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CICLO XXI

INVOLVEMENT OF AUXIN AND LTP PROTEINS IN THE REGULATION OF ROOT NODULE FORMATION IN *Medicago truncatula - Sinorhizobium meliloti* SYMBIOSIS

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RIASSUNTO

Questo progetto di dottorato ha avuto come obiettivi: i) valutazione del ruolo del'auxina di derivazione batterica nella simbiosi rizobio-leguminosa, che dà origine a noduli di tipo indeterminato, ii) lo studio funzionale di MtN5, una proteina di tipo "Pathogenesis Related", che viene indotta precocemente durante la nodulazione e che presenta omologie di sequenza con membri della famiglia delle Lipid Transfer Protein vegetali.

L'auxina (acido indol-3-acetico, IAA) è un ormone vegetale implicato in molti aspetti che riguardano la vita e lo sviluppo delle piante; un suo coinvolgimento nello sviluppo del nodulo radicale era stato ipotizzato già all'inizio del secolo scorso. Studi successivi hanno dimostrato un'inibizione del trasporto acropeto di IAA nella radice a seguito dell'infezione con rizobi, con un conseguente accumulo di fitormone a livello del sito di infezione. E' stato dimostrato che la maggior parte dei batteri della rizosfera che producono effetti di promozione sulla crescita della pianta, rizobi inclusi, possiedono vie biosintetiche per IAA. I rizobi sono in grado di sintetizzare auxina in coltura liquida e, molto probabilmente, mantengono questa capacità anche durante la nodulazione. Ad oggi, però, i dati riguardanti il ruolo dell'auxina batterica nello sviluppo dei noduli sono ancora controversi; sono stati infatti documentati sia effetti stimolatori che inibitori.

Molti degli eventi che stanno alla base dell'associazione simbiotica tra rizobi e leguminose devono ancora essere chiariti. Ad esempio, la natura e la funzione dei segnali ormonali scambiati tra ospite e simbionte non sono ancora stati compresi nel dettaglio, così come le differenze e i parallelismi nella risposta delle leguminose verso il simbionte e verso i patogeni della radice. A tal riguardo, recenti osservazioni hanno dimostrato che la repressione della via di segnalazione intracellulare dell'auxina risulta in una maggiore resistenza innata delle piante verso microrganismi patogeni.

Piante di *Medicago truncatula*, specie modello per le leguminose che producono noduli di tipo indeterminato, e *Medicago sativa* (erba medica), specie correlata di interesse agronomico, sono state nodulate sia con rizobi wild-type e che con rizobi in grado di iper-produrre IAA (*S. meliloti* IAA). I risultati ottenuti hanno dimostrato che piante nodulate con *S. meliloti* IAA producevano un numero maggiore di noduli (aumento del 50% in *M. sativa* e

aumento del 100% in *M. truncatula*) e un apparato radicale più ramificato. Inoltre il contenuto di auxina nei noduli prodotti da rizobi IAA è mediamente 10 volte superiore alla concentrazione dei noduli prodotti da rizobi wild-type. I livelli di espressione dei geni responsabili del trasporto di auxina è stato valutato mediante RT-PCR quantitativa (qRT-PCR) e il carrier di efflusso *MtPIN2* è risultato significativamente più espresso (circa 2 volte) nel tessuto radicale di piante nodulate con rizobi IAA rispetto alle radici infettate con il rizobio di controllo. Questi risultati suggeriscono che l'effetto di promozione osservato sulla nodulazione e sull'accrescimento della radice laterale siano dovuti alla produzione di IAA nel nodulo e ad una sua redistribuzione all'interno dell'apparato radicale.

E' stato ampiamente dimostrato che l'ossido nitrico (NO) agisce come secondo messaggero nell'induzione di radici laterali e avventizie stimolata da auxina. Considerando la comune organogenesi tra radici laterali e avventizie e noduli indeterminati, in questo lavoro abbiamo dimostrato che esiste un collegamento tra auxina e NO nella via di segnalazione che porta all'induzione del nodulo.

Per mezzo di uno screening preliminare, condotto mediante qRT-PCR e volto ad individuare geni differenzialmente espressi in piante nodulate con rizobi IAA e piante nodulate con rizobi wild-type, fu osservato che il gene *MtN5* era più espresso negli apparati radicali di piante infettate con rizobi iperproduttori di auxina. Il prodotto genico di *MtN5* è stato annotato come una Lipid Transfer Protein (LTP) putativa. Le LTP vegetali sono caratterizzate dalla capacità sia di legare lipidi *in vitro* che di inibire la crescita di microrganismi. In questo progetto di tesi è stato dimostrato che MtN5 possiede la capacità di legare lisolipidi e che, come molti altri membri di questa famiglia di proteine, possiede attività antimicrobica *in vitro,* in particolare contro *Fusarium semitectum*, *Xanthomonas campestris* e *S. meliloti*.

Lo studio del profilo di espressione conferma che MtN5 viene precocemente indotta durante la nodulazione e che è specificamente localizzata all'interno del nodulo radicale. Inoltre, l'infezione di piante con *F. semitectum* provoca l'accumulo di MtN5 nel tessuto radicale.

La funzione di *MtN5* nella nodulazione è stata studiata mediante la generazione di radici avventizie transgeniche, sia overesprimenti che silenziante per il gene di interesse. Le radici

silenziate per *MtN5* sviluppano circa la metà dei noduli rispetto a radici di controllo, mentre in radici transgeniche over-esprimenti *MtN5* il numero di noduli è risultato incrrementato del 300% rispetto al controllo. I risultati ottenuti dimostrano che *MtN5* facilita l'interazione simbiotica tra *M. truncatula* e *S. meliloti*, agendo probabilmente negli stadi precoci dell'infezione, e suggeriscono che MtN5 potrebbe avere un ruolo nella protezione dei noduli verso patogeni della radice. Ulteriori studi sono comunque necessari per ottenere una immagine più chiara del ruolo di *MtN5* sia nella simbiosi che nella risposta verso i patogeni.

ABSTRACT

The present thesis has had two main focuses: i) the evaluation of the role of bacteria-derived auxin in the symbiosis between rhizobia and legumes that bear indeterminate nodules, ii) the functional study of MtN5, a pathogenesis related protein which presents sequence homology with the members of the plant Lipid Transfer Proteins (LTP) family and is precociously induced during nodulation.

Auxin (indol-3-acetic acid, IAA) is a phytohormone involved in many aspects of plants growth and development; The role of auxin in the development of the rhizobia-legumes symbiosis was first hypothesised at the beginning of the twentieth century. More recent studies have demonstrated that auxin is accumulated at the site of infection as a consequence of the inhibition of the acropetal auxin transport in roots upon rhizobia inoculation. The production of IAA has also been documented in plant-associated rhizobacteria, including rhizobia, that have promoting effects on plants growth. When grown in liquid media, rhizobia can synthesize auxin and most likely they retain the same capability also during the nodule development. However, up to date, the data concerning the role of bacteria-derived auxin in the establishment of the symbiotic association are still contradictory, since both stimulatory and inhibitory effects have been documented.

Thus, there are still open questions in the understanding of the events that result in the establishment of the symbiosis. First of all the nature and the function of the hormonal signal(s) exchanged between the host and the symbiont are not thoroughly unfolded, as well as the parallelisms and the differences in the responses of legumes against root pathogens and root symbiont. In these regards, recent findings have pointed out that plants innate immunity results, at least in part, from the down-regulation of the auxin signalling pathway.

Medicago truncatula and *Medicago sativa* plants were nodulated with both wild-type and auxin hyper-synthesising rhizobia (*Sinorhizobium meliloti* IAA). The results obtained showed that plants nodulated with *S. meliloti* IAA produced a higher number of root nodules (50% more nodules in *M. sativa* and 100% more nodules in *M. truncatula*) and a more branched root apparatus. The root nodules elicited by *S. meliloti* IAA had a higher IAA content (at least 10-fold) when compared to control nodules. The expression levels of the auxin carriers were

evaluated and the efflux facilitator *MtPIN2* resulted more abundant (about 2-fold) in the root tissue of IAA plants when compared to wild-type plants These data suggested that such promoting effects on nodulation and lateral root growth might be due to the increased auxin content detected in IAA nodule produced by auxin hyper-synthesising rhizobia, as well as to a redistribution of the phytohormone in the root tissue.

It has been largely demonstrated that nitric oxide (NO) acts as second messenger in the auxin-induced pathway that leads to formation of lateral and adventitious roots. Since root nodules have the same organogenetic origin of lateral and adventitious roots, the possible connection between NO and root nodule induction was investigated and we demonstrated that NO participate in the signalling pathway for root nodule induction.

During a preliminary screening carried out by means of qRT-PCR, it has been found that *N5* gene of *M. truncatula* was more abundantly expressed in roots nodulated with *S. meliloti* IAA with respect to roots infected by wild-type rhizobia. The gene product of *MtN5* was annotated as putative Lipid Transfer Protein (LTP). LTPs are characterized by their ability to bind lipids *in vitro* and the majority of them exhibits antimicrobial activity. In this thesis, it has been demonstrated that the recombinant MtN5 protein is able to bind lysolipids and possesses inhibitory activity against *Fusarium semitectum*, *Xanthomonas campestris* and *S. meliloti*.

The studies of the expression pattern of both *MtN5* transcript and MtN5 protein confirmed that it is precociously induced during nodulation and revealed that it is specifically localized in the root nodule. In addition, when *M. truncatula* plants are infected with the root pathogenic fungus *F. semitectum,* MtN5 protein is accumulated in the root apparatus.

The function of *MtN5* in nodulation has been studied through the generation of transgenic adventitious roots, both over-expressed and silenced for the gene of interest. *MtN5*-silenced roots developed approximately 50% fewer nodules as compared to control roots, whereas in hairy roots over-expressing *MtN5* the nodule number was increased by about 300% with respect to control roots. Collectively the data indicate that *MtN5* facilitates the symbiotic interaction between *M. truncatula* and *S. meliloti*, probably acting in the early stages of rhizobia infection, and suggest that it might have a role in the protection of nodules against

root pathogen. However, further studies are needed to have a clear picture of the role played by *MtN5* in both symbiosis and defence response against pathogens.

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Introduction

1. Symbiosis

The acquisition of mineral nutrients is one of the major challenges for plant survival. In particular, the availability of the macronutrients nitrogen and phosphorus is of paramount importance to plants, since the lack of them can often limit plant growth. Several plant genera have overcome these nutrient limitations via symbiotic interaction with microorganisms. The most widespread symbiosis occurs between plants and mycorrhizal fungi, which help the plant in the uptake of nutrients and is particularly important for phosphorus capture. A more specialized symbiosis is established between certain genera of plants and nitrogen-fixing bacteria (e.g. legumes/rhizobia symbiosis). In this particular symbiotic relationship, the bacteria invade the plant root tissue and are harbored within a specialized organ, the root nodule, where atmospheric nitrogen is converted to ammonium, one of the bioavailable forms of nitrogen for plants. The nitrogen fixation is restricted to prokaryotes that possess the enzyme nitrogenase, which is responsible for nitrogen reduction. The nitrogenase is irreversibly inactivated by oxygen, therefore it needs an anoxic or microaerophilic environment to work properly. This is the main reason why nitrogen-fixing bacteria are housed in root nodules.

Nodule formation is a multistage process, involving rhizobia multiplication in the rhizosphere, recognition of the host plant, infection and penetration in the root tissues through tubule-like structures, called infection threads (IT). When the infection threads reach the nodule primordium, bacteria are released into plant cells through an endocytotic process; at this stage bacteria are called bacteroids. Bacteroids are enclosed in a plant-derived membrane

and the entire structure is termed symbiosome, which represents the functional unit for nitrogen fixation.

1.1 Invasion of plants roots

1.2.I Signals exchange

The almost completed genome of *Medicago truncatula*, the model plants for indeterminate legumes, and the completed genome of *Sinorhizobium meliloti*, its natural symbiont, have led to an in-depth dissection of the molecular mechanisms, underlying the establishment of the symbiosis.

Before the actual physical contact, plants and their hosts communicate through an exchange of signal molecules. The flavonoids (2-phenyl-1,4-benzopyrone derivatives) secreted by leguminous plants are the first signals to be exchanged by host and symbiont (Perret et al., 2000). Flavonoids perceived by rhizobia activate the transcription of genes deputated to the synthesis of nodulation factor (Nod factor). In particular, flavonoids bind the bacterial NodD proteins, which are transcriptional regulators of *nod* genes (Perret et al., 2000; Barnett and Fisher, 2006). Among NodD targets there are several *nod* genes encoding enzymes which are required for the biosynthesis of the Nod factors (Oldroyd and Downie, 2004). Nod factors are lipochitooligosaccharides constituted by a backbone of β-1,4-linked N-acetyl-D-glucosammine residues, which can vary in number between both bacterial species and also within the repertoire of a single species. The genes of the *nodABC* operon encode for proteins that are responsible for the synthesis of the Nod factor core structure (Perret et al., 2000). At the non-reducing terminal, Nod factor are N-acylated with the acyl chain of a fatty acid. The interaction with the host plant also induce the expression of *noe* and *nol* genes, which encode for enzymes responsible for further Nod factors modifications, including the addition of fucosyl, sulphuryl, acetyl, methyl, carboamyl and arabinosyl residues (Perret et al., 2000; Cook, 2004; Geurts et al., 2005; Oldroyd and Downie, 2004; Oldroyd and Downie, 2006). Such chemical modifications to this basic structure, the difference in the fatty acid chain length as well as in its degree of saturation produce specie-specific Nod factors, which

have an important role in defining the specificity of the interaction between rhizobia and their host plants (Dénarié et al., 1996; D'Haeze and Holsters, 2004).

Nod factors have a hormonal-like activity in the plant, since the perception of them by the host roots triggers the start of a developmental program, inducing multiple modifications that are essential for bacterial invasion. The mode of colonization of the root tissue is specific for the pair plant/prokaryotic partner. Two invasion strategies have been described so far (Sprent, 1989). In some legumes the root epidermal barrier is overcome by rhizobia at points of epidermal damage, where lateral roots emerge. In this case bacteria gain access to cortical cells through these infected cracks. However, in most legume/rhizobia interactions, the root hair cells play a pivotal role in allowing and facilitating the rhizobia penetration (Oldroyd and Downie, 2008).

Nod factor perception elicits a variety of biochemical responses in the root hair including changes in ion fluxes (rapid uptake of calcium ion, efflux of chloride and potassium ions), membrane depolarization, the oscillation of cytosolic calcium (spiking) and modifications in the cytoskeleton (Timmers et al., 1999; Sieberer et al., 2005; Cardenas et al., 2003; de Ruijter et al., 1999). Some specific genes associated with the early stages of nodulation (early nodulin genes, *ENOD*) are induced in these cells (e.g. *ENOD12* in *M. truncatula*) (Journet et al., 1994). Afterwards, the root hair curls, trapping rhizobia within a loop structure, termed colonized curled root hair (CCRH) (Esseling et al., 2003; Gage, 2004). Simultaneously, the root cortex cells are stimulated to re-enter the cell cycle and to divide, giving rise to the nodule primordia, which will be invaded by the symbionts (Foucher and Kondorosi, 2000). The cell division is also associated with the expression of *ENOD40* and *ENOD20* (Fang and Hirsch, 1998; Vernoud et al., 1999).

It is believed that Nod Factors are perceived by transmembrane LysM-type Ser/Thr receptor kinases (LYKs), which may act as heterodimeric Nod factor-binding complex and trigger the plant early responses to rhizobium (Fig. 1) (Radutoiu et al., 2003; Arrighi et al., 2006).

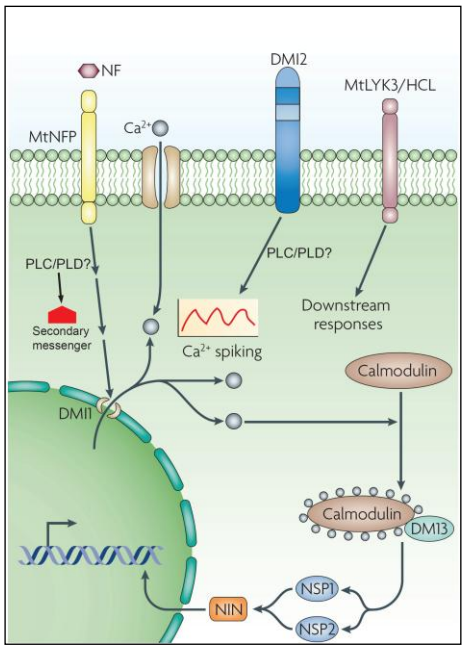

Figure 1. Downstream components of the Nod factor signal transduction system. A complete response of *Medicago truncatula* to Nod factor (NF) from *Sinorhizobium meliloti* requires multiple extracellular domain-containing cell-surface receptors, including the LysM family receptors MtNFP and MtLYK3/HCL. *DMI2* encodes a leucine-rich-repeat receptor kinase that is localized to the membrane and is required for tight root hair curling around the bacteria (Picture modified from Jones et al., 2007).

Many downstream components of the Nod factor-induced transduction pathway have been recently identified. The *MtDMI1* and *MtDMI2* genes (named after *M. truncatula Does Not Make Infection 1* and *2* mutants), and their orthologous genes in *Lotus japonicus CASTOR/POLLUX* and *SYMRK*, encode for a putative cation channel and a Leu-rich-repeats receptor-like kinase respectively and they are required for calcium spiking and nodulation (Endre et al., 2002; Stracke et al., 2002; Ané et al., 2004; Imaizumi-Anraku et al., 2005). The elimination of DMI1 causes the loss of calcium spiking after Nod factor perception, whereas DMI2 is required for the tight root hair curling around the bacteria (Ané et al., 2004; Riely et al., 2007). The *DMI3* gene encodes a Ca^{2+}-calmodulin dependent kinase (CCaMK) which is necessary for the induction of cell division in the root cortex (Mitra et al., 2004; Levy et al., 2004). The activation of DMI3 by both calcium and calmodulin suggests that it might integrate the calcium spiking into the Nod factor signaling pathway by decoding spiking frequency and amplitude. The role of *DMI* genes of *M. truncatula* is not restricted to nodulation, since mutants in these genes are also defective in the mycorrhizal association (Levy et al., 2004; Olàh et al., 2005), highlighting at least partial overlapping between the induction pathway of the two symbiosis.

Pharmacological and biochemical approaches have identified the importance of phospholipase C and phospholipase D in Nod factor signaling and calcium spiking (Charron et al., 2004), suggesting a probable involvement of a secondary messenger (e.g. phospholipids) which might act as link between the ligand perception at the plasma membrane and the induction of calcium spiking.

The perception of the calcium spiking by DMI3 is transduced to two transcriptional regulators belonging to the GRAS family, nodulation signaling pathway 1 and 2 (NSP1 and NSP2) (Kalo et al., 2005; Smit et al., 2005). There is emerging evidence that DMI3 and GRAS proteins form a complex, which can bind the DNA (Oldroyd and Downie, 2008).

1.2.II Infection threads development

In order to develop a functional symbiosis, bacteria must be internalized by plants in the root cortex cells, where they can start the nitrogen fixation. Rhizobia penetrate plant tissues by producing infection threads, which are tubule-like structures directed from the root hair towards the nodule primordium. Before invading plant cells, rhizobia adhere to the root hair epidermis; this process guarantees the enhancement of bacterial number in the rhizosphere and contributes to the selection of the most infective strain, since the infection is a clonal event that leads to a high number of symbionts within the nodule (Fujishige et al., 2006; Sanchez-Contreras et al., 2007). Plant lectins enhance the attachment of rhizobia to the root epidermis by binding surface polysaccharides (Smit et al., 1992). Rhizobia mutants that are unable to produce cyclic β-glucans cannot effectively colonize the root hair surface and result defective in nodulation (Yao et al., 2004; Zhang and Cheng, 2006).

Bacteria that are trapped within the CCRH at the root hair tip, and that can produce specific Nod factors and symbiotically effective polysaccharides, are able to induce the ingrowth of the root hair cell membrane resulting in the formation of a transcellular infection thread. The tip of the developing infection thread is the site of new membrane synthesis and it is proposed to involve the inversion of the growth direction normally showed by root hairs and most likely a reorganization of the cell polarity (Gage, 2004), which is coupled with the modification in the cell cytoskeleton (Timmers, 2008). Rhizobia divide at the growing tip of the infection thread, forming a column of bacteria. If more than one bacterial strain is entrapped in the same infection thread, then one single strain is selected out of the pool, thus resulting in a clonal invasion (Gage, 2002; 2004).

Rhizobia surface polysaccharides play a critical role during the infection by facilitating the infection thread formation (Pellock et al., 2000). The best understood interaction is that

between *M. truncatula* and *S. meliloti*, which produces two structurally distinct exopolysaccharides, the succynoglican (also termed exoploysaccharide I, EPS I) and the galactoglucan (ESP II). *S. meliloti* mutants unable to produce both EPS I and EPS II result unable to infect *M. truncatula*, whilst mutants lacking only the EPS II, despite being capable of initiating the infection, produce aberrant infection threads which do not result in any functional nodules (Jones et al., 2007). The active components of such polysaccharides are low molecular weight forms, suggesting that these fragments might act as signals most likely by affecting the plant cell defense response, although a positive signaling role is also possible (Gonzàlez et al., 1996; Urzainqui et al., 1992; D'Haeze and Holsters, 2004).

Once the infection threads have penetrated the root hair cell, the bacteria must induce new infection thread formation through the successive cell layers. The inward growth of infection threads is directionally influenced by the plant hormone cythokinin and by Nod factor-induced cell cycle reinitiation.

In developing indeterminate nodules, infection threads grow towards the central part of the nodule primordium (Foucher and Kondorosi, 2000). At this site, there are invasion competent cells that have just exited the mitotic phase (Mergaert et al., 2006) and have become polyploidy, due to the endoreduplication of their genome (Foucher and Kondorosi, 2000; Cebolla et al., 1999). Plant cell endoreduplication is required for the formation of functional nodules and is achieved by proteasome-dependent degradation of mitotic cyclins (Cebolla et al., 1999; Vinardell et al., 2003). The major advantage of polyploidy is both a higher transcription rate and a higher metabolic rate, with respect to cells with a 2n DNA content (Galitski et al., 1999).

1.2.III Endocytosis of rhizobia

Once infection threads, along with bacteria, have reached the target tissue, rhizobia must be internalized by plant cells in order to be accommodated into a suitable niche for nitrogen fixation. Every single plant cell endocytoses a single bacteria, which is then surrounded by an unwalled membrane compartment (named peribacteroid membrane) that originates from

the infection thread (Brewin, 2004). The entire unit, the single bacterium and the surrounding endocytic membrane, is known as symbiosome (Brewin, 2004). In indeterminate-type nodules, the bacterial cell and its surrounding membrane divide synchronously before bacteria differentiate into nitrogen-fixing bacteroids (Robertson and Lyttleton, 1984). This intense replication activity implicates that new-formed compartments are targeted with new lipidic and proteic materials, (e.g. ENOD8, ENOD16 and proteins involved in folding, transport and metabolism), that provide symbiosomes with a biochemical identity (Robertson and Lyttleton, 1984).

1.2.IV Bacteroids differentiation

The host plant exerts control over the bacteria within the symbiosome and, besides providing nutritional support and a suitable microenvironment for nitrogen fixation, it also provides specification for the differentiation program. Bacteroids that have been recently released in the host cells have a rod-shape, whereas during the differentiation their size increase about 30 times and they assume a more elongated or a Y shape (Patriarca et al., 2004). Furthermore, in legumes that produce indeterminate nodules, the host plant imposes the endoreduplication of the genome on invading bacterial cells, which might allow the achievement of higher metabolic rates to support nitrogen fixation (Mergaert et al., 2006; Galitski et al., 1999).

The differentiated bacteroids have been provided with a low oxygen microenvironment, thus they can express the enzymes of the nitrogenase complex and begin nitrogen fixation (Fischer, 1994). Along with bacteroids differentiation, transcriptional changes take place shifting the metabolism of bacteroids towards respiration and nitrogen fixation (Barnett and Fisher, 2006). The respiration is a fundamental aspect in the metabolism of bacteroids, since it is functional in the production of electrons and ATP, necessary for the reduction of nitrogen to ammonium (NH_4^+) (Poole and Allaway, 2000). The NH_4^+ is thought to be secreted and taken up by the host through ammonia transporters localized on the peribacteroid membrane (Prell and Poole, 2006; Day et al., 2001). There are indications that the assimilation of NH_4^+

by the plant occurs mainly through glutamine and asparagine synthetases (Prell and Poole, 2006; Poole and Allaway, 2000; Lodwig et al., 2003; de Bruijn et al., 1989).

A constant carbon supply, aiming to produce metabolites and energy, is required by bacteroids during differentiation and nitrogen fixation. In general, plants provide rhizobia with fixed carbon in the form of the dicarboxylic acids (e.g. malate), which are funneled to the tricarboxylic acid cycle in bacteroids (Poole and Allaway, 2000; Willis and Walker, 1998, Aneja et al., 2005).

Beside metabolites, also some plant-derived proteins are required to support nitrogen fixation. Leghaemoglobin is a plant protein which exhibits oxygen-binding properties and is responsible for the red colour of mature, nitrogen-fixing nodules (Ott et al., 2005). It has been proposed that leghaemoglobin is required for a functional fixation activity since it might grant the microaerophilic environment needed by nitrogenase to work properly. This hypothesis has been recently confirmed through RNAi experiments in *L. japonicus* (Ott et al., 2005).

1.2 Diversity in nodule structure

Two different nodule types characterized by a different structure and different position and activity of the nodular meristems have been studied in detail (Fig. 2). The indeterminate nodules are formed on most temperate legumes, e.g. pea (*Pisum sativum*), clover (*Trifolium* sp.), alfalfae (*Medicago sativa*) and barrel medic (*M. truncatula*). They are characterized by the organ initiation in both the inner cortex and the pericycle (Timmers et al., 1999). Indeterminate nodules are elongated (sometimes bilobated) because of the presence of a persistent meristems located at the apical part of the organ (Fig. 2A) (Hirsh, 1992). In the longitudinal section, indeterminate nodules display infected cells at different stages of development. The use of different cytological and molecular markers allows the definition of at least four distinct zones within the indeterminate nodule. From the distal region, the first zone to be found is the meristematic one, also called Zone I. The nodular meristem is a secondary meristem, which originates from outer cortex and pericycle cells. It is composed of undifferentiated, very small, cytoplasm rich cells surrounded by highly vacuolated cells (differentiated). The next region is the Zone II, also termed invasion or sub-meristematic

zone, and it is where the intracellular bacteria colonization takes place. The adjacent Zone II-III is termed interzone and is rich in amyloplast. Zone III mostly contains infected cells containing N-fixing symbiosomes, although some uninfected cells might occur. Zone IV, which is located in the most proximal region, is composed of senescent infected cells. The senescence is probably related to a reduction in the symbiosomes N-fixing activity.

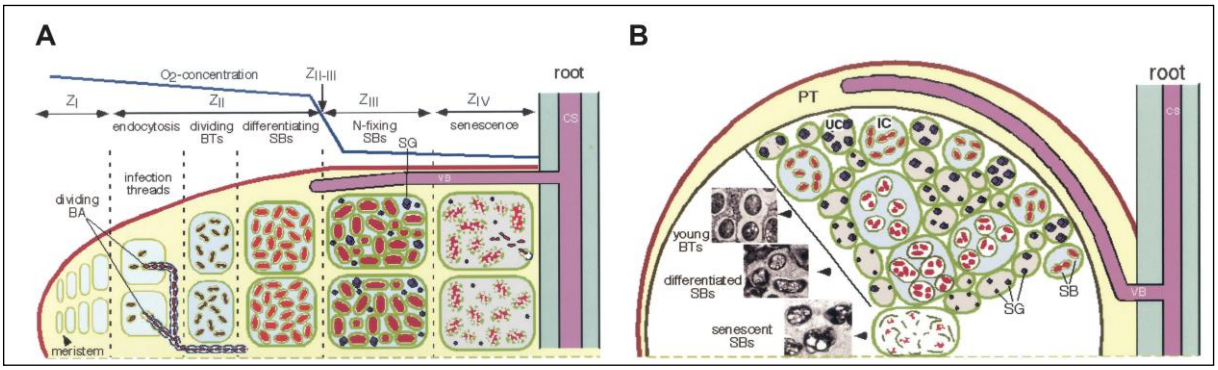

Figure 2. Nodules structure. A. Scheme of the indeterminate elongated nodule. The nodule zones (Z) are indicated. **B. Scheme of the determinate globose nodule.** BA, bacteria ; CS, central stele; VB, vascular bundle; PT, peripheral tissue; BTs, bacteroids; SBs, symbiosomes; SG, starch grain; IC, invaded cell; UC, uninvaded cell. (Picture modified from Patriarca et al., 2002).

The determinate nodules are formed on most (sub)tropical legume plants, including soybean (*Glycine max*), bean (*Phaseolus* sp.), and Japanese trefoil (*L. japonicus*) (Fig. 2B). They are initiated in the outer cortex by cell division and enlargement. Determinate nodules are also called globose nodules, since when they are fully developed, they have a spherical shape. Peripheral and cortical tissues are clearly distinguishable, but the internal zones, containing cells at different stages of development, are not as clearly recognizable as in indeterminate nodules (Patriarca et al., 2004). The central tissue of determinate nodules mostly consists of two different cell types, the infected cells and the uninfected ones. The infected cells display a central nucleus and their cytoplasm is not vacuolated but packed with symbiosomes. The uninfected cells are highly vacuolated and contain amyloplast, large peroxisomes and a very developed smooth endoplasmic reticulum (Patriarca et al., 2004). The activity of the meristems is temporally limited, therefore the cells of the central tissue are believed to differentiate at the same time and consequently the growth of the nodules is most likely to be due to cell expansion rather than to cell division (Patriarca et al., 2004).

2. Regulation of root nodule development

The present thesis is focused on the molecular mechanisms that control nodulation development. In particular, the role of the hormone auxin and some aspects of the defense responses elicited by rhizobia will be described in the following sections.

2.1 Phytohormones as regulators of nodulation

Molecular genetics has recently given the symbiosis field of research a huge amount of new information concerning the Nod factor transduction pathway. On the other hand, there is an abundance of evidence suggesting that infection and nodule organogenesis require the integration of the Nod factor signaling with the programs controlled by plant phytohormones.

The plant hormones abscisic acid (ABA), jasmonic acid (JA), ethylene and salicylic acid all act as negative regulators in the nodulation (Oldroyd et al., 2001; Penmetsa and Cook, 1997; Stacey et al., 2006; Sun et al., 2006; Suzuki et al., 2004), whereas auxin, cytokinin and gibberellic acid (GA) have positive effects on nodule development (Gonzalez-Rizzo et al., 2006; Murray et al., 2007; Ferguson et al., 2005).

Among those hormones that have inhibitory effects on nodule development, the best studied has been ethylene. Early investigations on pea highlighted the correlation between the levels of ethylene and the degree of nodulation, but the discovery of the *M. truncatula sickle* (*skl*) mutant revealed the full role of ethylene in nodulation (Penmetsa and Cook, 1997). The *skl* was initially identified as a supernodulating mutant which was able to produce approximately 10-fold more nodules than wild-type plants. The *skl* mutant is defective in the perception of ethylene and it was shown that *SKL* encodes for the *M. truncatula* homolog of *ETHYLENE INSESITIVE 2* (*EIN2*) that plays a key role in the ethylene transduction pathway of *Arabidopsis thaliana* (Oldroyd and Downie, 2008).

The ethylene precursor 1-amino-cyclopropane-1-carboxylic acid (ACC) reduces the number of cells that respond to Nod factor with calcium spiking (Oldroyd et al., 2001) suggesting that ethylene action could regulate early stages of the signaling pathway, determining the sensitivity of epidermal cells to Nod factors (Oldroyd et al., 2001; Penmetsa et al., 2003).

Furthermore, it was shown that ethylene affects the infection threads growth, since in the *skl* mutant the infection threads display an uncontrolled development throughout the cortex (Penmetsa and Cook, 1997), and is involved in defining the site of nodule initiation, opposite to the protoxilem pole (Heidstra et al., 1997).

JA negatively influences the Nod factor-induced calcium spiking and also the formation of the nodule. Like ethylene, JA seems to affect the number of epidermal cells able to induce the calcium spiking (Nakagawa et al., 2006; Sun et al., 2006). However, at low concentrations, JA can modulate the frequency of the calcium spiking (Sun et al., 2006), whilst Nod factor concentration does not have such effects (Ehrhardt et al., 1996).

Ethylene, JA, salicylic acid and ABA are all induced in response to environmental stresses, such as pathogen attack and drought. It was proposed that the regulation of nodulation by these hormones most likely allows the plants to control the extent of nodulation in order to preserve their health. The symbiosis represents a significant loss of fixed carbon for plants, therefore it is established only when the nitrogen concentration in the soil is limiting for the growth.

2.2 Auxin

The likely involvement of auxin in nodulation was first suggested after the discovery that nodulated roots contained a higher amount of auxin with respect to non-infected roots (Thimann, 1936). Afterwards, the spatial and temporal distribution of auxin was studied in legumes bearing both determinate and indeterminate nodules by means of auxin responsive promoters (e.g. *GH3* and *DR5*) fused to reporter genes (e.g. *GUS*). In plants that bear indeterminate nodules, the inoculation with rhizobia causes a decrease in the promoter response at the site of infection and below in the first 10 hours, whereas the promoter shows an increased activity at the site of nodule initiation after 24 h from inoculation, and the expression is further maintained in the pericycle and cortical dividing cells (Mathesius et al., 1998a; 1998b; Huo et al., 2006; van Noorden et al., 2006). The expression of the reporter gene disappears from the central tissue during the nodule differentiation, whereas it remains associated with both the meristematic tissue and the vasculature (Mathesius et al., 1998a;

1998b). Similarly, in determinate nodules the reporter expression is detected in the outer cortex dividing cells, whereas during nodule differentiation it is observed in the peripheral tissues and in the vasculature (Pacios-Bras et al., 2003).

2.2.I Auxin Biosynthesis

A. thaliana seedlings can synthesise indol-3-acetica acid (IAA) in leaves, cotyledons and roots, but the highest production capacity belongs to young leaves (Ljung et al., 2001).

The detailed elucidation of the molecular mechanisms of auxin synthesis in plants has been hindered by the complexity and apparent redundancy of the biosynthetic pathways *in vivo*. At least two kinds of auxin biosynthetic pathways are hypothesised, one is dependent on tryptophan (Trp), which is used as precursor, and the other is Trp-independent. Several Trp-dependent pathways, which are named after the intermediates, have been proposed: the indol-3-pyruvic acid (IPA) pathway, the tryptamine (TAM) pathway and the indol-3-acetaldoxime (IAOx) pathway (Woodward and Bartel, 2005). This is, however, an artificial classification, since (1) intermediates such as IAOx and indole-3-acetaldehyde occur in different pathways, which suggests putative interaction of these routes, and (2) several of the involved enzymes have wide substrate specificities (Kriechbaumer et al. 2007). Auxin biosybnthetic pathway intermediates such as IPA (Tam and Normanly 1998) and IAM (Pollmann et al. 2002), have been isolated from plants, but not all enzymes involved in their production have been identified yet. As such, questions about the extent to which these pathways contribute to IAA biosynthesis *in planta* require further investigation. In general, TRP-dependent IAA biosynthesis is thought to occur via deamination and decarboxylation steps (Glawischnig et al. 2000). In addition, although intermediate steps are still unknown, compelling evidence for a TRP-independent pathway was provided by TRP-auxotroph mutants in maize (Wright et al. 1991) and *A. thaliana* (Normanly et al. 1993) as well as numerous feeding studies with stable-isotope labeled TRP and TRP precursors (Östin et al. 1999; Sitbon et al. 2000). TRP-independent biosynthesis most likely use indole directly to synthesize IAA. Taken together, these lines of evidence indicate that multiple distinct pathways contribute to IAA formation in plant.

