PONTIFICIA UNIVERSIDAD CATÓLICA DE CHILE
Facultad de Ciencias Biológicas
Programa de Doctorado en Ciencias Biológicas
Mención Genética Molecular y Microbiología

TESIS DOCTORAL:

"*NSP-LIKE* AND *NIN-LIKE* GENES ARE INVOLVED IN THE ASSOCIATION BETWEEN *ARABIDOPSIS THALIANA* AND NITROGEN FIXING BACTERIA"

Por:

TATIANA DIANNELA KRAISER MIRANDA

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Tesis presentada a la Pontificia Universidad Católica de Chile como parte de los requisitos para optar al grado de Doctor en Ciencias Biológicas mención Genética Molecular y Microbiología.

Por

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This thesis is dedicated to my beloved father Gregorio Kraiser,
I wish you were here.

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ABSTRACT

Nitrogen acquisition in plants by the association with nitrogen fixing bacteria (NFB) have been mostly studied in legumes. Plant regulatory mechanisms are essential for a successful association and nodule development. Non-legume plants are unable to form nodules and it is unknown the extent to which they can or cannot establish functional associations with NFB and the molecular regulatory mechanisms involved. Our goal was to evaluate a functional association between *Arabidopsis thaliana* and NFB to develop a model system in which to study and identify molecular mechanisms underlying non-legumes and NFB association. Our results indicate Arabidopsis associates with NFB, some of which enhance nitrogen nutrition and plant growth through biological nitrogen fixation. In addition, we identified conserved plant transcription factors that are essential in the molecular mechanism required for functional association between non–legumes plants and NFB. We propose Arabidopsis and selected NFB as an excellent model system to identifying key regulatory networks in non-legumes and NFB association.

1. INTRODUCTION

Plants are sessile organisms and cannot escape adverse environmental conditions. In order to cope with constant and diverse challenges, plants must adjust their physiology, growth and development. One of the most important challenges for plants is to maintain an adequate nutrient supply under fluctuating environmental conditions. Nitrogen (N) is the mineral nutrient required in the greatest amount and its availability is a major factor limiting plant growth in natural (Marschner 1995) as well as agricultural environments (Galloway and Cowling 2002).

N is present in the biosphere in various chemical forms. Molecular nitrogen (N_2) represents approximately 80% of the atmosphere composition (Sanhueza 1982). However, plants cannot directly use this form of N. N_2 enters the biological N cycle in three main ways: through biological fixation (prokaryotic conversion of N_2 to ammonia), by atmospheric fixation (lightning and photochemical conversion of N_2 to nitrate), and by the Haber-Bosch industrial fixation of N_2 to produce ammonia (Marschner 1995). Once N is fixed as nitrate or ammonia, it can have two main fates: (i) nitrate and ammonia can undergo biochemical processes that transform them back to N_2 (Marschner 1995) or they can enter biological systems, and be reduced and/or assimilated for the biosynthesis of N-containing metabolites.

Amino acids, urea, small polypeptides and other N-containing biomolecules can be released back to the environment by secretion, excretion or by the decay of organic matter. These organic forms of N can also be used as N sources by plants and other organisms (Jones, Healeya et al. 2005).

Plants have evolved inorganic and organic N uptake systems to cope with the heterogeneous N availability in the soil. For nitrate (Crawford and Glass 1998) and ammonium (Ludewig, Neuhäuser et al. 2007), two uptake systems have been described: the low affinity transport systems (LATS) which operates at high nutrient concentrations (above 1 mM), and the high affinity transport systems (HATS) that predominates in the micromolar range (Wang, Siddiqi et al. 1993). Modulation of HATS and LATS function in coordination with changes in the pattern of growth and development allow plants to cope with heterogeneous N availability in the soil (Robinson 1994; Zhang and Forde 2000; López-Bucio, Cruz-Ramírez et al. 2003; Zhang, Rong et al. 2007; Vidal and Gutiérrez 2008; Forde and Walch-Liu 2009; Vidal, Tamayo et al. 2010). In this thesis, we will refer to these uptake systems as the autonomous pathway of N acquisition. In natural environments, plants can also interact and associate with many and functionally diverse microorganisms that can also contribute to an adequate N supply (Gage 2004; You, Nishiguchi et al. 2005). In this thesis, we will refer to these mechanisms as the association pathways. From this perspective, a single plant interacting with multiple microorganisms over time may be considered as an ecosystem (Pickett and Candenasso 2002; Martin, Tuskan et al. 2004). The integration of the autonomous and association pathways for N acquisition leads to an optimal plant's nutrition.

1.1 Autonomous pathways for N acquisition

1.1.1 N nutrient uptake systems – Molecular level

Two families of nitrate transporters, NRT1 and NRT2, have been identified in higher plants (Tsay, Chiu et al. 2007). Both gene families code for symporters that transport nitrate concomitantly with a proton (H^+) in a mechanism that is driven by pH gradients across membranes (Miller, Fan et al. 2007). The *NRT2* gene family codes for high-affinity nitrate transporters (Orsel, Chopin et al. 2006) while *NRT1* code for low-affinity nitrate transporters, with the exception of NRT1.1 (also known as CHL1) which is a dual-affinity transporter involved in both low and high affinity nitrate uptake (Wang, Liu et al. 1998; Liu, Huang et al. 1999; Liu and Tsay 2003). Two forms of nitrate HATS have been described, an inducible system that is stimulated by nitrate in the external medium (Crawford and Glass 1998) and a constitutive system which works even when plants have not been previously supplied with nitrate (Crawford and Glass 1998). The nitrate transporters studied in greatest detail are the *Arabidopsis NRT2.1* and *NRT1.1*. *NRT2.1* transcript is induced by low nitrate availability or N starvation and is repressed by high N provision (for example high ammonium or glutamine conditions) by a pathway involving the NRT1.1 transporter (Muños, Cazettes et al. 2004; Krouk, Tillard et al. 2006). The transport activity of NRT1.1 is regulated by phosphorylation of its Thr^{101}(Liu and Tsay 2003). Phosphorylated NRT1.1 functions as a high-affinity nitrate transporter and the dephosphorylated form of NRT1.1 functions as a low-affinity transporter (Liu and Tsay 2003).

Uptake of ammonium/ammonia is mediated by the AMT/MEP/Rh family of membrane proteins, found not only in plants but also in microorganisms and animals (von Wirén and Merrik 2004). In plants, members of the AMT1 family mediate ammonium

transport. These proteins have been described as ammonium uniporters that transport ammonium along the electrochemical gradient (Ludewig, von Wirén et al. 2002; Ludewig, Wilken et al. 2003) or as NH_3/H^+ cotransporters (Mayer, Dynowski et al. 2006). Ammonium uptake is known to be repressed by high external N and to be induced under N deficiency, by mechanisms that may act both at the transcriptional and post-transcriptional level (Lee, Purves et al. 1992; Gazzarrini, Lejay et al. 1999; Rawat, Silim et al. 1999).

Soil organic compounds can also contribute to plant N nutrition (Näsholm, Ekblad et al. 1998; Lipson and Näsholm 2001; Näsholm, Kielland et al. 2009). Amino acids represent the largest fraction of low molecular weight dissolved organic N in the soil (Jones, Healeya et al. 2005). The amino acid pool is dynamic because it is quickly taken up by plants and microorganisms (Jones and Hodge 1999). Several known and putative amino acid transporters have been described in plants (Lipson and Näsholm 2001). In Arabidopsis roots, three amino acid transporters have been identified with a role in the uptake of amino acids: lysine-histidine transporter 1 (LHT1), amino acid permease 1 (AAP1) and amino acid permease 5 (AAP5) (Hirner, Ladwig et al. 2006; Lee, Foster et al. 2007; Svennerstam, Ganeteg et al. 2008). LHT1 and AAP5 have different amino acid specificities, function at amino acid concentrations seen in field conditions and are thought to be important components of the root amino acid uptake system in Arabidopsis (Svennerstam, Ganeteg et al. 2008). Cationic amino acid transport is mediated by AAP5 while neutral and acidic amino acid transport is mediated by LHT1 (Svennerstam, Ganeteg et al. 2008). AAP1 has been shown to be important for root amino acid uptake only at high amino acid concentrations (Lee, Foster et al. 2007).

Urea is excreted into the environment by a variety of organisms and represents a readily available nitrogen source in soils. In addition, urea is one of the major N forms applied as fertilizer in agriculture. Physiological experiments have shown that plant roots can directly uptake urea from the soil (Krogmeier, McCarty et al. 1989; Gerendas, Zhu et al. 1998). The main transporter associated with urea uptake in Arabidopsis is AtDUR3, which co-transports urea and protons (Liu, Ludewig et al. 2003). AtDUR3 is a high-affinity urea transporter and its expression levels increase in N-deficient roots and decrease after re-supplementation with nitrate or ammonium (Kojima, Bohner et al. 2007).

Although the significance of proteins for plant nutrition remains to be determined, plants that are not mycorrhizal symbionts, including Arabidopsis, may use proteins as N source without obvious assistance from other organisms (Paungfoo-Lonhienne, Lonhienne et al. 2008). Two possible mechanisms could explain access of plants to N in soil proteins. Proteases present in root exudates may degrade proteins in the soil to amino acids (Paungfoo-Lonhienne, Lonhienne et al. 2008). Alternatively, intact proteins in the soil can be taken up by the root through unknown transporters or by endocytosis (Paungfoo-Lonhienne, Lonhienne et al. 2008).

1.1.2 Developmental adaptations for optimal N nutrition – Organism level

In addition to the regulation of the inorganic and organic N uptake systems (Fig. 1A), plants display considerable developmental plasticity in response to variations in the concentration and distribution of external nutrients. One of the most dramatic plant adaptations to ensure adequate N acquisition is the modulation of root system architecture (RSA) in response to N supply (Fig. 1B). Early studies by Drew et al., 1973 and Drew, 1975 in barley (*Hordeum sativum* L.) demonstrated that seedlings subjected to a local high

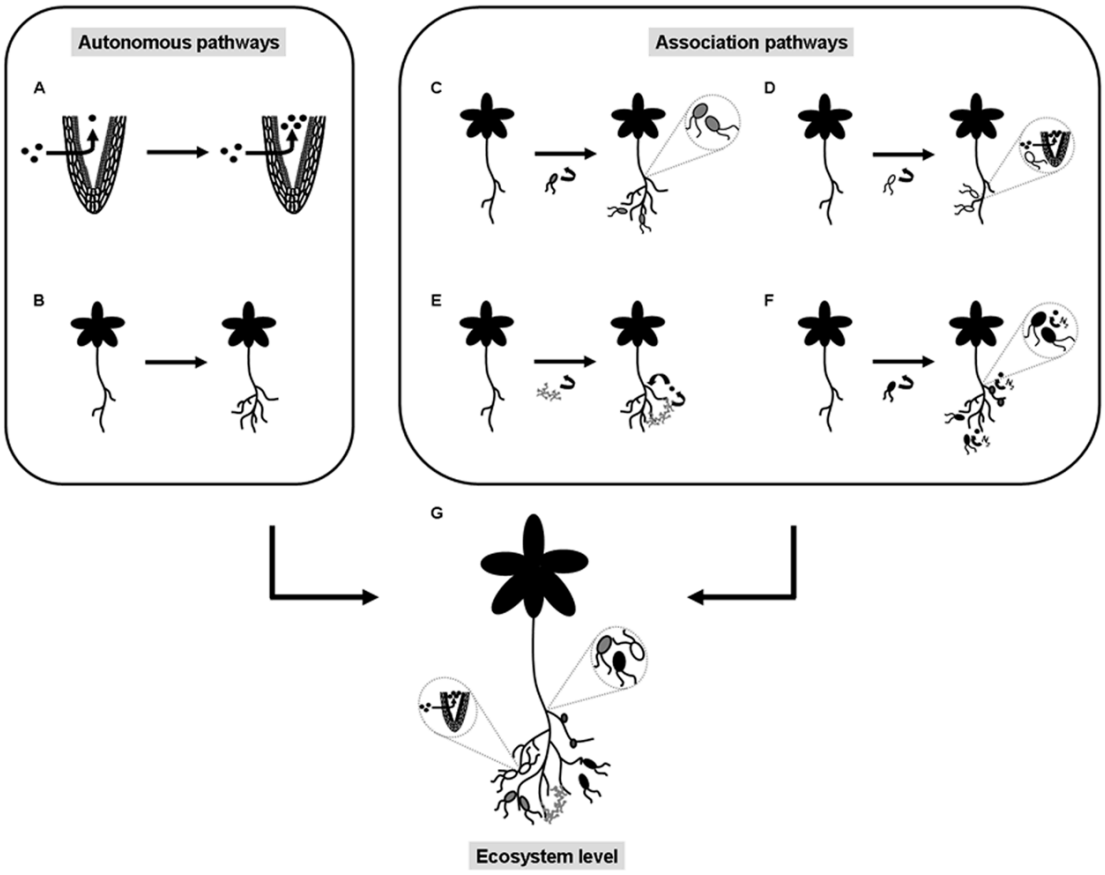

Figure 1: Simple model of pathways for N acquisition in plants. Autonomous pathways regulate (A) N (black circle) uptake and/or (B) root system architecture (RSA). Association pathways allow plants to associate with (C) endophytic and/or (D) plant growth promoting bacteria (PGPB). These bacteria improve plant N nutrition by increasing root surface area (grey bacteria) and/or N uptake (white bacteria). (E) Plant association with mycorrhizal fungi improves plant N nutrition by modification of RSA. These fungi can also facilitate transfer of available N to the plant. (F) Plant association nitrogen fixing bacteria (NFB) (black bacteria). Bacteria can be in the rhizosphere, inside the plant tissue forming, or not part of a nodule. In all cases, NFB bacteria can fix atmospheric N_2 to NH_3 available for plant use. Ecosystem level (G): autonomous and association pathways coexist in plants in the environment and act simultaneously and coordinately to ensure an adequate N supply.

concentration of nitrate or ammonium had a dramatic proliferation of lateral roots (LRs) in the nutrient-rich zone. The proliferation of LRs within a localized nitrate-rich zone is a response that occurs in many plants and represents a common adaptation phenomenon (Robinson 1994; Hodge 2004). Additional effects of N supply on root architecture and root developmental plasticity include changes in primary root growth (Walch-Liu, Liu et al. 2006; Walch-Liu and Forde 2008; Vidal, Araus et al. 2010), lateral root (LR) initiation (Little, Rao et al. 2005; Remans, Nacry et al. 2006b; Gifford, Dean et al. 2008) and LR elongation (Zhang and Forde 1998; Zhang, Barlow et al. 1999; Vidal, Araus et al. 2010). Although different phenotypic impacts of N supply/source in plants have been identified, the N sensors and signaling pathways mediating these effects have yet to be fully characterized.

