EVIDENCE FOR MULTIPLE FUNCTIONS OF A *Medicago truncatula* TRANSPORTER

Ying-Sheng Huang

Thesis Prepared for the Degree of

MASTER OF SCIENCE

UNIVERSITY OF NORTH TEXAS

December 2014

APPROVED:

Rebecca Dickstein, Major Professor
Brian Ayre, Committee Member
Daniel Kunz, Committee Member
Sam Atkinson, Chair of the Department of Biological Sciences
Mark Wardell, Dean of the Toulouse Graduate School

Huang, Ying-Sheng. Evidence for Multiple Functions of a *Medicago truncatula* Transporter. Master of Science (Biochemistry), December 2014, 76 pp., 10 tables, 12 figures, references, 98 titles.

Legumes play an important role in agriculture as major food sources for humans and as feed for animals. Bioavailable nitrogen is a limiting nutrient for crop growth. Legumes are important because they can form a symbiotic relationship with soil bacteria called rhizobia that results in nitrogen-fixing root nodules. In this symbiosis, rhizobia provide nitrogen to the legumes and the legumes provide carbon sources to the rhizobia.

The *Medicago truncatula NPF1.7/NIP/LATD* gene is essential for root nodule development and also for proper development of root architecture. Work in our lab on the *MtNPF1.7/MtNIP/LATD* gene has established that it encodes a nitrate transporter and strongly suggests it has another function. *Mtnip-1/latd* mutants have pleiotropic defects, which are only partially explained by defects in nitrate transport. MtNPF1.7/NIP/LATD is a member of the large and diverse NPF/NRT1(PTR) transporter family. NPF/NRT1(PTR) members have been shown to transport other compounds in addition to nitrate: nitrite, amino acids, di- and tri-peptides, dicarboxylates, auxin, abscisic acid and glucosinolates.

In *Arabidopsis thaliana*, the AtNPF6.3/NRT1.1(CHL1) transporter was shown to transport auxin as well as nitrate. *Atchl1* mutants have defects in root architecture, which may be explained by defects in auxin transport and/or nitrate sensing. Considering the pleiotropic phenotypes observed in *Mtnip-1/latd* mutant plants, it is possible that MtNPF1.7/NIP/LATD could have similar activity as AtNPF6.3/NRT1.1(CHL1).

Experimental evidence shows that the *MtNPF1.7/NIP/LATD* gene is able to restore nitrate-absent responsiveness defects of the *Atchl1-5* mutant. The constitutive expression of

MtNPF1.7/NIP/LATD gene was able to partially, but not fully restore the wild-type phenotype in the *Atchl1-5* mutant line in response to auxin and cytokinin. The constitutive expression of *MtNPF1.7/NIP/LATD* gene affects the lateral root density of wild-type Col-0 plants differently in response to IAA in the presence of high (1mM) or low (0.1 mM) nitrate. *MtNPF1.7/NIP/LATD* gene expression is not regulated by nitrate at the concentrations tested and *MtNPF1.7/NIP/LATD* does not regulate the nitrate-responsive *MtNRT2.1* gene. *Mtnip-1* plants have an abnormal gravitropic root response implicating an auxin defect. Together with these results, *MtNPF1.7/NIP/LATD* is associated with nitrate and auxin; however, it does not act in a homologous fashion as *AtNPF6.3/NRT1.1(CHL1)* does in *A. thaliana*.

ACKNOWLEDGEMENTS

I would like to express the deepest appreciation to my major professor, Dr. Rebecca Dickstein for her sponsorship, her great interest and her assistance in the pursuit of these studies and in the preparation of this thesis. Without her guidance and persistent help this thesis would not have been possible.

I would like to thank my committee members, Dr. Brian Ayre and Dr. Daniel Kunz, for all of their feedback and suggestions that have helped me complete my studies. I would also like to thank Dr. Kent Chapman for allowing me to use his camera station to take pictures for my root phenotype experiments any time during all these years.

I would like to thank all of the current lab members including Naudin Alexis, Khem Kadel, Dr. Vijay Veerappan, Ashely Scott, and David Burks and former lab members including Matthew Meckfessel, Mohammad Salehin, Rammyani Bagchi and Dr. Sarah Adeyemo for making Dickstein Lab a good team to work together.

I would like to thank Dr. Sarah Holt for teaching me how to do strawberry transformation and Dr. Swati Tripathy for teaching me how to do tobacco transformation.

In addition, I would like to thank Aswad Khadilkar and Sara Pruitt from Dr. Ayre's lab, Bikash Adhikari, Jantana Keereetaweep, and Yingqi Cai from Dr. Chapman's lab for their support and friendships.

Finally, my biggest thanks goes to my parents for their love and support at all times and Yi-Wen Wang, the love of my life, for everything she has done for me, especially during the time I was injured from the car accident. I couldn't have done any of this without them.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

ABA: Abscisic acid

BAP: 6-Benzylaminopurine (cytokinin)

DAT: Day after turning

ENOD: Early nodulin

Gln: L-Glumatine

IAA: Indole-3-acetic acid (auxin)

KCl: Potassium chloride

KNO_3: Potassium nitrate

L.japonicus: *Lotus japonicus*

LjLHK: *Lotus japonicus Lotus* histidine kinase

LR: Lateral roots

MtCRE: *Medicago truncatula* cytokinin receptor

MtDMI: *Medicago truncatula* does not make infection

MtERN: *Medicago truncatula* ERF required for nodulation

MtLYK: *Medicago truncatula* lysine motif domain containing receptor-like Kinase

MtNIN: *Medicago truncatula* nodule inception

MtNPF1.7NIP/LATD: *Medicago truncatula* nitrate peptide tranposter family 1.7/numerous infections and polyphenolics/lateral root-organ defective

MtNSP: *Medicago truncatula* nodulation-signaling pathway

M.truncatula: *Medicago truncatula*

NH_4Cl: Ammonium chloride

NOD: Nodulin

NPF: NRT1/PTR Family

NRT1/PTR: Nitrate transporter 1/peptide transporter

NS: no significant

OE: Over expression

PR: Primary roots

S.D.: Standard deviation

S.E.: Standard error

CHAPTER 1

INTRODUCTION

1.1 Nitrogen and Symbiotic Nitrogen Fixation

Nitrogen is an essential component of many major biomolecules, such as nucleic acids, amino acids, and many vitamins. Although 78.1% of the earth's atmosphere consists of nitrogen gas, plants cannot use nitrogen in this form. Bioavailable nitrogen is usually limited in soils. Legume plants have evolved a symbiotic relationship with several classes of soil bacteria collectively known as rhizobia that are able to reduce nitrogen gas into forms of nitrogen that plants can use in a process known as symbiotic nitrogen fixation. In the nitrogen fixing symbiosis with rhizobia, plant hosts supply photosynthates to the bacteria in exchange for reduced nitrogen, which allows plant legumes to thrive in soils with poor nitrogen content.

Medicago truncatula, commonly known as barrel medic, is a small legume native to the Mediterranean region. It has been chosen as a model organism for studying symbiotic nitrogen fixation among temperate legumes which form indeterminate nodules. *Lotus japonicus* has been chosen as a model organism for studying symbiotic nitrogen fixation in plants forming determinate nodules, which typically form on tropical legume roots. *M. truncatula* and *L. japonicus* are diploid, have relatively small genomes as compared to other legumes, are self-fertile, have prolific seed production in a relatively short generation time, and are capable of genetic transformation with *Agrobacterium species* (Handberg and Sstougaard, 1992; Cook et al., 1997; Cook, 1999; Léran et al., 2013). The genomes of *M. truncatula* and *L. japonicus* are currently partially sequenced and researchers are working progressively toward completion (Sato et al., 2008; Young et al., 2011; Tang et al., 2014).

Legume nodules are unique root organs, specifically formed for the nitrogen-fixing symbiosis with rhizobia, and as mentioned in the previous paragraph, have two major morphological types: indeterminate and determinate. Generally, indeterminate nodules are found on temperate legumes, such as *Medicago sativa*, *Trifolium repens*, *Pisum sativum*, and *M. truncatula*. Indeterminate nodules have persistent meristems, which are responsible for their cylindrical shape. Indeterminate nodule apical meristems are able to produce new cells that are continuously infected by rhizobia (Ferguson et al., 2010). Determinate nodules are usually found on the subtropical and tropical legumes, such as *Glycine max*, *Phaseolus vulgaris*, *Pongamia pinnata* and *L. japonicus*. Determinate nodules are usually spherical and have non-persistent meristems (Ferguson et al., 2010). Despite differences between indeterminate and determinate nodules, nodulation, the process of nodule formation, shares common developmental features in all legumes.

1.2 Overview of Nodule Development

Nodulation starts when legume plants release flavonoids into soil in response to low environmental nitrogen. This initiates communication between host plants and the rhizobia (Brewin, 1991). Flavonoids attract rhizobia to the root and trigger the activation of rhizobial *nod* (nodulation) genes which encode proteins that produce and secrete lipo-chito-oligosachharides, also as known as Nod factors (Long, 1996). In the current model of the Nod factor signaling pathway in *M. truncatula*, the MtNFP and MtLYK3/4 proteins are putative Nod factor receptors in the plant root hairs and responsible for Nod factor perception (Amor et al., 2003; Limpens et al., 2003; Smit et al., 2007). Thereafter, Nod factor perception stimulates a downstream signal transduction cascade in the plant root epidermis and root cortex, which involves 1) activation of

the *MtDMI2* gene that encodes a leucine-rich-repeat receptor-like kinase containing serine/threonine kinase domains, 2) a rapid influx of calcium ions through a calcium ion-channel protein localized in the nuclear membrane encoded by the *MtDMI1* gene, and 3) activation of the calcium-calmodulin kinase encoded by the *MtDMI3* gene mediated by induction of calcium fluxes ("calcium spiking")(Cullimore and Denarie, 2003; Mitra et al., 2004; Oldroyd and Downie, 2004; Peiter et al., 2007; Venkateshwaran et al., 2008). In *M. truncatula*, upon activation of the MtDMI3 protein, four transcription factors: MtNSP1, MtNSP2, MtERN1 and MtNIN, work together to activate the production of cytokinins in root epidermal cells and regulate the early nodulation *(ENOD*) genes (Wais et al., 2000; Oldroyd and Long, 2003; Smit et al., 2005; Oldroyd, 2007), and are also required for cell division events in the root cortex (Heckmann et al., 2006). Cytokinins, working as mobile signals, are then delivered from the epidermal layer to the inner cortical cells (Oldroyd, 2007). Perception of cytokinins by their receptors that are encoded by the *MtCRE1*/*LjLHK1* gene in the root cortex induces cortical cell division and formation of the nodule primordia (Gonzalez-Rizzo et al., 2006; Murray et al., 2007; Oldroyd, 2007).

Following the perception of flavonoids from host plants, rhizobia form micro-colonies on the surface of the root hairs and secrete Nod factors (Brewin, 1991; Hirsch, 1992; Brewin, 2004). Paralleling with initiation of cortical cell division and formation of nodule primordia, perception of Nod factors causes root hair cells to undergo morphological changes in the root epidermis. Root hairs swell, branch, and curl to trap rhizobia, which stimulates the formation of infection threads. The infection thread is a cylindrical tube-like structure that grows and invaginates through root hair cells, carrying rhizobia to the newly formed nodule primordium (Rae et al., 1992). Once the rhizobia arrive at the nodule primordium cells, the rhizobia are released into

them through a process resembling endocytosis, leading to formation of symbiosomes. In a symbiosome, the endocytosed rhizobia are called bacteroids. They differentiate and mature into forms capable of fixing nitrogen (Brewin, 1991; Brewin, 2004). As the nodules develop and grow, the differentiation of nodules into distinct zones occurs (Vasse et al., 1990; Crespi and Galvez, 2000), as illustrated in Figure 1. The most distal region of the nodule, known as Zone I, is the meristematic zone, where cells continually divide; nodules continually grow and expand outward from the meristematic zone. The meristematic zone contains no bacteria; hence, no bacterial infection or invasion occurs there. The region proximal Zone I is Zone II, also as known as the infection zone where the bacteria enter the host cells and are released, forming symbiosomes. Adjacent to Zone II is the Interzone II-III where endocytosed rhizobia complete their differentiation into the nitrogen-fixing forms. Zone III, known as the nitrogen fixation zone, is adjacent to the Interzone II-III. The most proximal region of the nodule is Zone IV, also known as the senescent zone, where aging bacteroids and host cells die and are recycled by the plant (Vasse et al., 1990).

1.3 Nitrogen Assimilation

As mentioned at the beginning, bioavailable nitrogen is usually limited in soil. Bioavailable nitrogen is usually of two forms: nitrate, the most oxidized form, and ammonia, the most reduced. Nitrate is the main inorganic nitrogen source that plants use in soils. It also functions as a signaling molecule involved in metabolism and development of plants (Krouk et al., 2010). In plants, nitrogen assimilation is regulated by a complicated network of transporters, reductases, synthetases, and transaminases in response to internal and environmental signals (Glass et al., 2002; Stitt et al., 2002; Vidal and Gutiérrez, 2008; Gojon et al., 2009), which

regulate the availability of carbon and energy for nitrogen assimilation and root development to control nitrogen uptake in the roots. Nitrate in the soil can be taken up by the plant roots and assimilated into plant cells in three sequential steps: (1) Nitrate is transported across the plasma membrane via high and low affinity transporters in response to varied concentrations of nitrate in the soil. (2) Reduction events occur, which convert nitrate to nitrite and nitrite to ammonium via the operation of nitrate reductase and nitrite reductase. Alternately, nitrate may be transported to the leaves and reduced and assimilated there. (3) Ammonium assimilation into organic nitrogen takes place by sequential activities of the enzymes, glutamine synthetase and glutamate synthase (Marquez et al., 2005).

1.4 Nitrate Transporters in Plants

In higher plants, three protein families of nitrate transporters have been identified, the Chloride Channel (CLC), NRT2, and NPF/NRT1(PTR) transporter families (Tsay et al., 2007; Zifarelli and Pusch, 2010; Bagchi et al., 2012; Léran et al., 2014). Proteins in the CLC family were found to be associated with nitrate transport between cytosol and organelles (Zifarelli and Pusch, 2010). Proteins in NRT2 family are high-affinity nitrate transporters, and there are 7 NRT2 transporters in *A. thaliana* (Tsay et al., 2007). Recent studies also showed AtNRT2.1 functions as a nitrate sensor to coordinate the development of the root system with nutritional cues by repressing lateral root initiation, which is independent of nitrate-uptake ability (Little et al., 2005; Tsay et al., 2007). There are 53 NPF/NRT1(PTR) transporters found in *A. thaliana*, and these two families (NRT2 and *NPF*/*NRT1*(*PTR*)) are distinct from each other, with no sequence homology found between them (Tsay et al., 2014). Many proteins found in NPF/NRT1(PTR) family that have been investigated so far are low-affinity nitrate transporters or di/tripeptide

transporters. An exception is the AtNPF6.3/NRT1.1 (CHL1) transporter, which is a dual-affinity nitrate transporter in *A. thaliana* (Liu et al., 1999). Whether the AtNPF6.3/NRT1.1(CHL1) transporter is truly a dual-affinity transporter or simply a low-affinity transporter has recently been called into question (Glass and Kotur, 2013). Recent data show that NPF/NRT1(PTR) family transporters have diverse substrate transport capacity (Léran et al., 2014).

1.5 NPF/NRT1(PTR) Family and Substrates Transported by NPF Family Members

Most members of Nitrate Transporter 1/Peptide Transporter (NRT1/PTR) family have 12 transmembrane domains and are predominantly localized to the plasma membrane. This family was recently renamed the Nitrate Peptide Transporter (NPF) family (Léran et al., 2013, 2014). As mentioned in Section 1.4, many of the members in NPF/NRT1(PTR) family are low affinity nitrate transporters but some were found to transport peptides and other ionic species as well. There are 53 NPF/NRT1(PTR) members found in *A. thaliana*, 93 NPF/NRT1(PTR) members in *Oryza sativa*, 80 NPF/NRT1(PTR) members in *M. truncatula*, and similar numbers of NPF/NRT1(PTR) members in other plant species (Léran et al., 2014). Studies demonstrated that NPF/NRT1(PTR) members couple H^+ transport across membranes down the H^+ gradient to transport nitrate (Tsay et al., 2007), nitrite (Sugiura et al., 2007), di-or tri-peptides (Waterworth and Bray, 2006; Tegeder and Rentsch, 2010), dicarboxylates (Jeong et al., 2004), auxin (Krouk et al., 2010), ABA (Kanno et al., 2012), and glucosinolates (Nour-Eldin et al., 2012). Furthermore, some NPF/NRT1(PTR) members are able to transport more than one substrate: nitrate/IAA in the case of AtNPF6.3/NRT1.1(CHL1) (Krouk et al., 2010), nitrate/ABA in the case of AtNPF4.6/NRT1.2 (Huang et al., 1999; Kanno et al., 2012), nitrate/glucosinolates in the case of AtNPF2.9/NRT1.9 and AtNPF2.9/NRT1.9 (Fan et al., 2009; Wang and Tsay, 2011; Nour-Eldin et

al., 2012), or nitrate/nitrite in the case of AtNPF3.1/Nitr (Sugiura et al., 2007).

1.6 Roles of Auxin and Cytokinin in Nodulation

Auxin has been known to play a role in nodulation for a long time; however, proteins that control auxin flux during nodulation have yet to be identified. A local accumulation of auxin in the nodule initiation zone can be triggered by inoculation of legumes with rhizobia or by Nod factor treatment (Mathesius et al., 1998; Pacios-Bras et al., 2003). Transcripts of *MtAUX1*, encoding a putative auxin influx transporter, were found to accumulate after inoculation with rhizobia. Polar auxin transport was found to be disrupted at the nodule primordium emergent site (de Billy et al., 2001). Flavonoids, as mentioned previously, secreted during nodulation, inhibit polar auxin transport (Jacobs and Rubery, 1988). Reduction of nodulation and polar auxin transport were observed when chalcone synthase, the gene encoding the flux-generating step in flavonoid biosynthesis, was silenced (Mathesius et al., 1998; Wasson et al., 2006). In 2013, Turner., et al reported that ectopic expression of miR160 which silences a set of repressive auxin response factor transcription factors, resulted in auxin hypersensitivity and cytokinin hyposensitivity that leads to inhibition of symbiotic nodule development in soybean (Turner et al., 2013).

Cytokinin, another important plant hormone, has been known to be involved in root nodule symbiosis. Expression of an *Agrobacterium tumefaciens* cytokinin biosynthetic gene in rhizobia mutants that fail to make Nod factors resulted in development of nodule-like structures in alfalfa roots (Cooper and Long, 1994). The loss-of function *Ljlhk-1* mutant, a homolog of *MtCre1*, defective in cytokinin perception, displayed numerous infection threads, but formed very few nodules (Gonzalez-Rizzo et al., 2006; Murray et al., 2007). Exogenous cytokinin treatment to *L. japonicus* roots was able to induce formation of nodule primordia (Desbrosses

and Stougaard, 2011). It is clear from these studies that both auxin and cytokinin are required for nodulation. However, many of the receptors, transporters, and enzymes involved in auxin and cytokinin action during nodulation remain to be discovered.

1.7 Roles of Auxin and Cytokinin in Root Development

Auxin and cytokinin in *A. thaliana* root development are involved in regulating vascular differentiation, lateral root initiation, root apical dominance and root gravitropism and have recently been reviewed (Aloni et al., 2006; Overvoorde et al., 2010; Perilli et al., 2010). In roots, polar auxin transport is achieved via auxin influx transporters (e.g. AUX1) and auxin efflux transporters (PINs) and results in an auxin gradient with a maximum around the quiescent center. Auxin promotes lateral root initiation and primordium development by mediating cell cycle activation. In roots, auxin is transported from the shoots towards the root tips via the root vasculature, and then auxin transported to the root tips is redirected toward the base of the root via the outer cell layers for lateral root initiation and subsequent lateral root primordium development (Fukaki and Tasaka, 2009). In addition to auxin transport, the auxin signaling mediated by AUX/IAA, ARF, and SCF^{TIR1} proteins is required for lateral root development (Fukaki and Tasaka, 2009; Overvoorde et al., 2010). AUX/IAA proteins are important components of auxin and cytokinin balance in roots, which repress transcription mediated by Auxin Responsive Factors (ARFs). ARFs can either act as transcription activators or repressors. In the presence of auxin, the SCF^{TIR1} binds to auxin and AUX/IAA proteins, leading to proteasome-mediated degradation of AUX/IAA proteins. Then ARF proteins are released from AUX/IAA suppression and consequently trigger auxin responses by acting as auxin transcription factors and promoting transcription of auxin sensitive genes. In *A. thaliana*, lateral root initiation

is from a pair of differentiated pericycle cells adjacent to the protoxylem, which is mainly regulated by auxin (De Smet, 2012). Auxin signals transported to the protoxylem percicycles cells mediated by AUX1 or PINs trigger degradation of the AUX/IAAs (IAA14/SLR) involved in lateral root initiation via SCF^{TIR1} and proteasome, leading to activation of ARF7/ARF19 function to trigger the transcriptions of genes required for lateral root initiation (LBD16/ASL18, LBD29/ASL16 and other genes) (Fukaki and Tasaka, 2009).