2.2.II Auxin transport

Auxin is synthesized locally, mainly in the shoots, and it is delivered throughout the whole plant in a directional, cell-to-cell, fashion. This movement is called polar auxin transport (PAT) (Friml and Palme, 2002) and is explained at molecular level by the chemiosmotic model (Raven, 1975; Rubery and Sheldrake, 1974), which proposes the involvement of auxin influx and efflux carriers. IAA present in protonated form in the apoplastic space can both freely enter the cell and be actively transported by means of influx carriers, but it is subsequently trapped within the cytoplasm because of its higher pH compared with that of apoplast (Fig. 3). Therefore the de-protonated IAA needs active efflux carrier(s) to exit the cell. One of the most important features of this hypothesis is that the efflux carrier(s) are positioned asymmetrically on the plasma membrane (PM), allowing the directionality of auxin transport. Several molecular components that could take part in this process have been identified.

The main contribution to the understanding of PAT comes from the molecular genetic approach on *A. thaliana*, which led to the identification of candidate genes encoding influx and efflux carriers.

AUXIN RESISTANT 1 (AUX1) is the only well described auxin import carrier (Bennett et al. 1996; 1998; Swarup et al. 2000). The *AUX1* gene displays similarities with plant amino acid permeases, consistent with a role for AUX1 in the cellular uptake of auxin (Fig. 3) (Marchant et al., 1999; Swarup et al. 2001). Carrier-mediated auxin transport was proposed for the movement of IAA against concentration gradient and/or to prevent the IAA diffusion in the neighboring cells, in order to create, maintain and control auxin gradients within cells and tissues (Hellmann et al., 2003; Reinhardt et al., 2003; Swarup et al., 2001).

The PIN FORMED (PIN) protein family consists of eight members, which are involved in the mediation of auxin efflux (Friml and Palme, 2002; Paponov et al., 2005; Teale et al., 2006). PIN proteins possess two important characteristics that suggested their involvement in PAT. Firstly, they are mainly expressed in auxin-transporting cells and tissues and, secondly, they

are asymmetrically positioned on the PM, consistent with IAA flux direction (Friml et al., 2002a, 2002b, 2003; Galweiler et al., 1998; Müller et al., 1998).

In plants, PIN proteins show a constitutive recycling between PM and endosomes (Geldner et al., 2001) and they have different subcellular localization, including apolar, basal and apical, depending on the PIN protein and the cell type (Fig. 3) (Wiśniewska et al., 2006). PIN1 typically displays a basal (root-tip facing) position in the inner cells of both shoots and roots, PIN2 has a apical (shoot facing) localization in root epidermal and lateral root cap cells, and PIN3 is usually laterally localized at the inner side of shoot endodermis cells (Galweiler et al., 1998; Müller et al., 1998; Friml et al., 2002b). The different localization of distinct polar transporters within the same cells suggested the presence of a polarity determinant in the protein sequence (Wiśniewska et al., 2006). The polarity signal most likely directs PINs to the distinct apical or basal targeting machineries, depending on the phosphorylation status. It was demonstrated that a Ser/Thr protein kinase PINOID (PID) (Friml et al., 2004) as well as a protein phosphatase 2A (PP2A) (Michniewicz et al., 2007) act on PINs phosphorylation playing a pivotal role in apical-versus-basal targeting of the efflux carriers.

The PIN proteins are not the only components able to mediate auxin efflux. Another group of putative auxin transport facilitators is represented by the MULTIDRUG RESISTANCE (MDR)/P-glycoprotein (PGP) protein family, which includes MDR1, PGP1, PGP2, PGP4 and PGP19 (Fig. 3) (Brown et al., 2001; Gil et al., 2001; Murphy et al., 2000; 2002; Noh et al., 2001; 2003). The *A. thaliana mdr1* mutant showed a reduction in the PAT which is more severe in the *mdr1pgp1* double mutant, suggesting a role for these MDR proteins in auxin distribution (Noh et al., 2003). Recently, it was demonstrated that PIN1 and PGP1 proteins co-localize and interact with each other (Blakeslee et al., 2007), but the functional relationship between them still remains unclear.

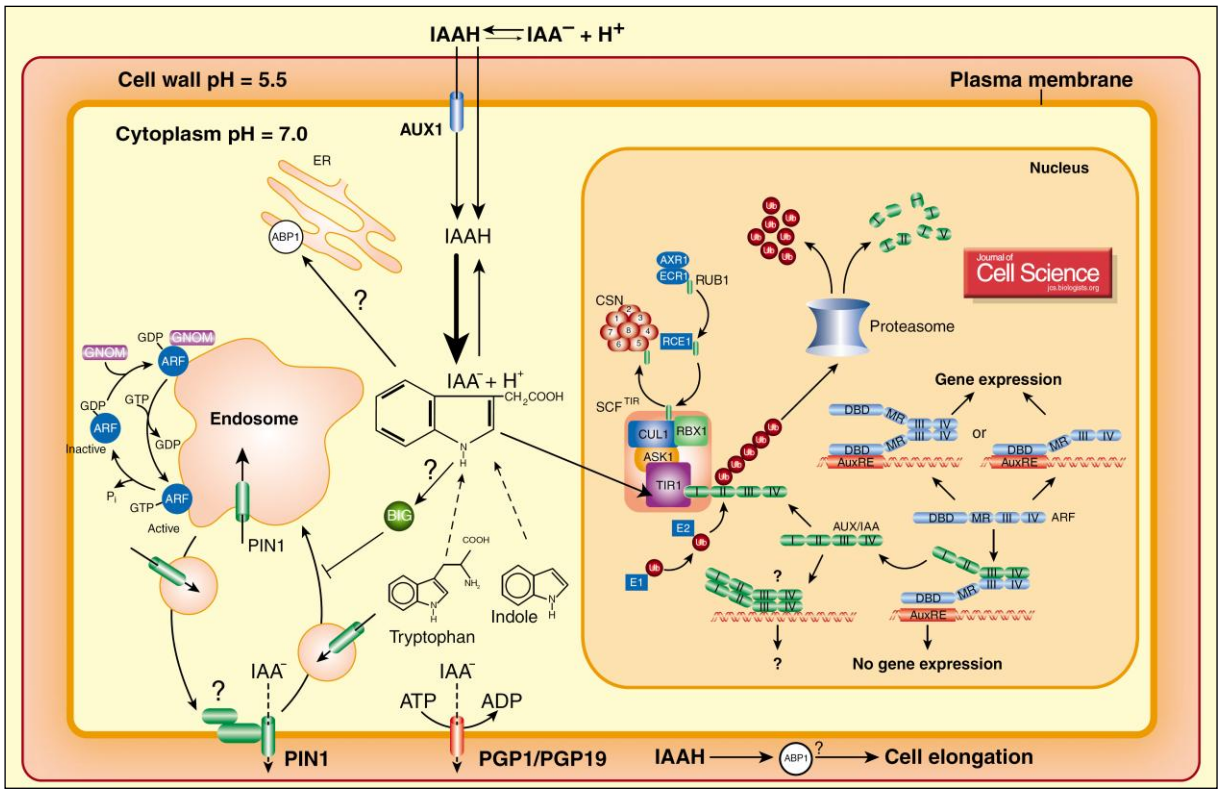

Figure 3. Schematic representation of auxin transport and signaling at cellular level (Picture modified from Paciorek and Friml, 2006).

2.2.III Auxin signaling

Auxin biosynthesis, metabolism (i.e. conjugation and degradation) and transport together ensure that appropriate IAA levels are accumulated in specific cells and tissues to drive plant development. Auxin acts on cells by altering genes expression and causing the accumulation of at least three families of transcripts, *i.e.* the *AUXIN/INDOLE-3-ACETIC ACID* (Aux/IAA) family, *SMALL AUXIN-UP RNAs* (SAURs) family and *GRETCHENHAGEN*-3 (GH3) family (Abel and Theologis, 1996; McClure and Guilfoyle, 1987; Hagen and Guilfoyle, 1985). The promoter regions of several auxin responsive genes contain one or more auxin responsive elements (*AuxRE*), (Ulmasov et al., 1995). The isolation of the *AuxRE* led to the identification of the AUXIN RESPONE FACTOR 1 (ARF1) in *Arabidopsis* (Ulmasov et al., 1997); subsequently, it was discovered that *A. thaliana* genome encodes for 23 *ARF* genes (Remington et al., 2004; Okushima et al., 2005). ARFs display a conserved N-terminal domain that mediates the interaction with the *AuxRE* and they also contain a C-terminal conserved dimerization element (Domain III and IV). Between the conserved C-terminal and

N-terminal ends, there is a variable domain which can be either glutamine (Q)-rich or serine (S)-rich. This difference is thought to confer the characteristic of either transcriptional activator (Q-rich) or transcriptional repressor (S-rich) (Tiwari et al., 2003).

The hormonal regulation of ARFs is achieved through the interaction with the Aux/IAA proteins. In *A. thaliana*, the *Aux/IAA* gene family encodes for 29 auxin-induced proteins showing a nuclear localization, most of which have a common four-domain structure (Remington et al., 2004; Overvoorde et al., 2005). Aux/IAA proteins show structural similarities with ARF proteins: their C-terminal end contains the domains III and IV which mediate the heterodimerization between Aux/IAA and ARF. This interaction is required to repress the transcriptional activity of Q-rich ARF in the absence of the hormonal stimulus. The transcriptional repression is mediated by the domain I, which is positioned at the N-terminal end of the protein, but the underlying molecular mechanisms have not yet been fully elucidated. The removal of Aux/IAAs enable ARFs to activate gene transcription. Most Aux/IAAs have a very short half-life, which decreases in the presence of auxin (Zenser et al., 2001); a 13 amino acid conserved motif in the domain II functions as degron sequence, targeting the Aux/IAA proteins for ubiquitin-mediated proteolisis (Fig. 3) (Ramos et al., 2001; Gray et al., 2001). Proteins that contain the degron sequence are an efficient target of the SCF^{TIR1} E3 ubiquitin ligase complex (Dharmasiri and Estelle, 2004).

The SCF complexes are the largest family of ubiquitin ligase in plants and they are formed of Skp1, cullin, an F-box protein and the small Ring protein Rbx1. The cullin acts as a scaffold and it binds Skp1 at the N- terminus and Rbx1 at the C- terminus. Skp1 binds the F-box protein, which functions as substrate-specific adaptor, recruiting protein for the ubiquitinilation (Zheng et al., 2002).

Mutations in the *TRANSPORT INHIBITOR RESPONSE1* (*TIR1*) gene of *A. thaliana* led to the discovery of TIR1, a protein belonging to the F-box family which was shown to interact with the core structure of the SCF complex (Gray et al., 1999; 2002). The SCF^{TIR1} complex mediates the ubiquitinylation of the Aux/IAA repressors and it was recently found that auxin

and Aux/IAA bind to the same pocket on TIR1, with IAA increasing the otherwise low affinity of TIR1 towards Aux/IAA.

2.3 Role of auxin in root nodule organogenesis

Root nodule initiation requires the reprogramming of pericycle and cortical cells, which means that they are stimulated to enter the cell cycle again, abandoning the quiescent state (G0 phase for the cortical cells and G2 phase for the pericycle cells), to originate a new meristematic center (Foucher and Kondorosi, 2000; Roudier et al., 2003).

The dividing cells form the so-called nodule primordium, which will eventually be invaded by rhizobia. The cells adjacent to the nodule primordium located in the central cortex divide to form a new meristem which will give rise to the nodule parenchyma, vascular traces, vascular endodermis and nodule endodermis (Hirsch, 1992).

2.3.I Auxin and cytokinin in founder cells specification.

The cell division is mainly controlled by two crucial plant phytohormones, auxin and cytokinin, which regulate the progression of cells through the cell cycle (Kondorosi et al., 2005). Concentrations and auxin to cytokinin ratio both play a pivotal role in determining whether and where cells are about to enter the mitotic phase in plants.

The findings that the auxin transport inhibitor N-1-naphthylphthalamic acid (NPA) induces the formation of spontaneous nodules without rhizobia infection (Hirsch et al., 1989) and that rhizobia, as well as purified Nod factor, NPA and flavonoids, inhibit the expression of the GH3 reporter in white clover (Mathesius et al., 1998b), suggest that rhizobia infecting legumes bearing indeterminate nodules might interfere with the auxin flux before the onset of cell division. This hypothesis is further supported by the measurements of radiolabeled auxin transport in the inoculated root tissue of both *Vicia sativa* and *M. truncatula*, which show that rhizobia as well as specific Nod factors can produce the inhibition of the polar auxin transport (Boot et al., 1999; van Noorden et al 2006; Wasson et al., 2006). In contrast, the same experiments carried out on legumes bearing determinate nodules, such as *L. japonicus*, do

not reveal any inhibition in the auxin transport following rhizobia inoculation (Pacios-Bras et al., 2003).

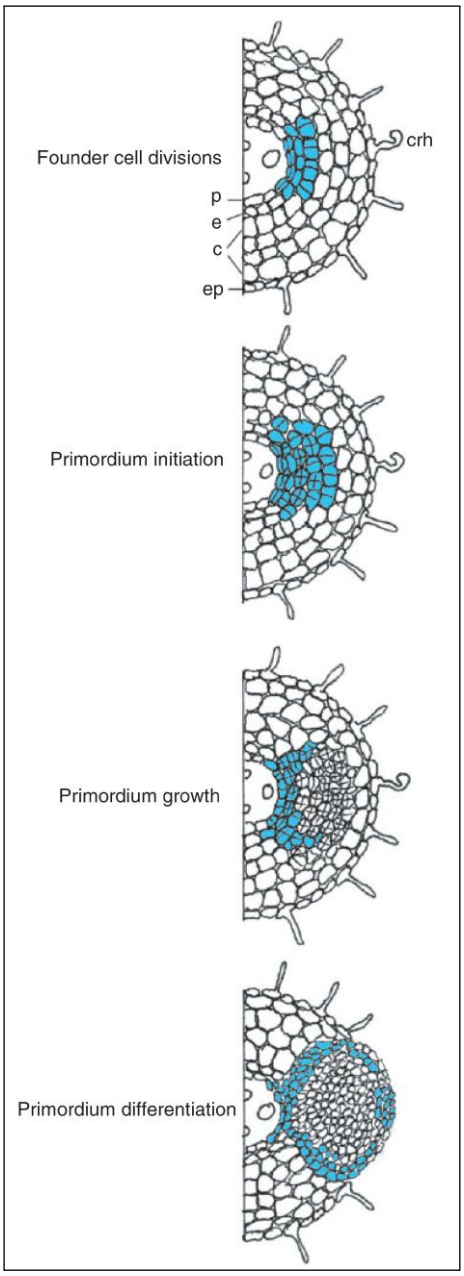

Figure 4. Indeterminate nodule organogenesis. Organogenesis of indeterminate nodules involves the first founder cell divisions, primordium initiation, primordium growth and primordium differentiation. The expression of the auxin responsive promoter *GH3* is shaded in light blue. Expression patterns are modelled on data from *Medicago truncatula* and white clover (*Trifolium repens* L.). p, pericycle; e, endodermis; c, cortex; ep, epidermis; crh, curled roots hair, indicating the infection site of rhizobia. (Picture modified from Mathesius, 2008).

Two well characterized regulators of auxin transport are ethylene and flavonoids (Burg and Burg, 1966; Jacobs and Rubery, 1988) and they are both induced during the early stages of nodulation (Mathesius et al., 1998a; Ligero et al., 1986). Ethylene is known to be a negative regulator of nodulation (Guinel and Geil, 2002), therefore its role in modulating the auxin flux in the early stages of nodulation seems unlikely. Flavonoids accumulation is specifically induced in the nodule precursor cells after the application of both rhizobia and purified Nod factors (Mathesius et al., 1998a) and they are also accumulated following the cytokinin treatment (Mathesius et al., 2000a). The involvement of flavonoids in auxin flux inhibition was further investigated through an RNAi approach. *M. truncatula* hairy root depleted of flavonoids resulted unable to establish the symbiosis and the auxin transport inhibition induced by rhizobia was abolished, confirming that flavonoids are required in the early stages of nodulation (Wasson et al., 2006). However, a similar study carried out on legumes that give rise to determinate nodules clarified that in this experimental model, isoflavonoids are required as Nod factors inducers in rhizobia but they do not affect the establishment of the symbiosis (Subramanian et al., 2006). In addition, no auxin flux inhibition in determinate

legumes has ever been documented (Subramanian et al., 2006; Pacios-Bras et al., 2003). This might suggest that auxin flux inhibition represents a peculiar mechanism of indeterminate legumes (Subramanian et al., 2006; Wasson et al., 2006).

The reduction of auxin transport in the root changes the auxin fluxes along with the auxin to cytokinin ratio at the site of nodule initiation and in the tissue below (Fig. 4). There is strong evidence that rhizobia can induce the cytokinin signaling, which is required for the cell division and for the expression of the cytokinin-inducible *ENOD40* (Fang and Hirsch, 1998; Gonzalez-Rizzo et al., 2006; Murray et al., 2007). In addition, a few hours before the onset of cell division, the early nodulin *ENOD40* is localized in the inner cortex and pericycle, supporting a role for cytokinin in specifying the nodule founder cells (Charon et al., 1997; Mathesius et al., 2000a).

On the other hand, both the auxin transport inhibition by NPA and the constitutive activation of the cytokinin receptor (*MtCRE1*) have been documented as sufficient conditions to generate nodule-like structure, despite the lack of infection (Hirsch et al., 1989; Tirichine et al., 2007). These observations might suggest a model where the simultaneous drop of auxin concentration and the increase in the cytokinin synthesis and/or response could be necessary to define whether and where a nodule is initiated (Mathesius, 2008). Although there is still no evidence that functionally link auxin and cytokinin, it was observed that cytokinin can alter PIN expression (Laplaze et al., 2007) and can cause the accumulation of auxin and flavonoids in the dividing cortical cells.

2.3.II Auxin and cytokinin in nodule initiation and differentiation.

After 24 h from inoculation, the inhibition in auxin transport is followed by an increase in auxin concentration in all the cell layers at the site of nodule initiation in both indeterminate (Fig. 4) (Mathesius et al., 1998b) and determinate nodules (Fedorova et al., 2000). Furthermore, a strong induction of two hydrolase, which are thought to release free and active auxin from the conjugate forms, has been documented in *M. truncatula* after 24 hours from inoculation (Campanella et al., 2008).

After this increase in concentration, auxin is localized in the actively dividing cells, i.e. pericycle and inner cortex in indeterminate nodule primordia (Mathesius et al., 1998b; van Noorden et al., 2007) and outer cortex in determinate nodule primordia (Pacios-Bras et al., 2003). Therefore, in contrast to what happens in the founder cells specification, in the initiation of both determinate and indeterminate nodule, auxin seems to play a similar role, which might possibly stimulate cell division (Roudier et al., 2003).

The retention of IAA within the dividing cells could be related to the spatially overlapping accumulation of flavonoids in the precursor cells and nodule primordia. Some flavonoids as well as other phenolic compounds can inhibit the activity of peroxidase and auxin hydrolase (Furuya et al., 1962; Grambow and Langenbeck-Schwich 1983), and in white clover, these flavonoids accumulated in the inner cortical cells protecting auxin from the peroxidase-mediate breakdown (Mathesius, 2001).

Besides the action of flavonoids, the IAA might be accumulated in the nodule primordia also because of the involvement of auxin transporters. *MtLAX* genes, encoding for cell influx facilitators, are strongly expressed in the young nodule primordia of *M. truncatula* (de Billy et al., 2001). Likewise, the expression of *PIN1* and *PIN2* is mainly localized in the early *M. truncatula* nodules and their silencing by means of RNAi led to a significant reduction in the number of nodules (Huo et al., 2006).

Furthermore, proteomic studies aiming to compare the root response in *M. truncatula* to rhizobia inoculation and to a 24 hours treatment with IAA showed that there is a significant overlap (around 80%) in the protein changes following the two applications, suggesting that increased auxin levels in the root might mediate many of the responses of the root towards rhizobia (van Noorden et al., 2007). The intervention of IAA during the nodule initiation was also corroborated by the fact that the application of the auxin action inhibitor p-chlorophenoxyisobutyric acid (PCBI) significantly reduces the numbers of nodules developed (van Noorden et al., 2006).

However, the action of auxin during the nodule initiation is also linked to other plant hormones, e.g. cytokinin and GA. Cytokinin are probably involved in sustaining the cell

division in the nodule primordia; the cytokinin responsive promoter *ARR5* (*Arabidopsis Response Regulator 5*) was shown to be active in the young primordia of *L. japonicus* (Lohar et al., 2004), likewise in *M. truncatula* the cytokinin-inducible *ENOD40* localizes in the actively dividing cells (Crespi et al., 1994). Furthermore, cytokinin-insensitive plants fail in nodules initiation (Gonzalez-Rizzo et al., 2006; Murray et al., 2007).

The role played by auxin during the early stages of rhizobia infection could also be related to the action of GA. It is well known that GA biosynthesis in plants requires the presence of IAA (Ross et al., 2000) and it has also been observed that the addition of GA to nodule extracts can stimulate auxin production. These observation and the fact that GA-deficient pea mutants are impaired in nodules initiation (Ferguson et al., 2005) suggest that GA and auxin might act synergistically during nodule primordia formation.

As the nodule primordium starts differentiating, the GH3 promoter activity is retained only in the peripheral cell layers, whereas it disappears from the central tissue (Fig. 4). Such decrease in IAA might be regulated by the activity of peroxidases that destroy the auxin accumulated within the nodule (Fedorova et al., 2000; Mathesius, 2001). In mature nodules the GH3 expression is only detectable in the vasculatures and in the apical meristem (Mathesius et al., 1998b; Pacios-Bras et al., 2003). This expression pattern matches with that of *MtAUX1* (de Billy et al., 2001) supporting the role of auxin in the vasculature differentiation (Aloni et al., 2006) and in the maintenance of meristem (Roudier et al., 2003; Kondorosi et al., 2005).

2.3.III Auxin and the control of root nodule number.

In legumes the number of nodules is regulated by several mechanisms. When nitrogen is sufficient to sustain the growth, then plants choose to uptake nitrate and ammonium over the more costly establishment of a symbiotic relationship. It is known that both nitrate and ammonium can inhibit the symbiosis by affecting different stages of the infection, e.g. nodule development and nitrogen fixation, but the exact mechanisms involved in this inhibition are still mostly unknown (Streeter, 1988).

Plants also have an internal, systemic regulatory mechanism which limits the number of nodules developed on the root. This was termed autoregulation of nodulation (AON) and it relies on a leucine-rich repeat receptor-like kinase (LRR-RLK), also known as nodulation autoregulation receptor kinase (NARK) (Kinkema et al., 2006). Following the first few infection events, the AON stops further nodule formation on the root, most probably to limit the metabolic cost of symbiosis establishment and maintenance. An early event during nodule formation sends a signal, termed Q, towards the shoot where it, or a derivative signal, is perceived by NARK. NARK in turn causes another signal, the shoot-derived inhibitor (SDI), to move to the root and to inhibit further nodulation (Kinkema et al., 2006). However, these long distance messengers have not been identified yet.

Auxin is known to act as a long distant signal in the regulation of lateral root induction, therefore the possible role of IAA in the AON was explored (van Noorden et al., 2006). van Noorden and colleagues (2006) demonstrated that in the *M. truncatula sunn* (*super numeric nodules*) mutant (Schnabel et al., 2005) the IAA transported from the shoots is three times more with respect to wild-type plants (van Noorden et al., 2006). After rhizobia inoculation long distance auxin transport in wild-type seedlings is decreased and it is in a good relation with the onset of AON (van Noorden et al., 2006). However, no inhibition in auxin transport takes place in the mutants, suggesting that SUNN protein, which is a LRR-RLK, might participate in regulating the auxin flux in response to rhizobia inoculation. Furthermore, the treatment of *M. truncatula sunn* with NPA causes a drop in the number of nodules produced to a level similar to untreated wild-type seedlings (van Noorden et al., 2006), strongly suggesting that auxin could be at the base of AON mechanism.

Nevertheless, it is still unclear whether parallel to the inhibition of auxin flux, a distinct SDI acts in controlling the number of nodulation events, and if such SDI is either dependent or not on SUNN activity (Mathesius, 2008).

It is also worth noting that the long distance auxin transport in the AON is regulated independently of the local transport inhibition that occurs at the root tip after rhizobia inoculation. The *sunn* mutant displays local inhibition transport following rhizobia infection as

in wild-type plants, despite the impairment in the long distance transport inhibition (van Noorden et al., 2006).

The number of nodules on the root is also regulated by ethylene and this is demonstrated by the *M. truncatula sickle* (*skl*) mutant, which is ethylene-insensitive and displays a hypernodulation phenotype (Penmetsa and Coock, 1997).

Ethylene can have several roles in controlling the nodulation, such as a defense response to limit rhizobia infection (Penmetsa and Coock, 1997; Praytino et al., 2006a; Penmetsa et al., 2008) and the regulation of auxin transport. In the hypernodulating *skl* mutant, following rhizobia infection auxin transport is not altered whereas the increase in IAA concentration that takes place after 24 hours is higher and is coupled with an over-expression of *PIN2* (Praytino et al, 2006b). These observation might suggest that ethylene could control nodule number by reducing auxin accumulation at the site of nodule initiation (Mathesius, 2008). Furthermore, the interaction between ethylene and auxin during nodulation might also be sustained by the finding that the roots of *sunn* mutant are less sensitive to ethylene than wild-type roots (Penmetsa et al., 2003).

2.4 Auxin synthesized by the symbiont

Auxin is often referred to as a phytohormone and is synthesized by all higher plants (Woodward and Bartel, 2005). However, many plants-associated soil bacteria are able to produce auxin and this feature could be part of the mechanism underlying their ability to manipulate the growth of host plants (Spaepen et al., 2007). Auxin synthesis has been shown in non-symbiotic plant growth-promoting rhizobacteria (Dobbelaere et al., 1999), in symbiotic nitrogen-fixing cyanobacteria (Sergeeva et al., 2002) and in the actinomycete *Frankia* (Wheeler et al., 1984). There is evidence that bacterial-derived IAA can alter the architecture of the root apparatus in non-legume plants. Auxin synthesised by the plant growth-promoting rhizobacterium *Azospirillum brasilense* has been shown to stimulate root growth in wheat (Dobbelaere et al., 1999) and *Pseudomonas putida*-derived IAA can induce the elongation of root in canola (Xie et al., 1996).

Furthermore, many *Rhizobium* species have the capacity to synthesise IAA through different biochemical pathways (Badenoch-Jones et al., 1983; Theunis et al., 2004) and this could constitute at least a part of a strategy to thwart the auxin fluxes in plant roots that are required for nodule organogenesis.

Some experimental evidence points to a role of rhizobia-derived auxin in the establishment of the symbiosis between plants and bacteria. It has been demonstrated that exudates from bean plants (i.e. flavonoids) can stimulate IAA synthesis in rhizobia, which most likely use the tryptophane secreted by the roots as precursor in the biosynthesis (Kefford et al., 1960; Prinsen et al., 1991; Theunis et al., 2004). However, the knocking-down of IAA synthesis in rhizobia did not significantly affect the capacity to nodulate bean plants (Spaepen et al., 2007).

Bacteroids of plants inoculated with *Bradirhizobium japonicum* mutants characterized by different IAA synthesis capability showed a positive correlation between the amount of auxin synthesised by the free-living symbiont and the concentration of IAA detected within the nodules, suggesting that the auxin found in the nodules might be, at least in part, of prokaryotic origin (Hunter, 1987).

Several pieces of research have proposed a role for rhizobia-derived auxin in affecting the nodulation efficiency in legumes bearing determinate nodules. 5-methyltryptophane resistant mutants of *B. japonicum* that produce high amounts of IAA have been shown to produce a lower nodule mass and a lower nodule number when compared to wild-type rhizobia (Hunter, 1987). However, another study has shown that the inoculation of soybean with catabolic mutants of *B. japonicum* that produced elevated amounts of IAA (about 30-fold higher than wild-type rhizobia) and IPA increases the nodule volume and the root weight in comparison with plants inoculated with wild-type *B. japonicum* (Kaneshiro and Kwolek, 1985). A similar promoting effect on determinate nodule development was also suggested by the fact that IAA-deficient mutants of *B. japonicum* had a significantly reduced ability to induce root nodules with respect to wild-type symbionts (Fukuhara et al., 1994). However, the effects of

an increased synthesis of auxin in the symbiont and its efficiency in the induction and development of indeterminate nodules have not been investigated yet.

The application of exogenous auxin can enhance nodulation on both *M. truncatula* and *Phaseolus vulgaris* when added at very low concentrations (up to 10^{-8} M), whereas higher concentrations have inhibitory effects (Plazinski and Rolfe, 1985; van Noorden et al., 2006). Furthermore, it was noted that nodule-like structure could also be induced in non-legume plant by the application of IAA and that the resulting organ might be colonized by diazotroph microorganisms such as *Azospirillum*, which apparently infects the plant via crack entry (Christiansen-Weniger, 1998). Therefore it could hypothesised that the production of auxin by microsymbionts might be a general and ancient mechanism to alter root architecture and to induce nodule-like structures in plants.

2.5 Root nodules and lateral roots development

Many similarities exist between the development of lateral root and root nodules. Both organs are formed post-embryonically from endogenous cells that are stimulated to divide. The profound analogies between the two developmental processes suggested the hypothesis that nodulation, which is a relatively recent event in the evolution, evolved from a developmental pathway already existing in plants and devoted to the specification of lateral root formation (Mathesius et al., 2000b). This hypothesis is supported by the origin of nodules in a *Parasponia* sp. and in actinorhizal plants from modified lateral roots (Hirsch and LaRue, 1997), as well as by the presence of hybrid structures between nodules and lateral roots in legumes (McIver et al., 1993). Furthermore, several genes that are expressed during nodule formation are also expressed during lateral root development, e.g. *ENOD40* (Yang et al., 1993) and *ENOD12A* (Bauer et al., 1996). There is evidence that activation and division of the pericycle cells are at the origin of both lateral roots and indeterminate root nodules. It has been shown that in *M. sativa* the pericycle cells division precedes the activation of the inner cortical cells division during nodule initiation (Timmers et al., 1999). However, differently from root nodules, lateral root initiation is developmentally regulated since the founder cells are probably specified in the root meristem. It is thought that the lateral root

founder cells are kept in a meristematic state, that is they remain competent to divide in an otherwise differentiated root tissue. The founder cells are mostly found in the G2 phase of the cell cycle, whereas the pericycle cells, not belonging to the founder pool, are mainly blocked in the G1 phase (Beeckman et al., 2001; Roudier et al., 2003). On the other hand, root nodules in many legumes are initiated *de novo* at unspecified times during plant development and they are finally generated by the activation of both pericycle and cortical cells and typically have peripheral vascular bundles.

Despite the striking parallelism, the in-depth investigation of these two organogenetic programs has suggested that the major difference resides in the specification of the founder cells, whilst their development might be regulated in a similar way (Mathesius, 2008). This conclusion is supported by the different requirement for auxin and cytokinin at the outset of organogenesis. High auxin concentrations increase the number of lateral roots and they inhibit the initiation of root nodules (van Noorden et al., 2006). Cytokinins have the opposite effect, since they inhibit the lateral root initiation, increase the number of nodules (Lohar et al., 2004) and can also induce the generation of spontaneous nodules (Gonzalez-Rizzo et al., 2006).

The developmental mechanism of lateral root formation has been studied in great detail in the last few years and it was found that the correct auxin localization and the subsequent responses are crucial for this developmental program (Casimiro et al., 2003; De Smet et al., 2006; Fukaki et al., 2007). The extensive study of *Arabidopsis* mutants impaired in lateral root formation highlighted the fact that such mutants also display auxin-related defects (Casimiro et al., 2003). The application of exogenous auxin (Himanen et al., 2002) and the overproduction of the endogenous IAA (Boerjan et al., 1995; Celenza et al., 1995) result in an increase in the lateral root number, whereas when plants are grown in media containing NPA fail to initiate lateral roots (Casimiro et al., 2001). Furthermore, the presence of the phytohormone and/or the response to auxin was shown just before and during the division leading to the formation of LR primordium (Benkovà et al., 2003). It has also been reported that several auxin-induced genes resulted expressed during the lateral root initiation

(Marchant et al., 2002; Tatematsu et al., 2004). Therefore, auxin is clearly required for the initial cell division and might be involved in the G1-to-S transition (Himanen et al., 2002).

Besides the role of auxin, the involvement of downstream components of the signal pathway has received great attention in the past few years (Lau et al., 2008). Recent findings have shown that nitric oxide (NO) plays a role in the induction pathway of both adventitious and lateral root (Correa-Aragunde et al., 2004; Pagnussat et al., 2003). In lateral rooting, NO is produced in the pericycle cells, which will give place to the primordia, indicating that NO is required during the early stages of the induction and supporting the hypothesis of a signalling cascade involving NO as a secondary messenger downstream of auxin (Correa-Aragunde et al., 2004).

Indications of a possible occurrence of NO in the symbiosis between legume and rhizobia have been reported. The production of nitric oxide was detected both at the early stages (4 h after inoculation) of the interaction between *L. japonicus* and *Mesorhizobium loti* (Shimoda et al., 2005) and at later time points (from 24 to 48 h after inoculation) in the *Medicago-Sinorhizobium* system (Baudouin et al., 2006). These data might suggest that NO is evoked transiently during the very first stages of the symbiotic interaction (Baudouin et al., 2006). NO is also produced in functional nodules where its detection was mainly associated with the fixation zone, where a sub-set of plant cells are infected by bacteroids. Nevertheless, the presence of bacteroids within a cell is not sufficient per se for NO production, suggesting that specific physiological conditions might be required for NO production (Baudouin et al., 2006). These findings might propose a role for NO in the symbiosis, although the origin of NO in nodules and its function still needs further investigation (Baudouin et al., 2006).

3. Plant defence responses and symbiosis

The colonization of root by rhizobia does not result in an efficient plant defence reaction, normally induced by pathogenic microorganisms (Djordjevic et al., 1987; McKhann and Hirsch, 1994; Baron and Zambryski, 1995). However, plant reaction to the invading rhizobacteria includes responses that are common to pathogenic interactions, for instance

the expression of proteins that are structurally related to defence proteins as well as the generation of reactive oxygen species (ROS) (Gamas et al., 1998; Santos et al., 2001). The transcriptome analysis of *M. truncatula* root nodules has led to the discovery that more than 750 plant genes are differentially expressed during the establishment of the symbiosis (El Yahyaoui et al., 2004). Among these genes are several transcripts related to pathogen response that are significantly induced during early stages of infection. For istance, genes encoding cystein-rich peptides with anti-microbial activity are well represented. Some of these peptides belong to the Plant Lipid Transfer Proteins family.

3.1 Lipid transfer proteins

Plant Lipid Transfer Proteins (LTPs) were discovered about 30 years ago (Kader, 1975) and they were named after their ability to transfer phospholipids from a donor to an acceptor membrane *in vitro* (Kader, 1996).