In higher plants, *NITRATE REGULATED 1* (*ANR1*) gene was the first described regulatory factor involved in modulating root architecture in response to a localized nitrate supply (Zhang and Forde 1998). *ANR1* encodes a member of the MADS-box transcription factor gene family and was found in a reverse genetic screen designed to isolate genes whose expression is induced in nitrate-rich patches (Zhang and Forde 1998). Transgenic plants in which *ANR1* was repressed, display a decreased root growth response to a localized nitrate supply (Zhang and Forde 1998).

Reverse genetics approaches have suggested that NRT1.1 and NRT2.1 are components of the N signaling pathway. The role of these transporters has been supported mostly by work focusing on the effect of N availability in the modulation of RSA. Remans and colleagues (Remans, Nacry et al. 2006a) found that NRT1.1 mutant plants exhibit a strongly decreased root colonization of localized high nitrate supply and this effect is mediated by ANR1. In addition to the stimulatory effect on LRs growth, nitrate also

antagonizes the L-Glu effect on primary root elongation and this requires NRT1.1 (Walch-Liu and Forde 2008). In addition, NRT2.1 has been implicated in LR initiation control in response to a low nitrate supply (Remans, Nacry et al. 2006b) and in LR repression in response to high C/N ratio (Little, Rao et al. 2005).

The phytohormone auxin plays an important role in the modulation of RSA in response to N. Studies with maize suggested that inhibition of root growth by high nitrate supply is correlated with reduced auxin concentration in the roots (Tian, Chen et al. 2008). It has been proposed that the auxin long-distance signal from shoot to root regulates the inhibition of early LR development by high rates of nitrate supply in Arabidopsis seedlings (Forde 2002; Walch-Liu, Ivanov et al. 2006). Gifford and colleagues (Gifford, Dean et al. 2008) found a regulatory module including miR167 and its target the AUXIN RESPONSE FACTOR 8 (ARF8) involved in regulation of LR initiation and emergence in response to nitrate. More recently, a regulatory module that includes miR393 and the auxin receptor *AFB3* was shown to mediate both LR and primary root growth in response to nitrate treatments in Arabidopsis roots (Vidal, Araus et al. 2010). *AFB3* expression was induced directly by nitrate and miR393 expression was induced by N metabolites generated after nitrate reduction. Because increased levels of miR393 lead to down-regulation of the *AFB3* mRNA levels, this regulatory module provides a simple molecular mechanism to control root system architecture in response to internal and external N availability (Vidal, Araus et al. 2010). There is also evidence that abscisic acid (ABA) plays a central role in mediating the regulatory effects of high nitrate concentrations on root branching in Arabidopsis. ABA signaling mutants *abi4-1, abi4-2* and *abi5* are insensitive to repression of LRs growth by high nitrate, and the ABA biosynthesis mutants (*aba1-1, aba2-3, aba2-4 and aba3-2*) show a

reduced sensitivity to this high nitrate repression (Signora, De Smet et al. 2001). The authors propose that there are two regulatory pathways mediating the inhibitory effects of nitrate in Arabidopsis roots. One pathway is ABA-dependent and involves ABI4 and ABI5, whereas the second pathway is ABA-independent (Signora, De Smet et al. 2001).

1.2 Association pathways for N acquisition – Ecosystemic Level

1.2.1 Plant growth promoting bacteria and N nutrition

Nutritionally beneficial plant-bacteria interactions (i.e. mutualistic symbiosis) can increase nutrient accessibility, uptake or both (Bertrand, Plassard et al. 2000; Park, Lee et al. 2009). Bacteria that contribute to plant nutrition have positive effects on plant growth and are generally referred to as plant growth promoting bacteria (PGPB). Some PGPB can produce phytohormones such as indole acetic acid, cytokinins and gibberellins, increasing hormone levels inside the plant (Long, Schmidt et al. 2008; Islam, Madhaiyan et al. 2009). PGPB can also decrease ethylene levels enzymatically by 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Onofre-Lemus, Hernández-Lucas et al. 2009). By modulating hormone levels, PGPB can influence root morphology, increasing LR length and hair number and length (Persello-Cartieaux, David et al. 2001). For example, *Azospirillum* spp. bacteria secrete high quantities of auxins which could be an important factor contributing to the stimulation of root development in plants (Spaepen, Vanderleyden et al. 2007). *Pseudomonas thivervalensis* bacteria colonizes and promotes root development in *A. thaliana* (Achouak, Sutra et al. 2000; Persello-Cartieaux, David et al. 2001). *Arabidopsis* mutant plants in the auxin influx transporter gene *AUX1* were insensitive to the effect of *P. thivervalensis* suggesting a role for

bacterial auxin in inducing morphological modifications of roots (Persello-Cartieaux, David et al. 2001). *Bacillus megaterium* can promote growth of *A. thaliana* and *Phaseolus vulgaris* (common bean, *Fabaceae*) (López-Bucio, Campos-Cuevas et al. 2007). *B. megaterium* increases root development independent of auxin or ethylene, because mutant plants defective in either auxin or ethylene signaling still show increased root growth when are inoculated with the bacterium (López-Bucio, Campos-Cuevas et al. 2007). Mutant plants in the cytokinin receptors revealed that the integrity of the cytokinin signaling pathway was essential for the bacterial effect in the plant and suggested that the increased root growth and plant growth promotion are due to cytokinins action (Ortiz-Castro, Valencia-Cantero et al. 2008).

The increased nutrient acquisition observed in response to PGPB inoculation can be explained not only by branching and enlargement of the root surface area (Fig. 1C), but also by increasing nutrient uptake (Fig. 1D) (Bertrand, Plassard et al. 2000). Studies with the PGPB genus *Achromobacter* in association with *Brassica napus* (Rapeseed, *Brassicaceae*) revealed that this bacterium increases plant growth by stimulating nitrate uptake by the plant (Bertrand, Plassard et al. 2000). Electrophysiological measurements of nitrate net flux with ion-selective microelectrodes showed that inoculation resulted in a specific increase of net nitrate influx, in the root zone that was morphologically similar in inoculated and non-inoculated plants (Bertrand, Plassard et al. 2000). *Phylobacterium* strain STM196 affects both RSA and N nutrition in Arabidopsis (Mantelin, Desbrosses et al. 2006). This bacterium elicits an increase in root branching and plant N status promoting plant growth under different N concentrations (Mantelin, Desbrosses et al. 2006). *Phylobacterium* inoculation leads to the abolishment of the inhibition of LRs elongation by high nitrate supply. This

bacterium is able to optimize plant growth independently of the external nitrate concentration (Mantelin, Desbrosses et al. 2006). However, the molecular mechanism by which this bacterium exerts this effect on the plant is still unknown.

Beside bacteria, other microorganisms such as mycorrhizal fungi can modify RSA and increase the area of interaction with the soil contributing to better nutrient acquisition (Fig. 1E). Many studies of arbuscular mycorrhizal (AM) fungi-plants associations have shown that AM induce modification of RSA (Berta, Trotta et al. 1995; Gamalero, Trotta et al. 2004; Gutjahr, Casieri et al. 2009). The importance of this association to plant nutrition has been mainly studied in the context of phosphorus uptake (Fitter and Hay 2002; Plassard and Dell 2010). However, a few studies have addressed the importance of mycorrhizal fungi for N nutrition. The AM fungi *Glomus intraradices* can increase inorganic N and total N content uptake ability of carob trees (*Ceratonia siliqua, Fabaceae*) as compared to plants without this fungus (Cruz, Green et al. 2004). Such an increase in plant N uptake was observed only in carob trees growing at low levels of N (Cruz, Green et al. 2004). Stable isotope labeling experiments showed that inorganic N is uptaken by the AM fungi and then transferred to the plant roots (Fig. 1E) (Govindarajulu, Pfeffer et al. 2005).

1.2.2 Plant interactions with nitrogen fixing bacteria for N acquisition

The best known example of beneficial plant-bacteria association for N nutrition occurs in nodulating plants (Fig. 1F) (Sprent and James 2007). Nodulating plants are able to obtain an important part of the N required to sustain their growth and development from nitrogen fixing bacteria (NFB) symbionts (Materona and Danso 1991). NFB are able to reduce atmospheric N_2 to ammonium by the action of an evolutionarily conserved enzyme complex called nitrogenase. This complex is composed by two enzymes: the dinitrogenase and the

dinitrogenase reductase (Joerger, Wolfinger et al. 1991; Zehr, Jenkins et al. 2003; Zhang, Hurek et al. 2007). Both bacteria and archea are able to carry out N fixation (Zehr, Jenkins et al. 2003). On a global scale, this process represents the incorporation to the biosphere of some 110 and 140 Tg per year on land or in the ocean, respectively (Galloway et al. 2004). BNF is a crucial process from the environmental and agricultural points of view, just second to photosynthesis in importance for the maintenance of the biosphere.

The symbiotic interaction with NFB occurs in plants of the *Fabaceae* family (legumes) and also in the plant genus *Parasponia* (*Cannabaceae*) (Sprent and James 2007). Nodulating plants can interact with bacteria of the genera *Rhizobium*, *Mesorhizobium*, *Sinorhizobium*, *Bradyrhizobium* and *Azorhizobium* of the *Rhizobiaceae* family (Gage 2004). Legumes can also associate with some strains of the *Methylobacterium, Cupriavidus, Shinella, Devocia* and *Burkholderia* genus (Chen, Laevens et al. 2001; Sy, Giraud et al. 2001; Rivas, Willems et al. 2003; Chen, de Faria et al. 2005; Lin, Wang et al. 2008). Members of the *Betulaceae, Casuarinaceae, Myricaceae, Elaegnaceae, Rhamnaceae, Rosaceae, Coriariaceae* and *Datisticaceae* nodulate with the actinomycetal genus *Frankia* (Gage 2004; Sprent and James 2007).

The legumes:NFB association is highly regulated by plant's genes. In response to plant flavonoids, NFB bacteria secrete lipochito-oligosaccharide commonly named Nod factors. In the plant, perception of Nod factors by Nod factors receptors (NFR) and SymRK proteins activates the symbiotic transduction pathways in which decodification of calcium spiking leads to the activation of essential transcription factors: *Nodulation signaling pathway* 1 (NSP1) and NPS2 (Fig. 2) (Kalo, Gleason et al. 2005; Smit, Raedts et al. 2005; Heckmann, Lombardo et al. 2006). Together and forming a complex, NSP1 and NSP2 regulated the

expression of a secondary transcription factor: *nodule inception* (NIN), that is require for both bacteria infection and nodule organogenesis (Fig. 2) (Schauser, Roussis et al. 1999; Smit, Raedts et al. 2005; Hirsch, Kim et al. 2009). Mutant plants in either of these transcription factors are unable to develop a nodule and therefore to associate with NFB (Oldroyd 2013).

In addition to the importance of nodulation for plant nutrition, beneficial plant-bacteria interactions for N nutrition are also observed within plant species that do not nodulate (Fig. 2F) (Stone, O'Callaghan et al. 2001; Chi, Shen et al. 2005; Perin, Martínez-Aguilar et al. 2006a; Rosenblueth and Martínez-Romero 2006). Interactions between non-nodulating plants and NFB are functional associations which have received considerable less attention than interactions leading to nodule formation (Egener, Hurek et al. 1998; Iniguez, Dong et al. 2004; You, Nishiguchi et al. 2005). However, NFB can colonize the rhizosphere of the plant, as shown for the *Burkholderia* genus found associated to the rhizosphere of tomato plants (Caballero-Mellado, Onofre-Lemus et al. 2007). NFB have also been shown to colonize plant tissues and exhibit an endophytic lifestyle (Hurek, Reinhold-Hurek et al. 1994b; Reinhold-Hurek and Hurek 1998; You, Nishiguchi et al. 2005; Rosenblueth and Martinez-Romero 2006). Endophytes capable of fixing N have been isolated from a wide diversity of non-nodulating plants in an order of up to 10^8 cells per gram of tissue (Reinhold-Hurek and Hurek 1998; Chi, Shen et al. 2005; Perin, Martinez-Aguilar et al. 2006b). The endophytic population can vary depending on environmental factors such as the type of soil as well as plant characteristics such as genotype and developmental stage (Kuklinsky-Sobral, Araujo et al. 2004; Rosenblueth and Martinez-Romero 2006). Comparison of the rhizospheric and the

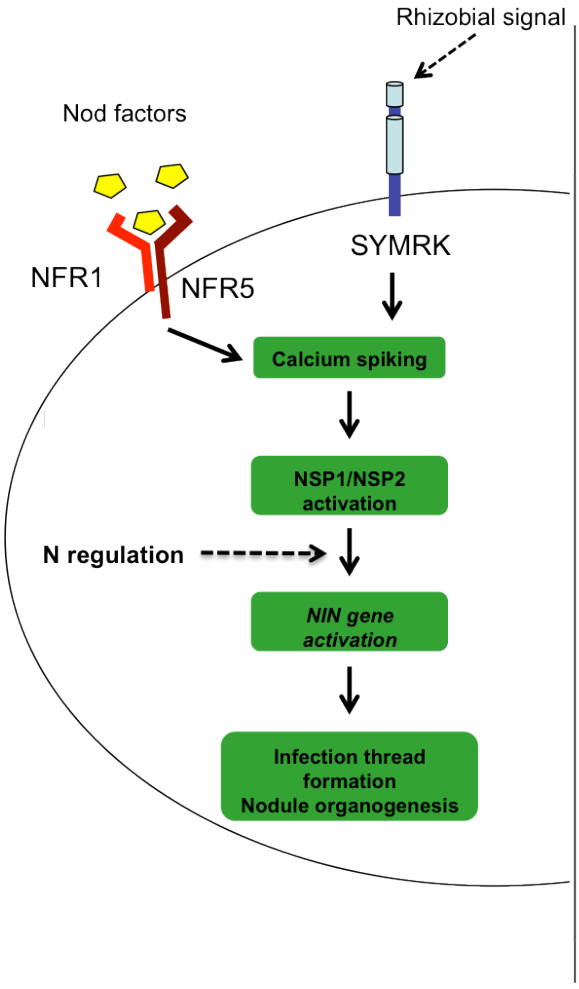

Figure 2: Symbiotic transduction pathway. Nod factors and other rhizobial signals are secreted by nitrogen fixing bacteria (NFB) and sensed by plants receptors such as NFR1/NFR5 complex and SYMRK, respectively. Perception of the bacterial signals triggers calcium oscillations that activate the induction of the primary transcription factors NSP1 and NSP2. Both transcription factors form a regulatory complex that regulates the expression of NIN. The secondary transcription factor NIN activates essential processes to the NFB entry and to the establishment of the symbiotic association such as infection thread and nodule organogenesis. The activation of this transduction pathway depends on N availability. In the presence of this macronutrient NIN genes are unable to be induced by the NFB.

endophytic bacterial communities of cucumber plants (*Cucurbitaceae*) revealed higher diversity in the rhizospheric population as compared with the endophytic population (Mahaffee and Kloepper 1997). In most cases, endophyte taxa can be found in the rhizosphere. However, there are examples of bacteria with strict endophytic lifestyle and that can only be isolated from plants, such as *Azoarcus* sp. BH72, *Herbaspirillum* and *Acetobacter* species (Reinhold-Hurek and Hurek 1998).

