Genetic constraints that determine rhizobium-root nodule formation in *Parasponia andersonii*

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TO MY DEAR HUSBAND

SASAN

Chapter1

General Introduction

Biological nitrogen fixation

Nitrogen (N) is the seventh most abundant element on earth, and in living cells it is required in large amounts as it is a component of proteins, nucleic acids and other cellular constituents. About 80% of the earth atmosphere is made up of di-molecular nitrogen (N_2) gas (Lum and Hirsch, 2002). However, N_2 gas cannot be used by most organisms, including plants (Vance, 2001). Plants can only utilize reduced forms of nitrogen like ammonium (NH_4^+) and nitrate (NO_3^-) (Hirel *et al.*, 2007). They acquire these forms of fixed nitrogen for example by; 1) application of chemical fertilizer, 2) the release of these compounds during decomposition of organic matter, 3) atmospheric nitrogen that is converted into ammonia and nitrate by lightning and entering soil by rainfall and/or 4) biological nitrogen fixation.

Biological nitrogen fixation can be conducted by a limited number of prokaryotes. Some of these bacteria can conduct nitrogen fixation in a free-living state, whereas others can do so only in association with plants. As biological nitrogen fixation is an energy demanding biochemical reaction, highest efficiencies of nitrogen fixation are obtained when the bacteria can retrieve carbon from a host plant. In return, the microbe can deliver ammonia, providing a selective advantage to the plant under nitrogen limiting conditions. Ultimately, such interactions can evolve in a stable symbiosis, giving profit to both partners. Among the best studied nitrogen-fixing symbiosis is the interaction between legumes and rhizobia.

Symbiotic nitrogen-fixing rhizobia represent 15 genera; 13 within the *α-proteobacteria* (among which *Rhizobium, Sinorhizobium, Mesorhizobium* and *Bradyrhizobium* are most prominent) and 2 genera within *β-proteobacteria* (*Burkholderia* and *Cupriavidus*, respectively). All these bacteria combine two genetic traits; namely a set of nitrogen fixation (*nif*) genes that encode the nitrogenase enzyme complex, and a set of nodulation (*nod*) genes that allow them to synthesize lipo-chitooligosaccharide (LCO) molecules (also known as Nod factors). These LCOs are in structure very similar to LCOs that are produced by *Arbuscular Mycorrhizal* (AM) fungi of the order Glomerales (Gough and Cullimore, 2011). Secreted LCOs can act as signaling molecules to establish infection of plant roots. AM fungi are obligate biotrophs that need to form feeding structures –known as arbuscules - in root cortical cells. In these cells, the fungus supplies minerals, especially phosphates, to the plant to retrieves carbohydrates. The AM fungus-plant symbiosis is widespread. It is estimated that more than 80% of today's plant species can establish an AM symbiosis (Parniske, 2008). Detection of AM fungi in fossilized plant records indicates

that the association with AM fungi evolved more than 400 million years ago, and possibly was important for the evolution of land plants in mid-Paleozoic era (Read *et al.*, 2000). By contrast, the N_2-fixing rhizobium symbiosis in legumes is much younger, presumably originating 60 million years ago (Sprent, 2008). Production of LCOs by symbiotic rhizobia suggests that these can activate the LCO signaling pathway of many plant species. However, N_2-fixing rhizobium symbiosis evolved only in two lineages; the legume family (Fabaceae) and the genus *Parasponia* in the Cannabis family (Cannabaceae).

Rhizobia require specialized organs –known as nodules- to commit nitrogen fixation. Nodules are generally formed on the root of the plant. Legume nodules are highly differentiated organs that consist of a large central zone surrounded with several vascular bundles to facilitate transport of nutrients. The central zone of the nodule is surrounded by a layer of suberized cells that form an oxygen barrier (Nap and Bisseling, 1990). Cells of the central zone can contain hundreds of rhizobia that are differentiated in nitrogen fixing organelle-like structures. These structures are named symbiosomes and produce ammonia (Roth and Stacey, 1989). Legumes evolved several mechanisms to further optimize the symbiotic interaction, some of which are lineage specific. For example, many legumes evolved a mechanism to select specific rhizobial species or even strains (Martínez-Romero, 2009). Additional mechanisms control nodule number or terminate the symbiosis when sufficient exogenous nitrates can be retrieved from soil, or when rhizobia remain in default (Streeter and Wong, 1988). In contrast to legumes, *Parasponia* root nodules are less sophisticated. *Parasponia* nodules have a single –central- vascular bundle and bacteria remain in thread-like structures, known as fixation threads (Behm *et al.*, 2014). Additionally, *Parasponia* is highly promiscuous for rhizobium, and even makes nodules with strains that default in nitrogen fixation (Op den Camp *et al.*, 2012).

LCO signaling pathway

By exploiting *Lotus japonicus* (*Lotus*) and *Medicago truncatula* (*Medicago*) as research models, insight has been obtained in the genetic networks underlying the formation of root nodules. Especially, the LCO induced genetic network controlling nodule formation and bacterial invasion has been uncovered (Geurts *et al.*, 2005; Geurts *et al.*, 2016). Rhizobium LCOs have a basic structure consisting of a backbone of three to five N-acetyl-glucosamine residues that are acylated at the non-reducing amino group with a fatty acid of 16–20 C-atoms in length (C16 to

C20). Additional substitutions to the backbone and/or unsaturated bounds in the acyl chain can be present and play a role in the host specificity of the symbiosis (D'Haeze and Holsters, 2002).

In legumes, rhizobium LCOs are recognized by a heterodimeric complex of two types of LysM (Lysin motif) receptor kinases (LysM-RLK), which activate a signaling pathway that results in activation of a transcriptional network (Broghammer *et al.*, 2012). These receptors are named LjNFR1 and LjNFR5 in *Lotus* and MtLYK3 and MtNFP in *Medicago*. LysM domains are predicted to be sites of interaction with N-acetyl-glucosamines-containing compounds (Steen *et al.*, 2005). In case of LjNFR1/MtLYK3 and LjNFR5/MtNFP it is found that these receptors bind LCOs. LjNFR1 and LjNFR5 receptor proteins directly bind to the LCOs with high affinity (Broghammer *et al.*, 2012). All LysM-RLKs contain three diverged LysM domains. In *Medicago* two main LysM-RLK classes are recognized, named LysM-I (containing *LYK* genes, including *MtLYK3*) and LysM-II (containing *LYR* genes as well as *MtNFP*). Genes of both classes are distinct in their intron/exon organization, and whether a canonical kinase domain is present. *LYK* genes contain 10 to 12 exons, and encode proteins with functional kinase domains. In contrast, *LYR* genes are generally single exon genes, and contain a kinase domain that lacks an activation loop. Such kinases are considered to be inactive. LYR proteins therefore most probably will function in a heterodimeric complex. Studies with of LjNFR1/LjNFR5 and MtLYK3/MtNFP indicated that such complexes are indeed formed, which suggests that LCO signaling is committed by such heterodimeric complex (Madsen *et al.*, 2003; Madsen *et al.*, 2011; Arrighi *et al.*, 2006; Pietraszewska-Bogiel *et al.*, 2013) Besides MtLYK3 and MtNFP, the LysM-RLK MtLYR3 of *Medicago* has been identified as a high affinity LCO binding protein (Malkov *et al.*, 2016). This suggests that possibly different type of receptor complexes can be formed to fine tune symbiotic LCO signaling.

Besides *LjNFR1/MtLYK3* and *LjNFR5/MtNFP*, a series of other genes have been identified to be essential for LCO signaling including: *LjSYMRK/MtDMI2*, *LjCASTOR*, *LjPOLLUX/MtDMI1*, *LjNUP133*, *LjNUP85*, *LjNENA*, *CCaMK* and the transcriptional regulators *LjCYCLOP/MtIPD3, NIN, MtERN*, *NSP1* and *NSP2*. Knockout mutations in any of these genes led to major defects in, or even complete loss of, nodule initiation (Kistner *et al.*, 2005; Oldroyd *et al.*, 2009; Parniske, 2008).

LjSYMRK/MtDMI2 encodes a trans-membrane receptor-like kinase protein with three Leucine-rich repeat (LRR) domains in the predicted extracellular region. It is located in the plasma

membrane and studies in *Lotus* show that it interacts with LjNFR1 and LjNFR5 (Chen *et al.*, 2012). Mutation in the *LjSYMRK/MtDMI2* gene abolishes rhizobium LCO signaling. Activation of the LCO receptors in conjunction with *LjSYMRK/MtDMI2* induces intracellular signaling, which is associated with oscillations of the calcium concentration in the perinuclear region. For this a perinuclear cation channel (Lj*CASTOR* and Lj*POLLUX*/*MtDMI1*) and 3 components of the nuclear pore including; *Lotus* NUCLEOPORIN 85 (*LjNUP85*), *LjNUP133* and *LjNENA* are essential (Groth *et al.*, 2010; Saito *et al.*, 2007; Kanamori *et al.*, 2006). The LCO induced calcium oscillation signal is –most probably- decoded by a nuclear localized calcium and calmodulin dependent kinase CCaMK (also named MtDMI3 in *Medicago*). Activation of CCaMK triggers a transcriptional network, starting with the interacting protein LjCYCLOPS/MtIPD3, yet the only known target of CCaMK. By phosphorylation, LjCYCLOPS is released from the complex with CCaMK, and become an active transcription factor. LjCYCLOPS binds to the promoter of the *ERN1* and *NIN* gene and activates its transcription in a phosphorylation-dependent manner (Singh *et al.*, 2014; Kistner and Parniske, 2002). Mutations in these genes interfere with LCO signaling, resulting in a nodulation phenotype. Interestingly, all genes from *LjSYMRK/MtDMI2* down to the activation of *LjCYCLOPS/MtIPD3* are also important for AM-symbiosis.

NIN (*NODULE INCEPTION*) is a nodulation-specific gene that encodes a transcription factor and acts downstream of the LCO signaling pathway. Nodule organogenesis and infection are fully blocked in *nin* knockout mutants (Marsh *et al.*, 2007; Schauser *et al.*, 1999). Downstream targets of *NIN* include genes encoding subunits of the Nuclear Factor Y (NF-Y) CCAAT-box-binding heterotrimeric transcription factor complex. The NF-Y complex consists of the three distinct proteins called NF-YA, NF-YB and NF-YC, all encoded by a small gene family (Kahle *et al.*, 2005). In legumes several members of these proteins have been found to commit a function in rhizobium symbiosis. In *Lotus*, *LjNF-YA1* and *LjNF-YB1* are transcriptional targets of *LjNIN* and promoting cortical cell divisions (Soyano *et al.*, 2013). Knock down studies of *MtNF-YA1* in *Medicago* revealed that a symbiotic NF-Y transcription complex commits functions that are essential in nodule development. *MtNF-YA1* RNAi nodules vary in size, but are always smaller than wild type nodules. Small *MtNF-YA1* knock down nodules lack a meristem, instead contain only fully infected cells (Xiao *et al.*, 2014). This suggests that *MtNF-YA1* is required for formation and maintenance of the nodule meristem in *Medicago*.

ERN belongs to the APETELLA2 / Ethylene Response Factor (AP2/ERF) family of transcription factors. MtERN1 Studies in *Medicago* showed that MtERN1 in conjunction with two GRAS-Type transcriptional regulators, MtNSP1(Nodulation Signaling Pathway1) and MtNSP2, is required for rhizobium LCO induced expression of the early nodulin gene, *MtENOD11*, in the root epidermis (Cerri *et al.*, 2012). Knockout mutations in *MtNSP1* or *MtNSP2* are unable in functional LCO signaling, whereas this is not the case for *Mtern1* knockout plants. A knockout mutation in *MtERN1* hampers nodulation, but mutant plants show early symbiotic responses including rhizobial infection. This suggest the existence of overlapping expression patterns with different gene namely *MtERN2*, a close homolog of *MtERN1*. Genetic analysis on these genes indicate that *MtERN1Mt/ERN2* coordinately induce rhizobial infection and nodule organogenesis (Cerri *et al.*, 2016).

MtNSP1 has been shown to bind to the promoter of *MtENOD11,* which requires MtNSP2 (Hirsch *et al.*, 2009). Yeast-two hybrid studies as well as split YFP studies indicate that MtNSP1 and MtNSP2 function in homo- and heteromeric complexes (Heckmann *et al.*, 2006). It is also reported that *MtNSP1* is involve in arbuscular mycorrhizal symbiosis, though not absolutely essential (Delaux *et al.*, 2013). Furthermore, a recent study showed that *nsp2* mutants in *Medicago* do not respond to Myc- LCOs and are colonized less than wild-type plants by the AM fungus *Rhizophagus irregularis* (Maillet *et al.*, 2011). In addition, *MtNSP1* and *MtNSP2* also have been shown to be involved in strigolactone biosynthesis. In this study a Mt*nsp1Mtnsp2* double mutant showed reduced colonization by AM fungi, which probably is caused by reduced strigolactone secretion, a compound that triggers branching of fungal hyphae (Liu *et al.*, 2011).

The rhizobium and AM fungal symbiosis share signaling and cellular processes

Genetic studies in *Pea* and later, in *Medicago* and *Lotus* have revealed that a major part of the rhizobium LCO signaling pathway is shared with the signaling pathway that is activated by AM fungi Therefore, this genetic signaling network is named the common symbiosis signaling pathway, or symbiotic toolkit (Parniske, 2008). Components of the common signaling pathway are *SYMRK* (Endre *et al.*, 2002; Stracke *et al.*, 2002), *Castor* and *Pollux* (Ané *et al.*, 2004; Imaizumi-Anraku *et al.*, 2005) *CCaMK* (Lévy *et al.*, 2004; Mitra *et al.*, 2004; Tirichine *et al.*, 2006) and *Cyclops* (Messinese *et al.*, 2007; Chen *et al.*, 2008; Yano *et al.*, 2008), *NSP1* (Takeda *et al.*, 2013) and *NSP2* (Maillet *et al.*, 2011). Mutations in these genes affect both endosymbiotic

interactions. In legumes, no LCO receptors have been found to commit a function in rhizobium as well as endomycorrhizal symbiosis. However, like rhizobia, AM fungi produce LCOs. These are named Myc factors and are structurally similar to Nod factors (Gough and Cullimore, 2011). This suggests that the receptors for Myc factors will be similar to those for Nod factors.

Parasponia plants are the only non-legumes that can also establish a nodule symbiosis with rhizobium. Knock down of the *Parasponia* LCO receptor gene, *PaNFP*, results in a block of intracellular infection nodules (den Camp *et al.*, 2011). Knock down of this receptor also blocks arbuscule formation by AM-fungi, whereas the roots are still intercellularly colonized (den Camp *et al.*, 2011). *Parasponia* has only a single *NFP* (LysM-type like) gene, whereas in legumes this gene experienced a duplication event. As will be described in more detail below, the *Parasponia*-rhizobium symbiosis evolved relatively recent in comparison to the legume-rhizobium symbiosis. Therefore, it seems probable that in *Parasponia,* the *PaNFP* LCO receptor didn't neofunctionalized to function exclusively in rhizobium symbiosis, but this receptor still has its ancestral function, which is perception of Myc factors. Such role of *NFP*-like genes in mycorrhizal symbiosis is supported by studies in tomato on *SlLYK10*. *SlLYK10* is the ortholog of *PaNFP*. In tomato, absence of mycorrhization was observed in knock down mutants of *SlLYK10* (Buendia *et al.*, 2015). This suggests that a controlling arbuscule formation in AM symbiosis is the ancestral function of *PaNFP*/*SlLYK10* genes.

Activation of the common symbiosis signaling pathway –either by rhizobium or AM-fungi- induces cellular processes that lead to intracellular accommodation of the microbe. In both symbioses this involves the formation of a host membrane structure by which the microbe is guiding into the cells (Gutjahr and Parniske, 2013; Ivanov *et al.*, 2012). Anytime the microbe remains bound by plant derived membrane structures; symbiosomes or arbuscules (Limpens *et al.*, 2009), These symbiotic membrane compartments facilitate nutrient exchange between both partners (Geurts and Vleeshouwers, 2012). Studies in *Medicago* have revealed that specific vesicle-SNAREs (soluble N-ethylmaleimide sensitive factor attachment protein receptor) belonging to the VAMP72 (vesicle-associated membrane protein) family are involved in symbiosome as well as arbuscule formation. VAMP72 proteins are active in exocytotic pathways and mark secretory vesicles that accumulated in the cellular entry point of the microbe (Ivanov *et al.*, 2012). This shows that despite of morphological differences, rhizobium and mycorrhizal

fungi trigger similar cellular responses. So, in addition to the LCO signaling pathway, also the pathway controlling symbiosome formation has been co-opted from the AM fungal symbiosis.

Root nodule symbiosis is not limited to nitrogen fixing rhizobia. Also some gram positive filamentous *Frankia* bacteria can establish a nodular nitrogen fixing symbiosis on a selective group of ~220 plant species. These so-called actinorhizal plants belong to eight families of the Fabid clade, suggesting multiple evolutionary origins of this symbiosis (Diédhiou *et al.*, 2014; Doyle, 2011). Although it remains unclear whether *Frankia* species produce LCOs or use an alternative signal to communicate with their host plants, reverse genetic studies indicated that –at least part of- the common symbiosis signaling pathway is used to establish the *Frankia*-actinorhizal plant symbiosis. Studies in the actinorhizal plants *Casuarina glauca* and *Datisca glomerata* revealed the symbiotic functioning of *CgSYMRK/DgSYMRK*, *CgCCaMK* and *CgNIN*. (Gherbi *et al.*, 2008; Markmann *et al.*, 2008; Clavijo *et al.*, 2015). Furthermore, *Frankia* induced signaling triggers a Ca^{2+} oscillation response in *C. glauca* and *Alnus glutinosa* (Granqvist *et al.*, 2015; Chabaud *et al.*, 2016). These findings strongly suggest that evolution of a nitrogen-fixing endosymbiosis with either rhizobium or *Frankia* was guided by genetic constraints.

Assuming that AM fungi infect their various hosts by a conserved mechanism, it implies that the genes encoding components for LCO signaling as well as the symbiosis related exocytosis pathway will be widespread in the plant kingdom. This is well supported by the genome sequence analysis of several plant species. Species that are able to establish an AM symbiosis harbor putative orthologues of the symbiosis genes identified in legumes, whereas often these genes are absent in AM non-hosts (Delaux *et al.*, 2014; Bravo *et al.*, 2016). For example in *Arabidopsis,* which is non-host for AM fungi, most genes of the common signaling pathway as well as *MtNFP*/*LjNFR5* have been lost. This further supports the idea that genetic constraints determined the evolution of the rhizobium endosymbiosis. However it arise the question why not more plant species have gained the rhizobium symbiosis trait? Taken into account the recent research on *Parasponia*, comparative studies of *Parasponia* and legumes as well as *Parasponia* and its non-nodulating sister species *Trema* can answer this question, and may pave the way for future transfer of rhizobium symbiosis to the other non-legume plants.

Effect of fixed nitrogen sources on legume root nodule formation

Legumes only form root nodules under nitrogen limiting condition, whereas the addition of

excessive amounts of combined nitrogen suppress nodulation (Barbulova *et al.*, 2007). Also, nitrogen fixation can cease in existing nodules by addition of an exogenous fixed nitrogen source. Legumes control the total number of nodules formed, because the maintenance of nitrogen fixing rhizobia is energy demanding (Reid *et al.*, 2011; Schnabel *et al.*, 2011). Competence of leguminous plants to form nodules is determined by physiological conditions. The mechanisms by which fixed nitrogen suppress the different steps of root nodule formation is only partially understood (Barbulova *et al.*, 2007).

The best studied mechanism to control nodule numbers is autoregulation of nodulation (AON). AON is a negative feedback regulation by which the number of nodules and the nodulation zone are tightly restricted. Mutants in AON have lost the ability to control nodule number and therefore form numerous nodules (so-called super- or hypernodulators). AON mutants have been studied in several legumes like *Pea*, *Soybean*, but also the model legumes *Medicago* and *Lotus*. This has resulted in the identification of a CLAVATA1-like LRR receptor kinase (LRR-RLK) named *HYPERNODULATION ABERRANT ROOT FORMATION1* (*LjHAR1*) in *Lotus* (Kawaguchi *et al.*, 2002), *NODULE AUTOREGULATION RECEPTOR KINASE* (*GmNARK*) in *Soybean* (Olsson *et al.*, 1989) and *SUPER NUMERIC NODULES* (*MtSUNN*) in *Medicago* (Elise *et al.*, 2005). *LjHAR1*, *GmNARK* and *MtSUNN* are most probable orthologous genes that upon mutation results in a supernodulating phenotype. *Ljhar1, Gmnark* and *Mtsunn* knockout mutants also have lost the ability to suppress nodulation in presence of exogenous fixed nitrogen (Barbulova *et al.*, 2007; Carroll *et al.*, 1985; Magori *et al.*, 2009). Grafting experiments showed that *LjHAR1*, *GmNARK* and *MtSUNN* are only essential in the shoot and are key factors in systemic root-to-shoot-to-root negative feedback regulation of nodulation. The encoded LRR-RLK recognizes CLV3/embryo-surrounding region (CLE) peptides (Hirakawa *et al.*, 2008). CLEs are a group of small (12–13 amino acids) secreted peptides that are derived from the C-terminal region of pre-proteins. 39 *LjCLE* genes have been identified in the *Lotus* genome, three of them, *LjCLE-RS1*, *LjCLE-RS2* and *LjCLE3*, are significantly up-regulated in *Lotus* nodulated roots. Transcription of *LjCLE-RS1* and *LjCLE-RS2* is responsive to exogenous nitrate and rhizobial inoculation (Okamoto *et al.*, 2009). When *LjCLE-RS1* or *LjCLE-RS2* is ectopically expressed in roots of the *Ljhar1* mutant using an *Agrobacterium rhizogenes*-mediated transformation system, both transformed and non-transformed roots show a super-nodulation phenotype. This indicates that these two genes have suppressive effect on nodulation in a

LjHAR1 dependent manner (Okamoto and Kawaguchi, 2015). *LjCLE-RS2* was found to be strongly up-regulated in response to nitrate. Therefore it is hypothesized that LjCLE-RS2 translocates from root-to-shoot and directly binds to the LjHAR1 receptor (Okamoto *et al.*, 2013). Based on these findings, a model for the long-distance nitrate inhibition of nodulation has been proposed in *Lotus*. In this model, the LjCLE-RS2/LjHAR1 long-distance signaling system is shared between nitrate inhibition and AON (Okamoto and Kawaguchi, 2015).

A different study in *Medicago,* found that MtCLE peptides control nodulation not only systematically, but also locally (Mortier *et al.*, 2010). In this study, 25 *MtCLE* genes in the *Medicago* genome have been identified. Two genes, *MtCLE12* and *MtCLE13,* negatively regulate nodulation. *MtCLE12* and *MtCLE13* have a high degree of similarity with *LjCLE-RS1* and *LjCLE- RS2* (Mortier *et al.*, 2010). Similarity in sequence and expression profiles suggests that *MtCLE13* and *LjCLE-RS1/LjCLE-RS2* might exert a comparable function, respectively. Studies in *Soybean* on *GmNARK* revealed similar results as described for *Lotus* and *Medicago*. *Gmnark* knockout mutants are affected in both AON and nitrate regulation of nodulation suggesting this gene is a common component of both regulatory mechanisms(Reid *et al.*, 2011). Candidate CLE peptides in *Soybean* that commit symbiotic functions are GmRIC1, GmRIC2, and GmNIC1. Among these, *GmNIC1* is strongly induced by nitrate and possessed the conserved proline residues close to the C-termini, the part similar as found in MtCLE13 and LjCLE-RS1/2 (Okamoto *et al.*, 2009).

CLE-CLAVATA-type LRR-RLK signaling does not function exclusively in symbiosis, but act as signaling module in plant organ development (Okamoto *et al.*, 2009). This holds also for genes that control root nodule formation. For example, an increased number of emerged lateral roots was reported in the *Lotus Ljhar1* mutant under both symbiotic and non-symbiotic conditions (Wopereis *et al.*, 2000). The *compact root architecture 2* (*Mtcra2*) is another *Medicago* mutant which is affected in a CLAVATA1-like Leucine-Rich Repeat Receptor-Like Kinase (LRR-RLK). *Mtcra2* mutant plants form an increased number of lateral roots with a reduced number of symbiotic nitrogen-fixing nodules (Huault *et al.*, 2014). These finding reveal the related function of the CLE-CLAVATA1 type signaling modules in lateral root development and autoregulation mechanism.

The role of plant hormones in legume root nodule formation

Rhizobium root nodule formation is causally linked with formation of a local maximum of indoleacetic acid (IAA), the natural auxin in plants (Mathesius *et al.*, 1998; Pacios-Bras *et al.*, 2003; Huo *et al.*, 2006; van Noorden *et al.*, 2007; Takanashi *et al.*, 2011; Imanishi *et al.*, 2014; Chiu *et al.*, 2005; Perrine-Walker *et al.*, 2010; Suzaki *et al.*, 2012). Based on auxin responsive reporter constructs as well as quantification studies, it is known that IAA is not uniformly distributed in the plant root. The highest concentrations are found in the plant cells undergoing cell divisions –e.g. the root apical meristem and lateral root primordia, whereas also in the elongation zone relative high amounts of auxin are detected (Pacios-Bras *et al.*, 2003; Larkin *et al.*, 1996; Deinum *et al.*, 2012). Auxin is accumulating at the sites of nodule development, which implies that LCO signaling interferes with auxin homeostasis in the plant root. The mechanism by which such local auxin maximum is formed remains unknown; however it is unlikely that LCOs trigger such response cell autonomously. This because LCOs are extremely immobile and stick within walls of epidermal cells, whereas in most legumes the auxin maximum is formed in cortical and pericycle cell layers (Deinum *et al.*, 2012; Marhavý *et al.*, 2013). This suggests that upon perception of LCOs a secondary signal will released from the epidermis that triggers an auxin maximum in lower layers. Quantitative modelling suggests that an auxin maximum can be formed by local inhibition of auxin efflux transport; e.g. by interfering with the functioning the PIN auxin efflux carrier proteins (Deinum *et al.*, 2012). Several compounds are known that have such function; including the plant hormones cytokinin and strigolactones as well as flavonoids.

The plant hormone cytokinin is implicated in the regulation of many physiological processes during plant development, growth, and adaptation to environmental conditions (Martín *et al.*, 2000; Mok and Mok, 2001). Active cytokinins accumulate upon LCO signaling (van Zeijl *et al.*, 2015; Frugier *et al.*, 2008; Gonzalez-Rizzo *et al.*, 2006; Lohar *et al.*, 2006; Tirichine *et al.*, 2007). It has been reported that a gain-of-function mutation in the cytokinin receptor LjLHK1 of *Lotus* and MtCRE1 of *Medicago* triggers spontaneous root nodule organogenesis (Tirichine *et al.*, 2007; Ovchinnikova *et al.*, 2011). This indicates that perception of the phytohormone cytokinin is a key element in nodule formation in legumes, which is underlined with experiments in which application of exogenous cytokinin induces formation of nodule primordia (Heckmann *et al.*, 2011; Cooper and Long, 1994). In the opposite manner, reduced cytokinin accumulation and/or perception blocks nodulation (Murray *et al.*, 2007; Gonzalez-Rizzo *et al.*, 2006). This

demonstrates unequivocally that cytokinin signaling is necessary and sufficient to induce cortical cell divisions and nodule organogenesis.

Besides cytokinins, also the biosynthetic pathways of strigolactones and flavonoids are activated upon LCO signaling. Both are known to have the capacity to interfere with PIN functioning. However, knockout mutants in strigolactone biosynthesis genes have only a weak symbiotic phenotype, suggesting that strigolactone functioning is less essential for root nodule formation Interestingly, it was found that exogenous application of flavonoids can complement the knockout phenotype of *Mtcre1*. This suggests that flavonoid act downstream of or in parallel of cytokinins (Ng *et al.*, 2015).