Cytokinin produced locally or transported via the vascular system is perceived by the AHK2, 3, and 4 receptors in *A. thaliana* (Hirose et al., 2008). Binding of cytokinin activates phosphotransfer proteins, which leads to phosphorylation of type B and type A Arabidopsis Response Regulator (ARR) in the nuclei. Type B ARRs are responsible for positive cytokinin responses while type A ARRs are responsible for negative cytokinin response. During root development, cytokinins are associated with the auxin signaling pathway. In *A. thaliana*, cytokinin activates *SHY2/IAA3* gene expression, leading to production of SHY2 protein that represses the expression of *PIN1*, *3*, and *7* auxin efflux transporter genes as well as the major cytokinin biosynthetic gene *IPT5* (Dello Ioio et al., 2008; Ruzicka et al., 2009). Hence, cytokinin disrupts the PIN-dependent auxin gradient and inhibits its own biosynthesis. The balance of cytokinin and auxin regulates SHY2 levels and contributes to root meristem development. During root development, auxin positively promotes lateral root formation while cytokinin disrupts PIN auxin efflux transporter to repress the elongation of lateral root primordia without influencing formation of founder cells (Heckmann et al., 2011). However, high auxin can also trigger an ethylene response that inhibits lateral root development. High auxin leads to the build-up of the ethylene precursor, 1-aminocyclopropane-1 carboxylic acid (ACC), which triggers an associated inhibitory response of lateral root development. Ethylene stimulates auxin

biosynthesis and increases auxin transport to root tips and to the elongation zone via AUX1 and PIN2, leading to inhibition of cell elongation and overall root growth (Růzicka et al., 2007; Ivanchenko et al., 2010). The interactions between ethylene and auxin in lateral root development are complicated and more work needs to be done to fully understand them.

1.8 Roles of *A. thaliana* NPF6.3/NRT1.1 (CHL1) protein

AtNPF6.3/NRT1.1 (CHL1), a dual affinity nitrate transporter, was shown to play an important role in regulating lateral root development in response to different nitrate conditions in *A. thaliana* and was suggested to participate in a nitrate signaling pathway (Munos et al., 2004; Little et al., 2005; Remans et al., 2006; Wang et al., 2009). In 2009, Ho et al. showed that the AtNPF6.3/NRT1.1(CHL1) transporter is involved in the primary *A. thaliana* nitrate response by examining *AtNRT2.1* gene expression in wild-type plants and the *Atchl1-5* mutant in different nitrate conditions (Ho et al., 2009). In this study, the *Atchl1-5* mutant was used because it is a deletion mutant in which no AtNPF6.3/NRT1.1(CHL1) protein is present and both nitrate uptake and the primary nitrate response are defective (Tsay et al., 1993; Munos et al., 2004). Ho et al. (2009) also used an uptake and signaling decoupled mutant (*Atchl1-9*) to demonstrate that nitrate transport activity is not required for the sensing function of AtNPF6.3/NRT1.1(CHL1), suggesting AtNPF6.3/NRT1.1(CHL1) is itself a nitrate sensor and transport activity is not required for the sensor function (Ho et al., 2009). Moreover, Krouk et al. (2010) showed that AtNPF6.3/NRT1.1(CHL1) not only transports nitrate but also transports auxin (Krouk et al., 2010). An exogenous supply of auxin was able to recover retarded lateral root development in wild-type plants grown on media containing no nitrate, suggesting that AtNPF6.3/NRT1.1(CHL1) regulates lateral root growth by repressing accumulation of auxin

(Krouk et al., 2010). Mutation of *AtNPF6.3/NRT1.1(CHL1)* promotes auxin accumulation in lateral roots, leading to enhanced lateral root development, suggesting that an AtNPF6.3/NRT1.1(CHL1)-dependent nitrate signal pathway could be associated with auxin transport (Krouk et al., 2010). In legumes, nitrogen-induced genes responsible for regulation of root architecture and nodulation have not yet been identified. Nitrogen sensing and signaling may be a different story because of the capacity of legumes to develop symbiotic nitrogen-fixing nodules (Yendrek et al., 2010).

1.9 Roles of M. truncatula NPF1.7/NIP/LATD Gene

The *NPF1.7/NIP/LATD* gene in *M. truncatula* is required for normal development of the primary root, lateral roots and symbiotic root nodules. Previous studies showed that the *Mtnip-1*, *Mtnip-3* and *Mtlatd* mutants had defects in root architecture and nodulation (Veereshlingam et al., 2004; Bright et al., 2005; Teillet et al., 2008; Harris and Dickstein, 2010; Yendrek et al., 2010). From a phylogenic study (Yendrek et al., 2010), the *MtNPF1.7/NIP/LATD* gene was shown to encode a protein belong to NPF/NRT1(PTR) family of transporters. Gene expression studies (Yendrek et al., 2010) showed the *MtNPF1.7/NIP/LATD* gene is not regulated by nitrate, but is regulated by the plant hormones cytokinin, auxin, and abscisic acid (ABA); in addition, regulation of root architecture of the *Mtnpf1.7/nip/latd* mutants had a decreased response to nitrate, suggesting *MtNPF1.7/NIP/LATD* has a possible role in nitrate signaling or sensing (Yendrek et al., 2010; Bagchi et al., 2012). Expression of *MtNPF1.7/NIP/LATD* in *Xenopus laevis* oocytes conferred on them a pH-dependent nitrate uptake ability at low nitrate concentrations which suggests that *MtNPF1.7/NIP/LATD* encodes a high affinity nitrate transporter (Bagchi et al., 2012), and expression of *Mtnip-1* in *X. laevis* oocytes failed to

transport nitrate while *X. laevis* oocytes expressing *Mtnip-3* showed nitrate uptake ability (Bagchi et al., 2012). Dr. Bagchi obtained evidence that MtNPF1.7/NIP/LATD may transport auxin: *MtNPF1.7/NIP/LATD* RNA injected *X. laevis* oocytes contained less ^{3}H-IAA after one hour incubation compared to control water injected oocytes, indicating an efflux of IAA (Bagchi, PhD Thesis, 2013). This suggests that *MtNPF1.7/NIP/LATD* may encode an auxin efflux transporter (Bagchi, PhD Thesis, 2013). ABA was able to rescue lateral root defect in *Mtlatd* mutant but not the nodule phenotype (Liang et al., 2007). Others have suggested that MtNPF1.7/NIP/LATD may transport ABA. However, *X. laevis* oocytes expressing *MtNPF1.7/NIP/LATD* failed to transport ABA (Bagchi, PhD Thesis, 2013).

1.10 Objective of the Research

In this study, my aim was to address the hypothesis that MtNPF1.7/NIP/LATD functions as a nitrate sensor. An *A. thaliana* nitrate transporter mutant (*chl1-5*) and the wild-type Col-0 constitutively expressing the *MtNPF1.7*/*NIP*/*LATD* gene were compared to *Atchl1-5* and Col-0 control plants for a variety of responses associated with nitrate sensing. Specifically the *MtNPF1.7*/*NIP*/*LATD* gene was tested to see if it could restore *Atchl1-5* to the wild-type phenotype (Krouk et al., 2010). Further experiments were carried out with *M. truncatula* plants with mutations in *MtNPF1.7*/*NIP*/*LATD* and also ones that constitutively express *MtNPF1.7/NIP*/*LATD* to test whether the physiological hallmarks of normal nitrogen and auxin signaling are present in these plants.

CHAPTER 2

EFFECTS OF CONSTITUTIVE EXPRESSION OF THE *MtNPF1.7*/*NIP*/*LATD* GENE IN *A. thaliana* ON ROOT PHENOTYPES AND PLANT GROWTH IN RESPONSE TO DIFFERENT GROWTH CONDITIONS

2.1 Effects of Constitutive Expression of the *MtNPF1.7*/*NIP*/*LATD* Gene in the *A. thaliana* Nitrate Transporter *AtNPF6.3*/*NRT1.1 (CHL1)* Mutant *Atchl1-5* in Response to Nitrate

In order to determine if the *MtNPF1.7*/*NIP*/*LATD* gene might play a role nitrate sensing, the *A. thaliana chl1-5* mutant (Tsay et al., 1993; Krouk et al., 2010) was used. The *A. thaliana chl1-5* mutant was previously shown to have defects in nitrate sensing and root architecture in response to different nitrogen sources (see details in introduction). The experimental strategy was to determine *MtNPF1.7/NIP/LATD* could complement these nitrate sensing defects. Previous studies on the *Atchl1-5* mutant showed that it had a relatively enhanced lateral root growth in the absence or at low concentrations of nitrate (Krouk et al., 2010). Dr. M. Salehin, previously of the Dickstein lab, generated 2 lines of transgenic *A. thaliana chl1-5* mutant plants expressing the *MtNPF1.7*/*NIP*/*LATD* gene under the control of the constitutive *A. thaliana EF1α* promoter (Auriac and Timmers, 2007; Bagchi et al., 2012; Salehin et al., 2012). These 2 lines of transgenic plants, *Atchl1-5+ MtNPF1.7*/*NIP*/*LATD* line#11 and *Atchl1-5+ MtNPF1.7*/*NIP*/*LATD* line#12, the *Atchl1-5* mutant, and the control wild-type plant line, Col-0, were grown for 8 days on media containing different concentrations of nitrogen sources: 0.5 mM L-Glutamine, containing 2 amine groups per molecule, versus 1 mM KNO_3, and compared to those grown for 8 days on nitrogen-free media. Twenty plants of each line were grown for each condition and the lateral root density, defined as number of visible (>0.5 mm) lateral roots divided by length of primary

root (in mm), was measured for each individual plant. The results are presented in Figure 2. As can been seen in Figure 2, in low nitrate concentration or no nitrate conditions, *Atchl1-5* plants have a higher LR density than wild-type plants. No significant difference in lateral root density was observed in *Atchl1-5* versus wild-type Col-0 in the presence of 1 mM nitrate as compared to those grown on 0.5mM L-Glutamine and nitrogen free media. This result duplicates the work of Krouk et al., (2010) for the *Atchl1-5* mutant. We then compared *Atchl1-5*, wild-type, and the two transgenic lines for lateral root growth. For the two transgenic lines expressing *MtNPF1.7/NIP/LATD*, *Atchl1-5+ MtNPF1.7/NIP/LATD* #11 and *Atchl1-5+ MtNPF1.7/NIP/LATD* #12, there were similar patterns of lateral root density as for the wild-type plants. This suggests that the *MtNPF1.7/NIP/LATD* gene can restore these defects of the *Atchl1-5* mutation in response to nitrate.

It was reported that the *A. thaliana chl1-5* mutant plants were less sensitive to high nitrate than wild-type plants for their lateral root phenotype (Tian et al., 2009). In the work of Tian et al., (2009), lateral root growth was shown to be inhibited in response to high external nitrate and the reductions of LR length in wild-type plants were greater as compared to *Atchl1-5* plants in response to high nitrate concentration. To investigate whether the constitutive expression of the *MtNFP1.7/NIP/LATD* gene in the *A. thaliana chl1-5* mutant would complement the defects in *Atchl1-5* mutation for this phenotype, I studied the response of root growth in the two lines of transgenic plants. These two transgenic lines, the *Atchl1-5+ MtNFP1.7/NIP/LATD* #11 and *Atchl1-5+ MtNFP1.7/NIP/LATD* #12 mentioned earlier, the *Atchl1-5* mutant, and the control wild-type plant, Col-0, were tested. After germination, the plants were transferred to 1/2 MS agar medium without nitrogen salts, or containing varying nitrate concentrations (no nitrogen, 0.1 mM KNO_3, and 10 mM KNO_3) for 5 days. Twenty plants were grown for each condition. At 5

days, the length of the primary root, number of lateral roots and length of the lateral roots were measured for each individual plant. The averages and standard errors are plotted in Figures 3, 4, and 5. In the previous studies of Tian et al., (2009), the length of primary roots of both wild-type plants and *Atchl1-5* mutant plants did not differ in the high nitrate and low nitrate concentration. In addition, the reduction in length of the lateral roots was greater in wild-type plants in response to high nitrate concentration. Significant reductions in lateral root lengths were observed in the wild-type Col-0 plants and *Atchl1-5* mutant plants grown on media containing high nitrate concentration compared with those grown on media containing low nitrate concentration (Tian et al., 2009). However, I was unable to replicate these results, as shown in Figure 3, Figure 4, and Figure 5. As shown in Figure 3, for the all the plants grown in the presence of 0.1 mM nitrate, no significant difference of the length of primary roots was observed whereas the primary roots of *Atchl1-5* mutant plants grew longer in response to 10 mM KNO_3 compared to Col-0, *Atchl1-5+ MtNFP1.7/NIP/LATD* #11 and *Atchl1-5+ MtNFP1.7/NIP/LATD* #12 plants. In contrast to the work of Tian et al., (2009), no significant difference was observed in the lengths of primary roots in response to varied concentrations of KNO_3. In my results, the number of the lateral roots for the experimental and control plants grown in 0.1 mM KNO_3 was similar to that of the experimental and control plants grown in 10 mM KNO_3, as demonstrated in Figure 4. The number of lateral root was expected to be reduced in high nitrate concentrations (Tian et al., 2009). As can been seen in Figure 5, for the length of lateral roots, my results were very different from the work of Tian et al., (2009). My results showed that reduction in lateral root length was less in *Atchl1-5* mutant plants in response to high nitrate concentration. However, my results also showed that the *Atchl1-5* mutant primary root length was longer than that of the wild-type plant Col-0, and *MtNFP1.7/NIP/LATD* appeared to complement this defect in the transgenic lines in

media containing 10 mM KNO_3 (Figure 3). Similar results were obtained for lateral root number (Figure 4), but not for lateral root length (Figure 5). Overall, my data provides support for the *MtNFP1.7/NIP/LATD* gene's ability to complement phenotypes associated with a loss of nitrate sensing in the *Atchl1-5* mutant. The inability to replicate the results of Tian et al., (2009) might be due to a technical problem which could be improved, such as dealing with a smaller number of samples at one time. It is also possible that the results of Tian et al., (2009) could only work under certain conditions, beyond those that are spelled out in their published paper.

2.2 Effects of Constitutive Expression of the MtNPF1.7/NIP/LATD Gene in An *A. thaliana* Nitrate Transporter *AtNPF6.3/NRT1.1 (CHL1)* Mutant *Atchl1-5* in Response to Auxin and Cytokinin

Previous studies on the *A. thaliana chl1-5* mutant showed that it had a relatively enhanced lateral root growth in the absence or at low concentrations of nitrate (Krouk et al., 2010). Krouk et al., (2010) showed that an exogenous supply of auxin was able to recover retarded lateral root development in wild-type plants grown on media containing no nitrate. These data and other experimental results led Krouk et al., (2010) to hypothesize that AtNPF6.3/NRT1.1(CHL1) regulates lateral root growth by repressing accumulation of auxin.

The experimental strategy used in my experiments was to determine if *MtNPF1.7/NIP/LATD* could complement these nitrate sensing defects. The lateral root density of two transgenic lines of *Atchl1-5* mutant transformed with a constitutive promoter expressing the *MtNPF1.7/NIP/LATD* gene, *Atchl1-5+ MtNPF1.7/NIP/LATD* #11 and *Atchl1-5+ MtNPF1.7/NIP/LATD* #12 was shown to have a similar pattern in response to varied concentrations of nitrogen sources (No nitrogen, 1 mM nitrate and 0.5 mM L-Glutamate) as

compared to lateral root density of wild-type plants as can been seen in Figure 2. This result suggests that the *MtNPF1.7/NIP/LATD* gene is able to restore these defects of the *Atchl1-5* mutation.

To address the question of whether MtNPF1.7/NIP/LATD might function in an analogous manner to AtNPF6.3/NRT1.1(CHL1), I examined whether the *Atchl1-5* mutant plants expressing the *MtNPF1.7/NIP/LATD* gene can recover retarded lateral root development by provision of an exogenous supply of auxin, similar to *Atchl1-5* plants expressing *AtNPF6.3/NRT1.1(CHL1).* Also, because the effects of auxin and cytokinin are different in lateral root development (Fukaki and Tasaka, 2009), the effects of exogenous supply of cytokinin were investigated in a parallel fashion with the effects of exogenous supply of auxin. Two lines of transgenic plants, *Atchl1-5+ MtNPF1.7/NIP/LATD* line#1 and *Atchl1-5+ MtNPF1.7/NIP/LATD* line#3, the *Atchl1-5* mutant, the control wild-type plant line, Col-0, and the transformation control plant line *Atchl1.5+AtNPF6.3/NRT1.1(CHL1)* , were grown for 8 days on media containing different concentrations of nitrogen sources: 0.5 mM L-Glutamine, 1 mM KNO_3, and compared to those grown for 8 days on the same media containing 10 nM IAA or 10 nM BAP (0.5 mM Gln+10 nM IAA; 0.5mM Gln+ 10 nM BAP; 1 mM KNO_3 +10 nM IAA; 1 mM KNO_3+ 10 nM BAP). Twenty plants of each line were grown for each condition and the lateral root density, defined as number of visible (>0.5 mm) lateral roots divided by length of primary root (in mm) was measured for each individual plant. The results are shown in Figure 6. As illustrated in Figure 6, the two lines of *A. thaliana chl1-5* mutant constitutively expressing *MtNPF1.7/NIP/LATD* gene behaved similarly to wild-type plants and the transformation control plants in the no nitrate condition with the auxin treatment. However, in the media containing 1 mM KNO_3 and 10 nM IAA, the lateral root density was lower in both lines of *A. thaliana chl1-5* mutant constitutively expressing

MtNPF1.7/NIP/*LATD* gene as compared to wild-type and *Atchl1-5* plants, suggesting constitutive expression of the *MtNPF1.7/NIP*/*LATD* gene in the *Atchl1-5* mutant can only partially complement the wild-type phenotype in response to auxin, at least in these conditions. On the other hand, the transformation control plant line *Atchl1.5+AtNPF6.3/NRT1.1(CHL1)* had a similar lateral root density with both the lines of *A. thaliana chl1-5* mutant constitutively expressing *MtNPF1.7/NIP*/*LATD* gene and the wild-type Col-0, which might be caused by constitutively expressing *AtNPF6.3/NRT1.1(CHL1)* gene because of losing its native regulating mechanism. However, the lateral root density of the *Atchl1.5+AtNPF6.3/NRT1.1(CHL1)* plant was not statistically different as compared to wild type plant Col-0 and *Atchl1-5* plants. In the media containing 0.5 mM Gln+10 nM BAP and 1 mM KNO_3+10 nM BAP, the overall lateral root density in all lines was lower (Figure 6). This result was expected because cytokinin has been shown to inhibit lateral root growth (Fukaki and Tasaka, 2009).

2.3 Effects of Constitutive Expression of the *MtNPF1.7/NIP/LATD* Gene in Wild-Type *A. thaliana* Col-0 on Root Architecture Responses in Different Growth Conditions

During the time that I was carrying out the experiments described above, former Dickstein lab members Drs. M. Salehin and R. Bagchi found that two lines of transgenic *A. thaliana chl1.5* constitutively expressing *MtNPF1.7/NIP/LATD* were larger in both shoots and leaves (Salehin et al, In preparation). Similar effects were seen when *MtNPF1.7/NIP/LATD* was expressed under the control of the constitutive *A. thaliana EF1α* promoter in wild-type Col-0 (Salehin et al, In preparation). In order to study how constitutively expressing *MtNPF1.7/NIP/LATD* can affect the root phenotypes in response to different growth conditions, two lines of transgenic *A. thaliana* Col-0 plant constitutively expressing *MtNPF1.7/NIP/LATD* were tested. Because auxin has previously been reported to participate in lateral root initiation and both cytokinin and abscisic

acid were shown to negatively regulate lateral root formation in *A. thaliana* (Fukaki and Tasaka, 2009), these three plant hormones were chosen to study.

Two lines of transgenic *A. thaliana* Col-0 plants constitutively expressing *MtNPF1.7/NIP/LATD* (Col-0+ *MtNPF1.7/NIP/LATD* L1 and Col-0+ *MtNPF1.7/NIP/LATD* L4), the wild-type Col-0, and the transformation control (Col-0+*AtNPF6.3/NRT1.1(CHL1)*) were germinated on 1/2MS-N media. This media is a basal media with no nitrogen source. After germination, all seedlings were transferred to different growth conditions for 14 days: the basal media as the control, and basal media containing a) 1mM NH_4Cl, b) 200 µM KNO_3, c)1 mM KNO_3, d)1 mM KNO_3+0.1 µM IAA, e)1 mM KNO_3+0.1 µM BAP, f) 1 mM KNO_3+0.2 µM ABA, and g) 10 mM KNO_3. Fifteen plants of each line were grown for each condition and the lateral root density, defined as number of visible (>0.5 mm) lateral roots divided by length of primary root (in mm) was measured for each individual plant. The results are shown in Figure 7. As seen in Figure 7, there was no significant difference among the genotypes in lateral root density when plants were grown in the no nitrogen condition, 1 mM NH_4Cl, 1 mM KNO_3, 10 mM KNO_3, and 1 mM KNO_3+0.2 µM ABA. Lateral root density in the transgenic *A. thaliana* Col-0 constitutively expressing *MtNPF1.7/NIP/LATD* lines was higher in the basal media containing 200 µM KNO_3, and 1 mM KNO_3+0.1 µM BAP as compared to wild-type Col-0 plants and the transformation control (Col-0+*AtNPF6.3/NRT1.1(CHL1)*). Lateral root density was lower in the transgenic *A. thaliana* Col-0 constitutively expressing *MtNPF1.7/NIP/LATD* lines in the basal media containing 1 mM KNO_3+0.1 µM IAA than it was in the Col-0 and the transformation control (Col-0+*AtNPF6.3/NRT1.1(CHL1)*) lines. These results suggest that MtNPF1.7/NIP/LATD directly or indirectly interacts with auxin and cytokinin in 1 mM KNO_3.