Non-nodulating plants and NFB can establish functional associations (Fig. 1F). *Azoarcus* sp. BH72 fixes N under microaerobic conditions. At nanomolar oxygen concentrations, these bacterial cells can shift into a state of higher activity of N fixation and respiratory efficiency in which intracytoplasmic membrane stacks (diazosomes) related to N fixation are formed, and the iron protein of the nitrogenase is highly enriched (Hurek, Reinhold-Hurek et al. 1994a). Transcriptional fusion of the nitrogenase *nifH* gene promoter to green fluorescent protein reported high levels of nitrogenase gene expression in *Azoarcus sp*. BH72 within rice roots (Egener, Hurek et al. 1998). In addition, molecular ecological methods were developed to assess *nifH* mRNA expression within Kallar grass (*Leptochloa fusca, Poaceae*) plants inoculated with this bacterium. Screening for the *nifH* gene by *nifH*-specific reverse transcription polymerase chain reaction in root mRNA, showed that *Azoarcus* sp. BH72 expresses nitrogenase genes inside the plant root system (Hurek, Handley et al. 2002). Dry weight, total N content, and $^{15}N/^{14}N$ ratio were determined in plants inoculated with either wild-type bacteria or a *nifK* mutant strain BHNKD4 (unable to fix N) (Hurek, Handley et al. 2002). In N-deficient conditions, plants inoculated with strain BH72 grew better and accumulated more N with a lower $^{15}N/^{14}N$ ratio than non-N_2-fixing control plants inoculated with the mutant strain (Hurek, Handley et al. 2002). Differences on N isotopic composition

suggests that the plants in both treatments had access to different nitrogen sources (Hurek, Handley et al. 2002). It has been shown that nitrogenase discriminates the heavier isotope (Hoering and Ford 1960; Hurek, Handley et al. 2002). Therefore, the accumulation of more N with a decreased abundance of ^{15}N suggests that the wild type bacteria can provide N for plant use.

The significance of BNF for wheat has been evaluated by the ^{15}N dilution technique (Iniguez, Dong et al. 2004). In this technique, plants are grown with ^{15}N isotopic labeled N sources and the increase in ^{14}N relative to ^{15}N content in the plant tissues under low N conditions is monitored over time. Wheat plants inoculated with NFB *Klebsiella pneumoniae* 342 assimilated up to 49% of the plant N from the atmosphere through BNF (Iniguez, Dong et al. 2004). Indeed, plants grown under N deficient conditions inoculated with a *nifH* mutant of *K. pneumoniae* (unable to fix N), showed signs of N deficiency, in contrast to plants inoculated with the wild type bacterium (Iniguez, Dong et al. 2004). Similar experiments showed that some varieties of sugarcane (*Sacharrum* spp*., Poaceae*) are also capable of obtaining a significant proportion of the required N from BNF (Boddey, Urquiaga et al. 1991). In fact, these plants can dispense N fertilization under good conditions of water and the supply of other nutrients (Boddey, Urquiaga et al. 1991).

1.3 N mediated regulation of autonomous and association pathways

With the advent of genomic technologies, our understanding of plant transcriptional changes occurring upon exposure to different N conditions has grown considerably. Genome-wide gene expression analyses using nitrate and other forms of N, such as nitrite, or glutamic acid revealed a large set of genes involved in a wide range of plant processes (Gutiérrez,

Gifford et al. 2007; Vidal and Gutiérrez 2008; Krouk, Crawford et al. 2010). Due to the importance of nitrate as primary N source for plants, the nitrate response has been the most thoroughly characterized. Roots are highly responsive to nitrate, with more than 1,000 genes responding rapidly at very low concentrations of externally added nitrate (Wang, Okamoto et al. 2003). Some of the transcriptional changes caused by nitrate treatments have been shown to correlate with changes at the protein level, as observed by two-dimensional gel electrophoresis analysis (Prinsi, Negri et al. 2009). The expression of many genes involved in the autonomous pathway is regulated by these N treatments (e.g. ammonium and nitrate transporters, genes involved in the control of RSA). However, these genome-wide experiments also show N regulation of many plant genes that may impact the association pathways. It has been reported that low levels of nitrate and ammonium stimulate nodulation, whereas high concentrations of these nutrients inhibit nodule formation (Eaglesham 1989; Zahran 1999)**.** The inhibitory effects of nitrate on different phases of nodulation, including the number of infection sites in the root, nodule development, N fixation in pre-existing nodules and nitrogenase activity have been well documented (Bisseling, van den Bos et al. 1978; Caetano-Anolles and Gresshoff 1991; Zahran 1999). Moreover, nitrate can significantly decrease the number of rhizobial cells adhering to plant roots, which is an important step for root infection (Dazzo and Brill 1978). Plant genes involved in perception of nodulating factors, such as *NFR1* and *NFR5,* as well as transcriptional regulators of nodulation, such as *NSP1* and *NSP2,* are also regulated by N in plants exposed to nodulating factors (Barbulova, Rogato et al. 2007). The transcription factor NIN was not induced by nodulating factors in the presence of nitrate or ammonium as compared to plants grown in the absence of N (Barbulova, Rogato et al. 2007). The lack of induction of NIN may represent an

important event in nitrate dependent inhibition of nodule development, since NIN factors are essential for nodule organogenesis (Fig. 2) (Schauser, Handberg et al. 1998; Borisov, Madsen et al. 2003; Oldroyd and Downie 2008). The effect of nitrate on NIN gene expression was not observed in the hypernodulation aberrant root formation (*har1*) mutant plants treated with nodulating factors or with NFB, suggesting that NIN expression is controlled by HAR1 and that the nitrate effect is mediated by HAR1 (Nishimura, Hayashi et al. 2002; Barbulova, Rogato et al. 2007). HAR1 is a key regulator involved in the systemic regulation that prevents nodule formation in the presence of nitrate. This process, termed auto-regulation of nodulation (AON) is a universal inhibitory control mechanism conserved among legumes (Carroll, McNeil et al. 1985; Krusell, Madsen et al. 2002; Nishimura, Hayashi et al. 2002; Searle, Men et al. 2003).

N is also an important regulatory factor of plant and NFB associations in non-nodulating plants. However, little is known about the molecular mechanisms involved. Rice (*Orysa* spp*., Poaceae*) plants treated with large doses of N fertilizers show a rapid decrease in NFB diversity in roots 15 days after treatment (Tan, Hurek et al. 2003). Similarly, sorghum plants (*Sorghum bicolor, Poaceae*) grown under high N fertilizer regimes showed decreased NFB associated to the rhizosphere (Coelho, Marriel et al. 2009). In sugarcane, high N fertilization caused a decrease in the colonization of the plant by *G. diazotrophicus* as compared to plants grown under low N fertilization (Fuentes-Ramírez, Caballero-Mellado et al. 1999). In addition, nitrate or ammonium leads to the repression of nitrogenase genes and to an inactivation of nitrogenase activity (Martin and Reinhold-Hurek 2002). Therefore, N fertilization not only has an effect in the diversity and in the number of NFB associated to the plant, but also has an effect in the activity of the associated bacteria. Although the regulatory

components of this interaction are unknown, *A. thaliana* (the best studied non-nodulating plant) and other non-nodulating plants have homologous genes to those involved in nodulation in nodulating plants such as NFR1, NFR5 and SYMRK receptors and the transcriptional factors NSP1, NSP2 and NIN (Schauser, Handberg et al. 1998; Stracke, Kistner et al. 2002; Radutoiu, Madsen et al. 2003; Kalo, Gleason et al. 2005; Smit, Raedts et al. 2005; Vernie, Moreau et al. 2008; Hirsch, Kim et al. 2009). In Arabidopsis, NSP1 and NSP2 homologous are codified by one gene (Heckmann, Lombardo et al. 2006). However, a recent whole-genome duplication event specific to Arabidopsis has given rise to four AtNLP pairs whose are the homologous genes of the legumes NIN transcription factors (Schauser, Wieloch et al. 2005). Whether the regulatory function of these genes has a role in non-nodulating plants and NFB interactions remains to be elucidated. According to this, *Arabidopsis* can be a good model to evaluate the role of these genes in non-nodulating plants, since it is the best plant system available so far for identifying and studying the role of gene functions.

1.4 Importance of BNF on agriculture and environment

Traditional agriculture is based in the use of chemical N fertilizer to support world nutritional needs and its around of 120 Tg/year (Gutierrez 2012). However, this type of fertilizer is quite damaging to the environment (climate, biodiversity, water, soil and air quality) and to human health. It is necessary to use new agricultural practices in order to sustainably meet an increasing demand for plant food. For this purpose, BNF is the most natural solution to increase crop yields the most environmental friendly way with significantly lower economic costs. BNF is the principal process incorporating available N

sources for living organisms in natural ecosystems and it represents the incorporation of 110 Tg of this macronutrient in lands environments (Olivares, Bedmar et al. 2013). However, to enhance BNF in non-nodulating plants it is necessary a deeper understanding of the molecular mechanisms underlying the association with NFB. Our goal was to stablish a model system to studied non-nodulating plant and NFB interactions at a molecular level. Here we used the non-nodulating plant *A. thaliana* to determine if it can functionally associate with NFB. We shows that under depleted N conditions, Arabidopsis associated with a NFB that is capable of providing available reduced N for plant nutrition. Additionally, we identified conserved and essential transcription factor in the plant for the association with the NFB.

Hypothesis

A. thaliana can establish a beneficial association with NFB and this association is regulated by plant genes.

General Aim

Evaluate association between *A. thaliana* and NFB and identify plant genes involved in the regulation of this interaction.

Specific Aims

1.- Determine if there is an interaction between NFB and *A. thaliana.*

2.- To establish the biological effect on the plant of the association with NFB.

3.- Determine plant genes involved in the association with NFB.

2. MATERIALS AND METHODS

2.1 Plants and bacterial materials

A. thaliana Columbia (Col-0) ecotype were used in all experiments. Plant mutants in NSP1 and NLPs genes were obtained from de Arabidopsis Biological Resource Center (https://abrc.osu.edu/) and were genotyped to obtain homozygous mutant lines for each gene (Table 1). Bacterial strains utilized are listed in Table 2.

Table 1: Arabidopsis mutant lines.

Mutated gene	**ABRC stock**
AtNSP1 **(At3g13840)**	Salk_036071C
AtNLP4 **(At1g20640)**	Salk_100786C
AtNLP8 **(At2g43500*)***	Salk_140298
AtNLP9 **(At3g59580)**	Salk_025839C

Table 2: Bacterial strain.

Bacterial strains	**References**
***Sinorhizobium meliloti* RmP110**	(Yuan, Zaheer et al. 2006)
Derivatives:	
RmP110-GFP	This study
RmP110-Δ*nifH*	This study
***Rhizobium etli* CFN42**	(Poupot, Martinez-Romero et al. 1995)
***Cupriavidus taiwanensis* LMG19424**	(Amadou, Pascal et al. 2008)
***Burkholderia xenovorans* LB400**	(Chain, Denef et al. 2006)
***Burkholderia vietnamiensis* G4**	(O'Sullivan, Weightman et al. 2007)
***Burkholderia phytofirmans* PSJN**	(Zuniga, Poupin et al. 2013)
***Cupriavidus pinatubonensis* JMP134**	(Ledger, Zuniga et al. 2012)

2.2 Surface sterilization of seeds and seedlings

Plant material was sonicated twice in sterile water for 3 min in a bath sonicator. After sonication, plants were treated with 70% ethanol for 3 min and then transferred to a solution of 10% sodium hypochlorite for 5 min and again with 70% ethanol for 30 s. Finally, plants were washed tree times with sterile water. Incubations were done in a shaker to ensure a constant movement of the samples.

2.3 DNA extraction and *nifH* amplification

DNA extraction from fourteen days Arabidopsis seedlings or cells culture grown under sterile conditions was performed with Wizard Genomic DNA Purification Kit (Promega). Amplification of *nifH* gene was performed using degenerated primer PolF: TGCGAYCCSAARGCBGACTC and PolR: ATSGCCATCATYTCRCCGGA to amplify a region of 360-bp. PCR amplification was carried out as described by Poly et al. 2001.

2.4 Isolation of bacteria from plant tissue

We grew approximately 1,500 Arabidopsis seedlings in hydroponic cultures under long-day (16/8-h light/dark) conditions at 22ºC (Percival incubators) using MS-modified basal salt media without N (Phytotechnology Laboratories) supplemented with 0.5 mM ammonium succinate and 3 mM sucrose. After fourteen days, plants were separated in shoot and root tissue and then surface sterilized. Both plant tissues were grinded and plated to grow in 1.5 % agar plates with 869 medium (Mergeay, Nies et al. 1985) ten times diluted (1 L of medium contains: 1 g tryptone, 0.5 g yeast extract, 0.5 g NaCl, 0.1 g D-glucose and 0,0345 g $CaCl_2.H_2O$) at 30° C.

