The lss supernodulation mutant in Medicago truncatula: Genetics, Characterization and Mapping

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THE \textit{lss} SUPERNODULATION MUTANT IN MEDICAGO TRUNCATULA: GENETICS, CHARACTERIZATION AND MAPPING

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Genetics

by
Arijit Mukherjee
December, 2007

Accepted by:
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ABSTRACT

The symbiotic association between legumes and nitrogen fixing rhizobia culminates in the formation of root nodules. Legumes control the extent of root nodule formation through a regulatory mechanism in which nodulating roots induce a shoot response that signals back to the roots, inhibiting further nodule initiation. Disruption of genes involved in this regulatory process lead to super or hypernodulation. The lss (like sunn supernodulator) mutant of Medicago truncatula has been identified as a naturally occurring supernodulation mutation in the Jemalong cultivar. The phenotype of lss is practically indistinguishable from the phenotype of the previously isolated sunn mutant with respect to root length, nodule number and shoot control of the phenotype. However, lss and sunn plants nodulate differently in the presence of nitrogen, endogeneous auxin (IAA) or ethylene. SUNN gene expression is also altered in lss shoots versus sunn shoots. F1 analysis of progeny from lss crossed with sunn demonstrated that the two mutations do not complement each other and the nodule and root phenotypes of the F1 are indistinguishable from either sunn or lss. Because, sequencing of the SUNN gene, the RLP1 gene and the region between them showed no mutations, the lss phenotype probably does not result from some lesion in this approximately 12 kb region. F2 plants from the lss/sunn cross segregated wild type plants in a ratio suggesting separate but linked genes, confirmed from mapping of the locus through a separate cross to the polymorphic ecotype A20. Analysis of segregating CAPS markers in the F2 from this cross determined that lss maps to an 800 kb area at the bottom of LG4, which also contains sunn. Methylation analysis by bisulfite sequencing of the SUNN promoter and
3’UTR in shoots and roots of *lss* showed no significant differential methylation from wild type to account for the *lss* supernodulation phenotype.
DEDICATION

I would like to dedicate this dissertation to my family. My grandparents, Amiya Kumar Mukherjee and Geeta Mukherjee, Nanda Dulal Goswami and Sudha Goswami. Thank you for your love and unending blessings. My parents, Arup Kumar Mukherjee and Namita Mukherjee. Thank you for your love, support and guidance. My brother, Anirban Mukherjee. Thank you for your love. I love you all. Thank You!
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CHAPTER ONE
LITERATURE REVIEW


Introduction

With the growth of the world population in the last century and its continued expansion in the ensuing future, the availability of food to meet the increasing demand depends to a great extent on nitrogen rich fertilizers. In most ecosystems, availability of nitrogen is the one of the primary restraints on plant production when compared to carbon, hydrogen and oxygen. This is due to the chemical nature of nitrogen. The most abundant form, N\textsubscript{2}, which makes up 78% 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 (Graham and Vance 2003; Smil 1997). Legumes overcome this limitation by forming a symbiosis with bacteria containing an enzyme that can break the triple bond of N\textsubscript{2} and incorporate the element into a more reactive form like ammonia. The culmination of this symbiosis is the formation of root nodules where nitrogen fixation takes place. In free-living bacteria, nitrogen fixation is limited by the presence of carbon. Also, the enzyme that fixes the atmospheric nitrogen,
nitrogenase, is irreversibly poisoned by oxygen. This constraint on nitrogenase activity is overcome in the nodules, where the bacteria get a regulated supply of carbon and the plant maintains a low oxygen tension in the nodules for efficient reduction of nitrogen. Formation of the symbiotically effective nodules depends on a coordinated molecular dialogue between the host plant and the bacteria (see Graham and Vance 2003; Smil 1997).

Some 40-60 million metric tons of N\textsubscript{2} are fixed by agriculturally significant legumes annually and another 3-5 million metric tons are fixed by legumes in natural ecosystems. Legumes are a rich source of proteins and are second only to Graminiae in their importance to human nutrition. They account for 27% of the world’s primary crop production (Graham and Vance 2003). Besides these food uses, legumes have immense significance in agro-forestry, maintenance of a balance in natural ecosystems, and several other industrial uses especially in the pharmaceutical and nutraceutical sectors (Morris 2005).

**Nodulation**

The nodule is the culmination of the symbiotic relationship between the host legume plant and nitrogen-fixing bacteria. The development of nodules starts with a signal exchange between the rhizobia and the legume root, followed by two processes in the plant root. In the first, the infection process, bacteria attach to susceptible root hairs and enter the host plant via a tube-like structure called an infection thread. In addition to root hair entry, bacteria can enter the plant through wounds in the epidermis or spaces...
between epidermal cells at lateral root junctions, although these are less common. The second process, nodule initiation, is characterized by cortical cell division leading to the formation of the nodule meristem and nodule primordium. The infection thread housing the bacteria (described below) makes its way through this actively dividing zone of cells. Finally, the bacteria are released into the plant cytoplasm in membrane bound symbiosomes inside the nodule (Hirsch 1992).

Initial signal exchange

Nodulation is a highly host-specific process, whereby specific rhizobial strains infect only certain legumes. The majority of rhizobia nodulate taxonomically defined plant groups, however, exceptions do exist. For example, *Rhizobium meliloti* infections are restricted only to species of *Medicago*, *Melilotus* and *Trigonella*, whereas *Rhizobium sp.* strain NGR234 nodulates a variety of legumes, ranging from perennials to annuals (Hirsch 1992; Pueppke and Broughton 1999).

The pre-infection stage begins with the host plant secreting substances such as flavonoids and betaines that serve as chemo-attractants of *Rhizobium* and *Bradyrhizobium spp* (Bais et al. 2006). These substances also induce *Rhizobium nod* genes, which are otherwise not expressed or expressed at very low levels in the free-living bacteria. Upon recognition, the flavonoids are bound by the bacterial NodD protein, which in turn induces the transcription of other bacterial nodulation genes involved in the synthesis of Nod factors (Bladergroen and Spaink 1998; Hirsch 1992).
Nod factors are the key bacterial signal molecules required for nodulation. The
Nod factors produced by different rhizobial species have a common basic structure. They
are lipo-chitooligosaccharides (LCOs), consisting of four to five
\( \beta-1,4 \)-linked \( N \)-acetyl-D-glucosamine residues, in which the \( N \)-acetyl group of the non-
reducing terminal sugar is replaced by an acyl chain. The proteins encoded by the
bacterial genes \( nodA \), \( nodB \), and \( nodC \) are involved in the synthesis of the LCO backbone
and these are common to all rhizobia (Cohn et al. 1998; Mylona et al. 1995). The
remaining Nod proteins are responsible for modifications of the Nod factor, and the types
of host plants nodulated by a given rhizobial species depends to some extent on the
chemical modification of the Nod factors (Dénarié and Debelle 1996). The LCO can be
modified by attachment of a sulphate, acetate or carbamoyl group(s), changes to the acyl
chain, addition of polysaccharides like arabinose, mannose or fucose and changes in the
chitin oligomer length. These modifications play a role in the ability of the bacterium to
interact with its host plant. For example, the \( O \)-acetate group as well as the structure of
the acyl chain is important for efficient infection (Downie and Walker 1999; Mylona et al.

Nod factors are essential for the induction of most symbiotic responses in the host
legume plant. Nod factors can induce a variety of effects including deformation of root
hairs, depolarization of the membrane potential in epidermal cells, changes in the calcium
flux in root hairs and the induction of early nodulin genes. The responses triggered by
purified Nod factor in root hairs precede the known morphological responses by hours or
days.
Nod factor perception and downstream physiological and morphological events

Root hair deformation

When rhizobia infect legume roots, they induce deformation and curling of root hairs and expression of several plant genes in epidermal cells. Rhizobia attach to root hairs all over the root, but the hairs that are most susceptible to rhizobial infection are recently emerged, young hairs in the elongation zone of the root. Rhizobia attach to susceptible root hairs in two steps. First, they loosely attach to a plant receptor through a calcium-binding bacterial surface protein known as rhicadesin. This is followed by a tight binding step mediated by cellulose fibrils or fimbriae (Hirsch 1992).

Entry of bacteria into the root hair occurs at the tip, where the cell wall is thinnest and more permeable than elsewhere (Bhuvaneswari et al. 1981). The process of root hair deformation varies from host to host, but in general root hairs deform within 3-18 hours after inoculation. In susceptible root hairs this morphological change is preceded by a depolarization of the plasma membrane, changes in calcium flux, proton efflux and rearrangements of actin filaments. This series of events eventually leads to root hair deformation and occurs within five to thirty minutes after inoculation with rhizobia (Stougaard 2000). The susceptible root hairs undergo several deformations assuming unusual shapes such as corkscrews, branches, twists and spirals. Some of these deformed root hairs coil 360° into curls referred to as shepherd’s crooks.
Calcium flux/spiking

Calcium, an important regulator of cellular activity in plants is implicated as a secondary messenger in plant-cell signal transduction. External stimuli like depolarization of root hair transmembrane potential, touch, pathogen elicitors, auxin and other stimuli have been shown to induce increases in free calcium (Sanders et al. 2002). Perception of Nod factors by the plant stimulates several physiological changes that play a role in nodule formation, including an increase in cellular calcium concentration.

Within the first few minutes of stimulation by Nod factors, there is a rapid influx of Ca\(^{2+}\), followed by a Cl\(^-\) efflux, and then efflux of K\(^+\) to balance the charge. This exchange of ions leads to alkalinization of the cytoplasm. The calcium influx triggers the activation channel that leads to the K\(^+\)/Cl\(^-\) movement (Downie and Walker 1999). This K\(^+\)/Cl\(^-\) movement, coupled with the movement of H\(^+\) ions, is responsible for the membrane depolarization. The Ca\(^{2+}\) flux is induced at the tip of root hair cells (Ehrhardt et al. 1996). The Nod factor induced Ca\(^{2+}\) flux has been observed in a variety of legumes including *Pisum sativum*, *Medicago sativum*, *Medicago truncatula* and *Phaseolus vulgaris* (Oldroyd and Downie 2004).

A few minutes after the initial Ca\(^{2+}\) flux, a wave of Ca\(^{2+}\) “spiking” occurs, in which the calcium ion concentration inside the cell rapidly oscillate up and down (Ehrhardt et al. 1996). Cytoplasmic oscillations of calcium, or calcium spiking, have been documented in root hairs of *M. sativa*, *M. truncatula*, *P. sativum* and *L. japonicus* (Cardenas et al. 1999; Ehrhardt et al. 1996; Shaw and Long 2003; Walker et al. 2000). This response appears to be common among legume hosts and is induced by very low
(picomolar) concentrations of Nod factor and are mostly observed in region of the cytosol associated with the nucleus (Felle et al. 1998). The spikes are characterized by a rapid increase in \( \text{Ca}^{2+} \) followed by a gradual decline. Some studies have shown that \( \text{Ca}^{2+} \) flux and spiking can take place independent of each other (Shaw and Long 2003). Also, the amount of Nod factor required for \( \text{Ca}^{2+} \) flux is much higher than that required for the induction of root hair deformation, \( \text{Ca}^{2+} \) spiking and downstream gene expression (Shaw and Long 2003). However, \( \text{Ca}^{2+} \) spiking can sustain itself at very low Nod factor concentrations, suggesting that the flux might not be as important for the downstream events as the \( \text{Ca}^{2+} \) spiking signal (Felle et al. 1999; Pingret et al. 1998). The information encoded by the calcium oscillatory frequency is suspected to be decoded by a protein that adjusts its activity based on the calcium oscillations, and the recently cloned \textit{DMI3} gene, described below, is a good candidate for that function.

\textit{Molecular Nod factor perception and downstream transcription events}

The initial morphological and physiological changes observed in symbiosis are the result of an exchange of molecular signals between the rhizobia and the host legume plant. Understanding how the plant perceives the bacterial signal, Nod factor, and transmits this signal to activate downstream events leading to infection and nodule organogenesis is fundamental to the understanding of the nodulation process. The development of legume model systems such as \textit{M. truncatula} and \textit{L. japonicus} resulted in the cloning of a number of genes identified through mutation as being involved in
nodulation, and this information has lent new insight into the molecular dialogue (Barker et al. 1990; Cook 1999; Handberg and Stougaard 1992).

Kawaguchi et al., (Kawaguchi et al. 2002) proposed that the Nod` phenotypes can be due to a mutation in Nod-factor signaling. Mitra et al., (Mitra et al. 2004b) showed that six out of the seven Nod M. truncatula mutants (nfp, dmi1, dmi2, dmi3, nsp1, nsp2) showed no transcriptional response to S. meliloti, implying that the encoded proteins are required for new transcription. The Nod` mutant hcl (Catoira et al. 2001) however, exhibits reduced transcriptional response to S. meliloti (Mitra et al. 2004b). Legume mutants that have lost all Nod factor responses or show different Nod-factor perception have been identified in Pisum sativum (PsSYM10), M. truncatula (MtNFP) and L. japonicus (LjNFR1 and LjNFR5) (Amor et al. 2003; Madsen et al. 2003; Radutoiu et al. 2003; Walker et al. 2000). The gene products encoded by LjNFR1 and LjNFR5 are proposed to play a critical role in Nod-factor perception. PsSYM10 and MtNFP are the putative orthologs of LjNFR5 in P. sativum and M. truncatula respectively (Madsen et al. 2003; Radutoiu et al. 2003). The Medicago truncatula HCL gene, the putative Medicago ortholog of NFR1, encodes LYK3, and is proposed to function as an entry receptor controlling infection (Smit et al. 2007). All of these genes encode receptor-like kinases with LysM domains in the extracellular portion of the protein, which might be involved in the binding of β-1,4-linked N-acetyl-D-glucosamine residues, and a kinase domain probably involved in signal transduction (Radutoiu et al. 2003). Although several factors point towards the LysM-receptor-like kinases as the receptors for Nod factor, biochemical proof is still lacking.
Other components of the Nod factor signaling that function downstream of the Nod factor receptors have been identified. The *dmi1*, *dmi2*, and *dmi3* (doesn’t make infections) mutants of *M. truncatula* are impaired in the early nodulation steps of root hair curling or infection (Catoira et al. 2000). In these mutants, Nod factor is perceived but the signal transduction is blocked. Orthologs of these genes have been found in other legume species. For example, *Pollox* and *Castor* in *L. japonicus* are orthologs of *MtDMI1*; *LjSYMRK* and *PsSYM19* are orthologs of *MtDMI2* in *L. japonicus* and *P. sativum* respectively; and *PsSYM9* in *P. sativum* is an ortholog of *MtDMI3* (Imaizumi-Anraku et al. 2005; Schneider et al. 2002; Schneider et al. 1999). The *MtDMI1* gene encodes a nuclear membrane protein with similarities to *Methanobacterium thermoautotrophicum* potassium channel (MthK) (Ane et al. 2004; Peiter et al. 2007), the *MtDMI2* gene encodes a leucine rich repeat receptor-like kinase (LRR-RLK) (Endre et al. 2002), and *MtDMI3* gene encodes a calcium and calmodulin-dependent protein kinase (Levy et al. 2004; Mitra et al. 2004a) which is downstream of the calcium spiking signal (Wais et al. 2000). Analysis of calcium responses in these mutants have shown that in *Mtdmi1*, *Mtdmi2*, *Pssym8* and *Pssym19* mutants, calcium spiking is blocked, whereas, *Mtdmi3* and *Pssym9* mutants show normal calcium spiking in presence of Nod factor (Catoira et al. 2000; Stracke et al. 2002; Wais et al. 2000). Thus, *MtDMI1* and *MtDMI2* and their orthologs act upstream of calcium spiking, whereas, *MtDMI3/PsSYM9* act downstream of calcium spiking suggesting the calcium/calmodulin-dependent protein kinases these genes encode perceive and transduce the calcium spiking signal (Levy et al. 2004; Mitra et al. 2004b).
Acting further downstream of the *MtDMI3* gene in Nod factor signal transduction are the *MtNSP1* and *MtNSP2* genes. In the *MtNSP* mutants, Nod factor induced gene expression is reduced, root hair deformation is modified and nodule initiation and infection are blocked. Calcium flux and calcium spiking are normal in these mutants (Catoira et al. 2000; Oldroyd and Long 2003). Thus, these genes act downstream of calcium spiking, but upstream of gene expression and cortical cell division. Both these genes have been shown to encode putative transcriptional regulators of the GRAS family (Kalo et al. 2005; Smit et al. 2005).

Also acting downstream of calcium spiking, the *LjNIN* gene and its orthologs *PsSYM35* and *MtNIN* might play a role in the final stages of Nod factor signaling by activating gene expression through induction of transcription factors (Borisov et al. 2003; Schauser et al. 1999). The *nin* mutants are characterized by excessive root hair curling and inability to form infection threads and nodule primordial. The *NIN* gene encodes a putative transcription factor (Schauser et al. 1999). It has been cloned recently and has been proposed to function downstream of early Nod Factor signaling pathway to coordinate and regulate the correct temporal and spatial formation of root nodules (Marsh et al. 2007).

**Nodule Development**

**Cortical cell divisions**

Following the preinfection stages, cortical cell divisions mark the initiation of nodule formation. Cortical cell divisions occur either in the inner or outer cortex of the
root depending on species, and the anticlinal divisions initiate the nodule formation. Nodules can be grouped into two broad categories, determinate and the indeterminate, based on characteristics of the meristem. Which type of nodule forms is dependent on the host legume plant and is dictated by the location of the initial cell divisions (Hirsch 1992).

Indeterminate nodules are characterized by a persistent nodule meristem, which causes them to be elongated and club shaped. Legume plants that form indeterminate nodules include alfalfa (*M. sativa*), barrel medic (*M. truncatula*), pea (*P. sativum*), clover (*Trifolium sp.*), and vetch. An indeterminate nodule is initiated by anticlinal cell divisions in the inner cortex of the root. The dividing cortical cells, which are separated from the root hair containing an infection thread by several layers, eventually gives rise to the nodule primordium. The cells adjoining the nodule primordium near the middle of the root cortex divide to form the nodule meristem. During the cortical cell division, the infection thread (described below) grows and leaves the root hair cell before penetrating the cortical cells (Newcomb 1981).

Before the infection thread penetration, a cytoplasmic bridge is formed in the outer cortical cells, which determines the path of the advancing infection thread. The infection thread makes its way through these cells into the inner cortical cells that comprise the nodule primordium. Finally, the infection thread invades the cells of the nodule primordium and these cells stop dividing and start differentiating. The infection threads then extend into the nodule meristem. The rhizobia which have been growing and dividing in the infection thread are released from the infection threads into the nodule
meristem cells. Rhizobia are released from wall-less branches of the infection thread, termed infection droplets, into the plant cytoplasm and the released bacteria are enclosed by a plant-derived membrane, known as the peribacteroid membrane via an endocytosis-like process (Hirsch 1992).

In contrast, determinate nodules are characterized by the lack of a persistent nodule meristem and are spherical in shape. Unlike the indeterminate nodules, cell divisions stop early in nodule development and the final form of the nodule is a result of cell enlargement rather than cell division (Newcomb et al. 1979). *L. japonicus*, soybean (*Glycine max*), and common bean (*Phaseolus vulgaris*) are examples of legumes that form determinate nodules. The rhizobial infection leads to anticlinal and hypodermal cell divisions, followed by cell divisions in the pericycle and the inner cortex, which are far away from the initial divisions. The two meristematically active regions, the outer and inner cortex, merge together and form the nodule (Hirsch 1992).

**Infection thread formation**

The initiation of the infection thread is triggered by several bacterial factors including exo-polysaccharides and lipo-polysaccharides. The infection thread is proposed to be a continuation of the plant cell wall. Rhizobial dissolution of the cell wall at a specific place (Callaham and Torrey 1981) and cell wall invaginations are some of the cell wall modifications proposed by different research groups (Nutman 1956). Once the rhizobia attach to the root hair, distinct cytoplasmic streaming occurs in response. The plasma membrane of the root hair invaginates and cell wall material is deposited around
the invagination and the rhizobia within. The newly formed cell wall, with the rhizobia inside is the infection thread. The internal space of the infection thread contains a matrix which is synthesized by both the plant and the rhizobia (Kijne 1992). The rhizobia travel from host cell to host cell via the infection thread and its branches before being released as membrane bound symbiosomes (Hirsch 1992). Brewin (Brewin 1991) suggested that infection threads are essentially intercellular symbiotic compartments.