LTPs are small peptides which can be grouped into two families. The LTPs of the first family, named LTP1, have molecular masses of approximately 10 kDa (Kader, 1996), whereas the protein belonging to the second family, termed LTP2, have an average molecular weight of 7 kDa. Both LTPs1 and LTPs2 present isoelectric points (pI) comprised between 9 and 10 and a signal peptide at the amino-terminal end, the length of which generally varies between 21 and 27 residues for LTP1 (Arondel et al., 1991; Suelves and Puigdomènech, 1997; Vignols et al., 1994) and from 27 to 35 amino acids for LTP2 (Garcia-Garrido et al., 1998; Kalla et al., 1994). The signal peptide is excised and targets the mature proteins towards the secretory pathway to be exported to the apoplast. Consistently, LTP1 of various plant species are localized at the cell wall (Thoma et al., 1993; Pyee et al., 1994; Tsuboi et al., 1992; Carvalho et al., 2004). As yet, there are no pieces of research elucidating the subcellular localization of proteins belonging to family 2 LTP.

3.1.I The structure

The primary structure of the mature LTPs of both families consists of a single polypeptide chain which is formed by 90-95 amino acids in the case of LTP1 family and approximately

70 residues in the case of LTP2 family (Douliez et al., 2001; Kader et al., 1996; Samuel et al., 2002). The members of both families are structurally related, since they present a common 8 cystein motif which is considered to be the characteristic signature of LTPs. These eight cysteins are engaged in the formation of four intramolecular disulfide bonds, that serve to stabilize the tertiary structure (Douliez et al., 2001;Castro et al., 2003; Liu et al., 2002).

Despite the strict conservation of the motif, however, there is a mismatch in the pairing of the cysteine residue for the formation of the disulfide bridges. In the LTP1 family, Cys3 pairs with Cys50 and Cys48 withCys87, whereas in the LTP2 family Cys3 pairs with Cys35 and Cys37 with Cys68 (Douliez et al., 2001; Liu et al., 2002; Samuel et al., 2002).

Although the two families exhibit a low overall sequence similarity (approximately 30% of identity), they display a similar three dimensional architecture, consisting of an hydrophobic cavity delimited by four α-helices, which are held in a compact fold by the four disulfide bonds (Yeats and Rose, 2008).

3.1.I.A LTP1 family

Three dimensional structures of several LTP1 have been solved by NMR and X-ray crystallography, revealing a globular and compact structure (Lee et al., 1998; Shin et al., 1995). The most important feature of the LTP1 is the presence of a flexible hydrophobic cavity that runs through the molecule's axis like a tunnel with two entrances, a small one and a large one (Lee et al., 1998; Shin et al., 1995).

Beside the perfectly conserved cysteines, LTPs1 have several other specific residues that are present in the majority of the members of this protein family. Small hydrophobic amino acids (i.e. Ile, Val, Leu, Ala) appear throughout the whole sequence and they are thought to participate in defining the hydrophobic character of the binding tunnel. Two tyrosine residues (Tyr16 and Tyr79) are well conserved in LTPs1. Studies concerning the side chain orientation and site direct mutagenesis experiments have demonstrated that Tyr16 is not involved in the lipid binding (Lullien-Pellerin et al., 1999). Some structures revealed that semi-conserved Tyr79 forms hydrogen bonds with the polar head of the phospholipid

(Charvolin et al., 1999; Tassin-Moindrot et al., 2000), but this is not always the case (Han et al., 2001; Cheng et al., 2004b). Another well conserved feature of LTP1 is the presence of two oppositely charged residues which are strategically positioned at the larger entrance of the hydrophobic tunnel, indicating a possible role in the interaction with lipids (Lee et al., 1998; Shin et al., 1995). In fact, the lipid molecules interact with the protein at the larger entrance and, whilst the hydrophobic chain stays buried inside the cavity, the carboxylic part remains exposed to the solvent.

In addition to the structural studies, the ligand binding properties of LTPs were investigated by means of equilibrium titration experiments. The ability of LTPs1 to bind glycerolipids (Lerche et al. 1997; Guerbette et al. 1999; Douliez et al. 2000), fatty acids (Tsuboi et al. 1992; Lerche et al. 1997; Douliez et al. 2000) and acyl-CoA (Ostergaard et al. 1993; Lerche et al. 1997) has been demonstrated. The analysis of titration curves revealed that the number of binding sites for each monomer was between one and two, most likely suggesting some kind of cooperativity in the binding. However, the technique used in these experiments (enhancement of intrinsic fluorescence) does not allow the drawing of such conclusions as it does not provide information about the concentration of free ligand, which is necessary to assess the cooperativity (Douliez et al. 2000). Nevertheless, the binding cooperativity hypothesis is also suggested by the X-ray resolution of two independent LTP1, one from rice (*Oryza sativa*) and one from wheat (*Triticum aestivum*), complexed with their own ligands, palmitic acid and L-α-myristoyl-phosphatidylcholine, respectively (Charvolin et al., 1999; Cheng et al., 2004b). In both structures, two lipids are shown to share the same binding tunnel with a tail-to-tail arrangement, probably having entered the cavity from opposite entrances and leaving their polar heads exposed to the solvent (Charvolin et al., 1999; Cheng et al., 2004b).

3.1.I.B LTP2 family

In comparison with the LTP1 family, there are considerably fewer structural studies for LTP2s. To date only the structure of two LTP2, one from rice (Samuel et al., 2002) and one from wheat complexed with L-α-palmitoyl- phosphatidyl glycerol (Pons et al., 2003; Hoh et

al., 2005), have been solved. As is the case of LTP1, also LTPs2 display four helices stabilised by four disulfide bonds. The globular structure encloses the internal hydrophobic cavity, which has the shape of a triangular hollow box, rather than a tunnel, and it is covered with amino acids such as Ile, Leu, Phe and Val (Samuel et al., 2002). Computational studies revealed that the binding cavity of LTP2 is more flexible than that of LTP1, allowing for the accommodation of a wider range of lipids, including sterols (Samuel et al., 2002; Pons et al., 2003; Cheng et al., 2004a; Hoh et al., 2005).

Recent mutational studies showed that some residues are important to both LTP2 structure and ligand binding. Site direct mutagenesis of Leu8, Phe36 and Val49 of rice LTP2 led to a significant alteration in the protein structure and in the lipid binding and transfer activity. Conversely, the mutation of Tyr45 to Ala resulted in a reduction in the lipid binding and transfer activity without affecting the structure. Although the affinity toward linear phospholipids remained unchanged, the Y45A mutant displayed significantly less affinity for planar sterol molecules (Cheng et al., 2007), suggesting that this aromatic residue might provide important indications about the nature of the *in vivo* ligand.

3.1.II Biological roles of LTPs

On the basis of their properties of transferring lipids, it was proposed that the LTPs are involved in all those functions where the intracellular movement of lipids is thought to be important, such as membrane biogenesis and turnover. However, all known plant LTPs are synthesised as precursors with an N-terminal signal peptide which targets the mature proteins towards the secretory pathway. These observations ruled out the initial hypothesis of an intracellular role of LTPs most likely in the lipid biosynthesis and different functions were suggested.

It has been proposed that LTPs might be involved in the synthesis of the cuticle. The cuticle is a structure which is composed of cutin, a polyester of hydroxyl-fatty acids (Heredia, 2003), and a variety of high melting point organic compounds generically termed waxes (Yeats and Rose, 2008). The lipid precursors of cuticle are synthesized within the epidermal cells and they must break through the hydrophilic cell wall to reach the developing cuticle. LTPs have

been proposed as carriers of these compounds. Although there is no direct evidence for this role, the positive correlation between the enhanced expression of LTPs1 and the increased accumulation of waxes (Hollenbach et al., 1997; Cameron et al., 2006) supports this hypothesis.

LTPs were also proposed to have a role in the regulation of plant growth and development. It has been demonstrated that a LTP1 from lily styles is necessary, along with polysaccharides, for pollen tube adhesion on an artificial stylar matrix (Park et al., 2000; Park and Lord, 2003). Furthermore, when the expression of the orthologous genes in *A. thaliana* was depleted, transgenic plants were defective in both vegetative and reproductive growth (Yeats and Rose, 2008). Interestingly, members of family 1 LTP isolated from tobacco and wheat have shown the ability to induce cell wall loosening and thus it was hypothesized that they might play a role in cell expansion and plant growth (Nieuwland et al., 2005). This appears to be a property shared at least by family 1 LTPs even though the mechanisms of action have not yet been elucidated (Nieuwland et al., 2005). To date, there is no experimental evidence indicating similar properties for family 2 LTPs.

The most widely investigated aspect of LTPs' biology is their potential as antibiotics. The antimicrobial activity of LTPs was discovered by the screening of proteinaceus extracts of plants, in order to find proteins that could inhibit the growth of phytopathogens *in vitro* (Terras et al., 1992, Molina et al., 1993, Segura et al., 1993, Cammue et al., 1995, Ge et al., 2003). Among the pathogens inhibited were both bacteria and fungi, although each LTP presented a relatively narrow spectrum of action as their activity strongly depends on the microorganism tested (Ge et al., 2003).

The most potent peptide belonging to LTP class was isolated from onion (*Allium cepa*) seeds, *Ace*-AMP1 (Cammue et al., 1995); it was able to inhibit the growth of all the fungi tested and of the Gram positive bacteria *Bacillus megaterium* and *Sarcina lutea*, but it did not have any effect on Gram negative bacteria (Cammue et al., 1995).

Although the mechanism of LTP toxicity is not entirely clear, it appears to be dependent, at least in part, on the ability to promote the membrane permeabilization of the pathogen, due

to loss of membrane integrity (Cammue et al., 1995; Regente et al., 2005). Indeed LTPs1 have been shown to interact with model membranes, such as monolayer composed of dipalmitoilphoshatidylglycerol (Subirade et al., 1996) and large unilamellar vesicles filled with fluorescent dyes (Caaveiro et al., 1997), and it has been also demonstrated that LTPs1 are able to permeabilize the yeast plasma membrane allowing the entrance of a small dye (SYTOX Green), which can penetrate only compromised membranes and binds nucleic acids with high affinity (Diz et al., 2006).

Supporting evidence for the antimicrobial activity came from the analysis of the expression patterns of LTPs, since many of them are up-regulated in plants upon pathogen challenge (Garcia-Olmedo et al., 1995, Jung et al., 2003). These feature resulted in the inclusion of LTP in the pathogenesis-related protein (PR) family (Van Loon and Van Strien 1999). Additional evidence has been provided by transgenic plants overexpressing LTPs, which were conferred an enhanced resistance against a wide range of plant pathogens (Mourgues et al., 1998, Iwai et al., 2002, Jung et al., 2005, Roy-Barman et al., 2006, Patkar and Chatoo, 2006, Yang et al., 2008). To date, there are no clear indications that also LTP2 can act as antimicrobial effectors, either *in vitro* or *in vivo*.

LTPs can also participate in the defence reaction as components of the signalling pathway that leads to the systemic acquired resistance. The first direct evidence of this role was obtained with the *dir1-1* mutant of *Arabidopsis thaliana*, which was impaired in the systemic acquired resistance (SAR) induction but not in the local response (Maldonado *et al*., 2002). The predicted sequence of DIR1 displays some similarities to family 2 LTPs, sharing such features as the eight cystein motif, a predicted signal peptide and the small size. However, the mature peptide has a pI of 4.5 which is much lower than that of the other LTPs2 (Maldonado et al., 2002). The overexpression of DIR1 in transgenic plants did not lead to a constitutive activation of SAR, therefore it was proposed that it was interacting with a second, not yet identified, signalling component in order to function in the SAR signalling pathway (Maldonado et al., 2002). Further characterizations, including the resolution of the structure

and the study of the biochemical properties of DIR1 were carried out and, on the basis its specific features, it was proposed as a new type of LTP (Lascombe *et al*., 2008).

Evidence for LTP1 acting as systemic resistance inducers originated from the observation that a *Nicotiana tabacum* LTP1 (NtLTP1) has the capability to bind jasmonic acid (JA) and the complex NtLTP1-JA is able to interact with the elicitin receptor on the plasma membrane. It was suggested that the binding with JA induces a conformational change on LTP that facilitate its recognition by the receptor. The application of the NtLTP1-JA complex to *Nicotiana tabacum* plants provides long distance protection against *Phytophthora parasitica* infection in tobacco plants (Buhot *et al*., 2004). However, it has not yet been demonstrated whether the NtLTP1-JA complex is the mobile signal for the systemic protection or whether the interaction with the elicitin receptor is required for the generation of a mobile signal (Buhot et al., 2004).

A possible role of LTPs in the symbiotic interaction between legumes and rhizobia has been suggested following the detection of genes encoding for putative LTPs, among early nodulins (Krause et al., 1994; El Yahaoui et al., 2004). A cDNA encoding for an LTP-like protein was isolated from a cDNA library constructed from RNA of *Vigna unguiculata* roots, one and four days after inoculation with rhizobia (Krause et al., 1994). The mRNA was absent in differentiated nodules and therefore it was concluded that the LTP gene was transiently expressed during the early stages of nodule development (Krause et al., 1994). The transcriptome analysis of *M. truncatula* nodulating root has shown that a gene encoding for a putative LTP, *MtN5*, is strongly induced during nodule formation (El Yahaoui et al., 2004).

Aim of the thesis

The first objective of this research is the evaluation of the potential effects of bacterial-derived auxin in the symbiotic relationship between legumes and rhizobia. In order to achieve this focus, a chimeric operon, encoding for a new auxin biosynthetic pathway, was generated. The gene construct consists of the bicistronic unit *iaaMtms2*, whose transcription was driven by the *rolA* promoter (Magrelli et al., 1994; Pandolfini et al., 2000). The chimeric gene was sub-cloned into the broad-host range vector pMB393 and mobilised into symbiotic rhizobia, obtaining auxin hyper-synthesising strains.

During a previous research project carried out in the same laboratory where I prepared my PhD thesis, auxin hyper-synthesising rhizobia was used to investigate the influence of auxin in the induction of determinate nodules. The data obtained on *Phaseoulus vulgaris* plants have demonstrated that an increase in the amount of auxin produced by rhizobia does not affect the formation of determinate nodules (Pii et al., 2007).

The present research was thus focused on leguminous plants bearing indeterminate nodules: *Medicago truncatula*, model plants for the indeterminate legumes, and *Medicago sativa*, a related species of agronomic interest.

Since regulation of auxin transport in rhizobia-infected roots is one of the key steps marking the early phases of the developing symbiosis (Mathesius, 2008), the perturbation of root auxin fluxes in plants infected with auxin hyper-synthesising strains, was investigated by studying the differential expression of genes affecting root polar auxin transport.

The second objective of this research has been the study of one of the pathogen related proteins induced during early phases of rhizobia infection, *MtN5* which shares sequence similarities with LTPs (lipid transfer protein) (El Yahyaoui et al., 2004). We studied the expression of MtN5 during the symbiotic interaction between *M. truncatula* and *S. meliloti*

and the pathogenic interaction with *Fusarium semitectum* and *Xanthomonas campestris*. In addition, we investigated its function using a RNA-interference-based suppression approach. Recently, it has been shown that plants can counteract bacterial pathogens by suppressing the intracellular auxin transduction pathway (Navarro et al., 2006), thus it has been hypothesised that many phytopathogens have evolved mechanisms to suppress plants innate immunity by stimulating plant IAA biosynthetic pathways or by producing auxin themselves (Robert-Seilaniantz et al., 2007). Therefore, besides evaluating the expression of MtN5 during symbiotic and pathogenic interactions, we tested the response of plants nodulated with both auxin hyper-synthesising rhizobia and wild type rhizobia to pathogenic microorganisms by assessing disease symptoms.

Materials and Methods

1. Microorganisms

Sinorhizobium meliloti 1021 is a streptomycin-resistant derivative of wild type field isolate SU47 (Meade et al., 1982). *S. meliloti* was grown at 28°C in LBMC medium (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl, 2.6 mM $MgSO_4$, 2.6 mM $CaCl_2$) supplemented with streptomycin 200 µg/ml. *Agrobacterium rhizogenes* ARqua1 (Quandt. *et al*., 1993) was grown at 28°C in TY medium (5 g/l tryptone, 3 g/l yeast extract, 6 mM $CaCl_2$, pH 7.2) supplemented with the appropriate antibiotics. *Fusarium semitectum* ISCF20a is a wild type field isolate (kindly gifted by Dr. M. Zaccardelli) and it was grown on solid medium PDA (SIGMA). *Xanthomonas campestris* pv. *alfalfa* was grown on YDA medium (10 g/l glucose, 5 g/l yeast extract, 5 g/l peptone).

2. Plasmids and gene constructs

2.1 IAA biosynthetic pathway

Standard techniques were used for the construction of recombinant DNA plasmids. The *rolAp-iaaMtms2* chimeric operon contains the bicistronic unit *iaaMtms2* under the control of *promintron*, the 85 bp-long intron of *rolA* gene of *Agrobacterium rhizogenes* which has promoter function in bacteria (Pandolfini et al., 2000). A 1773 bp-long DNA sequence spanning the coding region (1671 bp) of the *iaaM* gene (GenBank accession n. M11035) from *Pseudomonas syringae pv. savastanoi* (Yamada et al., 1985) and a 1452 bp-long DNA sequence spanning the coding region (1404 bp) of *tms2* (GenBank accession n. AH003431) from *Agrobacterium tumefaciens* (Klee et al., 1984), were cloned downstream of the *promintron* sequence (*rolAp*), connected by a 17 bp-long linker sequence. The *rolApiaaMtms2* construct was subcloned in the broad-host range plasmid pMB393 (Gage et

al., 1996) and introduced by electroporation into *S. meliloti* 1021 to obtain the IAA strains. *S. meliloti* 1021 harbouring the pMB393 plasmid containing the *promintron* sequence was used as control strain.

2.2 Constructs for MtN5 silencing and overexpression

The RNAi hairpin construct was built following the construct design previously described (Pandolfini *et al*., 2003). The 200 bp-long fragments homologous to the 5' end of *MtN5* coding sequence were obtained by PCR using the following primer: *MtN5* forward primer 5'–ATGTTGGCCGGCAGTCTAGCTGTT–3'; reverse primer 5'–GCTTTTTTGGCATTGATTCCATAA–3'. The chimeric gene was cloned into both the pBIN19 and pRedRoot (Limpens et al., 2004) binary vectors and the recombinant plasmids were mobilised into *A. rhizogenes* ARqua1.

In order to build the gene construct for *MtN5* over-expression, the coding sequence was PCR amplified (primers used: 5'–GGTACCATGGCACATTCTCAGGGCAA–3' and 5'–GGATCCTTAACAGTTGGAAGGTGTTTG–3') and sub-cloned in pGemT (Promega) and checked by sequencing. The *MtN5* coding region was *Kpn*I-*Bam*HI cloned in pRedRoot in-between the constitutive promoter *35S* from Cauliflower Mosaic Virus and the NOS terminator sequence. The recombinant vector was mobilized into *A. rhizogenes* ARqua1.

3. Plant growth and inoculation

Medicago truncatula cv. *Jemalong* and *Medicago sativa* ecotype *Romagnolo* seeds were scarified using fine grade sand paper sheets and sterilized in 5% commercial bleach for 3 min. Seeds were rinsed three times with sterile water and stored on 0.8% agar plates at 4°C for 2 days before placing in a growth chamber at 25°C for 7 days to allow germination. Germinated seedlings of *M. truncatula* were transferred in small pots and grown on a sand and perlite mixture (1:1) in a growth chamber at 22°C and 10-h light/14-h dark regimen under fluorescent lights giving an average irradiance of 120 µmol m^{-2} sec^{-1} of photosynthetically active radiation (PAR); the relative humidity was 65%. *Medicago sativa* seedlings were grown in small pots on sand and perlite mixture (1:1) in a greenhouse at day and night

temperatures of 24°C and 18°C, respectively, and 10 h light/14 h dark regimen. Once a week the *Medicago sp*. seedlings were supplemented with a nitrogen-free nutrient solution (0.13 mM KH_2PO_4; 0.3 mM $CaCl_2 \cdot 2H_2O$; 0.06 mM $MgSO_4 \cdot 7H_2O$; 0.2 mM K_2SO_4; 0.014 mM FeNa EDTA; 1.56 mM H_3BO_3; 1.24 mM $MnSO_4 \cdot H_2O$; 4.5 mM KCl; 0.11 mM $ZnSO_4 \cdot 7H_2O$; 0.1 mM $CuSO_4 \cdot 5H_2O$; 0.32 mM H_2SO_4; 2.1 mM $Na_2MoO_4 \cdot 2H_2O$). The seedlings were watered with sterile deionized water when necessary. For plant inoculation, *S. meliloti* was grown in liquid medium, collected by centrifugation, washed in sterile water, and then diluted in sterile water to 0.1 OD_{600} (approximately 10^8 cfu/ml). Ten ml of this suspension were used to inoculate seedlings at 10 and 24 days after germination. Leaves, roots and root nodules were collected 40 days after germination. At the end of each experiment, the presence of the recombinant plasmids in bacteroids was checked by PCR analysis on total DNA extracted from root nodules, using primers specific for the *iaaM* gene. The sequences of the oligonucleutides employed are the followings: *iaaM* forward primer: 5'-ATGTATGACCATTTTAATTCACCCAGT-3', *iaaM* reverse primer: 5'-CTGGGAGGAAAGCGCATCGCAC-3'.

3.1 NO scavenger assay

For cPTIO treatments, *M truncatula* seedlings were transferred after germination in 15 ml test tubes, containing nitrogen-free nutrient solution and perlite (1:2 vol/vol) and grown in a growth chamber at 24°C 16-h light/8-h dark regimen under fluorescent lights giving an average irradiance of 120 µmol m-2 sec-1 PAR; the relative humidity was 65%. Ten days old plants were inoculated using 1 ml of a bacterial suspension to an OD_{600} of 0.1. Two , 24 and 48 hours after inoculation, 1 ml of 1 mM 2-(4-carboxyphenil)- 4,4,5,5,-tetramethylimidazoline-1-oxyl-3oxide (cPTIO) (Sigma, St. Louis, MO, USA) was added to the nutrient solution; 1 ml of distilled water was used for negative controls. Nodules were counted 28 days after inoculation.

3.2 Micro-flood inoculation and MtN5 time-course

For primary root micro-flood inoculation assay and MtN5 time course assay, germinated seedlings of *M. truncatula* were placed in square Petri plates, containing slanted BMN agar medium (Engstrom *et al*., 2002) supplemented with 0.1 μM L-α-2-Aminoethoxyvinyl glycine (AVG). The plates were kept vertically in a growth chamber at 25°C and 10-h light/14-h dark regimen. Seven days after germination seedlings were micro-flood inoculated. *S. meliloti* was grown overnight in liquid LBMC medium, collected by centrifugation and suspended in 10 mM $MgSO_4$ to 0.2 OD_{600}. Micro-flood inoculation was performed by placing five drops (0.5 μl) of bacterial suspension on the surface of the root. For MtN5 time course assay, root apparatuses were collected 1, 3, 5, 7 and 14 days after inoculation. In micro-flood inoculation of primary root assay, root apparatuses were collected 14 days after inoculation.

3.3 Auxin treatment

Auxin treatment was carried out by placing 7 day old seedlings of similar length in a nitrogen-free mineral solution (see above) supplemented with 0.1 μM IAA. This concentration was chosen because it showed physiological effects on *M. truncatula*, including the stimulation of pericycle cells division (van Noorden et al., 2007). *M. truncatula* roots were harvested after 24 h of treatment and analyzed by western blot to check for MtN5 protein expression.

4. cPTIO toxicity assay on *S. meliloti*

The toxic effect of cPTIO on *S. meliloti* was verified by investigating the growth kinetic in the presence of the NO scavenger. Liquid cultures of both *S. meliloti* IAA and control strain were grown for 24 h at 28°C on nutrient medium LBMC supplemented with the suitable antibiotics. An aliquot of the grown cells were transferred to fresh RDM minimal medium (Vincent, 1970) supplemented with 0.5, 1 and 5 mM cPTIO. The cultures were incubated at 28°C with shaking at 180 rpm. The growth of *S. meliloti* was followed for 48 h by means of spectrophotometer measurements. The data reported represent the mean values of three independent assay.

5. IAA analysis

Root nodules (1 g FW) were collected from 40 day-old plants. IAA extraction was carried out as previously described (Mezzetti et al., 2004). 100 nmols of D5- IAA were added to the samples, as internal standard. TMS GC-MS analysis was performed on a Hewlett Packard 5890 instrument equipped with a HP-5 (Agilent technologies) fused silica capillary column (30 m, 0.25 mm ID, helium as carrier gas), with the temperature program: 70°C for 1 min, 70°C→150°C at 20°C/min, 150°C→200°C at 10°C/min, 200°C→280°C at 30°C/min, 280°C for 15 min. The injection temperature was 280°C. Electron Ionisation (EI) mass spectra were recorded by continuous quadrupole scanning at 70eV ionization energy. IAA analyses were carried out by Dr. A. Amoresano and Prof. P. Pucci (CEINGE, University of Naples "Federico II").

6. NO detection

Endogenous NO was detected with the fluorophore 4,5- diamino-fluorescein diacetate (DAF-2-DA). 4-aminofluorescein diacetate (4-AF DA) was used as a negative control. Nodules obtained with the control and IAA strains were incubated with 7.5 µM DAF-2DA (Calbiochem) or with the negative probe 4 AF-DA (Calbiochem) in 20 mM HEPES-NaOH, pH 7.5 (buffer A) for 30 min in the dark at 25°C. Thereafter, nodules were washed three times with buffer A for 15 min and fluorescence was detected with a Zeiss LSM 510 laser scanning confocal microscope exciting at either 488 or 543 nm. For emission in the green light, fluorescence was examined between 505 and 530 nm, while in the red light, fluorescence was collected at wavelengths >560 nm. Green fluorescence was quantified by measuring the medium pixel intensity in the confocal images for every single nodule analyzed. All the quantitative data were subjected to statistical evaluation (Student's *t* test). A $P < 0.05$ was considered statistically significant.

7. NOS-like activity assay

S. meliloti cells were grown on minimal medium (10mM phosphate buffer pH 6.3, 7.4 mM sodium succinate, 2.7 mM D-glucose, 0.8 µM nicotinic acid, 1 mM $MgSO_4$, 0.5 mM $CaCl_2$) supplemented with 0.1% NH_4Cl as nitrogen source. Cultures were incubated at 28°C with shaking at 180 rpm until reaching stationary phase, harvested by centrifugation at 5000xg for 20 min and resuspended in Homogenization Buffer (25 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM EGTA). Cells were disrupted by sonication for 1 min and the soluble fraction was obtained by centrifugation at 13000xg for 30 min. NOS-like activity was determined by the method based on the conversion of L-[^{3}H]arginine to L- [^{3}H]citrulline, by using the NOS Activity Assay Kit (Cayman Chemical, Ann Arbor, MI). Total protein concentration was measured by Bradford method and the enzyme activity was expressed as fmol arginine µg protein^{-1}h^{-1}.

8. RT-PCR analysis

Total RNA (2 µg) extracted from nodules was treated with 2 units of RQ1 DNase (Promega, Madison, WI) and then used as a template for a reverse transcriptase (Superscript II, Invitrogen, Carlsbad, CA) reaction primed with the oligonucleotide 5'-CTCCGTGTCCACCACACC-3' (Primer 1) complementary to the iaaM coding region +372 and +389 bp (Figure 1A, Results section). The complementary DNA was amplified with the forward primer 5'-ATGTATGACCATTTTAATTCACCCAGT-3' (Primer 2), corresponding to the region +1/+27 of the iaaM gene (+1 is the initiation of translation), and with the primer 5'-CTGGGAGGAAAGCGCATCGCAC-3' (Primer 3), complementary to the region +283/+304 of the *iaaM* gene.

8.1 Quantitative RT-PCR analysis

100 mg of frozen leaf and root tissues, collected from three different plants, were ground in liquid nitrogen and total RNA was isolated by using Rneasy Plant Mini Kit (QIAGEN), according to the manufacturer's protocol. Root samples were deprived of nodules before freezing. Five µg of total RNA were treated with 5 units of RQ1 DNase (1 U/µl) (Promega,

Madison, WI). All RNA samples were checked for DNA contamination before cDNA synthesis. Comparative PCR analysis was carried out using first strand cDNA obtained with oligo-dT primer and Superscript II (Invitrogen, Carlsbad, CA). The cDNA clones were amplified with gene-specific primers designed to give amplification products ranging from 100 to 150 bp. The nucleotide sequence of the gene-specific primers are the following: *MtPIN1* forward primer 5'-ATGGCTCTGCTGCTGCTGCTAA-3', reverse primer 5'-TCCAGATTGATCAGACGCTCC-3'; *MtPIN2* forward primer 5'-GCATGGGCGGTGGAAGTGGTAA-3', reverse primer 5'- TGGAAGGATCAACAGTGCCA-3'; *MtLAX*1 forward primer 5'-AAACAAGGCGAAGAAACAA-3', reverse primer 5'-ACAGCTAAACCAAGCATCAT-3', *MtLAX2* forward primer 5'-ATGTTGCCACAAAAACAAGG-3', reverse primer 5'-TGAATGAATGATCTTCCACC-3'; *MtLAX3* forward primer 5'-ATGACTTCTGAGAAAGTTGA-3', reverse primer 5'-CTTAGATAATTTGCCAGTAG-3'; actin forward primer 5'-AGATGCTGAGGATATTCAAC-3', reverse primer 5'-GTATGACGAGGTCGGCCAAC-3'.

The nucleotide sequences of the primers used for the qRT-PCR on *MtN5* and *PR1* genes are the followings: *MtN5*, forward primer 5'–ATGGCACATTCTCAGGGCAA–3', reverse primer 5'–GGTTTCTACCGGTAACGAATT–3'; *PR1*, forward primer 5'-ATGAGCTTTAGGTGTTTCAG-3', reverse primer 5'-ATGTTCTGGGGGATCAGAAA-3'. The couple of primers used to analyse the expression of *MtN5* in hairy roots was specifically chosen at the 3' end of the transcript to avoid the amplification of sequences derived from the hairpin construct. The nucleotide sequences of the primers used are the following: forward primer 5'- CTGCGGTTACAAGTCTGCCCTAAC -3', reverse primer 5'–GCGGATCCTTAACAGTTGGAAGGTGTTTG–3'.

The reaction mixture contained Platinum SYBR Green QPCR Supermix-UDG, ROX reference dye to correct for fluorescent fluctuations (Invitrogen, Carlsbad, CA) and 0.4 μM of each primer. UDG and dUTP were included in the mixture to prevent re-amplification of

carryover PCR products between reactions. The QRT-PCR was performed with ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) with the following cycling conditions: 2 min at 50°C, 2 min at 95°C, 40 cycles of 95°C for 30 sec, 56°C for 30 sec, 72°C for 30 sec and finally 72°C for 3 min. All quantifications were normalized to the actin gene as an endogenous control. For each amplification reaction, analysis of the product dissociation curve was performed to exclude the presence of nonspecific amplification. For each determination of mRNA levels, three cDNA samples derived from three independent RNA extractions were analysed. Relative quantification of transcript levels was carried out as previously described (Livak and Schmittigen, 2001).

9. Recombinant MtN5 expression and purification

The sequence (accession n. Y15371) corresponding to the mature MtN5 protein was amplified from cDNA obtained from mRNA of *M. truncatula* roots. The upstream primer was 5'–CATATG*CATCATCATCATCATCAC*GTTCAAATATGTAACATAGACCCAAATGAT–3' (*Nde*I site is underlined, His-tag is in italic) and the downstream primer was 5'–GGATCCTTAACAGTTGGAAGGTGTTTG–3' (*BamH*I site is underlined). The PCR product was double-digested with *Nde*I and *BamH*I and cloned into pET12b (Novagen), giving the recombinant plasmid pET12-N5. The PCR product was checked by sequencing. The recombinant vector pET12-N5 was mobilized into the host strain *E. coli* BL21 DE3 pLysS. MtN5 protein was purified from inclusion bodies using strong denaturing conditions (20 mM Tris HCl pH 8.0, 0,5 M NaCl, 5 mM imidazole, 6 M guanidine hydrochloride) and loaded on a Hi-trap column. MtN5 was refolded applying a linear gradient from 6 M to 0 M guanidine hydrochloride. 2 mg of the recombinant protein was used to produce polyclonal antibodies in rabbit. The polyclonal antibody was produced by PRIMM s.r.l. (Milano, Italy). The purified recombinant protein for libind binding assays and antimicrobial test, and the polyclonal antibody for western blot analysis were kindly provided by Prof. M. Crimi.

10. Lipid binding assay

Lipid binding capacity of MtN5 was assayed by monitoring tyrosine fluorescence. MtN5 protein was dissolved at a suitable concentration in 20 mM TrisHCl pH 7.8, 50 mM NaCl. Changes in fluorescence intensity were measured at 25°C with a Jasco FP-777 spectrofluorimeter using excitation wavelength at 275 nm and recording emission between 280 and 340 nm. Lysophosphatidil-choline dissolved in ethanol was added in a stepwise manner and fluorescence changes were measured after 2 min equilibration.

11. Antimicrobial activity

Antimicrobial activity of the MtN5 protein was assayed by microspectrophotometry on liquid cultures grown in micro-titre plates as described previously (Broekaert *et al*., 1990). Briefly, in a well of a 96-well plate 50 µl of microorganisms (*X. campestris*, *S. meliloti* and *F. semitectum*) cultures at a concentration of 10^4 cfu/ml was mixed with MtN5 recombinant protein at different concentrations. The plates were incubated for 72 h at a suitable temperature according to the microorganism. The antimicrobial effect was estimated as the difference between the optical density, measured at 595 nm, of the treated sample and that of the untreated sample.

12. Plant transformation

Transformation with *A. rhizogenes* ARqua1 was performed as previously described (Boisson-Darnier *et al*., 2001). Plants infected with ARqua1 were kept in square Petri dishes containing Fahraeus Modified Medium (1 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.7 mM KH_2PO_4, 0.8 mM Na_2HPO_4, 0.5 mM NH_4NO_3, 50 µM FeNaEDTA, 0.1 mg/L $MnSO_4$, $CuSO_4$, $ZnSO_4$, H_3BO_3, Na_2MoO_4) for three weeks. When the binary vector employed in the transformation was pBIN19, the Fahraeus Modified Medium (FMM) was supplemented with kanamycin 50 µg/ml for tranformants selection. Well-developed hairy roots were analysed by PCR for the presence of the hairpin gene construct. Up to 95% of the roots tested were positive for the gene construct insertion. The expression of hairpin construct was checked by RT-PCR (see

previous description). The cDNA was amplified with the forward primer 5'–CTGCGGTTACAAGTCCCTAAC–3', designed on *MtN5* coding region, and with the primer 5'–GCCAAGCTTGCGCGATGAAATCAAGTATCCAGT–3', complementary to 5' the region of the *rolC* termination sequence. When the binary vector harboured by *A. rhizogenes* ARqua1 was the pRedRoot, the antibiotic selection in the FMM was not necessary. In pRedRoot the antibiotic resistance-encoding gene was replaced with the gene encoding for the fluorescent protein DsRED1, which provides with a non-destructive selectable marker that allows the discrimination of transgenic roots from non-transformed ones, thus avoiding the use of antibiotics for transgenic root selection (Limpens et al., 2004). Transformed roots were checked using the Leica MZ16F fluorescence microscope using an appropriate filter setting for DsRED1 detection.

For nodulation experiments, plants were nitrogen starved on BMN medium supplemented with 0.1 μM AVG for 7 days and micro-flood inoculated as described above.

13. Plant infection

Fusarium semitectum strain ISCF 20 (Zaccardelli *et al*., 2006) was grown for a week on solid medium PDA (SIGMA). Ten plugs (6 mm diameter) excised from the margin of the fungus colony grown in the Petri plate were transferred to an Erlenmeyer flask containing 270 g of sterile sand, 30 g of corn meal and 60 ml of water. After 3 weeks of incubation at 25°C, the fungal suspension was diluted at 10% and 50% (v/v) with double-sterilised soil. 40 day-old plants, grown on sand and perlite as previously described, were transferred to pots containing, at the bottom, double-sterilised soil and the root apparatuses were covered with the diluted fungal suspension. Plants were collected 48 h after infection for further analysis.