2.5 Preparation of bacterial samples for inoculation

All bacteria were routinely grown in 869 diluted medium in an orbital shaker (200 rpm) for 12h at 30°C to an optical density (OD_{600nm}) value of 0.4. Cells were harvested by centrifugation, washed and resuspended in 5 mL with sterile water. The final solution of each

strain was homogeneously inoculated on 20 mL of 0.8% agar plates containing Murashige and Skoog (MS) basal salt mixture with or without nitrogen, according to the experiment.

2.6 Plant growth promotion and NFB screening

A. thaliana seedlings were grown vertically for seven days in complete MS medium under long-day (16/8-h light/dark) conditions at 22° C and then transferred to MS salt medium without nitrogen and inoculated or not with NFB bacteria (*B. xenovorans* LB400, *B. vietnamiensis* G4, *C. taiwanensis* LMG19424, *R. etli* CFN 42, *S. meliloti* RMP110) or non-NFB (*B. phytofirmans* PsJN, *C. pinatubonensis* JMP134). After seven days, plants were harvested and dried at 70° C for two days. Biomass was measured as dry weight of fifteen seedlings. Plants were weighted in an analytical balance (Sartorios) with a 0.1 mg of deviation in the measurements.

2.7 Nitrogenase inactivation

S. meliloti nifH gene was inactivated by the procedure described by Louie et al. (Louie, Webster et al. 2002). A 295-bp internal fragment of the *nifH* gene was amplified from RMP110 DNA by using the following primer pairs: NifH2For 5'-GAAGAGAACGGCGCTTACAA -3' and NifH2Rev 5'- GGATGAGCTTGGAATTGAGG -3'. The PCR product was cloned into pCR2.1-TOPO (Invitrogen). Final plasmid DNA (5 to 10 μg) was electroporated into electrocompetent RMP110 cells prepared as described by Louie et al. (2002). To verify interruption of the *nifH* gene sequence, PCR amplification and direct sequencing were carried out using primer pairs NifH1For 5'-

GTCCACGACCTCCCAAAATA -3' /M13Rev and M13For / NifH1Rev 5'- ATCTGC TCGTCGCTCTTCAT -3'.

2.8 N^{15} dilution assay

Arabidopsis seedlings were grown vertically in MS medium without N and supplemented with 5 mM KNO_3 isotopically labeled with 5% of ^{15}N. After seven days plants were washed with 0.01 mM $CaSO_4$ and transplanted to MS medium without N and supplemented or not with 2.5 mM NH_4NO_3 and inoculated with *S. meliloti* wild type or the derivative *nifH* mutant strains, or mock inoculated. Abundances of elemental N isotopes were measured in 14 days seedling by mass spectrometry at the Cornell University facility (http://www.cobsil.com)

2.9 Green fluorescence protein labeling and microscopy analysis

S. meliloti RMP110 cells were marked with the green fluorescence protein (GFP) marker gene by using plasmid pDG69. Electrocompetent cells of *S. meliloti* were prepared and electroporated in the presence of the pDG69 and then grow in the presence of tetracycline to select the transformed bacteria. Colonies of bacterial strains expressing GFP were verified by optical fluorescence microscopy with a Nikon Labophot 2 model microscope, equipped with a UV light source (100 W HBO Fluorescence lamp housing) and a B-2A fluorescent filter. Confocal microscopy was used to analyze the localization of *S. meliloti* on Arabidopsis seedling seven days after inoculation.

2.10 RNA isolation and gene expression analysis

RNA extraction from whole root and shoot tissue was performed with Ambion PureLink^{TM} RNA Mini Kit. cDNA synthesis was carried out using the Improm-II reverse transcriptase according to manufacturer's instructions (Promega). Gene expression analysis was carried out using the Brilliant SYBR Green QPCR Reagents on a Stratagene MX3000P qPCR system according to the instruction of the manufacturer. The RNA levels were normalized relative to *clathrin* (Atg4g24550).

2.11 Functional analysis

Seven days Arabidopsis seedlings (wild type or homozygous mutant lines) grown under sterile conditions in complete MS medium under long-day (16/8-h light/dark) at 22° C were transplanted to MS medium without nitrogen and inoculated or not with the NFB *S. meliloti*. After seven days plants were harvested and dried for 48 h at 70° C. Biomass was measured as plant dry weight.

3. RESULT

3.1 Arabidopsis is naturally associated with NFB.

To evaluate whether Arabidopsis can associate with NFB, we grew Arabidopsis plants under sterile conditions. After 14 days we isolated metagenomics DNA of surface sterilized seedlings and assess the presence of NFB by polymerase chain reaction (PCR) using universal primer pairs against the *nifH* gene. As shown in Fig. 3A, we were able to amplify *nifH* gene using DNA extracted from Arabidopsis seedlings. As controls, we determined that NFB are associated with whole plants but no signal is detected in DNA extracted from Arabidopsis cell cultures (Fig. 4). We performed an extraction control, which consisted in the DNA extraction protocol without the plant material. No amplification was observed in the extraction control indicating that the *nifH* signal comes from the plant (Fig. 4). Since we found the presence of NFB in plants growing under sterile conditions we evaluated the possibility of vertical transmission of the associated bacteria. For this purpose, we isolated metagenomics DNA of sterilized seeds and amplified *nifH* gene by PCR as indicated above. The amplification of *nifH* gene in surface sterilized seeds suggests NFB are inherited through

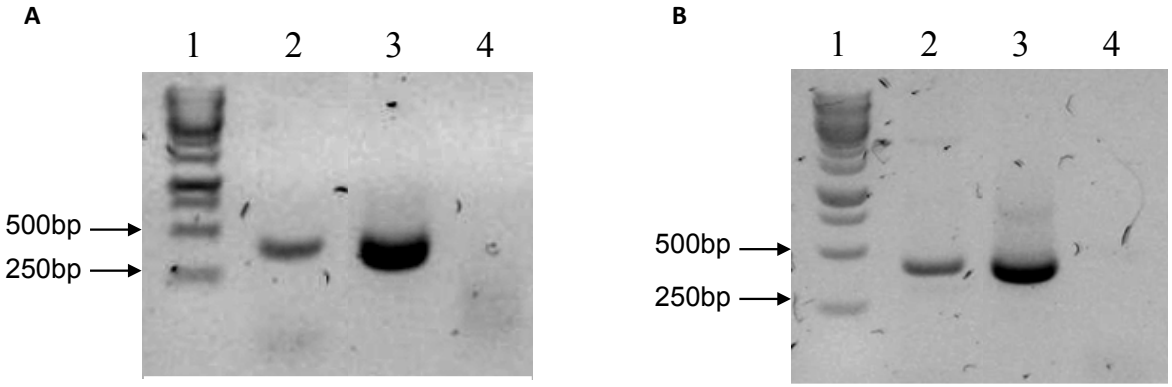

Figure 3: Detection of the *nifH* gene in Arabidopsis plant tissue. (A) *nifH* gene amplification of DNA obtained from Arabidopsis seedling grown under sterile conditions. 1: Ladder (1 Kb), 2: seedlings, 3: positive control (DNA of the NFB *S. meliloti*), 4: PCR negative control. (B) *nifH* gene amplification of DNA obtained from Arabidopsis seeds, after the sterilization process. 1: Ladder (1 Kb), 2: seeds, 3: positive control (DNA of the NFB *S. meliloti*), 4: PCR negative control.

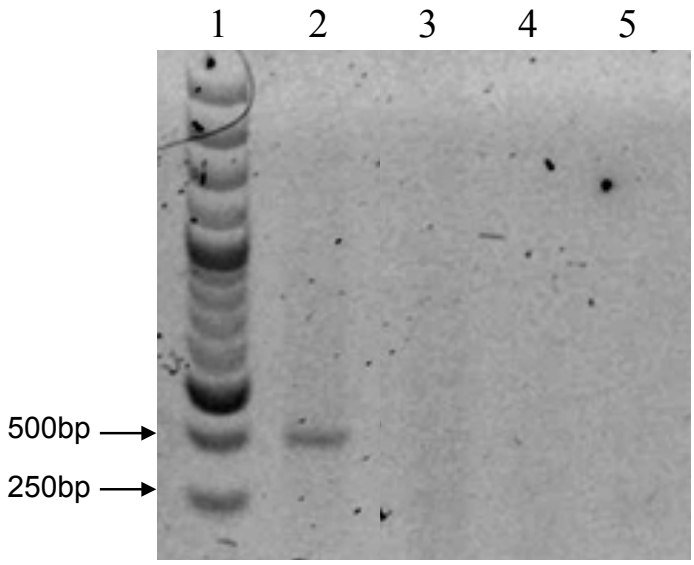

Figure 4: Assessment of *nifH* gene in Arabidopsis cell culture. (A) *nifH* gene amplification of DNA obtained from Arabidopsis cells grown under sterile conditions. 1: Ladder (1 Kb), 2: positive control (Arabidopsis seedling DNA), 3: cell culture, 4: extraction control, 5: PCR negative control. Extraction control consisted of DNA extraction without the addition of plant material.

generations (Fig. 3B). We verified that the amplification signal correspond to *nifH* gene by cloning and sequencing the PCR product. Alignment of the PCR sequence was performed using the NCBI BLASTN tool. As shown Fig. 5A, Blast result indicates that the PCR sequence from seedling has a 95% of identity with a gene encoding a nitrogenase iron protein (NifH). In the case of seeds, the PCR sequence has a 97% of identity with *nifH* (Fig. 5B). Together, our results suggest that *A. thaliana* is intimately associated with NFB.

In order to confirm the presence of bacteria that harbor the *nifH* gene, we isolated bacteria from shoot and root tissues of Arabidopsis seedlings growing under hydroponic conditions. By using PCR we amplified 16S rRNA and *nifH* gene. Sequencing the 16S rRNA gene of twelve isolates, allowed as to identify ten different genera of species and one non-identified bacterium associated to *A. thaliana* tissues (Table 2). We detected the presence of *nifH* gene in two of the twelve isolates: *Pontibacter sp*. and a non-identified bacterium (Table 2).

3.2 Functional association between *Arabidopsis thaliana* and NFB.

To determine whether *A. thaliana* establishes beneficial interactions with NFB, we assessed the effect of different NFB species on plant growth under N-limiting conditions. We selected five different NFB species shown to fix N in association with plants: *S. meliloti* RMP110 (Pichon, Journet et al. 1992), *R. etli* CFN42 (Poupot, Martinez-Romero et al. 1995), *C. taiwanensis* LMG 19424 (Marchetti, Catrice et al. 2011), *B. xenovorans* LB400 (Perin, Martinez-Aguilar et al. 2006) and *B. vietnamiensis* G4 (Perin, Martinez-Aguilar et al. 2006).

A

Uncultured bacterium clone IS64 nitrogenase iron protein (nifH) gene, partial cds

Sequence ID: gb|EU048122.1| Length: 368 Number of Matches: 1

Expect	Identities	Gaps
6e-158	339/355(95%)	2/355(0%)

```
Query  1    GATCCGAAAGCGGACTCGACCCGCCTGATCCTGCATGCCAAGGCGCAGGACACCATCCTT  60
            ||||||||| ||||||||||||||||||||||||||| ||||||||||||||||||||||
Sbjct  8    GATCCGAAGGCGGACTCGACCCGCCTGATCCTGCACGCCAAGGCGCAGGACACCATCCTT  67

Query  61   TCGCTGGCCGCCAACGCGGGCTCGGTGGAAGACCTCGAGCTCGAGGACGTGATGAAGGTC  120
            |||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  68   TCGCTGGCCGCCAACGCGGGTTCGGTGGAAGACCTCGAGCTCGAGGACGTGATGAAGGTC  127

Query  121  GGCTACAAGGACATCCGCTGCGTGGAGTCCGGTGGTCCGGAGCCAGGCGTCGGCTGCGCC  180
            |||||||| |||||||||||||||||||| ||||| |||||||| ||||||||||||||||
Sbjct  128  GGCTACAACGACATCCGCTGCGTGGAGTCGGGTGGCCCGGAGCCGGGCGTCGGCTGCGCC  187

Query  181  GGCCGCGGCGTGATCACCTCGATCAACTTCCTTGAGGAGAACGGCGCCTACGAGGACATC  240
            ||||| |||||||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  188  GGCCGTGGCGTGATCACCTCCATCAACTTCCTTGAGGAGAACGGCGCCTACGAGGACATC  247

Query  241  GACTACGTGTCCTACGACGTGCTGGGCGACGTGGTGTGCGGCGGCTTCGCGATGCCGATC  300
            |||||||||||||||||| |||| ||||||||||||||||||||||| ||||| |||||||
Sbjct  248  GACTACGTGTCCTACGACCTGCTCGGCGACGTGGTGTGCGGCGGCTTCGCCATGCCCATC  307

Query  301  CGCCGAGAACAAGGCGCAGGAAATCTACATCGTCATGTCCCGGCGAGATGATGGC  355
            ||| |||||||||||||||||||||||||||||||||||||| ||||| ||||||||
Sbjct  308  CGC-GAGAACAAGGCGCAGGAAATCTACATCGTCATGTCC-GGCGAAATGATGGC  360
```

B

Xanthobacter flavus clone CC1080A1 dinitrogenase reductase (nifH) gene, partial cds