Besides hormones with a positive effect on root nodule formation, several hormones have been implicated to have a negative effect on LCO signaling and root nodule formation. For example, abscisic acid and jasmonic acid are known to interfere with LCO induced calcium spiking (Ding *et al.*, 2008; Sun *et al.*, 2006). Also the gaseous plant hormone ethylene is known to have a negative effect on this physiological response, although it is not fully blocking it. Ethylene affects the maintenance of the response, as well as it changes the threshold concentration of LCO required for the induction of calcium spiking (Oldroyd *et al.*, 2001). Further, ethylene acts as inhibitor of nodule formation and bacterial infection. The application of ethylene, or ethylene-releasing compounds, inhibits nodule organogenesis in numerous species including *Phaseolus vulgaris* (Grobbelaar *et al.*, 1971), *Pea* (Drennan and Norton, 1972) *Trifolium repens* (Goodlass and Smith, 1979) *Melilotus alba* (Lee and LaRue, 1992) *Medicago* and *Lotus* (Penmetsa and Cook, 1997; Nukui *et al.*, 2000).

Like cytokinin. Several lines of evidence indicate that ethylene signaling is an integral part in root nodule formation. For example, local biosynthesis of ethylene opposite phloem poles is thought to provide positional information by locally inhibiting root cortical cell divisions (Heidstra *et al.*, 1997). Also there might be a low threshold of ethylene required for proper infection by rhizobium bacteria (Ferguson and Mathesius, 2003; Oldroyd *et al.*, 2001). The negative ethylene effect on nodule number could be accounted for invoking a specific ethylene regulation of infection thread growth (Oldroyd *et al.*, 2001). The role of ethylene in regulation of rhizobial symbiosis is supported with the identification of ethylene signaling mutant *ein2* in *Lotus* and *Medicago* (Miyata *et al.*, 2013; Chan *et al.*, 2013; Penmetsa and Cook, 1997). For example, the *Medicago Mtein2*/*sickle* knockout mutant makes the plant insensitive to ethylene

(Penmetsa and Cook, 1997), which dramatically effects nodule formation. In small root zones numerous nodules are formed that have lost spatial positioning in respect the stele of the root. Detailed analysis of the *Mtein2*/*sickle* mutant indicated that the read out of rhizobium LCO signaling is stronger. For example, root hair deformation in response to LCOs revealed a stronger response in *Mtein2*/*sickle* compared to the wild-type plants (Oldroyd *et al.*, 2001). Ethylene also has an effect on calcium spiking that may regulate the specificity of downstream responses. This effect could be either on the initiation of calcium spiking directly or that ethylene has the capacity to modulate the frequency of calcium spikes (Oldroyd *et al.*, 2001). This shows that ethylene acts upstream or at the point of calcium spiking in the LCO signal transduction pathway, suggesting that it can modulate both the degree and the nature of LCO pathway activation. Furthermore, the LCO concentration threshold that is required to activate calcium spiking is defined by the ethylene status (Oldroyd *et al.*, 2001). It is also known that ethylene signaling regulate auxin transport at certain stages of nodule development. Ethylene partially modulate auxin transporter through PIN gene expression, ultimately an increase in auxin transport results in higher nodule numbers (Prayitno *et al.*, 2006).

Whereas much is known about the molecular dialog of the legume-rhizobium mutualism, it has not been possible so far to answer the question why the rhizobium symbiosis trait is restricted to two lineages; legumes and *Parasponia*. Genomics studies showed that for most –if not all- symbiosis genes close homologs are present in all plant species that are able to establish an endomycorrhizal symbiosis (Parniske, 2008). To get insight in the evolutionary trajectory of the rhizobium symbiosis trait comparative systems are essential. A comparison of legumes to *Parasponia* for example may provide insights to what extend genetic constraints have guided evolution of rhizobium N_2-fixing root nodules. Studies in this direction revealed that *Parasponia* nodulates with rhizobium strains that also can form root nodules with legumes (Op den Camp *et al.*, 2012). Like with (most) legumes, the *Parasponia*-rhizobium symbiosis is driven by LCO signaling (Marvel *et al.*, 1987). Also one LysM-type LCO receptor has been identified, PaNFP, which showed to be orthologous to MtNFP/LjNFR5 of *Medicago* (den Camp *et al.*, 2011). Furthermore, it was found that an autoactive allele of CCaMK can trigger formation of root nodule-like structures in absence of rhizobium or LCOs (den Camp *et al.*, 2011). Taken together these data suggest that at least in part, both symbioses make use of identical signaling queues.

In addition to a Legume-*Parasponia* comparison to identify genetic constraints, a comparison of

symbiotic plants with non-symbiotic plants may uncover novel insights. For such comparison the plant species that differ in phenotype should be closely related. Furthermore, it is important that the non-nodulating plant species used in such comparison, does not represent a recent loss of the rhizobium symbiosis, but rather never gained it. In the legume family several non-nodulating plant lineages occur, but it remains elusive whether these represent a loss of nodulation or never gained it (Doyle, 2011). In legumes a non-nodulating plant species are represented by *Cercideae*, which form the most basal lineage in this family (Lavin *et al.*, 2005). This lineage diverged about 59 million years ago from the legume crown node, which represents all nodulating legumes. In contrast, the *Parasponia* lineage is considered to be much younger than the legume crown node (Behm *et al.*, 2014). Therefore, *Parasponia*, and its non-nodulating sister species of the genus *Trema*, may represent the best known comparative system of a nodulating and non-nondulating plant species.

Parasponia-rhizobium symbiosis

The genus *Parasponia* belongs to the Cannabaceae (Doyle, 1998). Cannabaceae (order Rosales) and Fabaceae (order Fabales) are only remotely related (figure 1), with a last common ancestor about 100 million years ago (Wang *et al.*, 2009). Therefore it is most probable that both lineages gained the *rhizobium*-nodule symbiosis independently.

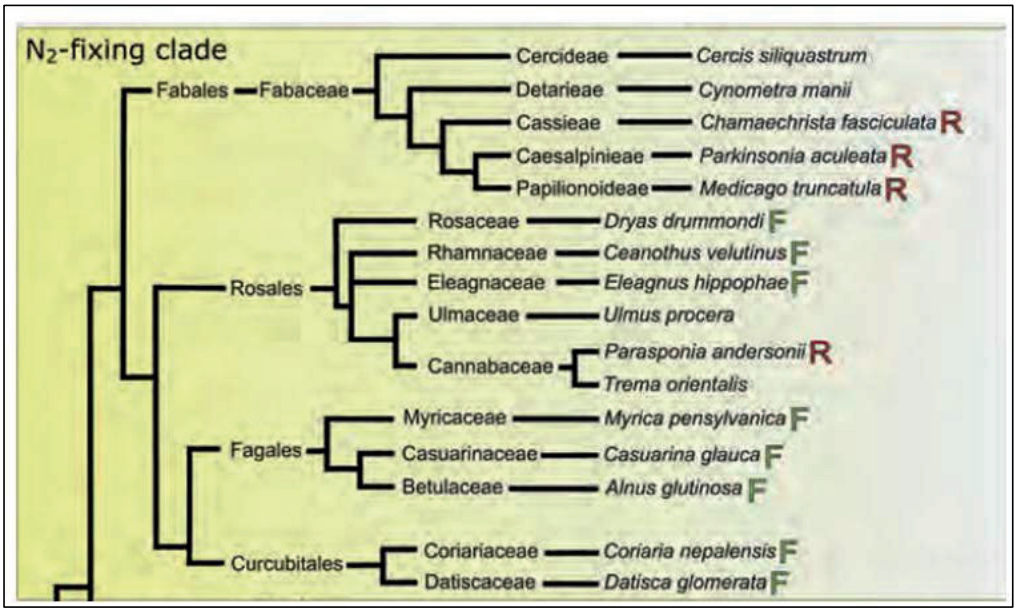

Figure 1. Phylogenetic representation of evolutionary distance between *Parasponia* and legumes. F: Frankia, R: Rhizobia.

Parasponia root nodules have a more basal appearance when compared to legume nodules. *Parasponia* nodules have a central vascular bundle with infected cells in the peripheral zone (Cao *et al.*, 2012) and a meristem at the tip. Cells proximal of this meristem are being invaded by rhizobia upon formation of infection threads. In contrast to most legume nodules the bacteria are not released into the cell as symbiosomes, but remain within thread-like structures. These, so-called fixation threads, differ from the penetrating infection threads in such a way that they have a cell wall that is significantly reduced in thickness. Also, fixation threads are somewhat wider than infection threads, allowing formation of double phyla of nitrogen-fixing bacteria. In contrast, in most legume nodules infection threads release rhizobia from the tip of the thread into the cytoplasm. At this point the host cell encloses the newly-released bacteria within a cell membrane, so that it is not actually in contact with the plant cytoplasm. These bacteria then differentiate into their symbiotic N_2-fixing organelle-like structures, and effectively become dependent on the host plant.

The structure of fixation threads somewhat resembles the structure of arbuscules formed by AM

fungi in the root cortical cells of their host plant. Overlap in both structures is support with the finding that *PaNFP* controls formation of both (den Camp *et al.*, 2011). Fixation threads are not unique to *Parasponia*. Several basal legumes host their symbiotic microbe in a similar way. For example, *Andira* as well as some *Chamaecrista* species form also fixation threads (also named persistent infection threads) (Faria *et al.*, 1987; Naisbitt *et al.*, 1992). *Andira* and *Chamaecrista* do not represent closely related legume lineages, but diverged ~58 million years ago. Fixation thread containing nodules harbor significantly less bacteria per cell when compared to nodules with symbiosomes (Behm *et al.*, 2014). Additionally, symbiosomes are deprived from cell wall material and have an enlarged surface of surrounding membrane when compared to fixation thread, which may be an advantage in nutrient exchange between both partners. Taken together it seems most probable that fixation threads resemble a more ancestral stage of intracellular rhizobium infection (de Faria *et al.*, 1986; Naisbitt *et al.*, 1992).

The *Parasponia*-rhizobium symbiosis is more basal when compared to the legume-rhizobium symbiosis. Not only the nodules are more basal in structure when compared to legumes, also the nitrogen fixating capacity is lower than in legumes by a 0.5 - 1.0 order of magnitude (Vessey *et al.*, 2004). The primitive nature of *Parasponia* root nodules is further underlined by the mode of rhizobium infection of the root. Infection of *Parasponia* by rhizobia occur by intercellular penetration of the root; a mechanism known as crack entry. Such mode of infection is less-sophisticated than root hair based intracellular infection as found in most legume species. Coinciding with crack entry is partner promiscuity in *Parasponia*, which allows nodulation by many rhizobial species, irrespective of their nitrogen fixation capacities (Op den Camp *et al.*, 2012).

Taken together, I argue that due to its young age and independent evolution, *Parasponia* provides a simple system to identify the genetic basis underlying rhizobium symbiosis evolution. Thereby the main question of my research is how this mutualistic interaction evolved in *Parasponia,* and which constraints have guided this evolutionary trajectory?

Forward evolution in Trema

Parasponia represents 5 tropical tree species that can be found on volcanic regions in the Malay archipelago. These species are phenotypically very similar to non-nodulating *Trema* species, which occasionally leads to incorrect species determination (Akkermans *et al.*, 1978). Although

Parasponia and *Trema* can be distinguished by some phenotypic features like their imbricate perianth lobes of the male flowers and by their intrapetiolar (Trinick, 1973). Recent phylogenetic studies using chloroplast genes such as *rbcL*, and *trnL* or the internal transcribed spacer region of 18S–26S nuclear ribosomal DNA as markers, indicated that the *Parasponia* lineages is nested in the *Trema* genus (Yang *et al.*, 2013). This suggests that *Parasponia* evolved only relatively recent, including its symbiotic capacities, from an ancestral *Trema* species. Such hypothesis is supported by the more restricted distribution of *Parasponia* when compared to *Trema*. The genus *Trema* includes 15 species that can be found in Asia, Australia, Africa and the Americas. Ever since the discovery of *Parasponia* (in 1973) as the first, and till now only, non-legume that independently evolved the nitrogen-fixing nodule symbiosis with rhizobium, it intrigued the scientific community. Comparing *Parasponia* not only to legumes, but also to *Trema* can help to characterize the genetics of the nitrogen fixing rhizobium root nodule trait.

Scope and outline of the thesis

The overall aim of this research was to identify the evolutionary trajectory by which *Parasponia* became able to establish the rhizobium nodule symbiosis. To investigate the nodulation process as a specific difference between *Parasponia* and *Trema*, a collection of techniques was needed, including plant transformation protocols. In **chapter 2,** I described a rapid *Agrobacterium rhizogenes*-mediated transformation method to generate composite plants carrying transgenic root for *Parasponia andersonii* and *Trema tomentosa*. This 'transient' transformation system allowed functional testing of transgenes. Unfortunately, this transient method was not very efficient for *Trema sp*., which forced me to develop a novel protocol for stable –*A. tumefaciens*-mediated transformation of *T. tomentosa* (**chapter 3)**. By this protocol, *T. tomentosa* plants carrying DR5::GUS were generated within a period of 6 months. Subsequently, the transgenic T0 lines were vegetatively propagated to study the DR5::GUS expression.

In chapter **4, 5 and 6** I studied the biology of the *Parasponia*-rhizobium symbiosis. Along the idea that *Parasponia*-rhizobium symbiosis evolved recently, I questioned whether *Parasponia* has gained mechanisms to control the interaction with its symbiotic partner. In this context I tested whether *Parasponia* can control root nodule formation in presence of an exogenous fixed nitrogen source (**chapter 4**). Considering that *Parasponia* is a tree with higher nitrate demand, I questioned which exogenous nitrate concentration will affect the nitrogen fixing symbiosis in

Parasponia? To do so, I examined the effect of a range of nitrate treatments on the rhizobium induced symbiotic responses in *Parasponia*. This revealed that nodule primordium formation and intracellular infection have different sensitivities to nitrates. Formation of nodules was optimal at 10 mM nitrate, whereas progression of intracellular infection was impaired at 20 mM nitrate. Specifically, the switch from infection to fixation thread formation was affected by exogenous nitrate. In line with this finding I argued that this effect of exogenous nitrate in the rhizobium symbiosis, is a novel invention in *Parasponia.*

Next I investigated whether the GRAS-type transcription factors NSP1 and NSP2 (**Chapter 5**) and ethylene signaling (**Chapter 6**), like in legumes, are co-opted by *Parasponia* to control root nodule formation. To do so, *Parasponia* orthologous genes to *Medicago NSP1*, *NSP2* and *EIN2* have been cloned and studied by RNAi using *A. rhizogenes* mediated transformation. This revealed that all these genes commit a symbiotic role, supporting the hypothesis that genetic constrains have guided evolution of rhizobium symbiosis in legumes and *Parasponia*.

In **Chapter 7** (General discussion) I place these findings in a broader context.

References

Akkermans, A., et al. (1978). "N2-fixing root nodules in Ulmaceae: *Parasponia* or (and) Trema spp.?" Plant and Soil **49**(3): 711-715.

Ané, J.-M., et al. (2004). "*Medicago truncatula* DMI1 required for bacterial and fungal symbioses in legumes." Science **303**(5662): 1364-1367.

Arrighi, J.-F., et al. (2006). "The *Medicago truncatula* lysine motif-receptor-like kinase gene family includes NFP and new nodule-expressed genes." Plant Physiology **142**(1): 265-279.

Barbulova, A., et al. (2007). "Differential effects of combined N sources on early steps of the Nod factor-dependent transduction pathway in Lotus japonicus." Molecular Plant-Microbe Interactions **20**(8): 994-1003.

Behm, J. E., et al. (2014). "Parasponia: a novel system for studying mutualism stability." Trends in Plant Science **19**(12): 757-763.

Bravo, A., et al. (2016). "Genes conserved for arbuscular mycorrhizal symbiosis identified through phylogenomics." Nature Plants **2**: 15208.

Broghammer, A., et al. (2012). "Legume receptors perceive the rhizobial lipochitin oligosaccharide signal molecules by direct binding." Proceedings of the National Academy of Sciences **109**(34): 13859-13864.

Buendia, L., et al. (2015). "The LysM receptor-like kinase SlLYK10 regulates the arbuscular mycorrhizal symbiosis in tomato." New Phytologist.

Cao, Q., et al. (2012). "Efficiency of Agrobacterium rhizogenes–mediated root transformation of Parasponia and Trema is temperature dependent." Plant Growth Regulation **68**(3): 459-465.

Carroll, B. J., et al. (1985). "A supernodulation and nitrate-tolerant symbiotic (nts) soybean mutant." Plant Physiology **78**(1): 34-40.

Cerri, M. R., et al. (2016). "The Symbiosis-Related ERN Transcription Factors Act in Concert to Coordinate Rhizobial Host Root Infection." Plant Physiology: pp. 00230.02016.

Cerri, M. R., et al. (2012). "Medicago truncatula ERN transcription factors: regulatory interplay with NSP1/NSP2 GRAS factors and expression dynamics throughout rhizobial infection." Plant Physiology **160**(4): 2155-2172.

Chabaud, M., et al. (2016). "Chitinase-resistant hydrophilic symbiotic factors secreted by Frankia activate both Ca2+ spiking and NIN gene expression in the actinorhizal plant Casuarina glauca." New Phytologist **209**(1): 86-93.

Chan, P. K., et al. (2013). "Classical Ethylene Insensitive Mutants of the Arabidopsis EIN2 Orthologue Lack the Expected 'hypernodulation'Response in Lotus japonicusF." Journal of Integrative Plant Biology **55**(4): 395-408.

Chen, C., et al. (2008). "OsIPD3, an ortholog of the Medicago truncatula DMI3 interacting protein IPD3, is required for mycorrhizal symbiosis in rice." New Phytologist **180**(2): 311-315.

Chen, T., et al. (2012). "A MAP kinase kinase interacts with SymRK and regulates nodule organogenesis in Lotus japonicus." The Plant cell **24**(2): 823-838.

Chiu, W.-L., et al. (2005). "Nitrogen deprivation stimulates symbiotic gland development in *Gunnera manicata*." Plant Physiology **139**(1): 224-230.

Clavijo, F., et al. (2015). "The Casuarina NIN gene is transcriptionally activated throughout Frankia root infection as well as in response to bacterial diffusible signals." New Phytologist **208**(3): 887-903.

Cooper, J. B. and S. R. Long (1994). "Morphogenetic rescue of Rhizobium meliloti nodulation mutants by trans-zeatin secretion." The Plant cell **6**(2): 215-225.

D'Haeze, W. and M. Holsters (2002). "Nod factor structures, responses, and perception during initiation of nodule development." Glycobiology **12**(6): 79R-105R.

de Faria, S. M., et al. (1986). "A new type of infected cell in root nodules of Andira spp.(Leguminosae)." Plant Science **45**(2): 143-147.

Deinum, E. E., et al. (2012). "Modeling a cortical auxin maximum for nodulation: different signatures of potential strategies." Front Plant Sci **3**: 96.

Delaux, P.-M., et al. (2014). "Comparative phylogenomics uncovers the impact of symbiotic associations on host genome evolution." PLoS Genet **10**(7): e1004487.

Delaux, P. M., et al. (2013). "NSP1 is a component of the Myc signaling pathway." New Phytologist **199**(1): 59-65.

den Camp, R. O., et al. (2011). "LysM-type mycorrhizal receptor recruited for rhizobium symbiosis in nonlegume Parasponia." Science **331**(6019): 909-912.

Diédhiou, I., et al. (2014). "Identification of potential transcriptional regulators of actinorhizal symbioses in Casuarina glauca and Alnus glutinosa." BMC plant biology **14**(1): 1.

Ding, Y., et al. (2008). "Abscisic acid coordinates nod factor and cytokinin signaling during the regulation of nodulation in Medicago truncatula." The Plant cell **20**(10): 2681-2695.

Doyle, J. J. (1998). "Phylogenetic perspectives on nodulation: evolving views of plants and symbiotic bacteria." Trends in Plant Science **3**(12): 473-478.

Doyle, J. J. (2011). "Phylogenetic perspectives on the origins of nodulation." Molecular Plant-Microbe Interactions **24**(11): 1289-1295.

Drennan, D. and C. Norton (1972). "The effect of ethrel on nodulation inPisum sativum L." Plant and Soil **36**(1-3): 53-57.

Elise, S., et al. (2005). "The Medicago truncatula SUNN gene encodes a CLV1-like leucine-rich repeat receptor kinase that regulates nodule number and root length." Plant Molecular Biology **58**(6): 809-822.

Endre, G., et al. (2002). "A receptor kinase gene regulating symbiotic nodule development." Nature **417**(6892): 962-966.

Faria, S. M. d., et al. (1987). "The occurrence of infected cells, with persistent infection threads, in legume root nodules." Canadian Journal of Botany **65**(3): 553-558.

Ferguson, B. J. and U. Mathesius (2003). "Signaling interactions during nodule development." Journal of Plant Growth Regulation **22**(1): 47-72.

Frugier, F., et al. (2008). "Cytokinin: secret agent of symbiosis." Trends in Plant Science **13**(3): 115-120.

Geurts, R., et al. (2005). "Nod factor signaling genes and their function in the early stages of Rhizobium infection." Current Opinion in Plant Biology **8**(4): 346-352.

Geurts, R. and V. G. Vleeshouwers (2012). "Mycorrhizal symbiosis: ancient signalling mechanisms co-opted." Current Biology **22**(23): R997-R999.

Geurts, R., et al. (2016). "What Does It Take to Evolve A Nitrogen-Fixing Endosymbiosis?" Trends in Plant Science **21**(3): 199-208.

Gherbi, H., et al. (2008). "SymRK defines a common genetic basis for plant root endosymbioses with arbuscular mycorrhiza fungi, rhizobia, and Frankiabacteria." Proceedings of the National Academy of Sciences **105**(12): 4928-4932.

Gonzalez-Rizzo, S., et al. (2006). "The Medicago truncatula CRE1 cytokinin receptor regulates lateral root development and early symbiotic interaction with Sinorhizobium meliloti." The Plant cell **18**(10): 2680-2693.

Goodlass, G. and K. Smith (1979). "Effects of ethylene on root extension and nodulation of pea (<i>Pisum sativum</i> L.) and white clover (<i>Trifolium repens</i> L.)." Plant and Soil **51**(3): 387-395.

Gough, C. and J. Cullimore (2011). "Lipo-chitooligosaccharide signaling in endosymbiotic plant-microbe interactions." Molecular Plant-Microbe Interactions **24**(8): 867-878.

Granqvist, E., et al. (2015). "Bacterial-induced calcium oscillations are common to nitrogen-fixing associations of nodulating legumes and non-legumes." New Phytologist **207**(3): 551-558.

Grobbelaar, N., et al. (1971). "The nodulation and nitrogen fixation of isolated roots of Phaseolus vulgaris L." Plant and Soil **35**(1): 203-214.

Groth, M., et al. (2010). "NENA, a Lotus japonicus homolog of Sec13, is required for rhizodermal infection by arbuscular mycorrhiza fungi and rhizobia but dispensable for cortical endosymbiotic development." The Plant cell **22**(7): 2509-2526.

Gutjahr, C. and M. Parniske (2013). "Cell and developmental biology of arbuscular mycorrhiza symbiosis."

Heckmann, A. B., et al. (2006). "Lotus japonicus nodulation requires two GRAS domain regulators, one of which is functionally conserved in a non-legume." Plant Physiology **142**(4): 1739-1750.

Heckmann, A. B., et al. (2011). "Cytokinin induction of root nodule primordia in Lotus japonicus is regulated by a mechanism operating in the root cortex." Molecular Plant-Microbe Interactions **24**(11): 1385-1395.

Heidstra, R., et al. (1997). "Ethylene provides positional information on cortical cell division but is not involved in Nod factor-induced root hair tip growth in Rhizobium-legume interaction." Development **124**(9): 1781-1787.

Hirakawa, Y., et al. (2008). "Non-cell-autonomous control of vascular stem cell fate by a CLE peptide/receptor system." Proceedings of the National Academy of Sciences **105**(39): 15208-15213.

Hirel, B., et al. (2007). "The challenge of improving nitrogen use efficiency in crop plants: towards a more central role for genetic variability and quantitative genetics within integrated approaches." Journal of Experimental Botany **58**(9): 2369-2387.

Hirsch, S., et al. (2009). "GRAS proteins form a DNA binding complex to induce gene expression during nodulation signaling in Medicago truncatula." The Plant cell **21**(2): 545-557.

Huault, E., et al. (2014). "Local and systemic regulation of plant root system architecture and symbiotic nodulation by a receptor-like kinase." PLoS Genet **10**(12): e1004891.

Huo, X., et al. (2006). "RNAi phenotypes and the localization of a protein:: GUS fusion imply a role for Medicago truncatula PIN genes in nodulation." Journal of Plant Growth Regulation **25**(2): 156-165.

Imaizumi-Anraku, H., et al. (2005). "Plastid proteins crucial for symbiotic fungal and bacterial entry into plant roots." Nature **433**(7025): 527-531.

Imanishi, L., et al. (2014). "Role of auxin during intercellular infection of Discaria trinervis by Frankia." Frontiers in plant science **5**.

Ivanov, S., et al. (2012). "Rhizobium–legume symbiosis shares an exocytotic pathway required for arbuscule formation." Proceedings of the National Academy of Sciences **109**(21): 8316-8321.

Kahle, J., et al. (2005). "Subunits of the heterotrimeric transcription factor NF-Y are imported into the nucleus by distinct pathways involving importin β and importin 13." Molecular and Cellular Biology **25**(13): 5339-5354.

Kanamori, N., et al. (2006). "A nucleoporin is required for induction of Ca2+ spiking in legume nodule development and essential for rhizobial and fungal symbiosis." Proceedings of the National Academy of Sciences of the United States of America **103**(2): 359-364.

Kawaguchi, M., et al. (2002). "Root, root hair, and symbiotic mutants of the model legume Lotus japonicus." Molecular Plant-Microbe Interactions **15**(1): 17-26.

Kistner, C. and M. Parniske (2002). "Evolution of signal transduction in intracellular symbiosis." Trends in Plant Science **7**(11): 511-518.

Kistner, C., et al. (2005). "Seven Lotus japonicus genes required for transcriptional reprogramming of the root during fungal and bacterial symbiosis." The Plant cell **17**(8): 2217-2229.

Larkin, P., et al. (1996). "Transgenic white clover. Studies with the auxin-responsive promoter, GH3, in root gravitropism and lateral root development." Transgenic research **5**(5): 325-335.

Lavin, M., et al. (2005). "Evolutionary rates analysis of Leguminosae implicates a rapid diversification of lineages during the Tertiary." Systematic biology **54**(4): 575-594.

Lee, K. H. and T. A. LaRue (1992). "Exogenous ethylene inhibits nodulation of Pisum sativum L. cv Sparkle." Plant Physiology **100**(4): 1759-1763.

Lévy, J., et al. (2004). "A putative Ca2+ and calmodulin-dependent protein kinase required for bacterial and fungal symbioses." Science **303**(5662): 1361-1364.

Limpens, E., et al. (2009). "Medicago N2-fixing symbiosomes acquire the endocytic identity marker Rab7 but delay the acquisition of vacuolar identity." The Plant cell **21**(9): 2811-2828.

Liu, W., et al. (2011). "Strigolactone biosynthesis in Medicago truncatula and rice requires the symbiotic GRAS-type transcription factors NSP1 and NSP2." The Plant cell **23**(10): 3853-3865.

Lohar, D. P., et al. (2006). "Transcript analysis of early nodulation events in Medicago truncatula." Plant Physiology **140**(1): 221-234.

Lum, M. R. and A. M. Hirsch (2002). "Roots and their symbiotic microbes: strategies to obtain nitrogen and phosphorus in a nutrient-limiting environment." Journal of Plant Growth Regulation **21**(4): 368-382.

Madsen, E. B., et al. (2011). "Autophosphorylation is essential for the in vivo function of the Lotus japonicus Nod factor receptor 1 and receptor-mediated signalling in cooperation with Nod factor receptor 5." The Plant Journal **65**(3): 404-417.

Madsen, E. B., et al. (2003). "A receptor kinase gene of the LysM type is involved in legumeperception of rhizobial signals." Nature **425**(6958): 637-640.

Magori, S., et al. (2009). "TOO MUCH LOVE, a root regulator associated with the long-distance control of nodulation in Lotus japonicus." Molecular Plant-Microbe Interactions **22**(3): 259-268.

Maillet, F., et al. (2011). "Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza." Nature **469**(7328): 58-63.

Malkov, N., et al. (2016). "Molecular basis of lipo-chitooligosaccharide recognition by the lysin motif receptor-like kinase LYR3 in legumes." Biochemical Journal **473**(10): 1369-1378.

Marhavý, P., et al. (2013). "Auxin reflux between the endodermis and pericycle promotes lateral root initiation." The EMBO journal **32**(1): 149-158.