Although many root hairs undergo deformation, not all of them form the familiar shepherd’s crook structure leading to infection threads and not all the infection events lead to N₂-fixing nodules. In fact the frequency of successful development all the way to symbiosomes is low, and most of the infection threads abort either in the root hair cells or in the root cortex (Penmetsa and Cook 1997). Studies have indicated that cells containing aborted infection threads and their adjoining cells have a high concentration of phenolic compounds in their cell walls. The infection thread abortion is associated with a hypersensitive-HR like response by the host legume plant (Hirsch 1992).

**Regulation of cortical cell division**

Some mutants in alfalfa, *L. japonicus* and white clover form spontaneous nodules even in the absence of rhizobia (Blauenfeldt et al. 1994; Tirichine et al. 2006; Truchet et al. 1989). These findings indicate that the host plant controls the initiation of cortical cell divisions that lead to nodule organogenesis. Spontaneous nodules are characterized by the absence of rhizobia, bacteroids or infection threads, but do show morphological features of rhizobial induced nodulation (Blauenfeldt et al. 1994; Tirichine et al. 2006). Also, pure
Nod factors are able to induce cortical cell divisions and lead to the formation of a complete, but empty nodule (Truchet et al. 1989).

Many plant developmental processes are mediated by phytohormones and nodulation is no exception. Multiple lines of research have shown that phytohormones such as auxin, cytokinin, ethylene, brassinosteroids, abscisic acid and jasmonic acid play a role in nodulation. Since some rhizobia synthesize hormones, a relationship between nodulation and hormones was presumed (Hirsch and Fang 1994). In 1973, Libbenga et al. suggested that gradients of both auxin and cytokinin are required for cortical cell divisions and nodule initiation (Libbenga et al. 1973). Caba et al. showed that the auxin:cytokinin ratio was lowered in hypernodulating mutant nts386 in soybean suggesting a role for this gradient (Caba et al. 1998). Synthetic polar auxin transport inhibitors have also been shown to induce pseudo-nodules and induce nodulin genes like ENOD2, ENOD12 (Hirsch et al. 1989b).

Auxin was suggested to play a role in nodule initiation and development as early as 1936 when Thimann observed elevated levels of auxin in pea nodules (Thimann 1936a) and many lines of evidence support Thimann’s postulation of a role for auxin. Specifically, nodules contain increased levels of auxin (Badenoch-Jones et al. 1984), the auxin responsive promoter (GH3) is expressed differentially during nodule initiation (Mathesius et al. 1998b), rhizobia inhibit auxin transport (Boot et al. 1999), AUXI-like auxin transport proteins (LAX genes) are expressed in M. truncatula developing nodule primordia (de Billy et al. 2001), and reduction of auxin efflux carrier gene expression (PIN genes) reduces nodule number (Huo et al. 2006).
Boot et al. (1999) showed that rhizobia manipulate auxin transport and thus alter the auxin:cytokinin ratio in the root (Boot et al. 1999). Mathesius et al. (1998) showed that in white clover the expression of the auxin responsive promoter $GH3$ was reduced below the site of inoculation between 12-24 hrs after spot inoculation with rhizobia or Nod factor. Between 24-48 hrs, there followed an increase in $GH3$ expression levels at the site of nodule initiation, leading to the proposal that auxin transport inhibition in the early stages of nodule development lowers the auxin:cytokinin ratio and triggers cell division. This decrease is proposed to be transient, based on the resumption of $GH3$ expression after 72 hours, presumably increasing auxin flow and suppressing further cell division (Mathesius et al. 1998b). These results were confirmed in $M. truncatula$ (Huo et al. 2006).

Mathesius et al. (1998) also showed that flavonoids are often located in the dividing and meristematic tissues of nodules (Mathesius et al. 1998a). Flavonoids can regulate auxin transport and therefore indirectly regulate cortical cell division by controlling auxin transport or accumulation (Brown et al. 2001). Wasson et al. (2006) have shown that silencing of the flavonoid pathway in $M. truncatula$ inhibits root nodule formation and affects auxin transport regulation by rhizobia (Wasson et al. 2006). In soybean, isoflavanones are essential for establishment of the symbiosis (Subramanian et al. 2006). Zhang et al. (Zhang et al. 2007) have shown that flavones are important for nodulation in $Medicago truncatula$. Thus, flavonoids and related compounds could play a role in regulation of cortical cell division, but more experimentation is needed. A further
role for auxin transport in nodule development at the level of the whole plant is discussed in the section on the regulation of nodulation.

Cytokinins are also important for new cortical cell divisions in nodulation. Several lines of evidence suggest that rhizobia alter the cytokinin levels in the root (Badenoch-Jones et al. 1987). Cytokinin can induce nodule initiation and the transcription of several early nodulin genes. Hirsch et al. showed that application of cytokinins in a rhizobia and Nod factor resistant mutant overcomes the mutant’s inability to nodulate (Hirsch et al. 1997). A reporter gene fused to the *Arabidopsis* cytokinin response regulator gene promoter *ARR5* is induced in nodule primordia (Lohar et al. 2004). Cooper and Long showed that synthesis of the cytokinin zeatin in a nodulation deficient mutant of *S. meliloti* is sufficient to induce nodule-like structures in alfalfa (Cooper and Long 1994). They did this by transferring the *Agrobacterium* trans-zeatin secretion gene into nodulation deficient mutants of *S. meliloti*, however the exact concentration of cytokinin was unknown and could be significant in determining if there is a stimulating or inhibiting effect on nodulation (Lorteau et al. 2001). Wopereis et al. showed that the *har1* mutant’s short root could be phenocopied in wild type plants by the application of cytokinin (Wopereis et al. 2000a). Recent further evidence that cytokinins play a role in nodule development comes from the discovery that a gain of function mutation in a cytokinin receptor gene in *L. japonicus* (*HIT1*) leads to the formation of spontaneous nodules in the absence of rhizobia while loss of function mutations in this same gene suppresses the hypernodulation phenotype of *har1* mutants and demonstrates the requirement of *HIT1* for the activation inception of regulator *Nin* and nodule
organogenesis (Murray et al. 2007; Tirichine et al. 2007). Recently, Gonzalez-Ricco et al., have shown that MtCRE1, a homologue of an Arabidopsis cytokinin receptor, is responsible for regulation of secondary root organogenesis. It has positive effect on nodule development and a negative impact on lateral root formation. Their findings also suggest the existence of a crosstalk between cytokinin and Nod factor signaling pathways (Gonzalez-Rizzo et al. 2006). Abscisic acid has also been shown to act in concert with cytokinins to affect root/shoot signaling (Davis and Zhang 1991). Several studies have indicated ABA suppresses nodule number (Nakatsukasa-Akune et al. 2005; Suzuki et al. 2004).

A number of mutants with defects in cell division and rhizobial release during nodule development have been isolated. For example, the *M. truncatula nip* (numerous infections and polyphenolics) mutant is characterized by small bump-like nodules on its roots that are blocked at an early stage of development and abnormal proliferation of infection threads, as well as indications of a host defense-like response. It has been speculated that *NIP* may have a regulatory role in root and nodule development (Veereshlingam et al. 2004). The *M. truncatula sli* (sluggish infections) mutant also undergoes aberrant nodulation and develops nodule primordia with rhizobia within infection threads and exhibit evidence of host defense response like the *nip* mutant (Dickstein, R., unpublished results). Cloning of mutants like these will shed light on the regulation of cell division in root nodule development.
**Nodule position and relation to lateral roots**

Legume nodules most frequently arise opposite protoxylem poles; in addition to originating opposite the phloem or between the xylem and phloem. This is very similar to the origin and distribution of lateral roots which, like determinate nodules, have an endogenous origin and usually develop from periclinal divisions of the pericycle cells (Dubrovsky et al. 2000). However, Libbenga et al. (Libbenga and Bogers 1974) showed that the site of origin of nodule primordia was different from that of lateral root primordia in indeterminate nodules and also that nodules differ from lateral roots physiologically. Indeterminate nodules lack a root cap which is present in lateral roots and also exhibit a peripheral arrangement of vascular tissues as opposed to a central arrangement in lateral roots.

There is evidence for uridine and ethylene as regulators of the positioning of root nodules. Smit et al. (Smit et al. 1995) and Libbenga et al. (Libbenga et al. 1973) showed that, in the presence of auxin and cytokinin, a uridine gradient from the protoxylem pole to the outer cortex and phloem poles determines the site of initial cell division. Ethylene regulation has also been demonstrated. Penmetsa and Cook showed that the ethylene insensitive *M. truncatula sickle* mutant is characterized by a random distribution of the nodules, versus location across from the xylem poles (Penmetsa and Cook 1997) and Heidstra, et al. showed that ACC oxidase, the enzyme that generates ethylene, is expressed in the cortical cells between xylem poles (Heidstra et al. 1997), suggesting ethylene prevents cell division from occurring between xylem poles and forces the location of the nodules to their normal sites across from the xylem poles.
The *M. truncatula LATD* gene is speculated to play a role in nodule and root (lateral) development (Bright et al. 2005). The *latd* mutant initiate nodule formation but do not complete it resulting in the formation of immature, non-nitrogen fixing nodules. Similarly lateral roots initiate but remain as short stumps. The primary root gradually ceases growth and forms an abnormal tip that resembles the mutant lateral root (Bright et al. 2005). The lateral root phenotype but not the nodule phenotype of these mutants can be rescued by application of ABA (Liang et al. 2007).

**Establishment of nitrogen fixation**

The rhizobium-legume symbiosis culminates in the formation of root nodules, whereby the bacteria within the nodule reduces the molecular nitrogen for the plant and the plant reciprocates by providing the bacteria with carbon-containing compounds. This is coordinated by multiple signals that are derived from both the host plant and the symbiotic bacteria. Therefore, if the bacteria fail to import dicarboxylic acid, they are unable to fix nitrogen inside the nodules (Udvardi et al. 1988). Several studies have looked into the factors essential for the final stages of the symbiotic process that support active nitrogen fixation by the bacteria. Genetic screens have identified plant symbiotic mutants that are either unable to initiate nodule development (*Nod*´) or are unable to support nitrogen fixation (*Fix*´). *Fix*´ mutants stand in contrast to the previously described *Nod*´ mutants that are characterized by absence of nodulation because *Fix*´ mutants respond to Nod factor and cells divide and differentiate to become nodules, but nitrogen fixation is not established.
Fix\(^-\) mutants have been identified in both *L. japonicus* and *M. truncatula* (Kawaguchi et al. 2002; Starker et al. 2006; Szczyglowski et al. 1998). These mutants form white, mostly ineffective nodules. Since, the Fix\(^-\) mutants are unable to support nitrogen fixation, in *M. truncatula* they are termed *defective in nitrogen fixation* (*dnf*) mutants (Starker et al. 2006). Starker et al. divided the *dnf* mutants into three groups based on their degree of inability to fix nitrogen and gene expression. All the *dnf* mutants, however, allow the initial events in nodulation that leads to the formation of the nodule primordium, nodule and cellular infection by the bacteria suggesting that these genes may be significant for symbiotic nitrogen fixation (Starker et al. 2006).

**Nodule number regulation**

It has been known for some time that nodule formation is inhibited by the availability of nitrogen in the form of nitrates or ammonium. The inhibitory effects of nitrate range from early nodulation events, such as root hair deformation, binding of the bacteria to the root hairs and growth of infection threads, to nodule primordium initiation (Streeter 1988). Some other secondary effects of nitrate include inhibition of nodulation by induction of ethylene (Caba et al. 1998), altering levels of flavonoids (Coronado et al. 1995) and phytohormones like auxin and cytokinins (Mathesius et al. 2000).

**Long distance signaling controlling nodule number**

Once nodules are formed, the rhizobia differentiate and convert atmospheric N\(_2\) into NH\(_3\), and supply the host legume with fixed nitrogen. In return the plant incurs a
metabolic cost in the form of carbon skeletons and nutrients supplied to the rhizobia across the peribacteroid membrane. The biological cost to the plant of nodule establishment and nitrogen transport is six grams of carbon in the form of dicarboxylic acids per gram of nitrogen it receives from the bacteria (Vance and Heichel 1991). In order to keep this metabolic cost down, the host legume plants possess mechanisms to not only form nodules, but also to avoid excessive nodulation, which might otherwise impair plant growth. Earlier nodulation events, or established nodules, suppress the formation of new nodules via a systemic feedback regulation known as ‘autoregulation of nodulation’ (AON) (Caetano-Anolles and Gresshoff 1991).

AON controls nodule number by a feedback mechanism that inhibits emergence of new nodules in the younger parts of the root system once an optimum number of nodules are formed (Pierce and Bauer 1983). Split-root experiments, in which the primary root of a plant is branched into two roots that can then be exposed to different treatments, have suggested that there exists a homeostatic control of nodule number throughout the root system. The inoculation of one side of the root system suppressed nodulation of the second side, after a delayed inoculation (Koslack and Bohlool 1984). The systemic nature of the AON is supported by the isolation of multiple mutants in which the inhibition of excessive nodulation is impaired. These mutants are characterized by the formation of greater number of nodules when compared to their wild type counterparts. A number of such mutants have been reported in soybean, pea, L. japonicus and M. truncatula termed super- or hyper- nodulating mutants.
The first discovered in this category of super-nodulating mutants are the soybean nitrogen tolerant symbiosis (nts) mutants, which develop large number of nodules even under high concentrations of nitrogen. (Carroll et al. 1985; Carroll et al. 1985a). The nts mutants were followed by supernodulating mutants in other legumes like sym29 in pea (Sagan and Duc 1996), har1 in L. japonicus (Szczyglowski et al. 1998; Wopereis et al. 2000a) and skl and sunn in M. truncatula (Penmetsa and Cook 1997; Penmetsa et al. 2003).

The ethylene-insensitive M. truncatula mutant sickle (skl) is characterized by increased nodule number but only in a limited area of the root. Thus in skl mutants, spatial distribution of nodules is impaired (Penmetsa and Cook 1997). Grafting experiments has shown that the supernodulation phenotype of skl mutant is root controlled (Prayitno et al. 2006). Also, comparative work with the sunn mutant has shown that skl and sunn function in different pathways (Penmetsa et al. 2003).

Grafting experiments with supernodulation mutants have shown that AON is mediated by a signal transduction cascade that involves both the root and the shoot. AON can even function in some cross-species grafts (Lohar and VandenBosch 2005). The har1/sym29/nts1/sunn, klavier, sym28, and nod4 are shoot-regulated autoregulation mutants, meaning that the genotype of the shoot graft determines the number of nodules that form on the roots (Delves et al. 1986; Krusell et al. 2002; Penmetsa et al. 2003). This suggests these mutants are impaired in the long distance shoot-to-root autoregulatory mechanism. On the other hand, nod3 and skl are root-regulated autoregulation mutants (Jacobsen and Feenstra 1984; Postma et al. 1988; Prayitno et al. 2006). These findings
imply that in the shoot-regulated mutants the root derived autoregulation signal is either not perceived and/or the shoot-derived autoregulation signal is not transmitted. Similarly, in the root-regulated mutants either the root derived autoregulation signal is not transmitted and/or the shoot derived signal is not perceived. Cloning and identifying these and other legume genes will be instrumental in developing a working model for such a long distance signaling roots and shoots.

*HAR1* and *SYM29* encode receptor-like kinase (RLK) proteins that contains leucine-rich repeats (LRRs), a single transmembrane domain and a serine/threonine kinase domain (Krusell et al. 2002; Nishimura et al. 2002a). The *nts* mutants also result from a mutation in the *HAR1* ortholog, termed *GmNARK* (*nodule autoregulation receptor kinase*) (Searle et al. 2003) as does *MtSUNN* (Schnabel et al. 2005). These four legume genes share a high degree of sequence similarity with the *Arabidopsis CLAVATA1* gene, which is involved in Shoot Apical Meristem (SAM) proliferation and maintenance (Clark et al. 1993). However, the legume supernodulation mutants do not display any of the *clv1*-like fasciated shoot phenotypes. In addition, *CLV1* is specifically expressed in the central region of the SAM (Clark et al. 1993) whereas the legume genes are widely expressed in several tissues but not in the shoot (Searle, 2003; Frugoli et al., unpublished data). These findings imply that although the legume genes encode LRR-RLKs like *CLAVATA1*, their function in nodule number regulation is not related to a role in the SAM. In Arabidopsis, the *CLV1* gene interacts with *CLV2* gene which has a high sequence similarity with *CLV1*, except that it lacks the kinase domain (Kayes and Clark 1998). The *CLV1* gene also interacts genetically with *CLV3*, a short peptide activating
the other downstream components that are involved in the SAM proliferation pathway (Clark 2001). This suggests that identification of other interacting partners of \( GmNARK/MtSUNN/LjHAR1/PsSYM29 \) will be important to gain an understanding of the pathway involved in nodule number regulation in legumes.

The light insensitive mutant \( astray \) in \( L. japonicus \) has twice the number of nodules as wild type plants. The \( ASTRAY \) gene encodes a potential transcriptional activator. The normal nodulation response of the \( astray \) mutant to nitrate and ethylene in contrast to other supernodulation mutants suggests it may be part of a second autoregulation mechanism (Nishimura et al. 2002b). Other supernodulating mutants with impaired AON have been isolated, though in most cases the nature of the molecular lesion is unknown. In \( L. japonicus \) the \( klavier \) mutant has a similar nodulation phenotype to \( har1 \) mutants, with the addition of abnormal leaf veins, delayed flowering and a dwarf phenotype (Oka-Kira et al. 2005). Other important uncloned supernodulation mutants include \( sym28, nodulation \ 4 \ (nod \ 4) \) and \( nodulation \ 3 \ (nod \ 3) \) from pea and \( skl \) from \( M. truncatula \) (Penmetsa and Cook 1997;Postma et al. 1988;Sagan and Duc 1996;Sidorova and Shumnyi 2003).

**Auxin and its role in nodulation**

Auxin is a phytohormone that is involved in several plant developmental processes that include cell division, elongation, differentiation and vascular bundle formation. Auxin is mostly synthesized as a weak acid (\( pK_a = 4.75 \)), indole-3-acetic acid (IAA), in the meristematic regions at the shoot apex and young leaves and is transported
to the roots by an active transport process (Blakeslee et al. 2005). Actively divided young leaves contain the highest concentrations of IAA (Ljung et al. 2001) and regulate the IAA biosynthesis in other parts. However, the auxin synthesized in the aerial parts of the plant is essential for normal plant development (Ljung et al. 2005). The auxin synthesized at these sites is redistributed throughout the plant to regulate a variety of developmental growth responses. Auxin is also synthesized in root tips.

**Polar Auxin transport**

Auxin transport in plants is conducted through both polar and non-polar mechanisms (Morris 2004). Non-polar auxin transport takes place through the phloem along a concentration gradient, where synthesized auxin is loaded into the phloem and passively translocated in the phloem sap to sink tissues (Morris 1996; Raven and Rubery 1982). Polar auxin transport involves complex regulation mechanisms and proceeds in a basipetal direction in the stem and in an acropetal direction in the root. The acropetal transport from the shoot to the root tip proceeds through xylem parenchyma cells and the basipetal transport in the root tip proceeds through the root cap and outer cell layers towards the root elongation zone (Lomax et al. 1995).

The first model for polar auxin transport (PAT) was proposed by Sheldrake and Raven (Raven 1975; Rubery and Sheldrake 1974). Their model is based on the observation that only the protonated form of IAA can move across the plasma membrane whereas the anionic form cannot. According to their model, IAA is mostly present in its protonated form in the acidic apoplast and can enter the cell through diffusion or active
import catalyzed by auxin influx carriers. The higher pH inside the cell causes the IAAH to deprotonate and eventual accumulation of IAA anionic form. The efflux of anions is then mediated by auxin efflux carriers through the plasma membrane. The directional auxin transport is a result of the asymmetric distribution of the auxin efflux carriers (Jones 1998).

**Auxin Influx Carriers**

Extensive research in *PAT* has identified a family of putative auxin influx carriers in *Arabidopsis*. AUX1 and its related LAX proteins are suggested to be the auxin influx carriers playing a role in active cellular auxin uptake (Bennett et al. 1996; Swarup et al. 2000; Yang et al. 2006). The *AUX1* gene encodes a transmembrane protein similar to the amino acid permeases and displays transport activity (Marchant et al. 1999; Swarup et al. 2001). AUX1 functions in a phloem-based auxin transport system in root basipetal auxin transport and in acropetal transport (Swarup et al. 2001). *M. truncatula* and other legumes also have a family of orthologous influx carriers, the *MtLAX* genes (deBilly, et al 2002, Schnabel & Frugoli 2004).