Sequence ID: gb|AY221812.1| Length: 324 Number of Matches: 1

Expect	Identities	Gaps
3e-150	310/319(97%)	0/319(0%)

```
Query  1    GACCCGCCTGATCCTGCATGCCAAGGCGCAGGACACCATCCTTTCGCTGGCCGCCAACGC  60
            |||||||||||||||| |||||||||||||||||||||||||||||||||||| |||||
Sbjct  3    GACCCGCCTGATCCTGCACGCCAAGGCGCAGGACACCATCCTTTCGCTGGCCGCGAACGC  62

Query  61   GGGCTCGGTGGAAGACCTCGAGCTCGAGGACGTGATGAAGGTCGGCTACAAGGACATCCG  120
            ||| |||||||||||||||||||||||||||||||||||||||||||||| ||||||||
Sbjct  63   GGGTTCGGTGGAAGACCTCGAGCTCGAGGACGTGATGAAGGTCGGCTACAACGACATCCG  122

Query  121  CTGCGTGGAGTCCGGTGGTCCGGAGCCGGGCGTCGGCTGCGCCGGTCGCGGCGTGATCAC  180
            |||||||||||| ||||| |||||||||||||||||||||||| || ||||||||||||
Sbjct  123  CTGCGTGGAGTCGGGTGGCCCGGAGCCGGGCGTCGGCTGCGCCGGCCGTGGCGTGATCAC  182

Query  181  CTCGATCAACTTCCTTGAGGAGAACGGCGCCTACGAGGACATCGACTACGTGTCCTACGA  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  183  CTCGATCAACTTCCTTGAGGAGAACGGCGCCTACGAGGACATCGACTACGTGTCCTACGA  242

Query  241  CGTGCTGGGCGACGTGGTGTGCGGCGGCTTCGCGATGCCGATCCGCGAGAACAAGGCGCA  300
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Sbjct  243  CGTGCTGGGCGACGTGGTGTGCGGCGGCTTCGCGATGCCGATCCGCGAGAACAAGGCTCA  302

Query  301  GGAAATCTACATCGTCATG  319
            ||||||||||||||||||
Sbjct  303  GGAAATCTACATCGTCATG  321
```

Figure 5: Alignment of *nifH* sequences. Verification of the *nifH*-like sequence was done by cloning and sequencing the PCR product. *nifH* sequence was amplified from sterilized (A) seedlings and (B) seeds Blastn was performed using NCBI blast tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Plant tissue	Blast best hit	Description	*nifH*
Shoot	*Arthrobacter* sp.	Gram +, Actinobacteria	-
	Environmental bacterium	Gram +, Actinobacteria	+
	Kocuria rhizophila	Gram +, Actinobacteria	-
	Microbacterium oxydans	Gram +, Actinobacteria	-
	Curtobacterium flaccumfaciens	Gram +, Actinobacteria	-
	Pontibacter sp.	Gram -, Bactereoidete	+
	Naxibacter intermedius	Gram -, β-Proteobacteria	-
Root	*Bacillales bacterium* NR100	Gram +, Firmicutes	-
	Kocuria sp. WPCB069	Gram +, Actinobacteria	-
	Arthrobacter sp.	Gram +, Actinobacteria	-
	Staphylococcus sp.	Gram +, Firmicutes	-
	Sphingobium yanoikuyae	Gram -, α-Proteobacteria	-

Table 3: Bacterial isolates associated with Arabidopsis. Bacteria were isolated from shoot and root tissue of Arabidosis seedling grown in hydroponic conditions. We identified Arabidopsis associated bacteria using the best Blast hit over 97 % of sequence identity (Blastn from NCBI). Amplification of *nifH* gene was performed for all bacteria; positive signal indicates the presence of the gene and negative signal indicates its absence.

We also tested two additional bacteria unable to fix nitrogen as controls. *Burkholderia phytofirmans* PsJN, known to enhance Arabidopsis growth (Zuniga, Poupin et al. 2013) and *Cupriavidus pinatubonensis* JMP134, a soil bacterium able to associate with plants but without a positive impact on plant growth (Ledger, Zuniga et al. 2012). Plants were grown on vertical plates with MS medium for seven days and were transferred to plates with the same MS medium but without N (MS-N) or MS-N inoculated with 10^4 colony forming units (CFU)/ml of different bacteria. We evaluated dry weight of the plants seven days after the transfer. Plant dry weight was significantly higher in the presence of *S. meliloti* as compared to non-inoculated medium under N-limiting conditions (Fig. 6A). Moreover, plant growth was comparable to that achieved in full MS medium, under our experimental conditions (Fig. 6A). These results indicate *S. meliloti* RMP110 can promote plant growth in the absence of an N source. Other NFB as well as the PGPB were not able to increase Arabidopsis dry weight indicating the mere presence of beneficial bacteria cannot account for this observation. Moreover, we did not observe the growth promoting effect when inoculated with dead *S. meliloti* (Fig. 6B). Together, these results suggest that plant growth promotion requires metabolically active bacterial cells and that it could be providing N for plant nutrition and growth.

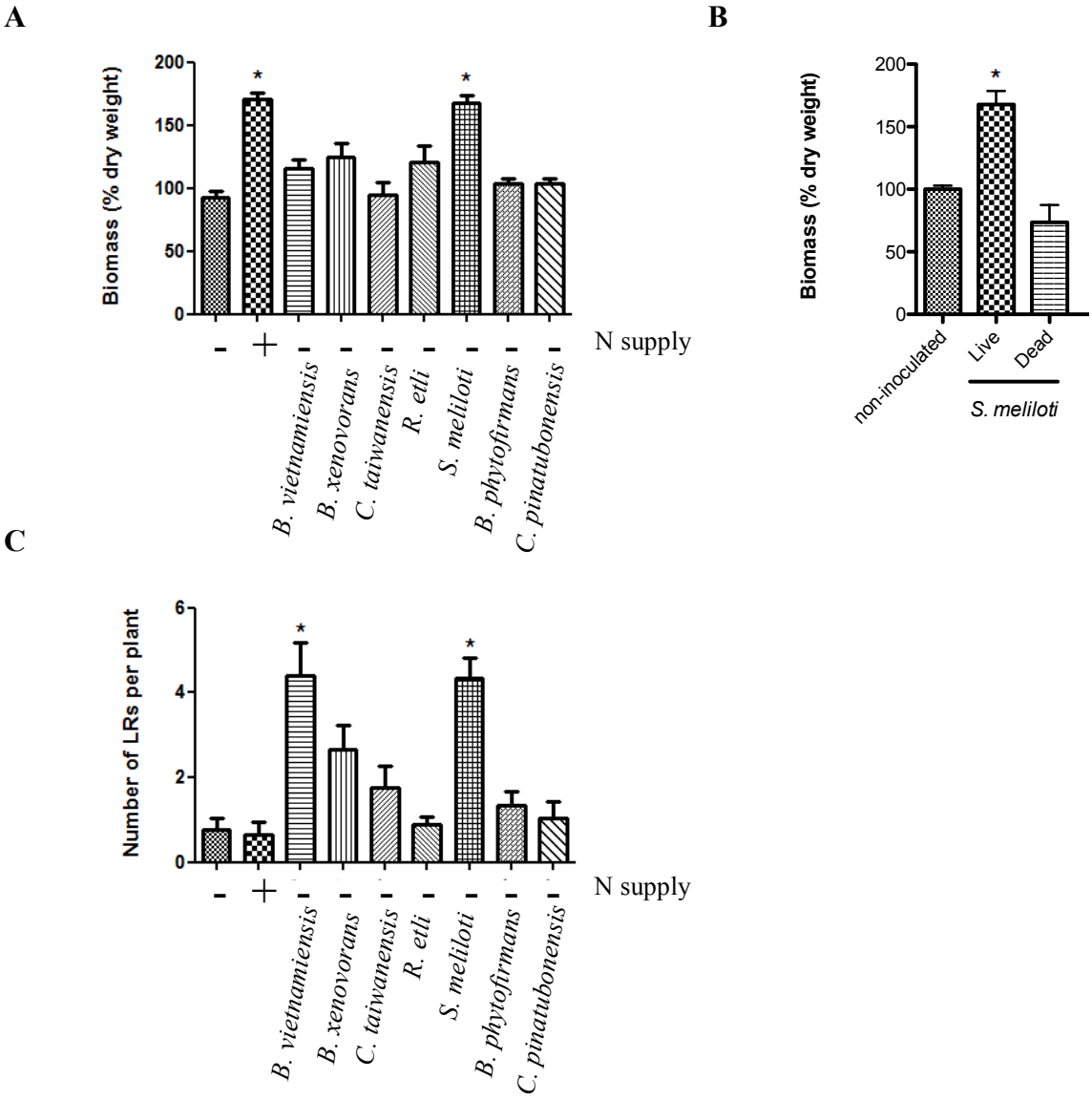

Figure 6: Effect of NFB on plant growth under limiting N conditions. (A) Biomass was measured seven days after transfer as dry weight of fifteen plants. Plants grown in complete MS salt medium for seven days were transplanted to MS medium with (40 mM NO_3 + 20 mM NH_4) or without nitrogen inoculated or not with different bacteria (NFB: *Burkholderia vietnamiensis* G4, *B. xenovorans* LB400, *Cupriavidus taiwanensis* LMG19424, *Rhizobium etli* CFN42, *Sinorhizobium meliloti* RMP110; non-NFB: *B. phytofirmans* PsJN, *C. pinatubonensis* JMP134). (B) Dry weight of plants grown in N-containing medium for seven days and that were transplanted to limiting N conditions and inoculated with live or dead *S. meliloti*, or non-inoculated. (C) Number of lateral root per plant. Plants were grew and treated in the same conditions that are detailed above for (A). Plotted values correspond to the mean of three independent biological replicates ± standard error. The asterisk indicates means that differ significantly as compared to non-inoculated plants grown without N ($P < 0.05$).

S. meliloti as well as *B. xenovorans* had an effect in the RSA by increasing the number of LR per plant (Fig 6C). In addition, an unspecific effect is also shown in Fig. 7, in which the presence of all bacteria increase root hair growth as compared with the non-inoculated medium. This response was triggered by NFB as well as non-NFB and thus it is independent of BNF (Fig. 7).

To further characterize the association of *S. meliloti* with *A. thaliana*, we generated a gfp::RmP110 strain. Plants were inoculated with the gfp tagged strain in the same conditions we indicated above. Confocal analysis of the root system of the plants, showed that *S. meliloti* is associated to epidermals cells and therefore to the root surface (Fig. 8). However, we could not find the bacterium when we analyzed internal parts of the plant tissue.

3.3 BNF promotes plant growth.

To determine whether BNF was required for plant growth promotion under N-limiting conditions, we generated a *S. meliloti* RMP110 mutant strain (Δ*nifH*) unable to fix N. As shown in Fig. 9A, *S. meliloti* RMP110 Δ*nifH* had a significantly reduced impact on plant growth as compared with the wild-type bacteria under N-limiting conditions (Fig. 9A). Other measurable phenotypes generated by the wild type bacteria were conserved in the mutant strain. Both bacteria have an effect on LR density and therefore increased surface absorption of nutrients and water (Fig. 10A). In addition, none of both strains had an effect on primary root length (Fig. 10B).

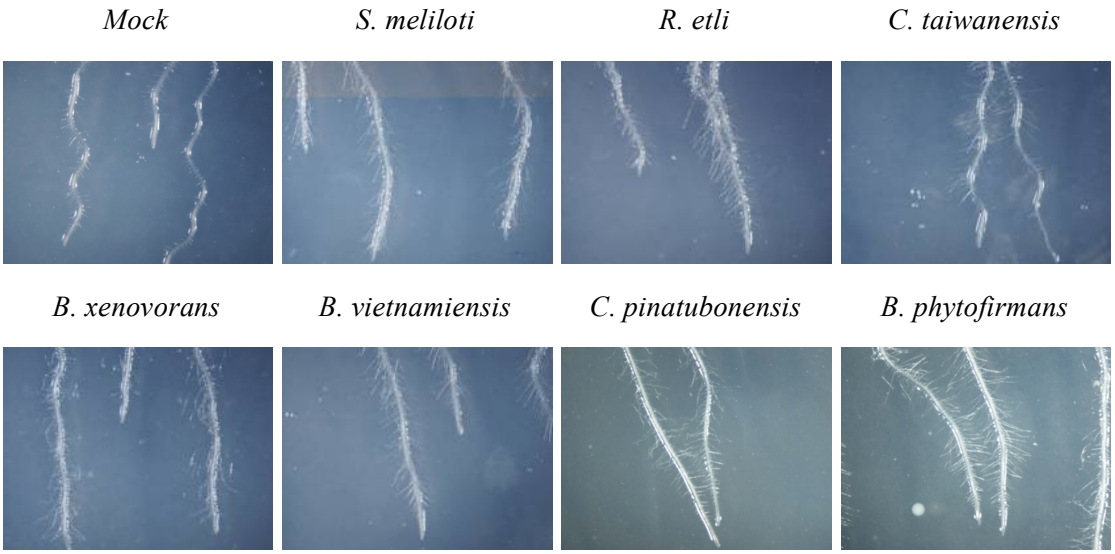

Figure 7: Effect of NFB on root hairs length. Plants grown in complete MS salt medium for seven days were transplanted to MS medium without nitrogen inoculated or not with NFB: *Sinorhizobium meliloti* RMP110, *Rhizobium etli* CFN42, *Cupriavidus taiwanensis* LMG19424, *Burkholderia xenovorans* LB400, and *B. vietnamiensis* G4, and non NFB: *B. phytofirmans* PsJN and *C. pinatubonensis* JMP134. Pictures were taken seven days after treatment.

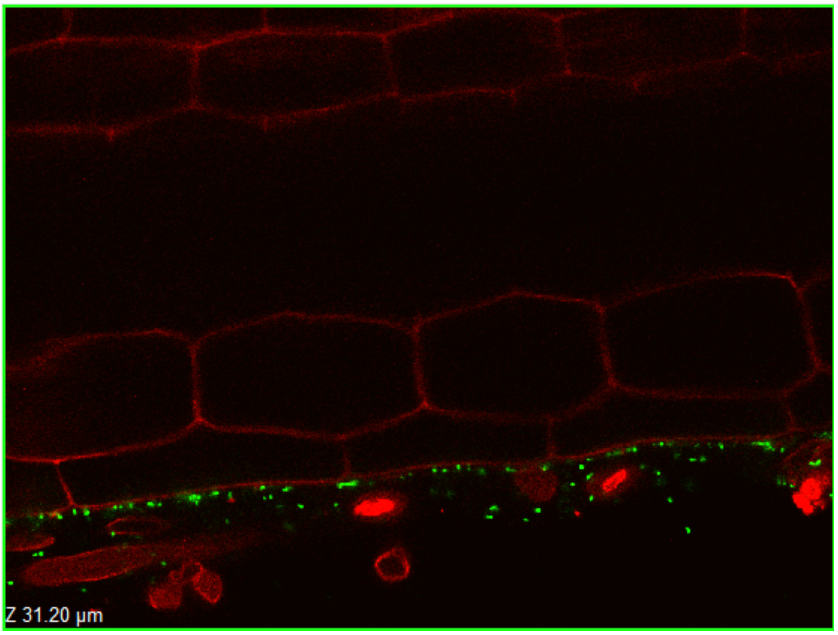

Figure 8: Epidermal localization of *Sinorhizobium meliloti* in Arabidopsis roots. Roots from Arabidopsis seedlings inoculated for seven days with a gfp-tagged *S. meliloti* cells were stained with propidium iodide and visualized by confocal microcopy using high performance filters.

