee A, Zhou X, Hall T, Frugoli JA (2011). The M. truncatula SUNN gene is expressed in vascular tissue, similarly to RDN1, consistent with the role of these nodulation regulation genes in long distance signaling. Plant Signal Behav. 7: 4–6.


CHAPTER 5

THE NON-SYMBIOTIC ROLE OF RDN AND CROSS SPECIES COMPLEMENTATIONS

ABSTRACT

In the previous chapters we described the *RDN* gene family as a group of uncharacterized proteins conserved across green plants and having a potential role in the secretory pathway. By their very nature, secreted proteins play multiple roles in plant development. In this chapter, we investigated the role of *RDN* genes outside of nodule autoregulation. Legumes are thought to have adopted part of the nod factor signaling pathway from the signaling pathway for more ancient and widespread mycorrhizal associations. We proposed that legumes and non-legume species may use analogous regulatory mechanisms to limit their symbiotic processes. To test this hypothesis, we examined whether *RDN* family members from a non-legume species were able to restore the rhizobial symbiotic properties of a *M. truncatula* *rdn1-2* mutant. We identified homologous genes from *Populus trichocarpa*, *Oryza sativa* and *Arabidopsis thaliana* and assayed them for cross-species complementation. Our results show that genes from *P. trichocarpa* (PtRDN1), *O. sativa* (OsRDN), *A. thaliana* (AtRDN2) and *M. truncatula* (MtRDN2) under CaMV 35S promoter can restore nodule number autoregulation, indicating that non-legumes use similar regulatory machinery and that other *RDN* genes in *M. truncatula* can function for *RDN1* when expressed at a high
level. However, under the control of the *MtRDN1* promoter, only *MtRDN1* and *OsRDN1* complemented the *rdn1-2* mutation. This suggests variation between the gene products either at the regulatory level or associated with the variable N-terminal region of the proteins, which can be compensated for by overexpression. Non-symbiotic roles of *RDN* genes were studied using *A. thaliana* lines with T-DNA insertions in *AtRDN2*. We identified allele specific increased lateral root number and branching density, and altered primary root development in the mutants. These findings further establish *RDN* as a family of proteins with previously uncharacterized regulatory functions playing a role in the pattern of root growth and lateral root development in plants.
INTRODUCTION

As mentioned in Chapter 1, two agriculturally important symbiotic systems are well studied; rhizobial and arbuscular mycorrhizal symbiosis (Zhu H et al. 2006). Most higher plants including the major cereal crops such as rice and wheat form only arbuscular mycorrhizal (AM) associations, in which the fungus colonizes the cortical cells to access carbon supplied by the plant and the fungus help the plant in the transfer of mineral nutrients, particularly phosphorus, from the soil (Harrison 1999; Sikes et al. 2010). AM is a very ancient symbiosis, more than 400 million years old, and more than 80% of the terrestrial plant species participate in it. In comparison, the more recent, evolutionary young bacterial and legume plant association is approximately 60 million years old (Remy et al. 1994; Godfroy et al. 2006). Unlike AM, the rhizobial symbiosis is limited to a few plant families within the clade of Eurosid I consisting of Fabales, Fagales, Cucurbitales and Rosales (Solitis et al. 1999; Limpens and Bisseling 2003; Brewin 2002; Zhu et al. 2006). Of these plant families, the most prominent group contributing high yields of fixed nitrogen to the natural ecosystem are legumes (Fabaceae). In nitrogen-limited conditions, leguminous plants interact with rhizobia, resulting in the formation of root-based organs in which the bacteria fix nitrogen for the plant (Geurts and Bisseling 2002; Mitra et al. 2004; Oldroyd and Downie 2004; Ferguson et al. 2010).
Rhizobial and AM symbioses share central genetic components in the early signaling pathway to trigger their symbiotic programming (Catoira et al. 2000; Geurts et al. 2005; Maillet et al. 2011). It has been reported that the Nod factor perception genes are unique for rhizobial symbiosis (Maillet et al. 2011; Rival et al. 2012). However, several key genes identified in *M. truncatula*, the *DMI1, DMI2* and *DMI3* genes downstream of Nod factor perception are shared between both rhizobial and AM symbiosis. *DMI3* was thought to be the last common protein acting as a switch, selectively activating the downstream transduction pathway leading either to nodulation or mycorrhization (Mitra et al. 2004). Quite recently, it has been reported that the downstream GRAS protein NSP2 is involved in mycorrhizal signaling, suggesting that NSP2 is also shared in the two symbiotic pathways (Maillet et al. 2011). The non-legume homologs of these genes are documented as essential for the AM symbiosis in non-legume mycorrhizal plants as well as functional in symbiosis in legume plants. For instance, rice *CCaMK* restores Nod-factor induction of the early nodulin gene *MtENOD11* and nodule formation in *M. truncatula* (Godfray et al. 2006). In addition, most of the symbiotic genes are also important for root branching (See Chapter 1). As an example, Nod-factor and a diffusible factor from AM fungi stimulate lateral root formation in *M. truncatula* via the *DMI1/DMI2* signaling pathway (Olah et al. 2005).

For the proteins analyzed in this chapter no prior data on sub-cellular localization was available except for that in Chapter 4. The study of protein subcellular localization has been important to elucidating protein functions (Scott et al. 2005; Lee et al. 2008).
RDN proteins have no well-known conserved functional domains except the N-terminal signal peptide suggesting a role in vesicle trafficking (Schnabel et al. 2011; Chapter 4). Since membrane trafficking is required for a variety of cellular functions and RDN is highly conserved even across primitive bryophytes to higher land plants, we speculate there should be a non-symbiotic role for RDNs. Since the development and structure of legume nodules resemble in some respects that of lateral roots, it has been proposed that their ontogeny may require the same molecular signals (Huo et al. 2006). In higher plants, there is accumulating evidence that all CLE (CLV3/ESR-related) precursors contain an N-terminal signal peptide and with a few exceptions, they all have a 14 aa conserved C-terminal motif from which the matured CLE peptide is derived (Meng et al. 2012). MtCLE12 and MtCLE13 in M. truncatula are known regulators of nodule formation (Mortier et al. 2010). In Arabidopsis, CLE-like peptides, the precursors of which are targeted to the secretary pathway, also control the pattern of root growth and lateral root development (Meng et al. 2012). Experimental evidence also implicates auxin in both nodulation and lateral root formation (Kuppusamy et al. 2009; Jin et al. 2012). Cytokinrin signaling mediated by the cytokinin receptor MtCRE1, leads to control of symbiotic nodule and lateral root organogenesis in opposing directions (Gonzalez-Rizzo et al. 2006). Although plant hormones, including auxin and cytokinin, appear to be key players in coordinating this cross talk, very few genes that cross-regulate root and nodule development have been identified thus far. A homolog of CELL DIVISION CYCLE16 (CDC16), a core component of the Anaphase Promoting Complex, is reported to be one of the key mediators in controlling the overall number of lateral roots.
and nodules (Kuppusamy et al. 2009). A potential nitrate transporter, the \textit{MtLATD/NIP} gene is also required for the establishment and maintenance of three root meristems; the primary root, lateral roots and symbiotic root nodules (Yendrek et al. 2010).