Dr. M. Salehin's unpublished data showed that the roots of the transgenic *M. truncatula* wild-type plant overexpressing *MtNPF1.7/NIP/LATD* also grew better in the low nitrate conditions in the aeroponic system, and Dr. M. Salehin's and our lab's collaborators' unpublished microarray data also reported that more genes were mis-regulated in the leaves of lines of *A. thaliana* Col-0 constitutively expressing *MtNPF1.7/NIP/LATD* when growing in 0.1 mM KNO_3 condition as compared to plants growing in 10 mM KNO_3 condition, showing that *MtNPF1.7/NIP/LATD* has a differential effect in the differing nitrate conditions. Figure 7 showed that AtCol-0+ *MtNPF1.7/NIP/LATD* plants had a statistically significant lower lateral root density in response to IAA in presence of 1 mM KNO_3 and had a higher lateral root density in 200 µM KNO_3 as compared to wild-type plants (Col-0) and the transformation control (Col-0+*AtNPF6.3/NRT1.1(CHL1)*). In order to study how transgenic *A. thaliana* wild-type plants constitutively expressing *MtNPF1.7/NIP/LATD* (Col-0+ *MtNPF1.7/NIP/LATD*) respond to the IAA and BAP in the low nitrate condition (200 µM), two lines of transgenic *A. thaliana* Col-0 plants constitutively expressing *MtNPF1.7/NIP/LATD* (Col-0+ *MtNPF1.7/NIP/LATD* L1 and Col-0+ *MtNPF1.7/NIP/LATD* L2), the wild-type Col-0, and the transformation control (Col-0+*AtNPF6.3/NRT1.1(CHL1)*) were used. Two lines of transgenic *A. thaliana* Col-0 plant constitutively expressing *MtNPF1.7/NIP/LATD* (Col-0+*MtNPF1.7/NIP/LATD* L1 and Col-0+*MtNPF1.7/NIP/LATD* L2), the transformation control (Col-0+*AtNPF6.3/NRT1.1(CHL1)*), and the wild-type Col-0 were germinated on the 1/2MS-N media, the basal media with no nitrogen source. After germination, all seedlings were transferred to different growth conditions for 14 days: the basal media as the control and the basal media containing 200 µM KNO_3, 200 µM KNO_3+0.1 µM IAA, 200 µM KNO_3+0.1 µM BAP, 1 mM KNO_3, 1 mM KNO_3+0.1 µM IAA, and 1 mM KNO_3+0.1 µM BAP. Fifteen plants of each line were grown for each condition and the

lateral root density, defined as number of visible (>0.5 mm) lateral roots divided by length of primary root (in mm) was measured for each individual plant. The results are shown in Figure 8. As expected from previous results (Figure 7), there was no statistically significant difference in the lateral root density between wild-type Col-0 plants, the transformation control (Col-0+*AtNPF6.3/NRT1.1(CHL1)*), and two lines of transgenic *A. thaliana* Col-0 plants constitutively expressing *MtNPF1.7/NIP/LATD* when plants were grown in 1 mM KNO_3. The two lines of transgenic *A. thaliana* Col-0 plants constitutively expressing *MtNPF1.7/NIP/LATD* had a higher lateral root density when growing in the 200 µM KNO_3 condition (Figure 8), similar to results obtained previously (Figure 7). However, auxin treatment in the 200 µM KNO_3 condition had an opposite effect as compared to 1 mM KNO_3 for lateral root density in transgenic *A. thaliana* Col-0 plants constitutively expressing *MtNPF1.7/NIP/LATD* as compared to the lateral root phenotype in response to auxin in 1 mM KNO_3 condition. As compared to wild-type Col-0 plants, transgenic *A. thaliana* Col-0 plants constitutively expressing *MtNPF1.7/NIP/LATD* were less sensitive to cytokinin at both nitrate concentration tested. Taken together and similar to previous experiments, these results show MtNPF1.7/NIP/LATD interacts with auxin and cytokinin in a nitrate concentration dependent manner. Because the effect is nitrate concentration sensitive, this suggests the possibility that MtNPF1.7/NIP/LATD may act as a nitrate sensor.

CHAPTER 3

EXPERIMENTS FOR DETERMINATION OF ROLES OF *MtNPF1.7/NIP/LATD* GENE IN *M. truncatula*

3.1 Response of *MtNRT2.1* Expression to Differing Nitrate Conditions in Wild-Type *M. truncatula* versus *Mtnip-1*

In *A. thaliana*, two nitrate transporters, AtNPF6.3/NRT1.1 (CHL) and AtNRT2.1 have been shown to function as nitrate receptors or "transceptors", able to function as both transporter and receptor (Gojon et al., 2011). As described in the introduction, both *AtNPF6.3/NRT1.1*(*CHL1*) and *AtNRT2.1* were shown to be regulated by nitrate and when mutated, fail to have nitrate-responsive genes respond normally to nitrate (Tsay et al., 1993; Little et al., 2005; Ho et al., 2009). Phenotypes of *A. thaliana* plants with mutations in *AtNPF6.3/NRT1.1(CHL1)* and *AtNRT2.1* suggest that they have nitrate sensing as well as transport defects: the most prominent of these are defects in root architecture which cannot be explained by simple transport defects alone (Munos et al., 2004; Little et al., 2005; Remans et al., 2006; Wang et al., 2009). Phenotypic analysis of *Mtnpf1.7/nip/latd* mutants showed marked root architecture defects as well as nodulation defects (Veereshlingam et al., 2004; Bright et al., 2005; Teillet et al., 2008), which cannot be explained by the finding that *MtNPF1.7/NIP/LATD* encodes a high-affinity nitrate transporter (Yendrek et al., 2010; Bagchi et al., 2012; Salehin et al., 2012). Phylogenetic analysis (Yendrek et al., 2010) showed that *MtNPF1.7/NIP/LATD* has close homologs in *A. thaliana*, *At1g52190* (*AtNPF1.2/NRT1.11*) and *At3g16180* (*AtNRT1.12*), which are low-affinity nitrate transporters (Tsay et al., 2007; Hsu and Tsay, 2013). These close homologs are regulated by nitrate, and double mutants are defective in high nitrate enhanced growth in leaves (Hsu and Tsay, 2013). Previously Yendrek et al (2010) showed that

MtNPF1.7/NIP/LATD expression was not responsive to nitrate, but this was only tested in the 10 mM KNO_3 condition. Thus, *MtNPF1.7/NIP/LATD* is an unusual gene: its mutants have some hallmarks of behaving as if they have lost nitrate sensing ability (root architecture), but unlike known Arabidopsis nitrate transceptor genes, *MtNPF1.7/NIP/LATD* so far does not appear to be nitrate responsive itself, although only one nitrate concentration was tested (Yendrek et al., 2010).

Here, I set up studies to determine two things: First, since *MtNPF1.7/NIP/LATD* has now been established as a high-affinity nitrate transporter, I wanted to determine if it was responsive to different, lower concentrations of nitrate than have been previously been tested. Second, I wanted to determine if the severe *Mtnip-1* mutant has defects in nitrate response in nitrate responsive genes. To do this, I chose *MtNRT2.1* as a target gene because it is a homolog of *AtNRT2.1* gene that shows a high expression in low concentration of nitrate (250 μM). *A. thaliana*. *AtNRT2.1* expression was shown to be strongly nitrate inducible, peaking at 3 to12 hours and subsequently declining (Okamoto et al., 2003). Therefore, in order to study gene expression in more depth, *MtNPF1.7/NIP/LATD* and *MtNRT2.1* gene expression were examined from the wild-type plant, A17, and the *Mtnip-1* mutant in varied concentrations of nitrate: 50 μM KNO_3, 250 μM KNO_3, and 1 mM KNO_3, and 5 mM KNO_3 and compared to respective concentrations of KCl as controls. The plants were grown in BNM media 5 days for nitrogen starvation after germination. Then nitrate treatment was applied for 2 hours, the time frame that Yendrek et al., (2010) used. As can been seen in Figure 9, nitrate had no obvious effects on *MtNPF1.7/NIP/LATD* expression in both A17 and *Mtnip-1* roots, extending previous work (Yendrek et al., 2010). The *MtNRT2.1* expression was sensitive in response to varied concentrations of nitrate as compared to the salt controls in A17 plants. The *MtNRT2.1*

expression also reported nitrate as compared to salt controls in *Mtnip-1*, similar to the wild type A17 response (Figure 10). These results show that MtNPF1.7/NIP/LATD does not regulate the nitrate-responsive *MtNRT2.1* gene and demonstrate that MtNPF1.7/NIP/LATD does not act in a homologous fashion as *AtNPF6.3/NRT1.1(CHL1)* does in *A. thaliana*.

3.2 Auxin-Dependent Gravitropic Responses in *Mtnip/latd* Mutant, *MtNPF1.7/NIP/LATD* OE and Wild-Type Plants

From phylogenic studies done several years ago (Yendrek et al., 2010) and more recently (Léran et al., 2013), the *MtNPF1.7/NIP/LATD* gene was shown to encode a protein belong to NPF/NRT1(PTR) family of transporters. Four proteins in NPF/NRT1(PTR) family have been shown to be able to transport more than one substrate: nitrate/IAA in the case of AtNPF6.3/NRT1.1(CHL1), nitrate/ABA in the case of AtNPF4.6/NRT1.2, nitrate/glucosinolates in the case of AtNPF2.9/NRT1.9, or nitrate/nitrite in the case of AtNPF3.1/Nitr (Léran et al., 2013). Gene expression studies (Yendrek et al., 2010) showed the *MtNPF1.7/NIP/LATD* gene was not regulated by nitrate, but regulated by plant hormones, such as cytokinin, auxin, and ABA. Dr. R. Bagchi's unpublished data suggested that MtNPF1.7/NIP/LATD is able to efflux auxin (Rammyani Bagchi, Thesis, 2013). In *A. thaliana*, some mutants having defects in auxin transport displayed a gravitropic phenotype in roots (Marchant et al., 1999; Lewis et al., 2007; Guyomarc'h et al., 2012).

In order to determine if *MtNPF1.7/NIP/LATD* has a role in gravitropic response, considering *MtNPF1.7/NIP/LATD* may be an auxin transporter, the wild-type A17, *Mtnip-1*, *Mtnip-3*, and *M. truncatula* wild-type A17 overexpressing *MtNPF1.7/NIP/LATD* (A17OE *MtNPF1.7/NIP/LATD* L4) plants lines were used. All plant lines were grown on BNM media

containing 5 mM ammonium nitrate on 100 x 15 mm square petri dishes for 4 days after germination. Then the petri dishes containing each experimental plant line were turned 90^{o}, and all plants were allowed to grow on the same petri dishes for 5 days. As can be seen in Figure 11, the curvature degree of the root in *Mtnip-1* was lower as compared to wild-type plant A17. There was no significant difference found in the curvature degree of the root in wild-type, *Mtnip-3*, and A17OE *MtNPF1.7/NIP/LATD* L4 plants. Thus, *Mtnip-1* plants have an auxin-related defect, one associated with auxin transport (Marchant et al., 1999; Lewis et al., 2007; Guyomarc'h et al., 2012).

CHAPTER 4

DISCUSSION

As previously described in Introduction, the *M. truncatula NPF1.7/NIP/LATD* gene is involved in root development and nodule formation. Plants with a mutation in the *MtNPF1.7/NIP/LATD* gene have defects in root architecture and nodulation (Veereshlingam et al., 2004; Bright et al., 2005; Teillet et al., 2008). The *MtNPF1.7/NIP/LATD* gene encodes a protein belong to the NPF/NRT1(PTR) transporter family (Yendrek et al., 2010). MtNPF1.7/NIP/LATD was found to be a high affinity nitrate transporter (Bagchi et al., 2012; Salehin et al., 2013), but unlike other nitrate transporters, its gene's expression is not regulated by nitrate (Yendrek et al., 2010). *MtNPF1.7/NIP/LATD* expression was found to be regulated by the phytohormones cytokinin, auxin and ABA (Yendrek et al., 2010). In addition, the root architecture of the *Mtnpf1.7*/*nip/latd* mutants had a decreased response to nitrate, suggesting a possible role in nitrate signaling or sensing (Yendrek et al., 2010; Bagchi et al., 2012). In addition to transporting nitrate, biochemical experiments conducted in the *X. laevis* oocyte system showed that expression of *MtNPF1.7/NIP/LATD* in oocytes conferred on them the ability to export IAA (Rammyani Bagchi, PhD thesis, 2013), which suggested that *MtNPF1.7/NIP/LATD* has dual functions.

In this study, my aim was to address the hypothesis that MtNPF1.7/NIP/LATD has biological *in planta* functions consistent with the biochemical activity of auxin transport. In order to determine the role of *M. truncatula NPF1.7/NIP/LATD* and its effects on root architecture and plant growth, several experiments were done, as elaborated in chapters 2 and 3.

As described in Introduction, Section 2.1 and 2.2, both MtNPF1.7/NIP/LATD and AtNPF6.3/NRT1.1(CHL1) belong to the NPF/NRT1(PTR) transporter family and are both nitrate

and auxin transporters. Root architecture in the *Mtnpf1.7/nip/latd* mutants had a decreased response to nitrate, suggesting a possible role in nitrate signaling or sensing (Yendrek et al., 2010; Bagchi et al., 2012). The *A. thaliana chl1-5* mutant was previously shown to have defects in nitrate sensing and root architecture in response to different nitrogen sources (Ho et al., 2009; Krouk et al., 2010) and this was attributed to a defect in nitrate-regulated auxin transport caused by a deletion of *AtNPF6.3/NRT1.1(CHL1)* (Krouk et al., 2010). To determine if the *MtNPF1.7/NIP/LATD* gene might function in an analogous manner to AtNPF6.3/NRT1.1(CHL1), several experiments were done. The experimental strategy was to determine *MtNPF1.7/NIP/LATD* could complement these nitrate sensing, auxin transport-associated defects. Krouk et al., (2010) proposed a model for coupling nitrate sensing by AtNPF6.3/NRT1.1(CHL1) in which auxin transport by AtNPF6.3/NRT1.1(CHL1) is inhibited by nitrate, which accounts for the differences in lateral root development in the presence and absence of nitrate. In this model, AtNPF6.3/NRT1.1(CHL1) favors the basipetal transport of IAA in lateral roots of wild-type Col-0 plants in absence of nitrate, which prevents IAA accumulation at the lateral root (LR) tips, leading to retarded lateral root development. This accounts for the observed retarded lateral root development in wild-type *A. thaliana* in the absence of nitrate (Krouk et al., 2010). Figure 12a illustrates the model (after Krouk et al 2010). In Figure 12, decreased lateral root density is represented as an idealized shorter lateral root and increased lateral root density is represented as an elongated lateral root. This is because decreased lateral root growth results fewer lateral roots that fit the criteria of lateral roots that were long enough to count in my assay. Conversely, increased lateral root growth is a result of lateral root primordia that have elongated from lateral root primordia into longer lateral roots. In the presence of 1 mM nitrate, the basipetal transport of auxin by AtNPF6.3/NRT1.1(CHL1) in wild-type Col-0 plants is

inhibited, which leads to IAA accumulation in the lateral root tips and enhanced lateral root growth (Figure 12b)(Krouk et al., 2010), the observed result. In the presence of both 0.5 mM L-Gln and 10 nM IAA, due to the exogenous supply of IAA, IAA accumulation is achieved at the lateral root tips of wild type Col-0 plants, promoting lateral root development (Figure 12c). Krouk et at., (2010) interpreted their data to suggest that AtNPF6.3/NRT1.1(CHL1) favors the basipetal transport of IAA in lateral roots of wild-type Col-0 plants in absence of nitrate. In the presence of 1 mM KNO_3 and 10 nM IAA, the basipetal transport of auxin away from the lateral root tips by AtNPF6.3/NRT1.1(CHL1) in wild-type Col-0 plants is inhibited, which leads to IAA accumulation in the lateral root tips and enhanced lateral root growth (Krouk et al., 2010) (Figure 12d). In the *Atchl1-5* mutant plants, lack of the basipetal transport of auxin by AtNPF6.3/NRT1.1(CHL1) results in auxin accumulation in the lateral root tips and enhanced lateral root development, regardless of the external nitrogen source, as is illustrated in Figures 12i, 12j, 12k, and 12l. In Figure 12i, in the presence of 0.5 mM L-Gln and the absence of nitrate, auxin transport by AtNPF6.3/NRT1.1(CHL1) in the lateral root tips of *Atchl1-5* plants does not occur due to the deletion mutation of *AtNPF6.3/NRT1.1(CHL1).* Thus, this leads to auxin accumulation in the lateral root tips and promotes lateral root development in *Atchl1-5* plants. In *Atchl1-5* plants, in the presence of 1 mM KNO_3, there is no AtNPF6.3/NRT1.1(CHL1) protein to be suppressed nitrate; thus auxin accumulation is achieved at the lateral root tips in *Atchl1-5* plants, which promotes lateral root development (Figure 12j). In *Atchl1-5* plants, in the presence of 0.5 mM L-Gln and the absence of nitrate, the exogenous supply of IAA causes IAA accumulation at the lateral root tips, promoting lateral root development (Figure 12k). In *Atchl1-5* plants, in the presence of 1 mM KNO_3 and 10 nM IAA, the exogenous supply of IAA causes IAA accumulation at the lateral root tips in *Atchl1-5* plants, promoting lateral root development

(Figure 12l).

Two transgenic lines of *Atchl1-5* mutant expressing *MtNPF1.7/NIP/LATD* (*Atchl1-5+ MtNPF1.7*/*NIP*/*LATD* #11 and *Atchl1-5+ MtNPF1.7*/*NIP*/*LATD* #12) displayed similar patterns of lateral root density as for the wild-type *A. thaliana* plants in the no nitrate condition (0.5 mM L-Glu) or no nitrogen condition. As can been seen in Figure 2, the lateral root density of two transgenic lines of *Atchl1-5* mutant expressing *MtNPF1.7/NIP/LATD* and wild-type Col-0 plants was lower in the no nitrate condition (0.5 mM L-Glu) or no nitrogen condition whereas lateral root density of *Atchl1-5* was insensitive to the conditions tested. These data suggest that the *MtNPF1.7*/*NIP*/*LATD* gene can complement these defects of the *Atchl1-5* mutation in response to absence of nitrate (Figure 2; compare Figures 12a and 12i to 12m). As diagrammed in Figure 12a, auxin transport is mediated by AtNPF6.3/NRT1.1(CHL1) at the lateral root tips of wild-type Col-0 in the presence of 0.5 mM L-Gln and the absence of nitrate, which results in retarded lateral root development. On the other hand, in the presence of 0.5 mM L-Gln and the absence of nitrate, auxin transport by AtNPF6.3/NRT1.1(CHL1) does not occur in *Atchl1-5* mutant plants, which leads to auxin accumulation at the lateral root tips and promotes lateral root development in *Atchl1-5* (Figure 12i). In the presence of 0.5 mM L-Gln and the absence of nitrate, in *Atchl1-5* mutant constitutively expressing *MtNPF1.7/NIP/LATD*, auxin transport is mediated by MtNPF1.7/NIP/LATD, leading to retarded lateral root development, which suggests that the *MtNPF1.7*/*NIP*/*LATD* gene can restore these defects of the *Atchl1-5* mutation in response to absence of nitrate as suggested in Figure 2 and modeled in Figure12m.