To demonstrate that growth promotion by *S. meliloti* was due to fixed N contributing to Arabidopsis nutrition, we grew plants for seven days under MS-N supplemented with 5 mM of KNO_3, which was isotopically labeled with 5% ^{15}N. Plants were then exposed to wild-type *S. meliloti* or the Δ*nifH* mutant in MS-N medium. Plants treated with MS-N medium supplemented with labeled KNO_3 in the presence of wild type *S. meliloti* RMP110 showed reduced ^{15}N isotopic proportion as compared to plants inoculated with Δ*nifH* or non-inoculated plants (Fig. 9B). This result indicates that part of the N content of the plant comes from the atmosphere in which ^{14}N isotope predominates. This result shows Arabidopsis growth promotion by *S. meliloti* is partly due to BNF.

The slight decrease in ^{15}N isotope incorporation of strain Δ*nifH* as compared with non-inoculated plants may be caused by plant promoting effects other than N acquisition. To confirm this possibility we evaluated the bacterium effect under sufficient N conditions (2.5 mM NH_4NO_3) when nitrogen fixation does not occur. Plants grown in N containing medium inoculated with both wild type and mutant bacteria have proportionally the same decrease in ^{15}N compared with non-inoculated plants grown in the same conditions (Fig. 9B). These results show *A. thaliana* can functionally associate with *S. meliloti* RMP110 to sustain plant growth under N-limiting conditions. This plant:bacteria interaction represents an excellent model system to address non-legume plant mechanisms to promote interactions with bacterial species leading to nutritionally relevant BNF.

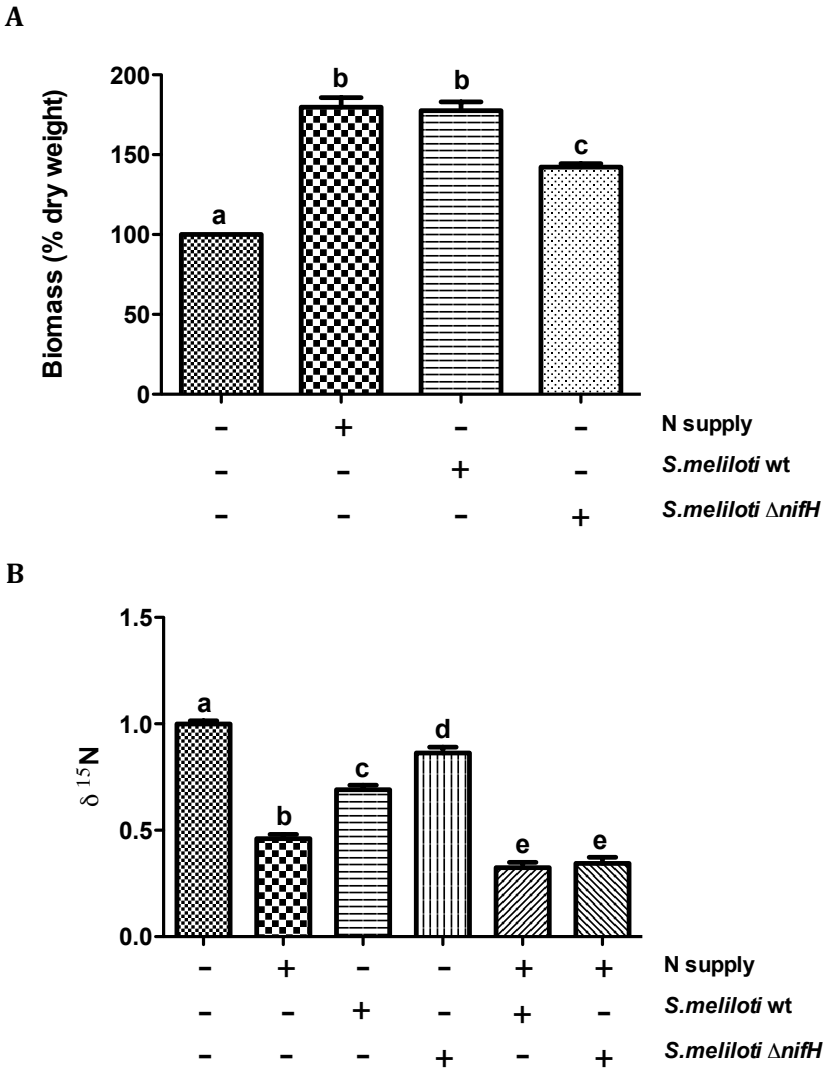

Figure 9: Nitrogen fixation enhances plant growth under N-limiting conditions. (A) Biomass was measured as dry weight of plants grown under sufficient/limiting N conditions inoculated or not with *Sinorhizobium meliloti* RMP110 wild type or with the mutant type unable to fix N. (B) Biological nitrogen fixation was measured by the ^{15}N dilution technique. Plants were grown in a N-containing medium isotopically labeled with 5% of ^{15}N. After seven days, plants were transferred to plates with the different treatments. Seven days after treatments, plants were harvested and dried. Mass spectrometry was used to determine the amount of N^{14} and N^{15}. δ ^{15}N represent the ^{14}N:^{15}N isotope ratio relative to the non-inoculated condition. Plotted values correspond to the mean of three independent biological replicates ± standard error. Different letters indicate significant differences among treatments. ($P < 0.05$).

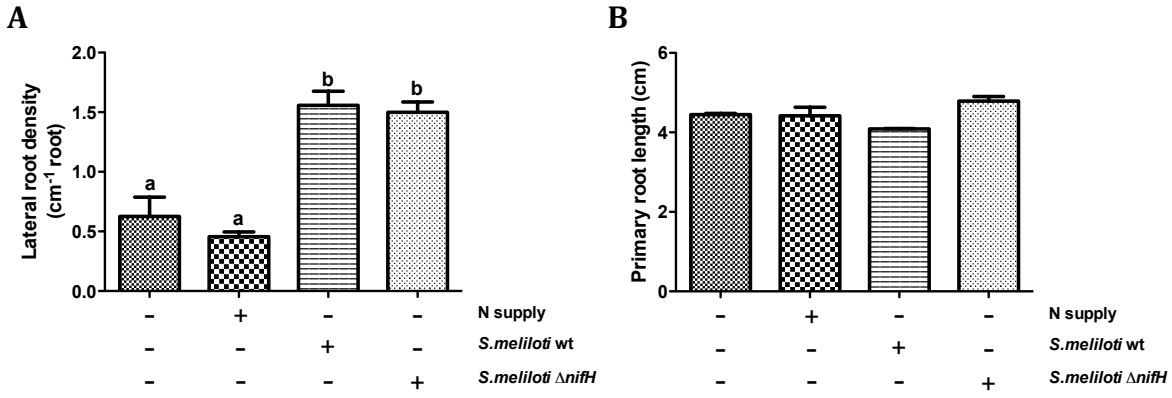

Figure 10: Effect of *Sinorhizobium meliloti* on root system architecture. (A) Lateral root density was measured in plants grown under sufficient/limiting N conditions inoculated or not with wild type *S. meliloti* RMP110 or with the mutant type unable to fix N. (B) Primary root length was measured in the same conditions mentioned above. Plotted values correspond to the mean of three independent biological replicates ± standard error. Different letters indicate significant differences among treatments. ($P < 0.05$).

3.4 Conserved genes between legumes and non-legumes are regulated by the presence of NFB in *A. thaliana*.

BNF in legume species depends on sophisticated mechanisms that control when and how symbiotic association is established. Genes involved in this interaction have been characterized in legumes (Oldroyd 2013). Key regulatory factors of this process are the NSP1 and NSP2 transcription factors (Oldroyd 2013). In order to determine whether comparable mechanisms may exist in Arabidopsis regulating interactions with *S. meliloti* RMP110, we analyzed the function of putative Arabidopsis NSP1 and NSP2 homologs, At3g13840 (AtNSP1) and At4g08250 (AtNSP2) genes, respectively (Kalo, Gleason et al. 2005; Smit, Raedts et al. 2005).

Expression of AtNSP1 and AtNSP2 genes was evaluated in plants grown under conditions of sufficient N (5 mM KNO_3), and then transferred to 2.5 mM NH_4NO_3 or to N-depleted medium inoculated or not with *S. meliloti*. Transcript levels were measured using real-time quantitative reverse transcription PCR (qRT-PCR). Our results show that AtNSP1 gene was induced when plants were transferred to bacteria-containing medium, but only when grown under N-depleted conditions (Fig. 11). This suggests that AtNSP1 might mediate the interaction between *S. meliloti* and Arabidopsis and that this interaction only occurs under N-limiting conditions. In contrast with its homolog, AtNSP2 did not present a transcriptional activation triggered by *S. meliloti* after three or seven days after inoculation under limiting N conditions (Fig. 11).

In legumes, NSP1 and NSP2 regulate the expression of NIN genes. The Arabidopsis genome encodes homologous genes for these essential regulators, which make up a gene family composed by nine Nin-like genes (NLPs) (Schauser, Wieloch et al. 2005).

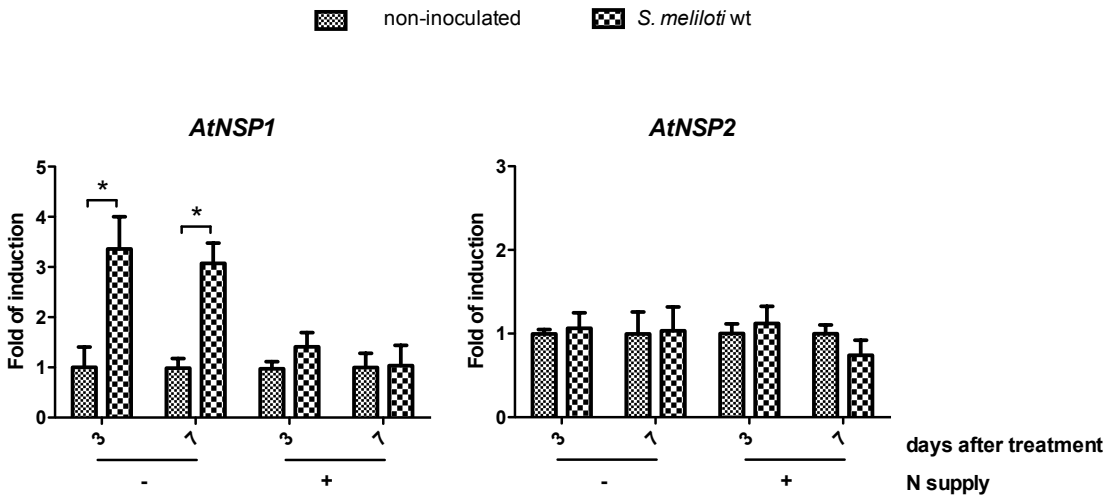

Figure 11: Transcriptional response of NSP1 and NSP2 to *Sinorhizobium meliloti* inoculation. Gene expression was measured by qRT-PCR at the third or seventh days post treatment. Plants were grown under sufficient (2.5 mM NH_4NO_3) or limiting N conditions and inoculated or not with NFB. Expression levels were normalized to the housekeeping gene *clathrin* and are presented as relative to untreated plants. Plotted values correspond to the mean of three independent biological replicates ± standard error. Asterisks indicate means that differ significantly as compared to non-inoculated plants ($P < 0.05$).

Phylogenetic analysis shows three clades shared between NLP genes and the legume NIN genes (Schauser, Wieloch et al. 2005). In order to determine the role of NLP genes in the interaction of Arabidopsis and *S. meliloti*, we analyzed the expression of the seven closest genes (NLP1: At2g17150, NLP2: At4g35270, NLP3: At4g38340, NLP4: At1g20640, NLP5: At1g76350, NLP8: At2g43500, NLP9: At3g59580) under the same experimental conditions described in Fig. 11. Results indicate that *AtNLP4, AtNLP8* and *AtNLP9* are regulated by NFB inoculation in plants grown without nitrogen source (Fig. 12), suggesting these genes might act with *AtNSP1* in order to regulate the interaction between Arabidopsis and *S. meliloti*.

A different expression pattern is observed in *AtNLP3*, which is induced upon bacterial inoculation under N sufficient conditions (Fig. 12). The expression of the other three NLPs evaluated is not altered in the presence of the NFB (Fig. 13).