In this chapter, we link multiple important roles of RDN in plant development. We show that the putative orthologs of \textit{MtRDN1} from \textit{Arabidopsis}, Populus and Oryza can restore nodule regulation in \textit{rdn1-2} mutants when expressed under the control of the CaMV 35S promoter, indicating that non-legume RDNs are able to recognize the early nodulation events and subsequently activate the downstream transduction pathway leading to nodule regulation. This finding predicts a potential molecular signaling in the plant shared between rhizobial and mycorrhizal symbiosis. However, transcomplementation is dependent on the strength of the promoter; the native promoter of \textit{MtRDN1} results in very low relative expression levels \textit{in planta} (Schnabel et al. 2011). Using T-DNA insertion Arabidopsis lines, we explore a non-symbiotic role of RDN in regulating lateral root number and branching density.
RESULTS

Evolutionary relationship among RDN proteins
In previous work, *in silico* analysis identified RDN1 as a member of a small gene family found across land plants and green algae (Schnabel et al. 2011). In *M. truncatula*, there are three *RDN* gene family members; *MtRDN1, MtRDN2*, and *MtRDN3*, but this three gene family occurs in both monocots and dicots, among plants that make symbiotic associations and those like Arabidopsis that do not. To investigate the function of RDN proteins in plant development not related to nodulation, we identified the closest homologs by amino acid sequence to MtRDN1 from *M. truncatula, P. trichocarpa, O. sativa*, and *A. thaliana*. Using multiple sequence alignment of the closest proteins from the subset in (Schnabel et al. 2011), we determined that PtRDN1 (PtA in Schnabel et al 2011), OsRDN1, AtRDN2 (At5g13500 in Schnabel et al. 2011) and MtRDN2 share 77%, 68%, 68%, and 72% amino acid identity with MtRDN1 respectively (Appendix Q). Interestingly, the C-termini of these proteins, which are rich in potential posttranslational modification sites and contain a proline-rich complexity region (Figure 5.1), were highly conserved across the species. In addition, the N-terminal signal peptides and the overlapping transmembrane regions, a proline-rich low complexity region, and potential phosphorylation sites are also highly conserved among the RDN proteins (Figure 5.1). The signal peptide, including the overlapping transmembrane domain, showed 17-71% amino acid identity score (Appendix N-A). However, outside the signal peptide, the N-termini had much lower sequence identities (6-32% identity score) and were the most variable region (Appendix N-B).
Figure 5.1. Multiple amino acid sequence alignment using Clustal W2. The predicted signal peptide (using SignalP) is shown in green underline and the transmembrane domain in red underline (from the Expasy database). Phosphorylation sites are boxed in red as determined by NetPhos 2.0. The variable region is boxed with a broken red line. The Expasy program also identified a conserved low-complexity region indicated with broken line black box.

As expected from previous analysis, a phylogenetic tree constructed of only these sequences identified PtRDN1 as the closest relative to MtRDN1 with a bootstrap value of 99 (Figure 5.2). As also might be expected, the monocot OsRDN1 from rice showed an earlier divergence.
Non-Legume RDNs Restore AON when overexpressed in an Mtrdn1 mutant

To address whether similarity in sequence translated to similarity in function, we did cross species complementation by transforming *M. truncatula rdn1-2* mutant roots with wild type copies of *MtRDN2*, *PtRDN1*, *OsRDN1* and *AtRDN2* and the roots examined for nodule number regulation. When these genes were expressed under the control of the CaMV 35S promoter, in each case the nodule regulatory defect of *rdn1-2* mutants was restored to a level consistent with rescue by *MtRDN1* (Figure 5.3), and in some cases even more. Plants transformed with *OsRDN1* or *PtRDN1* had statistically lower nodule numbers compared to the wild type control (Figure 5.3H). In contrast, the other *RDN* family member in *M. truncatula*, *MtRDN3*, did not rescue the *rdn1-2* mutation even when expressed under 35S promoter (Appendix O), suggesting a functional divergence of *RDN* gene paralogs in *M. truncatula*.
Figure 5.3. Non-legume RDNs complement the nodule autoregulation defective mutant, rdn1-2. A-G, Bright field image (left) and DsRED (transformation marker) image (right) of roots showing nodulation phenotypes on transgenic roots 21 days after inoculation with S. medicae. The CaMV 35S promoter was used in all expression constructs. (A) A17 (wild type) transformed with empty vector control. (B) rdn1-2 transformed with empty vector control. (C) rdn1-2 transformed with MtRDN1. (D) rdn1-2 transformed with MtRDN2. (E) rdn1-2 transformed with PtRDN1. (F) rdn1-2 transformed with OsRDN1. (G) rdn1-2 transformed with AtRDN2. (H) Average nodule number per transgenic root. N= (12-21) plants per genotype. Error bars are standard error of the mean. P<0.05 (Tukey Kramer test) was used to determine significance.

Restoration of AON is limited when RDN genes are expressed under the MtRDN1 promoter

The CaM 35S promoter is a strong, constitutive promoter, chosen to give high levels of gene expression in dicot plants; we reasoned that the similarity in nodule regulation responses between legume and non-legume RDNs may be due to the high expression levels obtained under this promoter. To investigate this, we expressed all the genes mentioned above except MtRDN3 under the control of the native MtRDN1 promoter used in Schnabel et al. (2011). We found that MtRDN1 restored AON and significantly reduced nodule formation under its own native promoter (Figure 5.4). However, only OsRDN1 could restore AON to the same level as MtRDN1 when expressed this way. Nevertheless, MtRDN2, PtRDN1 and AtRDN2 showed intermediate nodule number regulation similar to the AON defective mutant (Figure 5.4) suggesting these proteins have some function but cannot completely restore AON under the control of the MtRDN1 promoter. The differences in rescue could be due to differences in the nature of regulatory factors. Therefore, we constructed a phylogenetic tree to examine the evolutionary relationship between the promoter regions of the genes comparing 2000 bp upstream of each gene, with exception of AtRDN2 for which we used the whole upstream region of only 526 bp. We found that the MtRDN1 promoter is evolutionary
diverged from the rest of the group and predicted to be the ancestral promoter (Figure 5.5A). In addition, we scanned the promoter region of each gene using the SCOPE motif finder (version 2.1.0) and identified five novel cis-regulatory elements with the most occurrences among the promoters (Figure 5.5B). The motif with highest number of occurrences (Figure 5.5C) was found almost exclusively in the MtRDN2 promoter with only one occurrence in PtRDN1 and nowhere else. The remaining four were distributed among all the promoters and not exclusive to any one.

Figure 5.4. Cross species complementation of Mtrdn1-2 mutation with P. trichocarpa, O. satiava and A. thaliana homologs. Plants were transformed with expression constructs driven under MtRDN1 promoter. Nodules were counted 21 days after inoculation by S. medicae. N= (13-18) plants per genotype. Different letters indicate significant difference at P<0.05 significant level (Tukey Kramer test).
RDN Family Proteins have conserved Subcellular Localization

In Chapter 4, we reported the localization of the GFP tagged MtRDN1 protein in small moving punctate dots in the cytoplasm which co-localized with the Golgi-mCherry marker and the endocytic pathway dye FM4-64. Because all RDNs mentioned above have a predicted N-terminal signal peptide (Figure 5.1) and they all can rescue the *Mtrdn1*-2 AON phenotype (Figure 5.3), we hypothesized that the non-legume RDNs may have a similar subcellular localization. We studied this by cloning *MtRDN2* and *PtRDN1* into a Gateway™ binary vector (see Materials & Methods) and used...
Agrobacterium mediated hairy root transformation as above to determine localization in planta. In uninoculated *M. truncatula* hairy roots transformed with *p35S::MtRDN2-GFP* and *p35S::PtRDN1-GFP*, we observed both proteins in a punctate distribution at the cytoplasm as we had seen for *MtRDN1-GFP* (Figure 5.6 A&B). Similar localization results were obtained in *Nicotiana benthamiana* leaves transiently transformed with the same constructs (Figure 5.6C).

![Figure 5.6. In planta localization of RDN-GFP proteins](image)