Xanthomonas campestris pv. *alfalfae* was grown for 48 h at 28°C on liquid YDA medium. Bacterial cells were collected by centrifugation at 4500xg for 15 minutes and rinsed twice in 10 mM $MgSO_4$. 40 day-old *M. truncatula* plants, both non-nodulated and nodulated with either IAA-overproducing or control rhizobia strain, were kept for 24 h in high humidity to

allow stomata opening. Leaves were either infiltrate or spray-inoculated with a *X. campestris* suspension at 0.1 OD_{600}. In the first method, the abaxial surface of the leaf was infiltrated with 20 µl of pathogen suspension by means of a syringe. For the time course assay of infection at each time point three leaves in similar position on the plant were collected from three different plants. Each leaf was grinded in 1 ml of 10 mM $MgSO_4$ and the extract was plated on YDA. Plates were incubated at 28°C for 48 h. Negative controls were infiltrated with 20 µl of 10 mM $MgSO_4$.

In the nebulisation method, the whole aerial part of *M. truncatula* plants was spray-inoculated with approximately 5 ml of the *X. campestris* suspension at 0.1 OD_{600}. After infection, plants were kept in high humidity for 24 h and the symptoms of the infection were evaluated 12 days after the pathogen inoculation.

14. Western blot analysis

Total proteins were extracted by grinding frozen tissues in homogenizing buffer (30 mM Tris-HCl pH 8.2, 50 mM KCl, 0.5% TWEEN 20, 0.1% PVPP, 1 mM EDTA) supplemented with 0.04% 2-mecapto-ethanol and 0.1% plants protease inhibitor cocktail (Sigma). The soluble fraction was obtained by centrifugation at 12000 x g for 20' and the protein concentration was determined by Bradford method (Bradford, 1976). Each well was loaded with an equal amount of proteins which were then separated by a 15% Tris-Tricine SDS-PAGE and electro-blotted to PVDF membrane (Amersham). The membrane was probed with the primary polyclonal antibody against MtN5 and afterwards treated with the Alkaline Phosphatase conjugated secondary antibody. The immunoblot was placed in the alkaline phosphatase reaction buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) containing nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as colour developing reagent.

15. Statistical analysis

The mean values ± SE are reported in the figures. Statistical analyses were conducted using a Student's t-test.

Results

1. Auxin and Nodulation

1.1 A new biosynthetic pathway for auxin

In order to increase the auxin biosynthetic capacity of *Sinorhizobium meliloti*, a chimeric operon was produced and consisting of the *iaaM* gene from *Pseudomonas syringae* pv. *savastanoi* and the *tms2* gene from *Agrobacterium tumefaciens* (Figure 1A). The expression of this bicistronic unit was driven by a prokaryotic promoter (*rolAp*) which is the spliceosomal intron of the *rolA* gene of *Agrobacterium rhizogenes* (Magrelli et al., 1994; Pandolfini et al., 2000). The *iaaM* gene encodes for a tryptophan monooxygenase, which converts the tryptophan to indol-3-acetamide (IAM), whilst *tms2* codes for a IAM hydrolase, which is involved in the conversion of IAM in indol-3-acetic acid (IAA). The 85 bp-long *promintron* of the T-DNA gene *rolA* that behaves as a spliceosomal intron when *rolA* is expressed in plant

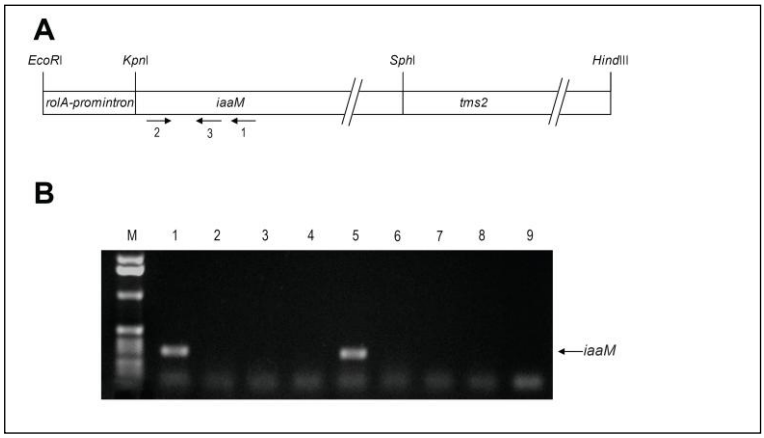

Figure 1. Expression of *rolAp-iaaMtms2* construct in indeterminate root nodules. **A**. Schematic drawing of the chimeric operon. Restriction endonuclease sites used for chimeric operon construction are reported. **B.** Agarose gel electrophoresis of RT-PCR product obtained from total RNA extracted from nodules (lanes 1 and 5) formed by *S. meliloti* IAA strain in *M. truncatula* and *M. sativa*, respectively. Lanes 3 and 7, RT-PCR performed on total RNA extracted from nodules induced by the control strain in *M. truncatula* and *M. sativa*, respectively. Lanes 2 and 4, RNA from nodules of *M. truncatula* induced by IAA and control strain, amplified without reverse transcriptase; lanes 6 and 8, RNA from nodules of *M. sativa* induced by IAA and control strain, amplified without reverse transcriptase. Lane 9, no-template control. The position of the primers used in RT-PCR analysis is indicated by arrows in the schematic drawing reported in panel A.

cells, acts as prokaryotic promoter in free-living rhizobia and in bacteroids inside nodules (Magrelli et al., 1994; Pandolfini et al., 2000).

The *rolAp-iaaMtms2* chimeric operon was introduced in the broad-host range vector pMB393 and the recombinant plasmids were mobilized into *S. meliloti* 1021 to generate auxin-overproducing strains (hereafter named IAA strains). Recombinant vectors containing only the *rolAp* promoter were transformed into rhizobia, with the aim of generating control strains. The expression of the chimeric operon in rhizobia-infected plants was tested through RT-PCR analysis. Total RNA was extracted from 40 day-old nodules of *Medicago truncatula* and *Medicago sativa* plants infected with either IAA or control strain. The cDNA was obtained by using a gene-specific oligonucleotide, positioned on the *iaaM* sequence, as primer for the reverse transcription; the results obtained from the subsequent PCR demonstrate that the chimeric operon is actively transcribed in the mature nodules of both *M. truncatula* and *M. sativa*, produced by the IAA strains (Figure 1B).

The total (free and conjugated) IAA concentration of 40 day-old root nodules was measured by GC-MS using deuterated IAA as internal standard. In control nodules of *M. sativa* the IAA concentration was 0.12 nmol/g FW, whereas the phytohormone was undetectable in the extract obtained from 1 g FW of *M. truncatula* root nodules. Accordingly to the detection limit of the method, it was estimated that the IAA content in control nodules of *M. truncatula* was below 0.010 nmol/g FW. In root nodules of *M. sativa* and *M. truncatula* produced by the IAA strain, the IAA content was 1.2 and 1.14 nmol/g FW, respectively. Therefore, the expression of the *rolA-iaaMtms2* chimeric operon within bacteroids resulted in at least a 10-fold increase in the auxin content of *Medicago* sp. root nodules.

1.2 IAA synthesis by rhizobia affects nodulation and root development

In order to assess the effects eventually caused on nodule formation and plant growth by the increased auxin biosynthetic capacity of bacteroids, roots and shoots growth and nodules number were evaluated in both *M. truncatula* and *M. sativa* plants inoculated with either IAA

or control *S. meliloti* strains. Three days after germination (dag), seedlings were transferred in pots containing sand and agriperlite and 10 dag they were inoculated with *S. meliloti* suspensions of either IAA or control strain. Samples were collected 40 dag.

The average number of nodules per plant had doubled in *M. truncatula* plants nodulated with the IAA strain when compared to plants infected with the control strain (Fig. 2A and 2C). A similar stimulatory effect was also observed in *M. sativa* plants (Fig. 2B and 2C), where the mean number of nodules produced by the IAA strain was approximately 50% higher than plant inoculated with the control strain (Fig.2C).

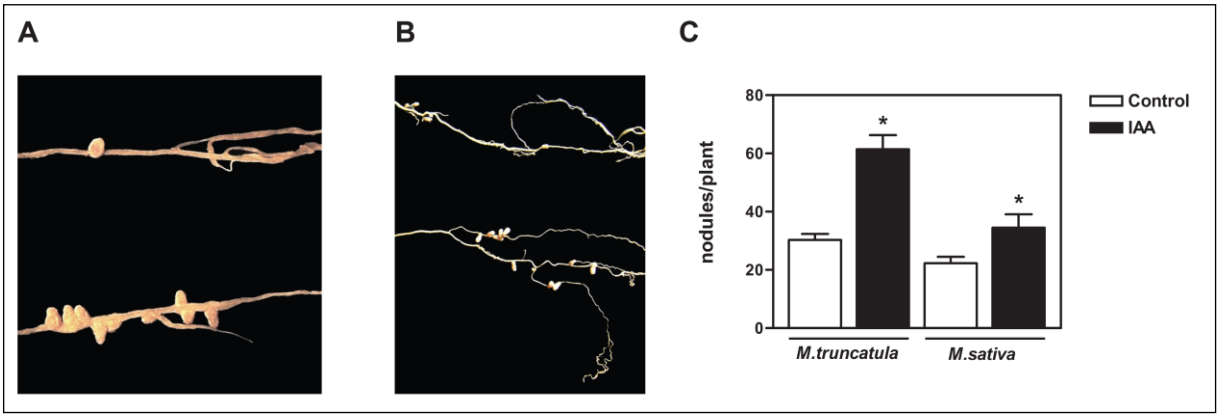

Figure 2. Nodulation in *M. truncatula* and *M. sativa* plants infected with *S. meliloti* IAA or control strain. **A.** *M. truncatula* root nodules: nodules induced by *S. meliloti* IAA strain (bottom) and nodules induced by the *S. meliloti* control strain (top). **B**. *M. sativa* root nodules: nodules induced by the *S. meliloti* IAA strain (bottom) and nodules induced by *S. meliloti* control strain (top). **C**. Number of nodules per plant. The values reported are means ± SE (n = ≥22). *, P < 0.05. Control: plants nodulated by the control strain. IAA: plants nodulated by the IAA strain.

The increased auxin synthesis within bacteroids had also effects on the development of root apparatuses. In *M. truncatula* plants inoculated with *S. meliloti* IAA the primary root length was on average 40% longer than that of plants bearing nodules elicited by the control strain (Fig. 3A and 3C), whereas in *M. sativa* the primary root growth resulted unaffected by the overproduction of IAA within bacteroids (Fig. 3B and 3C). The lateral root growth, which was calculated as weight of total root apparatus (expressed in mg FW) normalized to the length of the primary root (expressed in cm), was on average two times higher in *M. truncatula* plants nodulated by the IAA-overproducing strain (mean ± SE = 26 ± 2.6 mg/cm; n = 18) when

compared to plants nodulated with the control strain (mean ± SE = 13 ± 1.2 mg/cm; n = 18) (Fig 4A and 4B). A similar effect on lateral root growth was also observed in *M. sativa* plants infected with the IAA strain (Fig. 3B).

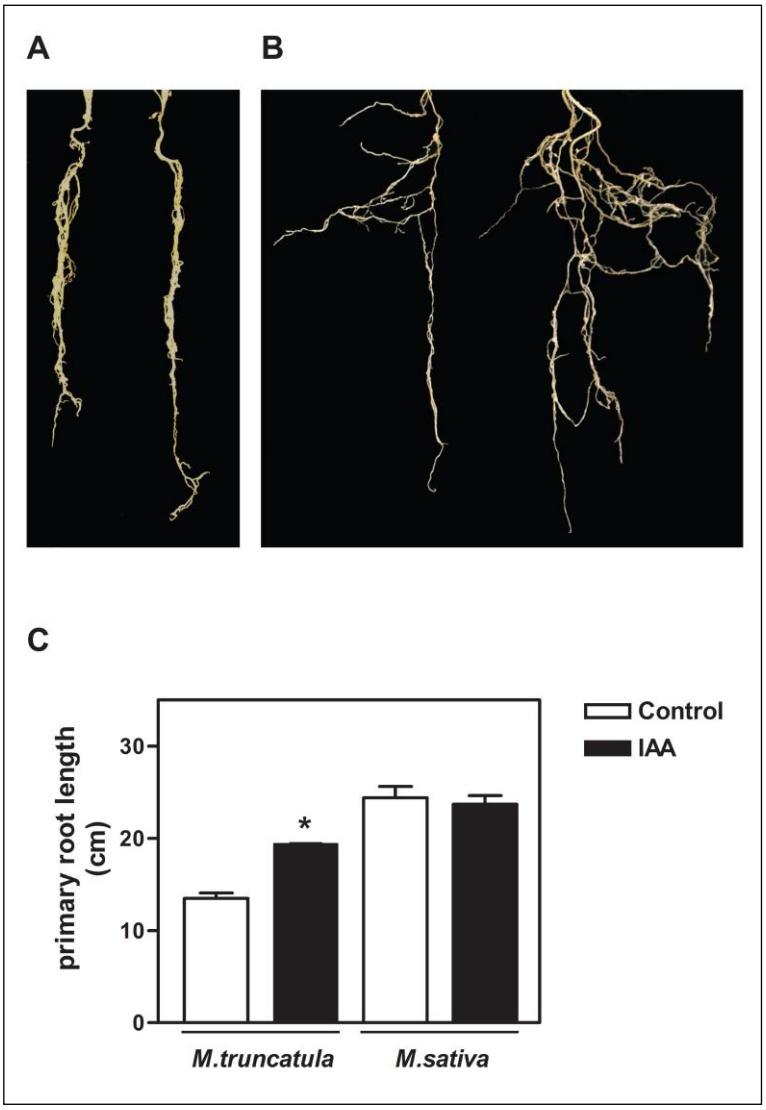

Figure 3. Root phenotypes of *M. truncatula* and *M. sativa* plants nodulated with *S. meliloti* IAA or control strain. A. *M. truncatula* primary roots of plants nodulated by the IAA strain (right) and roots of plants nodulated by the control strain (left) **B.** *M. sativa* roots of plants nodulated by the IAA strain (right) and roots of plants nodulated by the control strain (left). **C.** Primary root length. The values reported are means ± SE (n = ≥22). *, P < 0.05. Control: plants nodulated by the control strain. IAA: plants nodulated by the IAA strain.

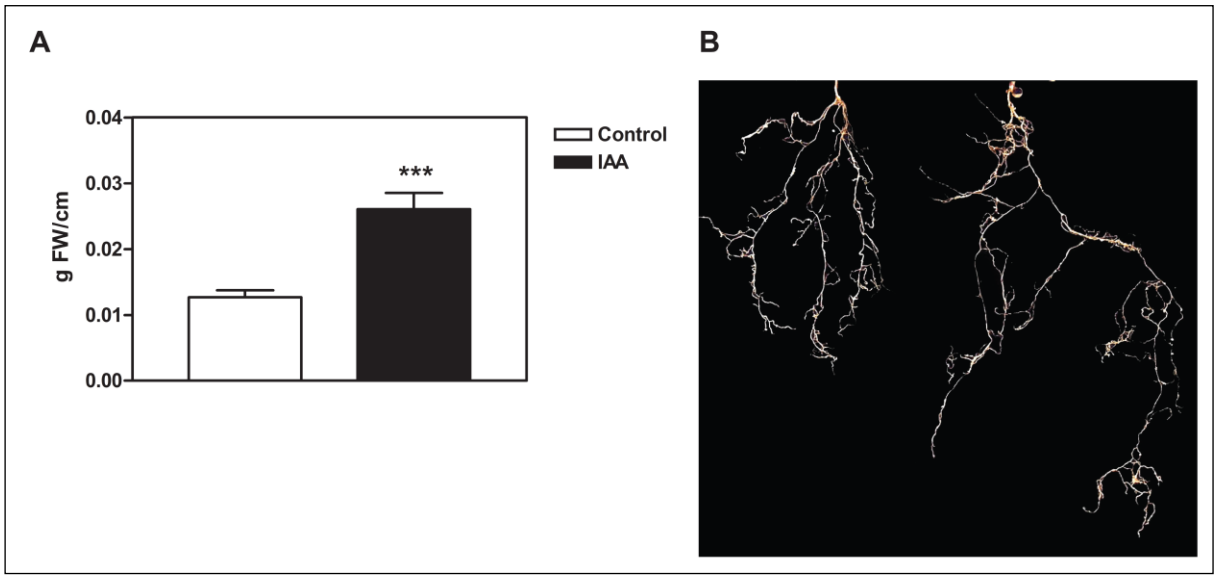

Figure 4. Lateral roots of *M. truncatula* plants. A. Quantitative evaluation of lateral root growth in *M.. truncatula* plants nodulated with either IAA-overproducing or control *S. meliloti* strain. Values reported are mean ± SE g FW/length of the primary root (n= 18; ***, P < 0.0001). **B.** Representative picture of lateral root growth in *Medicago truncatula* plants nodulated with control rhizobia strain (left) and IAA strain (right).

We also investigated the relationship between the number of lateral roots and the number of nodules present on them. In *M. truncatula* plants inoculated with the IAA-overproducing strain, these two parameters were significantly correlated (Fig. 5A), although they were not in plants bearing control nodules (Fig. 5B). Altogether, these data suggest that *S. meliloti* IAA has a greater capacity of nodule induction when compared to the control strain and that it has also a stimulatory effect on lateral root growth. These observations are further confirmed by the fact that when *M. truncatula* plants were grown in conditions that limit the lateral root growth (i.e. in 15 ml plastic tubes), no difference in the lateral root growth was detected (mean ± SD = 11 ± 3 and 10 ± 3 mg root FW/cm primary root length in IAA-overproducing and control strain inoculated plants, respectively), whereas a higher nodule density was observed in plants inoculated with *S. meliloti* IAA compared to those inoculated with control strain (mean ± SD = 0.12 ± 0.04 and 0.08 ± 0.02 nodules/mg root FW with IAA and control strain respectively; $P < 0.05$, n = 12) (Fig. 5C). Furthermore, micro-flood inoculation of the primary root of *M. truncatula* plants showed that *S. meliloti* IAA was able to induce the development of approximately 50% more nodules with respect to the control strain (Fig. 5D).

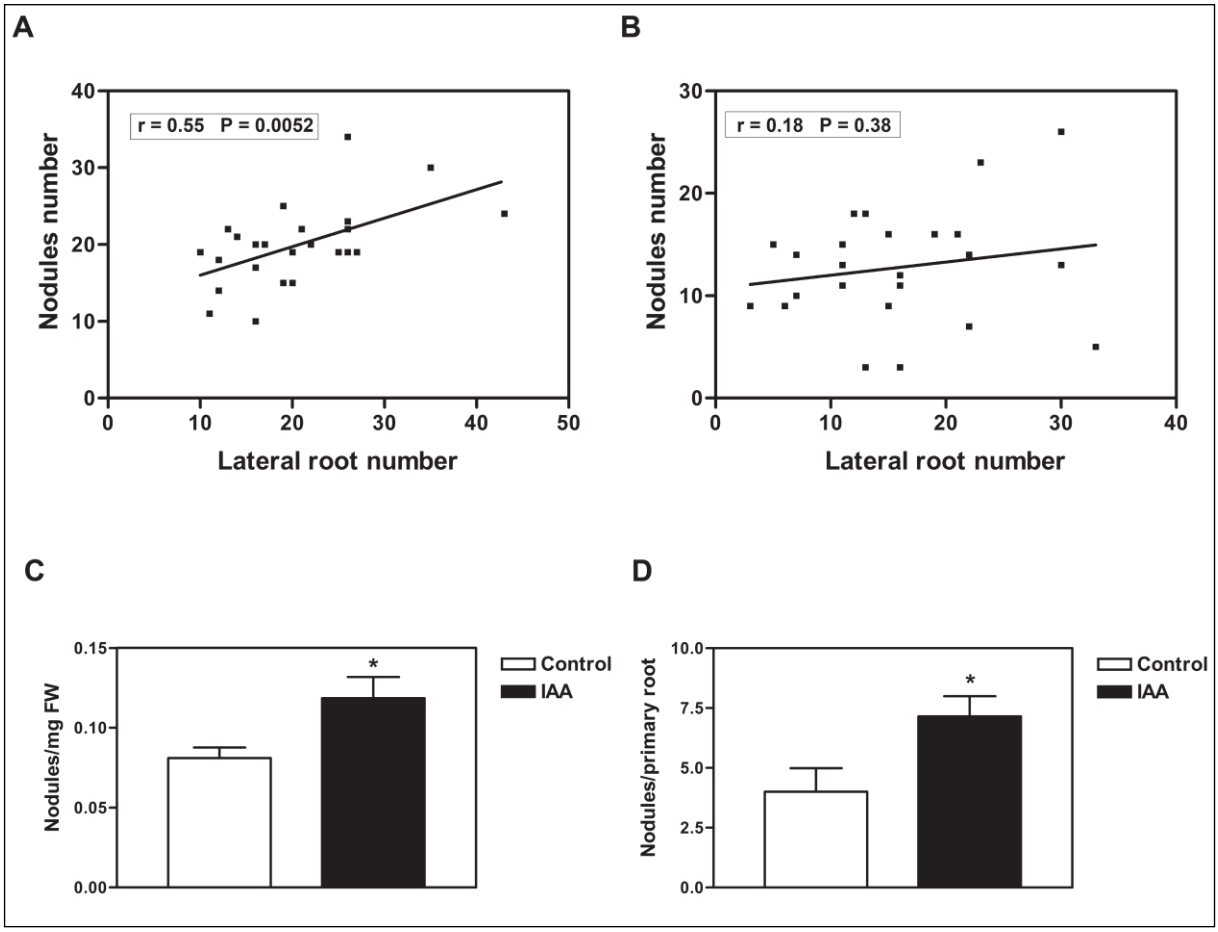

Figure 5. Lateral root and nodule number in *M. truncatula* plants. A. Statistical correlation between the number of lateral roots and the number of nodules developed on lateral roots in *M. truncatula* plants nodulated with *S. meliloti* IAA. **B.** Statistical correlation between the number of lateral roots and the number of nodules developed on lateral roots in *M. truncatula* plants nodulated with *S. meliloti* wild-type. **C.** Density of nodules, measured as number of nodules/root weight in *M. truncatula* plants grown in 15 ml plastic tubes. Values reported are means ± SE (n = 12; *, P < 0.05). **D.** Number of nodules developed by *M. truncatula* plants micro-flood inoculated on the primary root. Values reported are means ± SE (n = 19; *, P < 0.05). Control: plants nodulated by the control strain. IAA: plants nodulated by the IAA strain.

Thus, under conditions that limit the lateral root growth or in primary root inoculation experiments, the IAA strain still retained a higher capacity to induce nodule development, suggesting that the stimulatory effect on nodulation is not the consequence of the increased later root growth.

No significant differences were observed in the growth of the aerial parts (measured as shoot height) between plants nodulated with either IAA or control *S. meliloti* strain in both *M. truncatula* and *M. sativa* (Fig. 6A). These observations were also confirmed by the evaluation of dry matter production and the total protein concentration in the aerial parts that did not vary irrespectively of the *S. meliloti* strain used (Fig.6B).

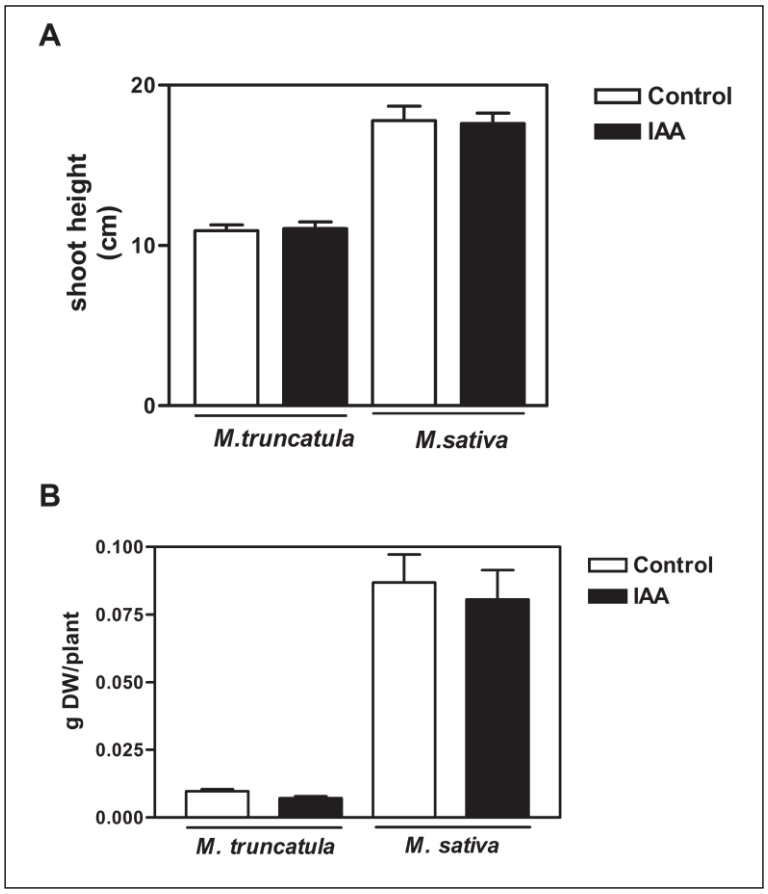

Figure 6. Shoot growth of *M. truncatula* and *M. sativa* plants infected with *S. meliloti* IAA or control strain. A. Shoot height. Values reported are means ± SE (n ≥ 22). **B.** Measure of the dry weight of the aerial part of *M. truncatula* and *M. sativa* plants. Values reported are means ± SE (n = 21). Control: plants nodulated by the control strain. IAA: plants nodulated by the IAA strain.

1.3 Auxin distribution

The polar auxin transport is crucial for the majority of auxin-related developmental processes and is based on the asymmetric distribution of specific auxin influx and efflux carriers (Friml, 2003). To investigate whether the rhizobia-derived auxin could influence the expression of plant auxin transporters, the steady state mRNA levels of selected putative influx and efflux carrier was evaluated by means of quantitative RT-PCR (qRT-PCR) analysis in *M. truncatula* plants nodulated by both IAA and control *S. meliloti* strains. Several members of the LAX and PIN gene families, involved in auxin transport, were identified in *M. truncatula* (de Billy et al., 2001; Schnabel and Frugoli, 2004). In particular, three auxin influx carrier genes, *MtLAX1*, *MtLAX2* and *MtLAX3*, known to be expressed in the nodulated roots (de Billy et al., 2001), and two efflux facilitator genes, the root-specific *MtPIN2* and *MtPIN1* expressed in the whole plant (Schnabel and Frugoli, 2004), have been studied.

The steady state mRNA levels of *MtPIN2* were significantly higher in the root tissue of plants bearing IAA-overproducing nodules when compared to plants nodulated with *S. meliloti* control strain (Fig. 7A). The expression of *MtPIN1* and of the other three transporters tested in roots nodulated with *S. meliloti* IAA did not significantly differ from that detected in roots bearing control nodules (Fig. 7A).

The same analysis was also performed on mRNA extracted from the aerial parts of *M. truncatula* plants nodulated with either IAA or control strain and the qRT-PCR did not highlight any statistically significant modification in transporter genes expression between the two samples (Fig. 7B).

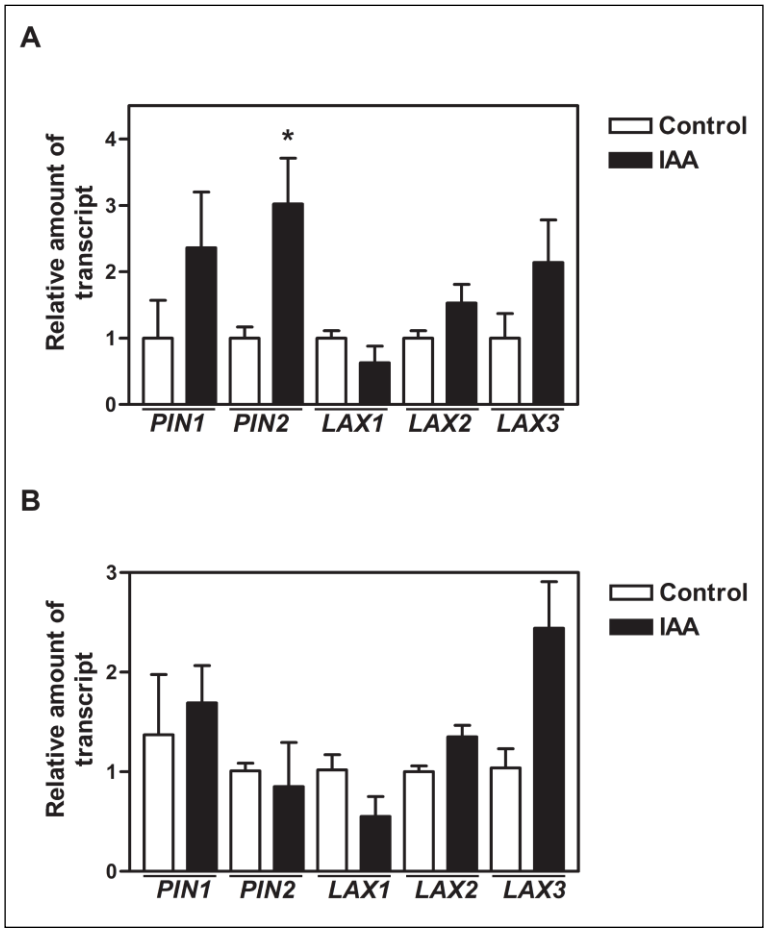

Figure 7. Expression of auxin carrier genes in *Medicago truncatula*. A. Expression levels in root tissue. **B.** Expression levels in shoot. Expression levels of auxin efflux (PIN1 and PIN2) and influx (LAX1, LAX2 and LAX3) carrier genes were evaluated by quantitative RT-PCR (QRT-PCR). The expression levels were normalized using actin as the endogenous control gene. Relative transcript level is the ratio between the expression levels in plants nodulated by the IAA strain and plants nodulated by the control strain. Relative transcript levels were calculated using the formula $2^{-(\Delta Ctiaa-\Delta Ctc)}$, where ΔCtiaa and ΔCtc is the difference between the threshold cycle of the gene tested and the threshold cycle of actin in IAA and control samples, respectively. The significance of the differences between control and IAA expression levels was evaluated using a Student's *t* test (n = 3). Mean values ± SE are reported. *, $P < 0.05$. Control: plants nodulated by the control strain. IAA: plants nodulated by the IAA strain.

2. Nitric Oxide (NO) and nodulation

The endogenous NO production was investigated in root nodules from *Medicago* plants nodulated by either the IAA or the control *S. meliloti* strain (Fig. 8), in collaboration with Prof. Crimi. The gaseous molecule NO was detected by means of the permeable NO-sensitive dye fluorophore 4,5-diaminofluorescein diacetate (DAF-2-DA). The results obtained clearly show that NO production is significantly enhanced in both *M. truncatula* and *M. sativa* IAA-overproducing nodules when compared to control nodules (Fig. 8). The increase in NO production was about 3 times and 2 times in *M. truncatula* and *M. sativa* respectively (Fig. 8C and 8D).

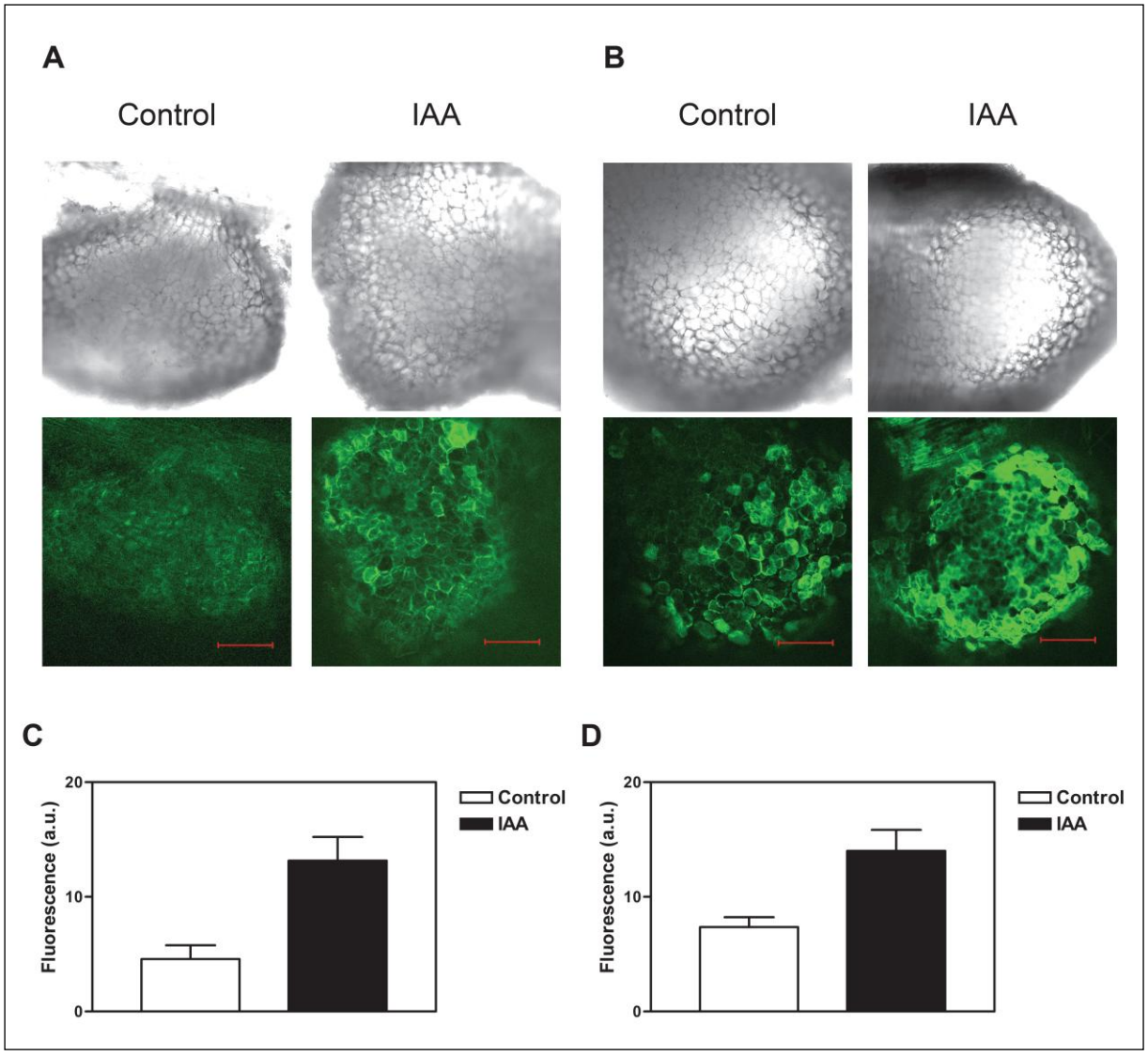

Figure 8. Evaluation of NO production in nodules. A. *M. truncatula* nodulated with *S. meliloti* IAA or control strain **B.** *M. sativa* nodulated with *S. meliloti* IAA or control strain. Upper panels: Microscopy. Bright-field images of the nodules (top) and the confocal laser scanning microscopy (CLSM) detection of endogenous NO in the same nodules (bottom, excitation at 488 nm, emission at 505–530 nm). Bars indicate 200 μm. Photographs are representative of results obtained from the analysis of nodules in three independent experiments. **C.** Analysis of fluorescence intensities in nodules induced in *M. truncatula* plants by IAA and control *S. meliloti* strain. **D.** Analysis of fluorescence intensities in nodules induced in *M. sativa* plants by IAA and control *S. meliloti* strain. Results are means ± SE (n ≥ 15); all data are statistically significant (P < 0.05). IAA: nodules produced by the *S. meliloti* IAA strain. Control: nodules produced by the *S. meliloti* control strain.