**Auxin Efflux Carriers**

The deprotonated IAA⁻ anion is unable to cross the plasma membrane without the aid of auxin efflux carriers (Delbarre et al. 1998; Rubery and Sheldrake 1974). The *Arabidopsis* family of *PIN* genes, consisting of eight members, has been identified as auxin efflux carriers (Benkova et al. 2003; Friml et al. 2002a; Friml et al. 2002b; Petrasek
et al. 2006). Besides the PIN proteins, certain multidrug resistance-like/P-glycoproteins (PGP) also function as auxin efflux carriers (Noh et al. 2001). The PINs and the PGPs, however, have been shown to function independently of each other as auxin efflux carriers (Geisler et al. 2005; Petrasek et al. 2006).

The PIN proteins contain 8-12 transmembrane domains that have similarity to other membrane transport proteins (Galweiler et al. 1998; Luschnig et al. 1998; Muller et al. 1998). Analysis of PIN function in *Arabidopsis* roots indicate that the PIN proteins function together to regulate auxin transport and the location of the PIN proteins is essential for polarity of auxin flow (Blilou et al. 2005). PIN1 is the primary mediator of auxin transport through vascular tissue to the root tip and PIN3, PIN4 and PIN7 aid this transport in the adjacent tissues in the lower root (Blilou et al. 2005). Once the auxin reaches the root tip, PIN2 redistributes it basipetally through cortical and epidermal cells (Benkova et al. 2003; Blilou et al. 2005). PIN2, PIN3 and PIN7 mediate lateral redirection of basipetally transported auxin back into PIN1-mediated auxin transport stream (Blilou et al. 2005; Friml 2003) and the process is repeated. PIN cycling is inhibited by polar auxin transport inhibitors (PATIs) like *N*-(*1-napthyl)*phthalamic acid (NPA), and 2,3,5-triiodobenzoic acid (TIBA) (Geldner et al. 2001). *M. truncatula* also has ten *PIN* genes, nine of which are expressed in roots (Schnabel & Frugoli 2004)

**Auxin and Nodulation**

Thimann’s postulation in 1936 that IAA is involved in nodule development in peas (Thimann 1936b) suggested the first scientific association of auxin with nodulation.
Root nodules were shown to have higher IAA content than uninfected root tissue. Certain species of alfalfa can make spontaneous nodules, implying that a second signal independent of Nod Factor is capable of inducing spontaneous nodulation (Joshi et al. 1991). Polar auxin transport inhibitors (PATIs) have been shown to cause pseudonodules in alfalfa (Hirsch et al. 1989a) and non-nodulating mutants of white sweet clover (Wu et al. 1996). These findings indicate that auxin could be involved in the early nodule primordial formation process (Hirsch and LaRue 1997) and it could act as a downstream signal for nodule development after the recognition of a rhizobia-derived signal.

Auxin levels have been shown to be elevated in the nodules of a variety of plant species viz., *P. sativum* (Badenoch-Jones et al. 1984), *P. vulgaris* (Federova et al. 2000) and *A. glutinosa* (Wheeler et al. 1979). Increased auxin levels have also been indicated in nodule-like structures of non-legumes after application of synthetic auxin, 2,4-dichlorophenoxyacetic acid (2,4-D) (Ridge et al. 1992).

Boot et al., 1999 showed that endogenous auxin can be transported during nodulation in response to rhizobia. Several groups have shown that in early nodulation events in *Vicia sativa* (vetch) and white clover, auxin transport is inhibited prior to nodulation (Boot et al. 1999; Mathesius et al. 1998b). Polar auxin transport was inhibited in roots during nodulation as with treatment with NPA. Later, Huo et al (Huo et al. 2006) have shown that disruption of expression of *MtPIN1, MtPIN2, MtPIN3* and *MtPIN4* through RNAi led to a reduction in nodule numbers, indicating a role of these auxin efflux carriers in nodule development. Thus, PIN proteins control auxin redistribution in nodule development. Recently, *PIN1* and *PIN2* expression has been shown to be
increased 24 hours post inoculation in the supernodulating skl mutant (Prayitno et al. 2006).

As mentioned previously, the auxin to cytokinin ratio in the roots has been shown to play a role for the initiation of cortical cell divisions and nodule formation (Libbenga et al. 1973). In nts386, the hypernodulating mutant in soybean, the auxin to cytokinin ratio was lower, suggesting the significance of this balance in nodule number regulation (Caba et al. 1998). Several experiments have implied that rhizobia manipulate auxin levels and thereby regulate nodulation. These findings led Gresshoff to propose the ‘auxin burst control’ hypothesis, which states that in soybean, a determinate legume, the nodules that are initiated send a signal to shoot, which leads to a burst of auxin flow from the shoot to the root, leading to the inhibition of nodule formation (Caetano-Anolles and Gresshoff 1991). Ferguson and Mathesius (Ferguson and Mathesius 2003), showed that a local inhibition of auxin transport is necessary for nodule initiation. Work with the supernodulating sunn mutant from Medicago truncatula indicated that the supernodulation phenotype could be due to a defect in auxin transport, based on a defect in auxin flux detected in the sunn mutant (van Noorden et al. 2006). Taking a cue from Gresshoff’s model, VanNoorden, et al., have suggested a model in which a signal is sent to the shoot following nodule initiation. This signal then gets perceived by the LRR-RLK encoded by SUNN/HAR1/PsSYM29/GmNARK in the shoot, and leads to a decrease in auxin flux from the shoot to the root. This reduction in auxin flux prevents further nodule formation, as not enough auxin is present for nodule initiation. In the supernodulation mutant sunn, auxin flux from shoot to root is not reduced and according to this model
nodule number regulation is impaired. In the root controlled supernodulating $skl$ mutant, a different defect in auxin transport is observed (Prayitno et al. 2006), adding weight to this model.

These suggest that there may be two independent auxin transport systems-local auxin transport is necessary for nodule initiation and long distance auxin transport is related to autoregulation of nodulation (Prayitno et al. 2006). Looking at auxin loading patterns in other supernodulating mutants might be useful to establish this model of auxin’s influence on nodule number regulation.

**Auxin and lateral roots**

Auxin’s role in nodulation overlaps with the development of other root structure like lateral roots and root galls. These require similar induction of new cell divisions and differentiation as root nodules. Bhalerao et al., (Bhalerao et al. 2002) showed that auxin is necessary for lateral root induction and Himanen et al., (Himanen et al. 2002) showed that auxin accumulates in lateral root primordia as in root nodules.

Components of polar auxin transport have been shown to play a role in lateral root formation (Casimiro et al. 2001; Reed et al. 1998). For example, AUX1, an auxin influx carrier, has been reported to mediate IAA distribution between source and target tissues and thereby promote lateral roots (Marchant et al. 2002). The $aux1$ mutant in *Arabidopsis* has reduced number of lateral roots (Hobbie and Estelle 1995). Auxin influx carriers also play a role in lateral root formation. *PIN* mutants, $pin3$, $pin1$ and $pin7$, display changes in lateral root numbers (Benkova et al. 2003). $Pin3$ has reduced number of lateral roots;
pin7 has increased numbers and overexpression of pin1 leads to an increase in lateral root numbers (Benkova et al. 2003).

In supernodulation mutants, har1 (L. japonicus) and nts (G. max), there is an increase in lateral root numbers when uninoculated (Searle et al. 2003; Wopereis et al. 2000b). However, in other supernodulation mutants astray (L. japonicus) and sunn (M. truncatula), there is no observed difference in lateral roots. These findings suggest that there might be additional regulators or different signals involved in lateral root formation and nodule formation.

**Ethylene and its role in nodulation**

Ethylene is a gaseous phytohormone that has multiple roles in developmental processes and fitness responses, including germination, flower and leaf senescence, fruit ripening, programmed cell death, responsiveness to stress and pathogen attack and root nodulation (Bleecker and Kende 2000; Johnson and Ecker 1998).

Ethylene is transiently induced by rhizobia during nodule initiation in alfalfa (Ligero et al. 1986) and soybean (Suganuma et al. 1995), but this is not observed in pea (Lee and LaRue 1992a). Billington et al (Billington et al. 1979) suggested that these increases could be due to the triggering of the defense responses emitted by the invading bacteria. However, ethylene mostly functions as a local inhibitor of nodule formation in most legumes. Ethylene or ethylene releasing compounds (for example, ACC, a precursor of ethylene) is inhibitory to nodulation in several species like pea (Drennan and Norton 1972; Lee and LaRue 1992b) and M. truncatula (Penmetsa and Cook 1997). Yet ethylene
fails to alter nodulation in *G. max* (soybean), a determinate nodulator (Schmidt et al. 1999). Thus, indeterminate and determinate nodulators respond differently to ethylene.

Ethylene has been shown to be involved in negatively regulating infection thread growth (Penmetsa and Cook 1997) and also controls the radial positioning of cortical cell division (Heidstra et al. 1997; Penmetsa et al. 2003). The *skl* mutant in *Medicago truncatula* is impaired to ethylene perception and is characterized by reduced leaf and petal senescence, increased root length, sustained infection thread growth, hypernodulation and presence of cell divisions across radial circumference of the root (Penmetsa and Cook 1997; Penmetsa et al. 2003).

The addition of ethylene biosynthesis inhibitors like L-\(\alpha\)-(2-aminoethoxyvinyl)-Gly (AVG) and silver ions (Ag\(^+\)) have been shown to increase nodule number in alfalfa (Caba et al. 1998), pea (Lee and LaRue 1992a) and *Medicago truncatula* (Oldroyd et al. 2001). These findings also suggest that ethylene is involved in inhibiting nodulation in some way.

Several theories behind the mechanisms by which ethylene inhibit nodulation have been postulated. Mellor and Collinge (Mellor and Collinge 1995) suggested that ethylene induces plant chitinases and these in turn are inhibitory to Nod factors, thereby limiting nodulation. Oldroyd et al (Oldroyd et al. 2001) proposed that ethylene’s action was in the early steps of nodulation by inhibiting frequency of calcium spiking. Another possibility is that ethylene is involved in regulation of defense responses during the infection process (Penmetsa and Cook 1997). But as Guinel and Geil (Guinel and Geil 2002) suggested, ethylene is likely to be involved at several stages of nodulation.
**Ethylene and Auxin**

Genetic and physiological studies have indicated that complex interactions among hormonal pathways exist controlling the final developmental outcome. Ethylene and auxin have been shown to synergistically regulate root hair growth and differentiation (Pitts et al. 1998) and root growth (Rahman et al. 2001). One interaction mode occurs at the level of biosynthesis of each hormone by the other. For example, ethylene has been shown to inhibit auxin transport (Burg and Burg 1966; Morgan and Gausman 1966; Suttle 1988) and also auxin biosynthesis (Stepanova et al. 2005). Auxin on the other hand induces ethylene synthesis by upregulation of ACC synthase, the enzyme required for the synthesis of ACC which is a precursor for ethylene production (Abel et al. 1995). Other cases where ethylene and auxin are involved in a crosstalk are apical hook formation (Li et al. 2004), gravitropic bending of roots (Roman et al. 1995) and root growth (Swarup et al. 2002).

The interaction of auxin and ethylene in nodulation was tested in the ethylene-insensitive supernodulation mutant *skl* by Prayitno et al (Prayitno et al. 2006). Their results indicate that both long distance and local auxin transport regulation is altered in *skl* during nodulation. They have proposed that ethylene modulates auxin transport by decreasing auxin accumulation in the nodulation zone by affecting *PIN1* and *PIN2* (auxin efflux carriers) expression. However, the initial local auxin transport inhibition that is necessary for nodule initiation is independent of ethylene signaling. SKL also plays a role in inhibiting long distance auxin transport to the roots in response to rhizobia and
regulates nodule number but this regulation is different from that by *SUNN*, as *SKL* acts in the root and *SUNN* acts in the shoot (Prayitno et al. 2006).

**Epigenetics**

In addition to mutation, gene expression can be changed by epigenetic mechanisms, and some of the data presented later explore the possibility of this event in a nodulation mutant. Nucleotide sequence is not the only determinant for heritable information on the chromosome. Epigenetic information based on DNA methylation or state of the chromosomes is also heritable during cell propagation. Currently epigenetics is defined as, “The study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence” (Russo et al. 1996). Epigenetics lead to altered states of gene expression (Kakutani 2002) and novel phenotypes. The two major types of methylation associated with epigenetics are *DNA methylation* and *Protein methylation*. All four nucleotides can be methylated, but the most prevalent form of DNA methylation is cytosine methylation (Martienssen and Colt 2001). In this process, methyl groups are added to the position 5 of cytosines, catalyzed by cytosine methyltransferases. Several genes have been found in *Arabidopsis* that encode methyltransferases and control the initial establishment and maintenance of the methylation patterns (Martienssen and Colt 2001). Protein methylation is characterized by addition of methyl groups to their lysine and arginine residues. Protein methylation is well represented in the histone proteins. Histone methylation can lead to gene repression or activation of repressed genes (Fischle et al. 2003). Both DNA methylation and histone
methylation can alter chromatin structure and lead to a range of specific epigenetic consequences that can contribute to altered phenotypes, including paramutation (Chandler et al. 2000), gene silencing (Martienssen and Colt 2001) and genomic imprinting (Li 2002). However, histone methylation cannot be stably inherited across generations like DNA methylation (Bird 2002).

**Epigenetics in plants**

Plants have been a great system to study epigenetic inheritance, and several important findings include transposable elements (Comfort 2001), paramutation (Chandler et al. 2000), small interfering RNAs (siRNAs) (Hamilton and Baulcombe 1999) and DNA methylation. In plants, cytosine methylation patterning is the most commonly observed, especially within the trinucleotide motif 5’ CXG 3’ (where G is guanine and X is any nucleotide) and 5’ CG 3’. Assymetric methylation patterns of cytosine residues are also present (Bender 2004). Differences in methylation patterns can lead to altered levels of gene expression and include variation in transcriptional level that ultimately can confer phenotypic effects (Kakutani et al. 1999; Ronemus et al. 1996). Variation in gene expression can cause alterations in chromatin structure and these constitute a mechanism for gene regulation (Johnson et al. 2002). Cytosine hypermethylation usually leads to gene silencing (Martienssen and Colt 2001). Demethylation studies in *Arabidopsis*, caused either by a mutation or chemical induction, have revealed that loss of methylation results in developmental differences like flowering
Alleles that differ from each other in the patterns of methylation of DNA nucleotides of the gene, rather than stable nucleotide mutations are termed as epialleles (Kalisz and Purugganan 2004). Effects of several epialleles have been documented over the years. These epialleles are mostly mitotically and meiotically stable and therefore can be passed on from one generation to the next (Kakutani et al. 1999). Jacobsen and Meyerowitz (Jacobsen and Meyerowitz 1997) were the first to document one such example. They characterized unstable alleles of the SUPERMAN (SUP) gene. SUP encodes a transcriptional activator that determines floral whorl boundaries. Mutant screens identified seven independently isolated stable alleles of this locus, called clark kent (clk). Fine mapping and complementation by SUP transgene indicated that clks were allelic to other sup mutations, however, the clk mutants had a wildtype SUP gene (Jacobsen and Meyerowitz 1997). The clks were later found to be heavily methylated and transcriptionally silenced. The epigenetic state was stable between generations except that the clk mutants sometimes reverted to wild-type at a very low frequency (Jacobsen and Meyerowitz 1997). Similar epigenetic silencing of the SUP gene was observed in met1 mutants and MET1 (Methyltransferase 1) antisense lines. Here, the SUP gene was hypermethylated however, overall DNA methylation level was reduced (Jacobsen et al. 2000). The late flowering FWA homeodomain gene is similar to SUPERMAN. FWA is a monogenic flowering-time loci that was previously found by conventional mutagenesis and linkage analysis (Koornneef et al. 1991). The FWA epiallele confers a late flowering
time phenotype and is a result of demethylation of the FWA promoter (Soppe et al. 2000). Another example of an epiallele affecting developmental pattern is BAL, which was mapped to the clustered disease resistance (Ling et al.) genes (Stokes et al. 2002). The epigenetic overexpression state of the BAL locus reverts at a high frequency to the normal silent state after exposure to EMS or irradiation (Stokes et al. 2002). In these cases of methylation-associated epiallelic inheritance, there is no evidence of gender bias in transmission.

Epigenetic silencing via methylation has been shown to be associated with gene duplications and repeated sequences as well. The PHOSPHORIBOSYLANTHRANILATE ISOMERASE gene family of several Arabidopsis ecotypes includes the unlinked genes (PAI1-PAI3). The PAI2 gene is hypermethylated and silenced in ecotypes that have an inverted duplication of similar sequences PAI1-PAI4 at a different locus (Bender and Fink 1995; Melquist et al. 1999). In ecotypes that lack the duplicated PAI1-PAI4, PAI2 is not methylated.

Naturally occurring epialleles have been found in Linaria vulgaris. The epialleles of the CYCLOIDEA gene render a radially symmetric floral phenotype as opposed to the bilaterally symmetric flowers in the wild types (Kakutani 2002). Naturally occurring epialleles are also found in Zea mays, where the epialleles are found in the R, B, P1 and P pigmentation genes that cause altered seed and vegetative tissue pigmentation (Chandler et al. 2000).
Scope of this thesis

Most of the legume-rhizobia interaction studies to date concentrated on the initial stages involved in nodulation. Nodule number regulation is one key aspect of nodulation, whereby earlier nodulation events, or already formed nodules, suppress the formation of new nodules once an optimum number of nodules form. So far only a few supernodulation mutants have been characterized and published. In this thesis, I report the identification, characterization, genetics and mapping information of the lss, supernodulation mutant in *Medicago truncatula*.

I characterized the *lss* mutant phenotypically in comparison to the previously isolated supernodulation mutant, *sunn*, which results from mutation in a leucine rich repeat receptor like kinase (LRR-RLK). I have shown how the *lss* mutants respond to treatment with known nodule number regulators including nitrate, auxin (IAA) and ethylene in comparison to wild type and *sunn*. I investigated how *lss* and *sunn* interact with one another, by crossing *lss* with different *sunn* alleles. I have also looked at how *SUNN* gene expression is affected in *lss* shoots and roots contrasted with wild type and *sunn* shoots and roots. I determined where the *lss* locus is positioned in *Medicago truncatula* using a mapping strategy. Finally, I looked at the methylation pattern of the *SUNN* promoter and its 3’UTR in *lss* shoots and roots and compared them to wild type using bisulfite sequencing.
CHAPTER TWO
MATERIALS AND METHODS

**Plant material**

The seeds of various genotypes and ecotypes of *Medicago truncatula* were scarified in concentrated sulfuric acid (A.C.S. 93-98%) by vortexing for eight minutes in 15 or 50 ml sterile plastic tubes, depending on the quantity to be planted/ plated. Following an eight minute treatment with concentrated sulfuric acid, the seeds were washed in distilled water five times and then rinsed once in 50% commercial bleach (12% sodium hypochloride) followed by a final wash with distilled water five times. Seeds were then imbibed in water, shaking at 50 rpm for 3-4 hours at room temperature. Following imbibition, the seeds were plated by suspending over sterile water, via surface tension, on the lid in sterile 15 ml Petri dishes and vernalized for at least two days in the dark at 4°C before being germinated in darkness at 25°C for 24 hours. After 24 hours at 25°C, germinated seedlings were grown on plates, caissons or soil as appropriate to a given experiment.

**Plant growth**

Plants were grown either on an aeroponic chamber (hereafter referred to as a *caisson*), soil or on plates depending on the purpose of the experiment.

Plants used for phenotypic analysis and comparison, transcript profiling and mapping purposes were grown in an aeroponic chamber on nodulation media as in
Penmetsa and Cook (1997) with the exception of experiments testing nitrogen tolerance in which 10mM NH$_4$NO$_3$ was added to the media (see Table 1). Plants were inoculated with wild type *Sinorhizobium meliloti* strain ABS7 (Leong et al. 1985) grown on TY containing 15 µg/ml tetracycline overnight at 30°С to an OD$_{600}$ of 0.9, four days after they were loaded onto the caisson, unless otherwise mentioned.