**Figure 5.6. In planta localization of RDN-GFP proteins** from (A) *M. truncatula* (MtRDN2-GFP) and (B) *Populus* (PtRDN1-GFP) in Mtrdn1-2 transgenic roots (C) tobacco leaf infiltration with PtRDN1-GFP. The red in the tobacco epidermal cells is chloroplast autofluorescence. Images obtained using an Axiovert 200 M fluorescence microscope with Apotome (Carl Zeiss). The green dots are the moving organelles to which MtRDN2 and PtRDN1 localized.
AtRDN2 regulates lateral root number in Arabidopsis
Since we found that AtRDN2 can rescue the nodule autoregulation defective mutant Mtrdn1-2 (Figure 5.3 G and H) despite the fact that Arabidopsis makes neither rhizobial nor mycorrhizal associations, and we observed a partial rescue with the same gene expressed under the control of the MtRDN1 promoter (Figure 5.4), we began a search for non-symbiotic roles of RDN proteins. We obtained a collection of T-DNA insertion mutants in the three RDN genes in A. thaliana; AtRDN2 (At5g13500) an ortholog by sequence of MtRDN2, AtRDN3 (At5g25265) an ortholog by sequence of MtRDN3 and AtRDN (At2g25260) with no direct sequence ortholog in M. truncatula. We grew seed stocks from the Arabidopsis Stock Center and identified lines homozygous for the T-DNA insertions through testing by polymerase chain reaction (PCR) for presence and absence of the inserts. The homozygous lines were then grown on half strength MS media for 10 days before root phenotypes analyzed. The lines with T-DNA insertions in AtRDN3 and AtRDN (Appendix P) did not display any obvious shoot or root phenotypic differences from wild type plants. However, three allelic mutant lines harboring T-DNA insertions in the AtRDN2 genomic region (Figure 5.7) displayed an increased lateral root phenotype (Figure 5.8).
As no mutation in this genomic region that causes any phenotype had been previously described, we designated these new mutations Atrdn2-1 (insertion on the last intron), Atrdn2-2 (insertion on the 5’UTR exon region) and Atrdn2-3 (on the promoter region) (Figure 5.7). The lateral root number phenotype is consistently strong for the AtRDN2-1 mutant allele (Figure 5.8 B&C). In contrast, the other two mutant alleles showed inconsistent and weak lateral root phenotypes (Figure 5.8 B&C). As for AtRDN3 and AtRDN insertion lines, we did not observe any obvious shoot phenotypes on these mutant lines.
Figure 5.8. *AtRDN2* mutants have lateral root phenotypes. (A) Lateral root phenotypes of representative plants from the wild type (COL-0) and T-DNA insertion lines *Atrdn2-1* and *Atrdn2-2*. (B) Mean lateral root number per plant for plants in A and *Atrdn2-3* mutants. (C) Mean branching density for plants in B; defined as the number of visible lateral roots per cm of branching root length. The error bars indicate SE of the mean (n = 20 for all genotypes). Tukey Kramer simultaneous comparison t-test was used for statistics analysis (p<0.05). Different letters represent significant differences.

To further dissect the cause of the lateral root phenotype we measured the primary root length of 10 days old seedlings and the primary root growth rates per day, 4 days after incubating plates vertically in the growth chamber. There was no significant difference in the rate of root growth in *Atrdn2-1* plants and *Atrdn2-2* plants when compared to wild type plants (Figure 5.9A). However, the primary root length of 10 day old seedlings of *Atrdn2-2* was significantly longer than the wild type and the *Atrdn2-1* plants despite no change in growth rate in the first six days (Figure 5.9B).
Figure 5.9. Root growth parameters. (A) Mean rate of root growth per day calculated from the root tip displacement of the wild type and T-DNA insertion mutant lines. N=8-10. (B) Mean primary root length of 10 days old plants from A. * indicates significant difference (P<0.05) (Tukey Kramer test).
We also examined the transcript abundance of AtRDN2 in the wild type and mutant plants at 10 days post germination using qRT-PCR. Using primers designed to amplify the first exon (Figure 5.10) we determined that the T-DNA insertion did not affect the expression level of AtRDN2 in the Atrdn2-1 and Atrdn2-3 alleles when examined (Figure 5.11A). However, using primers spanning the last intron (Figure 5.10) the transcription level of AtRDN2 in Atrdn2-1 is lower than wild type (Figure 5.11B). Interestingly, for either primer pair (the first exon and spanning the last intron) we observed increased transcript abundance of AtRDN2 in the Atrdn2-2 mutant (Figure 5.11A&B) suggesting that the T-DNA insertion in the 5’UTR region caused increased AtRDN2 message which could lead to the longer primary root phenotype mentioned above (Figure 5.9B).

![Gene structure of AtRDN2 gene with primer locations.](image)

**Figure 5.10.** Gene structure of AtRDN2 gene with primer locations. Translated regions of exons are shown as gray boxes. 5’ and 3’ untranslated regions are shown as black boxes. The start and stop codons are represented as ATG and TAA respectively. The primer pairs used for real time PCR are F1&R1 on the first exon and F2&R2 spanning the last intron.
Figure 5.11. Expression of AtRDN2 in the Atrdn2 T-DNA insertion mutant lines by quantitative RT-PCR. The whole plant is used for RNA extraction. The relative expression level of AtRDN2 was determined using (A) Primers on the first exon, and (B) Primers spinning the last intron. Transcript levels are normalized to Arabidopsis β-ACTIN-2 levels and the Pfaffl method (Pfaffl 2001) was used for relative quantification of three biological and technical replicates.

The T-DNA insertion in Atrdn2-2 does not Co-segregate with the Lateral Root Phenotype in one F2 family

Because the Arabidopsis T-DNA insertion lines may contain multiple insertions in a single line, we crossed the Atrdn2-1 (with strong lateral root phenotype) to wild type (Col) plants and examined the segregation of the T-DNA insertion with the lateral root phenotype in the F2 plants. We first identified four independent heterozygous F1 plants.
by PCR for further analysis. In the F2 population of one of these plants, the T-DNA insertion did not co-segregate with a lateral root phenotype (Figure 5.12 A&B). Both the lateral root number and the branching density were similar to the wild type plants in plants both heterozygous and homozygous for the T-DNA insertion. One possibility is that an additional mutation in Atrdn2 lines causes the observed root defects, but until the other F2 populations are examined this is simply preliminary data.

Figure 5.12. Mean lateral root number and mean branching density of F2 segregating plant populations from COL-0 x Atrdn2-1(At5g13500). (A) Lateral root numbers of Col-0, Atrdn2-1, and Col-0/Atrdn2-1 plants segregating in the F2 population. (B) Branching density of plants in A; defined as the number of visible lateral roots per cm of branching root length. The error bars are standard errors (N=4-11 for each genotype).
DISCUSSION

Despite their broad evolutionary distribution across monoctos, dicots and even moss the RDN gene family is highly conserved in gene number and sequence (Schnabel et al. 2011), as well as motifs and potential modification sites (Fig 5.1, 5.2, Supplemental Fig S5.1) and subcellular localization (Figure 5.6). Until Schnabel et al. (2011) and Chapter 4 of this work, no function had been assigned to any member of the family. As noted previously, root symbioses with AM fungi and nitrogen-fixing bacteria share common signaling components, suggesting that the nitrogen-fixing root nodule symbioses evolved from the ancient AM symbiosis (Kistner and Parniske, 2002) and nearly all the “nodulation” genes cloned thus far, either the common SYM genes or genes required only for rhizobial symbiosis, have putative orthologs in non-legumes (Zhu et al. 2006). Genes in the nodule autoregulation pathway are also found in non-nodulating plants (Nishimura et al. 2002; Searle et al. 2003; Schnabel et al. 2005; Schnabel et al. 2011).

These finding are supported by the cross species complementation assay reported here-putative orthologous RDN genes could rescue the Mtrdn1-2 AON phenotype when expressed at high levels (Figure 5.3), implying the biochemical function of the protein product produced by non-nodulating poplar, rice and Arabidopsis RDN genes is conserved. Even another M. truncatula RDN (MtRDN2) could rescue the phenotype when expressed at a high level (Figure 5.3), but this was not true for all M. truncatula RDNs; MtRND3 did not give rescue (Appendix O) but this RDN is more dissimilar in structure to MtRDN1 than the tested RDNs from other species (Appendix Q). The
rescue by MtRDN2 combined with the fact that MtRDN2 is wild type in the rdn1-2 mutant suggests that the high expression level from the CaM 35S promoter may overcome small differences in functionality among the paralogs to allow rescue. When expressed under the native promoter (pMtRDN1), only the putative rice ortholog could rescue the phenotype to the same level as MtRDN1, but all the genes tested reduced nodule number significantly more than the empty vector control (Figure 5.4). As noted in the introduction, rice orthologs of other genes involved in symbiosis have been shown to rescue legume symbiosis mutants; a calcium and calmodulin dependent protein kinase required for AM symbiosis in rice restored nodulation in the nodule defective MtDMI3 mutant (Godfroy et al. 2006; Chen et al. 2007), suggesting an equivalent role for the protein in non-legumes. Rice and poplar both establish AM associations, while Arabidopsis does not, so simple conservation of a common pathway cannot fully explain the results in Figure 5.4, but the Arabidopsis gene may still retain some of the function critical for symbiosis despite the loss of AM symbiosis in most Brassicaceae. It may be that differences in regulation of expression combined with small differences in biochemical function separate the legume RDNs from the non-legume RDNs, but that does not negate the fact that RDNs must have roles outside symbiotic regulation.