Markmann, K., et al. (2008). "Functional adaptation of a plant receptor-kinase paved the way for the evolution of intracellular root symbioses with bacteria." PLoS Biol **6**(3): e68.

Marsh, J. F., et al. (2007). "Medicago truncatula NIN is essential for rhizobial-independent nodule organogenesis induced by autoactive calcium/calmodulin-dependent protein kinase." Plant Physiology **144**(1): 324-335.

Martín, A. C., et al. (2000). "Influence of cytokinins on the expression of phosphate starvation responsive genes in Arabidopsis." The Plant Journal **24**(5): 559-567.

Martínez-Romero, E. (2009). "Coevolution in Rhizobium-legume symbiosis?" DNA and cell biology **28**(8): 361-370.

Marvel, D. J., et al. (1987). "Rhizobium symbiotic genes required for nodulation of legume and nonlegume hosts." Proceedings of the National Academy of Sciences **84**(5): 1319-1323.

Mathesius, U., et al. (1998). "Auxin transport inhibition precedes root nodule formation in white clover roots and is regulated by flavonoids and derivatives of chitin oligosaccharides." The Plant Journal **14**(1): 23-34.

Messinese, E., et al. (2007). "A novel nuclear protein interacts with the symbiotic DMI3 calcium-and calmodulin-dependent protein kinase of Medicago truncatula." Molecular Plant-Microbe Interactions **20**(8): 912-921.

Mitra, R. M., et al. (2004). "A Ca2+/calmodulin-dependent protein kinase required for symbiotic nodule development: gene identification by transcript-based cloning." Proceedings of the National Academy of Sciences of the United States of America **101**(13): 4701-4705.

Miyata, K., et al. (2013). "Two distinct EIN2 genes cooperatively regulate ethylene signaling in Lotus japonicus." Plant and Cell Physiology **54**(9): 1469-1477.

Mok, D. W. and M. C. Mok (2001). "Cytokinin metabolism and action." Annual review of plant biology **52**(1): 89-118.

Mortier, V., et al. (2010). "CLE peptides control Medicago truncatula nodulation locally and systemically." Plant Physiology **153**(1): 222-237.

Murray, J. D., et al. (2007). "A cytokinin perception mutant colonized by Rhizobium in the absence of nodule organogenesis." Science **315**(5808): 101-104.

Naisbitt, T., et al. (1992). "The evolutionary significance of the legume genus Chamaecrista, as determined by nodule structure." New Phytologist **122**(3): 487-492.

Nap, J.-P. and T. Bisseling (1990). "Developmental biology of a plant-prokaryote symbiosis: the legume root nodule." Science(Washington) **250**(4983): 948-954.

Ng, J. L. P., et al. (2015). "Flavonoids and auxin transport inhibitors rescue symbiotic nodulation in the Medicago truncatula cytokinin perception mutant cre1." The Plant cell **27**(8): 2210-2226.

Nukui, N., et al. (2000). "Effects of ethylene precursor and inhibitors for ethylene biosynthesis and perception on nodulation in Lotus japonicus and Macroptilium atropurpureum." Plant and Cell Physiology **41**(7): 893-897.

Okamoto, S. and M. Kawaguchi (2015). "Shoot HAR1 mediates nitrate inhibition of nodulation in Lotus japonicus." Plant Signaling & Behavior **10**(5): e1000138.

Okamoto, S., et al. (2009). "Nod factor/nitrate-induced CLE genes that drive HAR1-mediated systemic regulation of nodulation." Plant and Cell Physiology **50**(1): 67-77.

Okamoto, S., et al. (2013). "Root-derived CLE glycopeptides control nodulation by direct binding to HAR1 receptor kinase." Nature communications **4**.

Oldroyd, G. E., et al. (2001). "Ethylene inhibits the Nod factor signal transduction pathway of Medicago truncatula." The Plant cell **13**(8): 1835-1849.

Olsson, J. E., et al. (1989). "Lack of systemic suppression of nodulation in split root systems of supernodulating soybean (Glycine max [L.] Merr.) mutants." Plant Physiology **90**(4): 1347-1352.

Op den Camp, R. H., et al. (2012). "Nonlegume Parasponia andersonii deploys a broad rhizobium host range strategy resulting in largely variable symbiotic effectiveness." Molecular Plant-Microbe Interactions **25**(7): 954-963.

Ovchinnikova, E., et al. (2011). "IPD3 controls the formation of nitrogen-fixing symbiosomes in pea and Medicago Spp." Molecular Plant-Microbe Interactions **24**(11): 1333-1344.

Pacios-Bras, C., et al. (2003). "Auxin distribution in Lotus japonicus during root nodule development." Plant Molecular Biology **52**(6): 1169-1180.

Parniske, M. (2008). "Arbuscular mycorrhiza: the mother of plant root endosymbioses." Nature Reviews Microbiology **6**(10): 763-775.

Penmetsa, R. V. and D. R. Cook (1997). "A legume ethylene-insensitive mutant hyperinfected by its rhizobial symbiont." Science **275**(5299): 527-530.

Perrine-Walker, F., et al. (2010). "Auxin carriers localization drives auxin accumulation in plant cells infected by Frankia in Casuarina glauca actinorhizal nodules." Plant Physiology **154**(3): 1372-1380.

Pietraszewska-Bogiel, A., et al. (2013). "Interaction of Medicago truncatula lysin motif receptor-like kinases, NFP and LYK3, produced in Nicotiana benthamiana induces defence-like responses." PLoS ONE **8**(6): e65055.

Prayitno, J., et al. (2006). "The ethylene-insensitive sickle mutant of Medicago truncatula shows altered auxin transport regulation during nodulation." Plant Physiology **142**(1): 168-180.

Read, D., et al. (2000). "Symbiotic fungal associations in 'lower'land plants." Philosophical Transactions of the Royal Society of London B: Biological Sciences **355**(1398): 815-831.

Reid, D. E., et al. (2011). "Inoculation-and nitrate-induced CLE peptides of soybean control NARK-dependent nodule formation." Molecular Plant-Microbe Interactions **24**(5): 606-618.
Reid, D. E., et al. (2011). "Molecular mechanisms controlling legume autoregulation of nodulation." Annals of Botany **108**(5): 789-795.

Roth, L. and G. Stacey (1989). "Bacterium release into host cells of nitrogen-fixing soybean nodules: the symbiosome membrane comes from three sources." European journal of cell biology **49**(1): 13-23.

Saito, K., et al. (2007). "NUCLEOPORIN85 is required for calcium spiking, fungal and bacterial symbioses, and seed production in Lotus japonicus." The Plant cell **19**(2): 610-624.

Schauser, L., et al. (1999). "A plant regulator controlling development of symbiotic root nodules." Nature **402**(6758): 191-195.

Schnabel, E., et al. (2011). "ROOT DETERMINED NODULATION 1 regulates nodule number in M. truncatula and defines a highly conserved, uncharacterized plant gene family." Plant Physiology: pp. 111.178756.

Singh, S., et al. (2014). "CYCLOPS, a DNA-binding transcriptional activator, orchestrates symbiotic root nodule development." Cell Host & Microbe **15**(2): 139-152.

Soyano, T., et al. (2013). "Nodule inception directly targets NF-Y subunit genes to regulate essential processes of root nodule development in Lotus japonicus." PLoS Genet **9**(3): e1003352.

Sprent, J. I. (2008). "60Ma of legume nodulation. What's new? What's changing?" Journal of Experimental Botany **59**(5): 1081-1084.

Steen, A., et al. (2005). "AcmA of Lactococcus lactis is an N-acetylglucosaminidase with an optimal number of LysM domains for proper functioning." FEBS Journal **272**(11): 2854-2868.

Stracke, S., et al. (2002). "A plant receptor-like kinase required for both bacterial and fungal symbiosis." Nature **417**(6892): 959-962.

Streeter, J. and P. P. Wong (1988). "Inhibition of legume nodule formation and N2 fixation by nitrate." Critical Reviews in Plant Sciences **7**(1): 1-23.

Sun, J., et al. (2006). "Crosstalk between jasmonic acid, ethylene and Nod factor signaling allows integration of diverse inputs for regulation of nodulation." The Plant Journal **46**(6): 961-970.

Suzaki, T., et al. (2012). "Positive and negative regulation of cortical cell division during root nodule development in Lotus japonicus is accompanied by auxin response." Development **139**(21): 3997-4006.

Takanashi, K., et al. (2011). "Involvement of auxin distribution in root nodule development of Lotus japonicus." Planta **234**(1): 73-81.

Takeda, N., et al. (2013). "CERBERUS and NSP1 of Lotus japonicus are common symbiosis genes that modulate arbuscular mycorrhiza development." Plant and Cell Physiology **54**(10): 1711-1723.

Tirichine, L., et al. (2006). "Deregulation of a Ca2+/calmodulin-dependent kinase leads to spontaneous nodule development." Nature **441**(7097): 1153-1156.

Tirichine, L., et al. (2007). "A gain-of-function mutation in a cytokinin receptor triggers spontaneous root nodule organogenesis." Science **315**(5808): 104-107.

Trinick, M. (1973). "Symbiosis between Rhizobium and the non-legume, Trema aspera." Nature **244**: 459-460.
van Noorden, G. E., et al. (2007). "Overlap of proteome changes in Medicago truncatula in response to auxin and Sinorhizobium meliloti." Plant Physiology **144**(2): 1115-1131.

van Zeijl, A., et al. (2015). "Rhizobium lipo-chitooligosaccharide signaling triggers accumulation of cytokinins in Medicago truncatula roots." Molecular Plant **8**(8): 1213-1226.

Vance, C. P. (2001). "Symbiotic nitrogen fixation and phosphorus acquisition. Plant nutrition in a world of declining renewable resources." Plant Physiology **127**(2): 390-397.

Vessey, J. K., et al. (2004). "Root-based N-2-fixing symbioses: Legumes, actinorhizal plants, Parasponia sp and cycads." Plant and Soil **266**(1-2): 205-230.

Wang, H., et al. (2009). "Rosid radiation and the rapid rise of angiosperm-dominated forests." Proceedings of the National Academy of Sciences **106**(10): 3853-3858.

Wopereis, J., et al. (2000). "Short root mutant of Lotus japonicus with a dramatically altered symbiotic phenotype." The Plant Journal **23**(1): 97-114.

Xiao, T. T., et al. (2014). "Fate map of Medicago truncatula root nodules." Development **141**(18): 3517-3528.
Yang, M.-Q., et al. (2013). "Molecular phylogenetics and character evolution of Cannabaceae." Taxon **62**(3): 473-485.

Yano, K., et al. (2008). "CYCLOPS, a mediator of symbiotic intracellular accommodation." Proceedings of the National Academy of Sciences **105**(51): 20540-20545.

Chapter2

Efficiency of *Agrobacterium rhizogenes*–Mediated Root Transformation of *Parasponia* and *Trema* Is Temperature Dependent

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Chapter 2

Abstract

Parasponia trees are the only non-legume species that form nitrogen-fixing root nodules with rhizobium. Based on its taxonomic position in relation to legumes (*Fabaceae*), it is most likely that both lineages have gained this symbiotic capacity independently. Therefore, *Parasponia* forms a bridging species to understand the evolutionary constraints underlying this symbiosis. However, absence of key technologies to genetically modified *Parasponia* seriously impeded studies on these species. We employed *Agrobacterium rhizogenes* to create composite *Parasponia andersonii* plants that harbor transgenic roots. Here, we provide an optimized protocol to infect *P. andersonii* as well as its non- symbiotic sister species *Trema tomentosa* with *A. rhizogenes*. We show that the transformation efficiency is temperature dependent. Whereas the optimal growth temperature for both species is 28°C, the transformation is most efficient when co-cultivation with *A. rhizogenes* occurs at 21°C. By using of this optimized protocol up to 80% transformation efficiency can be obtained. These robust transformation platforms will provide a strong tool to unravel the *Parasponia–rhizobium* symbiosis.

Keywords: *Parasponia, Trema*, Agrobacterium transformation, Symbiosis, Transgenic root, Composite plant.

Introduction

Legumes (*Fabaceae*) are known for their protein-richness and many legume crops are cultivated to provide a protein source for humans. Legume crops have been used in ancient agriculture; e.g. in Neolithic China soybean was grown next to millet (Lee *et al.*, 2007; Guo *et al.*, 2010). Legumes can accumulate such high protein content in leaves and seeds due to a unique endosymbiosis with soil born nitrogen-fixing rhizobium bacteria. This symbiosis results in formation of novel root organs, the so- called root nodules. In nodules, rhizobium is hosted intracellularly and produces ammonia from atmospheric N_2. This is the most important biological process by which fixed nitrogen is produced in agriculture (Dawson and Hilton, 2011). The very high efficiency of N_2-fixation in legume nodules makes it an already long lasting goal for researchers to transfer this symbiotic capacity to important non-legume crops like rice and wheat (Burrill and Hansen, 1917; Beatty and Good, 2011).

Outside the *Fabaceae*, there is only a single plant genus, *Parasponia*, which can establish a similar symbiosis with rhizobium. *Parasponia* belongs to the *Celtidaceae* (order Rosales) (Yesson *et al.*, 2004), but molecular phylogenetic studies combine this (sub) family with *Cannabaceae* and *Urticaceae*, resulting in a new (super) family of *Cannabaceae* (Sytsma *et al.*, 2002; II, 2003). *Parasponia* and *Fabaceae* are only remotely related and had a last common ancestor 100 million years ago (Wang *et al.*, 2009). This suggests that both lineages evolved rhizobium symbiosis independently. Such independent evolutionary events provide a unique tool to unravel the molecular evolutionary mechanisms underlying the rise of endosymbiosis with rhizobium. Furthermore, studies on *Parasponia* can teach us how to transfer this important agricultural trait to non- legume crops. However, absence of key technologies to genetically modify *Parasponia* plants seriously has impeded studies in such directions.

In the *Parasponia* genus only 5 species are recognized, all of which are tropical trees with their native distribution in the Malay Archipelago, including Indonesia, Malaysia and Papua New Guinea (Trinick, 1973; Akkermans *et al.*, 1978; Becking, 1992). Upon initial discovery it was misclassified and named *Trema* due to strong phenotypic resemblance to these species, which

are unable to engage a rhizobium symbiosis (Trinick, 1973). The close relation of *Trema* and *Parasponia* genera is supported by molecular phylogenetic studies using the chloroplast genes *rbcL*, and *trnL* or the internal transcribed spacer (ITS) region of 18S– 26S nuclear ribosomal DNA as markers (Sytsma *et al.*, 2002; Yesson *et al.*, 2004). In these studies insufficient resolution is obtained to discriminate *Parasponia* as a monophyletic group from non-symbiotic *Trema* species. As *Parasponia* is the only non-legume species able to establish a symbiosis with rhizobium, it suggests that it has gained this capacity relatively recent, most likely just after the split from *Trema*.

The relative young age of the *Parasponia*-rhizobium symbiosis is further supported by the rather primitive nature of the root nodules. *Parasponia* nodules have the appearance of modified lateral roots with a central vascular bundle and infected cells in the peripheral zone (Trinick, 1979). Furthermore, the infection process is also rather primitive. Rhizobium enters the *Parasponia* root intercellularly by crack entry and only when bacteria reach a nodule primordium, intracellular infection occurs (Becking, 1992). Once inside a cell, the infection thread will branch and the newly formed threads have a very thin cell wall. These so-called fixation threads are filled with rhizobia that can fix nitrogen (Trinick, 1979) (Fig. 1c). Fixation threads resemble the endomembrane compartments containing highly branched hyphae formed by arbuscular mycorrhiza (AM) fungi, called arbuscules. Both structures form a continuum with the plasma membrane. Recent studies in *P. andersonii* revealed that both, AM arbuscules and rhizobium fixation threads, require the LysM-type receptor kinase PaNFP that in legumes is known to function as receptor for rhizobium secreted lipochitooligosaccharides, named Nod factors (den Camp *et al.*, 2011). In legumes this receptor is not essential for mycorrhization, possibly due to subneofunctionalization after a gene duplication event (Young *et al.*, 2011). These data are well in line with the idea that the *Parasponia*- rhizobium symbiosis is relatively young, and it clearly illustrates that *Parasponia* has the potential to provide insight in the core mechanisms controlling symbiosis (Streng *et al.*, 2011).

Genetic studies revealed that the evolutionary constraints underlying a rhizobium nodule symbiosis are largely based on the signaling machinery essential for mycorrhization. As the vast majority of plants can establish the endosymbiosis with endomycorrhizal fungi, it suggests that the genetic machinery to establish a rhizobium nodule symbiosis is in principle widespread in the plant kingdom. This, combined with the ability to transfer lateral roots into a

rhizobium hosting organ raises the intriguing question why most plants have not yet evolved a rhizobium nodule symbiosis. As *Parasponia* could provide the answer a Chinese-Dutch consortium has started the sequencing of the *P. andersonii* genome. However, to fully exploit this sequence information it will be essential to have an efficient transformation procedure available.

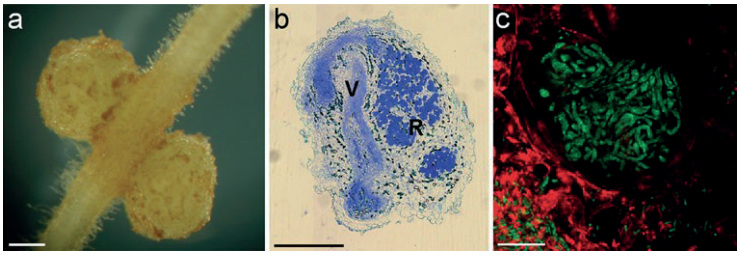

Fig. 1 *Parasponia* root nodules. a Nodules on *P. andersonii* roots induced by *Sinorhizobium* sp. NGR234. *Bar* 1 mm. b Structure of a *P. andersonii* nodule with central vascular bundle (V) and peripheral lobes with rhizobium infected cells (R). Bar 1 mm. c Confocal microscope image of an infected *P. andersonii* nodule cell filled with fixation threads. Rhizobia inside these fixation threads express the fluorescent marker gene GFP. Cells are counter-stained with FM-64. Bar 1mm.

Genetic transformation mediated by A. *tumefaciens or A. rhizogenes* has been developed for many plant species. In legumes especially co-transformation with *A. rhizogenes* has been used. In this system the *root inducing locus* (*rol*) genes of *A. rhizogenes* are transferred to the host and induce the formation of so-called hairy roots, even though the phenotypic appearance of these roots are often indistinguishable from untransformed roots (White *et al.*, 1985). The gene of interest can be co-transferred with the *rol* genes. *A. rhizogenes* mediated transformation leads to composite plants with a non-transgenic shoot carrying several transgenic roots that are the result of independent transformation events. For a number of legume species this method has been optimized, which resulted in rapid and effective protocols to generate composite plants (Boisson-Dernier *et al.*, 2001; Limpens *et al.*, 2004; Colpaert *et al.*, 2008; Estrada-Navarrete *et al.*, 2006). Since a composite plant may contain co-transformed as well as non-co-transformed roots, generally a fluorescent protein (e.g. DsRed or GFP) is used as non-destructive selectable

marker. Taken together, these protocols provide a solid platform for studies on legume root biology and root-microbe interactions, such as rhizobium and endomycorrhizal symbioses. Here, we aim to establish a similar platform for *P. andersonii* and its non-nodulation sister species *Trema tomentosa*.

Results and discussion

Agrobacterium rhizogenes-mediated root transformation of Parasponia andersonii is temperature dependent

Parasponia andersonii is a tropical woody plant, and the optimal temperature for growth of seedlings or micro-propagated shoots is 28°C (Davey *et al.*, 1993; Webster *et al.*, 1995). Shoot cuttings can be efficiently rooted at this temperature. Therefore we tested whether *A. rhizogenes* would induce the formation of transgenic roots at 28°C. For this experiment we used micropropagated plantlets that had already formed roots and the *A. rhizogenes* strain MSU440 that harbored a control binary vector containing DsRed1 as selectable marker. Plantlets were infected with *A. rhizogenes* after removing the root system and were inoculated at the wound surfaces. Plantlets and *A. rhizogenes* were co-cultivated for 5 days on EKM medium and subsequently transferred to emergence medium that is rich in nutrients and contains cefotaxime to kill *A. rhizogenes*. These plants formed about 3 new roots within a 3 week time period. However, the number of transgenic roots expressing *DsRed1* was very low. We analyzed 222 plants and only 8 contained one or more transgenic roots based on red fluorescence (Fig. 2a, b; Table 1). As the transformation efficiency (% of composite plants with a transgenic root) was only 3.6 %, this method was not really suitable for research and we aimed to optimize it.

Boisson-Dernier et al. (2001) had previously shown that a mild temperature during co-cultivation is beneficial for *A. rhizogenes*-mediated transformation of *M. truncatula*. Therefore, we tested whether a lower co-cultivation temperature could improve the efficiency of *A. rhizogenes* mediated transformation of *P. andersonii*. Again, we used micropropagated plantlets that had already formed roots.

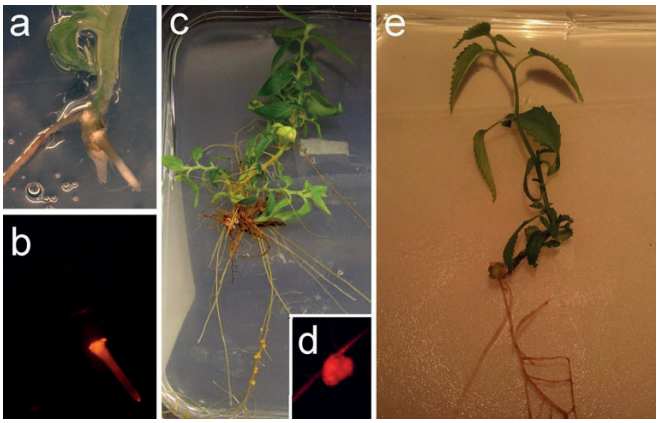

Fig.2 Micropropagated *Parasponia andersonii* and *Trema tomentosa* plantlets with transgenic roots. a, b *P. andersonii* plantlet carrying a transgenic root, which can be distinguished based on *DsRED1* expression causing red fluorescence (b). c, d *P. andersonii* plantlet carrying a transgenic root that is nodulated with *Sinorhizobium NGR234*. Transgenic root nodules can distinguished based on red fluorescence (d). e Rooted *T. tomentosa* plantlet.

Table 1: Transformation efficiencies of *P. Andersonii* and *T. tomentosa*

		Co-cultivation with Agrobacterium		Root emergence		Result	
Species	Plant material	Temperature (°C)	Time (days)	Temperature (°C)	Time (days)	Efficiency (%)	Number of transgenic
Parasponia	Rooted shoots	28	5	28	21	3.6	8/222
andersonii	Rooted shoots	21	5	28	21	70.3	83/118
	Unrooted shoots	21	5	28	21	61.3	57/93
	Rooted shoots	21	5	21 and 28	7 and 14	92.7	51/55
	Rooted shoots	21	5	21	21	94.1	64/68
	Unrooted shoots	21	5	21 and 28	7 and 14	85.5	47/55
	Unrooted shoots	21	5	21	21	84.0	42/50
Trema	Rooted shoots	21	5	21 and 28	7 and 14	61.5	24/39
	Unrooted shoots	21	5	21 and 28	7 and 14	37.1	23/62

Transformation efficiency is given as fraction or percentage of plantlets with one or more transgenic roots. Starting material used is either micropropagated rooted shoots or unrooted shoots. Given are the various temperatures of *A. Rhizogenes* co-cultivation and the time/temperature or growth on cefotaxime containing nutrient rich medium

P.andersonii was co-cultivated with A. *rhizogenes at* 21°C on EKM medium. Five days after inoculation plants were transferred to emergence medium (including cefotaxime) and placed back at 28 °C, which is the optimal temperature for growth of *P. andersonii*. Three weeks after transfer, the transformation efficiency of micropropagated plantlets was 70.3 % (Table 1). Based on these findings we conclude that co-cultivation at lower temperature significantly increases the transformation efficiency of *P. andersonii*.

Next, we tested whether prolonged growth at 21°C can further improve the transformation efficiency. To do so, we performed two types of experiments. After co-cultivation at 21 °C for 5 days, plants were transferred to emergence medium and kept for 1 or 3 additional weeks at 21 °C. Both growth conditions improved transformation, resulting in almost 100 % efficiency (Fig. 2; Table 1). So, extending the period that plants are grown at 21°C is the key to further increase the transformation efficiency. However, transgenic roots grew very slow at 21 °C and large calli developed at the base on the root system when grown at this temperature for 3 weeks. Therefore, we conclude that the best way to obtain proper transgenic roots in an efficient manner is by co-cultivation at 21 °C for 5 days and an additional week at 21 °C when grown on cefotaxime containing nutrient rich emergence medium, prior transfer to 28°C. These transgenic roots could also be nodulated when grown on plates by *Sinorhizobium* sp. NGR234 (Fig. 2c, d).

Transformation of unrooted Parasponia andersonii shoots

In the above described method we used rooted plantlets as source for transformation. These plantlets were obtained upon *in vitro* micropropagation as described by Davey et al. (1993) with a few modifications. As *P. andersonii* is a tree, we decided to use woody plant medium (WPM, (Lloyd, 1980) instead of MS medium. Generated shoots were subsequently rooted on auxin containing rooting medium; a procedure that takes 2 weeks. We raised the question whether unrooted shoots could be used directly for *A. rhizogenes* -mediated root transformation. This would be attractive as shoots can be obtained in high numbers with low labour input. The initial protocol for rooted plantlets (5 days at 21°C, 3 weeks at 28°C) resulted in a transformation efficiency of 61.3 % (Table 1). Either method of prolonged growth at 21°C resulted in a higher transformation efficiency of 85 % (Table 1). Taken

together, we conclude that also unrooted shoots can be used to generate composite plants carrying transgenic roots with efficiency comparable to rooted plantlets. By doing so, the time period of experimentation is shortened by about 2 weeks.

Micropropagation and root transformation of Trema tomentosa

To enable comparative studies with *Parasponia* we aimed to develop a similar root transformation system for the closely related non-nodulating sister species, namely *T. tomentosa*. We set up an *in vitro* micropropagation system similar as used for *P. andersonii*. The *in vitro* micropropagation method as applied for *P. andersonii* could be adopted with marginal changes for *T. tomentosa* (see ‘‘Materials and methods’’). We used the optimized protocol for *A. rhizogenes*-mediated transformation of *P. andersonii* and used either rooted or unrooted *T. tomentosa* shoots as starting material. The efficiency of rooted *T.tomentosa* plantlets was 61.5 % (Fig. 2e; Table 1). But when unrooted shoots are used an efficiency of only 37.1 % was obtained (Table 1). Most of untransformed shoots had big calli at the wound site of *A. rhizogenes* infection. This may decrease the transformation efficiency. Still the majority of the rooted plantlets could be transformed and therefore we concluded that rooted plantlets are a better starting material for *A. rhizogenes*-mediated transformation of *T. tomentosa*.

Conclusion

We developed a relatively fast and highly efficient method for *A. rhizogenes*-mediated roots transformation for *Parasponia* and *Trema* species. We show that in case of *P. andersonii* and *T. tomentosa transformation* with *A. rhizogenes* MSU440 at 21°C is most efficient despite the fact that the optimal growth temperature of these tropical species is 28°C. This is in line with previous *A. rhizogenes*- based root transformation studies on *M. truncatula* and supports the conclusion that Agrobacterium mediated DNA transfer is temperature dependent (Dillen *et al.*, 1997; Kondo *et al.*, 2000; Boisson-Dernier *et al.*, 2001; Salas *et al.*, 2001). The efficient transformation protocols will be an important tool for functional analysis of *Parasponia* and *Trema* genes by reverse genetics. This technology will facilitate the use of the *Parasponia* genome sequence that will become available in the near future and so it will be instrumental in unraveling the evolutionary mechanism by which *Parasponia* obtained the ability to

establish a N_2 fixing symbiosis with Rhizobium.