It has been demonstrated that some plants associated rhizobacteria are able to generate NO from the conversion of L-arginine to L-citrulline, by means of an NO synthase (NOS) activity (Cohen and Yamasaki, 2003). A radiolabelled L-arginine-based conversion assay was applied aiming to evaluate the production of NO by free-living *S. meliloti* at stationary phase of growth; it was observed that the IAA strain, grown under aerobically conditions and with

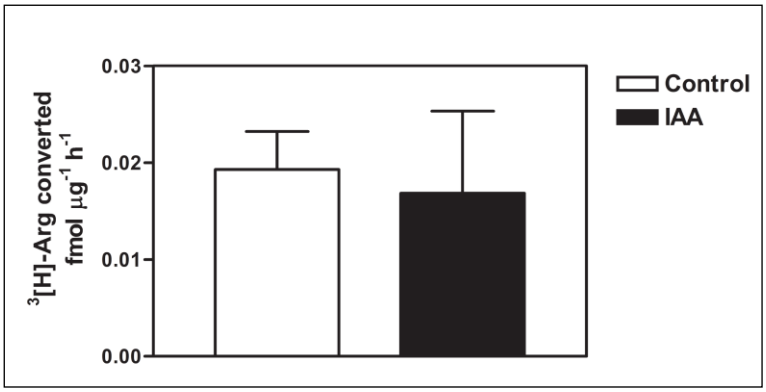

Figure 9. NOS-like activity assay. NOS-like activity was determined by the method based on the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline, by using the NOS Activity Assay Kit (Cayman Chemical, Ann Arbor, MI). Total protein concentration was measured by Bradford method and the enzyme activity was expressed as fmol arginine μg protein^{-1}h^{-1}. Values reported are means ± SE (n = 3). IAA: *S. meliloti* IAA strain. Control: *S. meliloti* wild-type.

ammonium salts as nitrogen source, can use L-arginine as substrate (Fig. 9). The same enzymatic activity is retained, to the same extent, by the control strain as well (Fig. 9). These data suggest that free-living *S. meliloti* can produce NO through an NOS-like activity and demonstrate that the two strains do not exhibit significant difference in the amount of NO generated (Fig. 9). Such NO-forming activity in bacteroids might contribute to the NO production within the nodule.

In order to asses a possible link between NO and indeterminate nodule formation, the effects of the NO scavenger, 2-(4-carboxyphenil)-4,4,5,5,-tetramethylimidazoline-1-oxyl-3oxide

(cPTIO) on nodulation were evaluated. *M. truncatula* plants grown on N-free nutrient solution were inoculated with IAA and control *S. meliloti* strains and 1 mM cPTIO was applied after 2, 24 and 48 hours . The NO depletion due to cPTIO treatment caused a significant reduction in

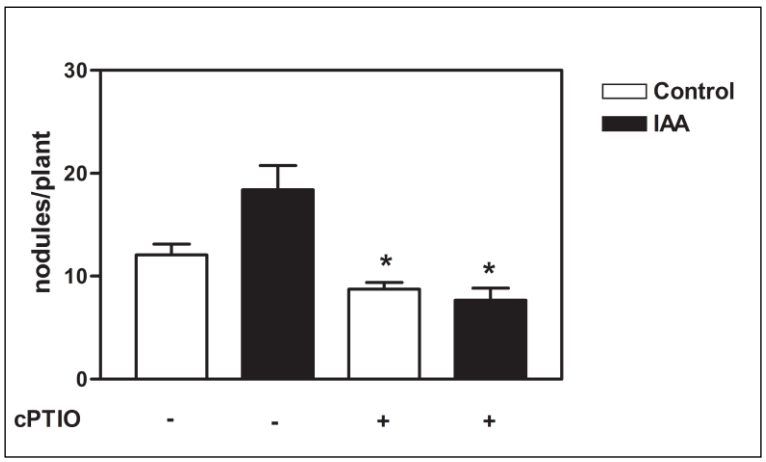

Figure 10. Effects of the NO scavenger cPTIO on nodulation. Nodule number of *Medicago truncatula* plants inoculated with IAA and control strain and treated with 1 mM cPTIO. Results are means ± SE (n = at least 12; *, P < 0.05). cPTIO treatments are significantly different from respective controls, (P < 0.05). IAA: nodules produced by the *S. meliloti* IAA strain. Control: nodules produced by the *S. meliloti* control strain.

the number of nodules developed by plants inoculated with either the IAA or control *S. meliloti* strain (Fig. 10). These data demonstrate that the NO depletion inhibit the indeterminate nodule development and completely abolish the stimulatory effects due to the IAA-overproduction in rhizobia. Moreover, the decrease in the number of nodules was not attributable to a toxic effect of cPTIO on the symbiont, since the growth of *S. meliloti* was not affected by the scavenger, even when it was applied at a 5 times higher concentration with respect to that adopted in the NO depletion assay (Fig. 11).

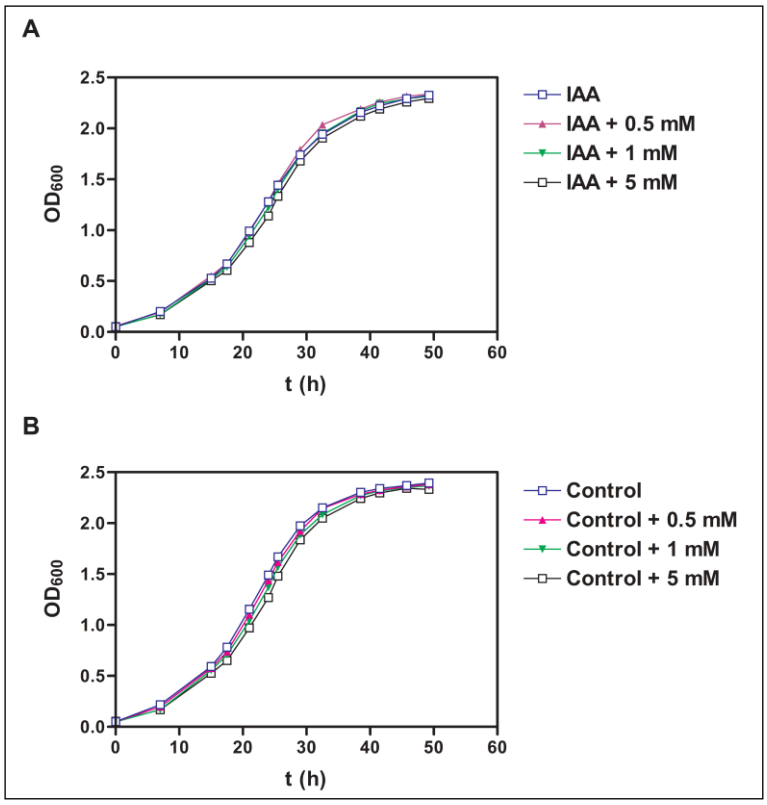

Figure 11. Effect of cPTIO on *S. meliloti* growth. A. Growth curves of *S. meliloti* IAA in minimal medium supplemented with 0.5, 1 and 5 mM cPTIO. **B.** Growth curves of *S. meliloti* wild type in minimal medium supplemented with 0.5, 1 and 5 mM cPTIO. Each point represents the average value of three measurement and is reported as mean ± SE. IAA: *S. meliloti* IAA strain. Control: *S. meliloti* wild-type.

3. Auxin, Rhizobium symbiosis and plant defence responses: a complicated interplay

The symbiosis with nitrogen-fixing bacteria is characterized by the close association between legumes and rhizobia, and, in the early phases of rhizobia-host interaction, some bacterial determinants activate plant innate immune responses. However, plant defence is generally depressed during the symbiotic interaction in order to allow root colonization. Nevertheless, it has been observed that some defence reactions can take place in legumes-rhizobia symbiosis and they are thought to be part of the mechanism that controls the extent of the infection (Mithöfer, 2002). The transcriptome analysis of *M. truncatula* root nodules highlighted that two transcripts connected to biotic defence responses are significantly induced during infection: the pathogenesis related protein 1 (PR1), which is known to be involved in SAR, and *MtN5*, which shares sequence similarities with LTPs (El Yahyaoui et al., 2004).

It has recently been demonstrated that *A. thaliana* plants react against pathogenic bacteria by repressing the auxin signalling pathway, through the agency of the miR393, and that the

application of exogenous auxin can enhance plants susceptibility to bacteria (Navarro et al., 2006). It was then hypothesised that pathogenic microorganisms could have evolved mechanisms to circumvent plants' innate immunity by stimulating plant IAA biosynthetic pathways or by producing auxin themselves (Robert-Seilaniantz et al., 2007). Following on from these observations, we wondered whether the increased auxin synthesis in the root nodule might affect the plant response against both rhizobia, thus allowing a higher level of nodulation, and pathogenic microorganisms, which would result in a higher sensitivity to pathogens. We have investigated whether the expression of the two early-induced genes, PR1 and MtN5, putatively involved in *M. truncatula* response against pathogens, was affected in plants nodulated by *S. meliloti* IAA strain. A qRT-PCR was carried out on both root and shoot samples of 40 day-old *M. truncatula* plants nodulated by either the IAA or the control *S. meliloti* strain (Fig. 12A and 12B). The expression of *PR1* did not significantly differ in both roots and shoots of *M. truncatula* plants nodulated with *S. meliloti* IAA and control strain (Fig. 12A). The steady state mRNA levels of *MtN5* resulted significantly higher (about 100%) in the root apparatuses of plants nodulated with *S. meliloti* IAA as compared with roots of plant infected with the control strain, whereas the relative abundance of *MtN5* transcript in the shoots did not show any significant difference between plants bearing IAA-overproducing nodules and those bearing control nodules (Fig. 12B).

The mature MtN5 has been expressed as recombinant protein in *E. Coli* (Fig. 12C) (see Matherials and Methods section) and used to produce polyclonal antibodies. In *M. truncatula* protein extracts such antibody detects a single principal band with the same apparent molecular mass of the recombinant protein (Fig. 12D). This polyclonal antibody has been employed to study the expression of MtN5 protein in plant tissues. The western blot analysis performed on total root proteins extracted from plants nodulated with the two different rhizobia strains confirmed the data obtained from the analysis of steady state mRNA levels (Fig.12D). The influence of exogenous auxin on *MtN5* expression was evaluated in 7-day-old plants both non-nodulated and nodulated with *S. meliloti* wild-type, by treating the seedlings with 1 μM IAA for 24 hour. The western blot analysis (Fig. 12E) demonstrated that the

amount of MtN5 protein in the root apparatus of both nodulated and non-nodulated plants was not affected by the auxin treatment. These data collectively indicate that the over-production of auxin within the bacteroids did not negatively affect the expression of *PR1* and *MtN5* genes, when compared to plants nodulated with control rhizobia.

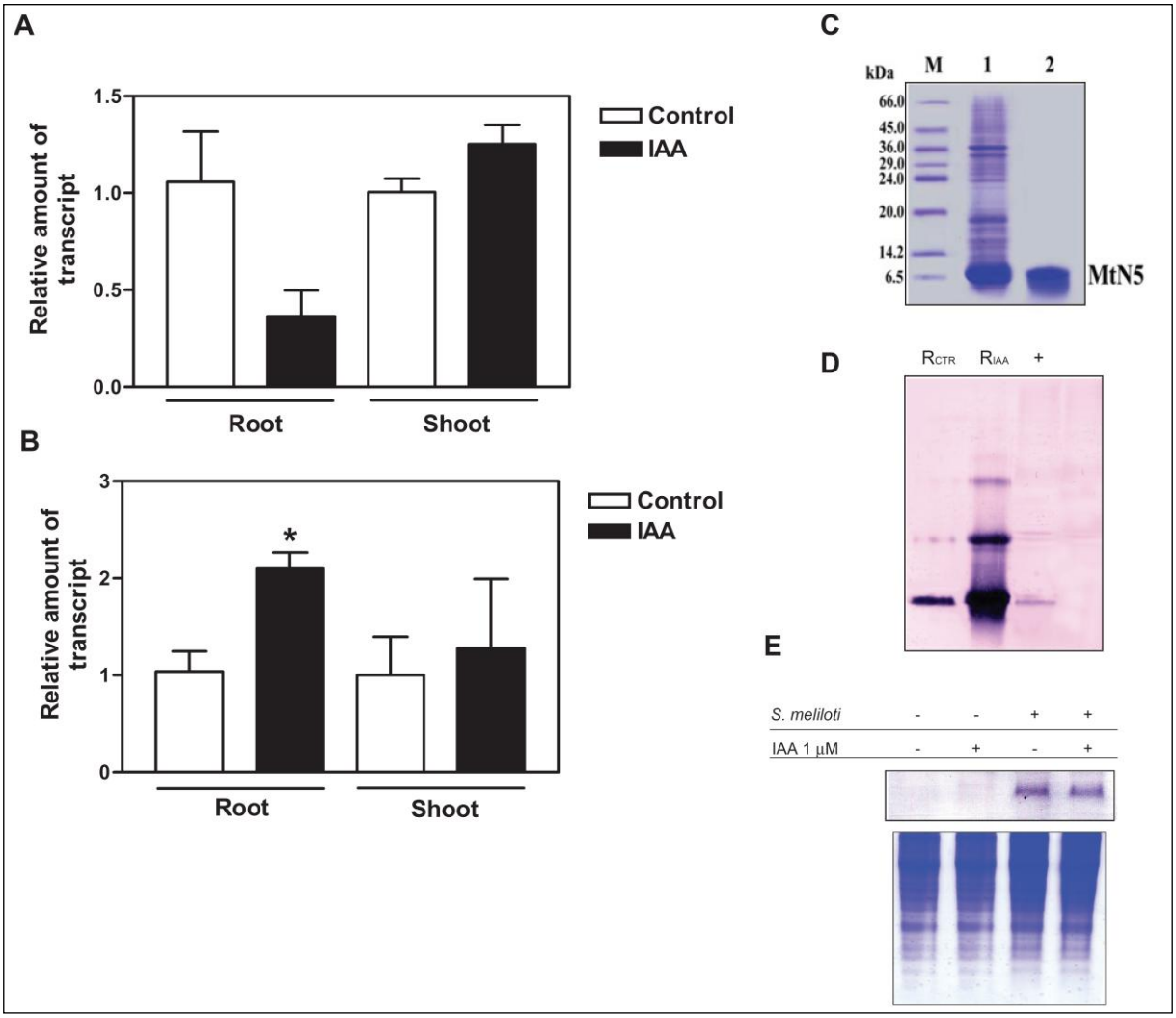

Figure 12. *PR1* and *MtN5* expression upon rhizobia infection. A. Expression pattern of *PR1* gene in root apparatus and aerial part of *M. truncatula* plants nodulated with either *S. meliloti IAA* or control strain assessed by qRT-PCR. **B.** Expression pattern of *MtN5* gene in root apparatus and aerial part of *M. truncatula* plants nodulated with either *S. meliloti IAA* or control strain assessed by qRT-PCR. **C.** Expression and purification of recombinant MtN5 protein (Coomassie staining). Lane1: *E.coli* BL21 lysate. Lane 2: Purified MtN5. **D.** Western blot analysis carried out on the whole root apparatus of plants nodulated with *S. meliloti IAA* (R_{IAA}) and with *S. meliloti* wild type (R_{CTR}). The protein extracts from the root apparatuses were quantified by Bradford method and an equal amount of protein was loaded in each lane. +, positive control, i.e. recombinant MtN5 protein. **E.** Effect of exogenous IAA on MtN5 levels in roots. *M. truncatula* plants either non-nodulated or nodulated with *S. meliloti* control strain were treated with IAA 1 µM. after 24 h of treatment the total proteins were extracted and quantified by Bradford method. An equal amount of protein was loaded in each lane, as assessed by Coomasie staining (lower panel). Negative controls are represented by plants kept for 24 h in nutritive mineral solution, without IAA added. IAA: plants nodulated with *S. meliloti* IAA strain. Control: plants nodulated with *S. meliloti* wild-type.

3.1 MtN5

MtN5 gene (Y15371) was isolated by means of a subtractive hybridization approach (Gamas et al., 1996) carried out on *M. truncatula* roots infected with *S. meliloti*. *MtN5* was identified as an early nodulin gene, strongly induced in the early phases of nodulation, and thus recognized as a precocious marker of the symbiotic association (Gamas et al., 1996). *MtN5* was annotated as a putative lipid transfer protein (LTP), on the basis of its predicted amino acid sequence that contains the eight cystein motif, considered to be the signature of plant LTPs (El Yahauoi et al., 2004).

MtN5 encodes for a 643 base-long mRNA which produces a putative protein of 102 amino acid residues (Fig 13). On the basis of the protein sequence (CAA75593), the molecular mass is estimated to be approximately 10.8 kD with an isoelectric point of about 9. MtN5 protein is predicted to have a 27 residue-long signal peptide, which is excised (Fig.12C) and most likely targets the mature protein towards the secretory pathway, consistently with what has been already observed for other members of this protein family. The mature protein has a molecular weight of 8 kD, thus positioning MtN5 in-between the family 1 LTPs(10 kD) and the family 2 LTPs (7 kD).

>Y15371 *Medicago truncatula* mRNA for *MtN5* gene

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TTAGCTAAGCATTTGGTTAATTAATTAGGAGTATGGCACATTCTCAGGGCAAAGCTTTGGCGCAGTGGAT
GATAGGAGCACTGCTCTTTGCCATGTTGGCCGGCAGTCTAGCTGTTCAAATATGTAACATAGACCCAAAT
GATCTAAAACAATCGTGTAGTAAATTCGTTACCGGTAGAAACCCGCCAAGGGCAGATGAGGCTTGCTGTG
GTGTTTTGCGCCGTGCCAATCTTCCTTGCCTCTGCGGTTACAAGTCTGCCCTAACTTATTATGGAATCAA
TGCCAAAAAAAGCTTTGGCCTTGCCCGGTCAATGTGGTCTGCAAACACCTTCCAACTGTTAAGTGAGGTGT
AGGTCTCCTCCATTAAAAGCAATTCTTCTTCATGTGTATTGCTAACACTAGAGAAGCTTTAAGCAGCATT
TGAAGCCTTTCCAAATAAAAAAAGTAGTTTATTTTTATCAGTGTGTTTTGTCCATATGTTTGCAAGCAAA
TATGTGGTGTATCAGTGTCTGATGTTGTAGTATTTGATGTACAAGAATACATGTCTCAAAAATATCAAGA
AGACATCGATCATCCTTCCCATTGCAACCTGTAGTTGGTTTAATAAAAAATGTTTTTCCTTAAAAAAAAA
AAAAAAAAAAAA
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>CAA75593 MtN5

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MAHSQGKALAQWMIGALLFAMLAGSLAVQICNIDPNDLKQSCSKFVTGRNPPRADEACCGVLRRANLPCL
CGYKSALTYYGINAKKALALPGQCGLQTPSNC
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Figure 13. MtN5 sequences. Upper panel. Nucleotide sequence of the mRNA encoded by *MtN5* gene. **Lower panel.** Amino acid sequence of MtN5 protein.

The sequence of the mature MtN5 protein was compared with the sequences of proteins belonging to the two different LTP families (type 1 and type 2) (Fig. 14A and 14B). The multiple sequence alignment with representative members of LTP1 family showed that the homology is restricted to the eight cysteine motif and a few other residues (14.5% identity, 34.2% similarity). However, considering the overall homology , MtN5 showed higher identity and similarity with type 2 LTPs (21% identity, 38% similarity) (Fig. 14B). Besides the eight strictly conserved cysteine, MtN5 displays several residues (i.e. L12, P25, L44, Y54 and I56) which were demonstrated to be crucial for the folding and the lipid binding activity of LTP2 (Fig. 14B) (Cheng *et al.*, 2007). In addition to these typical conserved amino acids found in the known LTPs2, in the alignment shown in figure 14B we can observe that MtN5 presents some peculiar characteristics with respect to the other LTPs2. When the sequences were aligned to maintain the position of the conserved cysteine motif, it was necessary to introduce two gaps in the other members of the LTP2 family, corresponding to residues I7-D8 and D29-E30 of MtN5. Thus, MtN5 looked like a peculiar type 2 LTP. Interestingly the alignment between DIR1, recently defined as a new type of LTP (Lascombe *et al*., 2008), and MtN5 (Fig. 14C) highlighted a higher homology degree (37% identity, 83% similarity) with respect to the other type 2 LTPs, even though the isoelectric point of DIR1 is acidic (pI 4.5) whereas MtN5 is a basic protein (pI 9.0).

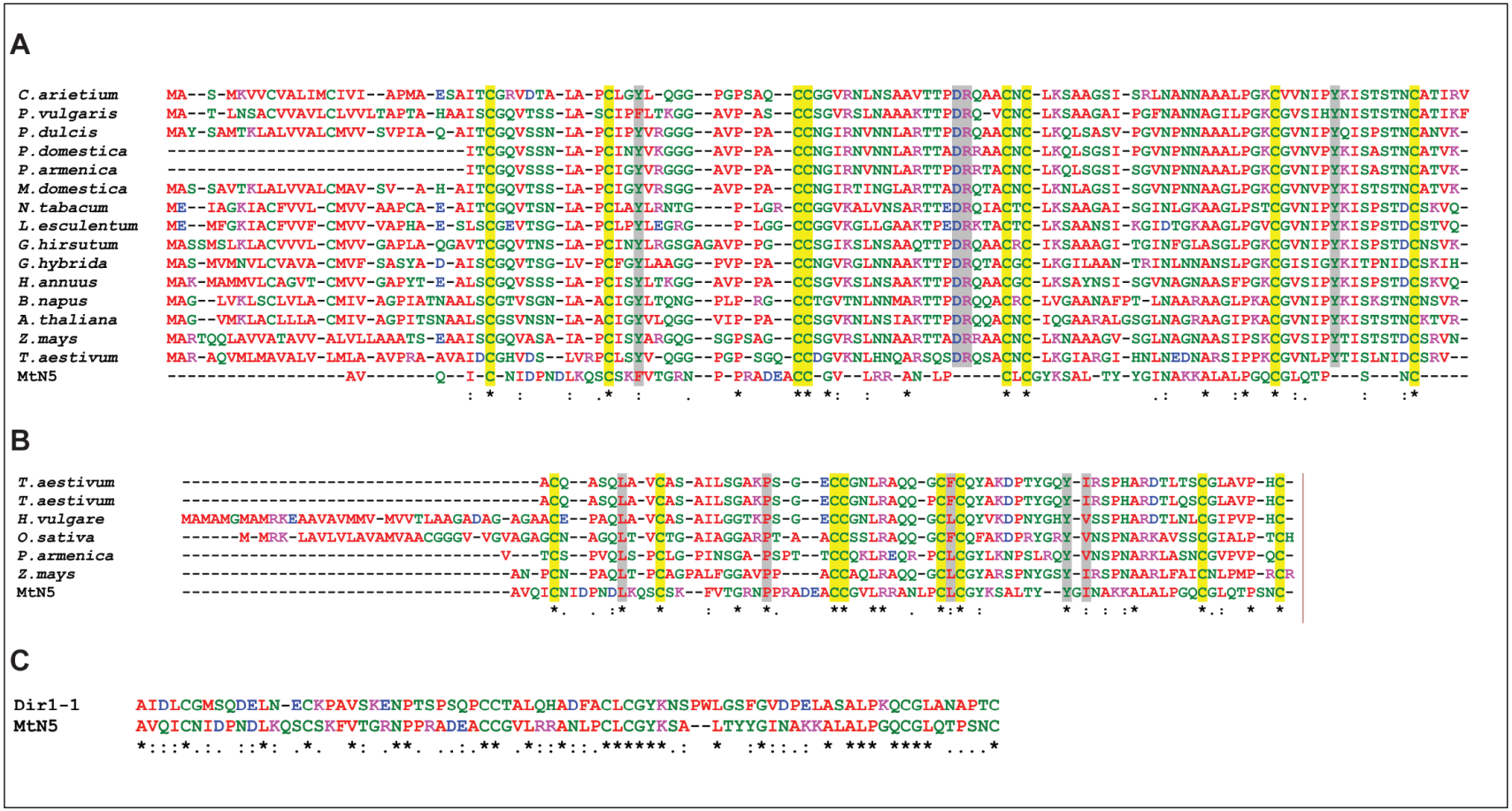


Figure 14. Comparison of the amino acid sequences of MtN5 and various plant LTPs. The sequence of the proteins were obtained from SWISS-PROT and aligned with the Clustal W (Thompson et al., 1994). The residues highlighted in yellow indicate the eight cysteine motif, whereas the residues that are fundamental for the folding and the lipid binging activity are boxed in light grey. **A.** Multiple sequence alignment between members of LTP1 family and MtN5. The data bank accession numbers of the protein are the following: *Cicer arietinum* (O23758), *Phaselus vulgaris* (O24440), *Prunus dulcis* (Q43017), *Prunus domestica* (P82534), *Prunus armeniaca* (P81651), *Malus domestica* (Q9M5X7), *Nicotiana tabacum* (Q42952), *Lycopersicon esculentum* (P93224), *Gossypium hirsutum* (Q9FVA5), *Gerbera hybrida* (Q39794), *Helianthus annuus* (Q39950), *Brassica napus* (Q42614), *Arabidopsis thaliana* (Q42589), *Zea mays* (P19656), *Triticum aestivum* (Q8GZB0). **B.** Multiple sequence alignment between members of LTP2 family and MtN5. The data bank accession numbers of the proteins are listed as follows: *Triticum aestivum* (P82900 and P82901), *Hordeum vulgare* (P20145), *Oryza sativa* (P83210), *Prunus armeniaca* (P82353) and *Zea mays* (P83506). **C.** Pairwaise alignment between MtN5 and *Arabidopsis thaliana* DIR1 (Q8W453).

Phylogenetic analysis was conducted to assess the relationships between MtN5 and several well characterized LTPs from other plant species, also including *A. thaliana* DIR1. The phylogenetic tree shown in figure 15 suggests that the proteins analyzed could be divided into three groups, two of which consist of family 1 and family 2 LTPs. MtN5 and DIR1 grouped independently from the two major LTP families, despite displaying a higher degree of homology with family 2 LTPs.

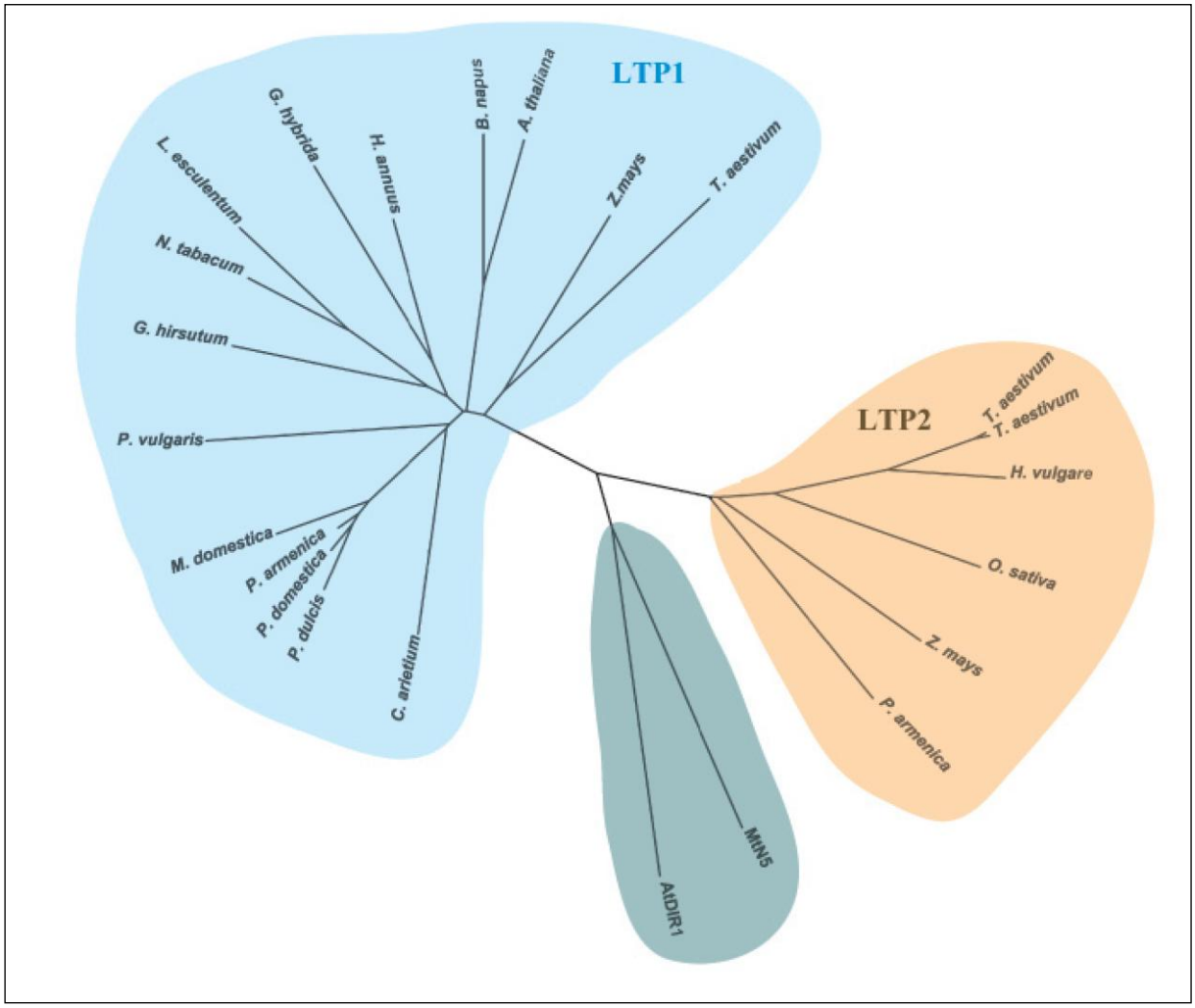
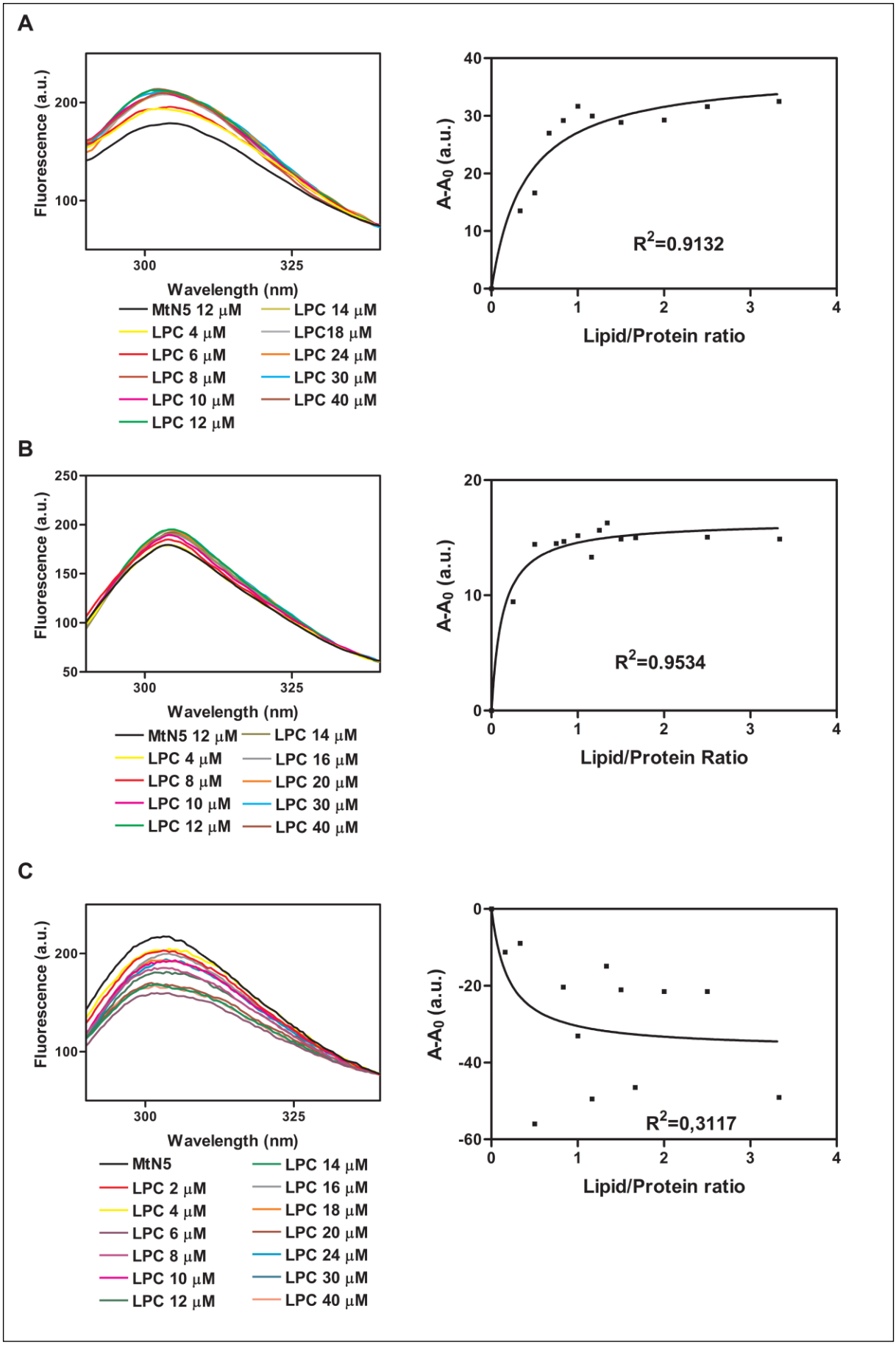


Figure 15. Phylogenetic tree. The sequence of the proteins were obtained from SWISS-PROT and the phylogentic tree was built with the Clustal W (Thompson et al., 1994). The protein sequence used in the phylogenetic analysis are listed as follows. For LTP1: *Cicer arietinum* (O23758), *Phaselus vulgaris* (O24440), *Prunus dulcis* (Q43017), *Prunus domestica* (P82534), *Prunus armeniaca* (P81651), *Malus domestica* (Q9M5X7), *Nicotiana tabacum* (Q42952), *Lycopersicon esculentum* (P93224), *Gossypium hirsutum* (Q9FVA5), *Gerbera hybrida* (Q39794), *Helianthus annuus* (Q39950), *Brassica napus* (Q42614), *Arabidopsis thaliana* (Q42589), *Zea mays* (P19656), *Triticum aestivum* (Q8GZB0). For LTP2: *Triticum aestivum* (P82900 and P82901), *Hordeum vulgare* (P20145), *Oryza sativa* (P83210), *Prunus armeniaca* (P82353) and *Zea mays* (P83506). DIR1 (Q8W453).