One possibility is the regulation of lateral root formation. Again, as noted in the introduction, nodules are similar to lateral roots in that their site of origin occurs largely opposite the protoxylem poles and involves pericyclic and endodermal cell proliferation.
(Callaham and Torrey, 1977). The two organs differ in that the cortical cells opposite
the protoxylem poles are stimulated to undergo divisions and these cortical cell
derivatives are incorporated into the developing primordium in nodule organogenesis,
while lateral roots don’t arise from cortical cells, which instead are crushed and
displaced by the lateral root primordium as they develop from cellular derivatives of the
pericycle and endodermis (Callaham and Torrey, 1977). Since the development and
structure of legume nodules resemble in some respects that of lateral roots, it has been
proposed that their ontogeny may require the same molecular signals (Nutman 1948;
Ferguson and Mathesius 2003; Huo et al. 2004; Kuppusamy et al. 2009; Gonzalez-
Rizzo et al. 2006). Members of the CLE gene family of small regulatory peptides have
been shown to regulate nodule number in M. truncatula (Mortier et al. 2010) and
overexpression of CLE genes in Arabidopsis results in long roots or long and wavy
roots that also showed altered lateral root patterning (Meng et al. 2012). The
orthologous hypernodulation mutants nts in soybean, harl in L. japonicus and sunn and
lss in M. truncatula) exhibit an excessive number of both nodules and lateral roots
(Wopereis et al. 2000; Schanbel et al. 2010). We took advantage of the availability of
Arabidopsis T-DNA insertion lines to look for a non-symbiotic role of RDNs and
seemed to have found one for AtRDN2. Compared to the wild type, the Atrdn2-1 line
showed increased lateral root number and a change in root length (Figure 5.8 & Figure
5.9B), which would suggest a function in common between nodules and lateral roots.
However, since our preliminary data from the outcross implies the T-DNA insertion
does not co-segregate with the lateral root phenotype initially observed (Figure 5.12), it
is likely another insertion in the mutant line causes the lateral root phenotype. Nevertheless, the experiment was on a small number of plants and the results need to be further confirmed using other seed from the crosses and including the original mutant line to be sure the lack of phenotype was not due to an unknown environmental change. We also plan to test complementation of the original mutation with the \( \text{AtRDN2} \) gene.

In support of a root phenotype, the allelic variant \( \text{Atrdn2-2} \) exhibited a significantly longer primary root phenotype compared to wild type plants (Figure 5.9B). This phenotype is the opposite of that observed when \( \text{MtRDN1} \) is disrupted- a short root phenotype was observed in \( \text{Mtrdn1} \) null mutants both in the presence or absence of rhizobia (Schnabel et al. 2011). Since expression analysis showed an increased \( \text{AtRDN2} \) transcript level specifically in \( \text{Atrdn2-2} \) lines (Figure 5.11), we conclude the T-DNA insertion in the 5’UTR is affecting transcriptional regulation. Thus if disruption of the gene causes short roots, it could be logically argued that overexpression can cause longer roots. Altogether, our results add to the growing body of evidence that legumes have recruited pre-existing root regulation mechanism for symbiosis development. Therefore there was no need for a major evolutionary adaptation to allow the functioning of the signaling pathway leading to nodule autoregulation.
MATERIALS AND METHODS

Plant Materials and Growth Conditions
Medicago truncatula cv. Jemalong A17 and the AON defective mutant rdn1-2 (Schnabel et al. 2011) were used for transcomplementation. Seeds were acid scarified and imbibed as described in Schnabel et al. (2010) and vernalized in dark at 4°C for 2 days on Harrison Modified Farhaeus (HMF) media (Huo et al. 2006) covered with two half round Whatman filter papers (GE Healthcare, USA). Seeds were then germinated in the dark at room temperature for 1 day, transferred to a new HMF plates, and incubated vertically in 25°C with 16hrs light: 8hrs dark in a growth chamber for five days. These 5 days old seedlings were used for Agrobacterium mediated hairy root transformation.

Arabidopsis wild type Columbia (Col-0) and T-DNA insertion lines of the same genetic background At5g13500 (SALK_047668), At5g13500 (SAIL_846_A03/CS837758) and At5g13500 (SALK_054259) were used to study the non-symbiotic role of RDN (Arabidopsis Biological Resource Center (ABRC)). For agar plate assays, Arabidopsis seeds were treated with 70% ethanol for 2 minutes, sterilized for 5 minutes in 20% bleach (Chlorox) containing final concentration of 1.2% sodium hypochlorite and 0.1% Tween-20, washed three times with sterilized distilled water, shaken for 20 minutes on a rotating platform and then planted on solid half strength MS media containing: 0.5-X Murashige and Skoog Basal medium w/ vitamins (Phytotechnology Laboratories®, Shawnee Mission, KS ), 0.5% (wt/vol) sucrose, 10mM MES, and 0.6% (wt/vol) Agar
(Caisson Laboratories, North Logan, UT), pH 5.7-5.8. After 3 days at 4°C, plates were placed vertically in growth chamber (23°C, 16hrs light and 8hrs of dark) for 10 days. Ten days old seedlings were evaluated for lateral root number, root length, and branching density. For crossing Col-0 and At5g13500-047668 to investigate the segregation of the T-DNA insertion with the lateral root phenotype, 10 day old seedlings were transferred to a soil system (Farrad potting media) and grown in a greenhouse.

**Bacterial Strains and Growth Conditions**
The *Agrobacterium rhizogenes* strain ArQua1 (Quandt *et al.* 1993) was used to transform all *M. truncatula* plants. The binary expression constructs were transformed into ArQua1 by electroporation (BIO-RAD Micropulser™, Hercules, USA) and grown for two days at 30°C under spectinomycine selection. Individual colonies from the spectinomycine plate were selected and grown over night in 1 ml liquid LB. Out of the 1 ml liquid culture, 950 µl was plated on two spectinomycine plates each with 475 µl liquid culture and the remaining 50 µl was used for making a liquid culture for plasmid isolation to confirm plasmid identity. *Sinorhizobium medicea* ABS7 strain (Bekki *et al.* 1987) was used to inoculate plants for root nodulation. The growth conditions for ABS7 are the same as our previous report (Schnabel *et al.* 2011).
Plasmid Construction
The Gateway™ cloning system which is based on phage lambda-based site-specific recombination was used to clone all the constructs. cDNA prepared from *Medicago truncatula, Populus trichocarpa,* and *Oryza sativa,* were used as template to amplify MtRDN1, MtRDN2 & MtRDN3, PtRDN1, and OsRDN1 genes respectively. The primers used are listed in Table 5.1. PCR products from each reaction were purified using Zymoclean gel DNA recovery kit (Zymo Research, USA) and cloned into the pDONR-221 vector using BP cloning (based on att phage recombination sites) per the manufacturer’s instruction (Invitrogen™, Carlsbad, USA). *AtRDN2* cloned in the same Gateway™ pDONR-221 vector was purchased from the *Arabidopsis thaliana* ATOME1 ORFEOME library (CNRGV-INRA, Castanet-Tolosan, France). DH5α and DH10β *E. coli* competence cells were used for transformation. The transcriptionally silent entry clones were grown under kanamycin selection and their identity was confirmed by restriction mapping and sequence analysis. To generate expression clones using the CaM 35S promoter, cloning was performed between the entry clones and the pK7FWG2 destination (expression) vector (Karimi *et al.* 2002) creating a C-terminal eGFP fusion to all the proteins. In addition, the pK7FWG2-R destination vector containing the DsRED fluorescent reporter gene was altered to use the MtRDN1 promoter to drive expression by removing the 35S promoter through restriction digestion with HindIII and SpeI and replacement with the promoter used in Schnabel *et al.* (2011). The positive expression clones were selected under spectinomycin and expression vectors containing the proper constructs were transformed into *Agrobacterium rhizogenes* strain Arqua1 (Quandt *et al.* 1993). Hairy root
transformation was performed in plants carrying the rdn1-2 mutant allele and the wild type control.