The two lines of *Atchl1-5* mutant constitutively expressing *MtNPF1.7/NIP/LATD* (*Atchl1-5+ MtNPF1.7*/*NIP*/*LATD #1* and *Atchl1-5+ MtNPF1.7*/*NIP*/*LATD #3*) had a similar response to auxin in no nitrate conditions as wild-type Col-0 plants, but not in media containing 1 mM KNO_3

and 10 nM IAA (Figure 6, modeled in Figures 12o, and 12p). This shows that constitutive expression of the *MtNPF1.7/NIP/LATD* gene in the *Atchl1-5* mutant can only partially complement the wild-type phenotype in response to auxin in the conditions tested, confirming that MtNPF1.7/NIP/LATD has an auxin-transport associated role, but may not operate by the same mechanism as AtNPF6.3/NRT1.1(CHL1) (wild-type Col-0 in Figures 12c and 12d; *Atchl1-5+MtNPF1.7/NIP/LATD* in Figures12o and 12p). We propose that in the 0.5 mM L-Gln and the absence of nitrate condition, MtNPF1.7/NIP/LATD might efflux IAA in cells of the lateral root tips in the two lines of *Atchl1-5* mutant constitutively expressing *MtNPF1.7/NIP/LATD* (*Atchl1-5+ MtNPF1.7/NIP/LATD #1* and *Atchl1-5+ MtNPF1.7/NIP/LATD #3*), and by doing so, it might prevent IAA accumulation at the root tips, as is modeled in Figure 12m, which represents lateral root density of *Atchl1-5* mutant constitutively expressing *MtNPF1.7/NIP/LATD* in response to 0.5 mM L-Gln. In other words, *Atchl1-5* plants expressing *MtNIP1.7/NIP/LATD* could have retarded lateral root development in the presence of 0.5 mM L-Gln and the absence of nitrate, a similar phenotype found in wild-type Col-0 plants (Figure 12a), but for a different reason. In the presence of 1 mM KNO_3, the lateral root density of the two lines of *Atchl1-5* mutant constitutively expressing *MtNPF1.7/NIP/LATD* (*Atchl1-5+ MtNPF1.7/NIP/LATD #1* and *Atchl1-5+ MtNPF1.7/NIP/LATD #3*) displayed a similar pattern as compared to wild-type plants Col-0 (Figures 2 and 6). We hypothesized that MtNPF1.7/NIP/LATD effluxes IAA in the two lines of *Atchl1-5* mutant constitutively expressing *MtNPF1.7/NIP/LATD*, possibly leading to IAA accumulation at the root tips, which promotes lateral root development, as the result seen in Figures 2 and 6 (modeled in Figure 12n). In the condition with 0.5 mM L-Gln and 1 nM IAA, the lateral root density of one of the two lines of *Atchl1-5* mutant constitutively expressing *MtNPF1.7/NIP/LATD* (*Atchl1-5+ MtNPF1.7/NIP/LATD #1*) displayed a similar pattern as

compared to wild-type Col-0 plants, while the other line (*Atchl1-5+ MtNPf1.7/NIP/LATD #3*) displayed a similar lateral root density as the control *Atchl1-5* transformed with *AtNPF6.3/NRT1.1(CHL1).* In this case, the exogenous supply of IAA might lead to accumulation of IAA at the root tips of two lines of *Atchl1-5* mutant constitutively expressing *MtNPF1.7/NIP/LATD*, promoting lateral root development, producing the results seen in Figure 6 (modeled in Figure 12o). Interestingly, for the condition with 1 mM KNO_3 and 1 nM IAA, as we observed in Figure 6, in the two lines of *Atchl1-5* mutant constitutively expressing *MtNPF1.7/NIP/LATD*, it appears that nitrate has triggered an unexpected response of MtNPF1.7/NIP/LATD to IAA in which lateral root development was suppressed. The *Atchl1-5* control constitutive expressing *AtNPF6.3/NRT1.1(CHL1)* showed a similar response, although it was not statistically different from wild-type Col-0 plants or from *Atchl1-5* plants. Because it is generally accepted that lateral root density is a proxy for IAA transport in the roots (Reed et al., 1998), it seems unlikely that this is due to IAA accumulation (Figure 12p). With these transgenic lines (*Atchl1-5* mutant constitutively expressing *MtNPF1.7/NIP/LATD* or *AtNPF6.3/NRT1.1(CHL1)*), more experiments based on a similar experimental strategy, such as comparing other growth phenotypes of *Atchl1-5* mutant constitutively expressing *MtNPF1.7/NIP/LATD* to *Atchl1-5* mutant and Col-0, can be done to determine if *MtNPF1.7/NIP/LATD* can complement other *AtNPF6.3/NRT1.1(CHL1)* mutation related phenotypes, such as reduced lateral root growth within a nitrate-rich patch (Remans et al., 2006).

As previously mentioned in Section 2.3, Drs. M. Salehin and R. Bagchi found that transgenic *A. thaliana* plants constitutively expressing *MtNPF1.7/NIP/LATD* were larger in both shoots and leaves (Rammyani Bagchi, thesis, 2013; Mohammad Salehin, thesis, 2013). In addition, considering that *MtNPF1.7/NIP/LATD* may encode an auxin efflux transporter (Bagchi,

PhD Thesis, 2013) and ABA was able to rescue lateral root defect in *Mtlatd* mutant but not the nodule phenotype (Liang et al., 2007), several experiments were done to determine if this enhanced growth phenotype in *A. thaliana* plants (Col-0 and *Atchl1-5*) constitutively expressing *MtNPF1.7/NIP/LATD* correlates with these plant hormones and cytokinin, also known to be involved in cell growth. Plants were treated with exogenous supply of auxin, cytokinin, and ABA in different nitrogen sources as indicated in Figure 7 and Figure 8. Figure 7 represents the lateral root density in response to various nitrogen conditions with the exogenous supply of the plant hormones mentioned above in presence of 1 mM KNO_3, and Figure 8 represents the lateral root density in response to low and high concentration of nitrate with an exogenous supply of the IAA and BAP. *A. thaliana* Col-0 plants constitutively expressing *MtNPF1.7/NIP/LATD* had a lower lateral root density in response to IAA in presence of 1 mM KNO_3 and had a higher lateral root density in 200 µM KNO_3 as compared to wild-type plants (Col-0) and the transformation control (Col-0+*AtNPF6.3/NRT1.1(CHL1)*) plants (Figures 7 and 8). These results suggest that *MtNPF1.7/NIP/LATD* might interact with auxin and cytokinin in the nitrate sensing pathway. By comparing lateral root phenotypes in response to various treatments as indicated in Figure 6, 7 and 8, the lateral root density of wild-type *A. thaliana* Col-0 plants constitutively expressing *MtNPF1.7/NIP/LATD* were more similar to *Atchl1-5* mutant plants in response to nitrate alone as compared to other plant lines (wild-type Col-0, *Atchl1-5*, and *Atchl1-5+MtNPF1.7/NIP/LATD* plants) as modeled in Figure in Figures 12e-f for Col-0+*MtNPF1.7/NIP/LATD* plants and Figures 12i-j for *Atchl1-5* plants. However, in the presence of IAA either in the low or high nitrate condition, wild-type *A. thaliana* Col-0 plants constitutively expressing *MtNPF1.7/NIP/LATD* displayed a unique lateral root growth pattern, which is an enhanced lateral root density in the presence of 200 µM KNO_3 and 0.1 µM IAA and suppressive lateral root density in the presence

of 1 mM KNO_3 and 0.1 µM IAA (Figures 8 and 12g-h). Other plant lines tested (wild-type Col-0, *Atchl1-5*, and *Atchl1-5+MtNPF1.7/NIP/LATD* plants) respond to the no or low nitrate and 10 nM IAA conditions with no enhanced lateral root development (Figure 6 and modeled in Figures12c, 12k, and 12o), and to the high nitrate and 10 nM IAA with no suppressive lateral root development (Figures 12d, 12l, and 12p). A possible explanation is that in the presence of 200 µM KNO_3, MtNPF1.7/NIP/LATD might interfere with auxin transport associated by AtNPF6.3/NRT1.1(CHL1) in the root tips of Col-0 plants constitutively expressing *MtNPF1.7/NIP/LATD. AtNPF6.3/NRT1.1(CHL1)* is expressed in a narrow window in the lateral root primordia and in lateral root meristems (Krouk et al., 2010), while *MtNPF1.7/NIP/LATD* is constitutively expressed in these transgenic plants. As can been seen in Figures 7 and 8, the lateral root density of Col-0 plants constitutively expressing *MtNPF1.7/NIP/LATD* was higher compared to the lateral root density of wild-type Col-0 plants in the presence of 200 µM KNO_3. We hypothesize that this leads to IAA accumulation at the tips, which promotes lateral root development in Col-0 plants constitutively expressing *MtNPF1.7/NIP/LATD* (Figure 12e) because lateral root density has shown to be correlated with auxin (Reed et al., 1998). As demonstrated in Figures 7 and 8, the lateral root density of Col-0 plants constitutively expressing *MtNPF1.7/NIP/LATD* was similar to wild-type Col-0 plants and the transformation control plants (Col-0+*AtNPF6.3/NRT1.1(CHL1)*) in the presence of 1 mM KNO_3. In the presence of 1 mM KNO_3, nitrate inhibits IAA transport by AtNPF6.3/NRT1.1(CHL1) (Krouk et al., 2010), while the effect of nitrate on MtNPF1.7/NIP/LATD IAA efflux is unknown. In this situation, we hypothesize that IAA accumulates at the root tips of Col-0 plants constitutively expressing *MtNPF1.7/NIP/LATD* and promotes lateral root development (Figures 7 and 8, modeled in Figure 12f). In the presence of 200 µM KNO_3 and 0.1 µM IAA, the lateral root density of Col-0

plants constitutively expressing *MtNPF1.7/NIP/LATD* is higher. In this condition, MtNPF1.7/NIP/LATD might promote IAA transport to lateral root tips by an unknown mechanism. Considering that lateral root density correlates with auxin (Reed et al., 1998), we hypothesized that these environmental conditions, there is likely to be more IAA accumulation at the root tips of Col-0 plants constitutively expressing *MtNPF1.7/NIP/LATD* and we speculate this is the underlying cause of even more enhanced lateral root development (Figure 12g). Another hypothesis could be that under these conditions, AtNPF6.3/NRT1.1(CHL1) is active at transporting auxin (not inhibited by nitrate) and so is MtNPF1.7/NIP/LATD, which promotes more auxin accumulation at the lateral roots of Col-0 plants constitutively expressing *MtNPF1.7/NIP/LATD* (Figure 12g), suggesting a synergistic effect of AtNPF6.3/NRT1.1(CHL1) and MtNPF1.7/NIP/LATD.

As demonstrated in Figures 7 and 8, in presence of 1 mM KNO_3 and 0.1 µM IAA, Col-0 plants constitutively expressing *MtNPF1.7/NIP/LATD* had a suppressed lateral root development. In this condition, we hypothesize that 1 mM KNO_3 inhibits auxin transport by AtNPF6.3/NRT1.1(CHL1) at the lateral root tips of Col-0 plants constitutively expressing *MtNPF1.7/NIP/LATD* and, at the same time, nitrate may affect MtNPF1.7/NIP/LATD's ability to transport IAA. This possibly promotes IAA transport away from the root tips of Col-0 plants constitutively expressing *MtNPF1.7/NIP/LATD* and suppresses lateral root development, as illustrated in Figure 12h. This suggests that AtNPF6.3/NRT1.1(CHL1) and MtNPF1.7/NIP/LATD could be working in opposition (one is known to be an influx transporter and there is evidence that the other is an efflux transporter), and AtNPF6.3/NRT1.1(CHL1) is inhibited by nitrate, while MtNPF1.7/NIP/LATD is still able to export auxin from cells. These results (Figures 7 and 8: modeled in Figure 12h) share no similarity to other plant lines (Col-0,

Atchl1-5, Col-0+ *MtNPF1.7/NIP/LATD*, and *Atchl1-5+ MtNPF1.7/NIP/LATD*) tested, which suggests that MtNPF1.7/NIP/LATD transports IAA by a different mechanism from AtNPF6.3/NRT1.1(CHL1) and is consistent with Dr. R. Bagchi's data that MtNPF1.7/NIP/LATD effluxes IAA in *X. laevis* oocytes system (Rammyani Bagchi, PhD thesis, 2013). In contrast, AtNPF6.3/NRT1.1(CHL1) is reported to be an auxin influx transporter (Krouk et al., 2010), implying that the direction of auxin flow might explain the different phenotypes of plant lines tested. However, together with all the current results that we have had on *MtNPF1.7/NIP/LATD*, the retarded lateral root development of transgenic *A. thaliana* plants (Col-0+ *MtNPF1.7*/*NIP*/*LATD* versus *Atchl1-5+ MtNPF1.7*/*NIP*/*LATD*) in the presence of 1 mM KNO_3 and 0.1 µM IAA or 1 mM KNO_3 and 10 nM IAA (Figures 6 and 8; modeled in Figures 12h and 12p) cannot be fully explained by this hypothesis. In these experiments (Figures 6, 7, and 8), the concentration of the exogenous IAA treatment for experiments (Figure 6) involved *Atchl1-5+MtNPF1.7/NIP/LATD* lines was 10 nM, whereas the concentration of the exogenous IAA treatment for experiments (Figures 7 and 8) involved Col-0+ *MtNPF1.7/NIP/LATD* lines was 0.1 µM, which means that we cannot make a direct comparison between these experiments. However, the idea here is to compare how MtNPF1.7/NIP/LATD affects lateral root development in response to IAA in the presence or absence of nitrate. Since IAA transport by AtNPF6.3/NRT1.1(CHL1) is inhibited by nitrate in Col-0+ *MtNPF1.7*/*NIP*/*LATD* plants, while there is no functional AtNPF6.3/NRT1.1(CHL1) in *Atchl1-5+ MtNPF1.7*/*NIP*/*LATD* plants, these transgenic plants should behave in a similar pattern in response to IAA in presence of 1 mM KNO_3. However, it may be that concentration of IAA is important (Figures 6, 8, 12h and 12p). Although both transgenic plant lines (Col-0+ *MtNPF1.7*/*NIP*/*LATD* and *Atchl1-5+ MtNPF1.7*/*NIP*/*LATD*) had a suppressed lateral root development in presence of 1 mM KNO_3

and 10 nM IAA or 1 mM KNO_3 and 0.1 µM IAA, the retarded lateral root development is more severe in Col-0 plants constitutively expressing *MtNPF1.7/NIP/LATD* than in *Atchl1-5* plants constitutively expressing *MtNPF1.7/NIP/LATD* (Figures 12h and 12p).

Together with the results discussed above (Figures 2, 6, 7, and 8), the *MtNPF1.7/NIP/LATD* gene affects the lateral root density of the transgenic plants constitutively expressing *MtNPF1.7/NIP/LATD* (Col-0+ *MtNPF1.7/NIP/LATD* and *Atchl1-5 + MtNPF1.7/NIP/LATD*) differently in response to IAA in the presence of absence of nitrate. The mechanism of auxin-associated nitrate-sensing role of MtNPF1.7/NIP/LATD remains unclear. Clearly, auxin and nitrate are tightly associated with MtNPF1.7/NIP/LATD in lateral root development. Localization of the auxin response in these plants (Col-0 plants constitutively expressing *MtNPF1.7/NIP/LATD* and *Atchl1-5* mutant constitutively expressing *MtNPF1.7/NIP/LATD*) will help understand this phenotype. Introduction of auxin response markers in these plants (Col-0 plants constitutively expressing *MtNPF1.7/NIP/LATD* and *Atchl1-5* mutant constitutively expressing *MtNPF1.7/NIP/LATD*) will be a future work.

As discussed already in Section 2.1, Tian et al., (2009) showed that lateral root growth was shown to be inhibited in response to high external nitrate and the reductions of LR length in wild-type plants were greater as compared to *Atchl1-5* plants in response to high nitrate concentration. To investigate whether the constitutive expression of the *MtNFP1.7/NIP/LATD* gene in the *A. thaliana chl1-5* mutant would complement the defects in *Atchl1-5* mutation for this phenotype, I studied the response of root growth in the two lines of transgenic plants (*Atchl1-5+ MtNFP1.7/NIP/LATD* #11 and *Atchl1-5+ MtNFP1.7/NIP/LATD* #12) in 0.1 mM KNO_3 and 10 mM KNO_3. In the studies of Tian et al., (2009), the length of primary roots of both wild-type plants and *Atchl1-5* mutant plants did not differ in the high nitrate and low nitrate

concentration. In addition, the reduction in length of the lateral roots was greater in wild-type plants in response to high nitrate concentration. Significant reductions in lateral root lengths were observed in the wild-type Col-0 plants and *Atchl1-5* mutant plants grown on media containing high nitrate concentration compared with those grown on media containing with low nitrate concentration (Tian et al., 2009). However, these results could not be replicated as shown in Figures 3, 4, and 5. My results showed that reduction in lateral root length was less in *Atchl1-5* mutant plants in response to high nitrate concentration. However, my results also showed that the *Atchl1-5* mutant primary root length was longer than that of the wild-type plant Col-0, and *MtNFP1.7/NIP/LATD* appeared to complement this defect in the transgenic lines in media containing 10 mM KNO_3 (Figure 3). Similar results were obtained for lateral root number (Figure 4), but not for lateral root length (Figure 5). Overall, my data provides support for the *MtNFP1.7/NIP/LATD* gene's ability to complement phenotypes associated with a loss of nitrate sensing in the *Atchl1-5* mutant. The inability to replicate the results of Tian et al., (2009) might be due to a technical problem which could be improved, such as dealing with a smaller number of samples at one time. It is also possible that the results of Tian et al., (2009) could only work under certain conditions, beyond those that are spelled out in their published paper.

Our lab mainly focuses on *M.truncatula*. Considering this, experiments were also performed in *M.truncatula*, the native system for *MtNPF1.7/NIP/LATD*. As described in Section 3.1, in order to determine if *MtNPF1.7/NIP/LATD* is responsive to nitrate and if *Mtnip-1* mutant has defects in nitrate response in nitrate responsive genes, *MtNRT2.1* was used as a nitrate responsive gene marker in these experiments. The gene expression of *MtNRT2.1* and *MtNPF1.7/NIP/LATD* were studied in response to nitrate application. As previously mentioned in the introduction, *AtNRT2.1* is regulated by nitrate and mutant defective in *AtNRT2.1*, fail to have nitrate-

responsive genes respond normally to nitrate (Munos et al., 2004; Little et al., 2005; Wang et al., 2009). *MtNRT2.1* was chosen as a target gene because it is a homolog of *AtNRT2.1* gene that shows a high expression in low concentration of nitrate (250 μM) *A. thaliana*. *AtNRT2.1* expression was shown to be strongly nitrate inducible, peaking at 3 to12 hours and subsequently declining (Okamoto et al., 2003).Since in *A. thaliana*, the putative orthologous *AtNRT2.1* gene is differentially regulated in the *Atchl1-5* mutant, the comparison between the two species demonstrates that *MtNPF1.7/NIP/LATD* does not act in a homologous fashion in *M. truncatula* as *AtNPF6.3/NRT1.1(CHL1)* does in *A. thaliana* (Figure 9 and 10). The experiments showed that *MtNRT2.1* expression is regulated by nitrate and is regulated by nitrate in the *Mtnip-1* mutant similarly to WT, suggesting that *MtNPF1.7/NIP/LATD* does not regulate the nitrate-responsive *MtNRT2.1* gene. However, different time frames of the nitrate treatment are needed to be tested to confirm this point since only 2 hour treatment was tested in this study. Also, different nitrate responsive genes can be tested to have more detailed information about the potential role of *MtNPF1.7/NIP/LATD* in nitrate sensing since my tests only looked at one nitrate responsive gene and it could be that I picked one that might be differently regulated than others.

As previously mentioned above, Dr. R. Bagchi's data (Rammyani Bagchi, PhD thesis, 2013) suggested that *MtNPF1.7/NIP/LATD* effluxes auxin. In *A. thaliana*, many mutants having defects in auxin transport displayed a gravitropic phenotype in roots (Marchant et al., 1999; Lewis et al., 2007; Guyomarc'h et al., 2012). A gravitropism assay was done to determine if *MtNPF1.7/NIP/LATD* has a role in gravitropic response. The wild-type A17, *Mtnip-1*, *Mtnip-3*, and *M. truncatula* wild-type A17 overexpressing *MtNPF1.7/NIP/LATD* (A17OE *MtNPF1.7/NIP/LATD* L4) plants lines were grown to compare their root phenotypes in response to the gravity. As can been seen in Figure 11, the degree of curvature of the root in *Mtnip-1* was

lower as compared to wild-type plant A17. So far, there was only one nitrogen condition tested (5mM ammonium nitrate). Considering the results in the *A. thaliana* system, it is possible that auxin flux may be affected by ammonium or nitrate or both, and thus, similar assays should be carried out in environmental conditions in which the nitrogen supply conditions are varied.

In conclusion, the major findings of this study are:

1. The *MtNPF1.7/NIP/LATD* gene is able to restore nitrate-absent responsiveness defects of the *Atchl1-5* mutant (Figure 2).
2. The constitutive expression of *MtNPF1.7/NIP/LATD* gene was able to partially, but not fully restore the wild-type phenotype in the *Atchl1-5* mutant line in response to auxin and cytokinin (Figures 6).
3. The constitutive expression of *MtNPF1.7/NIP/LATD* gene affects the lateral root density of wild-type Col-0 plants differently in response to IAA in the presence of high (1 mM) or low (0.1 mM) nitrate (Figures 7 and 8).
4. The *MtNPF1.7/NIP/LATD* expression is not regulated by nitrate at the concentrations tested, and *MtNPF1.7/NIP/LATD* does not regulate the nitrate-responsive *MtNRT2.1* gene (Figures 9 and 10).
5. *Mtnip-1* plants have an abnormal gravitropic root response implicating an auxin defect (Figure 11).

CHAPTER 5

MATERIALS AND METHODS

5.1 Plant Material and Growth Conditions

For root phenotype experiments as shown in Figure 2 and 6, *A.thaliana* wild-type Col-0, Atchl1-5, and 2 lines of transgenic *A.thaliana chl1-5* mutant plants expressing the *MtNPF1.7/NIP/LATD* gene under the control of the constitutive *A.thaliana EF1α* promoter (*Atchl1-5+ MtNPF1.7/NIP/LATD* line#11 and *Atchl1-5+ MtNPF1.7/NIP/LATD* line#12) (Auriac and Timmers, 2007; Bagchi et al., 2012; Salehin et al., 2012) were used. All seeds were put in a closed-jar and sterilized by chlorine vapors (adding 1mL of HCl into 50 ml of bleach solution used in the lab) for 4 hours. After the surface-sterilization, all seedlings were transferring onto 1 % phytagel square petri dishes containing basal growth media with different nitrogen sources (0.5mM L-Gln, 1mM KNO_3 and nitrogen-free). All seeds were vernalized in the dark at 4° C for 2 days. After the seed vernalization, all plates were incubated vertically at 22° C with 16hrs/8hrs light/ dark regime for 8 days. Then the lateral root density and root architecture were measured and examined.