Materials and methods

In vitro micro-propagation of P. andersonii and T. Tomentosa

Parasponia andersonii and *Trema tomentosa* axillary buds were surface sterilized in 4 % hypochlorite (commercial bleach) for 7 min and subsequently washed 6 times by autoclaved water. Sterilized *P. andersonii* axillary buds were placed on propagation medium in Ø 10 cm round petri dishes (pH 5.8, 20 g/L sucrose, 2.4 g/L McCown Woody Plant Medium (WPM) including vitamins (Duchefa Biochemie, Haarlem, The Netherlands; WPM) (Lloyd, 1980) 1.0 mg/L 6-Benzylaminopurine, 0.1 mg/ L Indole-3-butyric acid, 0.8 % Daichin agar (Brunschwig Chemie, Amstedam, The Netherlands)). Sterile *T. tomentosa* axillary buds were placed on propagation medium with half the hormonal concentrations (pH 5.8, 20 g/L sucrose, 2.4 g/L WPM, 0.5 mg/L 6-Benzylaminopurine, 0.05 mg/L Indole-3-butyric acid, 0.8 % Daichin agar). Both were kept in an Elbanton growth cabinet at 28°C with a 16/8 h day/night regime. Within 4 weeks shoots emerged from the axillary buds calli. Shoots of 1.0 cm in length were cut and transferred to rooting medium (pH5.8, 20 g/L sucrose, 2.4 g/L WPM, 1.0 mg/L indole-3-butyric acid, 0.1 mg/L 1-naphthaleneacetic acid) in round Ø10 cm petri dished in-between two half-round Ø 8.5 cm filter papers (Machery-Nagel, Du¨ren, Germany). These plates were covered by aluminum foil (darkness induced roots) and kept in the same growth cabinet at 28°C for 2 weeks. Rooted shoots were kept on hormone free medium (pH 5.8, 20 g/L sucrose, 2.4 g/L WPM, and 0.8 % Daichin agar).

Bacterial strains

Agrobacterium rhizogenes strain MSU440 carrying the empty binary vector pRed Root that contains DsRed1 as selectable marker was used for the transformations (Lim- pens et al. 2004). The binary vector is available upon request from our laboratory.

Agrobacterium rhizogenes-mediated transformation micropropagated *P. andersonii* or *T. tomentosa* shoots (over 1.0 cm in length) or rooted plants were used for *A rhizogenes*

transformation. Roots or the basipetal tip of a shoot stem were removed from the plantlets/shoots with a razor blade and plants were subsequently placed on top of a Ø 8.5 cm half-round filter (Machery-Nagel, Du¨ren, Germany) on EKM medium Ø10 cm petri dishes (pH 6.6, 0.25 g/L MgSO4.7H2O, 0.12 g/L KH2PO4, 0.36 g/L K2HPO4, 0.25 g/L CaSO4.2H2O, 0.10 g/L Na2SO4, 0.03 g/L NH4NO3, 0.005 g/L Fe-citrate, 1.0 mg/L MnSO4, 0.25 mg/L ZnSO4.7H2O, 0.25 mg/L CuSO4.5H2O, 0.25 mg/L H3BO3, 0.005 mg/L Na2MoO4.2H2O, 0.8 % Daichin agar (Bruns- chwig Chemie, Amstedam, The Netherlands)). Plate grown *A. rhizogenes* strain MSU440 carrying pRedRoot was applied on the wounded surface. The most basipetal shoot portion was carefully punctured 2–3 times with a sterile needle (0.45 micron) that was first dipped in *A. rhizogenes*. Plates with plantlets were placed vertically in the growth cabinet. First, the wounded shoots were co-cultivated with *A. rhizogenes* on EKM (in Elbanton growth cabinet at either 21 or 28°C with 16/8 h light/dark cycle), and subsequently transferred to cefotaxime (300 mg/ml) containing nutrient rich emergence medium (EM) (3 mM MES pH 5.8 containing 2.5 g/L KNO3, 0.4 g/L MgSO4·7H2O, 0.3 g/L NH4H2PO4, 0.2 g/L CaCl2·2H2O, 10 mg/L MnSO4·4H2O, 5 mg/L H3BO3, 1 mg/L ZnSO4·7H2O, 1 mg/L KI, 0.2 mg/L CuSO4·5H2O, 0.1 mg/L NaMoO4·2H2O, 0.1 mg/L CoCl2·6H2O, 15 mg/L FeSO4·7H2O, 20 mg/L Na2EDTA, 100 mg/L myoinositol, 5 mg/L nicotinic acid, 10 mg/L pyridoxine HCl, 10 mg/L thiamine HCl, 2 mg/L glycine, 10 g/L sucrose, 0.9 % Daichin agar containing 300 mg/ml Cefotaxime Sodium (Duchefa Biochemie, Haarlem, The Netherlands)) (Elbanton growth cabinet at either 21 or 28 °C with 16/8 h light/dark cycle). On EM medium shoots were kept in-between two half-round filter papers; mind especially that both filters are in contact with the wounded surface. Petri dishes were closed with parafilm, but a small opening was left to enable aeration.

Detection of transgenic roots

Transgenic roots were selected based on red fluorescence, using a Leica MZIII fluorescence stereomicroscope (filter settings; excitation 565/30 and emission 620/60). The number of transformed roots was determined based on DsRed1 fluorescence.

Nodulation of P. andersonii composite plants

Parasponia andersonii plantlets with transformed roots were transferred to EKM supplemented

with 2 lM Aminoethoxyvinylglycine (AVG) in 120×9×120 cm square petri dishes of which the lower half was streaked with plate grown *Sinorhizobium sp*. NGR234. Plates were kept vertically in an Elbanton growth cabinet at 28°C with 16/8 h light/dark cycle. Nodules appeared within 4–6 weeks.

Acknowledgments

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References

Akkermans, A., et al. (1978). "N2-fixing root nodules in Ulmaceae: Parasponia or (and) Trema spp.?" Plant and Soil **49**(3): 711-715.

Beatty, P. H. and A. G. Good (2011). "Future prospects for cereals that fix nitrogen." Science **333**(6041): 416-417.

Becking, J. (1992). The Rhizobium symbiosis of the nonlegume Parasponia, Routledge: Chapman and Hall, NY, USA**:** 497-559.

Boisson-Dernier, A., et al. (2001). "Agrobacterium rhizogenes-transformed roots of Medicago truncatula for the study of nitrogen-fixing and endomycorrhizal symbiotic associations." Molecular Plant-Microbe Interactions **14**(6): 695-700.

Burrill, T. J. and R. Hansen (1917). "Is symbiosis possible between legume bacteria and non-legume plants?" Bulletin (University of Illinois (Urbana-Champaign campus). Agricultural Experiment Station); no. 202.

Colpaert, N., et al. (2008). "Composite Phaseolus vulgaris plants with transgenic roots as research tool." African Journal of Biotechnology **7**(4).

Davey, M., et al. (1993). "Effective nodulation of micro-propagated shoots of the non-legume Parasponia andersonii by Bradyrhizobium." Journal of Experimental Botany **44**(5): 863-867.

Dawson, C. J. and J. Hilton (2011). "Fertiliser availability in a resource-limited world: Production and recycling of nitrogen and phosphorus." Food Policy **36**: S14-S22.

den Camp, R. O., et al. (2011). "LysM-type mycorrhizal receptor recruited for rhizobium symbiosis in nonlegume Parasponia." Science **331**(6019): 909-912.

Dillen, W., et al. (1997). "The effect of temperature on Agrobacterium tumefaciens-mediated gene transfer to plants." The Plant Journal **12**(6): 1459-1463.

Estrada-Navarrete, G., et al. (2006). "Agrobacterium rhizogenes transformation of the Phaseolus spp.: a tool for functional genomics." Molecular Plant-Microbe Interactions **19**(12): 1385-1393.

Guo, J., et al. (2010). "A single origin and moderate bottleneck during domestication of soybean (Glycine max): implications from microsatellites and nucleotide sequences." Annals of Botany **106**(3): 505-514.

II, A. (2003). "An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG II." Botanical Journal of the Linnean Society **141**(4): 399-436.

Kondo, T., et al. (2000). "Transformation and regeneration of garlic (Allium sativum L.) by Agrobacterium-mediated gene transfer." Plant Cell Reports **19**(10): 989-993.

Lee, G.-A., et al. (2007). "Plants and people from the Early Neolithic to Shang periods in North China." Proceedings of the National Academy of Sciences **104**(3): 1087-1092.

Limpens, E., et al. (2004). "RNA interference in Agrobacterium rhizogenes-transformed roots of Arabidopsis and Medicago truncatula." Journal of Experimental Botany **55**(399): 983-992.

Lloyd, G. (1980). Commercially-feasible micropropagation of mountain laurel, Kalmia latifolia, by use of shoot-tip culture. Int. Plant. Prop. Soc.

Salas, M., et al. (2001). "Temperature influence on stable T-DNA integration in plant cells." Plant Cell Reports **20**(8): 701-705.

Streng, A., et al. (2011). "Evolutionary origin of rhizobium Nod factor signaling." Plant Signaling & Behavior **6**(10): 1510-1514.

Sytsma, K. J., et al. (2002). "Urticalean rosids: circumscription, rosid ancestry, and phylogenetics based on rbcL, trnL-F, and ndhF sequences." American Journal of Botany **89**(9): 1531-1546.

Trinick, M. (1973). "Symbiosis between Rhizobium and the non-legume, Trema aspera." Nature **244**: 459-460.

Trinick, M. (1979). "Structure of nitrogen-fixing nodules formed by Rhizobium on roots of Parasponia andersonii Planch." Canadian Journal of Microbiology **25**(5): 565-578.

Wang, H., et al. (2009). "Rosid radiation and the rapid rise of angiosperm-dominated forests." Proceedings of the National Academy of Sciences **106**(10): 3853-3858.

Webster, G., et al. (1995). "The nodulation of micro-propagated plants of Parasponia andersonii by tropical legume rhizobia." Journal of Experimental Botany **46**(9): 1131-1137.

White, F., et al. (1985). "Molecular and genetic analysis of the transferred DNA regions of the root-inducing plasmid of Agrobacterium rhizogenes." Journal of bacteriology **164**(1): 33-44.

Yesson, C., et al. (2004). "Phylogenetic framework for Trema (Celtidaceae)." Plant Systematics and Evolution **248**(1-4): 85-109.

Young, N. D., et al. (2011). "The Medicago genome provides insight into the evolution of rhizobial symbioses." Nature **480**(7378): 520-524.

Chapter 3

Agrobacterium tumefaciens-mediated Stable Transformation of *Trema tomentosa*

Maryam Seifi Kalhor, Marijke Hartog, Tingting Xiao, Ton Bisseling and René Geurts

Abstract

Tropical tree species of *Trema* genus are the closest relatives of *Parasponia* tree plants; the only known non-legume lineage of plant species that are capable of establishing a nitrogen fixing nodule symbiosis with rhizobium. To identify genetic elements that underlie the rhizobium symbiosis, comparative studies of *Parasponia* sp. and *Trema* sp. are markedly informative. Generally, *Agrobacterium rhizogenes*-mediated root transformation is used to conduct reverse genetic experiments in a root symbiosis context. However, such transient methods are prone for variation, as every transgenic root is the result of a *de novo* transformation event. Here, we present a protocol for *Agrobacterium tumefacien*s-mediated stable transformation of *Trema tomentosa.* To do so, we used a binary vector containing the auxin responsive reporter DR5::GUS and two selectable marker genes; *NPTII* encoding kanamycin resistance and the red fluorescent protein encoding gene *DsRED1*. By using culture conditions optimized for *A. tumefaciens* co-cultivation, callus formation and regeneration of plantlets, a transformation efficiency of 15% was obtained. *In vitro* propagated T0 plants display a DR5::GUS expression in leaves that is identical to observed in primary T0 plantlets. Taken together, the presented transformation method for *T. tomentosa* is efficient, and results in transgenic plantlets within a timeframe of only 6 months.

Keywords: *Trema tomentosa*, Co-cultivation, *Agrobacterium tumefacien*s, DR5::GUS.

Introduction

Nitrogen fixing rhizobium symbiosis is a well-known character of leguminous plants (Fabaceae). Beside legume plants, a comparable symbiosis also occurs on five tropical tree species of the *Parasponia* genus (Cannabaceae). In both legumes as well as *Parasponia* spp., the interaction with rhizobium results in formation of novel root organs; so-called root nodules. Rhizobium is hosted in the nodules cells, where rhizobium bacteria can find the proper conditions to convert atmospheric nitrogen (N_2) into ammonia (NH_4^+). The produced ammonia can be utilized by the plant. *Parasponia* genus and Fabaceae family had a last common ancestor that lived about 100 million years ago (Wang *et al.*, 2009), which makes it most probable that both lineages evolved rhizobium symbiosis independently. Several lines of evidence indicate that genetic constraints have guided evolution of symbiosis in both lineages. Root nodule formation in legumes and *Parasponia* sp. can occur with the same rhizobium bacteria, requires very similar bacterial lipo-chitooligosaccharide (LCO) signal molecules, and is controlled by a conserved symbiotic signaling network (den Camp *et al.*, 2011). Therefore, the *Parasponia* research model –alongside with legume model plants like *Lotus japonicus* and *Medicago truncatula*- provides a novel tool to obtain insights in the genetics underlying the nitrogen fixing endosymbiosis with rhizobium.
Parasponia is highly promiscuous as it will allow most LCO-secreting rhizobia to enter its roots by a mechanism known as crack entry (Op den Camp *et al.*, 2012) These rhizobium bacteria remain in the apoplast, and only will infect intracellularly when reaching nodule cells. Once inside a cell, the bacteria remain within infection thread-like structures known as fixation threads. Interestingly, it was noted that *Parasponia* nodules are less effective in nitrogen fixation when compared to nodules of legumes formed by the same bacteria (Behm *et al.*, 2014). This may be explained by the limited colonization efficiency of infection threads, when compared to most legume nodules that host rhizobia as organelle-like structures (known as symbiosomes). Rhizobia in fixation threads may also have less efficient exchange of nutrients when compared to legume symbiosomes. Further, *Parasponia* nodules contain only a single –centrally positioned- vascular bundle, whereas legume nodules have several peripheral vascular bundles, which may limit its transport capacity. Taken-together, it suggests that *Parasponia* root nodules are more primitive when compared to nodules formed on legume plants (Behm *et al.*, 2014).

Rhizobium LCOs are perceived by specific LysM-type receptor kinases. Studies in legumes and *Parasponia* indicated that –at least for one receptor- the same gene has been cooped to commit such symbiotic function (den Camp *et al.*, 2011)These receptors activate a highly conserved symbiotic signaling network that stretches from an additional LRR-type receptor down to transcriptional regulators. This symbiotic signaling pathway is highly conserved, and present in most plant species as it is essential also for endomyocrrhizal symbiosis (Roberts *et al.*, 2013). The importance of this symbiotic signaling network for root nodule formation is underlined by ectopic expression studies and the use of dominant alleles that trigger spontaneous root nodule formation in legumes and *Parasponia* (den Camp *et al.*, 2011). However, it remains elusive whether adaptations have occurred in any of symbiotic genes that is essential for the nodulation trait in legumes and/or *Parasponia*.

Despite of extensive research especially in model legumes, the essential genetic adaptations that allow plants to form nitrogen fixing root nodules have not yet been characterized. It may be because of the exclusive focus on symbiotic plants (e.g. legumes or *Parapsonia*) to dissect the genetics underlying rhizobium symbiosis. Rarely comparative studies with close relatives that are unable to form nitrogen fixing root nodules are committed.

We advocate the adaptation of *Trema tomentosa* as comparative plant system for *Parasponia*. *Parasponia* and *Trema* are closely related sister genera and recent molecular phylogenetic studies even suggest that the *Parasponia*-*Trema* lineage is paraphyletic (Akkermans *et al.*, 1978; Yang *et al.*, 2013). This suggests that *Parasponia* –including its symbiotic trait- is relatively young and evolved from an ancestral *Trema sp.* In contrast to *Parasponia* that is native to volcanic regions in the Malay Archipelago, *Trema* sp. are pantropical. In total 15 *Trema* species are recognized, however the taxonomy of this genus has only partially been resolved. We selected *T. tomentosa*, a species native to northern Australia, as workable model. *T. tomentosa* trees flower within 6-9 months under greenhouse conditions and can be propagated vegetatively *in vitro*. Additionally, it was shown that *T. tomentosa* harbouring transgenic roots can be obtained using *Agrobacterium rhizogenes*-mediated transformation (Cao *et al.*, 2012). By using this method to introduce the Cameleon calcium sensor, we could confirm that *T. tomentosa* doesn't show symbiotic responses in form of regular Ca^{2+} oscillation upon application of rhizobium LCOs, whereas this response is triggered in *Parasponia* (Granqvist *et al.*, 2015).

Although commonly used in legume and *Parasponia* research, *A. rhizogenes* mediated root transformation has several disadvantages. First, the generated roots represent primary transformed tissue that cannot be characterized in terms of position and number of T-DNAs integrated. Such roots therefore may vary in expression level of the transgene. By default, compound plants carrying transgenic roots can only be used transiently, in other words, experiments have to be conducted with roots of independent transformation events. An additional flaw of *A. rhizogenes* is the transfer of the *root inducing locus* (*rol*) genes. These genes are essential to trigger root formation in the plant, but they interfer with the plant root's hormone balance. As a result, *A. rhizogenes* transformed roots may differ in phenotype, and therefore such roots are not suitable for studying plant hormones. In the case of *Parasponia* and *Trema* we showed that transformation with *A. rhizogenes* had also technical difficulties for transformation efficiencies (Cao *et al.*, 2012) and overgrowth of tissue by this pathogenic bacterium. This let us to decide to investigate whether *Parasponia* and/or *Trema* can be transformed with *A. tumefaciens* to generate stable transgenic lines.

Here we present a protocol for *A. tumefaciens* mediated transformation of *T. tomentosa.* By using a binary vector containing the auxin responsive reporter DR5::GUS and two selectable markers; *NPTII* encoding kanamycin resistance and the RED FLUORSCENT PROTEIN encoding gene *DsRED*. We demonstrate that transgenic *T. tomentosa* plantlets could be generated within 6 month. These plantlets can be vegetatively propagated without losing transgene expression.

Results

Agrobacterium tumefaciens mediated transformation of Trema tomentosa

For *T. tomentosa* an *in vitro* propagation protocol has been established (Cao *et al.*, 2012). In this protocol shoot axillary buds are used as starting material. We tested whether we can use this material as starting material for *A. tumefaciens* mediated transformation. To do so, we made use of the *A. tumefaciens* strain AGL1 harbouring the binary plasmid, pT14DR5. This plasmid contains the auxin responsive synthetic promoter DR5 driving *GUS.* Additionally, it contains three selectable markers; namely *NPTII* that convers resistance to kanamycin, *DsREDI* driven by the constitutive *AtUBQ10* promoter of *Arabidopsis thaliana* and spectinomycin adenyltransferase gene *(AADA)* for bacteria selection in the backbone (Figure 1).

Axillary buds were either sliced in segments or used as a whole. All explants were immersed in an *A. tumefaciens* culture (O.D.600=0.6) for 30 min and placed on WPM culture medium supplemented with 1 ml acetosyringone (20 µg/ml) and incubated in the dark for 3 days at 21 °C for co-cultivation. Next, explants were transferred to regeneration medium containing cefotaxime (300 mg/litr) and kanamycin (50mg/1lite) and transferred to 28°C and a 16/8 h day/night regime.
To determine which auxin/cytokinin dose is most effective for inducing regeneration we tested 3 different combinations of 6-benzyladenine (BAP) and indole-3-butyric acid **(**IBA) (table 1). All three hormone combinations induced callus formation within four weeks after transfer. In all cases, white callus initially developed at the cutting sides of the explants (Figure 2a). However, some calli in both samples (split and whole meristems) turned brownish and obtained a more thick appearance and became overgrown with *Agrobacterium* (Figure 2b, c). Segmented apical buds showed more efficient callus formation when compared to whole buds (table 1). Also the calli formed on whole buds all turned brown within 4 weeks. These calli stopped growing and could not be investigated further.

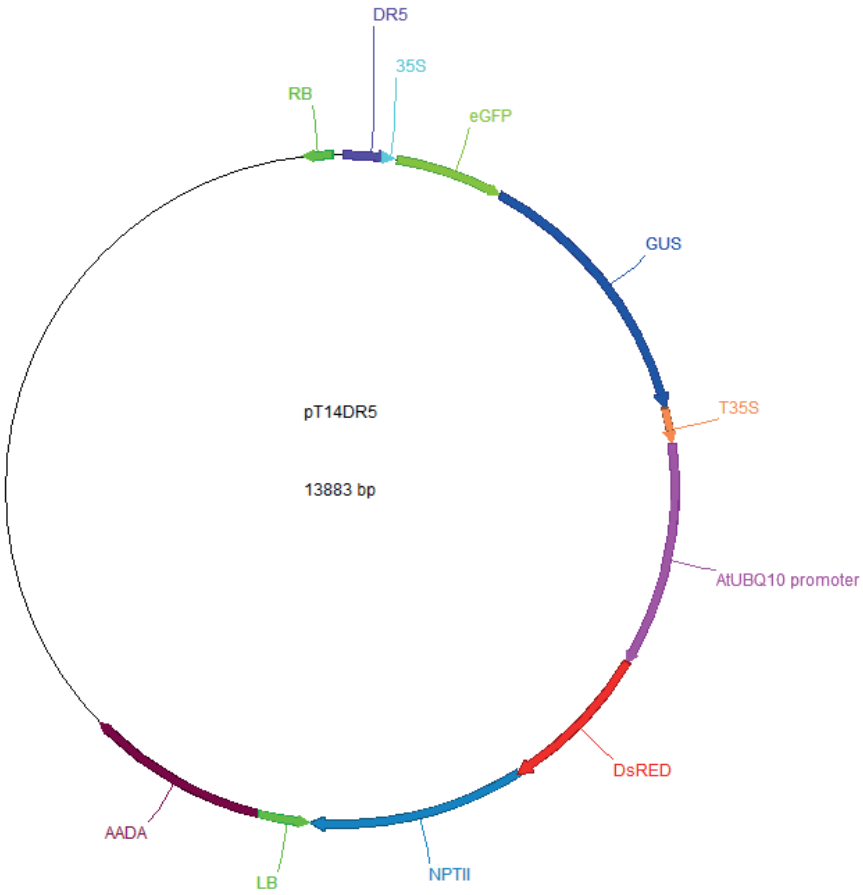

Figure 1. Map of vector pT14DR5containing a DR5::β-glucuronidase (GUS) reporter construct and three selectable markers; DsRED and neomycin phosphotransferase II gene (NPTII) for plant selection in between Left and Right T-DNA borders and spectinomycin adenyltransferase gene (AADA) for bacteria selection in the backbone.

Using segmented axillary buds as explants callus formation was observed on all three media, with comparable efficiencies (Table 1). However, on medium with 1.0 mg/l BAP and 0.1 mg/l IBA (M2) most calli maintained a greenish appearance (Figure 2d). We checked whether green calli were transgenic by monitoring red fluorescence. We noted that only on M3 medium (0.5

mg/l BAP, 0.05 mg/l IBA) *DsRED* expressing calli were found (5 out of 15) (Figure 2e and Table 2).

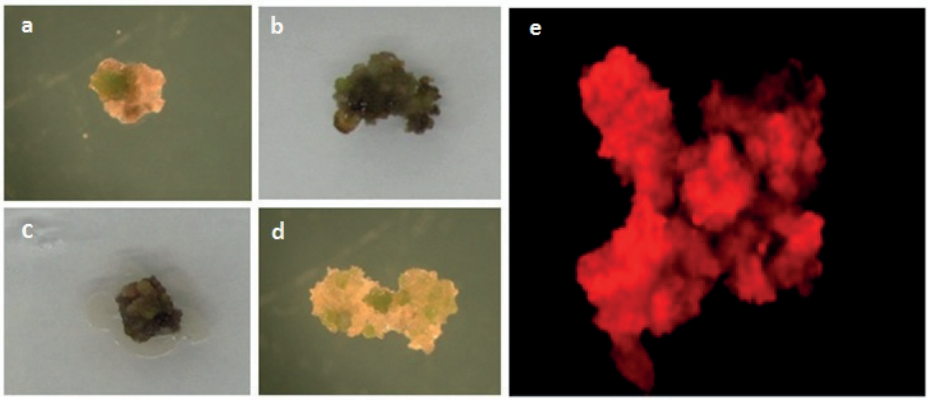

Figure 2. Callus induction from apical meristem culture of *Trema tomentosa*. a) Callus initiation from apical meristems cultured on WPM medium along with different combination of BAP and IBA b) Thick-brown tissues outgrowth from explants. c) Infected explants before developing callus. d) Green callus emerged after 4 weeks. e) Red florescent transgenic callus.

Table1. Effect of various media on callus induction from apical meristems derived from explants of *Trema. tomentosa*. Numbers indicate the total number.

Medium	Source of the callus							
	Spilt apical meristem				Full apical meristem			
	callus	BC	GC	TC	callus	BC	GC	TC
M1	20	15	5	0	5	5	0	0
M2	40	15	25	0	15	15	0	0
M3	30	15	15	3	0	0	0	0

Subculture media: M1: WPM2.5 mg/l BAP+0.25 mg/l IBA; M2: WPM+1.0 mg/l BAP+0.1mg/l IBA; M3: WPM+0.5 mg/l BAP+0.05mg/l IBA. BC: Brown Callus, GC: Green Callus, TC: Transgenic Callus.

Green calli (transgenic (M3 medium) and non-transgenic (M1 & M2 media)) were split in 4-5 pieces and divided over the same three hormone combinations as used for callus formation (M1, M2 & M3). Highest shoot regeneration efficiency occurred on M3 medium. On this medium 6 transgenic shoots were derived, originating from 5 primary calli (Figure 3c, Table 2). Also calli cultured on M2 medium formed shoots, although at lower efficiency, whereas on M1 medium no shoots could be regenerated (Figure 3, Table 2). All shoots emerged from calli cultured on M3 medium were confirmed to be transgenic based on DsRED1 mediated red fluorescence (Figure

4a, b). These shoots were isolated and transferred to rooting medium to induce root formation as described with Cao et al (Figure 4c) (Cao *et al.*, 2012). All roots were checked for red fluorescence. Four transgenic plants that originated from four different calli were transferred to the greenhouse.

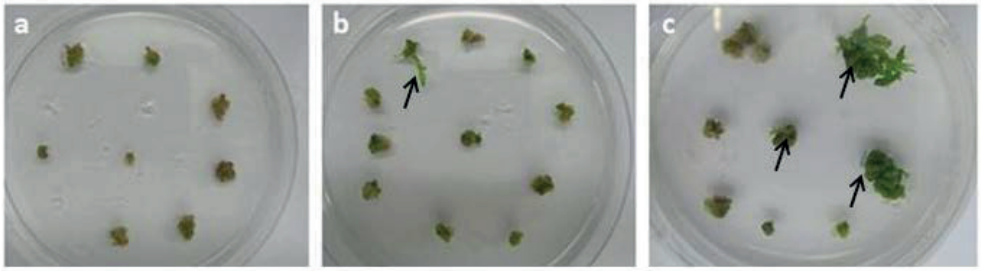

Figure 3. Shoot regeneration from callus in different shoot induction media. a) No shoot regenerate in M1 medium. b) M2 developed two shoots from two calli (in two different plates). c) Five calli on M3 medium form shoots (in three independent plates). The arrows indicate the shoots regenerated from callus.

Table 2. Effect of various media on Shoot and root regeneration from callus in *Trema tomentosa*.