Table 5.1. List of primers used to amplify MtRDN1, MtRDN2, MtRDN3, PtRDN1, and OsRDN to rescue the rdn1-2 mutation in M. truncatula.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers (from 5’ to 3’)</th>
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<tbody>
<tr>
<td>MtRDN1</td>
<td>GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGGGAGGGTAAAATCTCTACT</td>
</tr>
<tr>
<td></td>
<td>GGGGACCACCTTTGTACAAGAAAGCTGGGTCGCTTCTATTTAACGAATCCC</td>
</tr>
<tr>
<td>MtRDN2</td>
<td>GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGCACGAGCTTCACCGTTAC</td>
</tr>
<tr>
<td></td>
<td>GGGGACCACCTTTGTACAAGAAAGCTGGGTCCAATGTATCCCAGTTGGGGATGTT</td>
</tr>
<tr>
<td>MtRDN3</td>
<td>GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGGTGTTGTGGGAACATGTGTT</td>
</tr>
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<td></td>
<td>GGGGACCACCTTTGTACAAGAAAGCTGGGTCCGATGACCAGTTCGGAATATTT</td>
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<tr>
<td>PtRDN1</td>
<td>GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGGGCGAGCGTC</td>
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<td></td>
<td>GGGGACCACCTTTGTACAAGAAAGCTGGGTCGCCACTGTTCAATGAGTCCC</td>
</tr>
<tr>
<td>OsRDN</td>
<td>GGGGACAAGTTTGTACAAAAAAGCAGGCTATGAGCGG</td>
</tr>
<tr>
<td></td>
<td>GGGGACCACCTTTGTACAAGAAAGCTGGGTCCCTTTCTTCGTCCCACCCT</td>
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Agrobacterium-Mediated Plant Transformation

Seedlings were transformed as previously described (Limpens et al. 2004). The hypocotyls of five days old seedlings were cut and transformed by lightly scraping on the surface of LB plates densely grown with Agrobacterium rhizogenes strain Arqua1 (Quandt et al. 1993) containing the appropriate binary vector and antibiotic selections (at 30°C for 48hrs). After 5 days of co-cultivation with the Agrobacterium in the growth
chamber at 23°C (16 hrs of light and 8 hrs dark), the seedlings were transferred to nutrient rich hairy roots emergence media (Limpens et al. 2004) containing 300 μg/ml cefotaxime (Phytotechnology Laboratories®, Shawnee Mission, KS) sandwiched between two half round Whatman filter papers grown under the same growth conditions. Five days later, the top filter papers were removed from the plates and the seedlings were allowed to grow for additional five days on the same emergence medium placed vertically in the same growth chamber. For nodulation experiment non-transgenic roots were trimmed off and the entire plant transferred to Perlite as in Chapter 3. The plants were watered for 5 days with a 100-fold dilution of water-soluble 20:10:20 Peat-Lite Special fertilizer (Scotts Company, OH, USA). Fertilization was then withdrawn and the plants were hydrated with water alone an additional five days in order to induce nitrogen deficiency required for nodulation. Then plants were inoculated with S. medicae (OD600 nm=0.2) and data collected 21 days post inoculation.

**Quantitative Real time PCR**

Total RNA was isolated from plants grown on 0.5-XMS media with the RNeasy Plant Mini Kit (Qiagen, Maryland, USA) according to the manufacturer's instructions. RNA was extracted from 10 days old whole Arabidopsis plant tissue in three independent biological replicates. After 40 minutes of RNase free DNase (Promega, Madison, USA) treatment, the samples were purified through 3 mM NH₄Ac and 100% ethanol precipitation, the quality of RNA was checked and quantified with Biophotometer (Eppendorf, Hamburg, Germany). RNA (1 μg per 20 μl reaction mixture) was used for cDNA synthesis with the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, USA). The
cDNA samples were diluted two fold before use. All the qRT-PCR experiments were performed with iQTM SYBR® Green Super Mix and an iCycler iQ5 multicolor detection system (Bio-Rad, Hercules, USA) for amplification and detection. Each reaction was performed in triplicate and results averaged. The total reaction volume was 12.5 μL (10 μL of master mix including 0.175 μL each primer (0.35 μM final concentration), and 2.5 μL of cDNA). Cycle threshold (Ct) values were obtained with the accompanying software, and data were analyzed using Pfaffl method (Pfaffl 2001). Relative expression was normalized against the expression of Arabidopsis β-Actin2. Primers used for AtRDN2 are as follows: spanning the last intron forward: 5’AAATAGGAGAGTGGCGATTCG3’ and reverse: 5’CCAATTAGGGATAGTCGCAGTAG3’, on the first exon forward: 5’AGGAAAGCCAAGAGTTCCAGCA3’ and reverse: 5’AGCCACCCATATCAGAACCAGGAA3’ and for β-Actin-2 forward: 5’AGTGGGTCAAAACCAGGTATTGT3’ and reverse: 5’GATGGCATTGGAGGAGAGAAAC3’.

Microscopy and Imaging Analysis
To identify plants containing transgenic roots and remove the non-transgenic roots we used an Olympus SZX12 Dissecting Microscope using a DsRED filter (Chroma). Three weeks after inoculation with S. medicae, the nodules on transgenic roots were checked, counted and images of DsRED containing roots were processed and analyzed using the same Olympus SZX12 Dissecting Fluorescence Microscope fitted with an Olympus DP11 Digital Camera System. DsRed excitation and emission maxima of 554 and 586
nm, respectively were used DsRED root imaging. Larger scale pictures of Arabidopsis were photographed with a Nikon coolpix5000 camera. Subcellular localization images were taken from transgenic root sections using an Axiovert 200 M fluorescence microscope with Apotome under EC Plan Neofluar 100x/1.30 Oil Iris objective and AxioCamMR camera (Carl Zeiss, New York, USA). YFP band pass and FITC long pass filter settings (Chroma) were used to capture PrRDN1-GFP & MtRDN2-GFP, and the Chloroplasts images respectively.

**In silico Analysis**

Multiple alignment and calculation of amino acid sequence identity were performed using the default settings of Clustal W2 (http://www.ebi.ac.uk/clustalw2/). MAGA5.1beta was used to infer phylogenetic trees, estimate rates of molecular evolution, infer ancestral sequences, and test evolutionary hypotheses (http://www.megasoftware.net/). Phosphorylation sites were predicted using NetPhos 2.0 server (http://www.cbs.dtu.dk/services/NetPhos/). Promoter scanning for possible cis-acting elements was performed using SCOPE (http://genie.dartmouth.edu/scope). We used the Expasy database of the ELM resource for functional site prediction (http://elm.eu.org/) and the SignalP server for signal peptide cleavage site prediction (http://www.cbs.dtu.dk/services/SignalP/), and the non-redundant protein sequence NCBI database of blastP resource (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to examine the features associated with RDN gene and its protein families.
REFERENCES


CHAPTER 6

SUMMARY

The process of nodule formation and nitrogen fixation costs host plants extensive energy and carbon, and excessive formation of nodules in the root may impair the developmental needs of the host plant. Hence, the number of nodules formed in the roots of leguminous plants is systematically controlled by autoregulation of nodulation. Plants that are deficient in autoregulation display a hypernodulating phenotype. In this study we cloned, characterized and examined the functions of a Root Determined Nodulator 1 (RDN1) gene in *M. truncatula*.

Four hypernodulating alleles of *rdn1* were isolated from fast neutron bombardment M2 seedling collections by visual screening. PCR analysis revealed that three *rdn1* alleles (*rdn1*-1, *rdn1*-3, and *rdn1*-4) have large nested deletion ranging from 103-240kb. The *rdn1*-2 allele in the same genomic region had a 1kb indel in one of the candidate genes. The *RDN1* gene was identified by genetic mapping, transcript profiling, and phenotypic rescue by expression of the wild-type gene in *rdn1* mutants. Like other AON defective mutants, *rdn1* has a hypernodulation and short root phenotypes and leads to a partial nitrate tolerant nodulation. Grafting experiments demonstrated that RDN1 regulatory function occurs in the roots, not the shoots, and is essential for normal nodule number regulation. RDN1 is predicted to encode a 357aa protein of unknown function. The RDN1 promoter drives expression in the vascular cylinder, suggesting RDN1 may
be involved in initiating, responding to, or transporting vascular signals. Three RDN genes were identified from *M. thaliana* genome by BLAST analysis. RDN1 is a member of a small, uncharacterized, highly conserved gene family unique to green plants, including algae.