3.2 MtN5 lipid binding activity

Lipid binding properties of maize and wheat nsLTP have been investigated by means of equilibrium titration experiments using lysolipids as substrates (Gomar et al., 1996). The binding of lysolipids induces an enhancement in intrinsic fluorescence emitted by aromatic side chains, which are affected by the hydrophobic environment (Crimi et al., 2006). To probe the lipid-binding capacity of MtN5 protein, lyso-phosphatidylcholines (LPC), with various fatty acids chain lengths (LPC-C12, LPC-C16 and LPC-C18), were used. MtN5 presents three tyrosine residues (Y47, Y53 and Y54) in its amino acid sequence and it displayed a maximum of fluorescence emission at 305 nm in an aqueous environment. Upon the binding of LPC-C12 (Fig 16A) and LPC-C16 (Fig 16B), the MtN5 maximum of fluorescence emission at 305 nm remained unchanged (Fig 16A and 16B, left panels), however the relative fluorescence intensity increased as function of lipid concentration up to 12 μM; (Fig 16A and 16B). The fitting of the titration points, i.e. relative fluorescence intensity at 305 nm versus lipid/protein ratio, using a non-cooperative binding model revealed that the saturation of the lipid binding sites was reached when the molar ratio between MtN5 and the ligand was approximately 1:1, for both LPC-C12 and LPC-C16 (Fig 16A and 16B, right panels). In the binding assay carried out with LPC-C18 (Fig 16C), MtN5 did not display the increase in the intrinsic fluorescence emission observed when the ligands were LPC-C12 and LPC-C16. These findings might suggest that MtN5 is able to bind lipids, at least those having a fatty acid chain length less than or equal to 16 carbon units.

Figure 16. Lipid binding assay. A. Fluorescence emission spectra of MtN5 in the presence of increasing amount of LPC-C12 (left panel); titration points, i.e. increasing in fluorescence intensity ($A-A_0$) at 305 nm versus lipid/protein molar ratio (right panel). **B.** Fluorescence emission spectra of MtN5 with increasing amount of LPC-C16 (left panel); titration points, increasing in fluorescence intensity ($A-A_0$) at 305 nm versus lipid/protein molar ratio (right panel). **C.** Fluorescence emission spectra of MtN5 with increasing amount of LPC-C18 (left panel); titration points, increasing in fluorescence intensity ($A-A_0$) at 305 nm versus lipid/protein molar ratio (right panel). The fitting curves of the titration points were obtained with a non-cooperative binding model.


A
Fluorescence (a.u.)
200
100
300
325
Wavelength (nm)
MtN5 12 μM
LPC 4 μM
LPC 6 μM
LPC 8 μM
LPC 10 μM
LPC 12 μM
LPC 14 μM
LPC18 μM
LPC 24 μM
LPC 30 μM
LPC 40 μM
A-A0 (a.u.)
40
30
20
10
0
R2=0.9132
0
1
2
3
4
Lipid/Protein ratio
B
Fluorescence (a.u.)
250
200
150
100
50
300
325
Wavelength (nm)
MtN5 12 μM
LPC 4 μM
LPC 8 μM
LPC 10 μM
LPC 12 μM
LPC 14 μM
LPC 16 μM
LPC 20 μM
LPC 30 μM
LPC 40 μM
A-A0 (a.u.)
20
10
0
R2=0.9534
0
1
2
3
4
Lipid/Protein Ratio
C
Fluorescence (a.u.)
200
100
300
325
Wavelength (nm)
MtN5
LPC 2 μM
LPC 4 μM
LPC 6 μM
LPC 8 μM
LPC 10 μM
LPC 12 μM
LPC 14 μM
LPC 16 μM
LPC 18 μM
LPC 20 μM
LPC 24 μM
LPC 30 μM
LPC 40 μM
A-A0 (a.u.)
0
-20
-40
-60
R2=0,3117
0
1
2
3
4
Lipid/Protein ratio

3.3 MtN5 exhibits antimicrobial activity in vitro.

To study whether the recombinant MtN5 exerts antimicrobial activity, we tested in collaboration with Prof. Crimi its action on *Xanthomonas campestris* pv. *alfalfa*, a bacterial pathogen that attacks the aerial part of *Medicago* species, on *Fusarium semitectum*, which is a soil borne fungus that infects the root apparatuses of *Medicago* plants, and on *S. meliloti*, the *M. truncatula* symbiont. The action of MtN5 was compared to the effects caused by the same amount of bovine serum albumin (BSA) on the growth of the microorganisms tested. We observed that the growth of *F. semitectum* in the presence of MtN5 was slower than in untreated fungal culture, also at the lower concentrations (starting from 0.5 μM), and that the effect was dependent on the amount of protein added to the medium up to 10 μM (Fig. 17A). MtN5 also affects *X. campestris* growth; the bacterial growth inhibition displayed a dose-dependent response, and the effect was detectable starting from 0.4 μM (Fig. 17B). In addition, MtN5 displayed antibiotic activity also against *S. meliloti*, the natural host of *Medicago* plants (Fig. 17C). Interestingly, the inhibitory effect against rhizobium was obtained at a concentration higher than 1 μM. Thus the lowest effective concentration is higher as compared to those detected for *F. semitectum* and *X. campestris*.

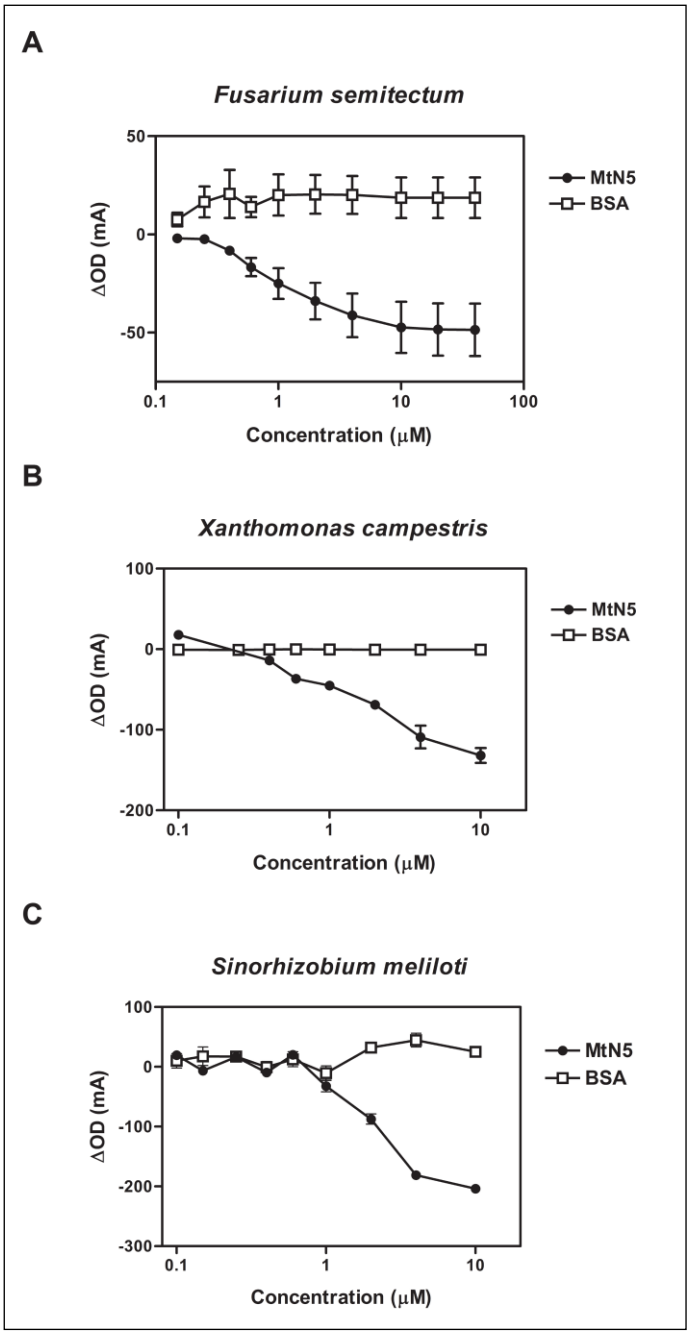


Figure 17. Dose response curves of MtN5 in *in vitro* antimicrobial assays. The effect of MtN5 was compared with bovine serum albumin (BSA). The microbial growth was determined by measuring the absorbance at 595 nm after 72h of incubation with different concentration of MtN5 protein. **A.** Effect of MtN5 on *F. semitectum* growth. **B.** Effect of MtN5 on *X. campestris* growth. **C.** Effect of MtN5 on *S. meliloti* growth. ΔOD_{595} represents the difference in the optical density between the untreated and the treated samples.

3.4 MtN5 is specifically expressed in root nodules during rhizobial symbiosis

The expression pattern of *MtN5* was evaluated in 40-day-old *M. truncatula* plants by means of quantitative RT-PCR (Fig. 18A). In plants nodulated with wild-type rhizobia, *MtN5* gene was highly expressed in the root nodules, where the level of expression is 7-fold higher with respect to the transcript level detected in either non nodulated roots or in nodulated roots deprived of nodules (Fig. 18A). The aerial part of both non-nodulated and nodulated plants

displayed an extremely low level of *MtN5* mRNA with respect to root tissue (2% and 18% in non nodulated and in nodulated plants respectively, as compared to non-nodulated roots).

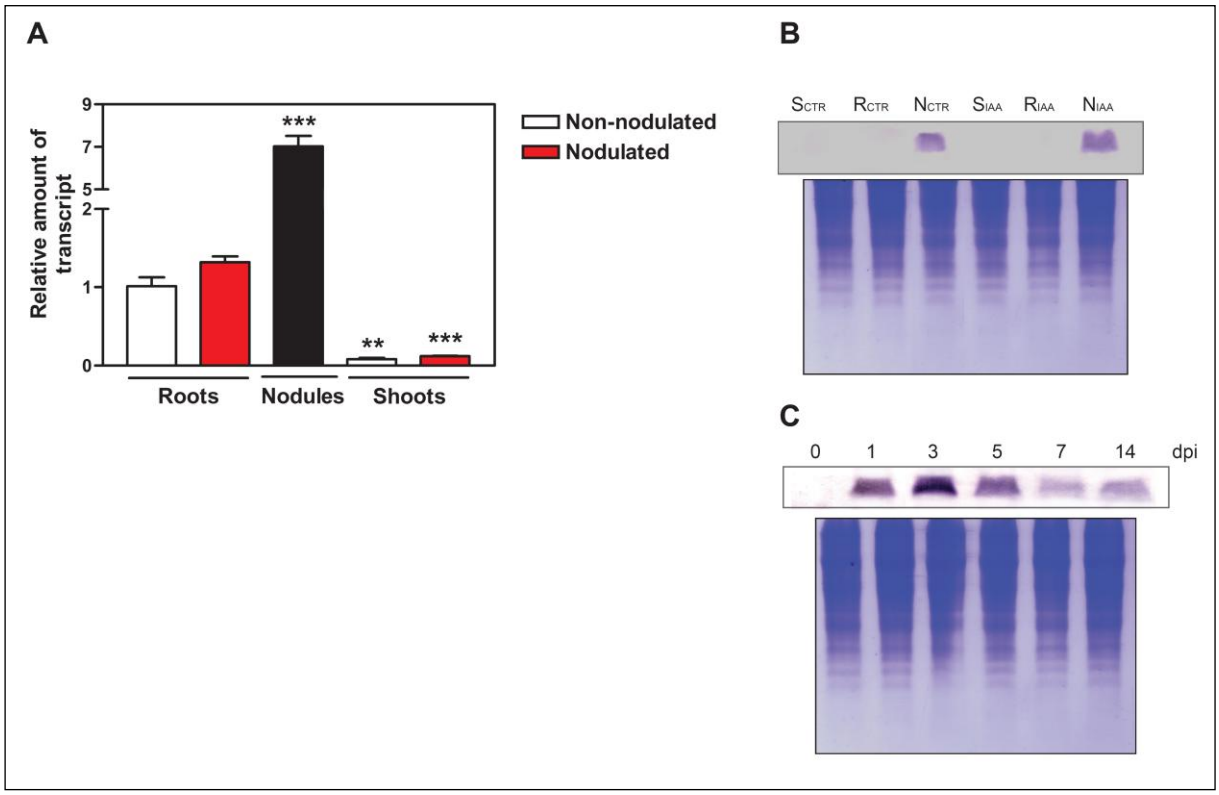


Figure 18. Expression pattern of MtN5 in *M. truncatula* plants. A. Expression of *MtN5* in nodulated and non-nodulated roots, and in root nodule was evaluated by qRT-PCR. Total mRNA was extracted from 40-day-old plants. The expression levels were normalized using actin as endogenous control gene and the relative expression ratios were calculated using non inoculated root as calibrator sample. The values reported are means ± SE (n = 3; **, P<0.01; ***, P<0.001). **B.** Western blot analysis carried out on the total protein extract from shoots (S), roots deprived of nodules (R) and nodules (N) of 40-day-old *M. truncatula* plants inoculated with either *S. meliloti* IAA or wild type. The proteins extracted were quantified by Bradford method and an equivalent amount of proteins was loaded in each lane, as assessed by Coomassie staining (lower panel). **C.** Western blot analysis carried out on the total protein extract from root apparatuses after micro-flood inoculation with *S. meliloti* wild type. Roots were collected 1, 3, 5, 7 and 14 days after inoculation, total protein content was extracted from the whole root apparatus and quantified by Bradford method. An equivalent amount of proteins was loaded in each lane, as shown by Coomassie staining (lower panel). IAA: plants nodulated with *S. meliloti* IAA strain. CTR: plants nodulated with *S. meliloti* wild-type.

We also evaluated the level of MtN5 protein in 40-day-old *M. truncatula* plants nodulated by either IAA-overproducing and control *S. meliloti* strain: in both IAA and control plants, MtN5 protein was detectable only in the nodules (Fig. 18B), suggesting that root nodules represent the organ where MtN5 is preferentially accumulated. Although we had observed a higher amount of MtN5 protein in roots nodulated with *S. meliloti* IAA when compared to control

roots (Fig. 12C), the quantities of MtN5 protein detected within the two type of nodule were not apparently different (Fig. 18B) .

MtN5 was classified as an early nodulin, whose mRNA is induced well before the onset of nitrogen fixation, maintained in mature nodules (14 days after inoculation) and also expressed in Nar nodules (Gamas et al., 1996). On the basis of these findings it has been proposed that the protein encoded by *MtN5* could play a role in root nodules organogenesis rather than in nitrogen fixation (Gamas et al., 1996). We studied the expression of MtN5 protein during different phases of rhizobia infection. 7-day-old seedlings were micro-flood inoculated with *S. meliloti* wild-type and the roots were collected 0, 1, 3, 4, 5 and 14 days after inoculation (dpi). MtN5 was detected starting from 1 dpi and it reached the highest concentration at 3 dpi. It is noteworthy that these two time points correspond to well characterized stages of rhizobia infection. At 1 dpi the infection threads are visible in the curled root hairs and at 3 dpi the infection threads invade the nodule primordia, which originate from the pericycle cells (Kuppusamy *et al*., 2004). The MtN5 protein content decreased after 3 dpi, and it remained stable between 7 and 14 dpi (Fig. 18C).

3.5 Silencing and overexpression of MtN5

The functional study of *MtN5* during the establishment of the symbiosis was undertaken by means of both silencing and overexpression approaches. A hairpin gene construct designed to silence the endogenous gene was produced and transferred to *M. truncatula* roots by *A. rhizogenes*-mediated transformation. As negative control, *M. truncatula* plants inoculated with *A. rhizogenes* harbouring the empty binary vector were used. *A. rhizogenes* generates adventitious, genetically transformed roots at the site of inoculation. Because other laboratories have previously observed that not all *M. truncatula* hairy roots selected by growing them on antibiotics were transformed, two different approaches were applied in the generation of transgenic root apparatuses. In the first case, the pBIN19 binary vector, encoding for the kanamycin resistance, was chosen and the transgenic state of each adventitious root was checked by PCR analysis; approximately 95% of the roots contained

the transgene (data not shown). Furthermore, the expression of the hairpin construct in the transformed roots was tested by means of RT-PCR (Fig. 19A).

The expression of the hairpin construct induced the silencing of the *MtN5* gene in the roots (Fig.19B). The steady state level of *MtN5* mRNA in silenced hairy roots was reduced by about 65% when compared with non-inoculated control roots and by 80% in comparison to

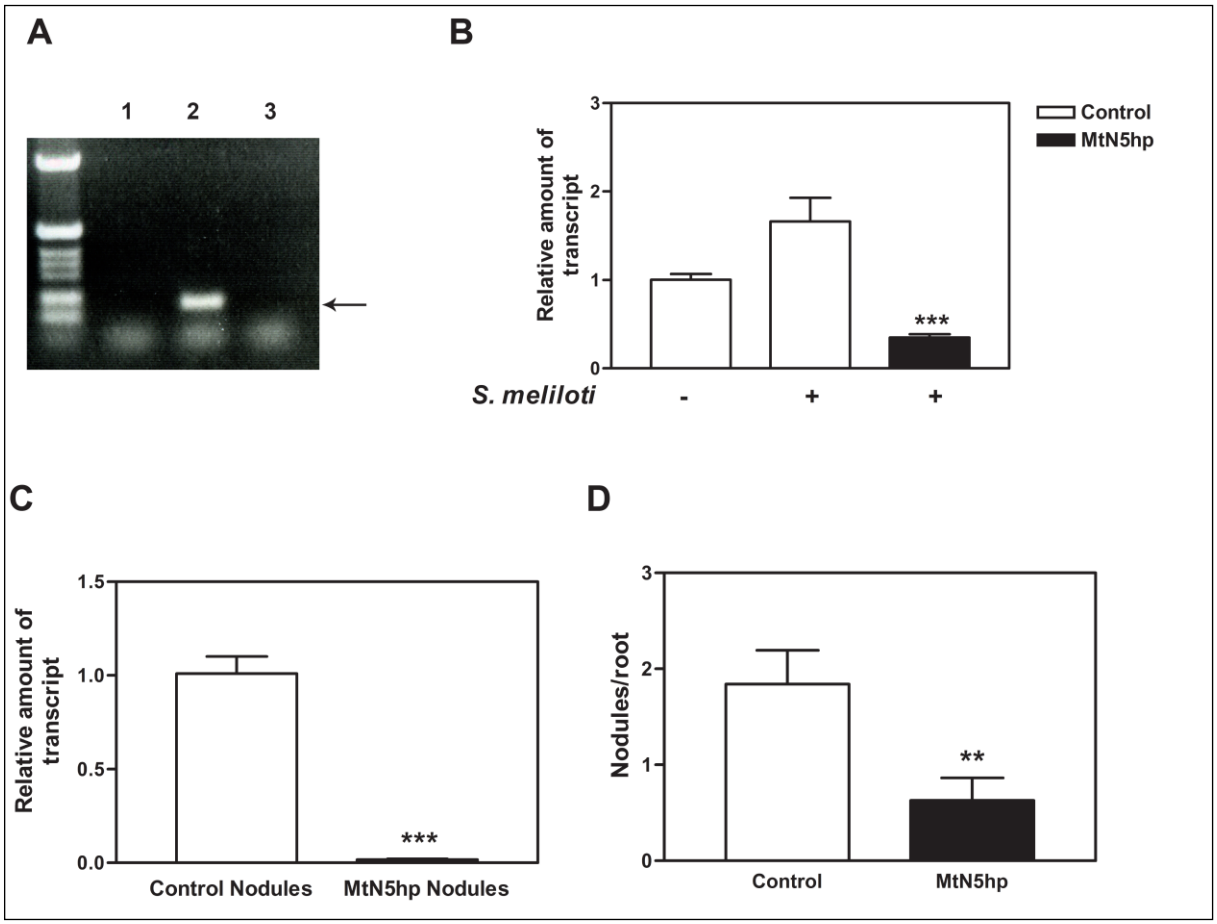


Figure 19. *MtN5* downregulation in transgenic *M. truncatula* roots. Control roots are hairy roots generated by *A. rhizogenes* carrying an empty pBIN19 vector, whereas MtN5hp roots are hairy roots generated by *A. rhizogenes* carrying the recombinant pBIN19 vector containing the hairpin construct. **A.** Agarose gel electroforesis of RT-PCR product obtained from total RNA extracted from control roots (lane 1) and from *MtN5* silenced roots (lane2). Lane 3. No-template control. **B.** Expression of *MtN5* in hairy roots. The expression level was evaluated by means of quantitative RT-PCR in MtN5hp roots inoculated with rhizobia, and in control roots, both non inoculated (-) and inoculated with rhizobia (+). The expression levels were normalized using actin as endogenous control gene. The relative expression ratios were calculated using non-inoculated control root as calibrator sample. The values reported are means ± SE (n = 3; ***, P<0.001). **C.** Expression of *MtN5* in root nodules generated on hairy control roots and on hairy N5hp roots. The expression levels were normalized using actin as endogenous control gene. The relative expression ratios were calculated using inoculated control root as calibrator sample. The values reported are means ± SE (n = 3; ***, P<0.001).**D.** Number of nodules per root. The values reported are means ± SE (n = 18; **, P<0.01).

control nodulated roots deprived of nodules (Fig. 19B). In addition, the *MtN5* expression has also been evaluated in the nodules, generated on both control and *MtN5*-silenced roots. In silenced nodules the relative amount of *MtN5* mRNA turned out to be reduced by about 98%

when compared to control nodules (Fig. 19C). The *MtN5*-silenced roots resulted impaired in nodulation, since they developed 50% fewer nodules with respect to the inoculated control roots (Fig. 19D).

In an independent test, a pBINPLUS (Van Engelen et al., 1995) derivative binary vector, named pRedRoot (Limpens et al., 2004), was chosen for harbouring the hairpin gene construct with the aim of silencing endogenous *MtN5*. In pRedRoot the antibiotic resistance-encoding gene was replaced with the gene encoding for the fluorescent protein DsRED1, which provides with a non-destructive selectable marker that allows the discrimination of transgenic roots from non-transformed ones, thus avoiding the use of antibiotics for transgenic root selection (Limpens et al., 2004). About 50% of the roots displayed DsRED1 expression, confirming the transformation efficiency reported by other groups using the same transformation protocol (Fig. 20A and 20B) (Limpens et al., 2004; Huo et al., 2006). Only nodules on roots expressing the fluorescent marker were considered for the analysis (Fig. 20A-F). The number of nodules developed by silenced roots was reduced by about 80% when compared to control adventitious roots, generated by transformation with the empty pRedRoot vector (Fig. 20G).

On the other hand, when the binary vector employed in the hairy roots generation was harboring the gene construct *35S::MtN5*, the transgenic adventitious roots contained about 7-fold more *MtN5* transcript than control adventitious roots (Fig. 20C-F, 20H) and developed about 300% more nodules with respect to control adventitious roots (Fig. 20G).

Nodules developed by MtN5hp and 35S::MtN5 transgenic roots appeared at maturation morphologically similar and of approximately the same size; the nodules were all apparently functional since they exhibit the typical red colour due to the presence of leghaemoglobin.

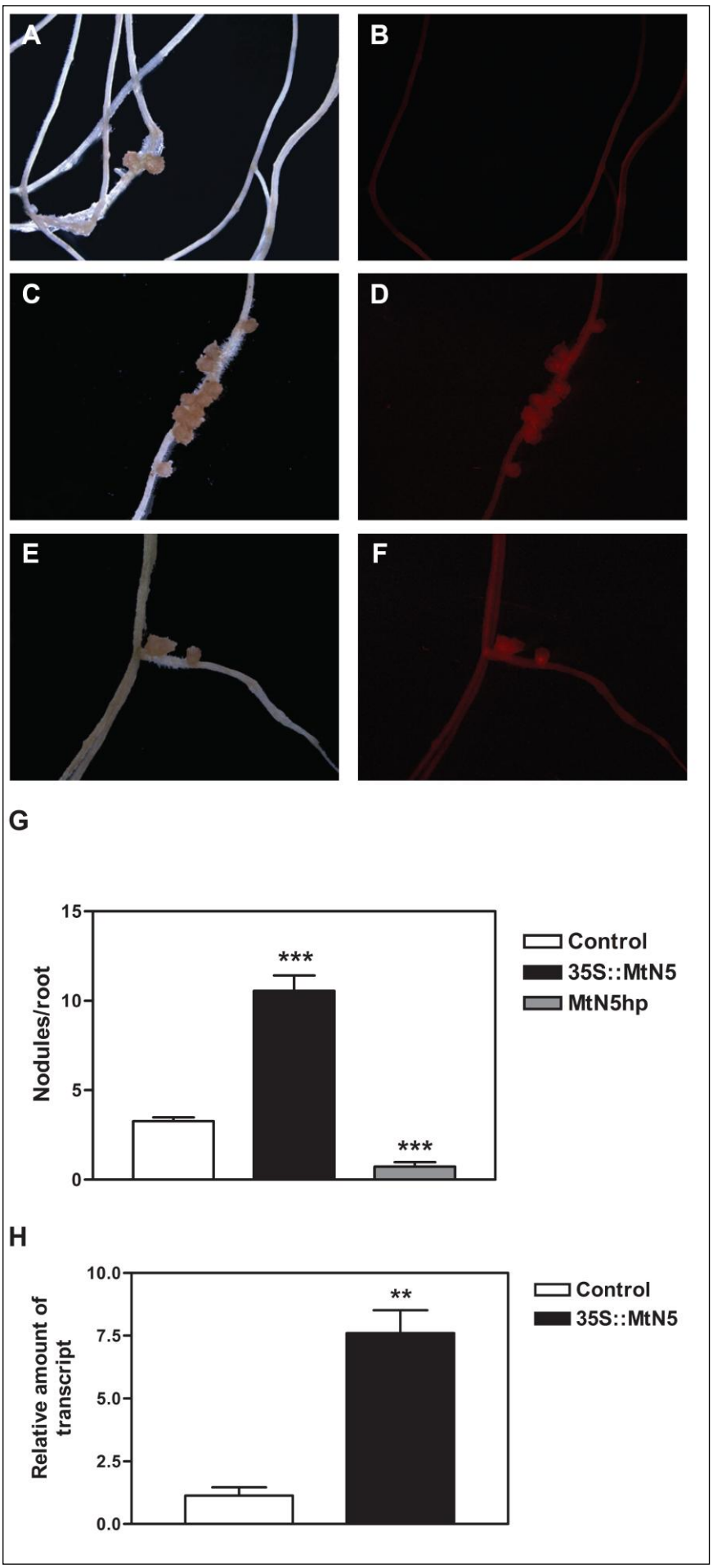

A
B
C
D
E
F
G
Nodules/root
15
10
5
0
***
***
Control
35S::MtN5
MtN5hp
H
Relative amount of transcript
10.0
7.5
5.0
2.5
0.0
**
Control
35S::MtN5

Figure 20. Effects of downregulation and overexpression of the endogenous *MtN5*. A. Bright field picture of *A. rhizogenes*-transformed hairy roots of *M. truncatula* using the pRedRoot binary vector harbouring the hairpin construct aiming at downregulating endogenous levels of *MtN5* mRNA. **B.** Same roots as shown in A panel using filter settings to visualize *DsRED1* fluorescence (red fluorescence). **C.** Bright field picture of *A. rhizogenes*-transformed hairy roots of *M. truncatula* using the pRedRoot binary vector harbouring the *35S::MtN5* gene construct aiming at increasing endogenous levels of *MtN5* mRNA. **D.** Same roots as shown in C panel using filter settings to visualize *DsRED1* fluorescence (red fluorescence). **E.** Bright field picture of *A. rhizogenes*-transformed hairy roots of *M. truncatula* using an empty pRedRoot binary vector as negative control. **F.** Same roots as shown in E panel using filter settings to visualize *DsRED1* fluorescence (red fluorescence). **G.** Number of nodules per root. The values reported are means ± SE. ***, P<0.001. **H.** Expression of *MtN5* in hairy roots. The expression level was evaluated by means of quantitative RT-PCR in both *MtN5* overexpressing and control roots inoculated with rhizobia and deprived of nodules. The expression levels were normalized using actin as endogenous control gene. The relative expression ratios were calculated using inoculated control root as calibrator sample. The values reported are means ± SE (n = 3; **, P<0.01). Control: adventitious roots generated by *A. rhizogenes* infection, carrying an empty pRedRoot vector. 35S::MtN5: adventitious roots generated by *A. rhizogenes* infection, carrying the recombinant pRedRoot vector containing the gene construct for the overexpression. MtN5hp: adventitious roots generated by *A. rhizogenes* infection, carrying the recombinant pRedRoot vector containing the hairpin gene construct.

3.6 MtN5 induction upon stresses

M. truncatula plants both inoculated, with either IAA-overproducing or control rhizobia strain, and non-inoculated were infected with *F. semitectum* ISCF20a. The root pathogenic fungus was used at two different concentrations (10% and 50% v/v) and the analyses on infected plants were carried out 48 hours after the infection.

In non-nodulated plants the production of MtN5 was elicited by *F. semitectum* at both concentrations (Fig. 21A). In non-nodulated plants treated with *F. semitectum* at 50%, the induction of MtN5 resulted lower than that obtained in the plants infected with the fungus at 10%. Also in plants nodulated with either IAA-overproducing or control strain upon treatment with 10% of fungus, we observed an increased amount of protein in the root apparatus of infected plants as compared with the root of untreated plants. The presence of MtN5 protein in root deprived of nodules (Fig. 21B) showed that in nodulated roots infected by *F. semitectum* at 10% MtN5 protein is localized both in the nodules and in the root tissues (Fig. 21A and 21B). Furthermore, MtN5 protein induction was restricted to the roots, as MtN5 was not detected in shoots of infected plants (data not shown).

F. semitectum is often isolated from plants with complex diseases; in legume crops, such as soybean and dry bean, *F. semitectum* may cause seed rot, collar rot and root rot (Zaccardelli et al., 2006). The plant response to fungus infection was evaluated through the symptoms displayed. For this purpose, leaves were subdivided in three groups: class I included healthy leaves, class II comprised closed leaves that showed wilting, and class III dead leaves. .

When plants were infected with 10% *F. semitectum*, *M. truncatula* nodulated with *S. meliloti* IAA resulted less affected by the fungus, i.e. a significantly higher frequency of healthy leaves and a lower frequency of dead leaves, as compared to both plants nodulated with control strain and non nodulated plants (Fig. 21C). However, when the concentration of fungus was increased to 50%, no significant differences were found between non-nodulated plants and plants infected with either IAA or control rhizobia strain in any classes analysed (Fig. 21D).

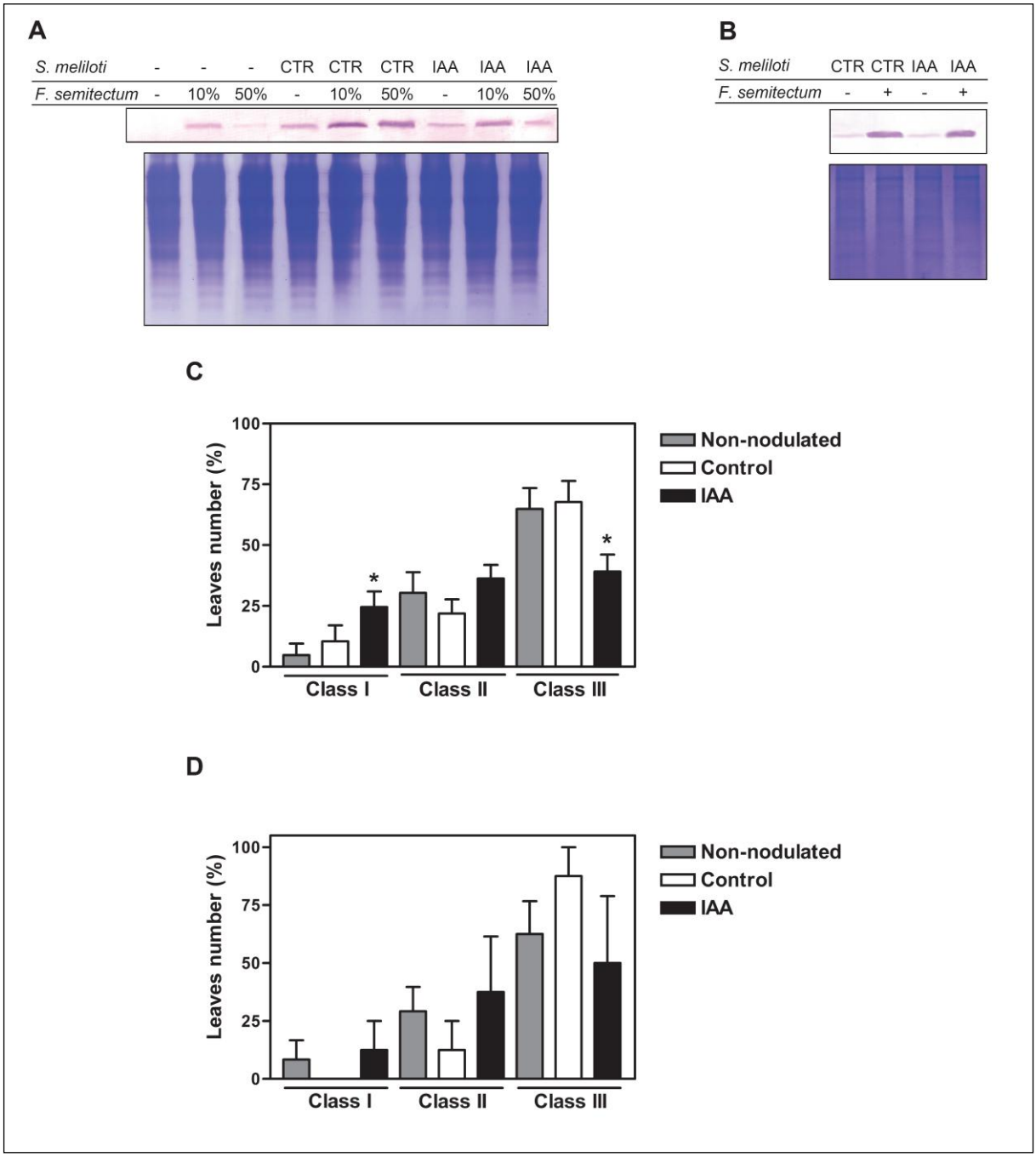


Figure 21. Effects of *F. semitectum* infection on *M. truncatula* plants. A. 40 day-old *M. truncatula* plants, both non-nodulated and nodulated with either *S. meliloti* IAA or control strain, were treated with two different concentrations (10% and 50% v/v) of *F. semitectum*. Total proteins were extracted from root apparatuses of both infected and non infected plants, quantified by Bradford method and analysed by western blot. An equivalent amount of proteins was loaded in each lane, as shown by Coomassie staining. **B.** 40 day-old *M. truncatula* plants, both non-nodulated and nodulated with *S. meliloti* wild type, were treated with *F. semitectum*. Total proteins were extracted from root apparatuses, deprived of nodules, of both infected and non infected plants, quantified by Bradford method and analysed by western blot. An equivalent amount of proteins was loaded in each lane, as assessed by Coomassie staining. **C.** The symptoms manifestation of *M. truncatula* plants treated with *F. semitectum* (10% v/v) were evaluated 48 h after infection. Data are reported as mean ± SE (n= 16; *, P < 0.05). **D.** The symptoms manifestation of *M. truncatula* plants treated with *F. semitectum* (50% v/v) were evaluated 48 h after infection. Data are reported as mean ± SE of the percentage of leaves (n= 4). Class I: healthy leaves. Class II closed leaves that showed wilting. Class III: dead leaves. IAA: plants nodulated with *S. meliloti* IAA strain. CTR, Control: plants nodulated with *S. meliloti* wild-type.

Also the effects of the leaf pathogenic bacteria *X. campestris* pv. *alfalfae* were tested on *M. truncatula* plants both non-inoculated and inoculated with either IAA over-producing and control *S. meliloti* strains.

M. truncatula plants were spray-inoculated with a *X. campestris* suspension and shoot samples were collected 12 days after infection (dai) to be further investigated for MtN5 elicitation upon pathogen challenge. The presence of MtN5 was not detected in the infected shoot tissue of both non-nodulated plants and *M. truncatula* plants bearing either IAA overproducing nodules or control nodules (data not shown).