Medium	Number of the callus with shoot	Number of the shoots	number of rooted shoot
M1	0	0	0
M2	2/30	2	2
M3	5/15	6	4

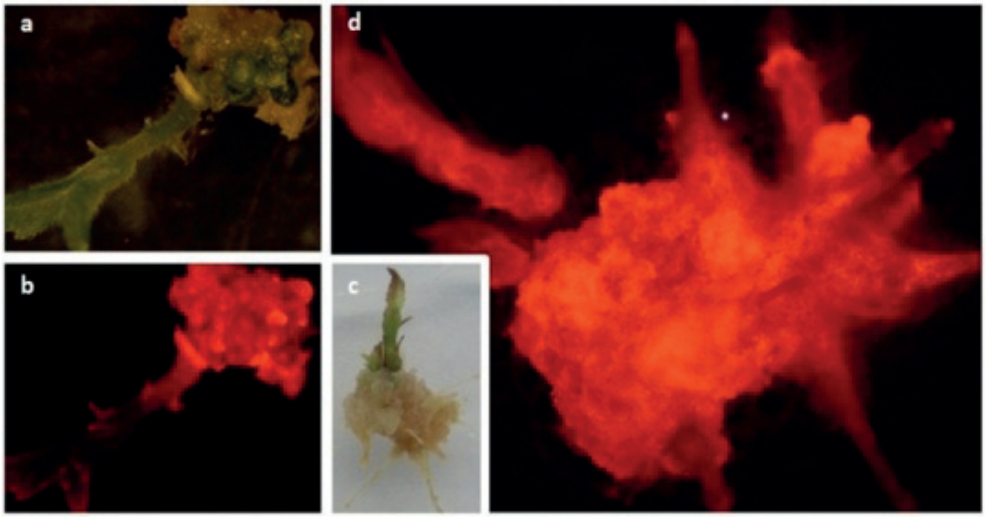

Figure 4. *Trema tomentosa* shoot and root regeneration from calli that originate from shoot apical meristem. a) Shoot regeneration from apical meristems cultured on WPM+ BAP and IBA for 8 weeks. b) Transgenic shoots can be distinguished based on *DsRED1* expression causing red fluorescence. c) Root formation of shoot, after 6 weeks cultivation. d) Red florescence in transgenic roots.

The auxin reporter DR5 is active in meristematic tissue of T. tomentosa

To check for transgene expression in the four selected transgenic plants we conducted a GUS assay on lateral roots and young leaves. In two out of four plants pattern of blue staining could be observed, with strong signal in the root apical meristem and at the rim of a developing leaf (Figure 5). These M0 plants were maintained in the greenhouse for seed production. DR5::GUS expression was investigated in more detail in transgenic M1 seedlings (Figure 6). Whole mount staining of 30 day old seedlings revealed a strong GUS signal in leaf vasculature, leaf rim, shoot apical meristem, and developing lateral roots (Figure 6). These sites, coincide with tissues known to accumulate high concentrations of auxin (Mattsson *et al.*, 2003; Kerk *et al.*, 2000).

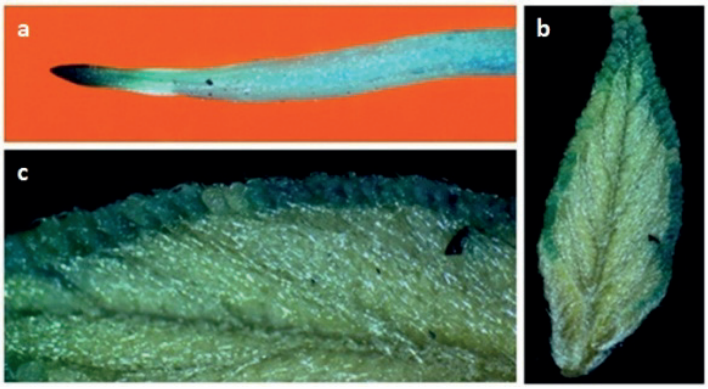

Figure 5. Bright field microscopy of the synthetic auxin-responsive reporter (DR5-GUS) expression in the root (a) and leaf (b, c) of transgenic *Trema tomentosa* resulting in extensive blue staining.

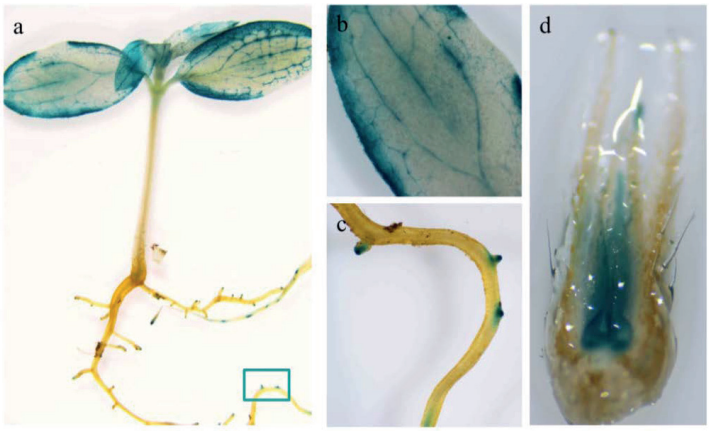

Figure 6. Bright field microscopy of the synthetic auxin-responsive reporter (DR5-GUS) expression in *Trema tomentosa* seedling (a-c) and apical meristem (d).

Auxin accumulates in Trema during lateral root development

For several plant species it is reported that lateral root initiation correlates with auxin accumulation (Casimiro *et al.*, 2001). Since lateral root primordia generally occur in a regular pattern we made use of this phenomenon and examined auxin response during lateral root formation in *T. tomentosa* using a DR5::GUS line. First auxin was increased in inner cortical cells and in the underlying pericycle cell layer, even before the observation of cell divisions

(Figure 7a). After induction of pericycle divisions the blue staining increased in these cells (Figure 7b), which will give rise to the lateral root primordium formation (Figure 7c-e). Interestingly, the DR5 signal in the associated cortex cell layers remained and extended to the epidermis (Figure 7e), even though none of these cells divided during lateral root development.

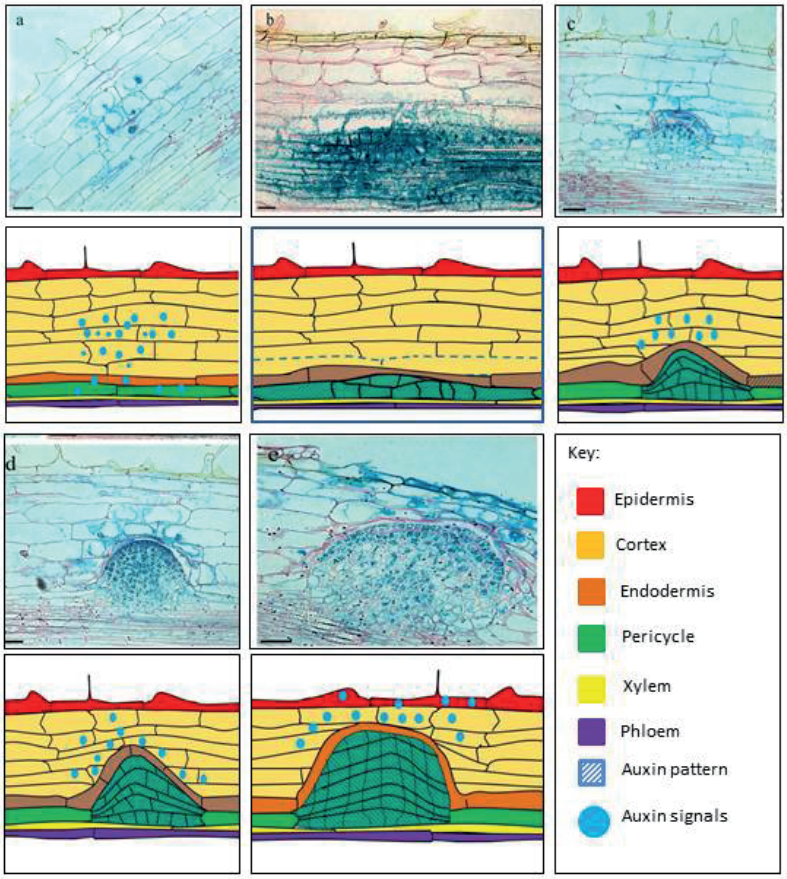

Figure7. Distribution of auxin during *Trema tomentosa* lateral root primordia development through spatial pattern of DR5::GUS expression during lateral root primordium initiation. (a) The DR5 activity gradient with the maximum at the primordium tip (inner cortical cell) is gradually established. (b) Cell division in pericycle and auxin accumulates in dividing cells (c-e) Auxin maximum in lateral root tip along with cell division. DR5 signals are visible in cortical and epidermal cell (d). Scale bars are: 25 µm.

Discussion

Here we present an efficient and relatively fast method to obtain stably transformed plant lines of *T. tomentosa*. By using lateral leaf buds as starting material and an optimized single medium for callus and shoot formation, transgenic plants can be obtained within 6 months. As *T. tomentosa* can be propagated vegetatively *in vitro*, T0 plants can be effectively cloned and used for experimentation. *Trema* sp. is the closest relative of *Parasponia* and is unable to form rhizobium nitrogen fixing root nodules. Therefore the presented transformation protocol of *T. tomentosa* will be a new and effective tool in comparative studies to underpin the genetics of the rhizobium symbiosis.

T. tomentosa is a tropical tree species that grows best at temperatures of > 28°C. However, in previous study we showed that T-DNA transfer of *A. rhizogenes* to *T. tomentosa* is most efficient at 21ºC (Cao *et al.*, 2012). In line with that we conducted co-cultivation at 21°C for 4 days, a temperature suboptimal for *T. tomentosa* growth. By doing so, transformation efficiencies up to 15% were achieved, which is sufficiently efficient to conduct experiments at larger scale.

Stable transformation offers four essential advantages over *A. rhizogenes* mediated root transformation. (I.) Transgenic lines can propagated, whereas this is not possible for compound plants carrying transgenic roots. In case of tree species however, generative propagation may be time consuming, or even impossible. However, in case of *T. tomentosa* this is not a problem, as this species flowers within 6 months and is self-compatible. Additionally, T0 plants can be vegetatively propagated. This further reduces the timespan between initial generation of transgenic plantlets and actual characterization of the transgenic line and subsequent phenotyping. (II.) An additional advantage of *A. tumefaciens* over *A. rhizogenes* is the absence of the transfer of the bacterial *rol* genes. This will allow conducting more reliably physiological studies. (III.) Also, stably transformed lines are suited for studies on shoot-root communication, which are impossible with chimeric compound plants that consist of a non-transgenic shoot carrying transgenic roots. In context of the rhizobium-symbiosis this is relevant as several studies indicated that shoot derived factors control root nodule formation (Mortier *et al.*, 2010; Soyano *et al.*, 2014). (IV.) A last advantage of stable transformation over *A. rhizogenes* is the possibility of re-transformation of an already transgenic line. In such case a different selection marker has to be used.

We introduced the auxin responsive reporter construct DR5::GUS into *T. tomentosa*. Using this reporter we detected transgene expression in meristematic regions in young developing leaves of primary transformed T0 plants. As the expression of the reporter is not affected upon vegetative propagation, it suggests that this *in vitro* propagation protocol is a fast way of propagating transgenic plants.

Root nodule organogenesis is associated with formation of a local auxin maximum in the plant root. By studying lateral root formation we showed that DR5::GUS can be used as auxin reporter also in *T. tomentosa.* Sectioning of young lateral root primordia revealed that DR5 activity is found not only in dividing pericycle cells that give birth to the primordium, but also in associated cortical cells that do not undergo cell divisions. This DR5 activity can be found even in the epidermal cells when the lateral roots start to emerge. This pattern is consistent with patterns reported in other species (Benková *et al.*, 2003). In line with this, a *T. tomentosa* DR5::GUS line can be used as a tool to study Rhizobium LCO signaling in non-symbiotic species.

In conclusion, the presented protocol for *T. tomentosa* stable transformation can be an effective replacement of the transient *A. rhizogenes* transformation protocol. Stably transformed lines will be an essential tool to forward engineer rhizobium symbiosis.

Materials and methods

Construction of DR5::GUS fusion construct

pENTR™/D-TOPO Cloning Kits (Invitrogen) and Gateway technology (Invitrogen) were used to generate the entry clone and genetic promoter-GUS construct (Karimi *et al.*, 2002) of *DR5::GUS* construct, respectively. First, 14 synthetic DR5 DNA fragment repeats (Ulmasov *et al.*, 1997) were inserted in the entry clone. Then, the entry vector was recombined into Gateway-compatible binary vector pKGW-RR, that contains GUS reporter and *AtUBQ10::DsRED1* as a selection marker (Limpens *et al.*, 2004), by using Gateway LR Clonase II enzyme mix (Invitrogen). Final construct was named pT14DR5.

Plant transformation

Apical meristems of *Trema tomentosa* plants were collected from the trees growing in the greenhouse and explants were divided in two groups. In one set apical meristems cut from the

middle (parallel to growth axis) and the other, full meristems were used for stable transformation. Before cutting, all meristems surface sterilized in 4% hypochlorite (commercial bleach) for 10 min and subsequently washed 6 times with sterile MQ water. *Agrobacterium tumefaciens* strain *AGL1* carrying the *DR5::GUS* fusion constructs was used for transformations.

A. tumefaciens AGL1 cultures harbouring construct pT14DR5 were grown overnight at 28°C on agar-solidified LB medium. The next day, *A. tumefaciens* was scraped from plate and resuspended in infiltration medium (McCown Woody Plant Medium (WPM) (Lloyd and McCown, 1980); Duchefa Biochemie, Haarlem, The Netherlands), 2.5 mg/L 6-benzylaminopurine (BAP), 0.25 mg/L indole-3-butyric acid (IBA), 20 mg/L acetosyringone, 0.02% silvet L-77 (v/v)) at OD_{600} = 0.6. Sterilized *T. tomentosa* axillary buds were immersed in this medium for 30 minutes. Thereafter, they were transferred to co-cultivation medium (WPM, 2.5 mg/L 6-benzylaminopurine (BAP), 0.25 mg/L indole-3-butyric acid (IBA), 20 mg/L acetosyringone, 0.8% Daichin agar (Duchefa Biochemie, Haarlem, The Netherlands) in Ø 10 cm round petri dishes. Plates were incubated in darkness at 28°C for three days. After three days, explants were transferred to selective media containing cefotaxime (300mg/L) and kanamycin (50mg/L). Explants were cultured on three different selective media to determine optimal hormone concentrations for tissue regeneration. Composition of these three media is listed below. Explants were incubated at 28°C under a 16/8 hour light/dark regime until regeneration was observed.

Hormonal treatments

Three different hormone combinations were performed to check the efficiency of callus, shoot and root regeneration.

M1: WPM + (2.5 mg/l) BAP + (0.25 mg/l) IBA

M2: WPM + (1.0 mg/l) BAP + (0.1mg/l) IBA

M3: WPM + (0.5 mg/l) BAP + (0.05mg/l) IBA

Regeneration of the plants

All emerged callus from explants transferred to the new fresh regeneration medium and kept at 28ºC. Within 8 weeks shoots emerged from the axillaries buds callus. Shoots of >1.0 cm in length were cut and transferred to rooting medium (20 g/L sucrose, 2.4 g/L WPM, pH: 5.8) in

round Ø10 cm Petri dish. These plates were covered by aluminium foil (darkness induced roots) and kept in the same growth cabinet at 28°C for four weeks. Rooted shoots were kept on hormone free medium (pH 5.8, 20 g/L sucrose, 2.4 g/L WPM, 0.8% Daichin agar) for three weeks before transferring to the pots in greenhouse.

Microscopy and Histology analysis

Transgenic roots and shoots were selected based on red fluorescence, using Leica MZIII fluorescence stereomicroscope (filter settings; excitation 565/30 and emission 620/60). The number of transformed roots was determined based on DsRed1 fluorescence.

For GUS activity, fresh lateral roots, young leaves and seedlings generated from the F1 seeds of *Trema tomentosa* plants were collected from greenhouse. All materials were immersed in GUS staining solution including 25 mM sodium phosphate buffer (pH 7.0), 2 mM 5-bromo-4-choro-3-indolylb- D-glucuronide cyclohexylamine salt (X-gluc), 0.5 mM ferricyanide, 0.5 mM ferrocyanide and 10 mM EDTA, vacuumed for 30 minutes and then incubated at 37 °C for overnight. For bleaching the tissues, the GUS staining solution was rinsed with 20, 50 and 70% ethanol respectively for 10 minutes. Stained tissue was checked with Leica MZIII fluorescence stereomicroscope for GUS expression. Fixation of material including roots and apical meristems was performed for 24 h at 4 ºC in 5% glutaraldehyde (v/v) and 3% sucrose (w/v) dissolved in phosphate buffer (pH 7.0). Subsequently an ethanol dehydration series was carried out.

Root Microsectioning

Transgenic roots were fixed in phosphate buffer solution (PBS) with 0.25% glutaraldehyde included. Vacuum was applied for 1-2 h until tissues sat on the bottom. The tissues were incubated at 4°C for overnight. After two times washing with PBS, dehydration steps were performed with 10%, 30%, 50%, 70%, 90% and 100% ETOH respectively for 10 min at room temperature for each step. Plastic infiltration was done in four steps, which included solution A (100ml Technovit7100, 1pack HrdnerI, 2.5ml PEG400):100% ETOH in 1:3, 1:1, 3:1 ratio respectively for 30-60 min in room temperature and finally treated with 100% solution A for overnight at 4°C. All materials were transferred into cupules and nodules located in the middle. Solution A was removed and polymerization solution (15ml Solution A, 1ml Hardener II) was added immediately. To remove air from the samples, cupules were covered with parafilm and

left for overnight at room temperature. After polymerization, holders were put on the blocks and holding solution (technovit3040: 2part powder, 1 part liquid) was added from the hole located in the centre of holder and kept for 15 min at room temperature. Finally sectioning of root segments were performed using a microtome machine and the samples were analysed by microscopy (Leica) after staining with toluidine blue (0.5%) buffer and washing with tap water for 5 minutes.

References

Akkermans, A., et al. (1978). "N2-fixing root nodules in Ulmaceae: Parasponia or (and) Trema spp.?" Plant and Soil **49**(3): 711-715.

Behm, J. E., et al. (2014). "Parasponia: a novel system for studying mutualism stability." Trends in Plant Science **19**(12): 757-763.

Benková, E., et al. (2003). "Local, efflux-dependent auxin gradients as a common module for plant organ formation." Cell **115**(5): 591-602.

Cao, Q., et al. (2012). "Efficiency of Agrobacterium rhizogenes–mediated root transformation of Parasponia and Trema is temperature dependent." Plant Growth Regulation **68**(3): 459-465.

Casimiro, I., et al. (2001). "Auxin transport promotes Arabidopsis lateral root initiation." The Plant cell **13**(4): 843-852.

Den Camp, R. O., et al. (2011). "LysM-type mycorrhizal receptor recruited for rhizobium symbiosis in nonlegume Parasponia." Science **331**(6019): 909-912.

Granqvist, E., et al. (2015). "Bacterial-induced calcium oscillations are common to nitrogen-fixing associations of nodulating legumes and non-legumes." New Phytologist **207**(3): 551-558.

Karimi, M., et al. (2002). "GATEWAY™ vectors for< i> Agrobacterium</i>-mediated plant transformation." Trends in Plant Science **7**(5): 193-195.

Kerk, N. M., et al. (2000). "Auxin metabolism in the root apical meristem." Plant Physiology **122**(3): 925-932.

Limpens, E., et al. (2004). "RNA interference in Agrobacterium rhizogenes-transformed roots of Arabidopsis and Medicago truncatula." Journal of Experimental Botany **55**(399): 983-992.

Mattsson, J., et al. (2003). "Auxin signaling in Arabidopsis leaf vascular development." Plant Physiology **131**(3): 1327-1339.

Mortier, V., et al. (2010). "CLE peptides control Medicago truncatula nodulation locally and systemically." Plant Physiology **153**(1): 222-237.

Op den Camp, R. H., et al. (2012). "Nonlegume Parasponia andersonii deploys a broad rhizobium host range strategy resulting in largely variable symbiotic effectiveness." Molecular Plant-Microbe Interactions **25**(7): 954-963.

Roberts, N. J., et al. (2013). "Rhizobial and mycorrhizal symbioses in Lotus japonicus require lectin nucleotide phosphohydrolase, which acts upstream of calcium signaling." Plant Physiology **161**(1): 556-567.

Soyano, T., et al. (2014). "NODULE INCEPTION creates a long-distance negative feedback loop involved in homeostatic regulation of nodule organ production." Proceedings of the National Academy of Sciences **111**(40): 14607-14612.

Ulmasov, T., et al. (1997). "Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements." The Plant Cell Online **9**(11): 1963-1971.

Wang, H., et al. (2009). "Rosid radiation and the rapid rise of angiosperm-dominated forests." Proceedings of the National Academy of Sciences **106**(10): 3853-3858.

Yang, M.-Q., et al. (2013). "Molecular phylogenetics and character evolution of Cannabaceae." Taxon **62**(3): 473-485.

Chapter4

Exogenous Nitrate Interferes with the Switch from Infection to Fixation Thread Formation in *Parasponia andersonii* Root Nodules

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Abstract

Parasponia trees are the only non-legume plant species that can form nitrogen fixing nodules with rhizobium bacteria. In legumes, rhizobium-induced nodule formation is tightly controlled and inhibited by exogenous fixed nitrogen. Species in the *Parasponia* lineage gained the rhizobium symbiosis trait more recent when compared to legumes. We questioned whether similar to legumes also *Parasponia* acquired mechanisms to control its symbiotic interaction in presence of exogenous fixed nitrogen. To get insight in the mechanism controlling nitrogen fixing symbiosis in presence of exogenous nitrogen, we studied nodule formation and intracellular rhizobium infection in *Parasponia andersonii* supplied with doses of KNO_3 ranging from 0.2 to 50 mM. We found that nodule primordium formation and intracellular infection have different sensitivities to nitrates. *P. andersonii* formed nodules at all tested nitrate concentrations, whereas progression of intracellular infection was impaired at 20 mM. Specifically, the switch from infection to fixation threads was affected, a process known to be controlled by the LysM-type receptor PaNFP. However, using *PaNFP* knockdown mutants and LCO responsive Ca^{2+} oscillation experiments indicate that exogenous nitrate doesn't affect LCO signaling. We conclude that *P. andersonii* employs a novel mechanism to control intracellular rhizobium colonization in presence of exogenous nitrates.

Introduction

Fixed nitrogen is an essential nutrient for plant growth, but in most ecosystems its availability is limited. Some plants species -like most legumes (*Fabaceae*) and species of the genus *Parasponia*- have overcome this problem by establishing a root nodule symbiosis with a diverse range of nitrogen fixing soil bacteria that are collectively known as rhizobia (Willems, 2006). *Parasponia* species and legumes obtained their symbiotic traits by convergent evolution at different moments in time. The nitrogen-fixing symbiosis trait in legumes is estimated to be ~60 million years old (Lavin *et al.*, 2005; Sprent, 2008; Doyle, 2011). In case of *Parasponia* the evolutionary origin of the nitrogen-fixing rhizobium symbiosis is not dated, however, two lines of evidence strongly suggests that the symbiosis trait in this lineage is relatively young when compared to legumes. First, the *Parasponia* genus is nested within -and only recently diverged from- the genus *Trema* (Yang *et al.*, 2013; Werner *et al.*, 2014). Second, the *Parasponia*-rhizobium symbiosis is far less sophisticated when compared to the legume-rhizobium symbiosis (Behm *et al.*, 2014). In case of legumes the relative long period of evolution has shaped the symbiosis trait in such a way that it has become highly efficient and robust. Legumes have gained mechanisms that link the N-status of the plant to its susceptibility for rhizobium. For example, in most legume species nodule formation is inhibited by moderate concentrations of exogenous fixed-nitrogen sources (Streeter, 1988). Such mechanism enables legume plants to exploit exogenous nitrogen sources, rather than to maintain an energetically costly symbiotic relation with rhizobium. As the *Parasponia*-rhizobium symbiosis evolved more recent and independent from legumes, we questioned to what extend similar mechanisms do occur in these species.

The negative effects of soil nitrates on symbiotic nitrogen fixation in legumes are known for a long time (Streeter, 1988). In model legumes *Lotus japonicus* and *Medicago truncatula* root nodule formation is fully inhibited by 5-10 mM exogenous fixed nitrogen (Sagan *et al.*, 1995; Barbulova *et al.*, 2007). Effects of exogenous nitrates can be categorized in three types of responses that differ in sensitivity; (i) reduced infection rates, (ii) decreased nitrogen fixation rates and (iii) decreased nodule mass or even total absence of nodules. Of these, infection seems to be least sensitive to exogenous nitrates (Streeter, 1988).

In legumes, bacterial infection and nodule primordium formation are tightly controlled by rhizobium induced lipo-chitoolichosaccharide (LCO) signaling. These rhizobial signals are perceived by a specific heterodimeric complex of two types of LysM domain containing receptor kinases, named LjNFR1/MtLYK3 and LjNFR5/MtNFP in *L. japonicus* and *M. truncatula* (Broghammer *et al.*, 2012; Moling *et al.*, 2014). These receptors subsequently trigger a plethora of symbiotic responses, ranging from nuclear Ca^{2+} oscillation, symbiotic gene expression, changes in the cytokinin and auxin homeostasis, root hair growth responses, and nodule primordium formation (Oldroyd, 2013; Miri *et al.*, 2016). Studies in different legume species showed that read-out of LCO signaling at least in part is affected by exogenous fixed nitrogen. For example, in *Vicia sativa* it was shown that LCO induced root hair growth responses are affected (Heidstra *et al.*, 1994), whereas studies in *M. truncatula* and *L. japonicus* revealed a strong inhibition of *NIN* expression; a key regulatory gene of bacterial infection and root nodule formation (Schauser *et al.*, 1999; Barbulova *et al.*, 2007; Marsh *et al.*, 2007).

In *Parasponia* species rhizobium bacteria enter the roots via crack entry. First upon reaching the dividing cells that form the nodule primordium, intracellular infection is achieved (Lancelle and Torrey, 1984). These infection threads have a similar appearance as in legumes. However, in contrast to most legumes the bacteria are not released as symbiosomes in the cytoplasm, but remain bound in so-called fixation threads. Fixation threads distinct from infection threads by relatively thin cell walls and more loosely packed bacteria (Price *et al.*, 1984). Similar to legumes, *P.andersonii* nodule primordium formation is triggered upon perception of rhizobium LCOs (Marvel *et al.*, 1987; Op den Camp *et al.*, 2011). The putative ortholog of LjNFR5/MtNFP in *P.andersonii* –PaNFP- was found to fulfil a symbiotic function. Knockdown of *PaNFP* by RNAi resulted in reduced nodulation efficiency, whereas the few nodules formed on *PaNFP* RNAi roots are defective in fixation thread formation (Op den Camp *et al.*, 2011). Taken together, the symbiotic functioning of LjNFR5/MtNFP orthologous genes in legumes as well in *P.andersonii* suggests that genetic constraints have guided evolution of rhizobium symbiosis in both lineages (Streng *et al.*, 2011; Geurts *et al.*, 2012).

It remains elusive whether *P.andersonii* gained additional mechanisms similar to control the interaction with rhizobium. Putting forward the relative short period of time in evolution when compared to legumes, we questioned to what extend *P.andersonii* gained a mechanism to control its symbiotic nitrogen fixing partner in presence of exogenous nitrates. By studying root nodule

formation at different doses of exogenous nitrate we found that *P. andersonii* employs a novel mechanism to control the living space of its endosymbiotic microbe by inhibiting formation of fixation threads.

Results

Dose-response relationship of P. andersonii nodulation efficiency and exogenous nitrate

In legumes, exogenous fixed-nitrogen acts on nodule initiation, infection and nitrogen fixation activity and the severity of the inhibition varies among species (Harper and Gibson, 1984; Streeter, 1988; Carroll and Mathews, 1990). To determine whether nodulation of *Parasponia* also is inhibited by nitrate, *P. andersonii* plantlets inoculated with *Bradyrhizobium elkanii* WUR3 were grown at 5 different KNO_3 concentrations (0.2, 1, 5, 10 and 20 mM). Plants were harvested at 8 weeks post inoculation. Highest shoot mass was obtained when the plant medium was supplemented with 20 mM KNO_3, whereas root biomass was highest when plants were grown at KNO_3 concentrations of 10 mM (Fig. 1A,B). This suggests that *P. andersonii* plants reduce their investment in root biomass at KNO_3 concentrations above 10 mM. Next, we determined the nodulation efficiencies of *P. andersonii* plants grown at the different doses of KNO_3. This showed that up to 10 mM nitrate, a positive effect on nodulation can be observed. Plants supplemented with this concentration had a 5 fold higher number of nodules when compared to plants that were grown with 0.2 mM KNO_3. At higher nitrate concentrations (20 mM KNO_3) nodulation efficiencies markedly drop (~90%) (Fig. 1C). However, at this concentration still up to 20 nodules per plant were formed, a nodulation efficiency comparable to plants grown on a hundred lower concentration (0.2 mM).