Analysis of the amino acid sequence showed no conserved domains except the N-terminal signal peptide, overlapping N-terminal transmembrane domain and C-terminal proline rich low complexity region. We localized RDN1 to a large number of tiny moving organelles in the cytoplasm of transgenic Medicago root cells and tobacco epidermal cells. Using different organelle specific markers we identified RDN1 as a Golgi localized protein. Staining with the endocytic tracer FM4-64 dye suggested a role of RDN1 in endomembrane system, possibly at trans-Golgi/early endosomes. The ruling out of ER, vacuole, peroxisomes and late endosome localizations may suggest a role for RDN1 in secretion. However, RDN1 did not co-localize with plasma membrane marker instead a partial association was observed, suggestive of a tethered protein reversibly associated with the PM. Present evidence of co-localization with secretory pathway components suggests that RDN1 may engage in vesicle trafficking. However, a connection of RDN1 to downstream elements of the secretory pathway is missing. Some possibilities include interaction with SUNN at the PM or involvement in trafficking cargo molecules back and forth from the PM (Figure 6.1).
We developed split root system and grafting techniques to investigate the timing of the long distance nodule autoregulation signal in *M. truncatula* and the role of *RDN1* and *SUNN* genes in the regulatory circuit. Split root inoculation confirmed that prior nodulation events do not affect later nodulation events in plants carrying mutations in *RDN1* and *SUNN* genes than did the wild type plants.

![Schematic representation of possible role of RDN1 in vesicle trafficking](image.png)

**Figure 6.1. Schematic representation of possible role of RDN1 in vesicle trafficking.** RDN1 may help protein cargo to move from TGN to PM and from PM to TGN. The potential candidate is the receptor kinase SUNN on the plasma membrane.

The full induction of the autoregulation of nodulation took three days in wild type plants and remains active at least for fifteen days. However, we report of evidence for the initiation of a second signal or accumulation of an initial signal associated with nitrogen fixing nodules ten days after the first root inoculation which suppressed
nodulation on the second root in mutant plants. Either nitrogen fixation or meristem activity cannot be ruled out as the possible cause of the signal.

Inverted-Y graft combinations of wild type plants and plants carrying mutations in RDN1 and SUNN demonstrated that RDN1 regulatory function occurs in the root before the shoot-derived suppression signal which involves SUNN. The phenotypes of the sunn/rdn1 double mutation and the shoot to root reciprocal grafting between sunn-4 and rdn1-2 support SUNN and RDN1 acting in the same pathway. Combined with our data that the SUNN message is down regulated in the rdn1 root background whereas RDN1 message level is normal in the sunn root background, we suggest RDN1 acts before SUNN and their possible interaction is in the root. Based on our data and other available experimental evidences, we propose a model that describes the roles RDN1 in nodule regulation (Figure 6.2).

Cross species complementation studies showed that genes from Populus (PtRDN1), Oryaza (OsRDN), Arabidopsis (AtRDN2) and Medicago (MtRDN2) under CaMV 35S promoter can restore nodule number autoregulation. These results add to the growing body of evidence that legumes have recruited pre-existing root developmental regulation mechanism for symbiosis development. Just as in nodule initiation, there was no need for a major evolutionary adaptation to allow the functioning of the signaling pathway leading to nodule autoregulation. We identified allele specific increased lateral root number and branching density, and altered primary root development in
Arabidopsis plants carrying mutations in an RDN gene. However, the lateral root phenotype didn’t co-segregate with the T-DNA insertion and needs further investigation. However, together these findings establish the RDNs as a family of proteins with previously uncharacterized regulatory functions playing a role in the pattern of root growth and lateral root development in plants.

**Figure 6.2. A proposed model for RDN1 functions in *M. truncatula*.** Rhizobia inoculation and exogenous application of nitrate initiate AON. Perception of Nod factor activates genes in the nod factor signaling pathway (Including nod factor perception (NFP), Doesn’t Make Infection (DMI1, DMI2, and DMI3), and downstream transcription factors (Nod factor Signaling Pathway (NSP1 and NSP2), Nodule Inception (NIN), and ERF Required for Nodulation1 (ERN1). This will initiate nodulation and the production of unknown root derived signal (Q). RDN1 in the root plays important role in the synthesis or release of Q and MtCLE12/MtCLE13 are the possible candidates of the root derived signal or its generation. The root derived signal (Q) is transported to the shoot and perceived by receptor kinases like SUNN. This will elicit the production and release of an unknown shoot derived inhibitory signal or
signals (R). In *M. truncatula*, the full induction of AON takes 3 days to suppress nodule formation and root development. And an unknown signal that has different route than the usual RDN1-SUNN root–shoot-root signaling will be released 10-12 days after inoculation. Known interactions are in solid lines and possible unknown interactions are in broken lines. Red lines are initiated by nitrate, black lines by rhizobia and broken purple lines due to the unknown second signal. Arrows with oval tips indicate inhibition.
APPENDICES
APPENDIX A

Nodulation on *rdn1* roots rescued with the Medtr5g089520 (RDN1) cDNA

Representative bright field (A, C, E, G, and I) and DsRed1 fluorescence (B, D, F, H, and J) images of nodulated hairy roots transformed with T-DNA carrying a UBQ10pro:DsRed1 reporter alone (empty vector, EV) or with 35Spro:RDN1 cDNA. A and B, A17 wild type transformed with EV. C and D, rdn1-1 transformed with EV. E and F, rdn1-1 transformed with 35Spro:RDN1 cDNA. G and H, rdn1-2 transformed with EV. I and J, rdn1-2 transformed with 35Spro:RDN1 cDNA. Bars = 1.0 mm.
APPENDIX B

PCR analysis of RDN1 expression

A, Locations of primers used in the analyses and predicted PCR product sizes. B, Specificity of primers used in RT-qPCR analysis and quality control of cDNA. Shown are the results of amplifications using primers from within RDN1 exon 2 (a-b), spanning RDN1 intron 2 (c-d), and from a non-coding sequence of M. truncatula chromosome 5 (primers JF1330 and JF1331). Primer pairs a-b and c-d produced single bands from cDNA and lacked visible primer-dimers, making them suitable candidates for qPCR analysis. The intron-spanning fragment is detected in cDNAs but not gDNA due to the large size of intron 2. The non-coding region primers yield the expected band only from gDNA and not from the cDNAs, demonstrating undetectable levels of gDNA in the cDNA samples. The non-coding region primers produce a primer-dimer product in the absence of target sequence as seen in the cDNA and no template samples. C, RDN1 expression in shoots and roots of wild type (A17) seedlings was detected using primers c and d. For all analyses cDNAs were synthesized from DNase-treated RNA isolated from nodulated plants.
APPENDIX C

The structure of the *PsNOD3* coding region in pea cultivar Rondo and the derived mutant *nod3*

The structure of the *PsNOD3* coding region in pea cultivar Rondo and the derived mutant *nod3*. A G to A transition mutation at the last base of an intron in Psnod3 (indicated by an arrow) shifts the splice site of that intron one base 3' (indicated by a /) resulting in a frame shift mutation with a premature stop three codons later. The alteration of the splice site was verified by sequencing nod3 cDNA (not shown). The full length Rondo transcript is predicted to encode a 357aa protein; nod3 would produce a protein of 128 aa. The sequence of the NOD3 gene was obtained using primers based on the MtRDN1 sequence.
## APPENDIX D

Alignment of the predicted sequences of RDN family proteins from twelve land plant species