Considering these findings, we can infer that MtN5 is a root specific protein which is induced in response to microorganisms that infect the root apparatuses. MtN5 is not expressed in the root tissue after wounding of the root, either (data not shown).

In addition, leaves of *M. truncatula* plants were either syringe-infiltrated to investigate the bacterial growth in the infected leaves and the spread of pathogens to non-infiltrated leaves, or spray-inoculated with a pathogen suspension in order to evaluate the global symptoms manifestation of *X. campestris* infection.

For bacterial growth measurements, infected leaves were collected at regular time points, grinded in a $MgSO_4$ solution and the extracts were plated on the nutrient medium for *X. campestris*. The curves representing the bacteria vital count demonstrated that bacterial load in both non-nodulated plants and in plants nodulated with *S. meliloti* wild type increased constantly during the first 12 days dai (Fig. 22A). In contrast, the curve representing the bacterial load in leaves of plants bearing IAA over-producing nodules showed that the number of bacteria started decreasing from 7 dai and was significantly lower at 12 dai when compared to infected leaves of both plants non-nodulated and inoculated with *S. meliloti* wild type at the same time point (Fig. 22A and 22B). Furthermore, the spread of bacteria to non-infiltrated leaves was analysed. In plants bearing IAA overproducing nodules the pathogenic bacteria were significantly less effective in reaching uninfected leaves in comparison to non-nodulated plants and of plants nodulated with control rhizobia strain (Fig. 22C).

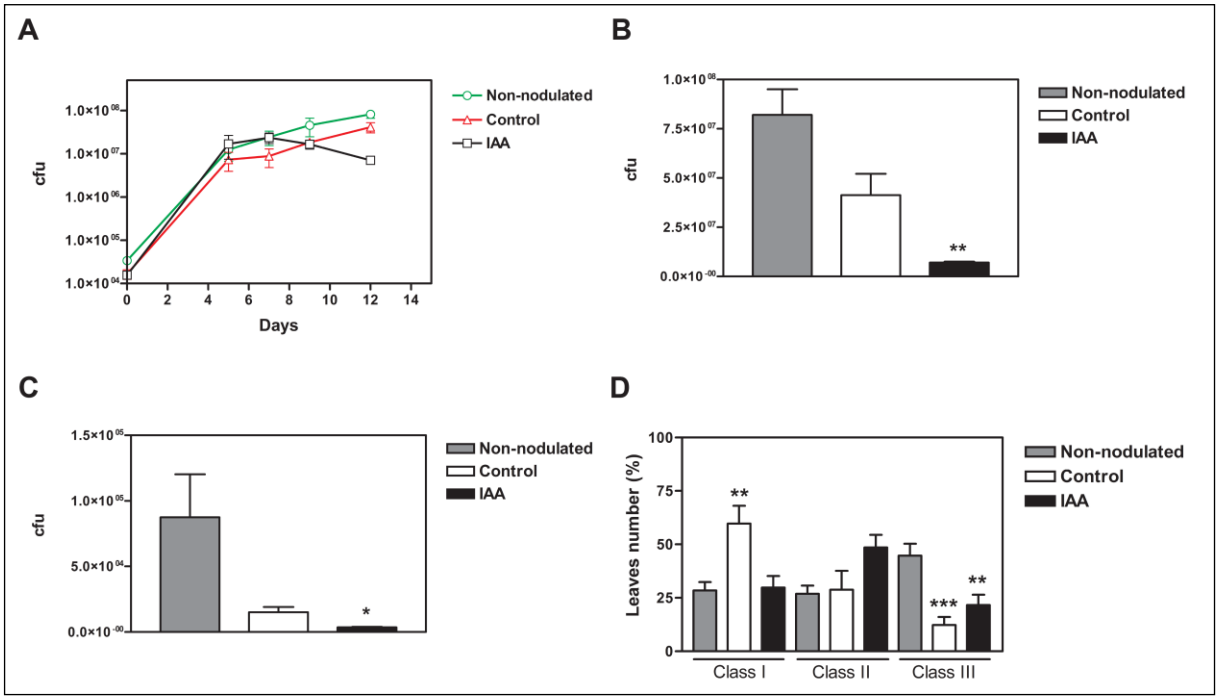


Figure 22. Effects of *X. campestris* infection of *M. truncatula* plants. A. Time-course evaluation of *X. campestris* infection in *M. truncatula* plants both non-nodulated and nodulated with either IAA-overpreoducing or control *S. meliloti* strain. The bacterial growth was followed for 12 dai and at each time point was estimated by vital count of *X. campestris* extracted from infiltrated leaves. Values reported are means ± SE (n = 3). **B.** *X. campestris* growth in infiltrated leaves at 12 dai. Values reported are means ± SE (n = 3; **, P < 0.01). **C.** Evaluation of pathogens spreading to non-infiltrated leaves. The bacterial growth was estimated by vital count of *X. campestris* extracted from non-infected leaves at 12 dai. Values reported are means ± SE (n = 3; *, P < 0.05). **D.** The symptoms manifestation of *M. truncatula* plants spray-inoculated with *X. campestris* were evaluated 12 dai. Data are reported as mean ± SE (n= 10; **, P< 0.01; ***, P < 0.001). Class I: healthy leaves. Class II symptomatic leaves (chlorosis and necrosis). Class III: dead leaves. IAA: plants nodulated with *S. meliloti* IAA strain. Control: plants nodulated with *S. meliloti* wild-type.

As in the case of *F. semitectum*, for the evaluation of the symptoms due to *X. campestris* infection the leaves have been subdivided in three groups: class I for healthy leaves, class II for symptomatic leaves (displaying chlorosis and necrotic spots) and class III for dead leaves. The data obtained show that the most evident difference was found in class III in which the frequency of dead leaves in non-nodulated plants is significantly higher when compared to the frequency of dead leaves nodulated in plants irrespectively of the rhizobia strain (IAA or wild type) used (Fig. 22D).

Collectively these data indicate that nodule-derived IAA does not negatively affect the responses of *M. truncatula* against *F. semitectum* and *X. campestris*.

Discussion

The development of nitrogen-fixing root nodules in leguminous plants requires a complex exchange of signals between the host and the compatible rhizobia strain. Several molecules, among which phytohormones, have been demonstrated to be implicated in this signalling process. In particular, it has been hypothesised that auxin might play a role in root nodules development. The earliest observations of the involvement of plant-derived auxin in nodulation were carried out at the beginning of the last century (Thimann, 1936) and subsequent studies determined that the inhibition of auxin basipetal transport in the root was required for nodule development (Mathesius et al., 1998b; van Noorden et al., 2006). Auxin, in the chemical form of indol-3-acetic acid, is also synthesised by rhizobacteria, those which have promoting effects on plants growth, as well as by symbiotic rhizobia. Up to now, the effects of rhizobia-derived auxin on nodulation have been investigated in several studies through rhizobia mutants, characterised by different abilities of IAA production (Hunter, 1987; Fukuhara et al., 1994; Kaneshiro and Kwolek, 1985), although the results that have been reported are rather contradictory. The findings of these studies are somehow difficult to interpret because of the limited molecular characterization of the rhizobia mutants.

On this basis, we adopted an alternative experimental approach in that we engineered *S. meliloti* for a new auxin biosynthetic pathway (*iaaMtms2*) to address the role of rhizobia-derived IAA in nodule development. We obtained a transgenic rhizobia strain that differs from the control strain only in the increased IAA synthesis capacity. The IAA-overproducing *S. meliloti* displayed an enhanced ability to nodulate both *M. truncatula* and *M. sativa*, which results in a 100% and 50%, respectively, increase in the number of nodules per plant when compared with plants infected with control *S. meliloti* strain. The expression of the chimeric

gene was driven by the *rolAp* promoter (Pandolfini et al., 2000) which enables bacteroids to synthesise auxin, and this produced an increase in the IAA content of nodules of at least 10-fold when compared to root nodules induced by control rhizobia. Nonetheless, the *rolAp* promoter is also transcriptionally active in free-living rhizobia, so it is likely that auxin synthesis takes place during the early phases of infection (e.g. root hair curling, infection threads development) and nodule initiation. Thus, the promoting effects of rhizobia-derived auxin on nodulation could be imputable to an implication in the early stages of infection as well as during nodule primordia development.

In the current model for indeterminate nodules initiation and organogenesis, the local accumulation of auxin at the site of infection is thought to be required to stimulate pericycle and cortical cells division (de Billy et al., 2001; Mathesius et al., 1998b). The higher auxin synthesising capacity of *S. meliloti* IAA strain might facilitate nodule formation by increasing the IAA content in the primordium. This interpretation concords with the observation of van Noorden and co-workers that in *M. truncatula sunn* mutant the content of auxin at the site of infection is about three times higher when compared to wild type roots (van Noorden et al., 2006). Yet, in our experimental model the average size of nodules produced by the IAA strain is not different from that of nodules induced by control strain, suggesting that the extra auxin synthesised by rhizobia mainly affects the efficiency of nodulation rather than nodules growth.

The regulation of auxin flux from the shoot to the plant root has been proposed as a mechanism to regulate the nodulation in legumes bearing indeterminate nodules. The model hypothesised by van Noorden and colleagues (2006) proposes that an inhibition of IAA transport occurs following the first infection events on the root. The decrease of auxin concentration in the root tissue might stop further nodule initiation. In the *M. truncatula sunn* hypernodulating mutant this mechanism is altered and auxin continues to be transported from shoots to roots sustaining hypernodulation (van Noorden et al., 2006). In our

experimental system, the enhanced nodulation phenotype observed is most likely due to the extra auxin loaded in the root from the bacteroids within the nodules.

M. truncatula and *M. sativa* plants bearing IAA overproducing nodules have a more developed root apparatuses, that also display a higher ramification, when compared to the root apparatuses of plants bearing control nodules. These are characteristic phenotypes observed in IAA overproducing mutants (Woodward and Bartel, 2005). The close similarity between lateral roots and root nodules has been already highlighted (de Billy et al., 2001; Mathesius et al., 1998b). In addition, following on from the observation of a strong correlation between the number of nodules and lateral root in pea plants, a possible overlap between the two signalling pathway for the organogenesis has been suggested (Ferguson et al., 2005). The data hereby reported concord with this hypothesis, since in *Medicago* plants bearing IAA overproducing nodules we observed, parallel to the enhanced number of nodules, a more developed lateral root. However, in plants nodulated with control rhizobia strain no correlation between the number of lateral root and the number of nodules present on them has been found. On the other hand, the significant correlation between the number of lateral root and the number of nodules displayed by *M. truncatula* plants infected by *S. meliloti* IAA suggests that the IAA overproducing rhizobia have a greater ability to nodulate the lateral root and besides having a promoting effect on lateral root growth.

In our experimental conditions, there is no evidence for the IAA overproducing rhizobia to have any positive effects on the growth and the biomass production of the aerial part either in *M. truncatula* or *M. sativa*. Therefore, under the growth conditions used in the experiments, there is no suggestion of an enhanced nitrogen fixation by the IAA strain. Nonetheless, it cannot be ruled out that plants bearing IAA-overproducing nodules grown under limiting conditions might exploit the more developed root apparatus (e.g. more efficient water and nutrient uptake).

Auxin transport is mediated by a set of asymmetrically-localized auxin influx and efflux carriers, which regulate the IAA distribution during the developmental programs that take place in plants (Benkova et al., 2003; Friml, 2003). It has been observed that the expression of *PIN* genes is either directly or indirectly regulated by auxin (Vieten et al., 2005) and that the variation of the expression levels of auxin carriers can affect the root development. Our data demonstrate that in *M. truncatula* plants bearing IAA-overproducing nodules, the expression of the efflux carrier *MtPIN2* is significantly increased when compared to its expression levels in roots nodulated with control strain. *MtPIN2* encodes for a root specific auxin efflux facilitator in *M. truncatula* and it is an ortholog of the auxin efflux carrier *PIN2* of *A. thaliana* (Friml, 2003), which has been shown to mediate auxin transport towards the root elongation zone (Schnabel and Frugoli, 2004). The enhanced expression of *MtPIN2* has been also detected in the hypernodulating *sickle* mutant of *M. truncatula* (Praytino et al., 2006b), whereas plants silenced for *MtPIN2* displayed a decreased nodulation (Huo et al., 2006). Therefore the result herein reported suggests that the promotion of the root growth as well as the enhancement of nodulation in plants bearing IAA-overproducing nodules might be due to both the increased auxin synthesis within nodules and the phytohormone redistribution in the root apparatus.

The involvement of NO in auxin-induced adventitious roots formation in cucumber and lateral roots formation in tomato has recently been reported (Correa-Aragunde et al., 2004; Pagnussat et al., 2003). These observations provided strong evidence that NO is a component of the auxin signalling pathway in these processes (Correa-Aragunde et al., 2004; Pagnussat et al., 2003). The present research demonstrates that *M. truncatula* and *M. sativa* root nodules can produce NO and that the NO production is increased when root nodules are elicited by IAA-overproducing rhizobia. In plant tissues, NO can be generated by both enzymatic and non-enzymatic systems (Crawford, 2006), whereas in rhizobia grown anaerobically it can be produced through the denitrification pathway (Watmough, 1999). According to the observations of Baudouin and co-workers (2006), the NO evoked in *M.*

truncatula root nodules is independent from the denitrification pathway of the symbiont. In addition, we observed that aerobically-grown *S. meliloti* can produce NO via a NO synthase-like activity, therefore the nitric oxide within nodules might be the outcome of both plant and rhizobia production. However, no significant difference in the NO production was detected between free-living IAA rhizobia and control strain, thus the increased NO levels in IAA-overproducing nodules is most likely to be the result of plant NO synthesis induced locally by the rhizobia-derived auxin. On the basis of these results, we propose a role for rhizobia-derived IAA in indeterminate nodules formation and we also demonstrate the involvement of NO in the auxin induced signalling pathway for nodulation. The latter conclusion is further strengthened by the observation that the NO scavenger cPTIO can reduce nodules formation, completely abolishing the stimulatory effects provided by the extra loading of auxin.

The establishment of the symbiotic relationship between rhizobia and leguminous plants requires an intimate contact between the two organisms. Despite resembling a pathogenic infection, the root colonization by rhizobia does not always activate the plant immune reaction that is normally elicited by pathogens (Djordjevic et al., 1987; McKhann and Hirsch, 1994; Baron and Zambryski, 1995). However, plants can activate several responses against invading rhizobia and they can consist of the expression of proteins structurally related to defence protein and the generation of ROS (Gamas et al., 1998; Santos et al., 2001). Recently, the transcriptome analysis of *M. truncatula* nodulating roots has led to the identification of genes that are differentially regulated during nodulation (El Yahyaoui et al., 2004). Among such differentially expressed transcripts, genes involved at different levels in pathogen responses have been found to be both up-regulated and down-regulated (El Yahyaoui et al., 2004). These findings are consistent with the need both to allow rhizobia infection and to control the extent of this infection, or to protect the nodules (carbon and nitrogen-rich organs) from pathogen attacks (El Yahyaoui et al., 2004).

It was found that plant resistance mechanisms against bacterial pathogen are mediated, at least in part, by the down-regulation of the plant auxin signalling pathway (Navarro et al., 2006). Many microorganisms are thought to evade plant innate immunity by synthesising auxin themselves or by stimulating auxin biosynthetic pathway in plants (Robert-Seilaniantz et al., 2007), therefore the introduction of a novel auxin synthesis pathway in *S. meliloti* could result in a higher susceptibility of *Medicago* plants towards rhizobia and pathogens infection. Following on from this, the expression of two genes induced during the early stages of nodulation was investigated in plants inoculated with IAA-overproducing rhizobia and compared to control plants. The pathogenesis related protein PR1, known to participate in SAR, did not show any statistically significant variation in the steady state levels of the transcript between the two samples tested, whilst *MtN5* resulted significantly induced in the root apparatus of plants nodulated with *S. meliloti* IAA. *M. truncatula* plants bearing IAA-overproducing nodules did not result more susceptible to either bacterial or fungal pathogens when compared to plants nodulated with *S. meliloti* wild type suggesting that nodule-derived auxin does not negatively influence the plant responses to pathogens. However, the plants bearing IAA-overproducing nodules do display less severe symptoms when infected by the root pathogen *F. semitectum*.

These observations prompted us to focus on the role of MtN5 in symbiosis and in pathogenic response.

MtN5 codes for a cysteine rich protein, which is classified as early nodulin since it is expressed during the early phases of the symbiotic association between *M. truncatula* and *S. meliloti* (Gamas et al., 1996; El Yahyaoui et al., 2004). MtN5 was annotated as putative plant LTP because of the presence of the typical eight cysteine motif in its amino acid sequence (El Yahyaoui et al., 2004). Although plant LTPs have not been provided with a clear biological role yet, they are characterized by the ability to bind lipids in vitro (Kader, 1996) and have been proposed to be involved in a series of different aspects of plants biology, such as the defence against pathogens, signalling cascades and developmental programs.

In the present research, we demonstrate that recombinant MtN5 can bind phospholipids *in vitro* and also exhibits a slight antimicrobial activity *in vitro* against, *F. semitectum*, *X. campestris* pv. *alfalfa* and *S. meliloti*.

On the basis of molecular mass, plant LTPs are grouped in LTP1 of about 9 kD and LTP2 of about 7 kD (Yeats & Rose, 2008). MtN5 protein has a molecular mass of about 8 kD and displays a higher sequence similarity with members of LTP2 family than with members of LTP1 family. Site directed mutagenesis studies carried out on LTP2 from *Oryza sativa* individuated a set of amino acids that are fundamental for the tertiary structure and the lipid binding activity of this protein (Cheng et al., 2007). The same positions are also conserved in MtN5 sequence, strengthening its resemblance to type 2 LTPs, even though MtN5 shows regions not conserved in the other members of the family. It is worth noting that MtN5 displays a higher degree of sequence homology with *A. thaliana* DIR1 (37% identity, 83% similarity), which was recently proposed as a peculiar LTP2 (Lascombe et al., 2008). Despite being closer to LTP2, the phylogenetic analysis demonstrates that MtN5 and DIR1 group independently from the two major LTP families.

Interestingly, Margaert and co-workers have identified a gene family (Nodule-specific Cysteine Rich, NCR), composed of members encoding for small (60-90 residues) cysteine rich peptides, which are upregulated during rhizobial infection. On the basis of their specific temporal and spatial expression profile, it has been proposed that they participate in nodule development (Mergaert et al., 2003). The specific role(s) of these cysteine-rich peptides, which generally possess antimicrobial activity, in the rhizobial infection and nodule development is not fully elucidated. The upregulation of these cysteine rich peptides has been explained either as a response that limits rhizobia invasion and spread or as a mechanism that protects root nodules from non-symbiotic microorganisms (Gamas et al., 1998, Mergaert et al., 2003, El Yahyaoui et al., 2004, Chou et al., 2006). In addition, it has

been observed that the induction of plant defence mechanisms is also involved in the control of nodule number (van Brussel et al., 2002).

MtN5 expression is an early event of rhizobia infection and it was shown to be dependent on Nod factor perception (Gamas et al., 1996). *MtN5* is not induced in root inoculated with *S. meliloti nodA* mutant, which is defective in Nod factors synthesis, and it is not expressed in *M. truncatula nfp* mutant, which is impaired in Nod factor perception (Gamas et al., 1996, El Yahyaoui et al., 2004). The results herein presented confirm that MtN5 protein is rapidly accumulated in the root tissue during the early stages of nodulation. Furthermore, we show that in the later stages MtN5 is predominantly localized in the nodule. This expression pattern might suggest that the role of MtN5 in the symbiotic relationship might be to promote nodule development and/or to protect the root nodules from pathogens attack. Nonetheless, the suppression of *MtN5* transcript results in a reduced nodulation whereas its overexpression increase the number of nodules produced by *S.meliloti*. This observation prompts us to rule out the putative involvement of MtN5 in the regulation of nodules number and in the limitation of rhizobia spread, even though it exhibits a certain antibiotic activity against *S. meliloti in vitro*. These observations concord well with the fact that *MtN5* does not belong to those genes that are down-regulated in the super-nodulating *M. truncatula* mutant TIR122 (El Yahyaoui et al., 2004).

It is worth noting that MtN5 reaches the higher concentration at 3 days after *S. meliloti* inoculation, corresponding to the stage of infection threads ramification which takes place just before the invasion of infection competent cells in the nodule primodium (Kuppusamy et al., 2004). This timing of expression might indicate the involvement of MtN5 in the early stages of rhizobia infection including the Nod factors perception and/or the formation of infection threads. Interestingly, phospholipids seem to act as signalling molecules in the establishment of the symbiosis (Charron et al., 2004). More recently it has been shown that lysophosphatidil-choline is involved in the signalling pathway of arbuscular mycorrhizal symbiosis (Drissner et al., 2007). We demonstrate that MtN5 can bind lysophosphatidil-choline and thus it could be involved in signalling processes during symbiosis. In this regard,

it is remarkable that MtN5 shares a high degree of homology with DIR1 which takes part in the signalling cascade for SAR activation in *A. thaliana*. However, considering that MtN5 is also expressed in mature nodules, we cannot exclude the fact that it might have a role in the later stages of nodules development too.

There is experimental evidence suggesting that LTPs could also be involved in the synthesis of cuticle, in particular, functioning as lipid carriers from the intracellular compartment towards the apoplast, where the cuticle is synthesised (Cameron et al., 2006). Following on from this idea, MtN5 could be involved in the targeting of new lipidic material during both the development of infection threads and symbiosome membranes. Such a role would be in good agreement with the higher number of nodules detected in root transformed with *35S::MtN5* gene construct.

The present results also demonstrate that MtN5 protein is rapidly accumulated in the root tissue after the infection with fungal root pathogens. Such induction upon *F. semitectum* inoculation might suggest that MtN5 can have a defensive role against root pathogenic fungi. It was demonstrated that plant LTPs can exert their antimicrobial activity either via permeabilization of pathogens' plasma membrane (Diz et al., 2006; Regente et al., 2005) or as lipid sensors in defence signalling pathway, as is the case of *A. thaliana* DIR1 (Maldonado et al., 2002). Lipid molecules (oxolipins, phosphatidic acid and N-acylethanolammines) are produced by plants as a consequence of pathogen attacks and act as secondary messengers in plant defence signalling (Wasternack and Parthier, 1997; Chapman, 2000; Munnik, 2001). Since MtN5 is not induced by either *X. campestris* infection or wounding, our findings also suggest that the expression of MtN5 protein is not a generic response to biotic and abiotic stresses, but is restricted to those microorganisms that interact with the plant at root level. The persistence of MtN5 in mature nodules could represent a form of rapid protection of the nodules against a fungal attack. Further studies are needed to have a clear picture of the role played by this gene during symbiotic interaction between *M. truncatula* and *S. meliloti.* In particular, it would be interesting to study the localization of MtN5 both within

the root nodule and in the root tissue during the different stages of rhizobia infection, and to identify which phases of the nodulation process MtN5 takes part in. In order to thoroughly dissect the involvement of MtN5 in the symbiosis, the employment of *MtN5*-silenced plants and mutants impaired in nodulation at different levels is required. In addition, the response to pathogenic microorganisms could be in-depth studied using *MtN5*-silenced and *MtN5*-overexpressed *M. truncatula* transgenic plants.

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Auxin and nitric oxide control indeterminate nodule formation

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Abstract

Background: Rhizobia symbionts elicit root nodule formation in leguminous plants. Nodule development requires local accumulation of auxin. Both plants and rhizobia synthesise auxin. We have addressed the effects of bacterial auxin (IAA) on nodulation by using *Sinorhizobium meliloti* and *Rhizobium leguminosarum* bacteria genetically engineered for increased auxin synthesis.

Results: IAA-overproducing *S. meliloti* increased nodulation in *Medicago* species, whilst the increased auxin synthesis of *R. leguminosarum* had no effect on nodulation in *Phaseolus vulgaris*, a legume bearing determinate nodules. Indeterminate legumes (*Medicago* species) bearing IAA-overproducing nodules showed an enhanced lateral root development, a process known to be regulated by both IAA and nitric oxide (NO). Higher NO levels were detected in indeterminate nodules of *Medicago* plants formed by the IAA-overproducing rhizobia. The specific NO scavenger cPTIO markedly reduced nodulation induced by wild type and IAA-overproducing strains.

Conclusion: The data hereby presented demonstrate that auxin synthesised by rhizobia and nitric oxide positively affect indeterminate nodule formation and, together with the observation of increased expression of an auxin efflux carrier in roots bearing nodules with higher IAA and NO content, support a model of nodule formation that involves auxin transport regulation and NO synthesis.

Background

The phytohormone auxin (indole-3-acetic acid, IAA) mediates several processes in plant growth and development such as tropic responses to light and gravity, general root and shoot architecture, organ patterning and vascular development [1]. A role for IAA in nodule development was first postulated in 1936 by Thimann [2], supported by the observation that root nodules have a higher IAA content than uninfected root tissue. Studies on nodule development performed with natural (flavonoids) and artificial (e.g. NPA) inhibitors of auxin transport, as well as direct and indirect measurements of IAA, have indicated that auxin accumulates at the site of nodule initiation during nodule formation [3-5].

Free-living rhizobia synthesize IAA [6] and most likely they retain a similar capacity to synthesize IAA during nodulation, because a positive correlation between IAA production in liquid culture and IAA content of the nodules has been demonstrated by using *Bradyrhizobium japonicum* mutants with different IAA synthesising capacities [7]. Several publications have addressed the putative

role of auxin produced by rhizobia in determinate nodule development and function [7-9]. 5-methyltryptophan-resistant mutants of *B. japonicum* that overproduce IAA caused, in comparison with wild type rhizobia, a lower nodule mass and a lower number of nodules in soybean [7]. However, another study [9] has shown that inoculation of soybean plants with a tryptophan catabolic mutant of *B. japonicum* that produced elevated amounts of IAA and IPA (indolyl-3-pyruvic acid) increases nodule volume and root weight compared to inoculation with wild type bradyrhizobia. A promoting effect of IAA on determinate nodule formation was also suggested by the observation that IAA-deficient *B. japonicum* mutants produced significantly less nodules than wild type strains [8]. To our knowledge, the effects of increased or reduced IAA synthesis by rhizobia on indeterminate nodule formation has not been investigated by genetic methods.

Nodule organogenesis and lateral root formation display some similarities. Both organs require auxin at development of the primordia and for the differentiation of the vasculature [10,11]. Furthermore lateral root initiation involves the formation of a dynamic auxin gradient in the primordia. Auxin gradient is formed by cellular efflux and requires asymmetrically localized IAA transporters, called PIN proteins [12]. The current model for nodule initiation is also based on the formation of an asymmetric auxin gradient [10].

The auxin signalling pathway and the role of downstream effectors have received great attention in the last years [13]. Recent experimental evidence has shown that NO plays a role in both lateral and adventitious root initiation [14,15]. In auxin-induced adventitious root formation, NO acts as a second messenger and operates downstream of IAA [15].

In this report, we have used *Sinorhizobium meliloti* and *Rhizobium leguminosarum* expressing an auxin-synthesising chimeric operon (*rolAp-iaaMtms2*) to study the effects of rhizobia-derived auxin on nodule formation. We show that auxin synthesised by rhizobia promotes nodulation and host root growth in plants bearing indeterminate nodules whilst no effect was observed in plants bearing determinate nodules. Furthermore, we show that NO is involved in both indeterminate nodule formation and lateral root growth.

Results

Expression of rolAp-iaaMtms2 *in* S. meliloti *increases nodule IAA content and alters root IAA polar transport*

In order to increase the auxin biosynthetic capacity of *S. meliloti*, we engineered a chimeric construct (Fig. 1A) containing the *iaaM* gene from *Pseudomonas syringae* pv. *savastanoi* and the *tms2* gene from *Agrobacterium tumefaciens* as a bicistronic unit under the control of the prokaryotic promoter (promintron) of the *rolA* gene of *Agrobacterium rhizogenes* [16,17]. The *iaaM* gene codes for a tryptophan monoxygenase, which converts tryptophan to indol-3-acetamide (IAM), while the *tms2* gene codes for a hydrolase involved in the conversion of IAM to IAA. The 85 bp-long intron of the T-DNA gene *rolA* has a dual function: it behaves as an intron when the *rolA* gene is expressed in plant cells and acts as a prokaryotic promoter in free-living rhizobia and in bacteroids inside nodules [16,17].

The *rolAp-iaaMtms2* chimeric operon was mobilized into *S. meliloti* strain 1021 to generate an auxin-overproducing strain (hereafter referred to as the IAA strain). RT-PCR analysis, carried out on total RNA extracted from 40 day-old nodules of *Medicago truncatula* and *Medicago sativa* plants infected by the IAA strain, demonstrated that the *rolAp-iaaMtms2* chimeric operon is transcribed in mature nodules (Fig. 1B).

The total (free and conjugated) IAA concentration of root nodules (1 g FW nodules collected from 40 day-old plants) was measured by GC-MS using deuterated IAA as an internal standard. In control nodules of *M. sativa*, the concentration of IAA was 0.12 nmol/g FW, whereas in extracts obtained from 1 g FW of *M. truncatula* control nodules, IAA was undetectable. Considering the detection limits of the method, we estimate that the value is below 0.010 nmoles for 1 g of tissue. In nodules of *M. sativa* and *M. truncatula* plants infected by the *S. meliloti* IAA strain, the IAA concentration was 1.2 and 1.14 nmol/g FW, respectively. Thus, the expression of the *rolAp-iaaMtms2* chimeric operon in bacteroids resulted in at least a 10-fold increase in root-nodule auxin content in *Medicago*. The polar transport of auxin, which is crucial for almost all auxin-related developmental processes, is based on the action and the asymmetric distribution of specific auxin influx and efflux carriers [18]. To investigate whether the auxin derived from rhizobia can affect the expression of auxin transporters, we have compared the steady state mRNA levels of selected putative influx and efflux carriers in *M. truncatula* plants bearing IAA overproducing and control nodules. Several members of the *LAX* and *PIN* gene families, directly involved in auxin transport, have been identified in *M. truncatula* [10,19]. In particular, we analysed the expression of three auxin influx carrier genes, *MtLAX1*, *MtLAX2* and *MtLAX3*, known to be expressed in nodulating roots [10] and two efflux facilitators *MtPIN* genes: *MtPIN2* expressed only in roots and *MtPIN1*, expressed in both roots and aerial parts [19] (Fig. 2).

The steady state mRNA levels of *MtPIN2* were significantly higher in roots nodulated by IAA rhizobia compared to roots nodulated by control rhizobia (Fig. 2). The steady state mRNA levels of *MtPIN1* and the three influx carriers

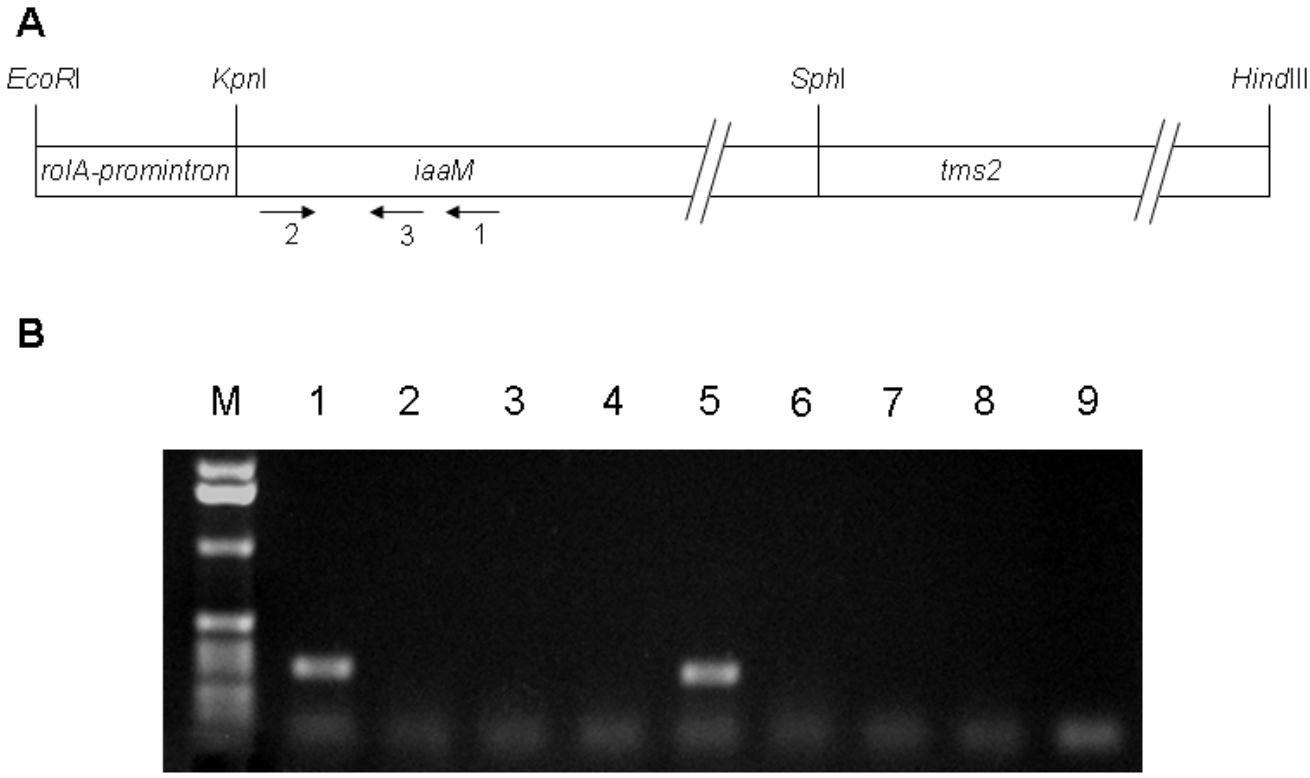

Figure 1

Expression of *rolAp-iaaMtms2* construct in indeterminate root nodules. A. Schematic drawing of the chimeric operon. Restriction endonuclease sites used for chimeric operon construction are reported. B. Agarose gel electrophoresis of RT-PCR product obtained from total RNA extracted from nodules (lanes 1 and 5) formed by *S. meliloti* IAA strain in *M. truncatula* and *M. sativa*, respectively. Lanes 3 and 7, RT-PCR performed on total RNA extracted from nodules induced by the control strain in *M. truncatula* and *M. sativa*, respectively. Lanes 2 and 4, RNA from nodules of *M. truncatula* induced by IAA and control strain, amplified without reverse transcriptase; lanes 6 and 8, RNA from nodules of *M. sativa* induced by IAA and control strain, amplified without reverse transcriptase. Lane 9, no-template control. The position of the primers used in RT-PCR analysis is indicated by arrows in the schematic drawing reported in panel A.

were not significantly modified. *MtPIN2* expression was not detectable in shoots, as already observed by Schnabel and Frugoli [19]. The mRNA levels of the other auxin transporter genes did not differ in the shoots of plants nodulated with either the IAA or control strains (data not shown).

IAA synthesised by rhizobia promotes nodulation and root development in legumes with indeterminate nodules

Root growth, nodule number and shoot growth of plants inoculated with either IAA or control *S. meliloti* strains were evaluated 40 days after germination. In *M. truncatula*, the average number of nodules per plant was doubled in plants infected by the IAA strain compared to plants infected with the control strain (Fig. 3A and Fig. 4A).

A stimulatory effect on nodulation was also observed in *M. sativa* where the mean number of nodules per plant produced by IAA strain was approximately 50% higher than in plants nodulated by the control strain (Fig. 3B and Fig. 4A). The weight and size of the nodules were on average identical regardless of whether plants were nodulated by the IAA or control strain (Fig. 3A and Fig. 3B and data not shown).