**Table 1. Nutrient composition for caisson**

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration</th>
<th>Component</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>K$_2$SO$_4$</td>
<td>0.52 mM</td>
<td>MgSO$_4$</td>
<td>0.25 mM</td>
</tr>
<tr>
<td>CaCl$_2$.2H$_2$O</td>
<td>1 mM</td>
<td>Na$_2$-EDTA.2H$_2$O</td>
<td>50 µM</td>
</tr>
<tr>
<td>H$_3$BO$_3$</td>
<td>30 µM</td>
<td>MnSO$_4$.H$_2$O</td>
<td>10 µM</td>
</tr>
<tr>
<td>ZnSO$_4$.7H$_2$O</td>
<td>0.7 µM</td>
<td>CuSO$_4$</td>
<td>0.2 µM</td>
</tr>
<tr>
<td>Na$_2$MoO$_4$.6H$_2$O</td>
<td>1 µM</td>
<td>CoCl$_2$</td>
<td>0.04 µM</td>
</tr>
<tr>
<td>FeSO$_4$.7H$_2$O$^*$</td>
<td>50 µM</td>
<td>K$_2$HPO$_4$ **</td>
<td>5.5 mM</td>
</tr>
<tr>
<td>KH$_2$PO$_4$ $^B$</td>
<td>5.5 mM</td>
<td>NH$_4$NO$_3$ ***</td>
<td>10 mM</td>
</tr>
</tbody>
</table>

* Freshly prepared before addition to the nutrient solution

** K$_2$HPO$_4$ and KH$_2$PO$_4$ are mixed and the pH is adjusted to 7.0

***Left out of media unless otherwise mentioned

Plants were also grown on plates containing growth media as in Huo, et al., (2006) as in Table 2. The lower two-thirds of the plates were wrapped in aluminum foil to prevent the roots from being exposed to excessive light and the plants were flood
inoculated with wild type *S.meliloti* strain ABS7 (Leong et al. 1985) four days after they were plated, unless otherwise mentioned. The plates were moved back to a 25°C growth chamber (16 hour photoperiod) till the completion of the experiment.

**Table 2. Nutrient composition for plates**

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration</th>
<th>Component</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.25 mM</td>
<td>MgSO$_4$</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>ZnSO$_4$.7H$_2$O</td>
<td>16.1 µM</td>
<td>H$_3$BO$_3$</td>
<td>51 µM</td>
</tr>
<tr>
<td>MnSO$_4$.H$_2$O</td>
<td>50 µM</td>
<td>Na$_2$MoO$_4$.2H$_2$O</td>
<td>1 µM</td>
</tr>
<tr>
<td>CuSO$_4$</td>
<td>0.1 µM</td>
<td>CoCl$_2$.6H$_2$O</td>
<td>0.05 µM</td>
</tr>
<tr>
<td>Na$_2$-EDTA.2H$_2$O</td>
<td>50 µM</td>
<td>FeSO$_4$.7H$_2$O*</td>
<td>13.9 mg/l</td>
</tr>
<tr>
<td>CaCl$_2$.2H$_2$O</td>
<td>2 mM</td>
<td>MES</td>
<td>390 mg/l</td>
</tr>
<tr>
<td>Agar**</td>
<td>11.5 g/l</td>
<td>AVG***</td>
<td>0.1 µM</td>
</tr>
</tbody>
</table>

Adjust pH to 6.5 with 1M KOH Add 250 ml of the media per plate

* Freshly prepared before addition to media

** Agar is added after the pH is adjusted to 6.5

***AVG, an ethylene scavenger, is added after the media is allowed to cool down to 55°C post autoclave.

For plants grown past 15 days, a greenhouse at 20-25°C with natural light supplemented with a 14:10 Light:Dark cycle was used. The plants were grown in Middleweight soil mix #3-B (Fafard, MA, USA) and were watered every 24 hours, with a
100-fold dilution of water soluble 20-10-20 Peat-Lite Special fertilizer (The Scotts Company, OH, USA).

**Plant phenotype scoring**

Plants grown on caissons or plates were scored for nodule number, root length and lateral root production ten days post inoculation with rhizobia, unless otherwise mentioned. Root length was measured with a ruler from just below the hypocotyl junction to the root tip. Nodules were counted either by naked eye or under a magnifying glass depending on the clustering pattern of the nodules. Lateral roots were also counted by naked eye.

**Genetic Crosses**

Artificial hybridization was performed using plants of various genotypes and ecotypes of *Medicago truncatula* as described by Pathipanawat et al. (1994) with slight modifications (Table 3). Emasculated buds 2 days prior to full anthesis were used for crossing purposes as they were most amenable to cross-pollination and least prone to self-pollination (Penmetsa and Cook 2000). Pollen was removed using a hand-held vacuum system from the pollen-recipient parent, and pollen from freshly tripped flowers of the pollen-donor parent was added to the stigma of the emasculated flowers using fine tweezers as in Penmetsa et al. (2000). When crossing phenotypically similar individuals that may represent an allelic series we used a male sterile floral homeotic mutant of *M. truncatula* cv. Jemalong, *Mtapetala*, to identify any false positives from our crosses. The
apetala mutant did not require emasculation and the effects of apetala mutation are restricted only to floral organs; nodulation is indistinguishable from that of wild type (Penmetsa and Cook 2000). In both of the above cases, the resulting pods were wrapped in surgical gauge and labeled to aid collection.

**Table 3. Genetic crosses**

<table>
<thead>
<tr>
<th>Pollen donor</th>
<th>Pollen recipient</th>
</tr>
</thead>
<tbody>
<tr>
<td>lss</td>
<td>A20</td>
</tr>
<tr>
<td>lss</td>
<td>A17-ap</td>
</tr>
<tr>
<td>lss</td>
<td>sunn-1-ap</td>
</tr>
<tr>
<td>sunn-1</td>
<td>lss-ap</td>
</tr>
<tr>
<td>sunn-2</td>
<td>lss-ap</td>
</tr>
<tr>
<td>sunn-4</td>
<td>lss-ap</td>
</tr>
</tbody>
</table>

**Application of treatment compounds in Agar**

To study the effect of various hormones and treatment compounds on root length, nodule number and lateral root number, seedlings of different genotypes were germinated and grown in vertically oriented sterile Bio-Assay Dishes (245x245x25, Nunc Brand Products) with 250 ml of the above agar based growth media, unless otherwise mentioned. The plates were sealed with hypoallergenic cloth tape (Kendall, MA, USA)
and the lower two-thirds of the plates were wrapped in aluminum foil to prevent the roots from being exposed to excessive light, and then the plates were placed in a 25°C growth chamber (16 hour photoperiod). These were flood inoculated with wild type *S. meliloti* strain ABS7 (Leong et al. 1985) five days after they were plated, unless otherwise mentioned. The plates were moved back to a 25°C growth chamber (16 hour photoperiod) until the completion of the experiment. The plants were later scored for root length, nodule numbers and lateral root numbers.

**Hormonal treatment**

N-1-Naphthylphthalamic acid (NPA) was purchased from Supelco, PA, USA and Indole-3-Acetic acid (IAA) was purchased from Sigma. For stock solutions, 1M NPA was dissolved in 1 mL DMSO and 10mM IAA was dissolved in 1mL of 50% ethanol. The stocks were filter sterilized during preparation.

For localized application of these compounds, NPA dissolved in DMSO was added to 0.8% agar with a final concentration of 0.1% and IAA dissolved in ethanol was added to 0.8% agar with a final concentration of 0.1% (Reed et al. 1998). The agar was allowed to harden in sterile Petri plates, before being cut in small cubes and applied 24 hours before rhizobial inoculation just below the root-shoot junction of seedlings that had root length of approximately 20 mm. To rule out any effect of DMSO, we used DMSO containing agar cubes with no NPA as controls. For NPA treatment in the absence of rhizobia, the growth media was supplemented with 5 mM of NH$_4$NO$_3$ to aid plant growth.
For non-localized application of NPA and IAA, the appropriate amount of the compound was added to the growth media after the agar cooled to 55°C, before pouring the media onto plates. In these treatments, seedlings were inoculated with rhizobia in the same manner as above. The plants were scored for root length and nodule numbers ten days after inoculation with rhizobia.

1-Aminocyclopropane-1-carboxylic acid (ACC) was purchased from Research Organics, OH, USA. A stock solution of 100 mM ACC was prepared in 1 mL distilled water. Different concentrations of ACC (0, 2.5 µM, 5 µM, 10 µM) were added to the growth media before pouring the media into plates. For ACC treatments, L-α-(2-aminoethoxyvinyl)-glycine (AVG) was omitted from the growth media, to prevent any inhibitory action of AVG on ethylene production from ACC.

**Genetic Mapping**

In order to genetically map *lss*, individuals homozygous for the *lss* mutation (in the genetic background of the cultivar Jemalong, from which A17 genotype was isolated) were hand emasculated and crossed with pollen from ecotype A20, as described by Schnabel et al., 2003. The resulting F1 progeny from the *lss* x A20 cross were grown up in the greenhouse and allowed to self pollinate yielding multiple, independent F2 populations consisting of thousands of seeds. Progeny from eight *lss* x A20 F1 plants were grown up and screened for the *lss* phenotype, based on the nodule number and root
Table 4. Molecular markers used for fine mapping of *lss*

<table>
<thead>
<tr>
<th>Marker name (type)</th>
<th>Primers (5’→3’)</th>
<th>T&lt;sub&gt;A&lt;/sub&gt; (°C)</th>
<th>Enzyme</th>
<th>A17</th>
<th>A20</th>
</tr>
</thead>
<tbody>
<tr>
<td>18L14L (CAPS)</td>
<td>CGTAACATTTCATTATCGCTGCTAT</td>
<td>55</td>
<td><em>StyI</em></td>
<td>412</td>
<td>232+180</td>
</tr>
<tr>
<td></td>
<td>AAGTAATCCGGTGGATTGTATTTTTCTCC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ES63B19R (CAPS)</td>
<td>GGTTTCTTCTCTGCAATATTCTATCTTTTTGT</td>
<td>52</td>
<td><em>AluI</em></td>
<td>306+132+7</td>
<td>438+7</td>
</tr>
<tr>
<td></td>
<td>AGAAGAGCTCGGTAAAGGTCAGTATC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ES75A2L (SNP)</td>
<td>CACATATGCTATAAGCTGAAATAAGTGG</td>
<td>53</td>
<td>–</td>
<td>514</td>
<td>515</td>
</tr>
<tr>
<td></td>
<td>TTTTGGTACCATATTGCACTTTGTAATAT</td>
<td></td>
<td></td>
<td>8 T’s at nt 335</td>
<td>9 T’s at nt 335</td>
</tr>
<tr>
<td>MiLAX5 (LP)</td>
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<td>50</td>
<td>–</td>
<td>564</td>
<td>508</td>
</tr>
<tr>
<td></td>
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<tr>
<td>ES19E14L (dCAPS)</td>
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<td>53</td>
<td><em>Sau3A</em></td>
<td>300+190+129+28</td>
<td>300+190+157</td>
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<tr>
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<tr>
<td></td>
<td>CCCCTGAGCTTGGTTTTGACTCT</td>
<td>53</td>
<td><em>AflII</em></td>
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<td>860+260</td>
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<tr>
<td></td>
<td>CTCCTGACATACGTGTTACG</td>
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<tr>
<td>ES72D11R (CAPS)</td>
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<td><em>PstI</em></td>
<td>357+116+35</td>
<td>357+151</td>
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<tr>
<td>ES10P20-2 (CAPS)</td>
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<td><em>BstUI</em></td>
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<td>351+46</td>
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<tr>
<td>ES61G8* (LP)</td>
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<td>54</td>
<td>–</td>
<td>290</td>
<td>234</td>
</tr>
<tr>
<td></td>
<td>GCCTCCCACGGCCTACCA</td>
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length. Plants assumed homozygous for the *lss* mutation based on the supernodulation phenotype were used to map the *lss* mutation by following the segregation of the mutant phenotype with cleaved amplified polymorphic sequence (CAPS) markers and other
molecular markers (Table 4) some of which were previously developed against available EST and BAC sequences (Schnabel et al. 2003).

The F2 mapping populations were grown on a caisson along with the parental controls and screened for the \textit{lss} mutant phenotype. DNA from these plants was isolated using Qiagen DNAeasy Plant Kits (Qiagen Inc, Valencia, CA) and FTA leaf press cards (Whatman Inc, NJ, USA). The genomic DNA was used as the template to amplify PCR fragments using primer sets specific for each marker followed by digestion with the appropriate enzyme. Gel electrophoresis and ethidium bromide staining was used to observe the genotype of the plant at that particular marker. Because each marker is inherited in a co-dominant fashion, all allelic combinations were readily scored. The resulting genotype assignments were observed and compiled in a color map that consisted of all plants with informative recombination events to visualize the distance between the markers that flank \textit{lss} (Kiss et al. 1998).

\textbf{Quantification of Gene Expression}

Total RNA was extracted from plant tissues using the Qiagen RNeasy Plant Mini Kit (Qiagen Inc, CA, USA) according to the manufacturer’s instructions. RNA was extracted from roots and shoots from 15 day-old-plants (10 days post inoculation) gathered in three independent biological replicates. For the time course experiment, tissues from plants at 0, 12, 24, 48 and 72 hours post inoculation were used for RNA extraction. Approximately 1-2 µg of RNA was used as the template for single strand cDNA synthesis in 20 µl reactions, using random hexanucleotide primers (InVitrogen, La
Jolla, CA) and Superscript Reverse Transcriptase II (InVitrogen, La Jolla, CA) following the manufacturer’s instructions. The same amount of RNA was used as starting material within an experiment.

In previous work (Schnabel et al. 2005), gene specific intron-spanning primers were designed to amplify a 523 bp fragment from the kinase domain of \textit{SUNN} cDNA. For \textit{RLP-1} expression analysis, intron spanning primers amplifying a 368 bp fragment from the 5’ end of \textit{RLP-1} were used, also from Schnabel et al., (2005). Similarly, for \textit{MtPIN1} and \textit{MtPIN2} expression studies, intron-spanning primers from Schnabel and Frugoli (2004) were used. All primers for expression analysis are listed in Table 5. For all expression studies, the \textit{M. truncatula SECRET AGENT} gene was used as an internal control as in Schnabel, et al. (2005).

Gene expression levels were quantified by Real-Time Quantitative PCR in a BIORAD iCycler. Detection of gene expression levels occurred in 30 µl PCR reactions, consisting of iQ SYBR Green Supermix (BioRad, Hercules, CA). Cycling conditions were 95°C for 3 minutes, followed 40 cycles of 95°C for 10 seconds, 58°C for 20 seconds, 72°C for 1 minute and 82°C for 10 seconds. Fluorescence data (excitation filter 480/20; emission filter 530/30) was collected at 82°C, a slightly higher temperature to prevent signal detection from primer dimers from interfering with the signal from the specific PCR products.

PCR threshold cycles (Ct) were determined from PCR baseline subtracted curve fit data using default parameters of the iCycler software package. A dilution series of a known template was used for each experiment to calculate the efficiency (\(E\)) of the PCR
for a given primer pair from the slope of the regression line from a plot of Ct versus log (template concentration) using the formula \( E = 10^{-1/\text{slope}} \). For each primer pair on a biological replicate, three technical replicates were used and average Threshold Cycles (Ct) values were calculated from these values.

**Table 5. Primers used in expression analysis**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer (5’→3”)</th>
<th>Expected band size (bp)</th>
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<tr>
<td>SUNN-1</td>
<td>5’-CCATGGCAAACGGAACAGACG-3’</td>
<td>523</td>
</tr>
<tr>
<td></td>
<td>5’-AGCACCACCTCCGAAAACATACATCACT-3’</td>
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</tr>
<tr>
<td>RLP-1</td>
<td>5’-ACCGGCAGCTTCCAACTG-3’</td>
<td>368</td>
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<tr>
<td></td>
<td>5’-GCGTAAGCATTATCGTAACCTAAACA-3’</td>
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</tr>
<tr>
<td>MtPIN1</td>
<td>5’-ATGGCTCTGCTGCTGCTGCTAA-3’</td>
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<td></td>
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<td>MtPIN2</td>
<td>5’-AAAGGGGCTACTCCGAGGACT-3’</td>
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</tr>
<tr>
<td></td>
<td>5’-GACTCATGTTCTAAATCAATTCAGTAAC-3</td>
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</tbody>
</table>

**Bisulfite Sequencing**

Genomic DNA was extracted from roots and shoots by the DNeasy Plant Mini Kit (Qiagen Inc, CA, USA) according to the manufacturers’ instructions. Between 0.4-1.0µg
of total genomic DNA was used for bisulfite conversion. The Epitect Bisulfite Kit (Qiagen Inc, CA, USA) was used for the conversion and clean up of DNA for methylation analysis, according to the manufacturers’ protocol. As a control, efficiency of conversion was tested using distinct primer sets for both unconverted and converted DNA strands for a gene, ASA1, from Arabidopsis, known to be constitutively unmethylated (Jeddeloh et al. 1998).

Primer sets were designed to bind at the same C-rich position, but with specificity for unconverted or converted DNA. The PCR products were extracted with phenol: chloroform (1:1) and precipitated with 70% ethanol as in Sambrook, et al. (2001) and cloned into the vector pGEM T Easy Vector System I (Promega, WI, USA) according to the manufacturers’ instructions. Positive clones were selected by blue/white screening on LB/ampicillin/IPTG/X-gal plates for plasmid isolation. The inserts were checked for the correct size by digesting the plasmid with EcoRI and viewing sizes by running on a 1% agarose gel stained with ethidium bromide. The appropriately sized inserts were sequenced with T7 primer using Big Dye 3.1 terminator cycle sequencing kit (Applied Biosystems, CA, USA) on an ABI Prism 3130 capillary sequencer (Applied Biosystems, CA, USA) by Clemson University Genomics Institute (CUGI). Between eight and ten colonies were picked from each transformation for sequencing. The sequences were analyzed using Lasergene software and BiQ Analyzer (Bock et al. 2005).
**Expression in lss/sunn heterozygotes**

*lss/sunn-2* F1 cDNA was analysed with *sunn-2* marker to determine if the *lss/sunn-2* heterozygote expressed either *lss* or *sunn-2* allele or both. cDNA from *lss/sunn-2* heterozygote and A17 wild type and genomic DNA from A17 and *sunn-2* were extracted as mentioned earlier in this section. These were used as templates to amplify a 414 bp fragment using primers 5’-AGAATCTGAAGGTTCTAAGCATTTTTT-3’ and 5’-GACACTCCACTACTTCCTCTCGCT-3’. The annealing temperature used was 57ºC. Following amplification the PCR products were digested with BsmAI (NEB, MA, USA) at 55ºC for 4 hours, which cuts only *sunn-2* allele. Following digestion, the samples were run on a 1% agarose gel and stained with ethidium bromide.

**Statistical Analysis**

Significance results (P values) were calculated using Student’s T test in Kaleidagraph (Synergey Software, PA).
Growth characteristics of \textit{lss}

Based on visual screen for altered nodule phenotype, Lucinda Smith from Sharon Long’s Laboratory (Department of Biological Sciences, Stanford University, CA) identified a naturally occurring supernodulation mutant, \textit{lss (like sunn supernodulator)}, from the Jemalong cultivar (from which the wild type A17 ecotype was derived) which is characterized by a loss of regulation of nodule number and by short root length (Fig 1). When grown under aeroponic conditions and inoculated with rhizobia, \textit{lss} had approximately 35 nodules in the primary nodulation zone, which was similar to the number observed in \textit{sunn}, a previously characterized supernodulation mutant (Penmetsa et al. 2003). Thus, \textit{lss} displayed a six-fold increase in the nodule number when compared to the wild type A17 genotype, which developed approximately 6 nodules under the same growth conditions (P < 0.05; Fig 1A). Root length measurements of the same plants indicated \textit{lss} had a short root phenotype, which was again very similar to reduced root length phenotype observed for \textit{sunn}. Wild type A17 roots were almost twice as long as either \textit{lss} or \textit{sunn}, 10 days after being inoculated with rhizobia (P < 0.025; Fig 1B).
Fig 1. Phenotype comparison of *M. truncatula* wild type, *sunn* and *lss* when grown aeroponically. A. Average nodule number of individual genotypes from 14 d-old plants grown aeroponically, inoculated with rhizobia 4 d after the start of the experiment. B. Average root length in cm of the same plants. Results are means (n=30-40) ± SE. Values denoted with an * or ** are significantly different from that of the wild type at the 0.05 and <0.025 confidence level, respectively (Student’s *t* test).