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Alignment of the predicted sequences of 43 RDN family proteins from twelve land plant species including the dicots *Medicago truncatula* (Mt), *Lotus japonicus* (Lj), soybean (*Glycine max*, Gm), grape (*Vitus vinifera*, GSVIVT), cucumber (*Cucumus sativus*, Cucsa), poplar (*Populus trichocarpa*, POPTR), and Arabidopsis (At); the monocots rice (*Oryza sativa*, Os), sorghum (*Sorghum bicolor*, Sb), and maize (*Zea mays*, GRMZM); the moss *Physcomitrella patens* (Moss); and the lycophyte *Selaginella moellendorfii* (Sm). ClustalW was used to perform the initial alignment which was then adjusted manually. The source of the sequences is provided in Supplemental Table 1. Sequence groups are indicated by color: RDN1 group (green), RDN2 group (blue), RDN3 group (orange), monocot groups (purple and red), other (black). Amino acid position is given along the right column. Signal peptides predicted by SignalP 3.0 (Bendtsen et al., 2004) are underlined; the N-terminal transmembrane segments predicted by TMHMM 2.0 (Krogh et al., 2001) are shown in italics. The alignment was used to generate the phylogenetic tree shown in Figure 7 and to assess the sequence conservation among RDN proteins shown in Figure 5. Sequence GmRDN1.2B from soybean is derived from a putative pseudogene sequence that contains a very large intron (10.9 kb) and a downstream frameshift mutation resulting in a predicted protein length of 178 aa; shown here is the predicted ancestral sequence of 369 aa in which the frameshift was reverted by addition of a single nucleotide to the presumed coding sequence (indicated by an underlined X in the GmRDN1.2B sequence).
APPENDIX E

Alignment of the predicted sequences of RDN family proteins from the green algae with RDN protein sequences from land plants

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| M ap 5      | 34  |
| Ol 1        | 34  |
| Ol 2        | 34  |
| Ol 3        | 34  |
| MtrON1      | 34  |
| MtrON2      | 34  |
| MtrON3      | 34  |
| Sm 150302   | 34  |
| Sm 231983   | 34  |
| Sm 235499   | 34  |

(+ Additional alignment data continues here)
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<tr>
<th>MtrBDN1</th>
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<tr>
<td>MtrBDN2</td>
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<td>DQQ</td>
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<td>EG-HGSS</td>
<td>RG 112</td>
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<tr>
<td>Ot 1 partial</td>
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<tr>
<td>Ot 2</td>
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<td>CQFWHRDFPKSFCETYGR1MNDL-LPMPYEEERKLLT7TG</td>
<td>VLYPK 251</td>
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<tr>
<td>Ot 3</td>
<td>EQGS</td>
<td>ITVARDCDBCCETCCKT-AK-</td>
<td>CQFWHRDFPAKNEFCGETG1LINDLFMYPEEERKLLT7TG</td>
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</table>

MtrBDN1: AEIMEINTNS- | KYHVAVTATDAYSQCRMCROMYIYKTC- | MP 89 |
MtrBDN2: PEHVKRTKTS- | RAPFHTLALTADIAHYNQCRMYKQFQ- | LP 95 |
MtrBDN3: PLKNSASS- | KRLYFTAVTASDDVYNTQCVHRWFYRKK- | SG 95 |
Sm 150302: PFSYKNFFKKE- | KEMNYTNTASSTPYNNQERMYWYQFQKD | KA 105 |
Sm 231983: EFSDNPGKK | KLFHTYVTSYQYSARQYCRMYKQKFQD- | OP 81 |
Sm 235499: FRDFAKARR | EERFRHTATASALYRNCQYCRMWMYVFQKD- | EA 95 |
Cr 1 | AEKQVVTETVTVTTLVYG | STLQNPYRMDVYTTAVGSNHQARHYYWFQGRQACL | 152 |
Cr 2 | PEASKFARYDGQFTQWSSIDVIDNADGQSDRACASKGAFHEVTSQAOAGAVNHQVRHYYWKRKGK- | ECQP 262 |
M sp 1 | RPEELPTGQVDTNEPDNQFSGNKMFPADYR | -GERGAMKHFVNHVTNTANVYQGFQWYQFQWYRVETERMERECDED | 293 |
Ot 1 | RATMTTCGDEAQGWSQFISYQDMDR | -GDPDRPQKHFVNNTTYNNAQYQGQAWQYQFQKQABG | AGG 236 |
M sp 2 | TKEKRDQNKGG- | NGKHYHLLTANADAVYVRQSRHYQFKKIA- | DP 181 |
Cr 3 | ATKTDRAFD | NTISHITGNGPYQNGFYGTYLVQYM- | P 112 |
M sp 3 | GASSGSGSGGDP | -PGSIHVMATSMGSPQCNQCTIRMFTLDR | 158 |
M sp 4 | GVYKALAKQVFDP | -PFKCLHYTITSSGNYMNQFRMQITGQYTLSKKA- | P 298 |
M sp 5 | QFPRAYKSGASSKDD | PFCLHMTSNGQMNQTPYQFQWRTKKA- | R 346 |
Ot 1 partial | EKYPFATGKGD | -LPKCLHTINTSSGNYNQSMRYSMYSLHAA- | P 192 |
Ot 2 | APKYESATOKT | -LPKCLHTVNSGNYMNRKVMSRGSLHAA- | D 208 |
Ot 2 | IFKYYAESTDLKT | -CLHTMITSNGAYMNQTPYQFQWTKKAASE- | K 296 |
Ot 3 | KASYDAESTLKT | -CVWHTMSNGQMNQTPYQFQWTKKAASE- | K 301 |
Alignment of the predicted sequences of 13 RDN family proteins from the green algae *Chlamydomonas reinhardtii* (Cr), *Micromonas* sp. RCC299 (M sp), *Ostreococcus tauri* (Ot), and *Ostreococcus lucimarinus* (Ol) with the predicted sequences of RDN family proteins from *Medicago truncatula* (Mt) and *Selaginella moellendorfii* (Sm). Amino acid position is given along the right column. A portion of the aligned sequences (underlined in the MtRDN1 sequence) was used to generate the phylogenetic tree shown in Figure 2.7 and to assess the sequence conservation among RDN proteins shown in Figure 2.5. Positions of absolute conservation among the sequences are indicated with *. The source of the sequences is provided in Supplemental Table S2.1.

| MtRDN1  | LVMQMXATANIIPYWDSLNRSL   | 357 |
| MtRDN2  | LVMQMXASEANIIQWDLTL       | 360 |
| MtRDN3  | LVMQMXAASANIIQWDSSE       | 360 |
| Sm 150302 | LVRMINEATANIIQWDSNG      | 372 |
| Sm 231983 | LVRMINEATANIIQWDSNG      | 344 |
| Sm 235499 | LVMQMXATANIIQWDSNG       | 360 |
| Cr 1     | LVRMINEATANIIQWDSNG      | 464 |
| Cr 2     | LVRMINEATANIIQWDSNG      | 570 |
| M sp 1   | LVRMINEATANIIQWDSNG      | 580 |
| M sp 2   | LVRMINEATANIIQWDSNG      | 454 |
| Cr 3     | LVRMINEATANIIQWDSNG      | 499 |
| M sp 3   | LVRMINEATANIIQWDSNG      | 459 |
| M sp 4   | LVRMINEATANIIQWDSNG      | 616 |
| M sp 5   | LVRMINEATANIIQWDSNG      | 666 |
| Ol 1     | LVRMINEATANIIQWDSNG      | 512 |
| Ol 2     | LVRMINEATANIIQWDSNG      | 458 |
| Ol 3     | LVRMINEATANIIQWDSNG      | 592 |
| Ol 4     | LVRMINEATANIIQWDSNG      | 592 |
APPENDIX F

Expression levels of RDN family genes of *M. truncatula* and *A. thaliana* in various tissues

A. Expression of RDN genes in *M. truncatula* as reported by Benedito *et al.* (2008) using the Affymetrix Gene Chip® Medicago Genome Array. MtRDN1 (Mtr.42387.1.S1_at), MtRDN2 (Mtr.40743.1.S1_at), and MtRDN3 (Mtr.45545.1.S1_at). B. Expression of RDN family genes of Arabidopsis as reported by Laubinger *et al.* (2008) using the At-TAX whole genome tiling array.
**APPENDIX G**

**RDN family genes identified in the genomes of twelve land plants and four algae**

<table>
<thead>
<tr>
<th>Gene Group</th>
<th>Protein Sequence Designation</th>
<th>Organism</th>
<th>Sequence Name in Genome Database</th>
<th>Database^a</th>
<th>Notes on database annotation</th>
</tr>
</thead>
</table>