Both *M. truncatula* and *M. sativa* plants bearing IAA-overproducing nodules had a more developed root apparatus in comparison with plants nodulated by the control strain (Fig. 3C and Fig. 3D). The lateral root growth (calculated as weight of total root apparatus/cm of primary root) was on average two times higher in *M. truncatula* plants nodulated by the IAA strain (mean value ± SE = 26 ± 2.6 mg/cm; n = 18) than those nodulated by the control strain (mean value ± SE = 13 ± 1.2 mg/cm, n = 18) (see also additional file 1). A similar increase in lateral root growth was observed in *M. sativa* (Fig. 3D).

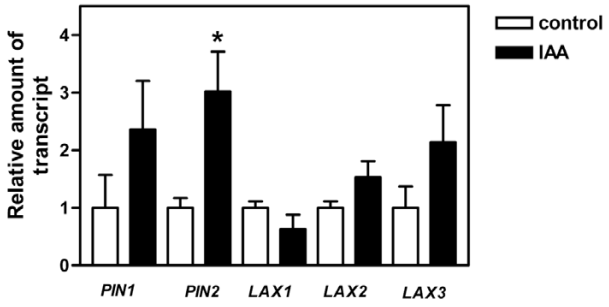

Figure 2
Expression of auxin carrier genes in *Medicago truncatula* roots. Expression levels of auxin efflux (PIN1 and PIN2) and influx (LAX1, LAX2 and LAX3) carrier genes were evaluated by quantitative RT-PCR (QRT-PCR). The expression levels were normalized using actin as the endogenous control gene. The QRT-PCR analysis was performed using a ABI Prism 7000 Sequence Detection System. Relative transcript level is the ratio between the expression levels in roots of plant nodulated by the IAA strain and roots of plant nodulated by the control strain. Relative transcript levels were calculated according to manufacturer's recommendations, using the formula $2^{-(\Delta Ctiaa-\Delta Ctc)}$, where ΔCtiaa and ΔCtc is the difference between the threshold cycle of the gene tested and the threshold cycle of actin in IAA and control samples, respectively. The significance of the differences between control and IAA expression levels was evaluated using a Student's *t* test (n = 3). Mean values ± SE are reported. *, $P < 0.05$. Control: plants nodulated by the control strain. IAA: plants nodulated by the IAA strain.

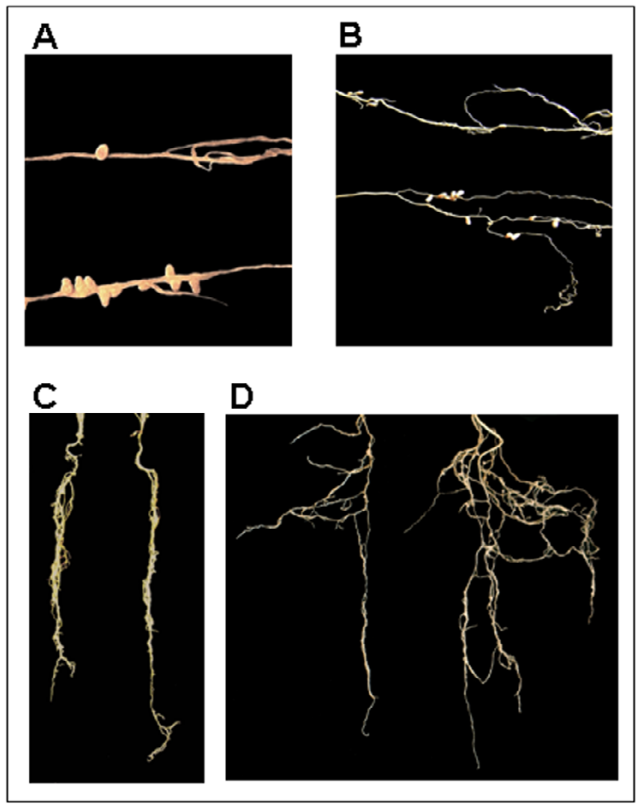

Figure 3
Phenotypes of *M. truncatula* and *M. sativa* plants nodulated with *S. meliloti* IAA or control strain. A. *M. truncatula* root nodules: nodules induced by *S. meliloti* IAA strain (bottom) and nodules induced by the *S. meliloti* control strain (top). B. *M. sativa* root nodules: nodules induced by the *S. meliloti* IAA strain (bottom) and nodules induced by *S. meliloti* control strain (top). C. *M. truncatula* roots of plants nodulated by the IAA strain (right) and roots of plants nodulated by the control strain (left) D. *M. sativa* roots of plants nodulated by the IAA strain (right) and roots of plants nodulated by the control strain (left).

We have also investigated the relationship between the number of lateral roots and the number of nodules present on them. The two parameters were significantly correlated in plants inoculated with IAA strain but not in plants inoculated with the control strain (see additional file 2). Altogether, these data suggest that IAA-overproducing rhizobia have a greater capacity to nodulate lateral roots and also a positive effect on lateral root formation.

When *Medicago* plants were grown under conditions that limit root growth – and in particular lateral root growth- (i.e. in 15 ml plastic tubes), a higher density of nodules was observed in plants inoculated by IAA strain compared to those inoculated by control strain (mean values ± SD: 0.12 ± 0.04 and 0.08 ± 0.02 nodules/mg root FW with IAA and control strain, respectively; $P < 0.05$, n = 12). Thus under conditions that limit root growth, IAA strain still retains a higher capacity to induce nodule formation. This suggests that the increase in lateral root growth is most probably a consequence of the increased synthesis of IAA in the nodules.

The primary root of *M. truncatula* plants bearing IAA-overproducing nodules was on average 40% longer than control plants, whereas in *M. sativa* nodulated by IAA strain, primary root growth was unchanged (Fig. 3C, Fig. 3D and Fig. 4B). No difference in growth of the aerial parts (measured as shoot height) was observed between plants nodulated by either the IAA or control strains. (Fig. 4C). This observation was confirmed by the evaluation of dry matter production and total protein concentration in aerial parts that did not vary in all the experiments (data not shown)

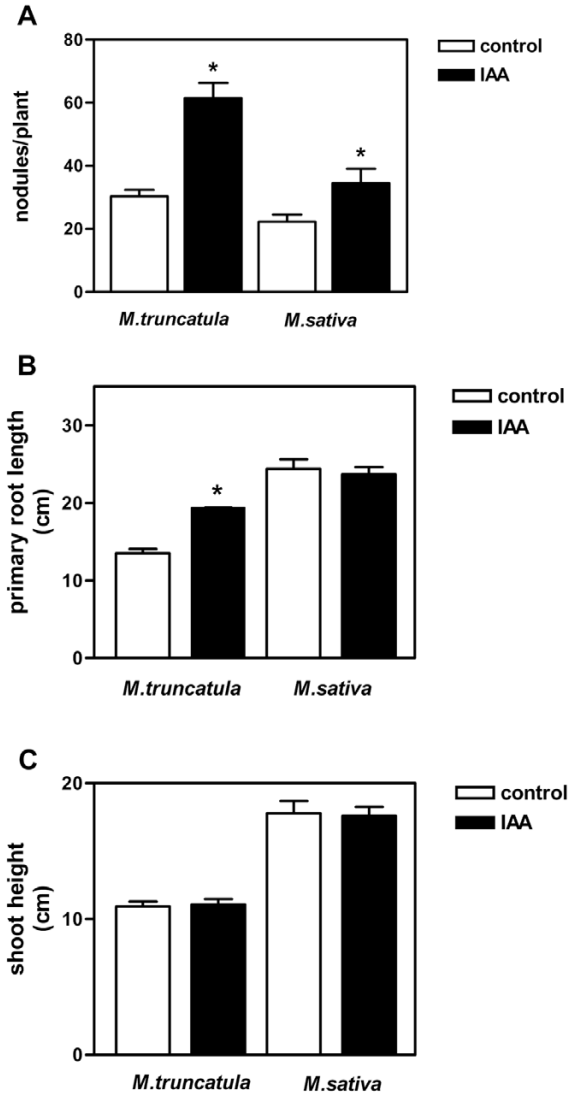

Figure 4
Effects of increased rhizobial IAA biosynthesis on nodulation and growth of *Medicago* plants. A. Number of nodules per plant. B. Primary root length. C. shoot height. The values reported are means ± SE (n = ≥22). The experiment was repeated three times with the same results. *, P < 0.05. Control: plants nodulated by the control strain. IAA: plants nodulated by the IAA strain.

Nodulation of legumes with determinate nodules is not affected by IAA-overproducing rhizobia

The *rolA* promintron is able to drive bacterial gene expression in determinate nodules (*Phaseolus vulgaris*) as shown by the *rolAp-GUS* gene construct (data not shown). In order to study the effect of increased IAA rhizobial synthesis on determinate nodules, *R. leguminosarum bv. phaseoli* harbouring the *rolAp-iaaMtms2* construct was used to inoculate *Phaseolus vulgaris* plants. Expression of the *rolAp-iaaMtms2* chimeric operon in bean nodules was proved by RT-PCR analysis (Fig. 5A).

Expression of the *rolAp-iaaMtms2* operon in mature determinate nodules of 30 days-old *Phaseolus vulgaris* plants results in a ten times higher concentration of IAA (0.034 nmol/g FW) compared to control nodules (0.003 nmol/g FW). Differently from *Medicago* species where an increase of IAA in the nodules was associated with enhanced nodulation and root growth (Fig. 3 and Fig. 4), the number of nodules and the root growth did not significantly differ in bean plants nodulated by IAA-overproducing rhizobia compared with control strain (Fig. 5B and data not shown).

NO is involved in the formation of indeterminate nodules

We also evaluated endogenous NO production in nodules, from *Medicago* plants produced by either the control or the IAA *S. meliloti* strain, loaded with the permeable NO-sensitive dye fluorophore 4,5-diaminofluorescein diacetate (DAF-2-DA). Figure 6 demonstrates that NO production is significantly increased in both *M. truncatula* and *M. sativa* IAA-overproducing nodules. The increase in NO production was about 3 times and 2 times higher in *M. truncatula* and *M. sativa*, respectively (Fig. 6A and Fig. 6B). The NO level in control nodules was higher in *M. sativa* compared to *M. truncatula* (Fig. 6A and Fig. 6B).

Some plant associated bacteria can generate NO from the conversion of L-arginine to L citrulline through an NO synthase activity [20]. Using DAF-2-DA to evaluate NO production in free-living *S. meliloti* at stationary phase of growth, we have observed that IAA-overproducing *S. meliloti* grown under aerobically conditions with ammonium salts as nitrogen source, generate, after arginine addition, a fluorescence signal similar to control strain (our observation). In agreement with this observation, NO synthase-like activity of IAA-overproducing and control rhizobia was not significantly different (see additional file 3).

This indicates that free living *S. meliloti* is able to produce NO and that wild type and IAA strain do not differ in NO production. This observation suggests that bacteroids can contribute to NO production in the nodule.

In order to assess a possible link between NO and indeterminate nodule formation, we tested the effect of the NO scavenger, cPTIO on *M. truncatula* plants inoculated by IAA and control *S. meliloti* strains. The plants, grown in plastic tubes on perlite supplemented with N-free nutrient solution, were treated with 1 mM cPTIO 2, 24 and 48 h after rhizobia inoculation. NO depletion by treatment with cPTIO caused a significant reduction in nodule

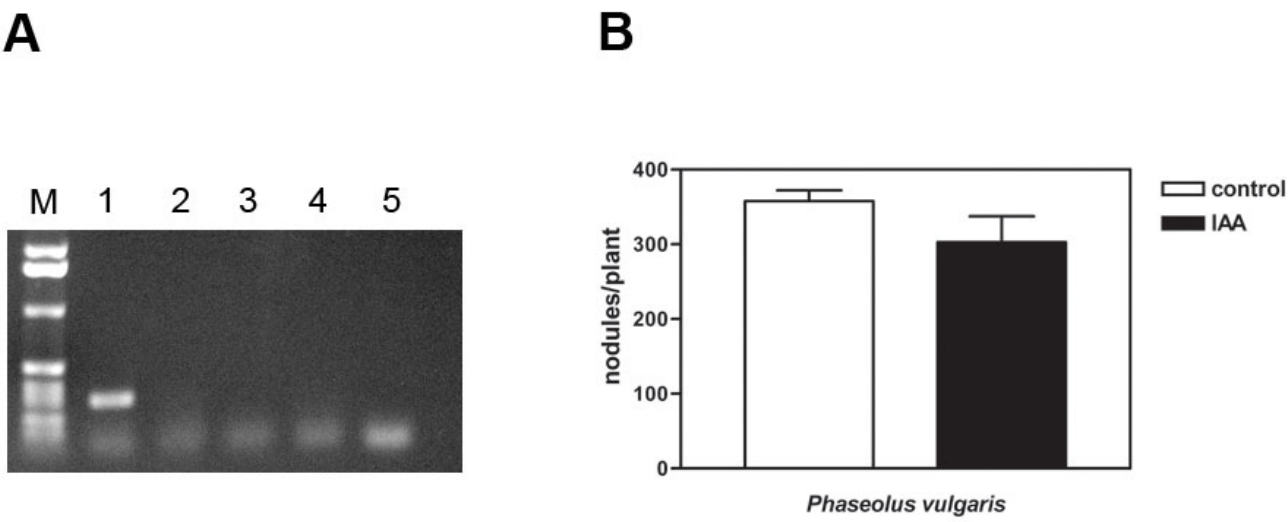

Figure 5
Expression of the *rolAp-iaaMtms2* chimeric operon in determinate root nodules. A. Agarose gel electrophoresis of RT-PCR product obtained from total RNA extracted from bean nodules (lane 1) formed by *Rhizobium leguminosarum* IAA strain. Lane 3, RT-PCR analysis performed on total RNA extracted from bean nodules induced by the control strain. Lanes 2 and 4 reaction without reverse transcriptase performed on total RNA extracted from bean nodules induced by IAA and control strain, respectively. Lane 5, no-template control. B. Number of nodules per plant. The values reported are means ± SE (n = 13). The mean values are not statistically different.

number (Fig. 7) in plants inoculated with either IAA-overproducing or control rhizobia. This finding demonstrates that NO depletion inhibited indeterminate nodule formation and completely abolished the auxin stimulatory effect on nodulation. Nitric oxide depletion inhibited the increase in lateral root growth caused by IAA-overproducing strain (data not shown), confirming previous data on the role played by NO in lateral root formation in tomato [14]. Primary root length and shoot growth were not affected by 1 mM cPTIO. Furthermore, the treatment with cPTIO has no effect on *S. meliloti* growth (see additional file 4).

Discussion

The formation of N_2-fixing root nodules in leguminous plants requires a complex exchange of signals between the host and the compatible rhizobia strain. Phytohormones, and in particular auxin, have been implicated in this process. Our data demonstrate that rhizobium-derived auxin promotes indeterminate nodule formation (*Medicago* sp.), whilst an increased synthesis of IAA within rhizobia does not affect determinate nodule formation (*Phaseolus vulgaris*).

The effects of IAA-overproducing and IAA-deficient rhizobia mutants on nodulation has been investigated in several previous studies that have reported rather contrasting results about the role of rhizobial IAA synthesis in nodule development [7-9]. Moreover, the results presented by those studies are somehow difficult to interpret due to the limited molecular characterization of the mutants. Our experimental approach is novel in that we have used rhizobia genetically engineered for a new (*iaaMtms2*) auxin biosynthetic pathway to address the role of rhizobia-derived auxin. Thus, the IAA-overproducing rhizobia strain differs from the control strain only for the increased auxin synthesising capacity. The *Sinorhizobium meliloti* strain that overproduces auxin has an enhanced ability to nodulate both *M. sativa* and *M. truncatula*, which results in a 50% and 100%, respectively, increase in the number of nodules per plant compared to the control strain. The use of the *rolA* promoter [17] to drive *iaaMtms2* expression enables the synthesis of auxin in bacteroids leading to an increase of auxin content within the nodules. The *rolA* promoter is active also in free living rhizobia [17]. Thus, it is likely that the synthesis of auxin takes place also during early phases of infection (e.g. root-hair curling and formation of the infection thread) and nodule initiation.

In the current model of both determinate and indeterminate nodule organogenesis, the local accumulation of auxin at the site of nodule initiation is thought to stimulate cellular division in the cortex and pericycle [10,4]. The higher auxin synthesising capacity of the IAA strain may facilitate nodule formation by increasing auxin levels within the nodule primordium. This interpretation is

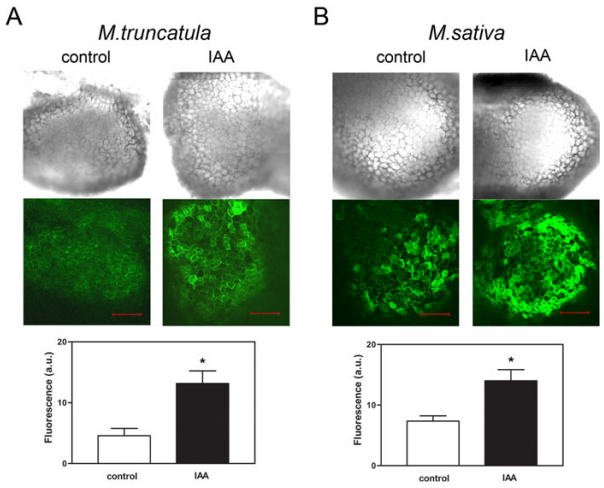

Figure 6
Evaluation of NO production in nodules. A. *M. truncatula* nodulated with *S. meliloti* IAA or control strain B. *M. sativa* nodulated with *S. meliloti* IAA or control strain. Upper panels: Microscopy. Bright-field images of the nodules (top) and the confocal laser scanning microscopy (CLSM) detection of endogenous NO in the same nodules (bottom, excitation at 488 nm, emission at 505–530 nm). Bars indicate 200 μm. Photographs are representative of results obtained from the analysis of nodules in three independent experiments. Lower panels: Analysis of fluorescence intensities in nodules induced by IAA and control *S. meliloti* strain. Results are means ± SE (from at least 15 nodules); all data are statistically significant ($P < 0.05$). IAA: nodules produced by the *S. meliloti* IAA strain. Control: nodules produced by the *S. meliloti* control strain.

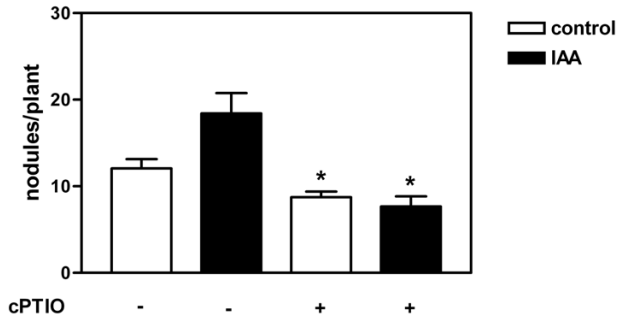

Figure 7
Effects of the NO scavenger cPTIO on nodulation. Nodule number of *Medicago truncatula* plants inoculated by IAA and control strain and treated with 1 mM cPTIO. Results are means ± SE (n = at least 12). cPTIO treatments are significantly different from respective controls, ($P < 0.05$). IAA: nodules produced by the *S. meliloti* IAA strain. Control: nodules produced by the *S. meliloti* control strain.

somehow in agreement with the observation that a *M. truncatula* supernodulating plant mutant contains three times more auxin than wild type at the site of nodule initiation [21]. Furthermore, the observation that the average size of IAA overproducing and control indeterminate nodules is similar indicates that rhizobia-derived auxin affects mainly nodule formation rather than nodule growth.

The auxin loading model of van Noorden and colleagues [21] suggests that the inhibition of auxin loading from shoot to root is the basis of the autoregulation of nodulation in indeterminate legumes. This mechanism is altered in the *sunn* hypernodulating mutant and consequently auxin continues to be transported from the shoot to the root and sustains supernodulation [21]. In our experimental system, the increased number of nodules obtained in *Medicago* plants is most likely the consequence of extra auxin loaded in the root from the bacteroids within the nodules.

Determinate nodules apparently do not inhibit the auxin transport from the shoot [5] and an hypernodulating plant mutant (soybean *nts*) does not show an increased auxin level in the root [22]. This finding has prompted van Noorden and colleagues [21] to propose that determinate and indeterminate nodules differ in the requirement, transport capacity or regulation of auxin transport [5,21]. In accordance with this hypothesis, our results show that an increased auxin synthesis in bacteroids does not affect determinate nodule formation in *Phaseolus vulgaris*.

M. truncatula and *M. sativa* plants bearing IAA overproducing nodules, compared to plants with control nodules, have a more developed root apparatus with abundant lateral roots, a characteristic trait observed in some mutants that overproduce auxin [1]. A striking similarity between lateral root and indeterminate nodule development has been already indicated [4,10]. Moreover, based on the observation of a strong correlation between nodule and lateral root number in pea, a possible overlap during early developmental pathways of the two organs has been suggested by Ferguson et al. [23]. Our data are somehow consistent with this hypothesis [23] since both more nodules and more developed lateral roots are observed in *Medicago* plants nodulated by IAA-overproducing rhizobia. However, in plants inoculated with the control strain we did not find any correlation between the number of lateral roots and the number of nodules present on the lateral roots (see additional file 2). On the other hand, the significant correlation between nodule and lateral root numbers detected in *M. truncatula* plants inoculated with IAA strain (see additional file 2) suggests that IAA-overproducing rhizobia have a greater capacity to nodulate lateral roots besides a positive effect on lateral root formation.

We did not observe any effects of IAA overproducing rhizobia on the growth and biomass production of the aerial parts of *M. truncatula* and *M. sativa*. Thus, under the growth conditions used in the experiments, there is no indication of an increased nitrogen fixation. However, we can not rule out that plants grown under limiting growth conditions might eventually take advantage (i.e. a more efficient water and nutrient uptake) from the more developed root apparatus induced by IAA overproducing rhizobia.

Auxin transport is mediated by asymmetrically-localized auxin influx/efflux facilitators that regulate auxin distribution during root and shoot growth [12,18]. Changes in the expression levels of auxin carriers can affect root development. In this study, we show that plants with IAA-overproducing nodules have increased expression of the root-specific *MtPIN2* gene, an ortholog of the auxin efflux carrier *PIN2* of *A. thaliana* [19] that mediates the transport of auxin towards the root elongation zone [18]. An increased *PIN2* expression has also been reported in the hypernodulating *sickle* mutant of *M. truncatula* [24] and nodulation was shown to be inhibited in *PIN2* silenced plants [25]. The aforementioned result suggests that the observed changes in nodulation and root growth are most likely the consequence of both the increased concentration of auxin in the nodules and the auxin redistribution in the root tissue.

The involvement of NO in auxin-induced adventitious root formation in cucumber and in lateral root formation in tomato has been recently reported, providing evidence that NO is a component of the auxin signalling pathway in these processes [14,26]. This work shows that NO is produced in root nodules of *M. truncatula* and *M. sativa* and that NO is increased in IAA-overproducing nodules. In plant tissues NO can be generated by enzymatic and non enzymatic systems [27], while rhizobia under anaerobic condition produce NO via the denitrification pathway [28]. According to a recent study [29] enzymes of the denitrification pathway do not contribute to NO generation during nodule development. We have observed that aerobically-grown stationary phase IAA-overproducing and wild type *S. meliloti* produce NO and possess NO synthase-like activity. Thus, NO level in nodules could be the result of both plant and bacterial production. However, no difference in NO production was observed in free living wt and IAA strains. Consequently, the increased NO level present in IAA-overproducing nodules is likely the result of plant NO synthesis induced locally by bacterial IAA. Based on these results, a role for IAA and NO in indeterminate nodule formation is hereby proposed. To our knowledge, the data showing that nodule NO biosynthesis is increased in plants with higher nodulation and that a NO scavenger reduces nodule formation represents the first experimental evidence of NO involvement in the auxin-signalling pathway controlling indeterminate nodule formation.

Conclusion

The data presented demonstrate, by using rhizobia engineered for a high production of auxin, that an increased bacterial auxin synthesis promotes the formation of indeterminate nodules, whereas it has no effect on determinate nodule formation. We also show that nitric oxide acts as a signal molecule in controlling, either directly and/or indirectly, nodule number in indeterminate legumes. These data indicate that indeterminate nodule formation involves regulation of both auxin and NO signalling.

Methods

Bacterial strains

Sinorhizobium meliloti 1021 is a streptomycin-resistant derivative of wild-type field isolate SU47 [30]. *S. meliloti* was grown at 28°C in LBMC medium (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl, 2.6 mM $MgSO_4$, 2.6 mM $CaCl_2$) supplemented with streptomycin 200 μg/ml. *Rhizobium leguminosarum bv. phaseoli* was grown at 28°C in YEM medium (mannitol 10 g/l, NaCl 0.1 g/l, $MgSO_4$ 0.2 g/l, KH_2PO_4 0.5 g/l, yeast extract 0.4 g/l).

Plasmids and gene constructs

Standard techniques were used for the construction of recombinant DNA plasmids. The *rolAp-iaaMtms2* chimeric operon contains the bicistronic unit *iaaMtms2* under the control of *promintron*, the 85 bp-long intron of *rolA* gene of *Agrobacterium rhizogenes* which has promoter function in bacteria [17]. A 1773 bp-long DNA sequence spanning the coding region (1671 bp) of the *iaaM* gene (GenBank accession n. M11035) from *Pseudomonas syringae pv. savastanoi* [31] and a 1452 bp-long DNA sequence spanning the coding region (1404 bp) of *tms2* (GenBank accession n. AH003431) from *Agrobacterium tumefaciens* [32], were cloned downstream of the *promintron* sequence (*rolAp*), connected by a 17 bp-long linker sequence. The *rolAp-iaaMtms2* construct was subcloned in the broad-host range plasmid pMB393 [33] and introduced by electroporation into *S. meliloti* 1021 and *R.l. phaseoli* to obtain the IAA strains. *S. meliloti* 1021 and *R.l. phaseoli* harbouring the pMB393 plasmid containing the *promintron* sequence was used as control strain.

Plant growth and inoculation

Medicago truncatula cv. *Jemalong* and *Medicago sativa* ecotype *Romagnolo* seeds were scarified using fine grade sand paper sheets and sterilized in 5% commercial bleach for 3 min. Seeds were rinsed three times with sterile water and stored on 0.8% agar plates at 4°C for 2 days before placing in a growth chamber at 25°C for 7 days to allow germination. *Phaseolus vulgaris* seeds were sterilized in 12% com-

mercial bleach for 7 minutes, rinsed and then imbibed in sterile water for 1 hour.

Germinated seedlings of *M. truncatula* were transferred in small pots and grown on a sand and perlite mixture (1:1) in a growth chamber at 22°C and 10-h light/14-h dark regimen under fluorescent lights giving an average irradiance of 120 μmol m^{-2} sec^{-1} of photosynthetically active radiation (PAR); the relative humidity was 65%. *Medicago sativa* and *Phaseolus vulgaris* seedlings were grown in small pots on sand and perlite mixture (1:1) in a greenhouse at day and night temperatures of 24°C and 18°C, respectively, and 10 h light/14 h dark regimen. Once a week the *Medicago sp*. and *P. vulgaris* seedlings were supplemented with a nitrogen-free nutrient solution (0.13 mM KH_2PO_4; 0.3 mM $CaCl_2 \cdot 2H_20$; 0.06 mM $MgSO_4 \cdot 7H_2O$; 0.2 mM K_2SO_4; 0.014 mM FeNa EDTA; 1.56 mM H_3BO_3; 1.24 mM $MnSO_4 \cdot H_2O$; 4.5 mM KCl; 0.11 mM $ZnSO_4 \cdot 7H_2O$; 0.1 mM $CuSO_4 \cdot 5H_2O$; 0.32 mM H_2SO_4; 2.1 mM $Na_2MoO_4 \cdot 2H_2O$). The seedlings were watered with sterile deionized water as necessary.

For plant inoculation, bacteria were grown in liquid medium, collected by centrifugation, washed in sterile water, and then diluted in sterile water to 0.1 OD_{600} (approximately 10^8 cfu/ml). Ten ml of this suspension were used to inoculate seedlings at 10 and 24 days after germination. Leaves, roots and root nodules were collected 40 days after germination. At the end of each experiment, the presence of the recombinant plasmids in bacteroids was checked by PCR analysis on total DNA extracted from root nodules.

For cPTIO treatments, *M truncatula* seedlings were transferred after germination in 15 ml test tubes, containing nitrogen-free nutrient solution and perlite (1:2 vol/vol) and grown in a growth chamber at 24°C 16-h light/8-h dark regimen under fluorescent lights giving an average irradiance of 120 μmol m^{-2} sec^{-1} PAR; the relative humidity was 65%. Ten days old plants were inoculated using 1 ml of a bacterial suspension to an OD_{600} of 0.1. Two hours after inoculation, 1 ml of 1 mM 2-(4-carboxyphenil)-4,4,5,5,-tetramethylimidazoline-1-oxyl-3oxide (cPTIO) (Sigma, St. Luois, MO, USA) was added to the nutrient solution; 1 ml of distilled water was used for negative controls. This treatment was repeated 24 and 48 hours after inoculation. Nodules were counted 28 days after inoculation. cPTIO (1 mM) has no effect on *S. meliloti* growth (see additional file 4).

IAA analysis

Root nodules (1 and 5 g FW for *Medicago* and bean plants, respectively) were collected from 40 day-old plants. IAA extraction was carried out as previously described [34]. 100 nmols of D^{5-} IAA were added to the samples, as internal standard.

TMS GC-MS analysis was performed on a Hewlett Packard 5890 instrument equipped with a HP-5 (Agilent technologies) fused silica capillary column (30 m, 0.25 mm ID, helium as carrier gas), with the temperature program: 70°C for 1 min, 70°C→150°C at 20°C/min, 150°C→200°C at 10°C/min, 200°C→280°C at 30°C/min, 280°C for 15 min. The injection temperature was 280°C. Electron Ionisation (EI) mass spectra were recorded by continuous quadrupole scanning at 70eV ionisation energy.

NO detection

Endogenous NO was detected with the fluorophore 4,5-diamino-fluorescein diacetate (DAF-2-DA). 4-aminofluorescein diacetate (4-AF DA) was used as a negative control, assuming that the green fluorescence detected corresponds to endogenous NO and not to unspecific reactions of the probe. Nodules obtained with the control and IAA strains were incubated with 7.5 μM DAF-2DA (Calbiochem) or with the negative probe 4 AF-DA (Calbiochem) in 20 mM HEPES-NaOH, pH 7.5 (buffer A) for 30 min in the dark at 25°C. Thereafter, nodules were washed three times for 15 min each with buffer A and fluorescence was detected with a Zeiss LSM 510 laser scanning confocal microscope exciting at either 488 or 543 nm. For emission in the green light, fluorescence was examined between 505 and 530 nm, while in the red light, fluorescence was collected at wavelengths >560 nm.

Green fluorescence was quantified by measuring the medium pixel intensity in the confocal images for every single nodule analyzed. All the quantitative data were subjected to statistical evaluation (Student's *t* test). A $P < 0.05$ was considered statistically significant.

RT-PCR analysis

Total RNA (2 μg) extracted from nodules was treated with 2 units of RQ1 DNase (Promega, Madison, WI) and then used as a template for a reverse transcriptase (Superscript II, Invitrogen, Carlsbad, CA) reaction primed with the oligonucleotide 5'-CTCCGTGTCCACCACACC-3' (Primer 1) complementary to the iaaM coding region +372 and +389 bp. The complementary DNA was amplified with the forward primer 5'-ATGTATGACCATTTTAATTCACCCAGT-3' (Primer 2), corresponding to the region +1/+27 of the iaaM gene (+1 is the initiation of translation), and with the primer 5'-CTGGGAGGAAAGCGCATCGCAC-3' (Primer 3), complementary to the region +283/+304 of the *iaaM* gene.

For quantitative RT-PCR analysis (QRT-PCR), leaf and root samples were frozen in liquid nitrogen immediately

after collection and stored at -80°C. Root samples do not include nodules that were detached from the roots before freezing.100 mg of pooled tissues, derived from three different plants, were ground in liquid nitrogen and total RNA was isolated by using Rneasy Plant Mini Kit (QIAGEN), according to the manufacturer's protocol. Five μg of total RNA were treated with 5 units of RQ1 DNase (1 U/μl) (Promega, Madison, WI). All RNA samples were checked for DNA contamination before cDNA synthesis. Comparative PCR analysis was carried out using first strand cDNA obtained with oligo-dT primer and Superscript II (Invitrogen, Carlsbad, CA). The cDNA clones were amplified with gene-specific primers designed to give amplification products ranging from 100 to 150 bp. The nucleotide sequence of the gene-specific primers are the following: *MtPIN1* forward primer 5'-ATGGCTCTGCTGCTGCTGCTAA-3', reverse primer 5'-TCCAGATTGATCAGACGCTCC-3'; *MtPIN2* forward primer 5'-GCATGGGCGGTGGAAGTGGTAA-3', reverse primer 5'-TGGAAGGATCAACAGTGCCA-3'; *MtLAX*1 forward primer 5'-AAACAAGGCGAAGAAACAA-3', reverse primer 5'-ACAGCTAAACCAAGCATCAT-3', *MtLAX2* forward primer 5'-ATGTTGCCACAAAAACAAGG-3', reverse primer 5'-TGAATGAATGATCTTCCACC-3'; *MtLAX3* forward primer 5'-ATGACTTCTGAGAAAGTTGA-3', reverse primer 5'-CTTAGATAATTTGCCAGTAG-3'; actin forward primer 5'-AGATGCTGAGGATATTCAAC-3', reverse primer 5'-GTATGACGAGGTCGGCCAAC-3'.

The reaction mixture contained Platinum SYBR Green QPCR Supermix-UDG, ROX reference dye to correct for fluorescent fluctuations (Invitrogen, Carlsbad, CA) and 0.4 μM of each primer. UDG and dUTP were included in the mixture to prevent re-amplification of carryover PCR products between reactions. The QRT-PCR was performed with ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) with the following cycling conditions: 2 min at 50°C, 2 min at 95°C, 40 cycles of 95°C for 30 sec, 56°C for 30 sec, 72°C for 30 sec and finally 72°C for 3 min. All quantifications were normalized to the actin gene as an endogenous control. For each amplification reaction, analysis of the product dissociation curve was performed to exclude the presence of non-specific amplification. For each determination of mRNA levels, three cDNA samples derived from three independent RNA extractions were analysed. Relative quantification of transcript levels was carried out as previously described [35].

Statistical analysis

The mean values ± SE are reported in the figures. Statistical analyses were conducted using a Student's t-test.

Authors' contributions

YP carried out nodulation experiments and molecular biology analysis; MC performed NO detection in nodules and participated in the manuscript preparation; GC performed the experiments on *Phaseolus*; AS design the gene constructs, participated in manuscript preparation; TP coordinated the study and wrote the manuscript. All authors read and approved the final manuscript.

Additional material

Additional file 1

***Root apparatus of* M. truncatula**. *Picture representing the stretched root apparatus.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2229-7-21-S1.pdf]

Additional file 2

***Nodule and lateral root numbers**. Correlation between nodule and lateral root numbers in plants inoculated by IAA and control strain.*
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Additional file 3

***NO synthase-like activity** Measurement of NO synthase-like activity in IAA-overproducing and control* S. meliloti *strains.*
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[http://www.biomedcentral.com/content/supplementary/1471-2229-7-21-S3.pdf]

Additional file 4

***Effect of cPTIO on* S. meliloti *growth**. Effect of cPTIO on* S. meliloti *growth and survival.*
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