When *lss*, wild type and *sunn* were grown on agar media plates as opposed to aeroponically, *lss* displayed a 6-to7-fold increase in nodule number when compared to nodule numbers in wild type A17, 10 days post inoculation with *S meliloti* ABS7 (P < 0.05; Fig 2A). In this instance, plants from the three genotypes were grown together on the same plate. Again, the nodule number phenotype of *lss* was very similar to that of *sunn*. Similarly, root length measurements of the same plants indicated *lss* to have a short root phenotype as observed for *sunn* (P < 0.01; Fig 2B).
Fig 2. Phenotype comparison of *M. truncatula* wild type, *sunn* and *lss* when grown on plates containing agar based media. **A.** Average nodule number of individual genotypes from 14 d-old plants grown on plates containing agar based media, inoculated with rhizobia 4 d after the start of the experiment. **B.** Average root length in cm of the same plants. Results are means (n=15 for all genotypes) ± SE. Values denoted with an * and ** are significantly different from that of the wild type at the 0.05 and 0.01 confidence level, respectively (Student’s *t* test).

Interestingly, when plants from the above three genotypes were each grown on separate plates, the *sunn* mutants produced a lower number of nodules than when grown on the same plate with wild type and *lss*. Wild type and *lss* mutants however, were not affected by this change of environment (Fig 3A). The root length of the plants grown under the two conditions, however, was not affected (Fig 3B).
Fig 3. Nodule number comparison between wild type, *lss* and *sunn* when grown on the same plates and when grown on separate plates. A. Average nodule number of wild type, *sunn* and *lss* when grown separately on plates (black) versus when grown together on plates (grey). Results are mean (n=15 for all genotypes) ± SE. B. Average root length in cm comparison of the same plants. Values denoted with an * and ** are significantly different from that of the wild type at the 0.05 and 0.01 confidence level, respectively (Student’s *t* test).

The root growth pattern of *lss*, wild type and *sunn* when grown aeroponically and inoculated with rhizobia 4-d after the start of the experiment indicates that post rhizobial infection, the rate of root growth is arrested in all cases (Fig 4A).
Fig 4. Growth of *lss*, wild type and *sunn* roots under aeroponic and plate conditions for 312 h. A. Root length in cm at 24 h intervals of *lss* (red), wild type (black) and *sunn* (blue) under aeroponic conditions for 312 h. B. Root length in cm at 24 h intervals of *lss* (red), wild type (black) and *sunn* (blue) on plates when grown together on the same plate containing agar based media for 312 h. C. Root length in cm at 24 h intervals of *lss* (red), wild type (black) and *sunn* (blue) on plates when grown separately (only one genotype) on plates containing agar based media for 312 h. Arrow indicates time of inoculation with rhizobia (96 h after start of the experiment). Results are means (n=15 for all genotypes) ± SE.
However, when the three genotypes were grown together on plates containing agar based media, root growth was dramatically arrested in *lss* as compared to either the wild type or the *sunn* plants (Fig 4B). In both conditions though, wild type root length is much longer than either *lss* or *sunn* plants. When the plants are grown exclusively (only one genotype) on plates *lss* roots appear to grow faster than wild type until rhizobial inoculation and then root growth is arrested (Fig 4C). Wild type roots were eventually longer than either *lss* or *sunn* plants.

**Wild type has longer roots than lss and sunn in the absence of rhizobia**

In order to investigate if the short root phenotype of *lss* mutants was related to rhizobial inoculation, *lss* was grown in the absence of rhizobia along side wild type A17 and *sunn* mutant plants. The plants were grown aeroponically and the nodulation media was supplemented with 5mM NH₄NO₃ to provide nitrate usually provided by rhizobia. Under these experimental conditions, 14 days after the start of the experiment, plants from the above 3 genotypes formed no nodules, as expected. Root length in *lss* was similar to *sunn* but shorter than wild type A17 (P < 0.005; Fig 5A). However, root length for all 3 genotypes appeared to be longer in the absence of rhizobia than in its presence. Lateral roots development share a similarity with nodule development (See Background). Accumulation of auxin at the site of future primordium is one such developmental similarity between lateral root formation and nodule development (Benkova et al. 2003). Rhizobia have been reported to inhibit auxin transport from shoot to root (van Noorden et al. 2006), so we were interested to see if absence of rhizobia had an impact on lateral root
development. Lateral root density (average lateral roots per cm) in lss was similar to wild type both of which are lower than sunn (P < 0.0005; Fig 5B). This was in contrast to the increased lateral root phenotypes as shown in the har1 mutants in L. japonicus (Wopereis et al. 2000b).

Fig 5. Phenotype comparison of M. truncatula wild type, sunn and lss grown in the absence of rhizobia. A. Average root length in cm of individual genotypes from 14 d-old plants grown aeroponically, in the absence of rhizobia. The media was supplemented with 5mM of NH₄NO₃ (see Text). Bars in grey indicate plants without rhizobia; bars in black indicate plants inoculated with rhizobia. B. Average lateral roots per cm from the same plants. Results are means (n=25 for A17 and sunn, n=40 for lss) ± SE. Values denoted with an * or ** are significantly different from that of the wild type at the 0.005 and 0.0005 confidence level, respectively (Student’s t test).
Effect of nitrate on nodule number and root length of lss

To determine whether nodulation in the lss mutant is more resistant to nitrate as observed in other AON mutants in *Medicago*, soybean, *L. japonicus* and pea, the effect of nitrate on nodulation was examined (Carroll et al. 1985a; Carroll et al. 1985b; Sagan and Duc 1996; Schnabel et al. 2005; Wopereis et al. 2000b). When inoculated with rhizobia after being grown aeroponically in the presence of 10 mM NH$_4$NO$_3$, wild type plants did not nodulate, however, lss plants did (Fig 6A). Nodulation in lss was observed to be insensitive to nitrate at this particular concentration (P < 0.1; Fig 6A).

The sunn mutants showed nodule development, as reported by Schnabel et al. (2005), however, nodule number was significantly reduced from the usual 5-10 fold increase observed in the absence of nitrate. Root length measurements of these same plants indicated that lss and sunn had shorter roots than wild type even in the presence of nitrate (P < 0.01; Fig 6B).
Fig 6. Phenotype comparison of *M. truncatula* wild type, *sunn* and *lss* grown in the presence of nitrate. A. Average nodule number of individual genotypes from 14 d-old plants grown aeroponically in the media supplemented with 10mM NH$_4$NO$_3$ and the plants inoculated with rhizobia 4 d after the start of the experiment. Bars in grey indicate nitrate treated plants; bars in black indicate plants with no nitrate treatment. B. Average root length in cm of the same plants. Results are means (n=30 for all genotypes) ± SE. Values denoted with an * or ** are significantly different from that of the wild type at the 0.1 and 0.01 confidence level, respectively (Student’s t test).

**The effect of auxin and an auxin inhibitor**

Auxin has been shown to be an important regulator of nodulation (see Background). van Noorden et al. (2006) reported altered auxin transport in *sunn* mutants and they have proposed that the supernodulation phenotype of *sunn* might be a result of
this altered auxin flux. In the root controlled supernodulating skl mutant, a different change in auxin transport was observed (Prayitno et al. 2006). Therefore, we decided to examine auxin’s effect on nodulation in lss mutants.

**The effect of IAA**

To determine the effect of the auxin IAA on nodule development and root growth in lss, plants were grown in presence of 10 µM IAA as mentioned in Materials and Methods and inoculated with rhizobia. Nodule number in all three genotypes was reduced under the experimental conditions when compared to untreated plants (Fig 7A). Nodulation in sunn however, was significantly reduced from no IAA controls but lss plants formed more nodules than either sunn or wild type plants (P < 0.05; Fig 7A). Root growth was significantly inhibited compared to untreated IAA controls (P < 0.0005; Fig 7B) in lss, wild type and sunn plants.

In the whole plate application of IAA, the entire plants were exposed to IAA treatment. As opposed to looking at effect of IAA on entire plants, examining the effect of local auxin involves application at a shoot/root junction and following the phenotypes. In order to test the application of auxin on nodule development and root growth of lss, agar cubes containing IAA were placed at shoot/root junctions of the experimental plants. Nodule number in lss mutants was much higher than in wild type plants (Fig 8A).
Fig 7. The effect of IAA on nodule number and root length in *M. truncatula* wild type, *sunn* and *lss*. **A.** Average nodule number of individual genotypes from 14 d-old plants grown on agar plates, supplemented with 10µM IAA. Plants were inoculated with rhizobia 4 d after being put on the plate (see Materials and Methods). Bars in black indicate IAA treated plants; bars in grey indicate untreated plants. **B.** Average root length in cm of the same plants. Control plants were grown on agar plates without IAA. Results are means (n=15 for all genotypes) ± SE. Values denoted with an * and ** are significantly different from that of the wild type at the 0.05 and 0.01 confidence level respectively (Student’s *t* test).

Root length of *lss* was lower than wild type under these conditions (Fig 8B).
Fig 8. The effect of local application of IAA on nodule number and root length in *M. truncatula* wild type and *lss*. A. Average nodule number of individual genotypes from 14 d-old plants grown on agar plates, inoculated with rhizobia 4 d after being put on the plate (see Materials and Methods). At the root shoot junction of each plant, an agar block containing 10 µM IAA was placed 1 d before inoculation with rhizobia. Bars in black indicate IAA treated plants; bars in grey indicate untreated plants. B. Average root length in cm of the same plants. Control plants were grown on agar plates without the IAA agar blocks. Results are means (n=15 for all genotypes) ± SE. Values denoted with an * and ** are significantly different from that of the wild type at the 0.05 and 0.01 confidence level, respectively (Student’s *t* test).
The effect of NPA- auxin transport inhibitor

N-(1-napthyl)phthalamic acid (NPA) is a known polar auxin transport inhibitor (PATI) (Geldner et al. 2001). Previously van Noorden et al., (2006) reported that nodule numbers in the sunn mutant were significantly reduced when grown in the presence of NPA. In supernodulating skl mutant, auxin transport was significantly reduced by NPA (Prayitno et al. 2006). To study the effect of NPA on nodulation and root growth in lss mutants, plants from the 3 genotypes lss, wild type and sunn were treated with an agar block containing NPA at the root shoot junction one day before inoculation with S. meliloti to block auxin transport from shoot to root. Nodule number in lss mutants was significantly higher than either wild type or sunn mutants for NPA treated plants (P < 0.1; Fig 9A). Root length of lss, wild type and sunn mutants was not affected upon treatment in the same plants (Fig 9B). NPA was dissolved in DMSO, before being added to the agar cubes for blocking auxin transport. In order to confirm that the effects were solely from NPA and not DMSO induced, we used agar cubes with only DMSO as another set of controls for the experiment.

To study the effect of NPA, in the absence of rhizobia, on root growth and lateral root number in lss mutants, the plants were not inoculated with rhizobia; instead the agar media was supplemented with 5 mM NH₄NO₃. Wild type, lss and sunn mutants did not nodulate even after 14 days from the start of the experiment confirming rhizobia were not present. Root lengths of lss and sunn were not significantly lower than wild type plants (P << 0.0005; Fig 10A). In fact root lengths of lss and sunn were longer in the absence of
rhizobia than in its presence. Lateral root numbers in wild type was higher than either lss or sunn mutants (P < 0.05; Fig 10B).

Fig 9. The effect of NPA on nodule number and root length of *M. truncatula* wild type, sunn and lss. A. Average nodule number of individual genotypes from 14 d-old plants grown on agar plates, inoculated with rhizobia 4 d after being put on the plate. At the root shoot junction of each plant, an agar block containing NPA dissolved in DMSO was placed 1 d before inoculation with rhizobia. B. Average root length in cm of the same plants. Control plants were grown on agar plates treated with agar blocks containing only DMSO. NPA treated plants are in black; DMSO treated controls are in stripes; control plants containing neither NPA nor DMSO are in grey. Results are means (n=15 for all genotypes) ± SE. Values denoted with an * and ** are significantly different from that of the wild type at the 0.1 and 0.01 confidence level respectively (Student’s *t* test).
Fig 10. The effect of NPA on root length and lateral roots of *M. truncatula* wild type, *sunn* and *lss*, in the absence of rhizobia. **A.** Average root length in cm of individual genotypes from 14 d-old plants grown on agar plates, supplemented with 5mM NH$_4$NO$_3$ (see Text). At the root shoot junction of each plant, an agar block containing NPA dissolved in DMSO was placed 1 d before inoculation with rhizobia. Bars in grey indicate plants without rhizobial inoculation; Bars in black indicate plants inoculated with rhizobia. **B.** Lateral roots per cm of the same plants. Results are means (n=15 for all genotypes) ± SE. Values denoted with an * are significantly different from that of the wild type at the 0.05 confidence level (Student’s $t$ test).

**The effect of ethylene on nodulation in lss**

Ethylene has been reported to be an inhibitor of nodulation (see Background). Several studies have indicated the effects of ethylene on nodulation are via regulation of auxin transport and in turn, auxin transport regulation often requires ethylene signaling
(see Background). The ethylene-insensitive skl mutant of *Medicago truncatula* has been reported to show altered auxin transport during nodulation (Prayitno et al. 2006). However, the supernodulation phenotype in *sunn* mutants was not a result of altered ethylene responses, as the responses were similar to wild type (Penmetsa et al. 2003). In order to study the effects of ethylene on nodule development and root growth in *lss* mutants, we compared *lss* mutants to wild type and *sunn* mutants upon treatment with ethylene precursor 1-amino cyclopropane-1-carboxylic acid (ACC). The *lss*, wild type and *sunn* plants were inoculated with rhizobia after being grown in the presence of different concentrations of ACC. Nodule number in *lss* mutants decreased upon increasing the concentration of ACC from 0 to 10 µM, being most drastic in presence of 10 µM ACC (Fig 11A). However, nodule number in *lss* mutants was significantly higher (P < 0.05; Fig 11A) than wild type and *sunn* mutants at all ACC concentrations. The *lss* mutants appeared to be responsive to ethylene, but less sensitive to ethylene than either wild type or *sunn*. Root lengths of the same plants were reduced in the presence of ACC. However, root length reduction was not dose dependent in case of *lss* mutants (Fig 11B). Wild type and *sunn* displayed similar response to ACC treatment in a dose dependent manner.
**Fig 11.** The effect of ACC on nodule number and root length of *M. truncatula* wild type, *sunn* and *lss*. 

**A.** Average nodule number of individual genotypes from 14-d-old plants grown on agar plates, supplemented with 0, 2.5 µM, 5 µM and 10 µM ACC. The plants were inoculated with rhizobia 4-d after being put on a plate. Bars in black indicate wild type (A17); bars in grey indicate *lss*; bars in light grey indicate *sunn*. 

**B.** Average root length in cm of the same plants. Control plants were grown on agar plates without ACC. Results are means (n=15 for all genotypes) ± SE. Values denoted with an * and ** are significantly different from that of the wild type at the 0.05 and 0.01 confidence level respectively.

**PIN1 and PIN2 expression does not change after inoculation with rhizobia over time**

In the event that auxin effects were due to changes in Polar Auxin Transport levels in *lss*, we examined the expression levels of *MtPIN1* and *MtPIN2* in *lss* roots upon rhizobial infection. Previously, *MtPIN1* and *MtPIN2* expression levels in the inoculation
zone of *skl* roots, 24 hrs post inoculation with rhizobia, were reported to be higher than in wild type roots. However, *MtPIN1* and *MtPIN2* expression in *sunn* roots were similar to wild type levels in the inoculation zone, 24 hrs post rhizobial inoculation (Prayitno et al. 2006). We performed a time course quantitative RT-PCR to look at the expression levels of *MtPIN1* and *MtPIN2* in samples generated from whole roots from *lss*, wild type and *sunn* plants collected at 0, 24, 48 and 72 hrs post inoculation with rhizobia. Taking into account the variation seen with only two replicates, *MtPIN1* mRNA levels in *lss* were similar to levels in both wild type and *sunn* roots at all the time points (Fig 12A). Likewise the expression level of *MtPIN2* in *lss* roots was similar to its expression level in wild type and *sunn* roots at all the time points (Fig 12B). These results suggest that *MtPIN1* and *MtPIN2* expression levels in *lss* roots are not altered from their expression in wild type roots upon rhizobial inoculation.
Fig 12. Expression analysis of \textit{MtPIN1} and \textit{MtPIN2} in wild type, \textit{sunn} and \textit{lss} roots at different time points with respect to rhizobial inoculation. \textbf{A.} Relative amount of \textit{MtPIN1} expression in \textit{sunn} and \textit{lss} roots compared to wild type roots at 0 hr, 24 hr, 48 hr and 72 hr post rhizobial inoculation. \textbf{B.} Relative amount of \textit{MtPIN2} expression in \textit{sunn} and \textit{lss} roots compared to wild type roots at 0 hr, 24 hr, 48 hr and 72 hr post rhizobial inoculation. Plants were inoculated with rhizobia 4 d after the start of the experiment. Total RNA was extracted from roots of plants at 0 hr, 24 hr, 48 hr and 72 hr post rhizobial inoculation. mRNA levels were determined by RT-PCR analysis. The gene, Secret Agent was used as an internal control (see Materials and Methods). Values are mean from two independent biological replicates each with three technical replicates. Values are normalized to wild type levels.
SUNN expression is greatly reduced in lss shoots compared to sunn and wild type shoots.

The effect of the SUNN gene on nodulation is a shoot-controlled phenotype (Penmetsa et al. 2003) and in lss mutants also the shoot genotype controls the root phenotype (grafting results; Lucinda Smith personal communication). Previously Schnabel et al., (2005) showed that SUNN is expressed in roots and shoots of sunn mutants. The expression of SUNN RNA in sunn mutant shoots was slightly lower than in wild type shoots however the level of SUNN RNA in sunn mutant roots was one-third of the level in wild type roots (Schnabel et al. 2005). In order to determine the expression levels of SUNN gene in lss mutant roots and shoots, quantitative RT-PCR was performed on samples generated from whole shoots and roots, 8 days after inoculation with rhizobia. Analysis revealed that SUNN gene is expressed in both roots and shoots of lss, wild type and sunn plants (Fig 13).

While the level of SUNN message in lss roots was one-fifth of the level in wild type roots and was close to the level of expression observed in sunn roots, in lss shoots, there was a drastic reduction of SUNN gene expression when compared to its expression in sunn and wild type shoots. The control gene used for the experiment did not permit comparison of expression levels between tissues.
Fig 13. Expression analysis of MtSUNN in wild type, sunn and lss roots and shoots.

A. Relative amount of SUNN expression in sunn and lss roots compared to wild type roots. B. Relative amount of SUNN expression in sunn and lss shoots compared to wild type shoots. Total RNA was extracted from roots and shoots of 12 d-old plants, inoculated with rhizobia 4 d after the start of the experiment. mRNA levels were determined by RT-PCR analysis. The gene, Secret Agent was used as an internal control (see Methods). Values are mean ± SE from three independent biological replicates each with three technical replicates. Values are normalized to wild type levels.

SUNN expression does not vary over time in lss shoots.