### RDN1 group

- **MIRLDN1**: Medtr9g09010<br>  - Mtr 3.5: six codon 5’ extension added to annotation<br>  - L. japonicus genome v2.5: six codon 5’ extension added to annotation
- **GmRDN1A**: Glyma01g44810<br>  - Glyma01g44810: first two introns of annotated version included in cDS (matches 356 aa protein instead of 298 aa)
- **GmRDN1B**: Glyma01g49070<br>  - Glyma01g49070: first two introns of annotated version included in cDS (matches 356 aa protein instead of 298 aa)
- **GmRDN1-2A**: Glyma01g46020<br>  - Glyma01g46020: first two introns of annotated version included in cDS (matches 356 aa protein instead of 298 aa)
- **GmRDN1-2B**: Glyma01g49070<br>  - Glyma01g49070: first two introns of annotated version included in cDS (matches 356 aa protein instead of 298 aa)

### RDN2 group

- **LjRDN2**: Lj0040141.1.LJ2F53500<br>  - L. japonicus genome v2.5: merged L. japonicus fragment with overlapping EST sequences to get full length coding sequence
- **GmRDN2A**: Glyma02g26410<br>  - Glyma02g26410: alternate start site used
- **GmRDN2B**: Glyma02g35400<br>  - Glyma02g35400: alternate intron junction used for one splice
- **Vv 2**: Vv04702290001<br>  - Vv04702290001: alternate intron junction used for one splice
- **Pt 1A**: POP1R 000128320<br>  - POP1R 000128320: alternate start site used
- **Pt 1B**: POP1R 000685230<br>  - POP1R 000685230: alternate start site used

### RDN3 group

- **LjRDN3**: Lj0040141.1.LJ2F53500<br>  - L. japonicus genome v2.5: merged L. japonicus fragment with overlapping EST sequences to get full length coding sequence
- **GmRDN3A**: Glyma03g37780<br>  - Glyma03g37780: alternate start site used
- **GmRDN3B**: Glyma03g32910<br>  - Glyma03g32910: alternate start site used
- **Vv 3**: Vv04702290001<br>  - Vv04702290001: alternate start site used
- **Pt 3A**: POP1R 000842160<br>  - POP1R 000842160: alternate start site used
- **Pt 3B**: POP1R 000627500<br>  - POP1R 000627500: alternate start site used
- **At5g1500**: At5g1500<br>  - At5g1500: alternate start site used

### Mosses (group 1)

- **Os 1**: Osm01g44000<br>  - Osm01g44000: alternate start site used
- **St 1**: SRA0F010449<br>  - SRA0F010449: alternate start site used

### Mosses (group 2)

- **Zn 1**: Zesm07g03156<br>  - Zesm07g03156: alternate start site used

### Algae group 1

- **Cn 1**: Cn01g0068000<br>  - Cn01g0068000: alternate start site used
- **Cr 2**: Cr01g16200<br>  - Cr01g16200: alternate start site used
- **Mn 3**: Mn01g02820<br>  - Mn01g02820: alternate start site used
- **Mg 1**: Mg01g07220<br>  - Mg01g07220: alternate start site used
- **Dh 1**: Dh01g16127<br>  - Dh01g16127: alternate start site used
### Table S1. \(RDN\) family genes identified in the genomes of twelve land plants and four algae (cont.)

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<tr>
<th>Algae group 2</th>
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<th>(Chlamydomonas reinhardtii)</th>
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<th>JGI Augustus v9/assembly v4</th>
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<tr>
<td>M sp 5</td>
<td>Micrornota sp. RCC299</td>
<td>EnGen.040001.0355</td>
<td>Micrornota sp. RCC299 v3.0</td>
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</tr>
<tr>
<td>Dh 1</td>
<td>Ostreococcus tauri</td>
<td>OSt2g11190</td>
<td>Ostreococcus tauri v2.0</td>
<td>incomplete at 5' end</td>
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<tr>
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**APPENDIX H**

**Primers used in RDN1 mapping, T-DNA vector preparation and cDNA analysis**

**Table SII.** Primers used in RDN1 mapping, T-DNA vector preparation and cDNA analysis.

<table>
<thead>
<tr>
<th>Application</th>
<th>Primer Name</th>
<th>Primer Sequence (5’ to 3’)</th>
<th>Primer Use</th>
<th>Location in gene relative to predicted RDN1 translation start</th>
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<tr>
<td>T-DNA preparation</td>
<td>RDN1cDNA-A</td>
<td>TCATGGTACCATGGGAGGTAAGAAAATCTCTACT</td>
<td>rescue construct</td>
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<tr>
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<td>RDN1cDNA-B</td>
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<td>rescue construct</td>
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<td>P2.1-F</td>
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<td>590 bp from @ bp 294: A17 = TAA; A20 = TAATAA; @ bp 328: A17 = T, A20 = C; @ bp 387: A17 = G; A20 = T Short sequence repeat marker</td>
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<td>h2_7n20d</td>
<td><a href="http://www.medicago.org/?genome">http://www.medicago.org/?genome</a></td>
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</tbody>
</table>
APPENDIX I

Shoot to root reciprocal grafted plants using inverted-Y grafting approach

Washed reciprocally grafted plants harvested from the soil ready for nodule counting showing large, healthy root systems.
Co-localization of MtRDN1-GFP with organelle specific mCherry tagged markers

Co-localization of MtRDN1-GFP with organelle specific mCherry vacuole marker (A) GFP channel (B) mCherry channel (C) merged image. (D-F) with peroxisome mCherry marker in the same channels (G-I) with late endosome mCherry marker in the same channels. Images obtained with Axiovert 200 M fluorescence microscope (Zeiss). For (A-C) we used 40x oil immersion objective and for (D-I) we used 100x oil immersion objective.
APPENDIX K

Microscopic images of nodules from perlite grown plants

Microscopic images of nodules from perlite grown wild type and AON defective mutants *rdn1-2* and *sunn-4*. Plants were grown in growth room conditions which are two days advanced in nodule development when compared to greenhouse conditions (data not shown). Images were taken 6 days after inoculation 8 days after inoculation. Color change from white to pink is an indication of leghemoglobin synthesis, a marker for active nitrogen fixing nodules.
APPENDIX L

Nodule numbers and fresh weight from perlite grown plants

Nodulation phenotypes of *A17*, *rdn1-2* and *sunn-4* mutants 15 days after inoculation with rhizobia in perlite system under greenhouse conditions. (A) Mean of combined total nodule and nodule primoridia number (5-6) (B) Mean of nitrogen fixing nodules only (counted based on the pink color (leghemoglobin)). (C) Nodule fresh weight averaged from 10 samples (3 nodules together weighted as one sample) for each genotype. Error bars are standard error of the mean.
APPENDIX M

Genetic interaction between SUNN and RDN1

Comparison of nodule number for wild type, single and double mutants of sunn and rdn1 alleles. Mean nodule number 10 days post inoculation on A17, rdn1-1, rdn1-2, sunn-1, sunn-4, sunn-1/rdn1-1 and sunn-4/rdn1-2 (n=3-6 plants per genotype). Bars are standard error of the mean.
Amino acid sequence alignment using N-terminal regions of RDN proteins from *M. truncatula*, *P. trichocarpa*, *O. sativa*, and *A. thaliana*. Colors indicate similarity (A) The signal peptide region together with the overlapping transmembrane domain. (B) The N-terminal region immediately next to the transmembrane domain. Alignments were made using Clustal W2.
MtRDN3 does not rescue the *Mtrdn1*-2 mutation

*Agrobacterium* mediated hairy root transformation performed to complement *Mtrdn1*-2 mutation using *MtRDN3* driven by the CaMV 35S promoter. (A) Average nodule number in plants transformed with an empty vector (EV) or *MtRDN3*. Bars indicate standard error of the mean n= 4-9 plants per genotype. (B) Representative images of transgenic nodulating root transformed with *MtRDN3*. (left) DsRed transformation marker, (right) Bright field image.
APPENDIX P

The gene structure of AtRDN3 (AT5G25265) and AtRDN (AT2G25260) indicating the T-DNA insertions sites.

Yellow triangles indicate the insertion sites. The gene structure was derived using PLAZA, a resource for plant comparative genomics (van Bel et al 2012).
APPENDIX Q

Pairwise Amino acid sequence identity among the RDN proteins

<table>
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<th>SeqA</th>
<th>Name</th>
<th>Length</th>
<th>SeqB</th>
<th>Name</th>
<th>Length</th>
<th>Score</th>
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<tbody>
<tr>
<td>1</td>
<td>MtRDN1</td>
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<td>2</td>
<td>MtRDN2</td>
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<td>351</td>
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