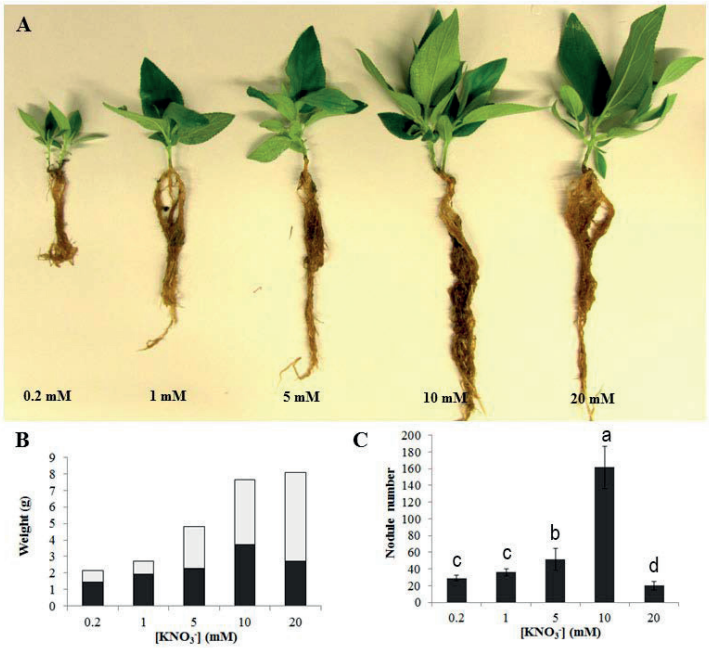

Figure 1. Effects of exogenous nitrate on *Parasponia andersonii* plants inoculated with *Bradyrhizobium elkanii* WUR3 and grown on different concentrations of KNO_3. (**A**) Plant phenotypes, and (**B**) shoot (white bar) and root (black bar) fresh weights 8 weeks post inoculation. (**C**) Average number of nodules per plant. (n=10, 8 weeks post inoculation).

To determine whether nodulation can be fully inhibited at higher concentrations of exogenously nitrate, we grew *P. andersonii* plants with 30 and 50 mM exogenous KNO_3. In such high doses of nitrate plant fresh weights marginally increased (Fig. S1B). Interestingly, the *P. andersonii* grown at 30 or 50 mM KNO_3 still formed a few root nodules, although less when compared to plants grown at lower nitrate concentrations (Fig. S1A). Taken together, it shows that *P. andersonii* plants can control root nodule initiation in response to high exogenous nitrate. However, *P. andersonii* is unable to fully block this developmental process that is triggered in response to rhizobia, even not if nitrogen seems no longer growth limiting factor.

Nitrate interferes with fixation thread formation

We noted that the nodule fresh weight negatively correlated with the exogenous nitrate concentration supplied to the plants (Fig. 2A). Therefore, we examined the effect of nitrate on

the nitrogen fixation capacity of nodules by measuring nitrogenase activity. This showed that nitrogenase activity decreased in a nitrate concentration-dependent manner. Nitrogenase activities decreased by 5 and 23-fold, respectively, in nodules isolated from plants supplied with 10 and 20 mM exogenous KNO_3^-, when compared to nodules of plants grown at 0.2 mM KNO_3^- (Fig. 2B). These results indicate that although *P. andersonii* is capable to form nodules in presence of a high concentration of exogenous nitrate, these nodules have lower nitrogen fixation efficiency.

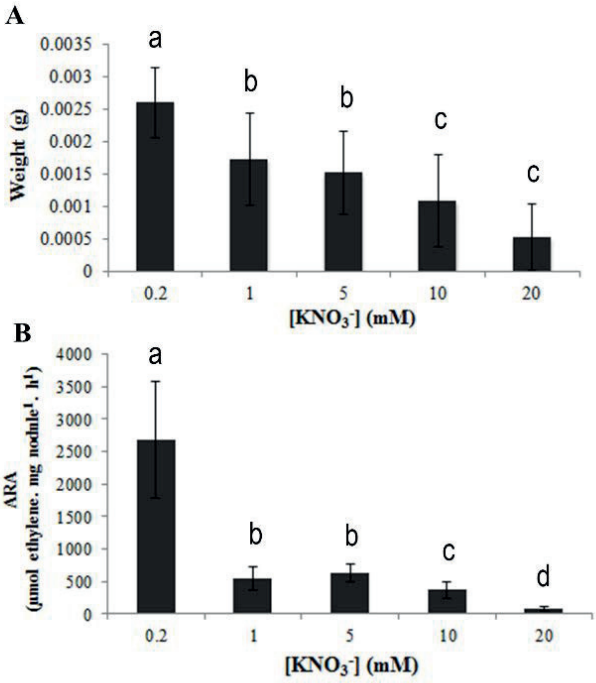

Figure 2. (A) Fresh weight of individual *Parasponia andersonii* nodules and (**B**) nitrogenase activity measured by acetylene reduction rates of nodules grown at different KNO_3 concentrations and inoculated with *Bradyrhizobium elkanii* WUR3. (n=10 plants / treatment).

We examined the nodule cyto-architecture to determine whether exogenous nitrate affects nodule development. Nodules grown on 0.2 mM and 20 mM were sectioned and studied by light and

transmission electron microscopy (TEM) (Fig. 3 and Fig. 4). The general anatomy of the nodules formed was similar irrespective of the exogenous nitrate concentration. As *P.andersonii* nodules have an apical meristem a gradient of developmental stages of infected cells could be distinguish along the longitudinal axis. Inward nodule cortical cells that surrounded the vascular bundle were infected, whereas outward nodule cortical cells remained uninfected (Fig. 3A). Cells directly adjacent to the meristem contained newly infected cells with infection threads (Fig. 3B). Using TEM it was noted that intracellular infection started from an apoplastic micro-colony that formed a cell wall bound infection thread (Fig. 4A). Such infection threads contained a single file of bacteria. Further penetration of these infection threads into the host cells coincided with fragmentation of the main vacuole (Fig. 4B). Infection threads eventually changed shape and became wider, now harbouring 2-3 files of bacteria (Fig. 4B, C). TEM studies revealed that at this stage the infection structures were largely deprived of a cell wall matrix; a hallmark for fixation threads (Compare Fig. 4A,B and Fig.4C) (Price et al., 1984). This change in infection morphology reflects the switch to the symbiotic stage.

Nodules formed in presence of 20 mM exogenous nitrate showed dramatic difference in infection (Compare Fig. 3A and 3D). Initial infection of nodule cortical cells was similar to nodules grown at 0.2 mM KNO_3. However, further development of intracellular infection threads towards fixation threads was seriously hampered (Fig. 3D-F, Fig 4D). Infected cells of nodules grown at 20 mM KNO_3 contained only infection threads, which remained relatively short and bounded in a dense cell wall (Fig. 3F, Fig 4E). Central vacuoles in these cells do not undergo fragmentation and remained intact (Fig. 4E).

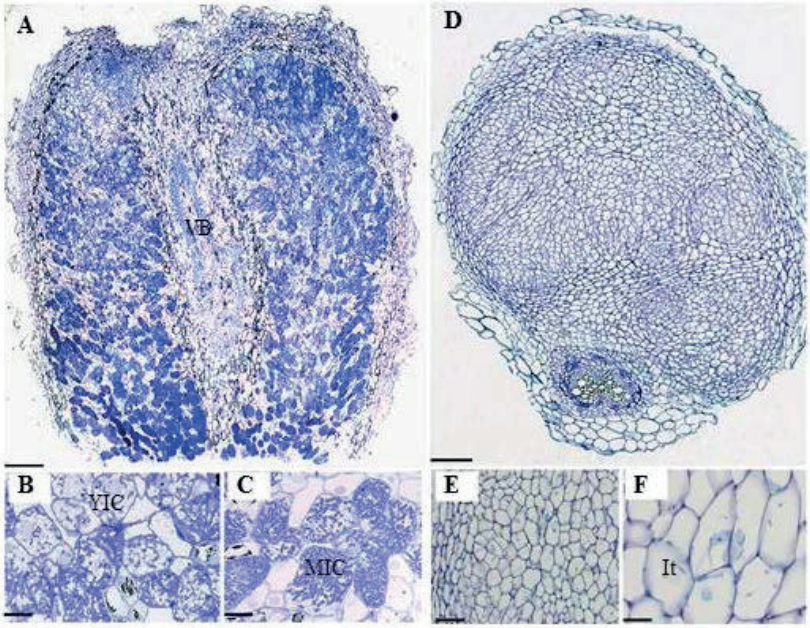

Figure 3. Sections of *Parasponia andersonii* nodules infected with *Bradyrhizobium elkanii* WUR3 and grown at 0.2 (**A-C**) or 20 mM (**D-F**) KNO_3. (**A**) Longitudinal section of a nodule formed at 0.2 mM KNO_3 with a central vascular bundle (VB). (**B**) Apical region of the nodule containing recently infected cells with infection threads and young infected cells (YIC). (**C**) Infected cells filled with fixation threads and mature infected cells (MIC). (**D**) Longitudinal section of a nodule formed at 20 mM KNO_3. (**E**) Apical region of the nodule containing recently infected cells with infection threads. (**F**) Cells in the central zone of the nodule containing only infection threads (It), but no fixation threads. Bars: A, D: 75 µm, B, C: 12, 5µm, E: 25µm F: 10µm.

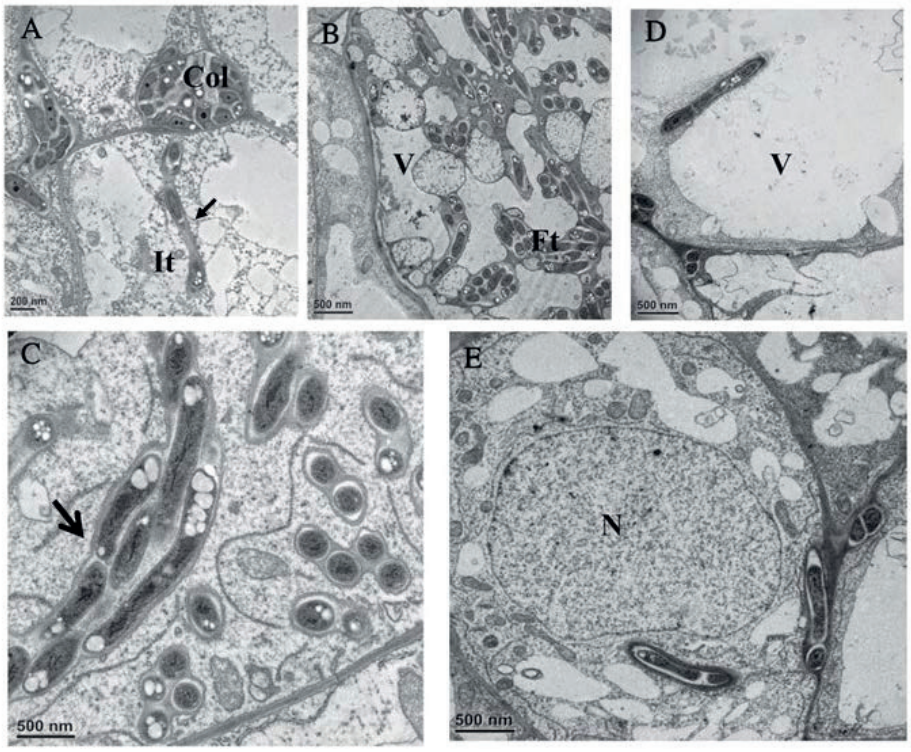

Figure 4. Transmission electron microscopy images of infected cells of *Parasponia andersonii* nodules infected with *Bradyrhizobium elkanii* WUR3 and grown at 0.2 (**A-C**) or 20 mM (**D, E**) KNO_3. (**A**) Infection thread with one file of rhizobia entering to the cell from the bacterial colony situated in apoplast. Note presence of cell wall of infection thread (arrow). (**B**) Cell containing infection threads with one file of bacteria, and fixation threads containing two files of bacteria. Note the vacuole fragmentation in cell. (**C**) Thin cell wall of fixation threads (marked by arrow). (**D, E**) The structure of nodules treated with 20 mM nitrate. (**D**) Infection threads entering a young nodule cell containing only small vacuoles. (**E**) Mature infected cell with developed vacuole containing only an infection thread. Col: bacteria colony in apoplast. It: infection thread, Ft: fixation thread, V: vacuole, N: nucleus.

Taken together, these data show that high fixed nitrogen does not prohibit nodule initiation and intracellular infection thread formation, but hampers formation of fixation threads. This block of intracellular infection coincides with occurrence of thick (and possibly rigid) infection thread cell walls, which may be the cause of infection suppression. This suggests that *P.andersonii* employs an active mechanism to control intracellular rhizobial colonization in case alternative sources of fixed nitrogen are available.

Exogenous nitrate doesn't interfere with LCO signaling in P. andersonii

In a previous study we showed that fixation thread formation in *P. andersonii* is controlled by the LysM-type receptor kinase PaNFP (Op den Camp *et al.*, 2011). PaNFP is orthologous to the rhizobium LCO receptors *LjNFR5*/*MtNFP* in *L. japonicus* and *M. truncatula*. In *M. truncatula* it was shown by nodule specific knock down that MtNFP controls the switch from infection thread growth to bacterial release (Moling *et al.*, 2014). As in legumes LCO signaling is sensitive to exogenous nitrate (Heidstra *et al.*, 1994, 1997; Barbulova *et al.*, 2007; Marsh *et al.*, 2007), we tested whether this is the case for *P.andersonii* as well. To investigate this, two experiments were conducted. First it was determined whether LCO-induced Ca^{2+}-oscillation response is affected by exogenous nitrate. To test this, *P. andersonii* plantlets carrying transgenic roots expressing the NupYC2.1 Ca^{2+} reporter were generated (Granqvist *et al.*, 2015). Compound plantlets carrying transgenic roots were grown on EKM-medium complemented with either 0.2 mM or 20 mM KNO_3. Subsequently, transgenic roots –selected based on green fluorescence- were treated with an LCO mixture of *Sinorhizobium fredii* NGR234 (~10^{-9} M). Ca^{2+}-oscillation could be observed in about 50% of the tested epidermal cells, irrespective of the nitrate concentration in the growth medium (Fig. 5). Likewise, the spiking frequency and amplitude is undistinguishable between both nitrate regimes. This suggests that inhibitory effect of exogenous nitrate acts independent - or downstream of- rhizobium LCO induced Ca^{2+} spiking.

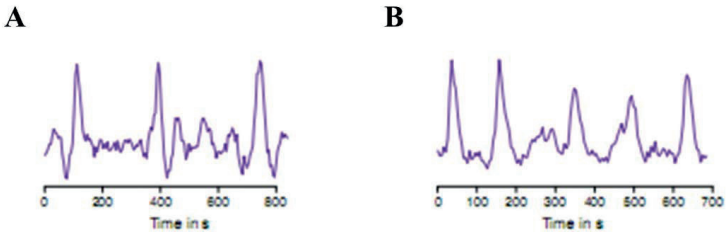

Figure 5. Ca^{2+} oscillation in *Parasponia andersonii* root hairs grown in 0.2mM KNO_3 (A) and 20mM KNO_3. Roots transformed with NupYC2.1 and treated with an LCO mixture of *Sinorhizobium fredii* NGR234 (10^{-9} M).

In a second experiment we determined whether exogenous nitrate may interfere with PaNFP functioning. First we determined whether expression of *PaNFP* is affected by exogenous nitrate. qRT-PCR using RNA isolated from roots of the plants grown on 0.2 and 20 mM KNO_3 showed that expression of *PaNFP* is not affected by exogenous nitrate (Fig. S2). Next it was determined

whether exogenous nitrate has an additive effect on the nodulation and infection phenotype of *PaNFP* knocked down roots. We have generated transgenic plants harbouring a *PaNFP* RNAi construct. Transgenic roots displayed a reduction of *PaNFP* mRNA of >80% (Fig. S3). Transgenic plants harbouring either *PaNFP* RNAi or an empty vector construct were grown on either 0.2 or 20 mM KNO_3 for 2 weeks, and subsequently inoculated with *Bradyrhizobium elkanii* WUR3. *PaNFP* RNAi roots formed significantly less nodules when compared to the empty vector control, and these nodules remained relatively small. However, nodulation efficiency of *PaNFP* RNAi plants was independent of the nitrate regime (Fig. 6).

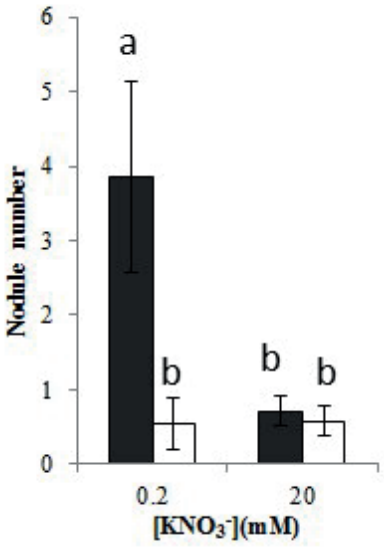

Figure 6. Nodulation efficiency of *PaNFP* RNAi knocked down roots (white bars) and control roots (black bars) at 0.2 or 20 mM KNO_3 (eight weeks post inoculation, *Bradyrhizobium elkanii* WUR3).

To determine whether the infection phenotype of these *PaNFP* RNAi nodules is affected by the nitrate regime, the structure of the nodules was studied by light microscopy (Fig.7). This showed that some, but not all cells in transgenic nodules were infected, but infection threads remain short and infected cells contained large vacuoles (Fig. 7C, H). This phenotype was irrespective of the nitrate regime (Fig. 7C-E and F-H), indicating that PaNFP and exogenous nitrate do not display

additive effects. Taken-together, these studies reveal that exogenous nitrate acts downstream or independent of PaNFP and LCO induced Ca^{2+}-oscillation signaling.

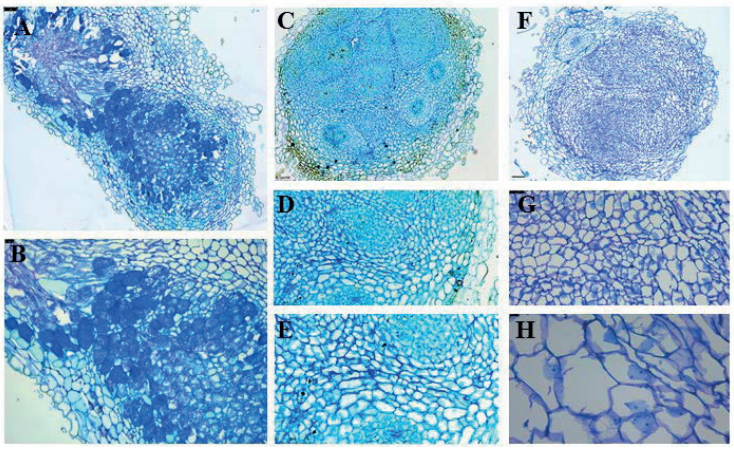

Figure 7. Sections of *PaNFP* knockdown nodules grown at 0.2 mM KNO3 (**C-E**) or 20 mM KNO3 (**F-H**) compare with non-transgenic control plants grown in 0.2mM (**A, B**). Magnifications (**D, E and G,H**) show that in infected cells only infection threads are present, independent of the nitrate regime. Bars: A, C, F: 75µm, B, D, G: 25 µm, H: µm 12.5, E: 10 µm.

Discussion

In legumes, exogenous nitrate has severe effects on the rhizobium symbiosis; ranging from premature nodule senescence, inhibition of nodule growth, inhibition of formation of new nodule primordia, reduced bacterial infection and reduced expression of -at least some- LCO-responsive genes. The severity of these responses vary among legume species (Harper and Gibson, 1984), suggesting that exogenous nitrate induced inhibition of nodulation. This represents an adaptation in the symbiotic trait which in part evolved independent from different legume lineages. Here we investigated the effect of exogenous nitrogen on the *Parasponia*-rhizobium symbiosis, which in evolutionary terms is relatively young. We showed that in presence of exogenous nitrate formation of fixation threads -the intracellular structure allowing exchange of nutrients- is most sensitive and inhibited at nitrate concentrations of ~20 mM. This suggests that *Parasponia*

employs an active mechanism to control intracellular rhizobial colonization when alternative sources of fixed nitrogen are available.

In *P.andersonii* also the nodulation efficiency is affected by exogenous nitrate. Surprisingly, nodulation efficiency is highest at 10 mM KNO_3, a concentration that in case of legumes generally is found to be inhibitory. At higher KNO_3 concentrations the nodulation efficiency of *P.andersonii* drops, but unlike many legumes nodulation is not inhibited completely, even in presence of 50 mM KNO_3. Strikingly, the few nodules that are formed contain many infected cells, suggesting that infection thread formation is not affected by high concentrations of exogenous KNO_3. Instead, the switch from infection threads with relative dense cell walls to fixation threads that are largely devoid from cell wall material is blocked. In nodules formed in presence of relative low exogenous nitrates, this developmental switch in intracellular infection structure occurs in the first 10-15 cell layers proximal to the nodule meristem. These cells enlarge in size when compared to uninfected cells. Formation of fixation threads coincides with fragmentation of the vacuole similar as seen in legumes (Gavrin *et al.*, 2014). However, in presence of exogenous nitrate, the vacuoles remain large, infection threads stop growing and do not progress to form a fixation threads. Taken-together, this indicates that nitrate-grown *P. andersonii* plants block specifically the cellular process underlying formation of an interface with reduced cell wall, which allows better nutrient exchange between both partners.

Fixation thread formation in *P. andersonii* nodules is controlled by the putative LCO receptor PaNFP (Op den Camp *et al.*, 2011). Most legumes do not form fixation threads, but rather release the rhizobia in a small membrane compartment, which are known as symbiosomes. These symbiosomes originate from cell wall free regions of infection threads. Studies in legumes revealed that several key genes in the rhizobium LCO signaling network are essential to commit this process. For example, in *M. truncatula* a reduced expression of the symbiotic receptors *MtNFP*, *MtDMI2* as well as a knockout mutation in *MtIPD3* results in absence of a cell wall free region in the infection threads and a block on symbiosome formation (Limpens *et al.*, 2005; Horváth *et al.*, 2011; Ovchinnikova *et al.*, 2011; Moling *et al.*, 2014). We tested the functional relation between *PaNFP* and exogenous nitrate in controlling fixation thread formation. Nodule infection phenotypes of *PaNFP* knockdown or wild type plants grown under high exogenous nitrate concentration are very comparable, suggesting that fixation thread formation is a strictly controlled in the rhizobium-*Parasponia* symbiosis. In legumes several studies have been

conducted to place the inhibitory effect of exogenous fixed-nitrogen in relation to rhizobium LCO signaling. The outcome of these studies varied depending on the species and which response was investigated. For example, in *Vicia sativa* it was shown that inhibition of LCO-induced root hair growth responses has a 24-36 h lag phase post combined nitrogen application (20 mM) (Heidstra *et al.*, 1994). Under such growth conditions, LCO-induced gene expression shows differential response to exogenous fixed nitrogen were some genes are still induced (e.g. *VsLB1*), whereas for others symbiotic expression ceased (*VsENOD5* and *VsENOD12*) (Heidstra *et al.*, 1997). Studies in *L. japonicus* revealed that inhibition of LCO-induced root hair growth responses is mainly triggered by ammonium, whereas nitrate interferes with symbiotic gene expression (e.g. *LjNIN*) (Barbulova *et al.*, 2007). Similarly, repression of *MtNIN* in presence of nitrate was reported for *M. truncatula* (Marsh *et al.*, 2007). We performed three types of experiments to elucidate the effect of exogenous nitrate on LCO signaling in *P. andersonii*; (I) LCO induced Ca^{2+} oscillation in root hairs, (II) *PaNFP* expression in roots and (III) additive effect on the *PaNFP* knock down phenotype. None of these experiments supported the idea that exogenous nitrate affects LCO signaling and/or *PaNFP* functioning.

Besides in *P.andersonii* root nodules, fixation threads form also in few legume species -e.g. *Andira* sp., *Chamaecrista ensiformis* and *C. flexuosa*- as well as in the nitrogen fixing symbiosis between filamentous actinobacteria of the genus *Frankia* and a small polyphyletic group of non-legume plant species (known as Actinorhizal plants) (Faria *et al.*, 1986; Naisbitt *et al.*, 1992; Pawlowski and Demchenko, 2012). In case of Actinorhizal symbiosis the effect of exogenous fixed nitrogen has been studied. These showed that fixed nitrogen affects nodule formation, nodule biomass and N_2-fixation, very similar as described for legumes (Huss-Danell, 1997). Also variation in sensitivity between different Actinorhizal species is observed. e.g. for *Alnus glutinosa*, *Casuarina cunninghamiana* and *Myrica cerifera* exogenous nitrate in concentration of 1 mM is inhibitory, whereas for *Elaeagnus angustifolia* no inhibitory effect is reported for concentrations up to 3 mM nitrate (Kohls and Baker, 1989). As the actinorhizal symbiosis is considered to be tens of million years old and possibly evolved multiple times independently in different lineages (Doyle, 2011; Werner *et al.*, 2014; Li *et al.*, 2015), the observed variation may reflect lineage-specific adaptations. Alternatively, it has been shown that the difference in sensitivity to exogenous nitrate correlates with the employed infection strategy of the plant. Actinorhizal plants with root hair based infection are more sensitive to the inhibitory effects of

nitrate than plants infected by crack entry (Kohls and Baker, 1989). This hypothesis is in line by our findings that *P.andersonii* root nodule formation is highly tolerant to exogenous nitrates.

The *Parasponia*-rhizobium symbiosis is considered to be relatively young when compared to legumes, and only evolved after emergence of the *Paraspona* lineage in the genus *Trema* (Yang *et al.*, 2013; Werner *et al.*, 2014; Behm *et al.*, 2014). Such young symbiosis may lack sophisticated mechanisms to control its symbiotic partner, and/or may even employ a different strategy than known from legumes. Our finding that formation of fixation threads, and the nitrogen-fixing stage of intracellular rhizobium in the nodules, is most strictly controlled in presence of exogenous nitrate shows that *P.andersonii* employs a novel strategy to control its symbiotic partner. In this way the energy demanding process of biological nitrogen fixation is prohibited, rather than the less energetic process of nodule formation. This finding provides new insights in the evolutionary plasticity on the nitrogen fixing symbiosis trait.

Materials and methods

Plant material and growth conditions and transformation

P. andersonii accession WU1 was propagated in *in vitro* cell culture as described previously (Cao *et al.*, 2012). Plantlets were transferred to the 14 cm round pots containing sterilized sand:granule mixture (v/v 1:1) supplemented with EKM medium with the required KNO_3 concentration (Cao *et al.*, 2012). Plants were inoculated with 10 ml *Bradyrhizobium elkanii* WUR3 at a final optical density of OD_{600}: 0.15 (Op den Camp *et al.*, 2012). Plants were grown under greenhouse conditions at 28°C, ~100% RH, 12/12 day/night regime and irrigated three times per week; once with nitrogen free EKM medium and two times with water. Non-inoculated plants were grown in the same condition as control. All plants were watered with EKM-medium minus N-source, whereas KNO_3 was used only once at the start of the experiment. Plant were harvested 8 weeks post inoculation (n=10 / treatment).

Fresh weights were determined immediately after harvesting. Nodules from each plant were removed from the root, counted and weighed. Dry weights were measured after desiccation in an oven at 60°C for 24h.

Transgenic compound *P. andersonnii* plants carrying either a *PaNFP* RNAi construct (Op den Camp *et al.*, 2011) or a cameleon NupYC2.1 construct (Granqvist *et al.*, 2015) were generated

using *Agrobacterium rhizogenes* mediated transformation (Cao *et al.*, 2012). Plants containing NupYC2.1 were kept *in vitro*. Calcium oscillation experiments were conducted as described by Granqvist et at 2015, *PaNFP* RNAi plants were grown in the sand:granule system as described above. Plants transformed with an empty vector considered as control and grown in the same condition with *PaNFP* RNAi knock down plants.

Nitrogenase activity

Nitrogenase activity was measured by using acetylene reduction assay (ARA) (Hardy *et al.*, 1973). Individual roots with attached nodules were placed in 35 ml bottle and sealed. 3.5ml volume of air was withdrawn with a syringe and replaced by 3.5 ml of acetylene. After 1h incubation in room temperature, 1ml of gas were taken from the bottle and injected into the gas chromatograph system. Ethylene production was determined by gas chromatograph (Chrompack Packard 438A equipped with Porapak N column (110 cm x 1.6 mm ID) using a N_2 as a carrier gas at a flow rate of 30 ml/min at 60 °C.