According to van Noorden et al. (2006) rhizobial inoculation affects local and long distance auxin transport in M. truncatula and they suggest the SUNN protein must regulate nodule numbers by altering auxin transport (van Noorden et al. 2006). Since we
found that *SUNN* expression is immensely reduced in *lss* shoots 8 days after inoculation with rhizobia (see previous results), to determine what happens to *SUNN* expression in *lss* shoots upon rhizobial inoculation, we performed a time-course quantitative RT-PCR on samples generated from whole shoots collected at 0, 24, 48 and 72 hrs post inoculation with rhizobia. Quantitative Real Time PCR suggested that *SUNN* expression increases in wild type shoots 24 h after rhizobial inoculation when compared to its levels in wild type shoots before rhizobial inoculation (Fig 14A). In the *sunn* mutants, we found a similar increase in the *SUNN* expression 24 h after rhizobial inoculation. The *SUNN* RNA in *lss* shoots at all the time points checked were almost nonexistent when compared to the levels observed in wild type and *sunn* shoots (Fig 14B). These findings indicate that rhizobial infection does not affect the level of *SUNN* expression in *lss* shoots or that the levels are so low a difference cannot be detected with Real Time PCR.
Fig 14. Expression analysis of MtSUNN in wild type, sunn and lss shoots at different time points with respect to rhizobial inoculation. A. Relative amount of SUNN expression at different time points (0, 24, 48 and 72 h post rhizobial inoculation) in wild type (black) and sunn (grey) shoots when compared to SUNN expression levels at 0 h post rhizobial inoculation in wild type and sunn shoots respectively. B. Relative amount of SUNN expression in sunn and lss shoots compared to wild type shoots. Plants were inoculated with rhizobia 4 d after the start of the experiment. Total RNA was extracted from shoots of plants at 0 hr, 24 hr, 48 hr and 72 hr post rhizobial inoculation. mRNA levels were determined by RT-PCR analysis. The gene, Secret Agent was used as an internal control (see Methods). Values are mean from three independent biological replicates each with three technical replicates. Values are normalized to wild type levels.
**RLP1 expression is lower in both shoots and roots of lss than in sunn**

*RLP1* (Receptor-like protein-1; Genbank AY769944) is an abbreviated copy of a *SUNN*-like sequence immediately upstream of *SUNN*. It shares 95% nucleotide sequence identity to the receptor and transmembrane domains of *SUNN*, but lacks a kinase domain (Schnabel et al. 2005). Quantitative RT-PCR detected similar expression of *RLP1* in shoots of *sunn* vs. wild type nodulating plants, and significant reduction in *RLP1* message in *sunn* roots (Fig 15).

This was similar to the pattern of *SUNN* gene expression observed in *sunn* shoots and roots vs wild type nodulating plants (Schnabel et al. 2005). Quantitative RT-PCR was performed to study the *RLP1* expression pattern in *lss* shoots and roots on samples generated from whole plants, 8 days after inoculation with rhizobia. *RLP1* expression levels in *lss* were lower than its levels in *sunn* shoots and roots (Fig 15).
Fig 15. Expression analysis of MtRLP1 in wild type, sunn and lss roots and shoots.

A. Relative amount of RLP1 expression in sunn and lss roots compared to wild type roots. B. Relative amount of RLP1 expression in sunn and lss shoots compared to wild type shoots. Total RNA was extracted from roots and shoots of 12 d-old plants, inoculated with rhizobia 4 d after the start of the experiment. mRNA levels were determined by RT-PCR analysis. The gene, Secret Agent was used as an internal control (see Materials & Methods). Values are mean ± SE from three independent biological replicates each with three technical replicates. Values are normalized to wild type levels.

Genetics & Mapping

lss segregates as a single, recessive locus

To determine the genetic nature of the lss locus, pollen from a polymorphic mapping ecotype A20 was used in crosses with homozygous lss individuals. F1 heterozygotes (4) derived from this cross had a wild type level of nodulation, indicating
*lss* is a recessive locus barring penetrance issues. F2 progeny (n=209) were scored for nodule number 10 days post rhizobial inoculation. Analysis revealed 44 plants that contained an estimated 35 nodules/plant which were similar to the known *lss* supernodulators grown as controls.

![Bar graph showing average nodule number and root length](image)

**Fig 16. Segregation of *lss* as a single, recessive locus.** A. Average nodule number of the wild type and supernodulating progeny segregating from two individual plants in the F2 generation in a cross between *lss* and a wild type mapping parent. Plants were inoculated with rhizobia 4 d after the start of the experiment and screened 10 d post rhizobial inoculation. B. Average root length in cm of the same plants. From the F2 population, 44 supernodulators segregated out from a total of 209 plants. Segregating plants are compared to *M. truncatula* wild type and known *lss* plants and results are indicated as mean (n=44 for supernodulators, n=165 for normal nodulators) ± SE. Values denoted with an * and ** are significantly different from that of the wild type at the 0.05 and 0.01 confidence level, respectively (Student’s *t* test).
The remaining 165 individuals had wild type level of nodulation (Fig 16). The phenotypic ratio of 165:44 closely approximates 3:1 ($\chi^2 = 1.73$, $P < 0.05$) and is consistent with a single gene.

**lss does not complement sunn-1/-2/-4 alleles**

To test if the *lss* locus was allelic to the previously described supernodulation mutant *sunn*, we crossed *lss* to *sunn-1/-2/-4* alleles and examined the phenotypes of the F1 populations. The *SUNN* gene encodes a leucine-rich repeat receptor like kinase (See Background). *Sunn-1* is a missense mutation in the kinase domain, *sunn-2* is a missense mutation in a residue between two leucine-rich repeats and *sunn-4* is result of truncation of almost the entire protein (Schnabel et al. 2005). F1 individuals from all three crosses were supernodulators similar to known supernodulators grown as controls ($P < 0.05$; Fig 17A). Root length of the same F1 individuals were significantly shorter than the wild type plants but were same as the supernodulating controls ($P < 0.05$; Fig 17B). These results show that *lss* does not complement *sunn-1/-2/-4* alleles in the F1 and this non-complementation is not allele specific.
Fig 17. Complementation analysis of lss with the different sunn alleles. A. Relative nodule number from the progeny, F1, of a cross between lss and sunn-1; lss and sunn-2; lss and sunn-4. B. Relative root length from the same F1 plants from A. Crosses were performed as mentioned in Materials and Methods. The F1 plants were inoculated with rhizobia 4 d after being loaded onto a caisson and were screened 10 d post rhizobial inoculation. Values are compared to M. truncatula wild type, sunn -1/-2/-4 and lss plants and results are mean (n=4 for lss X sunn-1; n=3 for lss X sunn-2; n=3 for lss X sunn-4) ± SE. Values denoted with an * and ** are significantly different from that of the wild type at the 0.05 and 0.01 confidence level respectively (Student’s t test).
In order to rule out a role for parental imprinting on this inheritance pattern, we performed a reciprocal cross where *sunn-1* mutant was the pollen donor, as opposed to *lss* homozygote. F1 individuals from this cross yielded progeny having supernodulation and very similar to the F1 individuals derived from the cross when *lss* homozygote was the pollen donor (Fig 18). Thus the origin of the alleles in the cross has no outcome on the lack of rescue of the phenotype.

**Fig 18. Pollen parent has no effect on the inheritance pattern in cross between *lss* and *sunn-1*.** A. Average nodule number of F1 progeny in cross between *sunn-1* and *lss*; and *lss* and *sunn-1*. A17, *sunn-1* and *lss* were grown as controls and the values are from 14 d old plants grown aeroponically, inoculated with rhizobia 4 d after the start of the experiment. B. Average root length in cm of the same plants. Results are means (n=4 for *lss* X *sunn-1*; n=2 for *sunn-1* X *lss*) ± SE. Values denoted with an * and ** are significantly different from that of the wild type at the 0.05 and 0.01 confidence level respectively (Student’s *t* test).
**SUNN, RLP1 and their intergenic region is wild type in lss**

Following the above results we set about to determine if *lss* was an allele of *SUNN*. But, upon sequencing of the entire *SUNN* gene including 1.3 kb of promoter sequence and 532 bp of 3’UTR region in *lss* mutants, we found no lesion. Next we sequenced the entire *RLP1* gene including 200 bp of its promoter in *lss*. *RLP1* sits just upstream of *SUNN* and has a high homology to receptor and transmembrane domains of *SUNN* but lacks a kinase domain (Schnabel et al. 2005). We still found no lesion in this region in *lss*. Finally, for completion, we sequenced the entire 4.8 kb region between *RLP1* and *SUNN* which included the 1.3 kb of the *SUNN* promoter, and yet again we found no lesion in *lss* plants. This suggests that the *lss* phenotype does not result from some DNA lesion in this approximately 12 kb region.

**Plants with normal nodulation segregate in F2 of lss/sunn-1 cross**

Based on the suspicion from the above sequencing that *LSS* and *SUNN* are two separate genes, we looked at the F2 population from the *lss* cross to *sunn-1*. In the F2 progeny, 29 plants having wild type level of nodulation segregated out of a total number of 308 individuals. The remaining 279 plants had an average of 29 nodules per plant, similar to the number of nodules in the known supernodulators, *lss* and *sunn-1* grown in the same experiment (Table 6). This finding suggests that *LSS* and *SUNN* are separate genes but the number of wild types we obtain is much lower than expected. We obtained 10% wild type plants segregating out of the F2 population, as opposed to the 31.25% which is expected due to two unlinked genes when plants heterozygous for both
mutations are supernodulators. Testing this hypothesis that the ratio of wild types: supernodulators in the F2 population due to two unlinked genes when plants heterozygous for both the mutations are supernodulators, on our experimental population led to a rejection of the hypothesis ($\chi^2 = 68.33$, $P >> 0.001$). The reason for this will become apparent upon discussion of mapping.

Table 6. Plants with wild type nodulation segregate in the F2 of lss/sunn-1 cross

<table>
<thead>
<tr>
<th>lss X sunn-1 F2 phenotype</th>
<th>Number of plants</th>
<th>Mean Nodule Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>29</td>
<td>5 +/- 7</td>
</tr>
<tr>
<td>Supernodulators</td>
<td>279</td>
<td>29 +/- 12</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Known Genotype</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>28</td>
<td>6 +/- 3</td>
</tr>
<tr>
<td>sunn-1</td>
<td>19</td>
<td>28 +/- 5</td>
</tr>
<tr>
<td>lss</td>
<td>20</td>
<td>35 +/- 7</td>
</tr>
</tbody>
</table>

**lss maps to the bottom of LG4**

An F2 population derived from a cross between lss (in the genetic background of Jemalong cv. from which the wild type A17 genotype is derived) and the polymorphic ecotype A20 was used to map the lss locus relative to CAPS markers corresponding to
known gene sequences. In initial mapping experiments, 22 individuals homozygous for the \textit{lss} mutation based on phenotype were identified in the mapping population and were analyzed to establish a linkage group association for the locus. This analysis was sufficient to assign \textit{lss} to a region of \textit{M.truncatula} linkage group (LG) 4, where the \textit{SUNN} gene also resides (Fig 19A&B). The same plants segregated randomly with markers from other linkage groups (Fig 19B). The small size of the initial mapping population placed \textit{lss} between PCT and DENP markers on the lower arm of LG 4 but did not permit precise positioning of \textit{lss} relative to several closely linked genetic markers. Therefore, the mapping population was expanded to 2342 plants and 449 individuals homozygous for the \textit{lss} lesion were analyzed to refine the relationship between \textit{lss} and flanking CAPS markers by looking for new recombinants in the area. The resulting linkage map (Fig 19C) placed \textit{lss} between ES10P20-2 and MtLAX5 (now ES61G8) which contained an undefined gap. Two plants of the 449 homozygotes were heterozygous for the parental ecotype at ES10P20-2 while displaying the \textit{lss} phenotype, while four plants were heterozygous for the parental ecotype at MtLAX5 while displaying the \textit{lss} phenotype. Markers between these two displayed the homozygous A17 genotype on all \textit{lss} homozygotes.

Based on sequence data from TIGR \textit{Medicago truncatula} database (http://www.tigr.org/tdb/e2k1/mta1/), we were able to build a contig overlapping with BAC mth2-78C5 (AC149293) and reduce the gap. The position of the contig was tested by designing a SNP marker, AJ25M19, on BAC mth2-25M19 (AC148346) and analyzing this marker on plants from the mapping population. In our analysis, we included plants
whose genotypes at ES10P20-2, MtLAX5, PCT and DENP had already been determined. Based on the genotypes obtained for these plants at AJ25M19, we were able to place this marker in relation to the other known markers. Once the contig’s position was confirmed, we analyzed the 4 homozygotes that were heterozygous for the parental ecotype at MtLAX5. This resulted in obtaining 2 plants that were heterozygous for the parental ecotype at AJ25M19 while displaying the lss phenotype. We were able to narrow the region to approximately 1400 kb and also jump the undefined gap. Upon expansion of the mapping population to include 692 homozygotes, we obtained 1 plant that was heterozygous for the parental ecotype at AJ16C13-B. This reduced the region to approximately 810 kb and this area is completely sequenced (Fig 19D). A list of the 175 genes predicted by FGENESH in this region appears in the Appendix.

In our mapping effort, we have analyzed 3662 total plants out of which 692 were homozygous for lss and displayed the lss phenotype. Unlike the initial cross to A17, chi-square analysis of proportion of homozygotes segregating for lss or containing only lss phenotypes was not consistent with the expected ratio of 3:1 ($\chi^2 = 72.74$, P $>> 0.001$) segregation in the F2 progeny of a cross between lss and A20. The low number of homozygotes that we obtained in the mapping population may be influenced by the number of albino plants that we observed in the F2 population. Albino plants segregate out from every A17 x A20 in a ratio that suggests a two gene interaction (Varma Penmentsa, personal communication). These plants die prematurely, so their nodulation pattern can’t be scored.
Fig 19A. *lss* maps to bottom of linkage group 4 of *M. truncatula*. Gross positioning of *lss* on LG 4 as determined by initial mapping using 22 homozygotes displaying the supernodulation phenotype (see Materials and methods). Distances on the left side of chromosome are in centimorgans based on the mapping population in A17XA20 public map. Markers are from http://www.medicago.org.
Fig 19 B. Color Map representation of *lss* co-segregating with markers at bottom of LG4. 20 randomly selected plants from the F2 population of *lss*/A20 cross exhibiting the *lss* supernodulation phenotype are represented in the above color map with their genotypes at each marker on LG4, LG1, LG3 and LG8. A (green) indicates A17 genotype; B (red) indicates A20 genotype; H (yellow) indicates Heterozygous genotype of each plant at that particular marker; (-) indicates plants of unknown genotype.
Fig 19 C & D. Fine mapping of \textit{lss}. Physical contig around the \textit{lss} locus after fine mapping in larger population (see Materials and methods). Numbers above the chromosome line indicate recombination events observed in homozygotes between markers in the population. Markers are described in the marker table (see Materials and Methods). BAC numbers refer to the \textit{Medicago truncatula} library. Distance in kb is determined from Medicago Sequencing Resources available at http://www.medicago.org.
**lss/sunn heterozygotes express both alleles but have an intermediate amount of SUNN expression in shoots**

When a plant is heterozygous for both the *lss* and *sunn* mutations, it displayed a supernodulation phenotype. To study what happens to SUNN expression in shoots when we have one mutated copy of each *LSS* and *SUNN*, quantitative RT-PCR was performed on samples generated from whole shoots from the F1 of a cross between *lss* and *sunn-2*. Analysis of data revealed that *lss/sunn-2* heterozygotes have an intermediate amount of *SUNN* RNA when compared to its levels in wild type and *lss* shoots (Fig 20A).

In order to determine, if this reduced expression was due to expression from one allele while the other was silenced or if both alleles are expressed at reduced levels, we analyzed the *lss/sunn-2* heterozygote cDNA with the *SUNN*-2 marker. This CAPS marker allows us to distinguish between wild type and mutant *SUNN* message by a change in size of the product after digestion with restriction enzyme. We found that the *lss/sunn-2* heterozygote expressed both the alleles but at reduced levels (Fig 20B).
**Fig 20. lss/sunn heterozygotes express both alleles but at reduced levels.** A. Relative amount of SUNN expression in lss/sunn heterozygote shoots compared to wild type and lss shoots. Total RNA was extracted from shoots of plants, inoculated with rhizobia 4 d after the start of the experiment. mRNA levels were determined by RT-PCR analysis. The gene, Secret Agent was used as an internal control (see Methods). Values are mean from a biological replicate with three technical replicates. Values are normalized to wild type levels. B. lss/sunn heterozygotes express both alleles. Lane 1; Hyperladder I. Lane 2; A17 genomic DNA. Lane 3; sunn-2 genomic DNA. Lane 4; A17 cDNA. Lane 5; lss X sunn-2 (F1) cDNA. All samples from Lanes 2-5 were analyzed with a sunn-2 marker, which is a CAPS marker that cuts with BsmAI restriction enzyme in sunn-2 but not in wild type. A band of size 414 bp is obtained from A17 after digestion with BsmAI and bands of sizes 329 bp and 85 bp are obtained from sunn-2.
**lss exhibits reversion in a randomly grown population**

In two separate experiments, Lucinda Smith (personal communication) and I isolated 3 revertants from randomly grown population of 600 lss homozygotes. The 3 revertants displayed a wild type level of nodulation and had longer roots when compared to the other supernodulators. In order to check the genotype of the 3 revertants, they were allowed to self and their progeny were examined.

![Fig 21. Wild types and supernodulators segregate in the progeny from the 3 lss revertants. A. Average nodule number of progeny from the 3 lss revertants. Plants with normal nodulation and supernodulation segregated in a 3:1 ratio. A17 and lss were grown as controls and the values are from 14 d-old plants grown aeroponically, inoculated with rhizobia 4 d after the start of the experiment. B. Average root length in cm of the same plants. Results are means (n=127 for wild type; n=39 for supernodulators; n=15 for control plants) ± SE. Values denoted with an * and ** are significantly different from that of the wild type at the 0.05 and 0.01 confidence level (Student’s t test).](image-url)
The progeny from the revertants produced plants that segregated with a wild type level of nodulation and supernodulators in a 3:1 ratio ($\chi^2 = 0.201$, $P < 0.05$; Fig 21). This suggests that the revertants were heterozygous for the \textit{lss} mutation.

\textit{The SUNN promoter and 3’UTR region is not differentially methylated in \textit{lss}}

So far we have observed that \textit{lss} does not complement any of the \textit{sunn} alleles but wild type plants segregate out in F2 of the \textit{lss/sunn} cross. The \textit{SUNN} gene and another 7 kb region upstream of \textit{SUNN} including \textit{RLP1} had no nucleotide difference in \textit{lss}. The \textit{SUNN} expression is dramatically reduced in \textit{lss} shoots and we also obtained revertants from a randomly grown population of \textit{lss} mutants. Such similarities have been reported between \textit{SUP} (\textit{SUPERMAN}) and its epiallele \textit{clk} (\textit{clark kent}) in Arabidopsis (Jacobsen and Meyerowitz 1997). This led us to consider if \textit{lss} was an epiallele of \textit{SUNN}. In our analysis we included 900 bp of the \textit{SUNN} promoter and 300 bases in the 3’UTR region of \textit{SUNN} to look at methylation pattern in wild type and \textit{lss}. Since, \textit{SUNN} is differentially expressed in \textit{lss} shoots, we looked at the methylation pattern of these regions in both shoots and roots. Bisulfite sequencing of one strand of these regions showed that the \textit{SUNN} promoter is heavily methylated in both wild type and \textit{lss}. In our analysis we looked at several clones that were obtained by amplification of the regions with specially designed primers after bisulfite conversion of the shoot and root DNA (See Materials and Methods). In our analysis, we used the BiQ Analyzer (Bock et al. 2005), which takes into account the percentage of conversion of C to Ts, or G to As and I used a minimum threshold conversion level of 50% in all cases to analyze the level of methylation.
Fig 22. A representation of methylation pattern in SUNN promoter in wild type and lss shoots and roots. 313 bp and 333 bp of the SUNN promoter analyzed by bisulfite sequencing in wild type and lss shoots and roots. The bars indicate the position of the CpG islands in the two fragments. Yellow is used to indicate the extent of methylation associated with that CpG island in the clones tested. Blue is used to indicate extent of non-methylation associated with that CpG island in the clones tested (see Materials and Methods).
The CpG islands were heavily methylated in both shoot and root tissues of wild type and *lss* plants but the pattern of non-CG methylation was variable with very less preference for sequence context. There was no significant differential methylation to suggest that altered methylation of the *SUNN* promoter led to the *lss* phenotype (Fig 22).
Growth characteristics: Similarities between lss and sunn

‘Autoregulation of nodulation’ (AON) refers to systemic feedback regulation in legumes whereby earlier nodulation events, or already formed nodules, suppress the formation of new nodules once an optimum number of nodules are formed (Caetano-Anolles and Gresshoff, 1991). So far only a few supernodulation mutants have been characterized and published including the orthologs sym29/ har1/ sunn/ nark (pea/ L. japonicus/ M.truncatula/ soybean) which are LRR receptor kinases, astray (a hy5 ortholog) and klavier (lesion unknown) in L. japonicus, nod3 (lesion unknown) in pea and skl (ethylene receptor) in M.truncatula (Carroll et al. 1985;Carroll et al. 1985a;Carroll et al. 1985b;Nishimura et al. 2002a;Nishimura et al. 2002b;Penmetsa and Cook 1997;Penmetsa et al. 2003;Sagan and Duc 1996;Sidorova and Shumnyi 2003;Szczyglowski et al. 1998;Wopereis et al. 2000b). These mutants not only form abundant nodules, but unlike wild type plants, form nodules in the presence of nitrate and have shorter root length than wild type plants in the presence or absence of rhizobia.