Composition of the basal growth media:

0.5mM $CaS0_4$, 0.5mM $MgCl_2$, 1mM KH_2PO_4, 2.5mM MES (pH 5.8), 50µM NaFeEDTA, 50µM H_3BO_3, 12µM $MnCl_2$, 1µM $CuCl_2$, 1µM $ZnCl_2$ and 0.03µM NH_4MoO_4

For the root phenotype experiments as shown in Figure 3, 4, and 5, *A.thaliana* wild-type Col-0, Atchl1-5, and 2 lines of transgenic *A.thaliana chl1-5* mutant plants expressing the *MtNPF1.7/NIP/LATD* gene under the control of the constitutive *A.thaliana EF1α* promoter (*Atchl1-5+ MtNPF1.7/NIP/LATD* line#11 and *Atchl1-5+ MtNPF1.7/NIP/LATD* #12) (Auriac and Timmers, 2007; Bagchi et al., 2012; Salehin et al., 2012) were used, and were sterilized as

previously described. After the surface-sterilization, all seedlings were germinated on 1/2 MS agar plates (0.6 % phytagel (w: v), pH 5.8 for 4 days). Thereafter the germinated seedlings of all plants were transferred to 1/2 MS agar medium without nitrogen salts, or containing varying nitrate concentrations (no nitrogen, 0.1 mM KNO_3, and 10 mM KNO_3) for 5 days. Then the length of the primary root, number of lateral roots and length of the lateral roots were measured.

Composition of 1/2 MS media:

1/2 MS major salts: 825 mg/L NH_4NO_3, 950 mg/L KNO_3, 220 mg/L $CaCl_2.H_2O$, 185 mg/L $MgSO_4.7H_2O$, and 85 mg/L KH_2PO_4

1/2 MS minor salts: 3.1 mg/L H_3BO_3, 11.15 mg/L $MnSO_4.4HO$, 4.3 mg/L $ZnSO_{4.4}H_2O$, 0.415 mg/L KI, 0.125 mg/L $Na_2MoO4.2H_2O$, 0.0125 mg/L $CoCl_2.6H_2O$, and 0.0125 mg/L $CuSO_4.5H_2O$

1/2 MS vitamins: 0.05 mg/L Thiamine (HCl), 0.25 mg/L Niacine, 1.0 mg/L Glycine, 0.25 mg/L Pyrodoxine (HCl), and 50 mg/L myo-inistol

Iron Stock (200X): 0.131 g/L Na_2EDTA and 0.1114 g/L $FeSO_4.7H_2O$

pH 5.8

For root phenotype experiments as shown in Figure 7 and 8, *A.thaliana* wild-type Col-0, two lines of transgenic *A.thaliana* Col-0 plant constitutively expressing *MtNPF1.7/NIP/LATD* (Col-0+ *MtNPF1.7/NIP/LATD* L1 and Col-0+ *MtNPF1.7/NIP/LATD* L2), and the transformation control (Col-0+*AtNPF6.3/NRT1.1*) plants were used, and all plants were sterilized as previously described. After the surface-sterilization, all plant seeds were vernalized on pertri dishes containing the germination media for 3 days in the dark at 4° C. Then all seedlings were germinated at 22° C with 16hrs/8hrs light/ dark regime for 3 days. Once seed germination was observed, all seeds were transferred to square petri dishes containing 1/2 MS-N basal media with

different nitrogen sources as indicated in the Figure 7 and 8 and allowed to grow vertically for 11 days. Then the lateral root density and root architecture were measured and examined.

Composition of the germination media:

1/2 MS-N media (as known as 1/2 MS nitrogen-depleted media):

1/2 MS major salts: 220 mg/L $CaCl_2.H_2O$, 185 mg/L $MgSO_4.7H_2O$, and 85 mg/L KH_2PO_4

1/2 MS minor salts: 3.1 mg/L H_3BO_3, 11.15 mg/L $MnSO_4$.4HO, 4.3 mg/L $ZnSO_{4.4}H_2O$, 0.415 mg/L KI, 0.125 mg/L $Na_2MoO4.2H_2O$, 0.0125 mg/L $CoCl_2.6H_2O$, and 0.0125 mg/L $CuSO_4.5H_2O$

1/2 MS vitamins: 0.05 mg/L Thiamine (HCl), 0.25 mg/L Niacine, 1.0 mg/L Glycine, 0.25 mg/L Pyrodoxine (HCl), and 50 mg/L myo-inistol

Iron Stock (200X): 0.131 g/L Na_2EDTA and 0.1114 g/L $FeSO_4.7H_2O$

1% sucrose

pH 5.8

0.2 % phytagel

Composition of 1/2 MS-N basal media:

1/2 MS-N media (as known as 1/2 MS nitrogen-depleted media):

1/2 MS major salts: 220 mg/L $CaCl_2.H_2O$, 185 mg/L $MgSO_4.7H_2O$, and 85 mg/L KH_2PO_4

1/2 MS minor salts: 3.1 mg/L H_3BO_3, 11.15 mg/L $MnSO_4$.4HO, 4.3 mg/L $ZnSO_{4.4}H_2O$, 0.415 mg/L KI, 0.125 mg/L $Na_2MoO4.2H_2O$, 0.0125 mg/L $CoCl_2.6H_2O$, and 0.0125 mg/L $CuSO_4.5H_2O$

1/2 MS vitamins: 0.05 mg/L Thiamine (HCl), 0.25 mg/L Niacine, 1.0 mg/L Glycine, 0.25 mg/L Pyrodoxine (HCl), and 50 mg/L myo-inistol

Iron Stock (200X): 0.131 g/L Na_2EDTA and 0.1114 g/L $FeSO_4.7H_2O$

1% sucrose

pH 5.8

0.8% phytagel

For the gene expression experiments as shown in Figure 9 and 10, *M. truncatula* wild-type A17 and *Mtnip-1* seedpods were ground to remove seeds. All seeds were scarified in concentrated sulfuric acid for 5 minutes, washed 5 times with autoclaved water, surface-sterilized with commercial bleach for 2 minutes, and washed 6 times with autoclaved water. Then all seeds were placed on a shaker and rinsed with autoclaved water every 40 minutes for 5 hours to imbibe them with water. All seeds were transferred onto petri dishes. Then all seeds were inverted and incubated in the dark at 4° C for 3 days for vernaliation. After the cold treatment, the plates were kept inverted and all seeds were germinated in the room temperature at the dark overnight. Next day, all seeds were grown hydroponically in BNM media until the primary root length of all plant roots reached 3 cm. Then all seedlings were transferred and grown hydroponically in BNM media containing different concentrations of nitrate as indicated in Figure 9 and 10 for 16 hours.

Composition of BNM media:

344mg/L $CaSO_4.2H_2O$ and 390 mg/L MES

200X Fe-EDTA: 3.73 g/L Na_2EDTA and 2.78 g/L $FeSO_4.7H_2O$

200X Nod Minors I: 620 mg/L H_3BO_3, 1.69 g/L $MnSO_4.H_2O$, and 920 mg/L $ZnSO_4.7H_2O$

200X Nod Minors II: 5.0 mg/L $CoCl_2.6H_2O$, 50.0 mg/L $Na_2MoO_4.2H_2O$, and 3.2 mg/L $CuSO_4$

pH 5.8

For the gravitropic phenotype experiments as shown in Figure 11, the wild-type A17, *Mtnip-1*, *Mtnip-3*, and *M. truncatula* wild-type A17 overexpressing *MtNPF1.7/NIP/LATD*

(A17OE*MtNPF1.7/NIP/LATD* L4) plants lines were used. All seeds were germinated as previously descried. After all seeds were germinated, they were transferred to square petri dishes contacting BNM media with 5 mM ammonium nitrate and grown vertically at 22° C with 16hrs/8hrs light/ dark regime for 4 days for the root establishment. Then square petri dishes containing each experimental plant line were turned 90^{o}, and all plants were allowed to grow on the same petri dishes for 5 days.

5.2 The Lateral Root Density Measurement

The pictures of all experimental plant roots were taken and the length of the primary roots was measured by ImageJ. All visible lateral roots per plant were counted. The lateral root density was calculated as a function of the visible lateral root number divided by the primary root length in mm.

5.3 The Root Curvature Measurement

The pictures of all experimental plant roots were taken every day and the root curvature of all experimental plant roots was measured by ImageJ.

5.4 Gene Expression Analysis

RNA samples of *M. truncatula* A17, and *Mtnip-1* were extracted from frozen plant material by grounding in liquid nitrogen and using Qiagen RNeasy Plant Mini Kit (catalog no. 74903). Extracted RNA samples were then treated with Ambion Turbo DNA-free Kit (catalog no. AM1907) to remove possible genomic DNA contamination and checked for gDNA contamination through PCR. RNA samples were then cleaned and concentrated by Qiagen

RNeasy MinElute Cleanup Kit (catalog no. 74204). Invitrogen SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (catalog no. 11752-050) was used to convert RNA samples to cDNA. All cDNA samples were used to determine the gene expression of *MtNPF1.7/NIP/LATD*, *MtNRT2.1* and *β-actin* (used as the housekeeping gene) by using iQ SYBR Green Supermix (catalog no. 170-8880). The primers used to study *MtNPF1.7/NIP/LATD* gene expression were NIPRT5F (5'-TCTCTCTGATTCTTACTTGGGTCGC-3') and NIPRT5R (5'-GGTTTTAGCACCGGGATCATGGCAG-3'). The primers used to study MtNRT2.1 gene expression were NRT2_F1 (5'-CCTGATGGTA ACCTTGGTGCCTTACAG-3') and NRT2_R3 (5'-CAGTG GTTAATTCAACTCCCATAGAGTAAC C-3'). The primers used as the housekeeping gene were *β*-actin_F (TCAATGTGCCTGCCATGTATGT) and *β*-actin_R (ACTCACACCGTCACCAGAATCC).

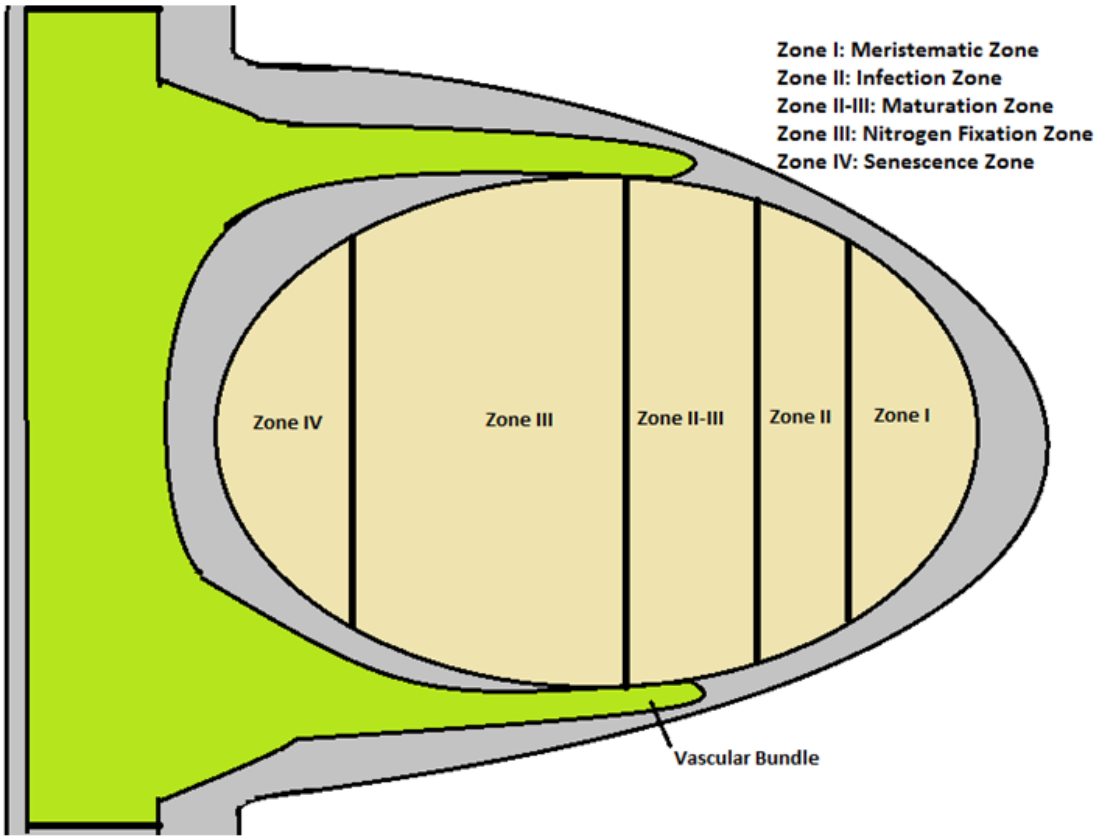

Figure 1. *Nodule zones and vascular bundle in indeterminate nodules.*

The most distal region of the nodule known as Zone I, is the meristematic zone where cells continually divide; nodules continually grow and expand outward from the meristematic zone. The meristematic zone contains no bacteria; hence, no bacterial infection or invasion occurs there. The region below Zone I is Zone II, also as known as the infection zone where the bacteria enter the host cells and are released forming into symbiosomes. Adjacent to Zone II is the Interzone II-III where endocytosed rhizobia complete their differentiation into the nitrogen-fixing forms. Zone III, known as the nitrogen fixation zone, is adjacent to the Interzone II-III. The most proximal region of the nodule is Zone IV, also known as the senescent zone where aging bacteroids and host cells die and are recycled by the plant (Vasse et al., 1990).

Lateral Root Density of Plants Grown in Differing N Sources

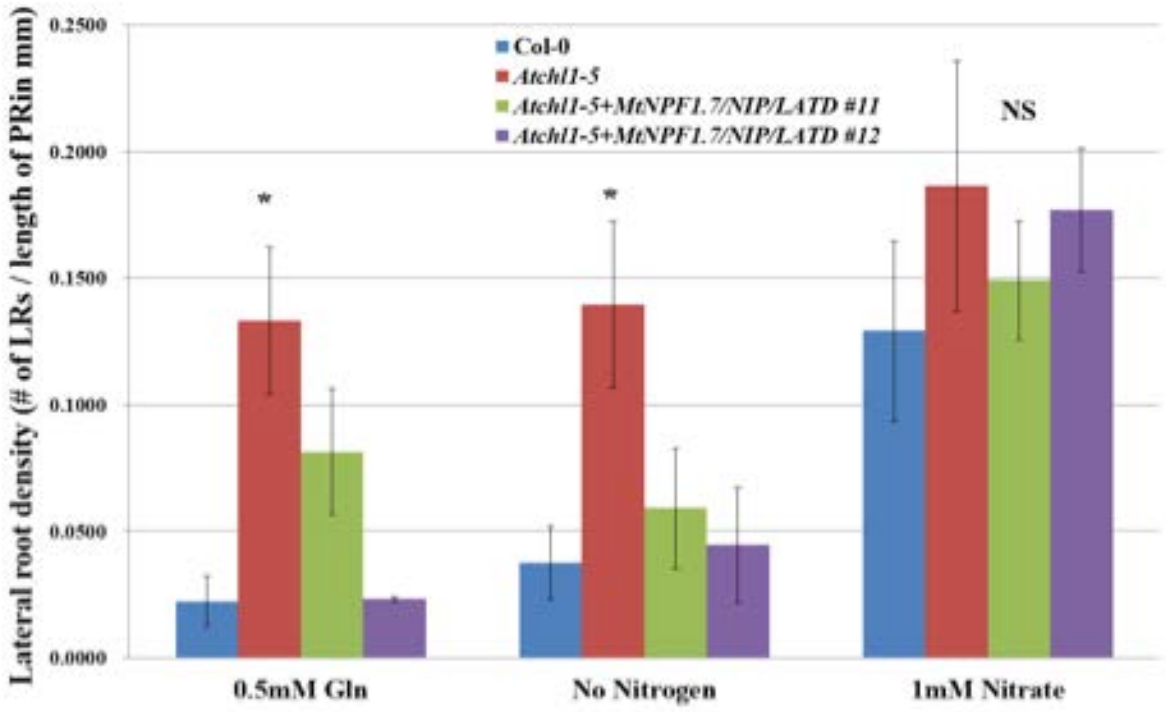

Figure 2. *Lateral root density of plants grown in differing N sources.*

Density of visible (>0.5mm) lateral roots of plants grown for 8 days on media containing nitrogen sources as indicated. Results (n=15-20) are representative of 4 independent experiments. Asterisks mark lateral root density significantly different from the wild-type plant, Col-0, using Student's t test that at $P < 0.05$.

Length of Primary Roots of Plants Grown in Differing N Sources

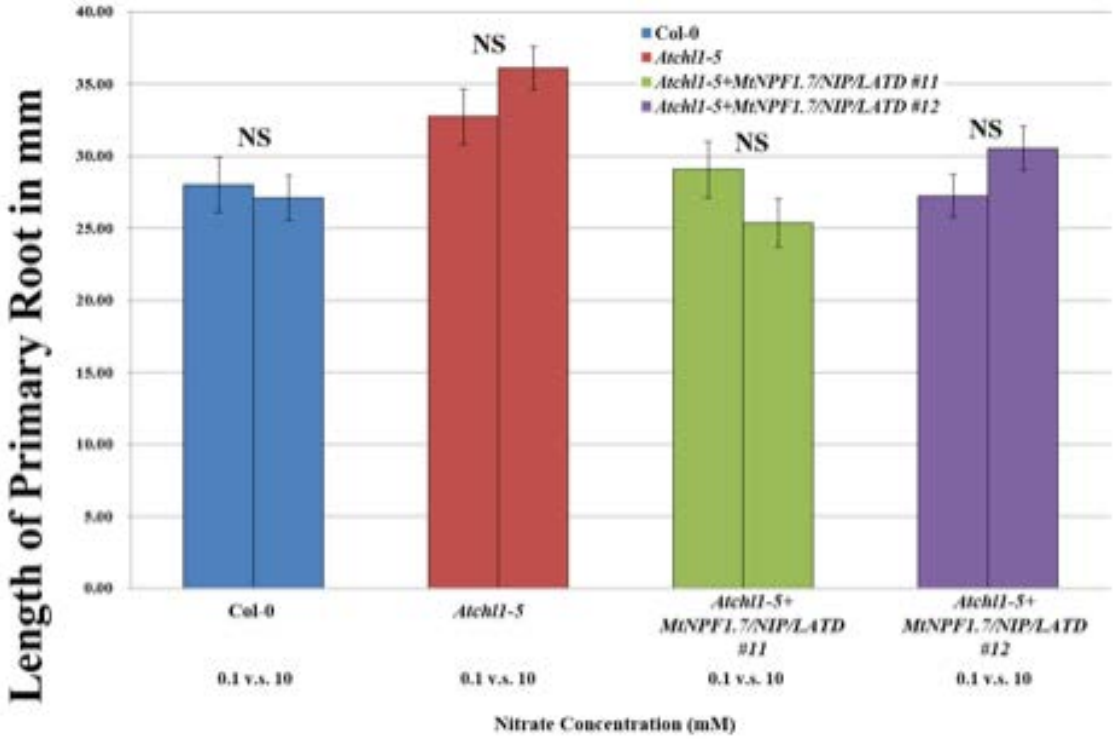

Figure 3. *Length of primary roots of plants grown in differing N sources.*

Length of primary roots in mm of plants grown for 5 days on media containing nitrogen source as indicated in the figure. Results (n=10-20) are representative of 2 individual experiments. NS represents as no significant difference as between low and high nitrate concentration.

Number of Visible Lateral Roots of Plants Grown in Differing N Sources

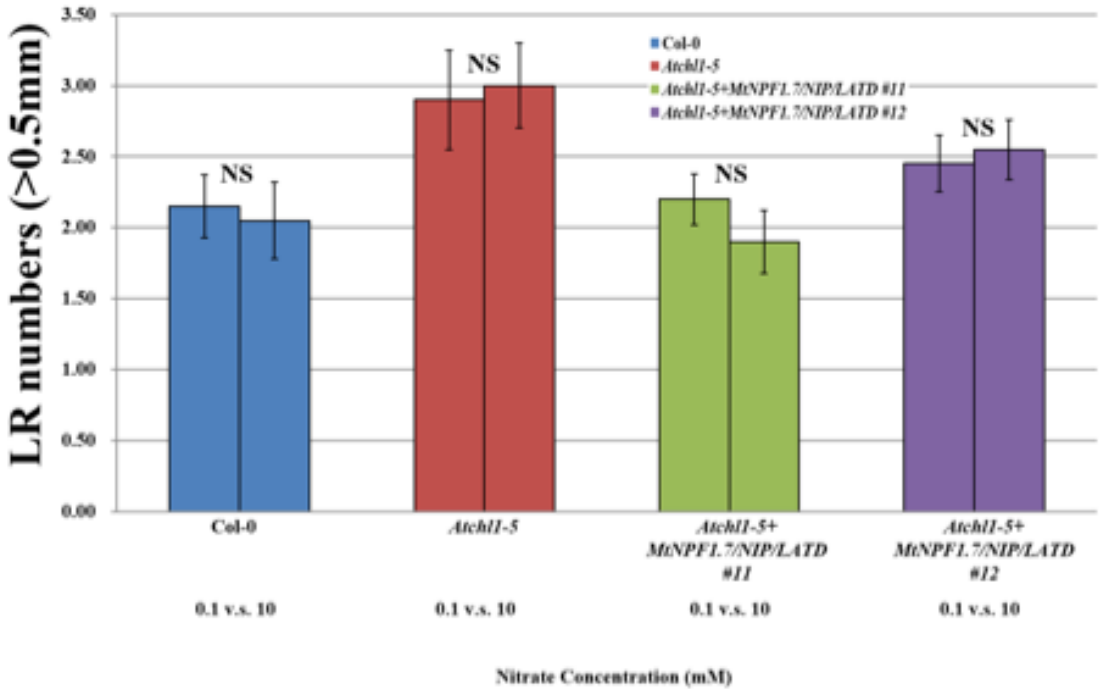

Figure 4. *Number of visible lateral roots of plants grown in differing N sources.*

Number of visible (>0.5 mm) lateral roots of plants grown for 5 days on media containing nitrogen sources as indicated in the figure. Results (n=10-20) are representative of 2 independent experiments. NS represents as no significant difference as between low and high nitrate concentration.