RNA extraction and Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from *P. andersonii* roots and treated with DNAse I using Qiagen plant RNAeasy kit (Qiagen; according to the manufacture's instruction). RNA concentration was quantified by NanoDrop spectrophotometer. cDNA was synthesized from 1 µg of total RNA using an i-script cDNA synthesis kit (Bio-Rad, Hercules, USA) as described in the manufacturer's protocol. Quantitive PCR reaction was performed in triplicate using primers for PaNFP and PaACTIN1 as described previously (Op den Camp *et al.*, 2011).

Nodule structure and ultrastructure

Nodules were embedded in Technovit 7100 according to the supplier' protocols (Heraeus-Kulzer, Wehrheim, Germany). Sections (4-6 µm) were cut on a microtome (RJ2035, Leica Microsystems), stained with toluidine blue (0.5%) and analysed by light microscopy (Leica AU5500B equipped with DFC425C Camera).

For transmission electron microscopy nodule tissue was prepared as described before (Fedorova *et al.*, 1999). Nodules were fixed in a 3% glutararaldehyde/4% paraformaldehyde mix, post-fixed by OsO_4 and embedded in LR Whine resin. Sections of 60 nm were prepared using a Leica

Ultracut microtome. Nickel grids were counterstained and examined using a JEOL JEM 2100 transmission electron microscope equipped with a Gatan US4000 4K×4K camera.

Acknowledgments

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Supplemental figures

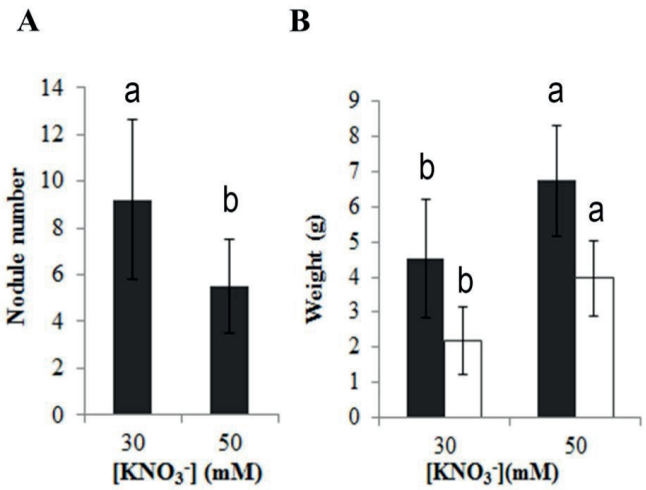

Figure S1. Effects of 30 mM and 50 mM KNO_3 on *Parasponia andersonii* inoculated with *Bradyrhizobium elkanii* WUR3 (eight weeks post inoculation). (A) Nodulation efficiency at different concentrations of exogenous nitrate. (B) Effect of exogenous nitrate on plant fresh weight (black bars: shoot fresh weight and white bars: root fresh weight) (n=10).

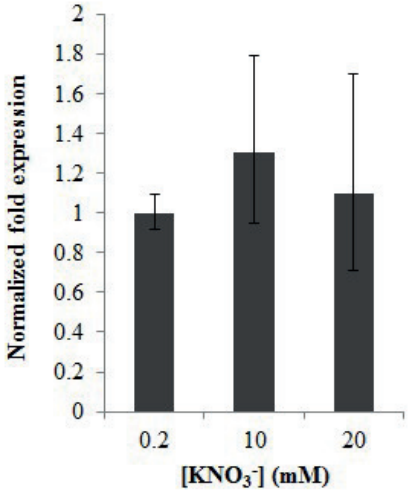

Figure S2. Relative expression of *PaNFP* in roots of *Parasponia andersonii* grown on 0.2, 10 and 20 mM KNO_3.

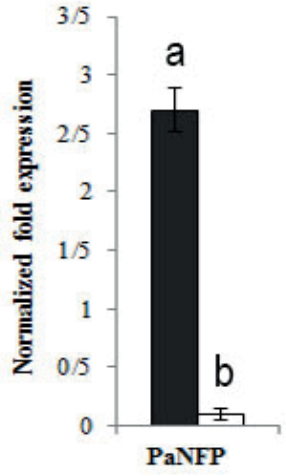

Figure S3. *PaNFP* expression level in non-transgenic roots (black bars) and *PaNFP* RNAi knockdown roots (white bars) grown at 0.2 mM KNO_3.

References

Barbulova A, Rogato A, D'Apuzzo E, Omrane S, Chiurazzi M. 2007. Differential effects of combined N sources on early steps of the Nod factor-dependent transduction pathway in Lotus japonicus. Molecular plant-microbe interactions 20, 994–1003.

Behm JE, Geurts R, Kiers ET. 2014. Parasponia: a novel system for studying mutualism stability. Trends in Plant Science 19, 757–763.

Broghammer a., Krusell L, Blaise M, *et al.* 2012. Legume receptors perceive the rhizobial lipochitin oligosaccharide signal molecules by direct binding. Proceedings of the National Academy of Sciences of the United States of America 109, 13859–13864.

Cao Q, Camp R den, Kalhor M. 2012. Efficiency of Agrobacterium rhizogenes–mediated root transformation of Parasponia and Trema is temperature dependent. Plant Growth Regulation 68, 459–465.

Carroll BJ, Mathews A. 1990. Nitrate inhibition of nodulation in legumes. In: Gresshoff PM, ed. The Molecular Biology of Symbiotic Nitrogen fixation. CRC Press, 159–180.

Doyle JJ. 2011. Phylogenetic perspectives on the origins of nodulation. Molecular plant-microbe interactions 24, 1289–95.

Faria SM De, Sutherland JM, Sprent JI. 1986. A new type of infected cell in root nodules Ofandira Spp. (Leguminosae). Plant Science 45, 143–147.

Fedorova E, Thomson R, Whitehead LF, Maudoux O, Udvardi MK, Day DA. 1999. Localization of H + -ATPases in soybean root nodules. Planta 209, 25–32.

Gavrin A, Kaiser BN, Geiger D, Tyerman SD, Wen Z, Bisseling T, Fedorova EE. 2014. Adjustment of Host Cells for Accommodation of Symbiotic Bacteria: Vacuole Defunctionalization, HOPS Suppression, and TIP1g Retargeting in Medicago. The Plant Cell 26, 3809–3822.

Geurts R, Lillo A, Bisseling T. 2012. Exploiting an ancient signalling machinery to enjoy a nitrogen fixing symbiosis. Current Opinion in Plant Biology 15, 438–443.

Granqvist E, Sun J, Camp RO Den, Pujic P, Hill L, Normand P, Morris RJ, Downie JA, Geurts R, Oldroyd GED. 2015. Bacterial-induced calcium oscillations are common to nitrogen-fixing associations of nodulating legumes and

non-legumes. New Phytol. 207, 551–558.

Hardy RWF, Burns RC, Holsten RD. 1973. Applications of the acetylene-ethylene assay for measurement of nitrogen fixation. Soil Biology and Biochemistry 5, 47–81.

Harper JE, Gibson a. H. 1984. Differential nodulation tolerance to nitrate among Legume species. Crop Science 24, 797.

Heidstra R, Geurts R, Franssen H, Spaink HP, Van Kammen A, Bisseling T. 1994. Root hair deformation activity of nodulation factors and their fate on Vicia sativa. Plant physiology 105, 787–797.

Heidstra R, Yang WC, Yalcin Y, Peck S, Emons a M, van Kammen A, Bisseling T. 1997. Ethylene provides positional information on cortical cell division but is not involved in Nod factor-induced root hair tip growth in Rhizobium-legume interaction. Development 124, 1781–1787.

Horváth B, Yeun LH, Domonkos Á, *et al.* 2011. Medicago truncatula IPD3 is a member of the common symbiotic signaling pathway required for rhizobial and mycorrhizal symbioses. Molecular Plant-Microbe Interactions 24, 1345–1358.

Huss-Danell K. 1997. Actinorhizal symbiosis and their N2 fixation. New Phytologist 136, 375–405.

Kohls SJ, Baker DD. 1989. Effects of substrate nitrate concentration on symbiotic nodule formation in actinorhizal plants. Plant and Soil 118, 171–179.

Lancelle SA, Torrey JG. 1984. Early development of Rhizobium-induced root nodules of Parasponia rigida. I. Infection and early nodule initiation. Protoplasma 123, 26–37.

Lavin M, Herendeen PS, Wojciechowski MF. 2005. Evolutionary rates analysis of Leguminosae implicates a rapid diversification of lineages during the tertiary. Systematic biology 54, 575–94.

Li H-L, Wang W, Mortimer PE, Li R-Q, Li D-Z, Hyde KD, Xu J-C, Soltis DE, Chen Z-D. 2015. Large-scale phylogenetic analyses reveal multiple gains of actinorhizal nitrogen-fixing symbioses in angiosperms associated with climate change. Scientific Reports 5, 14023.

Limpens E, Mirabella R, Fedorova E, Franken C, Franssen H, Bisseling T, Geurts R. 2005. Formation of organelle-like N2-fixing symbiosomes in legume root nodules is controlled by DMI2. Proceedings of the National Academy of Sciences of the United States of America 102, 10375–10380.

Marsh JF, Rakocevic A, Mitra RM, Brocard L, Sun J, Eschstruth A, Long SR, Schultze M, Ratet P, Oldroyd GED. 2007. Medicago truncatula NIN is essential for rhizobial-independent nodule organogenesis induced by autoactive Calcium/Calmodulin-Dependent Protein Kinase. Plant physiology 144, 324–335.

Marvel DJ, Torrey JG, Ausubel FM. 1987. Rhizobium symbiotic genes required for nodulation of legume and nonlegume hosts. Proceedings of the National Academy of Sciences of the United States of America 84, 1319–1323.

Miri M, Janakirama P, Held M, Ross L, Szczyglowski K. 2016. Into the Root : How Cytokinin Controls Rhizobial Infection. Trends in Plant Science xx, 1–9.

Moling S, Pietraszewska-Bogiel A, Postma M, Fedorova E, Hink M a., Limpens E, Gadella TWJ, Bisseling T. 2014. Nod factor receptors form heteromeric complexes and are essential for intracellular infection in Medicago nodules. The Plant Cell 26, 4188–4199.

Naisbitt T, James EK, Sprent JJI. 1992. The evolutionary significance of the legume genus Chamaecrista, as determined by nodule structure. New Phytologist 122, 487–492.

Oldroyd GED. 2013. Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants. Nature reviews. Microbiology 11, 252–63.

Op den Camp RHM, Polone E, Fedorova E, Roelofsen W, Squartini A, Camp HJMO Den, Bisseling T, Geurts R. 2012. Nonlegume *Parasponia andersonii* deploys a Broad Rhizobium Host Range Strategy Resulting in Largely Variable Symbiotic Effectiveness. Molecular Plant-Microbe Interactions 25, 954–963.

Op den Camp R, Streng A, De Mita S, *et al.* 2011. LysM-type mycorrhizal receptor recruited for rhizobium symbiosis in nonlegume Parasponia. Science (New York, N.Y.) 331, 909–912.

Ovchinnikova E, Journet E-P, Chabaud M, *et al.* 2011. IPD3 controls the formation of nitrogen-fixing symbiosomes in pea and Medicago Spp. Molecular plant-microbe interactions 24, 1333–44.

Pawlowski K, Demchenko KN. 2012. The diversity of actinorhizal symbiosis. Protoplasma 249, 967–979.

Price GD, Mohapatra SS, Gresshoff PM. 1984. Structure of nodules formed by Rhizobium strain ANU289 in the nonlegume Parasponia and the legume Siratro (Macroptilium atropurpureum). Botanical Gazette 145, 444.

Sagan M, Morandi D, Tarenghi E, Duc G. 1995. Selection of nodulation and mycorrhizal mutants in the model plant

Medicago truncatula (Gaertn.) after gamma-ray mutagenesis. Plant Science 111, 63–71.

Schauser L, Roussis A, Stiller J, Stougaard J. 1999. A plant regulator controlling development of symbiotic root nodules. Nature 402, 191–195.

Sprent JI. 2008. 60Ma of legume nodulation. What's new? What's changing? Journal of experimental botany 59, 1081–1084.

Streeter J. 1988. Inhibition of legume nodule formation and N2 fixation by nitrate. Critical Reviews in Plant Sciences 7, 1–23.

Streng A, op den Camp R, Bisseling T, Geurts R. 2011. Evolutionary origin of rhizobium Nod factor signaling. Plant Signaling & Behavior 6, 1510–1514.

Werner GDA, Cornwell WK, Sprent JI, Kattge J, Kiers ET, Werner. 2014. A single evolutionary innovation drives the deep evolution of symbiotic N2-fixation in angiosperms. Nature communications 5, 4087.

Willems A. 2006. The taxonomy of rhizobia: an overview. Plant and Soil 287, 3–14.

Yang M, Velzen R Van, Bakker F, Sattarian A. 2013. Molecular phylogenetics and character evolution of Cannabaceae. Taxon 62, 473–485.

Chapter 5

Symbiotic Functioning of the GRAS-TYPE Transcriptional Regulators *NSP1* and *NSP2* is Conserved in the Non-legume *Parasponia andersonii*

Maryam Seifi Kalhor, Ton Bisseling and René Geurts.

Abstract

GRAS proteins are plant-specific transcriptional regulators that play critical and diverse roles in plant development and signaling. Two genes of this family namely, *NODULATION SIGNALING PATHWAY1* (*NSP1*) and *NSP2,* have been identified in legumes to function in symbioses with rhizobium and endomycorrhizal fungi. Knockout mutations in either gene in pea (*Pisum staivum*), *Medicago truncatula* and *Lotus japonicus* block rhizobium induced root nodule formation and bacterial infection. In contrast, the mycorrhizal phenotypes of legume *nsp1* and *nsp2* knockout mutants are relatively weak and only observed in quantitative assays. This suggests that in legumes *NSP1* and *NSP2* have been recruited to commit an essential function in rhizobium symbiosis, whereas their functioning in endomycorrhizal symbiosis is partially redundant. We investigated the symbiotic functions of *NSP1* and *NSP2* in *Parasponia*, the only known non-legume lineage that acquired the rhizobium symbiosis by convergent evolution. RNAi mediated knockdown levels of *Parasponia andersonii PanNSP1* or *PanNSP2* correlated with reduced rhizobium nodulation efficiencies, including plants that were deprived from any nodule-like structure. However, knockdown of *PanNSP1* and *PanNSP2* only partially affected root mycorrhization. Only plants with highest knockdown levels showed a decrease in arbuscule formation. This suggests that *NSP1* and *NSP2* also in *P. andersonii* root nodule formation commit an essential function, whereas their functioning in endomycorrhization is less critical. Taken-together, we conclude that symbiotic functioning of *NSP1* and *NSP2* is conserved in legumes and in the non-legume *Parasponia*.

Keywords: GRAS proteins, endomycorrhizal fungi, *Parasponia andersonii, PanNSP1, PanNSP2.*

Introduction

Legume root nodules provide a niche to symbiotic rhizobium bacteria to convert atmospheric nitrogen into ammonia; a form that is accessible for plants. As this symbiosis is important in agriculture, it is intensively studied. Basic research provided insights in the underlying molecular mechanisms that drive the mutualistic relation between plant and microbe. Most prominently, it was found that signaling networks as well as cellular processes that are essential for root nodule formation, are co-opted from the much older -and more widespread- arbuscular mycorrhizal (AM) symbiosis (Parniske, 2008). Besides legumes, which form a large taxonomic family encompassing tens of thousands of species, a similar nitrogen fixing rhizobium root nodule symbiosis occurs also on five tropical tree species of the genus *Parasponia* (*Cannabaceae*) (Behm *et al.*, 2014). As the symbiosis trait in legumes and *Parasponia* evolved independently (Doyle, 2011), a comparative analysis may provide unique insights in the genetic constraints of this symbiosis.

Root nodule formation and rhizobium infection have been genetically investigated in the legume models *Medicago truncatula* and *Lotus japonicus*. This revealed that the rhizobium symbiosis has co-opted parts of the genetic network that plants used to establish an endomycorrhizal symbiosis. Genes that have a dual role in rhizobium and endomycorrhizal symbiosis encode a plasma-membrane localized LECTIN NUCLEOTIDE PHOSPHOHYDROLASE (LjLNP) (Roberts *et al.*, 2013), a protein complex of the LRR-type receptor kinase LjSYMRK/MtDMI2, a key enzyme of the mevalonate biosynthetic pathway (3-Hydroxy-3-Methylglutaryl CoA Reductase 1; MtHMGR1), a DNA-binding protein LjSIP1(Wang *et al.*, 2013; Endre *et al.*, 2002; Stracke *et al.*, 2002; Sun *et al.*, 2015), nuclear envelope localized cation channels LjCASTOR and LjPOLLUX/MtDMI1(Ané *et al.*, 2004; Imaizumi-Anraku *et al.*, 2005), a nuclear localized Calcium and Calmodulin-dependent protein kinase (CCaMK) (Ané *et al.*, 2004) and the transcription factors LjCYCLOPS/MtIPD3, NSP1 and NSP2 (Yano *et al.*, 2008; Maillet *et al.*, 2011; Horváth *et al.*, 2011; Delaux *et al.*, 2013). By studying the epistatic relationships among most of these genes a genetic network, stretching from the receptors down to the transcriptional regulators has been revealed (Geurts *et al.*, 2016). As this network controls both rhizobium as well as endomycorrhizal symbiosis it is named the common symbiosis signaling network.

Interestingly, the strengths of the phenotypes of knockout mutants of the genes in this network differ between both symbioses. This is most prominent for the transcriptional regulators NSP1 and NSP2. A knockout mutation in either gene in *L. japonicus*, *M. truncatula* or pea (*Pisum sativum*), blocks rhizobium infection and root nodule formation (Kaló *et al.*, 2005; Smit *et al.*, 2005; Heckmann *et al.*, 2011; Shtark *et al.*, 2016). In contrast, endomycorrhization phenotypes of *nsp1* and *nsp2* mutants in any of these species are relatively weak. Reduced colonization efficiencies and arbuscule formation could only be detected by sensitive quantitative assays in which a reduced number of mycorrhizal spores was used as inoculum (Maillet *et al.*, 2011; Lauressergues *et al.*, 2012; Delaux *et al.*, 2013; Takeda *et al.*, 2013; Shtark *et al.*, 2016). This let us to speculate that in legumes *NSP1* and *NSP2* are evolved to commit an essential function in rhizobium symbiosis, whereas their functioning in endomycorrhizal symbiosis is partially redundant.

NSP1 and NSP2 belong to the plant specific class of GRAS transcriptional regulators. In plants GRAS proteins are highly conserved and can be divided into several clades, each with distinct conserved domains and functions (Tian *et al.*, 2004; Lee *et al.*, 2008; Engstrom, 2011; Liu and Widmer, 2014) . NSP1 and NSP2 belong to two different clades; namely SHORT ROOT (SHR) and HAIRY MERISTEM (HAM), respectively (Kaló *et al.*, 2005; Smit *et al.*, 2007). Generally, GRAS proteins commit roles in shoot and root development, Gibberellin (GA3) signaling, phytochrome A signaling, abiotic stress response or symbioses. In latter case, besides NSP1 and NSP2, at least two additional symbiotic GRAS proteins have been identified that function in endomycorrhizal symbiosis in legumes; LjRAD1 and LjRAM1/MtRAM1(Gobbato, 2015; Zhang *et al.*, 2015). Furthermore, at least 6 GRAS proteins are transcriptionally induced in *M. truncatula* roots upon mycorrhization (Hogekamp *et al.*, 2011). Several of these symbiotic GRAS proteins showed to form heteromeric protein complexes, allowing an additional level of regulation. For example, it was found that MtNSP2 interacts with MtRAM1 and MtNSP1 to regulate the expression of the glycerol-3-phosphate acyl transferase MtRAM2, a gene essential for arbuscule formation by endomycorrhizal fungi (Gobbato *et al.*, 2012).

Studies in *M. truncatula* revealed that *MtNSP1* and *MtNSP2* control expression of several genes in a symbiotic context; including *MtENOD11* and *MtDWARF27* (*MtD27*) (Liu *et al.*, 2011). *MtD27* encodes a plastid localized carotenoid isomerase, which functions in the strigolactone biosynthetic pathway. Strigolactone biosynthesis is under the control of nutrient sensing

mechanisms, especially phosphate deficiency stress (Yoneyama *et al.*, 2012). Root-exuded strigolactones can activate AM-fungi, thereby promoting endomycorrization of the root (Akiyama *et al.*, 2005; Besserer *et al.*, 2006). As strigolactone biosynthesis in Medicago is –at least in part- controlled by transcriptional regulation of *MtD27* in a MtNSP1 and MtNSP2-dependent manner (Liu *et al.*, 2011; van Zeijl *et al.*, 2015), it may explain the reduction in root colonization by endomycorrhizal fungi in a Mt*nsp1Mt/nsp2* knockout mutant.

Studies in *Parasponia* have shown that evolution of rhizobium symbiosis is guided by genetic constraints (Geurts *et al.*, 2016). Similar to legumes the *Parasponia*-rhizobium symbiosis is founded on perception of rhizobium secreted lipo-chitooligosaccharide (LCO) signal molecules (Marvel *et al.*, 1987; den Camp *et al.*, 2011). In both lineages these LCOs are perceived by orthologous LysM-type receptors that activate the common symbiosis signaling network (Streng *et al.*, 2011). In *Parasponia* as demonstrated by ectopic expression of an autoactive allele of CCaMK, which leads to formation of nodule-like structures in absence of rhizobium (Op den Camp *et al.*, 2011).

We questioned whether *NSP1* and *NSP2* fulfil a symbiotic function in *Parasponia*. We identified single putative orthologs of *NSP1* and *NSP2* in the draft genome sequences of *Parasponia andersonii* and *Trema ortientalis*. By conducting RNAi in *P. andersonii* roots we demonstrate that *PanNSP1* and *PanNSP2* are essential for rhizobium root nodule formation. However, knockdown of *P. andersonii PanNSP1* and *PanNSP2* only partially affected mycorrhization. This suggests that *NSP1* and *NSP2* in *P. andersonii* root nodule formation commit an essential function, whereas their functioning in endomycorrhization is less critical. Taken together, we conclude that symbiotic functioning of *NSP1* and *NSP2* is conserved in legumes and the non-legume *Parasponia*.

Results

Identification of Parasponia andersonii NSP1 and NSP2

The GRAS protein family is largely conserved in plants and represents 12 distinct clades named according to a representative protein of *Arabidopsis thaliana* (Arabidopsis) (Liu and Widmer, 2014). NSP1 and NSP2 belong to two different clades; SHORT ROOT (SHR) and HAIRY

MERISTEM (HAM), respectively (Smit *et al.*, 2005; Kaló *et al.*, 2005; Engstrom, 2011). To identify the *Parasponia NSP1* and *NSP2* orthologous genes, the draft genome sequence assemblies of *P. andersonii* and *T. orientalis* were mined using the Arabidopsis members of both subclades as query. To create a robust phylogenetic structure similar searches were conducted in *M. truncatula* and *Glycine max* genome annotations Mt4.0v1 and Wm82.a2.v1.

The SHR clade contains 4 Arabidopsis genes. Besides *AtSHR*, these are *AtBLS1* (*BRASSINOSTEROID, LIGHT AND SUGAR* 1), At5G67411 and *AtSCL29* (*SCARECROW-LIKE 29*). The latter represents the putative ortholog of legume *NSP1*. Searching the *P. andersonii* and *T. orientalis* genome resulted in the identification of 5 orthologous genes, whereas for *M. turncatula* and *G. max* genes 5 and 12 genes could be identified, respectively. Phylogenetic analysis revealed 5 orthology groups (OGs) each containing at least one gene of *P. andersonii*, *T. orientalis*, *G. max* and *M. truncatula*. Arabidopsis genes only grouped in 3 OGs including *AtSCL29*, which represents the NSP1 OG. (Figure 1B). The putative *NSP1* orthologs of *P. andersonii* and *T. orientalis* were named accordingly *PanNSP1* and *TorNSP1*.

The HAM clade includes 5 Arabidopsis genes; *AtHAM1 to AtHAM4* and *AtSCL26*, respectively. The latter represents the putative ortholog of legume NSP2 (Kaló *et al.*, 2005). Blast searches in *P. andersonii*, *T. orientalis*, *M. truncatula* and *G. max* and subsequent phylogenetic reconstruction revealed 5 orthology groups, two of which were devoid of an Arabidopsis orthologous gene. *MtNSP2* clustered with a single *P. andersonii*, *T. orientalis* and Arabidopsis gene, but with four *G. max* and an additional *M. truncatula* paralog. The phylogenetic structure of the NSP2 OG suggests that *M. truncatula* and *G. max* share the duplication event (Figure 1A). The *P. andersonii* and *T. orientalis* genes were named accordingly *PanNSP2* and *TorNSP2*.

Pairwise alignment of the *PanNSP1* and *PanNSP2* proteins to their orthologs in *T. orientalis* showed that both gene orthologs are ~94% identical and shared a 65% (NSP1) and 59% (NSP2) identify with the *M. truncatula* proteins. Furthermore, the 5 conserved GRAS domains - leucine heptad repeat I (LHR-I), *VHIID*, *LHR-II*, *PFYRE* and SAW- were present in the *Parasponia* proteins. Taken together, this suggests that *PanNSP1* and *PanNSP2* represent functional GRAS-type transcriptional regulators.

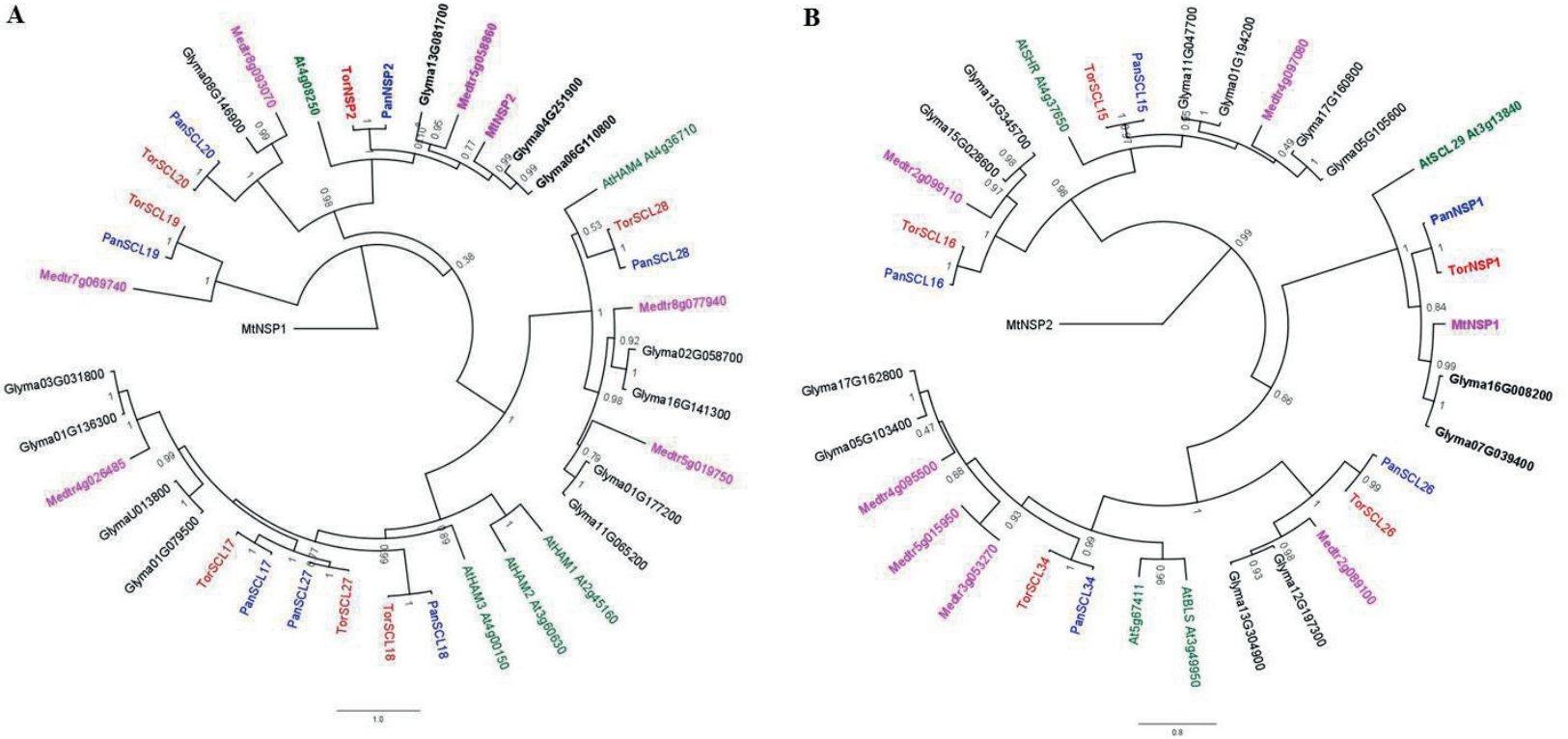

Figure1. Phylogenetic tree of the SHR clade of GRAS proteins, (A) NSP1against NSP2 as outgroup and (B) NSP2 with NSP1 as outgroup. Five orthology groups can be recognized among which is the clade containing MtNSP1. Tree has been constructed using FastTree (Geneious 8) and a ClustalW proteinalignment. Bootstraps were based on 100 replicates. Pan: *P. andersonii* (blue); Tor: *Trema orientalis* (red), Glyma: *Glycine max* (black) Phytozome: *G. max* Wm82.a2.v1), Mt/Medtr: *Medicago truncatula* (pink) (J. Craig Venter Institute: Mt4.0v1) and At: *Arabidopsis thaliana* (green) (TAIR). MtNSP2 (A) and MtNSP1 (B) was use as outgroup to root the tree.