3.5 Arabidopsis genes are essential for NFB promoting effect

In order to confirm that AtNSP1 and the AtNLP4, AtNLP8 and AtNLP9 genes mediate the interaction between Arabidopsis and *S. meliloti*, we analyzed NFB-promoted growth of Arabidopsis mutants on these genes. We genotyped and obtained homozygous mutant lines of *A. thaliana* in genes *NSP1* (salk_036071C), *NLP4* (salk_100786C), *NLP8* (salk_140298) and *NLP9* (salk_025839C) were obtained from the Arabidopsis Biological Resource Center (ABRC), and characterized. In addition, we first evaluated whether the mutation in *NSP1* and *NIN-LIKE* genes has an effect in plant growth in response to nitrogen. For this aim, plants were grown either in a N-containing or in N-free medium. Our results indicate that the growth in response to nitrogen availability of *nsp1*, *nlp9* and *nlp8* mutant

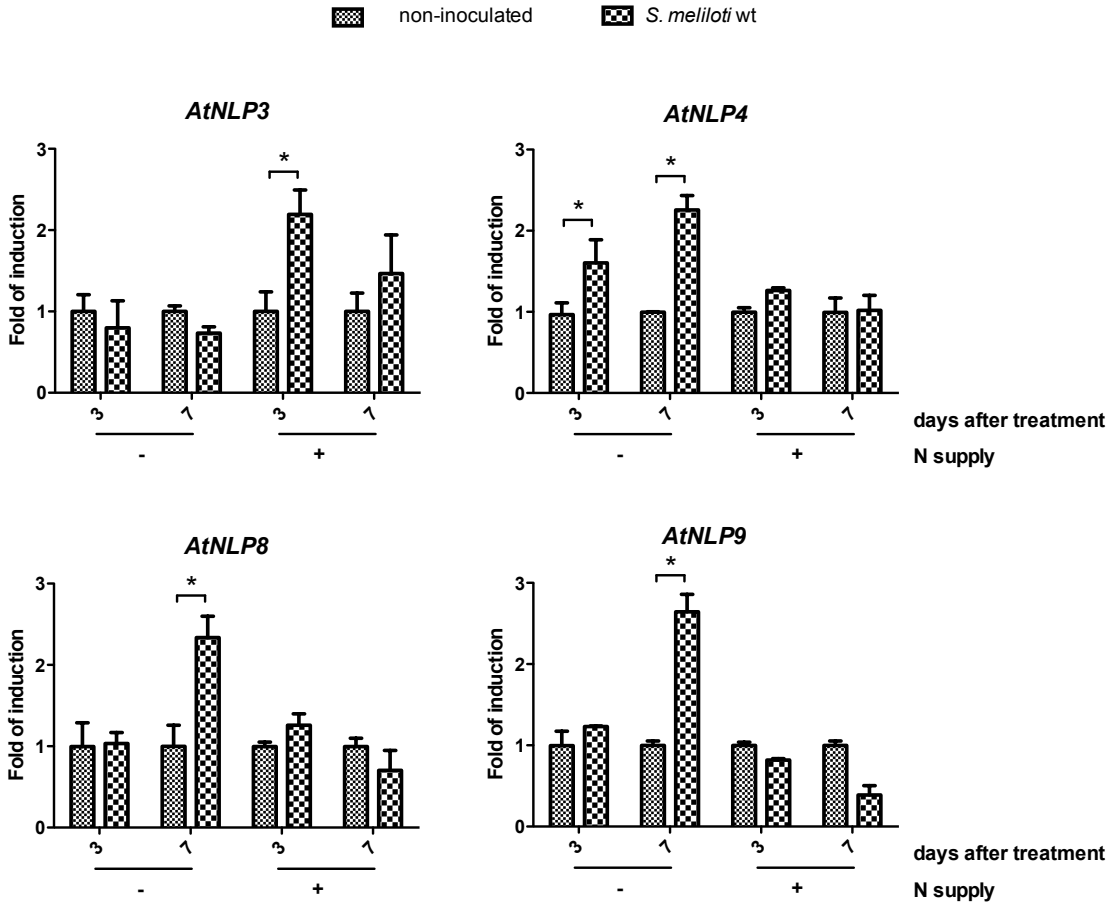

Figure 12: Nin-like transcription factor are induce upon *Sinorhizobium meliloti* inoculation. Gene expression was measured by qRT-PCR at the third or seventh days post treatment. Plants were grown under sufficient (2.5 mM NH_4NO_3) o limiting N conditions and inoculated or not with NFB. Expression levels were normalized to the housekeeping gene *clathrin* and are presented as relative to non-inoculated plants. Plotted values correspond to the mean of three independent biological replicates ± standard error. Asterisks indicate means that differ significantly as compared to non-inoculated plants ($P < 0.05$).

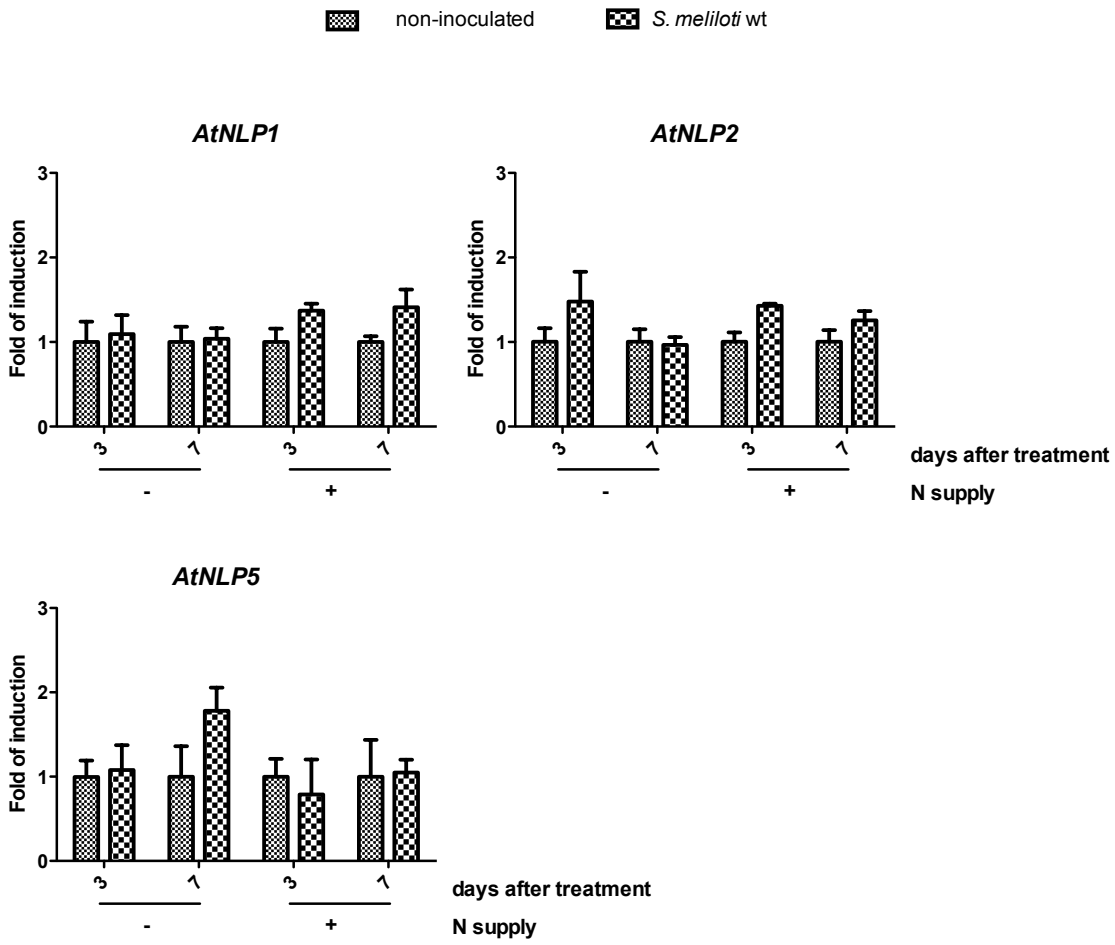

Figure 13: Response of NIN-like transcription factors to bacteria inoculation. Gene expression was measured by qRT-PCR at the third or seventh days post treatment. Plants were grown under sufficient (2.5 mM NH_4NO_3) or limiting N conditions and inoculated or not with NFB. Expression levels were normalized to the housekeeping gene *clathrin* and are presented as relative to non-inoculated plants. Plotted values correspond to the mean of three independent biological replicates ± standard error.

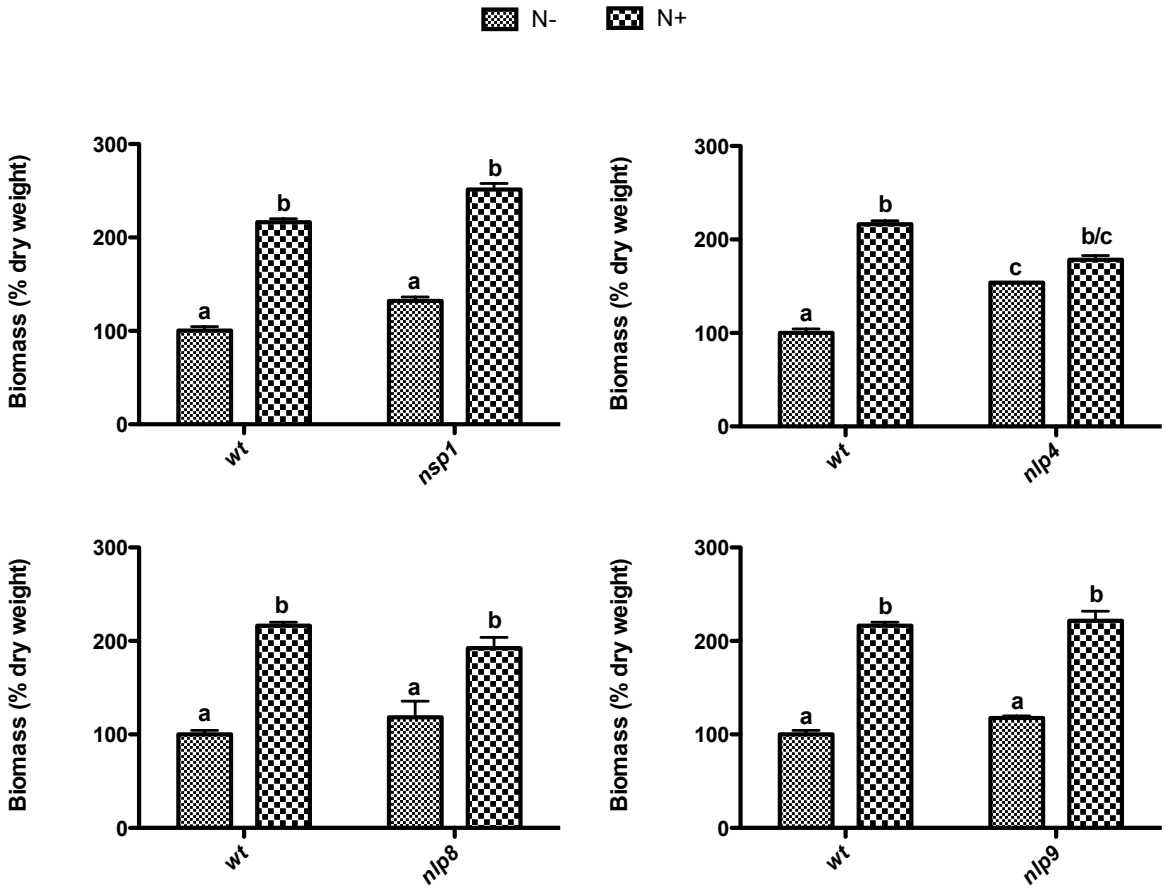

Figure 14: Growth of *nsp1* and *nlp* mutant plants under different N availability. Biomass expressed as dry weight was measured in wild type and mutant plants grown in a medium with sufficient N (N+, 2.5 mM NH_4NO_3) or without the macronutrient (N-). Plotted values correspond to the mean of three independent biological replicates ± standard error. Different letters indicate significant differences among treatments. ($P < 0.05$). All percentage values are respect to non-inoculated wild type plants.

plants is the same as the wild type plants (Fig. 14). All these plants have more biomass when grown in N-containing medium than in a medium without the macronutrient (Fig. 14). Unlike the others, *nlp4* plants grow better than wild type plants when grow under N limiting conditions (Fig. 14). However, *nlp4* plants achieve the same growth under different N availability (Fig. 14). These results, suggest that mutation of the *NLP4* gene affect the plant growth in response to N. In order to evaluated whether this genes are important to the beneficial effect of the bacteria, mutants and wild type plants grown under N limiting conditions were inoculated or not with wild-type *S. meliloti*. Our results show that the growth-promoting effect of *S. meliloti* is drastically reduced in *nsp1* mutants under limiting N conditions (Fig. 15). This suggests that this homologous gene of the primary transcription factor in the symbiotic legume:NFB interaction pathways has a conserved and essential role in mediating the functional association between Arabidopsis and *S. meliloti*. Consistent with this result, mutant plants in the candidates NLP genes were incapable to functional associates with *S. meliloti* (Fig. 15). Together our results indicate that *NSP1* and *NLP9* specifically affect plant growth in response to *S. meliloti*.

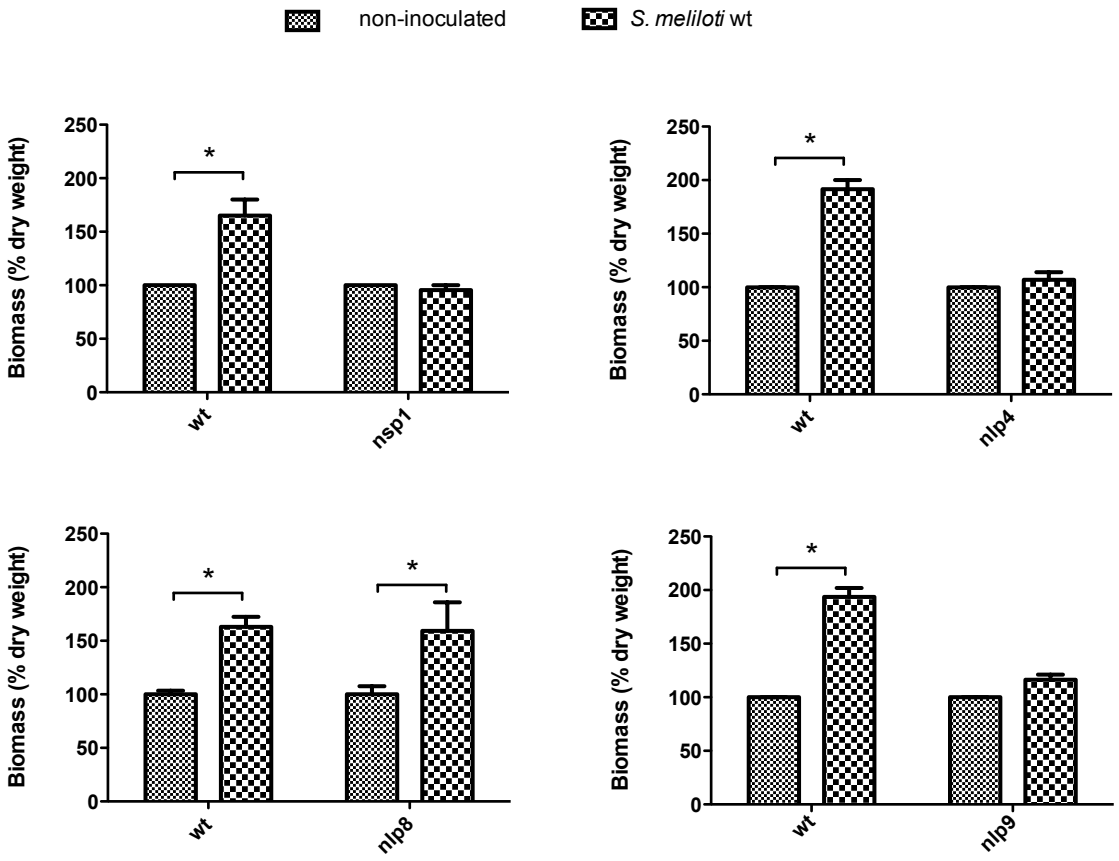

Figure 15: Arabidopsis homologs of essential regulatory transcription factors in legumes are required for the beneficial association with *Sinorhizobium meliloti* RMP110. Biomass expressed as dry weight was measured in wild type and mutant plants grown under limiting nitrogen conditions inoculated or not with the NFB, *S. meliloti*. Plotted values correspond to the mean of three independent biological replicates ± standard error. The asterisk indicates means that differ significantly as compared to non-inoculated plants ($P < 0.05$).