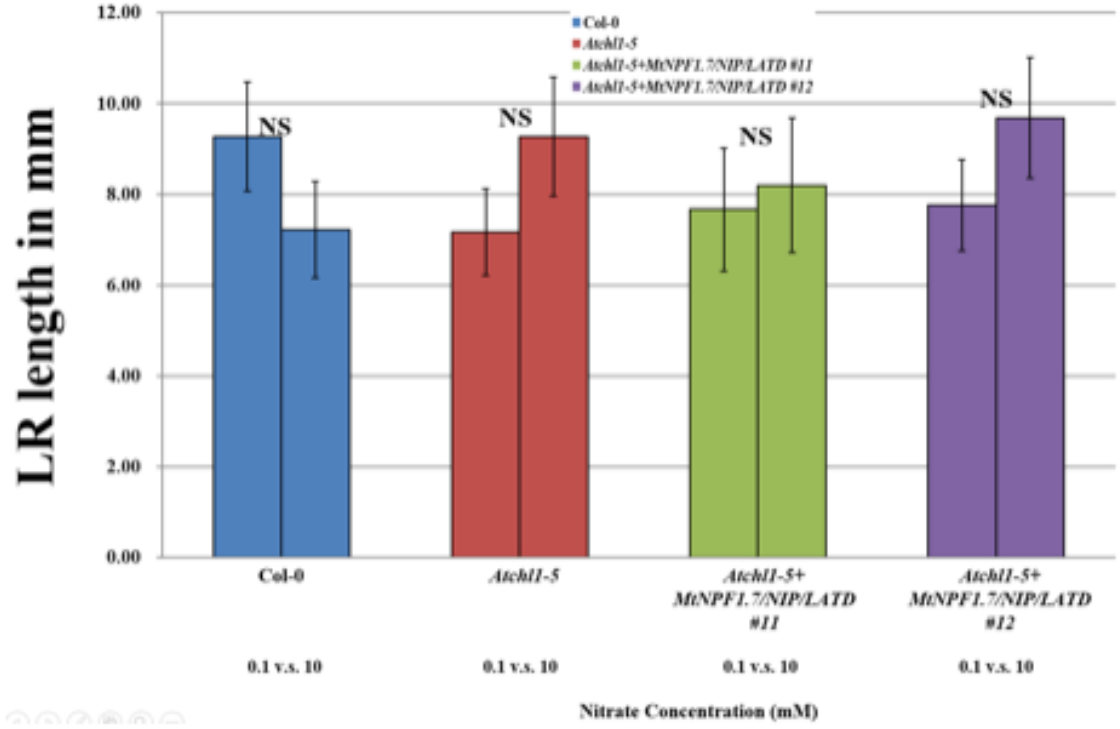

Figure 5. *Length of visible lateral roots of plants grown in differing N sources.*

Length of visible (0.5 mm) lateral roots of plants grown for 5 days on media containing nitrogen sources as indicated in the figure. Results (n=29-60) are representative of 2 independent experiments. NS represents as no significant difference as between low and high nitrate concentration.

Density of Visible Lateral Roots of Plants Grown in Differing N Sources

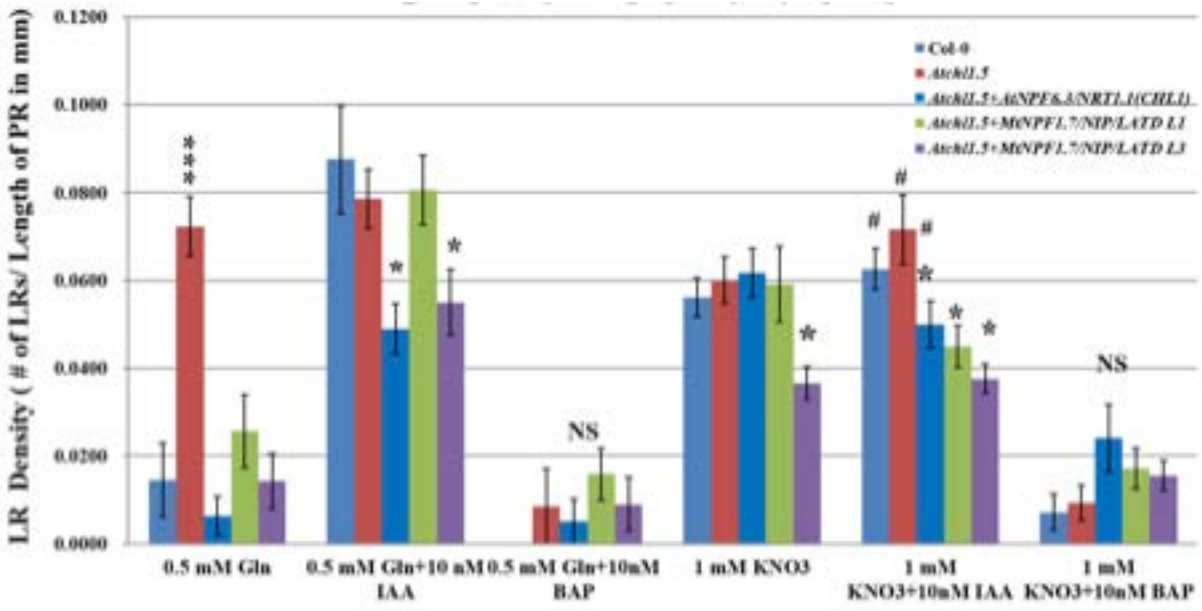

Figure 6. *Density of visible lateral roots of plants grown in differing N sources.*

Density of visible (>0.5mm) lateral roots of plants grown for 8 days on media containing nitrogen sources as indicated in the figure. Results (n=15-20) are representative of 2 independent experiments. Asterisks mark lateral root density significantly different from the wild-type plant, Col-0, using Student's t test that at $P < 0.05$. NS represents as no significant difference as between low and high nitrate concentration.

Density of Visible Lateral Roots of Plants Grown in Differing Conditions

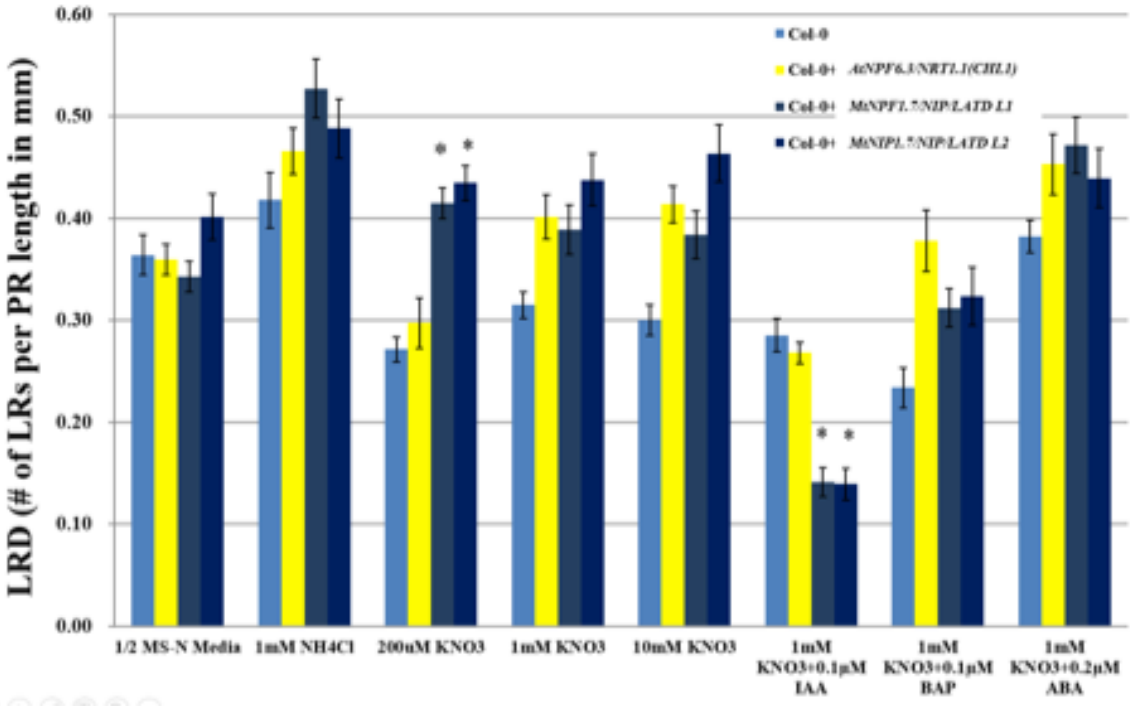

Figure 7. *Density of visible lateral roots of plants grown in differing conditions.*

Density of visible (>0.5mm) lateral roots of plants grown for 14 days on different growth conditions as indicated in the figure. Results (n=14-15) are representative of 3 independent experiments. Asterisks mark lateral root density significantly different from the wild-type plant, Col-0, using Student's t test that at $P < 0.05$.

Density of Visible Lateral Roots of Plants Grown in Differing Conditions

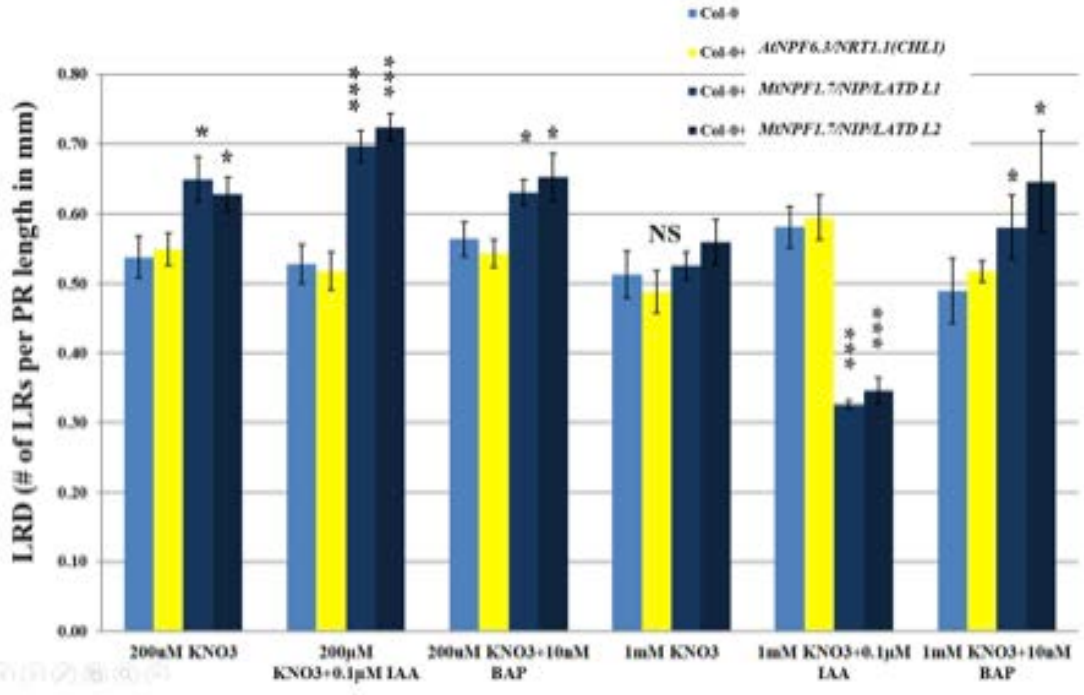

Figure 8. *Density of visible lateral roots of plants grown in differing conditions.*

Density of visible (>0.5mm) lateral roots of plants grown for 14 days on different growth conditions as indicated in the figure. Results (n=14-15) are representative of 3 independent experiments. Asterisks mark lateral root density significantly different from the wild-type plant, Col-0, using Student's t test that at $P < 0.05$. NS represents as no significant difference as between low and high nitrate concentration.

MtNPF1.7/NIP/LATD Gene Expression in Roots in Varied Concentrations of Nitrate

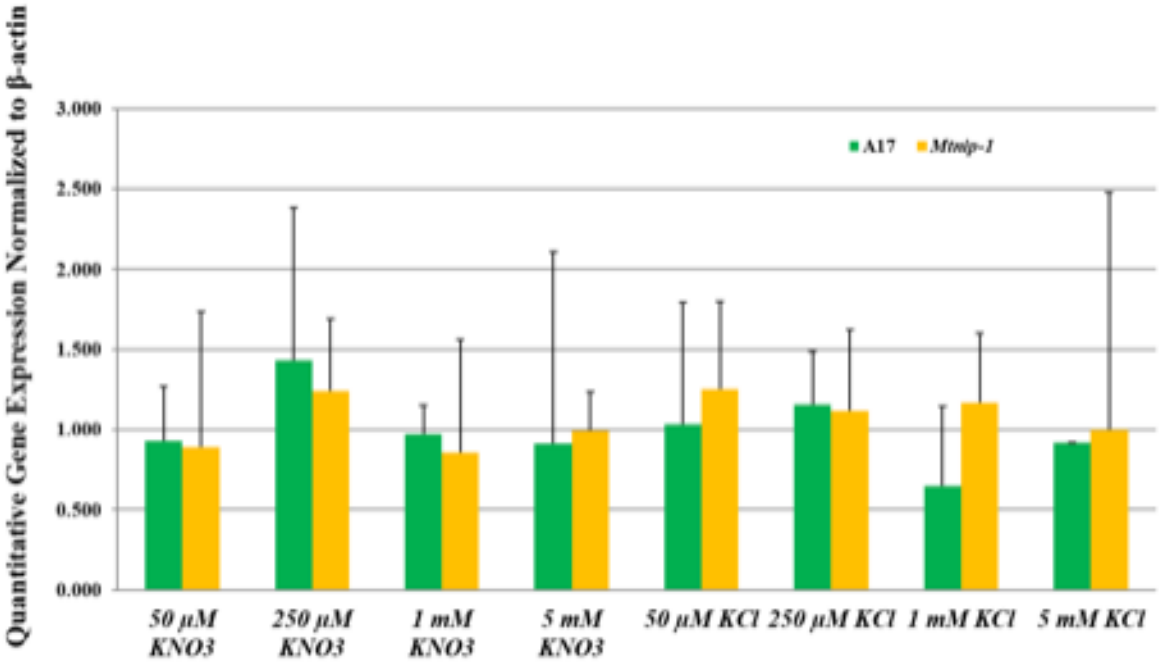

Figure 9. *MtNPF1.7/NIP/LATD gene expression in roots in varied concentrations of nitrate.*

Plants were grown in BNM media 5 days for nitrogen starvation after germination. Then nitrate treatment was applied for 2 hours. The *MtNPF1.7/NIP/LATD* gene expression was measured in Table 8 and plotted in Figure 9. As expected, level of nitrate had no obvious effects on both *MtNPF1.7/NIP/LATD* expression in both A17 and *Mtnip-1* roots, which is consistent with previous work. N=3.

MtNRT2.1 Gene Expression in Roots in Varied Concentrations of Nitrate

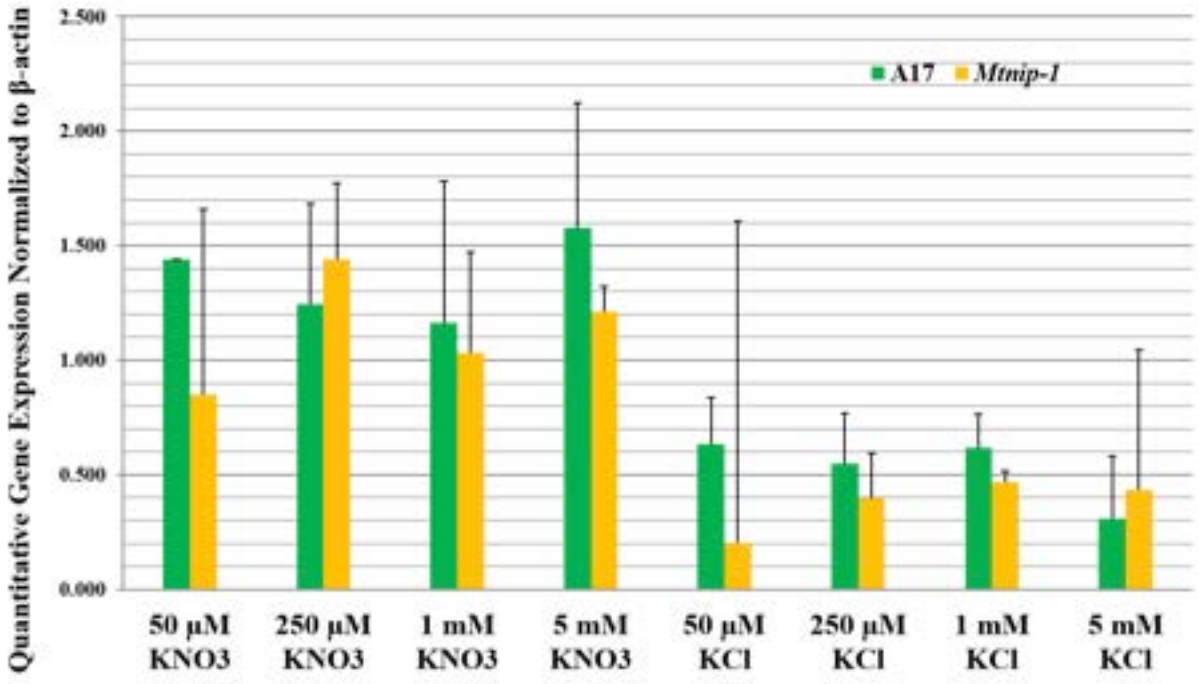

Figure 10. *MtNRT2.1 gene expression in roots in varied concentrations of nitrate.*

After germination, plants were grown in BNM media 5 days for nitrogen starvation. Then nitrate was treated for 2 hours. The *MtNRT2.1* gene expression was measured in Table 9 and plotted in Figure 10. *MtNRT2.1* is very sensitive to nitrate in both A17 and nip roots, suggesting that *MtNPF1.7/NIP/LATD* is not a nitrate sensor. Examination of another nitrate responsive gene is required since there is unpublished data suggesting *MtNPF1.7/NIP/LATD* as a nitrate sensor. N=3.

Curvature Degree of Plant Roots in Response to the Gravity

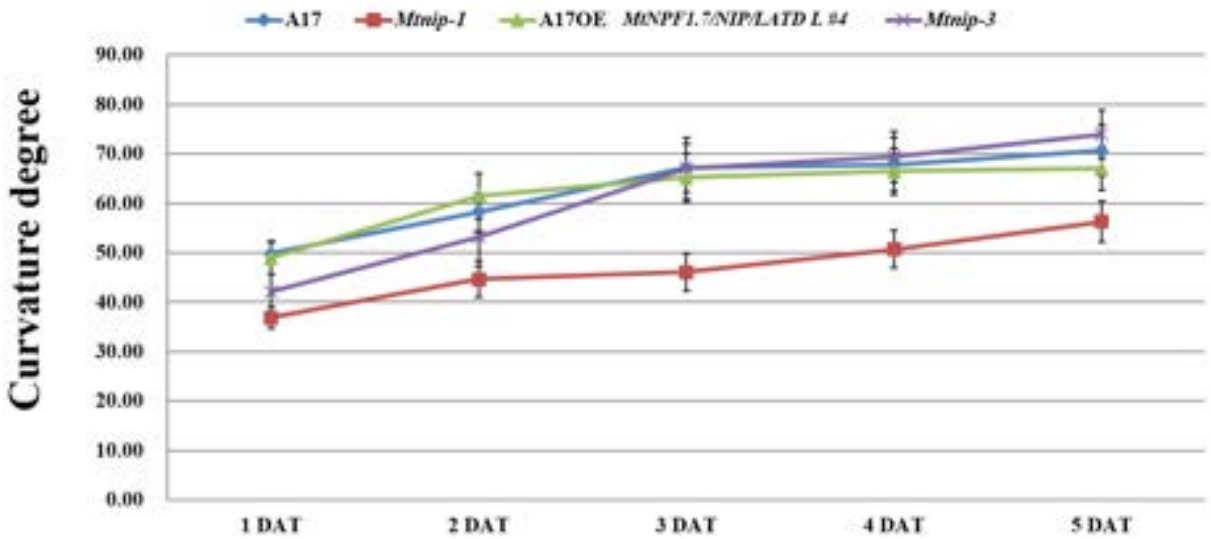

Figure 11. *Curvature degree of plant roots in response to the gravity.*

Curvature degree of plants roots of plants grown on BNM media containing 5 mM ammonium nitrate. All plants were grown on the square petri dishes for 4 days after germination and then turned 90º for another 5 days. Curvature degree of plants roots was measured in Table 10 and plotted in Figure 11. Results was from 12 independent plants. Two other independent experiments had similar results.