While all supernodulation mutants to date have been chemically induced, the lss supernodulation mutant is naturally occurring in origin. Based on our findings, the lss supernodulation mutant behaves very similarly to the sunn mutant in certain respects. The nodule number and short root phenotype displayed by lss mutants after rhizobial inoculation is very similar to the sunn phenotype when grown aeroponically (Fig 1;
Penmetsa et al. 2003; Schnabel et al. 2005) and on plates containing agar based media (Fig 2; van Noorden et al. 2006). The short root phenotype of lss could be attributed to curtailed cortical cell length as in L japonicus har 1 (Wopereis et al. 2000b) and M. truncatula sunn (van Noorden et al. 2006). When grown in absence of rhizobia under aeroponic conditions, wild type, lss and sunn had longer roots than in presence of rhizobia (Fig 5A). This indicates rhizobial inoculation and subsequent nodule formation does have a role in the short root length phenotype.

Besides forming abundant root nodules and displaying altered root length phenotypes, the supernodulation mutants are also characterized by the formation of nodules in the presence of nitrate (Carroll et al. 1985a; Carroll et al. 1985b; Schnabel et al. 2005; Wopereis et al. 2000b). When grown in presence of nitrate, wild type plants do not form nodules, but in sunn plants (Schnabel et al. 2005; van Noorden et al. 2006) and lss plants nodules are formed (Fig 6A). The root length phenotype in wild type, sunn and lss, however, is not affected by the presence of nitrate (Fig 6B), but, sunn and lss, still formed shorter roots than wild type in the presence of nitrate.

Previously performed grafting experiments have shown that lss phenotype is determined by the shoot genotype (Lucinda Smith, personal communication). This suggests there is a shoot to root signal in lss that determines the number of nodules that form in the roots. This shoot control of the root phenotype has also been observed in sunn (Penmetsa et al., 2003).
**Genetics and Mapping: Similarities between lss and sunn**

**lss does not complement sunn-1/-2/-4 alleles**

The *SUNN* gene encodes a leucine-rich repeat –receptor like kinase (LRR-RLK) consisting of 21 extracellular LRRs, a short transmembrane domain, and cytoplasmic serine/threonine kinase domain. The *SUNN* alleles include a missense mutation in the kinase domain (*sunn-1*), a missense mutation in a residue between two LRR (*sunn-2*), a truncation at the end of the kinase domain (*sunn-3*), an almost complete truncation of the protein (*sunn-4*) and a truncation of the kinase domain (*sunn-5*) (B. Seifert, pers comm.; Schnabel et al. 2005). Since *lss* does not complement *sunn-1/-2/-4* in the F1 progeny from any cross (Fig 17), *lss* could be an allele of *SUNN*.

**lss maps to the bottom of LG4**

The *lss* lesion segregates as a single, recessive locus (Fig 16). The lesion maps to the bottom of LG4 in 810 kb region between ES10P20-2 and AJ16C13-B markers (Fig 19C). This area also contains the *SUNN* locus. The reduced number of mapping plants exhibiting recombination events in this region was previously noted during the effort to map the *SUNN* locus (Schnabel et al. 2003).

*lss* does not complement *sunn-1/-2/-4* in the F1 progeny from any cross and *lss* maps to a region on the bottom of LG4 where the *SUNN* gene also resides. These findings tend to suggest that *lss* is an allele of *SUNN*.
Table 7. Summary of growth characteristics: Similarities between *lss* and *sunn"

<table>
<thead>
<tr>
<th>Phenotype</th>
<th><em>lss</em> compared to WT</th>
<th><em>sunn</em> compared to WT</th>
<th><em>lss</em> compared to <em>sunn</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Nodule number - (aeroponic)</td>
<td>Higher</td>
<td>Higher</td>
<td>Similar</td>
</tr>
<tr>
<td>Root length- (aeroponic)</td>
<td>Shorter</td>
<td>Shorter</td>
<td>Similar</td>
</tr>
<tr>
<td>Root length – (aeroponic; no rhizobia)</td>
<td>Shorter</td>
<td>Shorter</td>
<td>Similar</td>
</tr>
<tr>
<td>Nodule number- (plate)(^A)</td>
<td>Higher</td>
<td>Higher</td>
<td>Similar</td>
</tr>
<tr>
<td>Root length- (plate)(^A)</td>
<td>Shorter</td>
<td>Shorter</td>
<td>Similar</td>
</tr>
<tr>
<td>Root length- (plate)(^B)</td>
<td>Shorter</td>
<td>Shorter</td>
<td>Similar</td>
</tr>
<tr>
<td>Root length in presence of nitrate</td>
<td>Shorter</td>
<td>Shorter</td>
<td>Similar</td>
</tr>
</tbody>
</table>

\(^A\)- when wild type, *lss* and *sunn* are grown together on same plates; B- when wild type, *lss*, and *sunn* are grown exclusively on separate plates.

**Growth characteristics: Differences between *lss* and *sunn***

Despite the similarity of some phenotypes shared by *lss* and *sunn*, *lss* responds differently from *sunn* under certain conditions and displays genetic differences as well. For example, when wild type, *lss* and *sunn* are grown separately (only plants of one genotype are grown) on plates and inoculated with rhizobia, nodule number in *sunn* mutants is almost halved as compared to when grown together with wild type and *lss* plants (Fig 3A). A possible explanation for this would be a differential response between *sunn* and *lss* to ethylene or some other gaseous hormone released by plants in the plates. Also, like wild type, *lss* appears insensitive to the gaseous hormone responsible for reduction in nodule numbers in *sunn*. As a result, for all our phenotypic comparison
experiments on plates we have grown wild type, lss and sunn plants together on the same plates to minimize environmental fluctuations.

We have observed lss and sunn forms shorter roots than wild type under aeroponic conditions and plate conditions. However, the growth pattern of lss roots is different from sunn especially when grown on plates. In lss mutants, prior to rhizobial inoculation, root growth is as rapid as observed in wild type but much faster than in sunn roots (Fig 4B & 4C). In fact, when lss plants are grown separately on plates (only lss plants, no other genotype), lss roots appear to grow faster than wild type until rhizobial inoculation (Fig 4C). But once the lss plants are inoculated with rhizobia root growth is arrested very quickly versus wild type and sunn, which still continue to grow. Rhizobial inoculation causes inhibition of auxin transport from shoot to root (van Noorden et al. 2006), so this arrest in root growth in lss could be attributed to an inability to respond normally to a signal cascade triggered by rhizobial inoculation.

In contrast to previous reports (Penmetsa et al. 2003; Schnabel et al. 2005) and in accordance with the findings of van Noorden et al (2006), the average number of lateral roots per cm are increased in sunn, as compared to wild type in these experiments, while lss plants behaved like wild type plants (Fig 5B). In L. japonicus har1 and soybean nts mutants (the orthologs of sunn) lateral roots per cm are also increased (Day et al. 1986; Wopereis et al. 2000b).

When grown in the presence of nitrate, both sunn and lss forms nodules while wild type does not (Fig 6A). However, the extent to which sunn and lss nodulate differs. In sunn although nodules are formed, the number is reduced to a level resembling wild
type plants in the absence of nitrate (Schnabel et al. 2005; van Noorden et al. 2006). Thus, nodulation in *sunn* was inhibited by nitrate but not abolished. In contrast, *lss* still supernodulates in presence of nitrate with no reduction in nodule numbers (Fig 6A). Thus the perception of nitrate by *lss* mutants is different from other known supernodulation mutants. Nitrate has been reported to inhibit nodulation by induction of ethylene, altering levels of flavonoids and phytohormones like auxin and cytokinins (Caba et al. 1998; Coronado et al. 1995; Mathesius et al. 2000). This further supports the suggestion that ethylene response might play a role in the supernodulation phenotype in *lss* mutants. Based on these observations and additional results discussed below, we argue that *lss* represents a new and unique supernodulation mutant.

**Table 8. Summary of growth characteristics: Differences between *lss* and *sunn***

<table>
<thead>
<tr>
<th>Phenotype</th>
<th><em>lss</em> compared to WT</th>
<th><em>sunn</em> compared to WT</th>
<th><em>lss</em> compared to <em>sunn</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Rate of root growth before inoculation</td>
<td>Similar</td>
<td>Slower</td>
<td>Faster</td>
</tr>
<tr>
<td>Rate of root growth before inoculation&lt;sup&gt;A&lt;/sup&gt;</td>
<td>Similar</td>
<td>Slower</td>
<td>Faster</td>
</tr>
<tr>
<td>Nodule number- (plate)&lt;sup&gt;B&lt;/sup&gt;</td>
<td>Higher</td>
<td>Higher</td>
<td>Higher</td>
</tr>
<tr>
<td>Rate of root growth before inoculation&lt;sup&gt;B&lt;/sup&gt;</td>
<td>Faster</td>
<td>Slower</td>
<td>Faster</td>
</tr>
<tr>
<td>Inhibition of nodulation by nitrate</td>
<td>Not sensitive</td>
<td>Less sensitive</td>
<td>Not sensitive</td>
</tr>
</tbody>
</table>

<sup>A</sup>- when wild type, *lss* and *sunn* are grown together on same plates; <sup>B</sup>- when wild type, *lss*, and *sunn* are grown exclusively on separate plates.
**Differential response of lss and sunn towards hormonal treatments**

Plant hormones like auxin and ethylene have been reported to play a significant role in nodulation and AON (Prayitno et al. 2006; van Noorden et al. 2006). Ethylene and auxin are involved in a crosstalk which ultimately determines the fate of nodule number and root length (Prayitno et al. 2006). Ethylene has been documented to induce auxin synthesis (Stepanova et al. 2005) and inhibit auxin transport (Burg and Burg 1966). Auxin on the other hand upregulates the transcription of ACC synthase, the enzyme required for the synthesis of ACC, a precursor for ethylene (Abel et al. 1995).

The *sunn* mutant has altered long distance auxin transport, having high auxin flux from shoot to root irrespective of rhizobial presence (van Noorden et al. 2006). However, the *sunn* mutant has a wild type ethylene response characterized by reduced nodule number and arrested root growth (Penmetsa et al. 2003). This is in contrast to the ethylene-insensitive supernodulation mutant *skl* in which both long distance and local auxin transport regulation is altered (Penmetsa et al. 2003; Prayitno et al. 2006). Previously performed grafting experiments have shown that *lss* phenotype is determined by the shoot genotype (Lucinda Smith, personal communication).

Application of auxin (IAA) at a concentration of 10 µM has been reported to reduce nodule number and root length in *sunn* mutants in presence of rhizobia (van Noorden et al. 2006). When we grow plants in the presence of this concentration of IAA, *sunn* mutants have significant reduction in nodule numbers (Fig 7A). In *lss* mutants however, there is an observed reduction in nodule numbers when compared to untreated plants but they still produce twice as many nodules as *sunn* (Fig 7A). This implies that *lss*
and *sunn* have different sensitivities towards IAA applied in this manner. The *sunn* mutant already has a high auxin flux (van Noorden et al. 2006), and it may be that addition of extra IAA causes auxin levels to rise to an inhibitory concentration. Preliminary work has shown that *lss* does not have the high auxin flux found in *sunn* mutants before rhizobial inoculation (personal communication; Uli Mathesius). As a result when extra IAA is added, the inhibitory concentration of auxin is not reached and nodule number is not drastically affected in *lss*. Alternatively, application of external IAA might stimulate the synthesis of ACC, the precursor of ethylene which ultimately leads to inhibition of nodule numbers (Abel et al. 1995). The *sunn* mutants have been shown to be sensitive towards ethylene and *lss* appears to have an altered sensitivity towards ethylene. Application of local auxin however, does not reduce the nodule numbers in *lss* (Fig 8A).

NPA is a known inhibitor of Polar Auxin Transport (PAT) and it affects the efflux of auxin out of cell (Casimiro et al. 2001; Sabatini et al. 1999). NPA reduces nodule numbers in *sunn* (van Noorden et al. 2006) but *skl* is insensitive to the treatment (Prayitno et al. 2006). Our data indicates that NPA did not reduce nodule numbers in *lss* plants inoculated with rhizobia while nodule number was greatly reduced in *sunn* (Fig 9A). Thus, even if auxin transport is blocked by NPA in *lss* mutants, the supernodulation phenotype is not affected. This could mean that nodule number regulation in *lss* is similar to *skl*, even though the signal in *lss* is a shoot derived signal whereas, in *skl* the signal is a root derived signal (Penmetsa et al. 2003). Alternatively, while wild type plants decrease auxin flux upon inoculation with rhizobia, there is an increased flux of auxin from shoot to root in *lss* upon inoculation with rhizobia (Ulie Mathesius, personal communication).
and NPA at the concentration used is unable to sufficiently block this. Grown in the presence of NPA and the absence of rhizobia, neither lss nor sunn formed any nodules and their average lateral roots per cm were similar (Fig 10B).

Based on these results and previous reports on involvement of ethylene in nodule number regulation (Penmetsa et al. 2003;Prayitno et al. 2006), lss appears to have an altered response towards ethylene. Upon treatment of whole plants with increasing concentrations of ACC the average nodule numbers decreased in lss as the concentration was increased in a dose dependent manner compared to untreated plants, however nodule numbers in lss were significantly higher than sunn mutants or wild type (Fig 11A). In fact, nodulation in sunn mutants was greatly reduced (Penmetsa et al. 2003) and sunn appeared to be much more sensitive than lss. Previous work with the skl mutant reported the involvement of ethylene in nodule number regulation from a root derived signal (Penmetsa et al. 2003;Prayitno et al. 2006). If the lss phenotype is indeed ethylene-dependent as our results suggest, this would be the first case of ethylene regulation of nodule number from a shoot derived signal.

The sensitivity of lss towards NPA and ethylene makes it similar to the skl mutant. In the skl mutant, it was reported that ethylene acts via regulation of transcription of auxin efflux carriers, MtPIN1 and MtPIN2 in the root tips of nodulating plants (Prayitno et al. 2006). In the sunn mutants, there was no alteration of MtPIN1 and MtPIN2 expression levels either at 24 h or 120 h post rhizobial inoculation in the root tips (Prayitno et al. 2006). Neither MtPIN1 nor MtPIN2 were altered in their expression levels before or after rhizobial inoculation (0, 24, 48 and 72h post inoculation) in lss
when compared to their levels in wild type and **sunn** (Fig 12A & Fig 12B) although we focused on the entire root rather than only the root tips. Either the **lss** lesion does not affect regulation of transcription of *MtPIN1* or *MtPIN2* or looking at expression pattern of whole roots yields different results than following their expression in root tips.

**Table 9. Summary of growth characteristics to hormonal treatments**

<table>
<thead>
<tr>
<th>Phenotype</th>
<th><strong>lss</strong> compared to WT</th>
<th><strong>sunn</strong> compared to WT</th>
<th><strong>lss</strong> compared to <strong>sunn</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibition of nodulation by IAA</td>
<td>Less sensitive</td>
<td>More sensitive</td>
<td><strong>Less sensitive</strong></td>
</tr>
<tr>
<td>Root growth inhibition by IAA</td>
<td>Similar</td>
<td>Similar</td>
<td>Similar</td>
</tr>
<tr>
<td>Inhibition of nodulation by NPA</td>
<td>Less sensitive</td>
<td>More sensitive</td>
<td><strong>Less sensitive</strong></td>
</tr>
<tr>
<td>Inhibition of nodulation by ACC</td>
<td>Less sensitive</td>
<td>More sensitive</td>
<td><strong>Less sensitive</strong></td>
</tr>
<tr>
<td>Root growth inhibition by ethylene</td>
<td>Similar</td>
<td>Similar</td>
<td>Similar</td>
</tr>
<tr>
<td><em>PIN1</em> and <em>PIN2</em> expression- roots</td>
<td>Similar</td>
<td>Similar</td>
<td>Similar</td>
</tr>
</tbody>
</table>

(0, 24, 48, 72 h pi)

**Genetics and Mapping: Differences between **lss** and **sunn****

We found that **lss** does not complement *sunn-1/-2/-4* in the F1 progeny from any cross. Intergenic non-complementation can occur with alleles from the same gene, but it can also occur when two separate genes are acting in series or parallel in a signal cascade which is sensitive to the dosage strength (Hawley and Walker 2003). In such a case, the progeny from the cross will not rescue the wild type phenotype even when the plant is heterozygote for both the mutations. Since wild type plants segregate
out in the F2 population of a *sunn-1/lss* cross, we conclude the two loci are distinct (Table 6). However, only 10% of the plants segregating out in F2 population were wild type, a figure lower than the 31.25% expected from two unlinked genes when plants heterozygous for both mutations are supernodulators (based on the F1) (Table 6). But mapping showed that *LSS* and *SUNN* are tightly linked (Fig 19C). Based on how closely they map (0.2 cM), the number of wild type plants we obtain in the F2 of *lss/sunn-1* cross is much higher than the expected number of wild type plants that might result due to recombination between *lss* and *sunn*, estimated at one to two plants.

Since sequencing of the *SUNN* gene showed no lesion in *lss* mutants as did sequencing of *RLP1* and the region between, the *lss* phenotype probably does not result from some lesion in this approximately 12 kb region.

We have seen that *lss* maps to the bottom of LG4 in a region that also contains the *SUNN* gene. Although the reduced number of mapping plants exhibiting recombination events in this region was previously noted during the effort to map the *SUNN* locus (Schnabel et al. 2003), the suppression of recombination events is more severe in the *lss* mapping effort compared to the *sunn* mapping. The *lss* lesion is in the background of the Jemalong cultivar while the *sunn* mutation is in the background of A17 ecotype which was derived from the Jemalong cultivar in *Medicago*. It may be that differences, including genome rearrangement between Jemalong and A17 might be a possible reason for this enhanced suppression of recombination rate, especially since the A17 genotype has a rearrangement in this area versus the other parent of the mapping cross, A20, which is also derived from Jemalong (Kamphuis et al. 2007). Inversion of a DNA fragment in
this region might result in the low recombination rate that we observe. Work is currently underway to look at correlation between the genotype and the phenotype of the wild type plants that segregated out in the F2 population from the lss X A20 mapping cross, to rule out any effect of the Jemalong background. A southern analysis is also underway to observe possible rearrangements. The lss contig is located away from either the centromeric or the telomeric region (Fig 19A), so the lower recombination rate due to either of them is unlikely. The region containing the lss contig is sequenced entirely and it contains 175 genes (See Appendix) as predicted by FGENESH, of which a high percentage are repetitive sequences and retro-elements. This large percentage of repetitive sequences could be a possible explanation for the lower rate of recombination in the region. In maize, recombination rate is suppressed significantly in a region that is gene-poor and contains a large cluster of methylated transposons (Fu et al. 2002). A cross of lss to a different mapping parent is underway in an effort to eliminate ecotype effects and to narrow the region containing the gene.

**lss locus is genetically not stable**

Independent experiments have revealed that lss reverts from the supernodulating, short root phenotype to the normally nodulating, long root phenotype at a rate of 0.5% (Fig 21). In addition, in our efforts to map the lss locus, we obtained 692 homozygotes out of a total mapping population of 3662 plants. The number of expected homozygotes that should segregate out from a population of 3662 plants in the F2 progeny is 916 in a cross between lss and A20. Although, we lost a percent of plants that were albino, which
is due to a genetic incompatibility of two genes between the mapping parents, there still exists a probability that a percentage of the \textit{lss} homozygotes reverted back to wild type. Work is underway to detect any of such revertants in the mapping population. In the F2 progeny from the \textit{lss/sunn-1} cross, we obtained 29 wild types segregating in a total population of 308 plants which is much higher than the expected one to two wild type plants that would arise from a reversion event.

\textbf{SUNN expression is suppressed in \textit{lss} shoots}

Quantitative Real Time PCR study of expression levels of \textit{SUNN} gene has shown that \textit{SUNN} is expressed in both shoots and roots of \textit{lss} mutants. In \textit{sunn-1} mutants the level of \textit{SUNN} transcript in the shoots is similar to the level in wild type shoots (Schnabel et al. 2005), while the level of \textit{SUNN} transcript is decreased to almost one-third of the wild type level in \textit{sunn-1} roots. I obtained similar results examining \textit{SUNN} transcript levels in \textit{sunn-1} roots and shoots. In \textit{lss} roots however, while the same reduction of \textit{SUNN} transcript is observed, the level of \textit{SUNN} transcript is drastically reduced in shoots (Fig 13). Thus, the reduced amount of \textit{SUNN} transcript observed in \textit{lss} shoots may be sufficient to generate the \textit{sunn} supernodulation and short root length phenotype in \textit{lss}.