4. DISCUSSION

The main process incorporating N in natural ecosystems is BNF (Olivares, Bedmar et al. 2013). This macronutrient is one of the most limiting factors for plant growth and development and it has been seen that in N-limiting environments some plants have associations with NFB (Galloway and Cowling 2002; Oldroyd 2013). The most studied associations are those between legumes and *Rhizobiaceae*. However, less studied are the association of NFB with non-nodulating plants which include most of the plants that are used for human consumption. In order to obtain deeper understanding into the molecular basis of the association between non-legumes and NFB, we used *A. thaliana* as a model organism.

In this work we present evidence indicating that *A. thaliana* is naturally associated with NFB. Using culture-independent and culture dependent techniques we found the presence of bacteria carrying the *nifH* gene on seedlings grown under sterile conditions. Additionally, we detected the *nifH* gene in *A. thaliana* seeds suggesting a vertical transmission of the NFB. It has been reported in other plant species that bacteria are found within ovules and seeds (Mundt and Hinkle 1976). However, this is the first report of an association of *A. thaliana* grown under sterile conditions with bacteria. Even more, our work is the first in describing the presence of bacteria inside of the seeds and seedlings of this

model plant. Differences in the nucleotide sequence of *nifH* gene amplified from seedlings and seeds suggest the presence of different NFB associated to *A. thaliana*. This observation was confirmed by the isolation of two different NFB from *A. thaliana* seedlings. In other plant species, NFB have been isolated within plant tissue and has been found to have a functional association in plant growing under N-limiting conditions (Hurek, Handley et al. 2002).

Our results suggest that *A. thaliana* can interact with NFB. In order to evaluate the biological importance of this association, we selected five different NFB whose ability to fix nitrogen has been proven. Here we present evidence indicating that *A. thaliana* is functionally associated with *S. meliloti*. This bacterium has been widely studied in associations with the legume *Medicago truncatula*, being one of the most important models of NFB (Marsh, Rakocevic et al. 2007; Peiter, Sun et al. 2007). In the legume system, establishment of the symbiotic association between legume plants and the NFB requires the expression of specific plant and bacterial genes. In response to plant's flavonoids, the bacterium secrete Nod factors that are sensed by the plants. The recognition of the bacterial partner causes a root hair curling to entrap the NFB leading to the bacterial entry and the directed colonization of the nodule. Here we show that the exposure to NFB as well as non-NFB increase root hair growth in plants growing under nitrogen limiting conditions as compared with the non-inoculated plants. In these cases, the increase in root hair growth does not generate a curling effect, which it is apparently not necessary since *A. thaliana* is unable to form a nodule structure. Although NFB is mediating the increase of root hair growth, it is not a specific effect of this functional group of bacteria since non-NFB also produce the same effect.

Upon perception of Nod factors, the symbiotic transduction pathway is activated leading to nodule development and symbiosis. In legumes, the establishment of the association is generated only when plants grow under conditions of N deprivation. Both plants and bacteria have molecular mechanisms that prevent a functional association when N is present. In plants grown under sufficient N conditions, the induction of essential transcription factors such as NSPs and NLPs does not occur even when the NFB is present and therefore nodule development is not possible (Barbulova, Rogato et al. 2007). In the case of the NFB *S. meliloti*, fixes N only under N limiting conditions (Szeto, Nixon et al. 1987). In the presence of N, one of the master regulators (*ntrC*) of the *nif* operon is not expressed and therefore the nitrogenase is absent. In contrast, under limiting N conditions the expression of *ntrC* is induced and N fixation is able to occur (Szeto, Nixon et al. 1987). N fixation of *S. meliloti* has been mainly described in association with legumes within the nodule. However, there are few reports that indicate that this bacterium is also able to fix N under free-living conditions (Bedmar and Olivares 1979; Dreyfus, Elmerich et al. 1983). Our results, shows strong evidence suggesting that *S. meliloti* is fixing N in a nodule-free system. Under our experimental conditions, the bacterium is associated to the surface of the root (rhizoplane). The rhizoplane, is a zone rich in carbon source for bacterial growth due to plant exudates and also is an area with a reduced oxygen tension (Bramwell, Barallon et al. 1995). Therefore, lower levels of oxygen in the rhizoplane could facilitate N fixation by preventing the nitrogenase inactivation.

In our study, *S. meliloti* promotes plant growth in a medium without additional N source and thus suitable for N fixation. Our results show that this bacterium can be attached to the epidermal cells in Arabidopsis roots, suggesting that the bacterium is exerting its effect

from the rhizoplane. By mutational inactivation of nitrogenase we determined that plant growth promotion was due in part to BNF. We corroborated that BNF was happening through the ^{15}N dilution technique by comparing plants inoculated with wild type and Δ*nifH* mutant under limiting N conditions. Our results indicate that *S. meliloti* RMP110 is able to fix atmospheric N and make it available for plant nutrition. The remaining effect on plant growth of the Δ*nifH* mutant could be explained by additional promoting properties that have been described for this bacterium such as auxin biosynthesis (Kittell, Helinski et al. 1989; Spaepen, Vanderleyden et al. 2007). Here we show, that the inoculation of plants with the mutant strain promotes lateral root growth and this could explain the remaining effect on biomass. It has been shown that auxin directly impact the initiation of LR growth. Plants treated with this hormone or that overproduced it form more LR (Fukaki, Okushima et al. 2007). Consistently with auxin production in *S. meliloti*, both wild type and the mutant strain produce the same effect on the RSA of Arabidopsis seedlings that have been inoculated. An increase of the number of LR per plant produces an enlargement of the nutrient absorption surface and it is considered a bacterial plant growth promotion mechanism (Spaepen, Vanderleyden et al. 2007).

In legumes functional association with NFB is highly regulated. Molecular regulatory systems prevent the formation of nodules in plants grown in the presence of N (Eaglesham 1989; Barbulova, Rogato et al. 2007). Only under limiting N conditions and in the presence of the NFB, a symbiotic signal transduction pathway is activated and induces nodule development. Upon Nod factor recognition, calcium oscillation generates the induction of the primary transcription factors NSP1 and NSP2, which promotes NIN gene expression (Kalo, Gleason et al. 2005; Smit, Raedts et al. 2005). Our study describes the

importance of such transcription factors in a functional association of non-nodulating plants with NFB for the first time. In *A. thaliana*, orthologs for NSP1 and NIN genes are regulated by the presence of the bacterium only when plants are grown under limiting N conditions. Unlike the other NSP, NSP2 did not exhibit a transcriptional regulation on the time we evaluated. We do not discard a role of NSP2 in the association of non-legumes with NFB, since in legumes this transcription factor acts by forming a complex with NSP1 to regulate NIN expression. It has been described that plants mutant in *NSP2* could not develop a nodule (Kalo, Gleason et al. 2005). Besides the role of *NSP2* on nodulation, it was recently shown that this gene form part of the common symbiotic signaling pathways required for mycorrhizal fungi signaling (Maillet, Poinsot et al. 2011). Furthermore, *nsp2* mutant show a slower onset of mycorrhizal colonization (Maillet, Poinsot et al. 2011). By contrast, *NSP1* is specifically involved in nod factor signaling and nodulation, with not function during mycorrhiza formation. Unlike legumes and other non-legumes plants, Arabidopsis do not associated with mycorrhizal fungi (Catoira, Galera et al. 2000). Therefore, the lack of *NSP2* function in association with *S. meliloti* could be related with the ability of plants to associate with mycorrhiza fungi. We thought that in the case of plant that does not associates with this types of fungi, the molecular mechanisms in association with rhizobium could be reduce in a more basic elements that can account for a beneficial association with nitrogen fixing bacteria. The differences in the function of NSP2 in the association of legumes and non-legumes with NFB could be the reason of the ability of a plant to form a nodule structure.

In legumes, N is an important regulatory factor that determines the establishment of the association with the nitrogen-fixing partner. In the presence of this macronutrient and the NFB, the plant is unable to develop nodules (Barbulova, Rogato et al. 2007). In our study,

S. meliloti has a regulatory effect in the homologous legume genes only when plants were grown under limiting N conditions and it was abolished in the presence of the nutrient. This suggests that N availability is the main factor regulating plant susceptibility to respond to the bacterium.

Cross-complementation of legume mutants in *nsp1* and *nsp2* genes with rice (non-legume) homologs have proved that rice genes are functional and can reestablish infection thread and nodule organogenesis (Yokota, Soyano et al. 2010). However, there is no evidence of functionality for those genes in rice or others non-legume plants. By functional genomics approaches using insertional mutant plants for NSP1 and selected NLPs genes, we showed that some of these transcription factors are essential for the functional association between *A. thaliana* and *S. meliloti.* Homologous transcription factors are necessary to mediate plant growth promotion induced by the NFB. Specifically, mutation of *NSP1* and *NLP9* genes, that does not affect plant growth in response to N, directly affect the plant growth promotion effect of the bacterium. Unlike the others, *nlp4* plants have differences in growth levels achieved in relation to the wild type plants when grown at different nitrogen availabilities. This suggests, that the lost of the effect of the bacterium in *nlp4* plants could be due to an unspecific phenotype on plant growth in response to N. Our results indicate that the association between *S. meliloti* and the non-legume *A. thaliana*, is highly regulated by the plant. Our findings are consistent with the legume systems in which NSPs and NIN genes are essential for nodule development.

In summary, our study indicates that the NFB *S. meliloti* is able to interact with *A. thaliana* in order to provide the plant with N in N-deficient conditions and therefore can promotes plant growth (Fig. 16). We present the first evidence indicating the existence of

regulatory genes in a non-nodulating plants that mediates the association with a NFB (Fig. 16). We define *A. thaliana* and *S. meliloti* as an excellent model system to further understanding the molecular mechanisms underlying the association between non-legume plants and NFB.

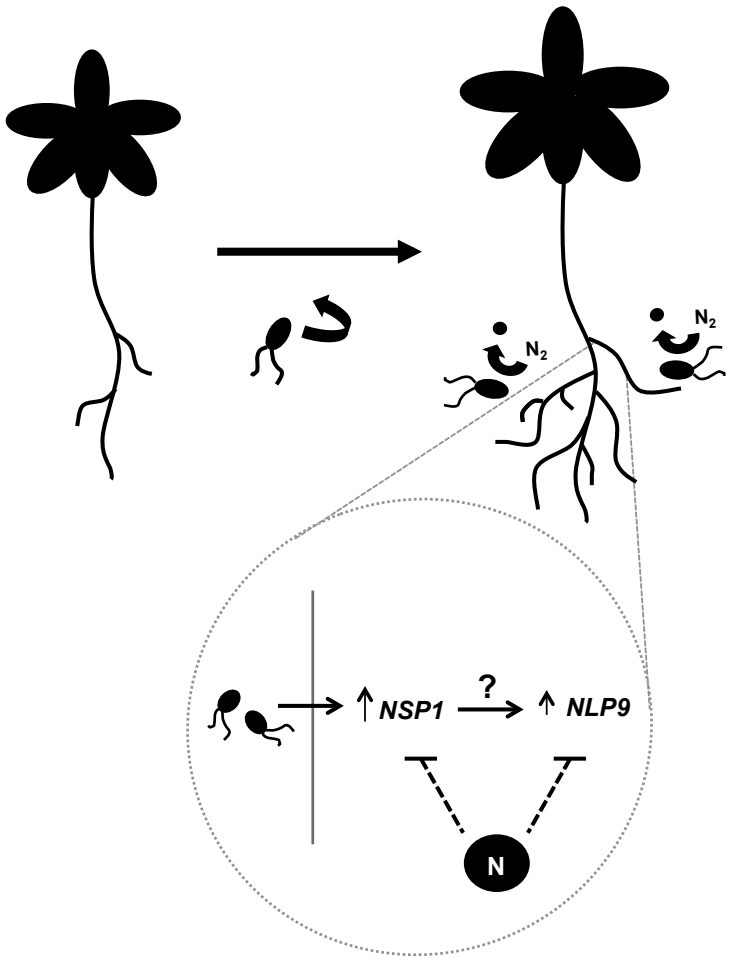

Figure 16: Model for the association between Arabidopsis and *Sinorhizobium meliloti*. Under N-limiting conditions, Arabidopsis can associate with the NFB *S. meliloti*. This bacterium can promote plant growth due to BNF and also by increasing the number of lateral roots. At a molecular level, plant genes (*NSP1* and *NLP9*) mediate the beneficial association with the bacterium. The expression of these essential transcription factors upon bacterial inoculation is mainly regulated by N availability in plant. Whether *NSP1* can directly regulate de expression of *NLP9* remains unknown.

5. CONCLUSIONS

In this thesis we discovered *Arabidopsis thaliana* can beneficially associate with *S. meliloti*. This bacterium can enhance growth of plants under N starvation conditions. Analysis of the association of Arabidopsis seedling with a strain unable to fix N showed that part of the plant growth promotion under N starvation was due to BNF. Our work provides evidence that the non-legume model plant *A. thaliana* can functionally associate with NFB *S. meliloti* to acquire N for plant nutrition. We identified plant regulatory factors involved in the establishment of this association. We showed that conserved regulatory genes exist in non-legumes that are essential for the functional association of plants with NFB. We identified AtNSP1 and AtNLP9 as essential molecular factors for plant growth promoting effect of this bacterium under N-starvation conditions. We postulated Arabidopsis and *S. meliloti* as model organisms to further studied the molecular mechanisms underlying the association between non-nodulating plants and NFB. Our work can have profound implications for developing new biotechnologies to reduce the use of fertilizers and contribute to a more sustainable and environmentally friendly agriculture.

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