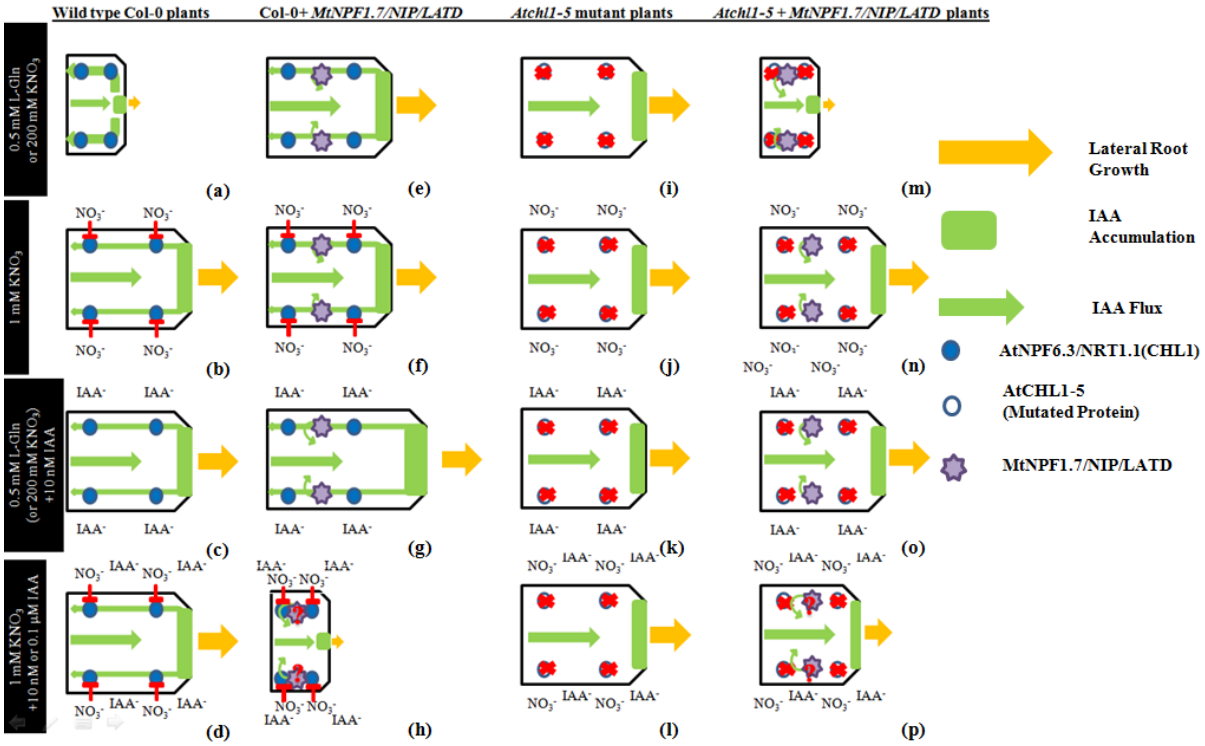

Figure 12. *Schematic model for MtNPF1.7/NIP/LATD and AtNPF6.3/NRT1.1(CHL1) control of lateral root development in A. thaliana in response to nitrate.*

Expanded model was developed starting with Krouk et al., (2010b)'s proposed model for AtNPF6.3/NRT1.1(CHL1) control of lateral root development in response to nitrate. The model depicts lateral root length as responding to the various treatments. It is important to recognize that lateral root length is a proxy for lateral root density, which is what my experiments measured. This is because nascent lateral roots do not elongate unless they stimulated to do so, and thus remain nascent. My experiments measured elongated lateral roots longer than 0.5 mm. Each box represents stunted lateral roots that don't emerge, thus leading to low lateral root density or emerged lateral roots, or super-emerged lateral roots, leading to higer lateral root density. Two scenarios are illustrated the nitrate-specific effect on lateral root development in response to 0.5 mM L-Gln or 1 mM KNO_3 in the growth media (1 mM external nitrogen in both cases). Figures 12a-d represent lateral root density of wild-type Col-0 plants in response to various treatments. Figures 12e-h represent lateral root density of Col-0+*MtNPF1.7/NIP/LATD* plants in response to various treatment. Figures 12i-l represent lateral root density of *Atchl1-5* mutant plants in response to various treatments. Figures 12m-p represent lateral root density of *Atchl1-5+MtNPF1.7/NIP/LATD* plants in response to various treatments. See text for more details.

APPENDIX

TABLES OF DATA PRESENT IN THE FIGURES

Lateral Root Density of Plants Growing in Differing N Sources

Growth condition	Col-0	S.D.	S.E.	*Atchl1-5*	S.D.	S.E.	*Atchl1-5+ MtNPF1.7/NIP/LATD #11*	S.D.	S.E.	*Atchl1-5+ MtNPF1.7/NIP/LATD #12*	S.D.	S.E.
0.5mM Gln	0.0223	0.0646	0.0101	0.1331	0.1432	0.0289	0.0813	0.1072	0.0250	0.0232	0.1018	0.0010
No Nitrogen	0.0375	0.0364	0.0144	0.1394	0.1080	0.0329	0.0590	0.0998	0.0240	0.0444	0.0427	0.0228
1mM Nitrate	0.1292	0.1590	0.0356	0.1864	0.1915	0.0495	0.1490	0.1047	0.0234	0.1768	0.1088	0.0243

Table I. *Lateral root density of plants growing in differing N sources.*

Density of visible (>0,5 mm) lateral roots of plants grown for 8 day on media containing nitrogen sources as indicated in Table 1 was measured and plotted as indicated in Figure 2. Results (n=15-20) are representative of 4 independent experiments.

Length of Primary Roots of Plants Grown in Differing N Sources

Growth Condition	Col-0	S.D.	S.E.	*Atchl1-5*	S.D.	S.E.	*Atchl1-5+ MtNPF1.7/NIP/LATD #11*	S.D.	S.E.	*Atchl1-5+ MtNPF1.7/NIP/LATD #12*	S.D.	S.E.
0.1 mM KNO3	28.00	8.68	1.94	32.75	8.61	1.93	29.07	7.57	1.95	27.25	6.48	1.45
10 mM KNO3	27.10	7.08	1.58	36.10	6.77	1.51	25.35	7.52	1.68	30.55	6.74	1.51

Table II. *Length of primary roots of plants grown in differing N sources.*

Length of primary roots in mm of plants grown for 5 days on media containing nitrogen sources as indicated in Table 2 was measured and plotted as indicated in Figure 3. Results (n=10-20) are representative of 2 individual experiments.

Number of Lateral Roots (>0.5 mm) of Plants Grown in Differing N Sources

Growth Condition	Col-0	S.D.	S.E.	*Atchl1-5*	S.D.	S.E.	*Atchl1-5+ MtNPF1.7/NIP/LATD* #11	S.D.	S.E.	*Atchl1-5+ MtNPF1.7/NIP/LATD* #12	S.D.	S.E.
0.1 mM KNO3	2.15	0.99	0.22	2.90	1.55	0.35	2.20	0.68	0.18	2.45	0.89	0.20
10 mM KNO3	2.05	1.19	0.27	3.00	1.34	0.30	1.90	0.97	0.22	2.55	0.94	0.21

Table III. *Number of lateral roots (>0.5 mm) of plants grown in differing N sources.*

Length of lateral roots (>0.5 mm) of plants grown for 5 days on media containing nitrogen sources as indicated in Table 3 was measured and plotted as indicated in Figure 4. Results (n=10-20) are representative of 2 individual experiments.

Length of Visible Lateral Roots of Plants Grown in Differing N Sources

Growth Condition	Col-0	S.D.	S.E.	*Atchl1-5*	S.D.	S.E.	*Atchl1-5+ MtNPF1.7/NIP/LATD* #11	S.D.	S.E.	*Atchl1-5+ MtNPF1.7/NIP/LATD* #12	S.D.	S.E.
0.1 mM KNO3	9.28	7.66	1.20	7.18	7.19	0.95	7.67	7.84	1.36	7.77	6.94	1.01
10 mM KNO3	7.23	6.36	1.05	9.27	10.20	1.31	8.20	8.99	1.48	9.68	9.52	1.33

Table IV. *Length of visible lateral roots of plants grown in differing N sources.*

Length of visible (0.5 mm) lateral roots of plants grown for 5 days on media containing nitrogen sources as indicated in Table 4 was measure and plotted as indicated in Figure 5. Results (n=29-60) are representative of 2 independent experiments.

Density of Visible Lateral Roots of Plants Grown in Differing N Sources

Growth Condition	Col-0	S.D.	S.E.	*Atchl1.5*	S.D.	S.E.	*Atchl1.5+ AtNPF6.3/NRT1.1*	S.D.	S.E.	*Atchl1.5+ MtNPF1.7/NIP/LATD L1*	S.D.	S.E.	*Atchl1.5+ MtNPF1.7/NIP/LATD L3*	S.D.	S.E.
0.5 mM Gln	0.0145	0.0322	0.0100	0.0723	0.0302	0.0100	0.0063	0.0155	0.0043	0.0257	0.0372	0.0100	0.0142	0.0240	0.0100
0.5 mM Gln+ 10 nM IAA	0.0876	0.0441	0.0100	0.0786	0.0295	0.0100	0.0490	0.0197	0.0057	0.0806	0.0296	0.0100	0.0550	0.0267	0.0100
0.5 mM Gln+ 10nM BAP	0.0000	0.0000	0.0000	0.0086	0.0298	0.0100	0.0051	0.0168	0.0051	0.0158	0.0262	0.0100	0.0090	0.0213	0.0100
1 mM KNO3	0.0562	0.0196	0.0000	0.0600	0.0203	0.0100	0.0617	0.0185	0.0056	0.0591	0.0383	0.0100	0.0365	0.0171	0.0000
1 mM KNO3+ 10nM IAA	0.0626	0.0212	0.0000	0.0716	0.0349	0.0100	0.0501	0.0176	0.0053	0.0450	0.0215	0.0000	0.0377	0.0145	0.0000
1 mM KNO3+ 10nM BAP	0.0072	0.0182	0.0000	0.0093	0.0179	0.0000	0.0240	0.0296	0.0076	0.0171	0.0203	0.0000	0.0155	0.0155	0.0000

Table V. *Density of visible lateral roots of plants grown in differing N sources.*

Density of visible (>0.5 mm) lateral rots of plants grown for 8 days on media containing nitrogen sources as indicated in Table 5 was measure and plotted as indicated in Figure 6. Results (n=15-20) are representative of 2 independent experiments.

Density of Visible Lateral Roots of Plants Grown in Differing Conditions

Growth Condition	Col-0	S.D.	S.E.	Col-0+ *AtNPF6.3/NRT1.1*	S.D.	S.E.	Col-0+ *MtNPF1.7/NIP/LATD* L1	S.D.	S.E.	Col-0+ *MtNPF1.7/NIP/LATD* L2	S.D.	S.E.
1/2 MS-N Media	0.364	0.075	0.019	0.359	0.059	0.015	0.343	0.058	0.015	0.401	0.088	0.023
1mM NH4Cl	0.418	0.101	0.027	0.466	0.088	0.023	0.527	0.112	0.029	0.488	0.112	0.029
200uM KNO3	0.271	0.046	0.012	0.297	0.096	0.025	0.415	0.059	0.015	0.435	0.066	0.017
1mM KNO3	0.315	0.050	0.013	0.401	0.085	0.022	0.389	0.094	0.024	0.437	0.099	0.026
10mM KNO3	0.300	0.059	0.015	0.414	0.070	0.018	0.384	0.091	0.024	0.464	0.109	0.028
1mM KNO3+ 0.1uM IAA	0.285	0.063	0.016	0.268	0.040	0.010	0.141	0.056	0.014	0.139	0.058	0.016
1mM KNO3+ 0.1uM BAP	0.234	0.076	0.020	0.378	0.117	0.030	0.312	0.072	0.019	0.324	0.110	0.029
1mM KNO3+ 0.2uM ABA	0.382	0.063	0.016	0.453	0.115	0.030	0.472	0.107	0.028	0.439	0.113	0.029

Table VI. *Density of visible lateral roots of plants grown in differing conditions.*

Density of visible (>0.5 mm) lateral rots of plants grown for 14 days on different growth conditions as indicated in Table 6 was measured and plotted as indicated in Figure 7. Results (n=14-15) are representative of 3 independent experiments.

Density of Visible Lateral Roots of Plants Grown in Differing Conditions

Growth Condition	Col-0	S.D.	S.E.	Col-0+ *AtNPF6.3/NRT1.1*	S.D.	S.E.	Col-0+ *MtNPF1.7/NIP/LATD* L1	S.D.	S.E.	Col-0+ *MtNPF1.7/NIP/LATD* L2	S.D.	S.E.
200uM KNO3	0.537	0.103	0.030	0.549	0.092	0.024	0.649	0.127	0.033	0.628	0.095	0.025
200µM KNO3+ 0.1µM IAA	0.528	0.098	0.028	0.518	0.105	0.027	0.696	0.085	0.022	0.724	0.075	0.019
200uM KNO3+ 10nM BAP	0.564	0.087	0.025	0.543	0.077	0.020	0.630	0.071	0.018	0.652	0.131	0.034
1mM KNO3	0.512	0.118	0.034	0.488	0.104	0.030	0.526	0.069	0.020	0.559	0.113	0.033
1mM KNO3+ 0.1µM IAA	0.581	0.103	0.030	0.595	0.106	0.032	0.326	0.021	0.006	0.346	0.063	0.018
1mM KNO3+ 10nM BAP	0.489	0.161	0.047	0.517	0.052	0.015	0.581	0.158	0.046	0.646	0.251	0.073

Table VII. *Density of visible lateral roots of plants grown in differing conditions.*

Density of visible (>0.5 mm) lateral rots of plants grown for 14 days on different growth conditions as indicated in Table 7 was measured and plotted as indicated in Figure 8. Results (n=14-15) are representative of 3 independent experiments.

MtNPF1.7/NIP/LATD Gene Expression in Roots in Varied Concentrations of Nitrate

Plant	50 µM KNO3	S.D.	S.E.	250 µM KNO3	S.D.	S.E.	1 mM KNO3	S.D.	S.E.	5 mM KNO3	S.D.	S.E.	50 µM KCl	S.D.	S.E.	250 µM KCl	S.D.	S.E.	1 mM KCl	S.D.	S.E.	5 mM KCl	S.D.	S.E.
A17	0.925	0.596	0.344	1.452	1.649	0.952	0.975	0.309	0.178	0.913	2.075	1.198	1.036	1.318	0.761	1.156	0.577	0.333	0.649	0.860	0.496	0.918	0.000	0.000
Mtnip-1	0.891	1.457	0.841	1.243	0.775	0.447	0.853	1.221	0.705	0.993	0.422	0.244	1.250	0.951	0.549	1.116	0.879	0.508	1.166	0.753	0.435	1.000	2.598	1.477

Table VIII. *MtNPF1.7/NIP/LATD gene expression in roots in varied concentrations of nitrate.*

Plants were grown in BNM media 5 days for nitrogen starvation after germination. Then nitrate treatment was applied for 2 hours. The *MtNPF1.7/NIP/LATD* gene expression was measured in Table 8 and plotted in Figure 9. As expected, level of nitrate had no obvious effects on both *MtNPF1.7/NIP/LATD* expression in both A17 and *Mtnip-1* roots, which is consistent with previous work. N=3.

MtNRT2.1 Gene Expression in Roots in Varied Concentrations of Nitrate

Plant	50 µM KNO3	S.D.	S.E.	250 µM KNO3	S.D.	S.E.	1 mM KNO3	S.D.	S.E.	5 mM KNO3	S.D.	S.E.	50 µM KCl	S.D.	S.E.	250 µM KCl	S.D.	S.E.	1 mM KCl	S.D.	S.E.	5 mM KCl	S.D.	S.E.
A17	1.440	0.000	0.000	1.243	0.764	0.441	1.161	1.077	0.622	1.579	0.941	0.543	0.634	0.352	0.203	0.547	0.382	0.221	0.618	0.251	0.145	0.309	0.468	0.270
Mnip-1	0.849	1.403	0.810	1.439	0.577	0.333	1.033	0.758	0.438	1.211	0.190	0.110	0.201	2.457	1.407	0.399	0.335	0.194	0.466	0.084	0.048	0.434	1.061	0.612

Table IX. *MtNRT2.1 gene expression in roots in varied concentrations of nitrate.*

After germination, plants were grown in BNM media 5 days for nitrogen starvation. Then nitrate was treated for 2 hours. The *MtNRT2.1* gene expression was measured in Table 9 and plotted in Figure 10. *MtNRT2.1* is very sensitive to nitrate in both A17 and nip roots, suggesting that *MtNPF1.7/NIP/LATD* is not a nitrate sensor. Examination of another nitrate responsive gene is required since there is unpublished data suggesting *MtNPF1.7/NIP/LATD* as a nitrate sensor. N=3.

Curvature Degree of Plant Roots in Response to the Gravity

Day After Turning 90°	A17	S.D.	S.E.	*Mtnip-1*	S.D.	S.E.	A17OE *MtNPF1.7/NIP/LATD* L#4	S.D.	S.E.	*Mtnip-3*	S.D.	S.E.
1 DAT	49.90	8.02	2.31	36.87	7.52	2.17	49.01	11.30	3.26	42.14	19.37	5.59
2 DAT	58.23	13.44	3.88	44.69	12.61	3.64	61.47	16.17	4.67	53.16	20.17	5.82
3 DAT	67.14	17.09	4.93	46.05	12.87	3.72	65.17	16.83	4.86	67.08	21.50	6.21
4 DAT	67.77	18.73	5.41	50.69	13.07	3.77	66.44	16.33	4.71	69.43	17.88	5.16
5 DAT	70.62	17.72	5.11	56.30	14.37	4.15	67.03	15.26	4.40	73.95	16.89	4.88

Table X. *Curvature degree of plant roots in response to the gravity.*

Curvature degree of plants roots of plants grown on BNM media containing 5 mM ammonium nitrate. All plants were grown on the square petri dishes for 4 days after germination and then turned 90º for another 5 days. Curvature degree of plants roots was measured in Table 10 and plotted in Figure 11. Results was from 12 independent plants. Two other independent experiments had similar results.

REFERENCES

Aloni R, Aloni E, Langhans M, Ullrich CI (2006) Role of cytokinin and auxin in shaping root architecture: regulating vascular differentiation, lateral root initiation, root apical dominance and root gravitropism. Ann Bot **97:** 883-893

Amor BB, Shaw SL, Oldroyd GED, Maillet F, Penmetsa RV, Cook D, Long SR, Denarie J, Gough C (2003) The NFP locus of Medicago truncatula controls an early step of Nod factor signal transduction upstream of a rapid calcium flux and root hair deformation. Plant Journal **34:** 495-506

Auriac M-C, Timmers ACJ (2007) Nodulation studies in the model legume Medicago truncatula: Advantages of using the constitutive EF1 alpha promoter and limitations in detecting fluorescent reporter proteins in nodule tissues. Molecular Plant-Microbe Interactions **20:** 1040-1047

Bagchi R (2013). Functiol chracterization of *MtNIP/LATD*'s biochemical and biological function. (Doctoral dissertation). Retrieved from ProQuest Disserations and Theses.