Since in both \textit{lss} and \textit{sunn}, the shoot derived signal determines the root phenotype, the low expression of \textit{SUNN} in roots may be due to a feedback regulation of \textit{SUNN} activity in the shoots. This is currently being investigated in the Long lab by examining expression levels of \textit{SUNN} in grafted plants. The dramatic reduction of \textit{SUNN} transcript in \textit{lss} shoots suggests that \textit{LSS} could be a regulator of \textit{SUNN} expression in
shoots. LSS might be involved as an element that is required for normal transcription of the SUNN gene in shoots.

In wild type plants, the SUNN transcript levels increased 24 h after rhizobial inoculation in shoots compared to SUNN transcript levels at 0 h (Fig 14A). This suggests that as nodulation progresses an increase in SUNN transcript might be important for nodule number regulation. While we observe the same spike in SUNN transcript levels after 24 h of rhizobial inoculation in sunn mutant shoots, the protein being made from the transcript is altered in an amino acid and probably lacks kinase activity (Schnabel et al. 2005). Since the amount of SUNN transcript in lss shoots was greatly reduced at all time points when compared to levels in wild type shoots at the same time points, we were unable to detect an increased transcription in response to rhizobia due to the consistently low levels of SUNN transcript in lss shoots (Fig 14B).

In the cross between lss and sunn-2, we obtained a plant heterozygous for both the mutations that yield a supernodulation and short root phenotype. This heterozygote plant had intermediate amount of SUNN transcript levels in shoots when compared to SUNN transcript levels to wild type and the low level in lss shoots (Fig 20A) again suggesting that the lss lesion, even when present in one copy, reduces SUNN expression. Because RT-PCR results suggested this expression pattern in the heterozygote was due to expression of both the alleles at reduced levels instead of the silencing of one allele (Fig 20) the lss locus acts in trans to reduce SUNN expression.

RLP1 expression levels in lss roots and shoots were reduced when compared to its expression levels in wild type roots and shoots (Fig 15) in a pattern similar to what was
observed in *sunn* mutants (Schnabel et al. 2005). This suggests that *RLP1* expression is regulated similarly in *lss* and *sunn-1* mutants.

**Is *lss* an epiallele of *SUNN*?**

Jacobsen and Meyerowitz (Jacobsen and Meyerowitz, 1997) isolated epigenetic alleles (see Background on Epigenetics Section) of the *SUP (SUPERMAN)* gene in *Arabidopsis* which is a determinant of floral whorl patterning. The epialleles of the *SUP* gene were phenotypically very similar to *sup* phenotype and did not complement *SUP*. The epialleles were heavily methylated and *SUP* was transcriptionally silenced (Jacobsen and Meyerowitz 1997). Because some of the phenotypes of *lss* suggested it might be an epiallele of the *SUNN* gene, we examined the methylation pattern of 900 bases of one strand from the *SUNN* promoter and 300 bases in the 3’UTR region of the *SUNN* gene in *lss* roots and shoots and compared this to wild type. The *SUNN* promoter is heavily methylated in both wild type and *lss* roots and shoots (Fig 22). There was no obvious change suggesting the *SUNN* promoter is differentially methylated in *lss* to generate the supernodulation phenotype. However, unpublished results in our lab with a GUS-reporter construct suggest *SUNN* is expressed in a discrete subset of tissues in the shoot (around the vasculature). Our template DNA for bisulfite sequencing included whole shoot tissues, a mix of tissues that are expressing *SUNN* and tissues that are not. This mixing of tissues could be a reason for not detecting a methylation difference and the possibility that *lss* is differentially methylated in *SUNN* promoter still exists. We are
exploring the possibility of using Dr. Steve Ellis’s laser-capture dissection microscope to isolate the specific tissues expressing \textit{SUNN} for further analysis.
Table 10. Summary of important root and nodule phenotypes of *lss* and *sunn*

<table>
<thead>
<tr>
<th>Phenotype</th>
<th><em>lss</em> compared to WT</th>
<th><em>sunn</em> compared to WT</th>
<th><em>lss</em> compared to <em>sunn</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Nodule number - (aeroponic)</td>
<td>Higher</td>
<td>Higher</td>
<td>Similar</td>
</tr>
<tr>
<td>Root length- (aeroponic)</td>
<td>Shorter</td>
<td>Shorter</td>
<td>Similar</td>
</tr>
<tr>
<td>Nodule number- (plate)(^A)</td>
<td>Higher</td>
<td>Higher</td>
<td>Similar</td>
</tr>
<tr>
<td>Root length- (plate)(^A)</td>
<td>Shorter</td>
<td>Shorter</td>
<td>Similar</td>
</tr>
<tr>
<td>Rate of root growth before inoculation(^A)</td>
<td>Similar</td>
<td>Slower</td>
<td><strong>Faster</strong></td>
</tr>
<tr>
<td>Nodule number- (plate)(^B)</td>
<td>Higher</td>
<td>Higher</td>
<td><strong>Higher</strong></td>
</tr>
<tr>
<td>Root length- (plate)(^B)</td>
<td>Shorter</td>
<td>Shorter</td>
<td>Similar</td>
</tr>
<tr>
<td>Rate of root growth before inoculation(^B)</td>
<td>Faster</td>
<td>Slower</td>
<td><strong>Faster</strong></td>
</tr>
<tr>
<td>Inhibition of nodulation by nitrate</td>
<td>Not sensitive</td>
<td>Less sensitive</td>
<td><strong>Not sensitive</strong></td>
</tr>
<tr>
<td>Inhibition of nodulation by IAA</td>
<td>Less sensitive</td>
<td>More sensitive</td>
<td><strong>Less sensitive</strong></td>
</tr>
<tr>
<td>Root growth inhibition by IAA</td>
<td>Similar</td>
<td>Similar</td>
<td>Similar</td>
</tr>
<tr>
<td>Inhibition of nodulation by NPA</td>
<td>Less sensitive</td>
<td>More sensitive</td>
<td><strong>Less sensitive</strong></td>
</tr>
<tr>
<td>Inhibition of nodulation by ethylene</td>
<td>Less sensitive</td>
<td>More sensitive</td>
<td><strong>Less sensitive</strong></td>
</tr>
<tr>
<td><em>SUNN</em> expression- roots (8 dpi)</td>
<td>Lower</td>
<td>Lower</td>
<td>Similar</td>
</tr>
<tr>
<td><em>SUNN</em> expression- shoots (8 dpi)</td>
<td>Lower</td>
<td>Similar</td>
<td><strong>Lower</strong></td>
</tr>
<tr>
<td><em>SUNN</em> expression- shoots</td>
<td>Lower</td>
<td>Similar</td>
<td><strong>Lower</strong></td>
</tr>
<tr>
<td><em>(0, 24, 48, 72 h pi)</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>RLP-1</em> expression- roots (8 dpi)</td>
<td>Lower</td>
<td>Lower</td>
<td><strong>Lower</strong></td>
</tr>
<tr>
<td><em>RLP-1</em> expression-shoots (8dpi)</td>
<td>Lower</td>
<td>Lower</td>
<td><strong>Lower</strong></td>
</tr>
</tbody>
</table>
**lss interacts genetically with sunn**

Crosses between *lss* and *sunn* alleles (*sunn-1/-2/-4*) indicated that *lss* interacts with *sunn* at a genetic level. *lss* does not complement any of the *sunn* alleles in the F1, however, the segregation of wild type plants in F2 in the *lss/sunn-1* cross indicates that they are distinct loci. The inability to rescue the wild type phenotype in F1 might be a case where functional alleles of both the loci are needed to acquire the wild type phenotype. This is best summarized in the figure below.

**Fig 23. SUNN activity determines fate of nodulation regulation.** Nodule number state based on the possible genotypes at *SUNN* and *LSS* loci. Black boxes indicate 1 functional *SUNN* allele; Grey boxes indicate 1 functional *LSS* allele; White box indicates non-functional alleles of both *LSS* and *SUNN*. “?” indicates genotype combinations with unknown phenotypes. See text for discussion.

When there are two functional copies of *SUNN* and *LSS* alleles, a wild type phenotype is obtained with normal nodulation. When one copy of *SUNN* is not
functional, an intermediate phenotype is obtained (Penmetsa et al. 2003). When the plant is heterozygous for both \textit{lss} and \textit{sunn} mutations “activity” of whatever the signal is falls too low and the plants supernodulate. Work is underway to determine the phenotype of a plant when the plant is heterozygous for only \textit{lss} mutation. Finally, when both copies of either \textit{LSS} or \textit{SUNN} are not functional a supernodulation phenotype is obtained. It will be interesting to observe the phenotype when a plant has both copies of \textit{LSS} and \textit{SUNN} nonfunctional, or essentially a double mutant for both the loci. Because the genes are closely linked with similar phenotypes and we do not know the molecular nature of \textit{lss}, we have yet to isolate the double mutant.

\textbf{What can \textit{LSS} be?}

\textit{Involvement in the nodule number regulation}

Based on \textit{LSS} behavior towards the different hormones and its interactions with \textit{SUNN}, the model (Fig 24) is a representation of our understanding of how \textit{LSS} is involved in a pathway that regulates nodule number. The known interactions are depicted in solid lines and the partially known interactions are depicted in broken lines. \textit{LSS} and \textit{SUNN} are both shoot derived signals in which nodule number regulation is altered. We know that \textit{LSS} affects \textit{SUNN} expression in the shoots and evidence is accumulating suggesting a feedback regulation of \textit{SUNN} activity on \textit{SUNN} transcription in the roots. Our results suggest that ethylene is involved in nodule number regulation via \textit{LSS} either directly or indirectly. Auxin flux from shoot to root is altered in \textit{sunn} mutants and preliminary results from the Mathesius lab suggest that \textit{lss} has an auxin flux defect as
well, but a different kind from that seen in *sunn*. Scenario A (Fig 24) speculates that *LSS* perceives ethylene signal in the shoot and it affects *SUNN* expression directly which in turn regulates auxin loading from shoot to root and thereby regulate nodule numbers in roots. Scenario B (Fig 24) speculates that *LSS* and *SUNN* are both involved in auxin loading from shoot to root but *LSS* might affect auxin flux through ethylene directly and thereby regulate nodule numbers. Therefore, it would be informative to determine if ethylene in some way affects the auxin transport in wild type and *lss*. Combined with enhanced mapping and methylation status experiments, this should help to establish the role of *LSS* in nodule number regulation and its position in the pathway with respect to other autoregulation signals.
Fig 24. Two tentative pathways of nodule number regulation involving *LSS* in *Medicago truncatula*. Known interactions are in solid lines; partially known interactions are in broken lines.; double ended arrow indicates a feedback. See text for explanation.

**Identity of LSS**

The *lss* locus maps to the bottom of LG4 of *Medicago truncatula* and is currently positioned in an 810 kb region which also contains the *SUNN* gene. Based on the 175 predicted genes (FGENESH) in this contig and *LSS* behavior it has a high possibility to be any of the following –

- LysM domain containing receptor like kinase (2)
- Protein/s of unknown function (4)
- Hypothetical protein/s (14)
- Isoflavone reductase; isflavone 3’-hydroxylase; isoflavone glycosyltransferase (5)
- Protein kinase (4)
- Transcription factor (4)
- Auxin/ethylene regulatory genes (6)

However, the possibility that LSS affects SUNN expression by differential methylation of the SUNN gene still exists. The revertants that we obtained from a randomly growing lss population may be because of hypomethylation of a methylated region in the SUNN gene. Alternatively, the transport of microRNAs in phloem has been shown to regulate auxin signals for lateral root development in Arabidopsis (Guo et al. 2005). So the role of a mobile microRNA in suppressing SUNN expression in lss shoots is also a possibility.
APPENDIX

Genes in the lss contig predicted by FGENESH

1- H+-transporting two-sector ATPase, C (AC39) subunit
2- Helix-loop-helix DNA-binding
3- Polynucleotidyl transferase, Ribonuclease H fold
4- LysM domain-containing receptor-like kinase 1; isoflavone 3'-hydroxylase
5- Tetratricopeptide-like helical
6- Zinc finger, CCCH-type
7- Aminoacyl-transfer RNA synthetase
8- Mitochondrial transcription termination factor-related
9- IMP dehydrogenase/GMP reductase, putative
10- RNA-binding region RNP-1 (RNA recognition motif)
11- Nucleic acid-binding, OB-fold
12- Protein of unknown function DUF889
13- Protein of unknown function DUF946
14- RNA-directed DNA polymerase (Reverse transcriptase)
15- RNA-binding region RNP-1 (RNA recognition motif); LysM domain-containing receptor-like kinase 3
16- Ribosomal protein L33; Nucleic acid-binding, OB-fold
17- RNA-directed DNA polymerase
18- Ribosomal protein L33; Nucleic acid-binding, OB-fold
19- hypothetical protein MtrDRAFT_AC174467g25v1; Leucine-rich repeat, plant specific
20- RNA polymerase alpha subunit
21- No significant similarity found
22- RNA-binding region RNP-1 (RNA recognition motif)
23- Ubiquitin
24- Polynucleotidyl transferase, Ribonuclease H fold
25- Peptidase arpartic
26- RNA-directed DNA polymerase (Reverse transcriptase)
27- Probable Histone 2B
28- Probable Histone 2B
29- beta glucosidase-like protein
30- Terpene synthase-like; Terpenoid synthase
31- Heat shock protein Hsp70
32- Chalcone and stilbene synthases
33- No predicted genes
34- No predicted genes
35- Peroxidase precursor
36- DDT; Homeodomain-related
37- Ribonuclease H
38- GH3 auxin-responsive promoter
39- hypothetical protein MtrDRAFT_AC182813g33v2
40- Helix-loop-helix DNA-binding
41- No predicted genes
42- Tetratricopeptide-like helical
43- isoflavone reductase
44- hypothetical protein MtrDRAFT_AC158497g41v2
45- isoflavone reductase
46- isoflavone reductase
47- Ribosomal RNA methyltransferase RrmJ/FtsJ
48- Leucine-rich repeat, plant specific; Auxin transporter-like protein 5 (AUX1-like protein 5) (MtLAX5)
49- probable retroelement pol polyprotein - , putative; nodulation receptor kinase
50- polyprotein-like, putative
51- Protein kinase, putative
52- AAA ATPase; ABC transporter, transmembrane region, type 1
53- lactate/malate dehydrogenase
54- Ubiquitin-conjugating enzyme, E2
55- hypothetical protein MtrDRAFT_AC149130g5v2
56- hypothetical protein MtrDRAFT_AC167711g40v28
57- Zinc finger, CCHC-type, putative
58- Short-chain dehydrogenase/reductase SDR
59- Short-chain dehydrogenase/reductase SDR
60- Ribonuclease H
61- Glycosyl transferase, family 28
62- Chaperonin Cpn60/TCP-1
63- Pentatricopeptide repeat
64- Cyclin-like F-box; F-box protein interaction domain
65- hypothetical protein MtrDRAFT_AC148396g9v2
66- Zinc finger, CCHC-type
67- class I KNOX homeobox transcription factor
68- No predicted genes
69- High mobility group proteins HMG-I and HMG-Y; Linker Histone
70- hypothetical protein MtrDRAFT_AC155885g6v2
71- Epsin, N-terminal; ENTH/VHS
72- hypothetical protein MtrDRAFT_AC155881g14v1; Protein kinase
73- 2-oxo acid dehydrogenase, lipoyl-binding site
74- RNA polymerase beta
75- 2-oxo acid dehydrogenase, lipoyl-binding site
76- Epoxide hydrolase
77- Polynucleotidyl transferase, Ribonuclease H fold
78- nodule-specific cysteine-rich peptide 209
79- Transcriptional factor B3
80- Ribonuclease H
81- Cellular retinaldehyde binding/alpha-tocopherol transport
82- hypothetical protein MtrDRAFT_AC140551g11v2
83- Cyclin-like F-box
84- squalene monooxygenase 2
85- O-methyltransferase, family 2; Dimerisation
86- Polynucleotidyl transferase, Ribonuclease H fold
87- Cellular retinaldehyde binding/alpha-tocopherol transport
88- HECT; Ubiquitin
89- Cellular retinaldehyde binding/alpha-tocopherol transport
90- Cellular retinaldehyde binding/alpha-tocopherol transport
91- hypothetical protein MtrDRAFT_AC147774g3v1
92- Terpenoid cyclases/protein prenyltransferase alpha-alpha toroid
93- Glycosyltransferase
94- No predicted genes
95- cAMP response element binding (CREB) protein
96- RNA-directed DNA polymerase (Reverse transcriptase)
97- hypothetical protein MtrDRAFT_AC155890g41v2
98- Pre-mRNA processing ribonucleoprotein, binding region
99- Protein kinase
100- (iso)flavonoid glycosyltransferase
101- RLP1 leucine-rich repeat receptor-like protein
102- hypothetical protein MtrDRAFT_AC153125g45v2
103- Transcriptional factor B3
104- CLV1-like receptor kinase- SUNN
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Polynucleotidyl transferase, Ribonuclease H fold
Ribonuclease H
Probable histone H2B
ankyrin repeats
acyl desaturase
auxin efflux carrier protein; transcription factor B3
Kinesin, motor region
Transcriptional factor B3
putative sucrose-H+ symporter
peroxidase precursor
hypothetical protein MtrDRAFT_AC160012g36v2; LRR
Putative non-LTR retroelement reverse transcriptase
No predicted genes
Root phototropism protein 3
Integrase, catalytic region
hypothetical protein MtrDRAFT_AC158502g23v2; LRR
gag-pol polyprotein, putative
No predicted genes
AML5; RNA binding proteins
lecithine cholesterol acyltransferase-like protein
Peptidylprolyl isomerase, FKBP-type
Protein of unknown function UPF0044
149- hypothetical protein MtrDRAFT_AC150800g41v2
150- IMP dehydrogenase/GMP reductase, related
151- IMP dehydrogenase/GMP reductase, related
152- Putative ion channel DMI-1
153- Polynucleotidyl transferase, Ribonuclease H fold
154- progesterone 5-beta-reductase, putative
    hypothetical protein MtrDRAFT_AC136139g1v2
155- No predicted genes
156- hypothetical protein MtrDRAFT_AC153125g21v2
157- phosphatidylinositol 3-kinase; LRR
158- Thioredoxin domain 2; Thioredoxin fold
159- RNA-directed DNA polymerase
160- Ribosomal protein S6e
161- Ubiquitin-conjugating enzyme, E2
162- TGF-beta receptor, type I/II extracellular region
163- Acetyl-CoA carboxylase carboxyltransferase beta subunit
164- Polynucleotidyl transferase, Ribonuclease H fold
165- RNA-directed DNA polymerase (Reverse transcriptase)
166- RNA-directed DNA polymerase, related
167- hypothetical protein MtrDRAFT_AC149642g29v2
168- No predicted genes
169- farnesyl-diphosphate farnesyltransferase
170- Pectinesterase; Pectinesterase inhibitor
171- ADP-ribosylation factor 1
172- Homeodomain-like; Protein kinase
173- Proteinase inhibitor I9, subtilisin propeptide
174- Homeodomain-like; Protein kinase
175- Proteinase inhibitor I9, subtilisin propeptide
REFERENCES


Thimann KV (1936b) On the physiology of the formation of nodules on legumes roots. PNAS 22: 511-514.


Subject:  Fwd: R: Re: book chapter
From: "Julia Frugoli" <jfrugol@CLEMSON.EDU>
Date:    Thu, December 6, 2007 3:52 pm
To:    "arijit mukherjee" <amukher@CLEMSON.EDU>
Priority:    Normal
Options:    View Full Header | View Printable Version | View Message details | Add to Addressbook | Bounce

Begin forwarded message:

> From: "anna.capasso8@tin.it" <anna.capasso8@tin.it>
> Date: February 2, 2007 7:52:55 AM EST
> To: <jfrugol@CLEMSON.EDU>
> Subject: R: Re: book chapter
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> Dear Julia
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> introduction to his thesis. We
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> 
> Best Regards
> 
> Anna
> 
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"Every step of the journey is the journey"

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