Bagchi R, Salehin M, Adeyemo OS, Salazar C, Shulaev V, Sherrier DJ, Dickstein R (2012) Functional assessment of the Medicago truncatula NIP/LATD protein demonstrates that it is a high-affinity nitrate transporter. Plant Physiol **160:** 906-916

Brewin NJ (1991) DEVELOPMENT OF THE LEGUME ROOT NODULE,

Brewin NJ (2004) Plant cell wall remodelling in the rhizobium-legume symbiosis. Critical Reviews in Plant Sciences **23:** 293-316

Bright LJ, Liang Y, Mitchell DM, Harris JM (2005) The LATD gene of Medicago truncatula is required for both nodule and root development. Molecular Plant-Microbe Interactions **18:** 521-532

Cook DR (1999) Medicago truncatula--a model in the making! Curr Opin Plant Biol **2:** 301-304

Cook DR, VandenBosch K, deBruijn FJ, Huguet T (1997) Model legumes get the nod. Plant Cell **9:** 275-281

Cooper JB, Long SR (1994) Morphogenetic Rescue of Rhizobium meliloti Nodulation Mutants by trans-Zeatin Secretion. Plant Cell **6:** 215-225

Crespi M, Galvez S (2000) Molecular mechanisms in root nodule development. Journal of Plant Growth Regulation **19:** 155-166

Cullimore J, Denarie J (2003) How legumes select their sweet talking symbionts. Science (Washington D C) **302:** 575-578

de Billy F, Grosjean C, May S, Bennett M, Cullimore JV (2001) Expression studies on AUX1-like genes in Medicago truncatula suggest that auxin is required at two steps in early nodule development. Mol Plant Microbe Interact **14:** 267-277

De Smet I (2012) Lateral root initiation: one step at a time. New Phytol **193:** 867-873

Dello Ioio R, Nakamura K, Moubayidin L, Perilli S, Taniguchi M, Morita MT, Aoyama T, Costantino P, Sabatini S (2008) A genetic framework for the control of cell division and differentiation in the root meristem. Science **322:** 1380-1384

Desbrosses GJ, Stougaard J (2011) Root nodulation: a paradigm for how plant-microbe symbiosis influences host developmental pathways. Cell Host Microbe **10:** 348-358

Fan SC, Lin CS, Hsu PK, Lin SH, Tsay YF (2009) The Arabidopsis nitrate transporter NRT1.7, expressed in phloem, is responsible for source-to-sink remobilization of nitrate. Plant Cell **21:** 2750-2761

Ferguson BJ, Indrasumunar A, Hayashi S, Lin MH, Lin YH, Reid DE, Gresshoff PM (2010) Molecular Analysis of Legume Nodule Development and Autoregulation. Journal of

Integrative Plant Biology **52:** 61-76

Fukaki H, Tasaka M (2009) Hormone interactions during lateral root formation. Plant Mol Biol **69:** 437-449

Fukaki H, Tasaka M (2009) Hormone interactions during lateral root formation. Plant Mol Biol **69:** 437-449

Glass AD, Britto DT, Kaiser BN, Kinghorn JR, Kronzucker HJ, Kumar A, Okamoto M, Rawat S, Siddiqi MY, Unkles SE, Vidmar JJ (2002) The regulation of nitrate and ammonium transport systems in plants. J Exp Bot **53:** 855-864

Glass AD, Kotur Z (2013) A re-evaluation of the role of Arabidopsis NRT1.1 in high-affinity nitrate transport. Plant Physiol

Gojon A, Krouk G, Perrine-Walker F, Laugier E (2011) Nitrate transceptor(s) in plants. J Exp Bot **62:** 2299-2308

Gojon A, Nacry P, Davidian JC (2009) Root uptake regulation: a central process for NPS homeostasis in plants. Curr Opin Plant Biol **12:** 328-338

Gonzalez-Rizzo S, Crespi M, Frugier F (2006) The Medicago truncatula CRE1 cytokinin receptor regulates lateral root development and early symbiotic interaction with Sinorhizobium meliloti. Plant Cell **18:** 2680-2693

Guyomarc'h S, Léran S, Auzon-Cape M, Perrine-Walker F, Lucas M, Laplaze L (2012) Early development and gravitropic response of lateral roots in Arabidopsis thaliana. Philos Trans R Soc Lond B Biol Sci **367:** 1509-1516

Handberg K, Sstougaard J (1992) *Lotus Japonicus*, an autogamois, dipolid legume species for classical and molecular genetics. The Plant Journal **2:** 487-496

Harris JM, Dickstein R (2010) Control of root architecture and nodulation by the LATD/NIP

transporter. Plant Signal Behav **5:** 1365-1369

Heckmann AB, Lombardo F, Miwa H, Perry JA, Bunnewell S, Parniske M, Wang TL, Downie JA (2006) Lotus japonicus nodulation requires two GRAS domain regulators, one of which is functionally conserved in a non-legume. Plant Physiol **142:** 1739-1750

Heckmann AB, Sandal N, Bek AS, Madsen LH, Jurkiewicz A, Nielsen MW, Tirichine L, Stougaard J (2011) Cytokinin induction of root nodule primordia in Lotus japonicus is regulated by a mechanism operating in the root cortex. Mol Plant Microbe Interact **24:** 1385-1395

Hirose N, Takei K, Kuroha T, Kamada-Nobusada T, Hayashi H, Sakakibara H (2008) Regulation of cytokinin biosynthesis, compartmentalization and translocation. J Exp Bot **59:** 75-83

Hirsch AM (1992) DEVELOPMENTAL BIOLOGY OF LEGUME NODULATION. New Phytologist **122:** 211-237

Ho CH, Lin SH, Hu HC, Tsay YF (2009) CHL1 functions as a nitrate sensor in plants. Cell **138:** 1184-1194

Hsu PK, Tsay YF (2013) Two Phloem Nitrate Transporters, NRT1.11 and NRT1.12, Are Important for Redistributing Xylem-borne Nitrate to Enhance Plant Growth. Plant Physiol

Huang NC, Liu KH, Lo HJ, Tsay YF (1999) Cloning and functional characterization of an Arabidopsis nitrate transporter gene that encodes a constitutive component of low-affinity uptake. Plant Cell **11:** 1381-1392

Ivanchenko MG, Napsucialy-Mendivil S, Dubrovsky JG (2010) Auxin-induced inhibition of lateral root initiation contributes to root system shaping in Arabidopsis thaliana. Plant J **64:** 740-752

Jacobs M, Rubery PH (1988) Naturally occurring auxin transport regulators. Science **241:** 346-

349

Jeong J, Suh S, Guan C, Tsay YF, Moran N, Oh CJ, An CS, Demchenko KN, Pawlowski K, Lee Y (2004) A nodule-specific dicarboxylate transporter from alder is a member of the peptide transporter family. Plant Physiol **134:** 969-978

Kanno Y, Hanada A, Chiba Y, Ichikawa T, Nakazawa M, Matsui M, Koshiba T, Kamiya Y, Seo M (2012) Identification of an abscisic acid transporter by functional screening using the receptor complex as a sensor. Proc Natl Acad Sci U S A **109:** 9653-9658

Krouk G, Crawford NM, Coruzzi GM, Tsay YF (2010a) Nitrate signaling: adaptation to fluctuating environments. Curr Opin Plant Biol **13:** 266-273

Krouk G, Lacombe B, Bielach A, Perrine-Walker F, Malinska K, Mounier E, Hoyerova K, Tillard P, Leon S, Ljung K, Zazimalova E, Benkova E, Nacry P, Gojon A (2010b) Nitrate-regulated auxin transport by NRT1.1 defines a mechanism for nutrient sensing in plants. Dev Cell **18:** 927-937

Lewis DR, Miller ND, Splitt BL, Wu G, Spalding EP (2007) Separating the roles of acropetal and basipetal auxin transport on gravitropism with mutations in two Arabidopsis multidrug resistance-like ABC transporter genes. Plant Cell **19:** 1838-1850

Liang Y, Mitchell DM, Harris JM (2007) Abscisic acid rescues the root meristem defects of the Medicago truncatula latd mutant. Dev Biol **304:** 297-307

Limpens E, Franken C, Smit P, Willemse J, Bisseling T, Geurts R (2003) LysM domain receptor kinases regulating rhizobial Nod factor-induced infection. Science (Washington D C) **302:** 630-633

Little DY, Rao H, Oliva S, Daniel-Vedele F, Krapp A, Malamy JE (2005) The putative high-affinity nitrate transporter NRT2.1 represses lateral root initiation in response to nutritional cues.

Proc Natl Acad Sci U S A **102:** 13693-13698

Liu KH, Huang CY, Tsay YF (1999) CHL1 is a dual-affinity nitrate transporter of Arabidopsis involved in multiple phases of nitrate uptake. Plant Cell **11:** 865-874

Long SR (1996) Rhizobium symbiosis: Nod factors in perspective. Plant Cell **8:** 1885-1898

Léran S, Muños S, Brachet C, Tillard P, Gojon A, Lacombe B (2013) The Arabidopsis NPF6.3/NRT1.1 Is a Bidirectional Transporter Involved in Root-to-Shoot Nitrate Translocation. Mol Plant

Léran S, Varala K, Boyer JC, Chiurazzi M, Crawford N, Daniel-Vedele F, David L, Dickstein R, Fernandez E, Forde B, Gassmann W, Geiger D, Gojon A, Gong JM, Halkier BA, Harris JM, Hedrich R, Limami AM, Rentsch D, Seo M, Tsay YF, Zhang M, Coruzzi G, Lacombe B (2013) A unified nomenclature of NITRATE TRANSPORTER 1/PEPTIDE TRANSPORTER family members in plants. Trends Plant Sci

Léran S, Varala K, Boyer JC, Chiurazzi M, Crawford N, Daniel-Vedele F, David L, Dickstein R, Fernandez E, Forde B, Gassmann W, Geiger D, Gojon A, Gong JM, Halkier BA, Harris JM, Hedrich R, Limami AM, Rentsch D, Seo M, Tsay YF, Zhang M, Coruzzi G, Lacombe B (2014) A unified nomenclature of NITRATE TRANSPORTER 1/PEPTIDE TRANSPORTER family members in plants. Trends Plant Sci **19:** 5-9

Marchant A, Kargul J, May ST, Muller P, Delbarre A, Perrot-Rechenmann C, Bennett MJ (1999) AUX1 regulates root gravitropism in Arabidopsis by facilitating auxin uptake within root apical tissues. EMBO J **18:** 2066-2073

Marquez AJ, Betti M, Garcia-Calderon M, Pal'ove-Balang P, Diaz P, Monza J (2005) Nitrate assimilation in Lotus japonicus. Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology **141:** S221-S221

Mathesius U, Schlaman HR, Spaink HP, Of Sautter C, Rolfe BG, Djordjevic MA (1998) Auxin transport inhibition precedes root nodule formation in white clover roots and is regulated by flavonoids and derivatives of chitin oligosaccharides. Plant J **14:** 23-34

Mitra RM, Gleason CA, Edwards A, Hadfield J, Downie JA, Oldroyd GED, Long SR (2004) A Ca2+/calmodulin-dependent protein kinase required for symbiotic nodule development: Gene identification by transcript-based cloning. Proceedings of the National Academy of Sciences of the United States of America **101:** 4701-4705

Munos S, Cazettes C, Fizames C, Gaymard F, Tillard P, Lepetit M, Lejay L, Gojon A (2004) Transcript profiling in the chl1-5 mutant of Arabidopsis reveals a role of the nitrate transporter NRT1.1 in the regulation of another nitrate transporter, NRT2.1. Plant Cell **16:** 2433-2447

Murray JD, Karas BJ, Sato S, Tabata S, Amyot L, Szczyglowski K (2007) A cytokinin perception mutant colonized by Rhizobium in the absence of nodule organogenesis. Science (Washington D C) **315:** 101-104

Nour-Eldin HH, Andersen TG, Burow M, Madsen SR, Jørgensen ME, Olsen CE, Dreyer I, Hedrich R, Geiger D, Halkier BA (2012) NRT/PTR transporters are essential for translocation of glucosinolate defence compounds to seeds. Nature **488:** 531-534

Okamoto M, Vidmar JJ, Glass ADM (2003) Regulation of NRT1 and NRT2 gene families of Arabidopsis thaliana: Responses to nitrate provision. Plant and Cell Physiology **44:** 304-317

Oldroyd GED (2007) PLANT SCIENCE: Nodules and Hormones. Science **315:** 52-53

Oldroyd GED, Downie JA (2004) Calcium, kinases and nodulation signalling in legumes. Nature Reviews Molecular Cell Biology **5:** 566-576

Oldroyd GED, Long SR (2003) Identification and characterization of nodulation-signaling

pathway 2, a gene of Medicago truncatula involved in Nod factor signaling. Plant Physiology (Rockville) **131:** 1027-1032

Overvoorde P, Fukaki H, Beeckman T (2010) Auxin control of root development. Cold Spring Harb Perspect Biol **2:** a001537

Pacios-Bras C, Schlaman HR, Boot K, Admiraal P, Langerak JM, Stougaard J, Spaink HP (2003) Auxin distribution in Lotus japonicus during root nodule development. Plant Mol Biol **52:** 1169-1180

Peiter E, Sun J, Heckmann AB, Venkateshwaran M, Riely BK, Otegui MS, Edwards A, Freshour G, Hahn MG, Cook DR, Sanders D, Oldroyd GED, Downie JA, Ane J-M (2007) The Medicago truncatula DMI1 protein modulates cytosolic calcium signaling. Plant Physiology (Rockville) **145:** 192-203

Perilli S, Moubayidin L, Sabatini S (2010) The molecular basis of cytokinin function. Curr Opin Plant Biol **13:** 21-26

Rae AL, Bonfante-Fasolo P, Brewin NJ (1992) Structure and growth of infection threads in the legume symbiosis with Rhizobium leguminosarum. The Plant Journal **2:** 385-395

Reed RC, Brady SR, Muday GK (1998) Inhibition of auxin movement from the shoot into the root inhibits lateral root development in Arabidopsis. Plant Physiol **118:** 1369-1378

Remans T, Nacry P, Pervent M, Filleur S, Diatloff E, Mounier E, Tillard P, Forde BG, Gojon A (2006) The Arabidopsis NRT1.1 transporter participates in the signaling pathway triggering root colonization of nitrate-rich patches. Proc Natl Acad Sci U S A **103:** 19206-19211

Ruzicka K, Simásková M, Duclercq J, Petrásek J, Zazímalová E, Simon S, Friml J, Van Montagu MC, Benková E (2009) Cytokinin regulates root meristem activity via modulation of the polar auxin transport. Proc Natl Acad Sci U S A **106:** 4284-4289

Růzicka K, Ljung K, Vanneste S, Podhorská R, Beeckman T, Friml J, Benková E (2007) Ethylene regulates root growth through effects on auxin biosynthesis and transport-dependent auxin distribution. Plant Cell **19:** 2197-2212

Salehin M (2013) Molecular and functional characterization of *Medicago truncatula NPF1.7* gene (Doctoral dissertation). Retrieved from ProQuest Disserations and Theses.

Salehin M, Huang YS, Bagchi R, Sherrier DJ, Dickstein R (2012) Allelic differences in Medicago truncatula NIP/LATD mutants correlate with their encoded proteins' transport activities in planta. Plant Signal Behav **8**

Sato S, Nakamura Y, Kaneko T, Asamizu E, Kato T, Nakao M, Sasamoto S, Watanabe A, Ono A, Kawashima K, Fujishiro T, Katoh M, Kohara M, Kishida Y, Minami C, Nakayama S, Nakazaki N, Shimizu Y, Shinpo S, Takahashi C, Wada T, Yamada M, Ohmido N, Hayashi M, Fukui K, Baba T, Nakamichi T, Mori H, Tabata S (2008) Genome structure of the legume, Lotus japonicus. DNA Res **15:** 227-239

Smit P, Limpens E, Geurts R, Fedorova E, Dolgikh E, Gough C, Bisseling T (2007) Medicago LYK3, an entry receptor in rhizobial nodulation factor signaling. Plant Physiology (Rockville) **145:** 183-191

Smit P, Raedts J, Portyanko V, Debelle F, Gough C, Bisseling T, Geurts R (2005) NSP1 of the GRAS protein family is essential for rhizobial Nod factor-induced transcription. Science **308:** 1789-1791

Stitt M, Müller C, Matt P, Gibon Y, Carillo P, Morcuende R, Scheible WR, Krapp A (2002) Steps towards an integrated view of nitrogen metabolism. J Exp Bot **53:** 959-970

Sugiura M, Georgescu MN, Takahashi M (2007) A nitrite transporter associated with nitrite uptake by higher plant chloroplasts. Plant Cell Physiol **48:** 1022-1035

Tang H, Krishnakumar V, Bidwell S, Rosen B, Chan A, Zhou S, Gentzbittel L, Childs KL, Yandell M, Gundlach H, Mayer KF, Schwartz DC, Town CD (2014) An improved genome release (version Mt4.0) for the model legume Medicago truncatula. BMC Genomics **15:** 312

Tegeder M, Rentsch D (2010) Uptake and partitioning of amino acids and peptides. Mol Plant **3:** 997-1011

Teillet A, Garcia J, de Billy F, Gherardi M, Huguet T, Barker DG, de Carvalho-Niebel F, Journet EP (2008) api, a novel Medicago truncatula symbiotic mutant impaired in nodule primordium invasion. Molecular Plant-Microbe Interactions **21:** 535-546

Tian QY, Sun P, Zhang WH (2009) Ethylene is involved in nitrate-dependent root growth and branching in Arabidopsis thaliana. New Phytol **184:** 918-931

Tsay YF, Chiu CC, Tsai CB, Ho CH, Hsu PK (2007) Nitrate transporters and peptide transporters. Febs Letters **581:** 2290-2300

Tsay YF, Schroeder JI, Feldmann KA, Crawford NM (1993) THE HERBICIDE SENSITIVITY GENE CHL1 OF ARABIDOPSIS ENCODES A NITRATE-INDUCIBLE NITRATE TRANSPORTER. Cell **72:** 705-713

Turner M, Nizampatnam NR, Baron M, Coppin S, Damodaran S, Adhikari S, Arunachalam SP, Yu O, Subramanian S (2013) Ectopic expression of miR160 results in auxin hypersensitivity, cytokinin hyposensitivity, and inhibition of symbiotic nodule development in soybean. Plant Physiol **162:** 2042-2055

Vasse J, Debilly F, Camut S, Truchet G (1990) CORRELATION BETWEEN ULTRASTRUCTURAL DIFFERENTIATION OF BACTEROIDS AND NITROGEN-FIXATION IN ALFALFA NODULES. Journal of Bacteriology **172:** 4295-4306

Veereshlingam H, Haynes JG, Penmetsa RV, Cook DR, Sherrier DJ, Dickstein R (2004) nip,

a symbiotic Medicago truncatula mutant that forms root nodules with aberrant infection threads and plant defense-like response. Plant Physiology (Rockville) **136:** 3692-3702

Venkateshwaran M, Riely BK, Peiter E, Otegui M, Sun J, Heckmann AB, Lougnon G, Edwards A, Freshour G, Hahn MG, Sanders D, Oldroyd GD, Downie AJ, Cook DR, Ane J (2008) The putative ion channel DMI1 localizes to the nuclear envelope and regulates nuclear calcium spiking during early symbiotic signaling. Phytopathology **98:** S163

Vidal EA, Gutiérrez RA (2008) A systems view of nitrogen nutrient and metabolite responses in Arabidopsis. Curr Opin Plant Biol **11:** 521-529

Wais RJ, Galera C, Oldroyd G, Catoira R, Penmetsa RV, Cook D, Gough C, Denarie J, Long SR (2000) Genetic analysis of calcium spiking responses in nodulation mutants of Medicago truncatula. Proceedings of the National Academy of Sciences of the United States of America **97:** 13407-13412

Wang RC, Xing XJ, Wang Y, Tran A, Crawford NM (2009) A Genetic Screen for Nitrate Regulatory Mutants Captures the Nitrate Transporter Gene NRT1.1. Plant Physiology **151:** 472-478

Wang YY, Tsay YF (2011) Arabidopsis nitrate transporter NRT1.9 is important in phloem nitrate transport. Plant Cell **23:** 1945-1957

Wasson AP, Pellerone FI, Mathesius U (2006) Silencing the flavonoid pathway in Medicago truncatula inhibits root nodule formation and prevents auxin transport regulation by rhizobia. Plant Cell **18:** 1617-1629

Waterworth WM, Bray CM (2006) Enigma variations for peptides and their transporters in higher plants. Ann Bot **98:** 1-8

Yendrek CR, Lee YC, Morris V, Liang Y, Pislariu CI, Burkart G, Meckfessel MH, Salehin

M, Kessler H, Wessler H, Lloyd M, Lutton H, Teillet A, Sherrier DJ, Journet EP, Harris JM, Dickstein R (2010) A putative transporter is essential for integrating nutrient and hormone signaling with lateral root growth and nodule development in Medicago truncatula. Plant Journal **62:** 100-112

Young ND, Debellé F, Oldroyd GE, Geurts R, Cannon SB, Udvardi MK, Benedito VA, Mayer KF, Gouzy J, Schoof H, Van de Peer Y, Proost S, Cook DR, Meyers BC, Spannagl M, Cheung F, De Mita S, Krishnakumar V, Gundlach H, Zhou S, Mudge J, Bharti AK, Murray JD, Naoumkina MA, Rosen B, Silverstein KA, Tang H, Rombauts S, Zhao PX, Zhou P, Barbe V, Bardou P, Bechner M, Bellec A, Berger A, Bergès H, Bidwell S, Bisseling T, Choisne N, Couloux A, Denny R, Deshpande S, Dai X, Doyle JJ, Dudez AM, Farmer AD, Fouteau S, Franken C, Gibelin C, Gish J, Goldstein S, González AJ, Green PJ, Hallab A, Hartog M, Hua A, Humphray SJ, Jeong DH, Jing Y, Jöcker A, Kenton SM, Kim DJ, Klee K, Lai H, Lang C, Lin S, Macmil SL, Magdelenat G, Matthews L, McCorrison J, Monaghan EL, Mun JH, Najar FZ, Nicholson C, Noirot C, O'Bleness M, Paule CR, Poulain J, Prion F, Qin B, Qu C, Retzel EF, Riddle C, Sallet E, Samain S, Samson N, Sanders I, Saurat O, Scarpelli C, Schiex T, Segurens B, Severin AJ, Sherrier DJ, Shi R, Sims S, Singer SR, Sinharoy S, Sterck L, Viollet A, Wang BB, Wang K, Wang M, Wang X, Warfsmann J, Weissenbach J, White DD, White JD, Wiley GB, Wincker P, Xing Y, Yang L, Yao Z, Ying F, Zhai J, Zhou L, Zuber A, Dénarié J, Dixon RA, May GD, Schwartz DC, Rogers J, Quétier F, Town CD, Roe BA (2011) The Medicago genome provides insight into the evolution of rhizobial symbioses. Nature **480:** 520-524

Zifarelli G, Pusch M (2010) CLC transport proteins in plants. FEBS Lett **584:** 2122-2127