Parasponia NSP1 and NSP2 are essential for root nodulation

First we determined whether *PanNSP1* and *PanNPS2* are expressed in roots of *P. andersonii* grown under susceptible conditions for root nodulation (EKM medium supplemented with 0.2 mM KNO_3). RNA was isolated from young growing roots and subsequently used as template in a qRT-PCR experiment to determine relative expression of *PanNSP1* and *PanNPS2* genes. This revealed that both genes are expressed in the root at comparable levels.

Next, we investigated whether *PanNSP1* and *PanNSP2* are essential for root nodule formation. To do so, gene specific RNAi constructs were made in the binary vector pK7GWIGW2 (II)-RR that includes a DsRED1 reporter as non-destructive selection marker. These *PanNSP1* and *PanNSP2* RNAi vectors were introduced in *P. andersonii* roots using *Agrobacterium rhizogenes*-mediated transformation. Plants with transgenic roots were selected based on red fluorescence. Of these plants non-fluorescent (non-transgenic) roots were removed prior transfer to pots. For each construct at least 5 RNAi knockdown plants were selected as well as a similar number of control plants (transformed with an empty vector construct) and subsequently inoculated with *Bradyrhizobium elkanii* WUR3. Eight weeks post inoculation roots were harvested to determine the nodulation efficiency of transgenic (red fluorescent) roots. Roots of control plants -transformed with empty vector- harbored ~15 nodules (in average). In contrast, nodule number on *PanNSP1* and *PanNSP2* knockdown roots varied, ranging between 0 and 15 nodules/plant (Figure 3A, B; Table 1)

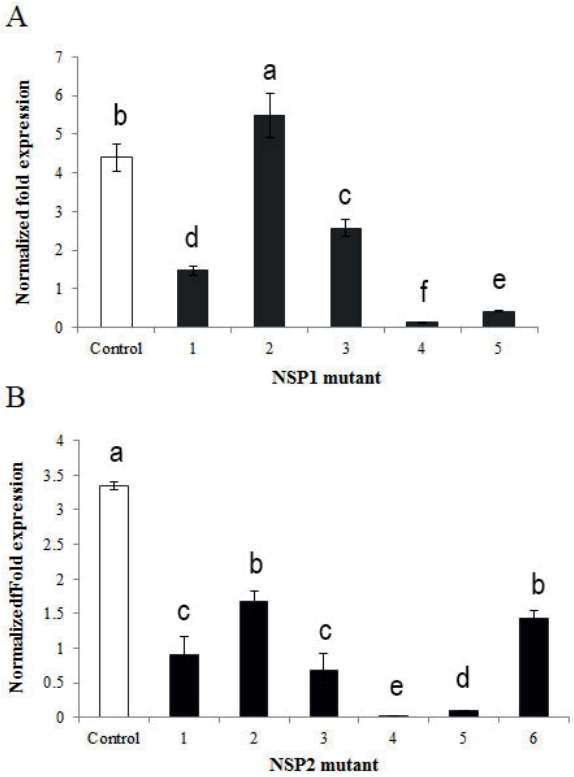

Figure2. *PanNSP* expression level in roots of mutants and control plants (harbouring empty vector) 8 weeks after inoculation with *Bradyrhizobium elkanii* (WUR3). (A) Level of transcription of *PanNSP1* in *Parasponia* RNAi knocked down roots (black bars) versus control root (transformed with empty vector, white bars). (B) Quantification of *PanN*SP2 in *Parasponia* knocked down roots (black bars) versus control roots (transformed with empty vector, white bars). Quantifications were normalized using stable expressed reference gene *PaACTIN.* Bars represent SD of three technical repeats.

To determine whether the reduction in nodule numbers correlated with the knock down levels of *PanNSP1* and *PanNSP2* expression, RNA was isolated from individual roots and expression level of both genes was determined by qRT-PCR. Correlation was found between level of knockdown and nodulation efficiency (Figure 3A, B). These finding demonstrate that *PanNSP1* and *PanNSP2* are essential for root nodule formation in *P. andersonii.*

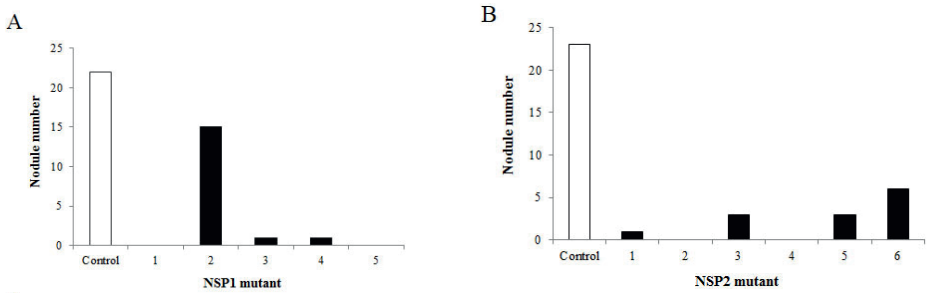

Figure 3. Nodule number in *PaNSP* mutants. (A) Reduced nodule number in *PanNSP1* RNAi knocked down mutants (black bars) compare with control plants (withe bars). (B) Reduced nodule number in *PanNSP2* RNAi knocked down mutants (black bars) compared with control plants (withe bars). Nodules counted 8 weeks after inoculation with *Bradyrhizobium elkanii* (WUR3).

Partial knock down of *PanNSP1* (e.g. plant 2 and 3) or *PanNSP2* (e.g. plant 3, 5 and 6) does not lead to a complete block of nodulation. Previous studies in *Lotus* suggest that LjNSP1 and LjNSP2 may have a function also in maintenance of symbiotic rhizobium in the root nodule (Heckmann *et al.*, 2006). To investigate whether *PanNSP1* and/or *PanNSP2* may have a similar function in *P. andersonii* we sectioned nodules formed on (partial) *PanNSP1* or *PanNSP2* knock down roots. In total we sectioned 5 *PanNSP1* RNAi and 8 *PanNSP2* RNAi nodules. All these nodules displayed a normal developmental phenotype, including intracellular infection (Figure 4). This suggests that *PanNSP1* and *PanNSP2* are essential in symbiotic signaling, but if a signaling threshold is achieved, nodule formation proceeds normally.

Table 1. Rate of infected plants in the wild type and *nsp1*, *nsp2* mutants.

	B.elkanii			*R.irregularis*		
	Number of inoculated plants	Number of nodulated plants	Ratio	Number of inoculated plants	Number of mycorrhized plants	Ratio
Wild type	10	10	1.00	6	6	1.00
Transgenic Control	8	8	1.00	8	8	1.00
NSP1	15	7	0.46	10	10	1.00
NSP2	15	12	0.8	11	11	1.00

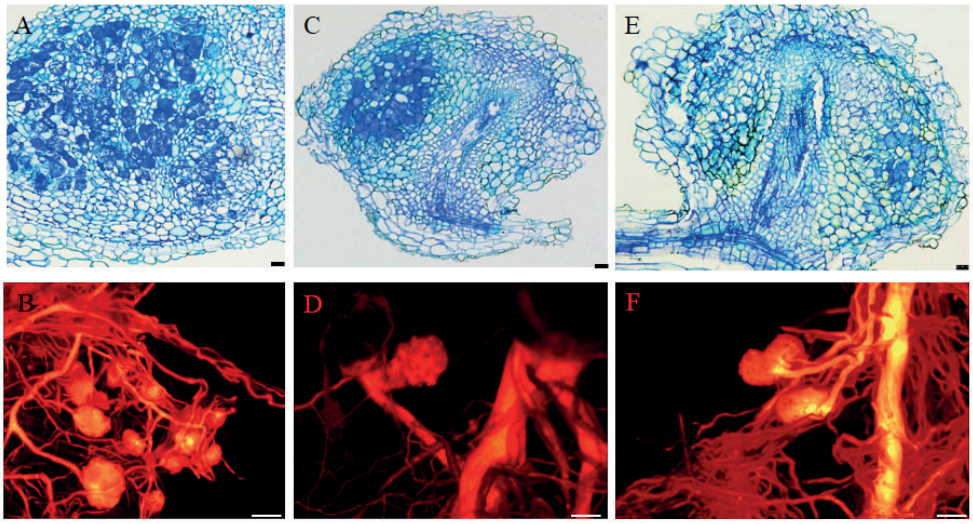

Figure 4. *Parasponia* nodules 8 weeks after inoculation with *Bradyrhizobium elkanii* (WUR3). (A) 4µm thin resin embedded section of control nodule (transformed with empty vector). (B) Transgenic nodule selected based on red fluorescence due to DsRED1 expression. (C) Longitudinal section of nodule originated from *PanNSP1* knocked down root. (D) Transgenic nodule selected based on red fluorescence due to DsRED1 expression. (E) Longitudinal section of nodule grown on *PanNSP2* knocked down root. (F) Transgenic nodule selected based on red fluorescence due to DsRED1 expression.

Parasponia PanNSP1 and PanNSP2 are involved in endomycorrhization

In order to determine whether *PanNSP1* and/or *PanNSP2* are involved in establishment of AM symbiosis, *P. andersonii* plants with knockdown and control roots were generated and grown in pots containing sterilized sand-granule (1:1) mixture as substrate. Plants were watered 3 times a week, once with Hoagland's solution (Hoagland, 1950) (0 µM PO_4) and twice with water. For each construct 15 RNAi knockdown plants were selected as well as a similar number of control plants (transformed with an empty vector construct) and subsequently inoculated with *Rhizophagus irregularis*. The percentage of root colonization was examined 4 weeks post inoculation. These studies revealed that *PanNSP1* and *PanNSP2* knockdown roots were all mycorrhized (Table 1). However, the fraction of *PanNSP1* RNAi root segments that were mycorrhized was significantly lower when compared to empty vector control roots or *PanNSP2*

RNAi roots (Figure 5A). Next, we determined the frequency of arbuscule formation in the infected root segments. This revealed that the number of arbuscules present in both -*PanNSP1* and *PanNSP2*- knockdown roots is significantly lower when compared to wild type control roots (Figure 5B). Taken together, these results indicate that both GRAS-type regulators are involved in -though not essential for- endomycorrhizal symbiosis.

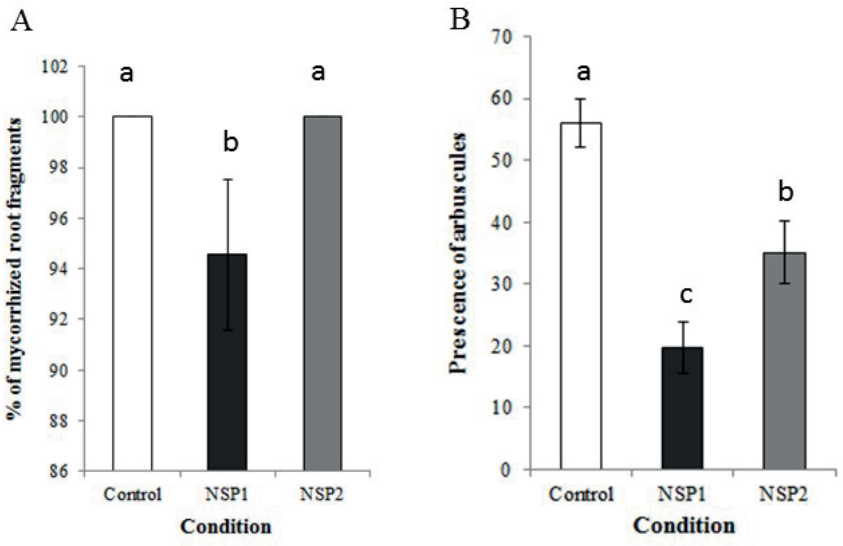

Figure 5. Analysis of *Parasponia* roots inoculated with *R. iregularis*.four weeks after inoculation (A) Percentage of mycorrhized root fragments in *PanNSP1* and *PanNSP2* RNAi knocked down mutants compare with control plants (transformed with empty vector). (B) Reduced level of arbuscul formation in mycorrhized fragments of *NSP1* and *NSP2* RNAi knocked down mutants compare with control plants (transformed with empty vector).

Discussion

In several legumes, it has been identified that the GRAS-type transcriptional regulators NSP1 and NSP2 are essential for rhizobium root nodule formation (Kaló *et al.*, 2005; Smit *et al.*, 2005; Heckmann *et al.*, 2006; Murakami *et al.*, 2007). Both proteins also play a promoting role in endomycorrhizal infection (Maillet *et al.*, 2011; Delaux *et al.*, 2013; Murakami *et al.*, 2013). Our studies revealed a very similar role of *NSP1* and *NSP2* in the non-legume *P. andersonii*. Complete knockdown of *PanNSP1* or *PanNSP2* expression blocks root nodule formation,

whereas mycorrhizal infection -although with a reduced efficiency- still occurs. As legumes and *Parasponia* gained the nodulation trait by convergent evolution, we conclude that recruitment of *NSP1* and *NSP2* in rhizobium root nodule formation is under tight evolutionary constraints.

We studied the phenotype on individual knockdown roots. In case of knockdown levels of >80% nodulation was almost completely inhibited. However, occasionally a single nodule was formed 8 weeks post inoculation. Based on the cytoarchitecture of these *PanNSP1* and *PanNSP2* RNAi nodules, we argue that these nodules most likely are functional. This finding is in line with phenotypes of *L. japonicus Ljnsp1* and *Ljnsp2* knock out mutants, where also occasionally functional nodules were found (Heckmann *et al.*, 2006; Murakami *et al.*, 2007). Such leaky phenotype is generally explained by gene redundancy scenario. Alternatively, both NSP proteins do not represent a core element in the rhizobium LCO signaling pathway, but rather facilitate dymbiotic signaling. This latter hypothesis is supported by genetic dissection studies of the rhizobium LCO induced signaling pathway in *L. japonicus,* which suggest that LjNSP1/LjNSP2 control several steps in the symbiosis signaling pathway. For example, LjNSP1 and LjNSP2 are required for rhizobium induced *LjNIN* expression (Smit *et al.*, 2007) a gene encoding a transcription factor that is essential as well as sufficient for root nodule formation. However, *LjNIN* induced nodule formation requires *LjNSP1* and *LjNSP2*, indicating that both GRAS proteins function upstream as well as downstream of *LjNIN* (Schauser *et al.*, 1999; Marsh *et al.*, 2007; Soyano *et al.*, 2013; Singh *et al.*, 2014; Lin *et al.*, 2014; Vernié *et al.*, 2015). A facilitator function may explain such phenotype. A facilitator function of *NSP1/NSP2* is also in line with the recent findings that both proteins control the carotenoid biosynthesis pathway (Liu *et al.*, 2011; van Zeijl *et al.*, 2015). Therefore we hypothesize that NSP1 and NSP2 are essential to create a physiological condition that facilitates LCO induced signaling and subsequent root nodule formation.

The finding that NSP proteins play a more prominent role in rhizobium root nodule formation that in endomycorrhization in independent lineages raises questions concerning the evolutionary trajectory of these proteins. Phylogenetic studies revealed that symbiotic *NSP1* and *NSP2* genes in legumes and *Parasponia* did not experience lineage specific duplication events. Furthermore, trans-complementation studies of *L. japonicus Ljnsp1* and *Ljnsp2* mutants with putative orthologs of *Nicotiana benthamiana* and/or rice (*Oryza sativum*) revealed that both proteins are

functionally conserved in higher plants (Heckmann *et al.*, 2006; Yokota *et al.*, 2010). This leaves only two possible scenarios concerning the evolutionary trajectory of both genes; adaptations in cis regulatory elements, or alternatively no symbiosis specific adaptations. Transcription levels and regulation in response to rhizobium LCOs was found to be important to control nodulation efficiency (Murakami *et al.*, 2013). Also it was noted that expression of *OsNSP2* in rice is relatively low (Liu *et al.*, 2011). However, since a direct comparison of functional promoters of *NSP1* and/or *NSP2* of nodulating and non-nodulating species has not been conducted, it remains elusive whether specific adaptations in cis regulatory elements of either of both genes were an essential step in evolution of root nodules.

The *Parasponia* lineage in the Cannabaceae family represents an independent event of evolution of nitrogen fixing symbiosis with rhizobium. When compared to legumes, the *Parasponia-rhizobium* symbiosis is relatively young, and subsequently less advanced (Behm *et al.*, 2014). We used *Parasponia* to get insight in the evolutionary constraints in rhizobium root nodule formation and identified the GRAS proteins *PanNSP1* and *PanNSP2* as key proteins in this process. In a previous study we identified the LysM-type receptor *PanNFP* and *PanCCaMK* as being essential for root nodule formation in *Parasponia* (den Camp *et al.*, 2011). Taken together, it suggests that evolution of rhizobium symbiosis is under severe genetic constraints. Such information is of considerable importance for any attempts to engineer a nitrogen fixing symbiosis on non-legume species other than *Parasponia*.

Materials and methods

In vitro Micro-propagation of Parasponia

*Parasponia andersoni*i buds were surface sterilized in 4% hypochlorite (commercial bleach) for 10 min and subsequently washed six times with sterile MQ water. Sterilized *P. andersonii* axillary buds were placed into propagation medium (20 g/l sucrose, 2.4 g/L McCown Woody Plant Medium (WPM) including vitamins (Duchefa Biochemie, Haarlem, The Netherlands; WPM) (Lloyd and McCown, 1980), 1.0 mg/L 6-Benzylaminopurine, 0.1 mg/L Indole-3-butyric acid, 0.8% Daichin agar, pH 5.8,) in Ø 10 cm round petri dishes. Plates were kept in an Elbanton growth cabinet at 28°C with a 16/8 h day/night regime. After four weeks shoots emerged from the axillary bud calluses. Shoots of >1.0 cm in length were cut and transferred to rooting medium

(10 g/L sucrose, 2.4 g/L WPM + 1.0 mg/L indole-3-butyric acid, 0.1 mg/L 1-naphthaleneacetic acid, pH5.8,) in round Ø10 cm Petri dishes. These plates were covered by aluminum foil (for root induction) and were kept in the same growth cabinet at 28°C for two weeks. Rooted shoots were kept on hormone free medium (20 g/L sucrose, 2.4 g/L WPM, 0.8% Daichin agar, pH 5.8,) for three weeks before transferring to the pots in greenhouse.

Constructs

The *PanNSP1* and *PanNSP2* RNAi constructs were made by cloning of 308 and 393 bp in pENTR-D-topo vector (PanNSP1F: TAAGCGAGAACAACATT, PanNSP1R: TTACTGTCATACTTTCTC; PanNSP2F: TTAACTATTTTGTCTTTC, PanNSP2R: TTCCTTATCTCCCTGGACA). Sequence hairpin construct was made by recombination of the amplified regions into the binary vector pK7GWIWG2 (II)-RR containing DsRED1 as selectable marker.

Rhizogenes Transformation

Agrobacterium rhizogenes, strain *MSU440*, containing the appropriate binary plasmid (all containing DsRED1 as selectable marker) was applied on the cut site of shoots with ~1 cm length. Two more wounds were produced with a needle dipped in *A. rhizogenes*. Inoculated plants were placed in line at EKM medium plates and half covered with sterile filter paper. The plates were sealed and positioned vertically at 21°C (16/8 h light/darkness). After one week the shoots were transferred to emergence medium in Daichin agar (0.9% w/v, Duchefa) containing 300 µg/ml Cefotaxime (Duchefa) and covered by a (half-) filter paper. Plants were grown for one week at 21°C, subsequently the filter paper was removed and the plates were placed vertically into a 28°C growth cabinet for 7-15 days. In this period new roots are formed that are co-transformed with the T-DNA of the binary vector. Co-transformed roots were selected based on red fluorescence. Subsequently, plantlets with transgenic roots were transferred to bigger plates containing McCown Woody Plant Medium with vitamins (Duchefa) and 300 µg/ml Cefotaxime and kept under the same growth conditions.

Nodulation assays

Plants harbouring transgenic roots were inoculated with *Bradyrhizobium elkanii* WUR3 by dipping the roots in a bacterial solution with an optical density of OD600 = 0.15. Subsequently, plants were placed in 14cm round pots containing sterilized sand-granule (1:1 v/v) mixture. Plants were fertilized once per week with EKM medium containing 0.2mM potassium nitrate and two times with water. Nodules were scored 8 weeks post inoculation.

RNA Isolation and qRT-PCR

P. andersonii root RNA was isolated from root samples using CTAB extraction buffer (2% CTAB, 2.5% PVP-40, 2 M NaCl, 100 mM Tris-HCl pH 8.0, 25 mM EDTA pH 8.0 and 2% of β-mercaptoethanol) followed by a phenol-chloroform-isoamyl alcohol (25:24:1, v/v/v) extraction on ice. Next the RNA was isopropanol precipitated at -20°C and washed with 70% ethanol. RNA was DNAseI treated and further purified on RNeasy Mini Spin columns (Qiagen). cDNA was synthesized from 1µg total RNA using the i-script cDNA synthesis kit (Bio-Rad, Hercules, USA) as described in the manufacturer protocol. Quantitative RT-PCR has been performed using SYBR green based detection (Bio-Rad, Hercules, USA). Experimental setup and execution have been conducted using a MyIQ optical cycler, according to protocol provided by the manufacturer (Biorad, Hercules, USA). Data analysis was performed using BioRad iQ5 software (BioRad). Baselines were set at 100 RFU to calculate Ct values. Relative expression of *PanNSP1*and *PanNSP2* in control and RNAi knockdown roots was normalized using a *P. andersonii* actin gene *PanACT1*.

Primers used:

PaACTIN-qF: CCTCATTGGAATGGAAGCAC,
PaACTIN-qR: TTCCAGGAAACATGGTGGAC
PanNSP1-qF: GTTCAAAGGCCGAGAGAGC
PanNSP1-qR: CTCGCACCACTTCTCTTTCC
PanNSP2-qF: CAAAGGTGGTGACAGTGGTG
PanNSP2-qR: GAACAGCGCCGAGTAGTAGTG

Endomycorrhization assay

Transgenic *P. andersonii* plant roots were inoculated with chemical Glomus inocolum. The plants were watered three times a week, once with Hoagland's solution (Hoagland medium according to (Ivanov et al 2012) and twice with water. To characterize mycorrhyzation efficiencies, *P. andersonii* roots were collected, and checked at the fluorescent macroscope for transgenic roots. Transgenic roots were then submerged in 10% KOH and heated at 90°C for 20 min. After two times rinsing the roots with water they were heated at 90 °C for 4 min in Trypan blue staining solution (2% Trypan blue in Lactoglycerol) and subsequently transferred to 30% glycerol. Root fragments were mounted on slides for examination. Mycorrhizal infection was quantified according to Pearson et al method (Tisserant *et al.*, 1993).

Nodule micro sectioning

Nodules were fixed in phosphate buffer solution (PBS) with 0.25% glutaraldehyde(Ivanov *et al.*, 2012) included. Vacuum was applied for 1-2 h until tissues sat on the bottom. The tissues were incubated at 4°C for overnight. After two times washing with PBS, dehydration steps were performed with 10%, 30%, 50%, 70%, 90% and 100% ETOH respectively for 10 min at room temperature for each step. Plastic infiltration was done in four steps, which included solution A (100ml Technovit7100, 1pack HrdnerI, 2.5ml PEG400):100% ETOH in 1:3, 1:1, 3:1 ratio respectively for 30-60 min in room temperature and finally treated with 100% solution A for overnight at 4°C. All materials were transferred into cupules and nodules located in the middle. Solution A was removed and polymerization solution (15 ml Solution A, 1 ml Hardener II) was added immediately. To remove air from the samples, cupules were covered with parafilm and left for overnight at room temperature. After polymerization, holders were put on the blocks and holding solution (technovit3040: 2 part powder, 1 part liquid) was added from the hole located in the centre of holder and kept for 15 min at room temperature. Finally sectioning of nodules was performed using a microtome machine and the samples were analysed by microscopy (Leica) after staining with toluidine blue (0.5%) buffer and washing with tap water for 5 minutes.

References

Akiyama, K., et al. (2005). "Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi." Nature **435**(7043): 824-827.

Ané, J.-M., et al. (2004). "Medicago truncatula DMI1 required for bacterial and fungal symbioses in legumes." Science **303**(5662): 1364-1367.

Behm, J. E., et al. (2014). "Parasponia: a novel system for studying mutualism stability." Trends in Plant Science **19**(12): 757-763.

Besserer, A., et al. (2006). "Strigolactones stimulate arbuscular mycorrhizal fungi by activating mitochondria." PLoS Biol **4**(7): e226.

Delaux, P. M., et al. (2013). "NSP1 is a component of the Myc signaling pathway." New Phytologist **199**(1): 59-65.

Den Camp, R. O., et al. (2011). "LysM-type mycorrhizal receptor recruited for rhizobium symbiosis in nonlegume Parasponia." Science **331**(6019): 909-912.

Doyle, J. J. (2011). "Phylogenetic perspectives on the origins of nodulation." Molecular Plant-Microbe Interactions **24**(11): 1289-1295.

Endre, G., et al. (2002). "A receptor kinase gene regulating symbiotic nodule development." Nature **417**(6892): 962-966.

Geurts, R., et al. (2016). "What Does It Take to Evolve A Nitrogen-Fixing Endosymbiosis?" Trends in Plant Science **21**(3): 199-208.

Gobbato, E. (2015). "Recent developments in arbuscular mycorrhizal signaling." Current Opinion in Plant Biology **26**: 1-7.

Gobbato, E., et al. (2012). "A GRAS-type transcription factor with a specific function in mycorrhizal signaling." Current Biology **22**(23): 2236-2241.

Heckmann, A. B., et al. (2006). "Lotus japonicus nodulation requires two GRAS domain regulators, one of which is functionally conserved in a non-legume." Plant Physiology **142**(4): 1739-1750.

Heckmann, A. B., et al. (2011). "Cytokinin induction of root nodule primordia in Lotus japonicus is regulated by a mechanism operating in the root cortex." Molecular Plant-Microbe Interactions **24**(11): 1385-1395.

Hogekamp, C., et al. (2011). "Laser microdissection unravels cell-type-specific transcription in arbuscular mycorrhizal roots, including CAAT-box transcription factor gene expression correlating with fungal contact and spread." Plant Physiology **157**(4): 2023-2043.

Horváth, B., et al. (2011). "Medicago truncatula IPD3 is a member of the common symbiotic signaling pathway required for rhizobial and mycorrhizal symbioses." Molecular Plant-Microbe Interactions **24**(11): 1345-1358.

Imaizumi-Anraku, H., et al. (2005). "Plastid proteins crucial for symbiotic fungal and bacterial entry into plant roots." Nature **433**(7025): 527-531.

Kaló, P., et al. (2005). "Nodulation signaling in legumes requires NSP2, a member of the GRAS family of transcriptional regulators." Science **308**(5729): 1786-1789.

Lauressergues, D., et al. (2012). "The microRNA miR171h modulates arbuscular mycorrhizal colonization of Medicago truncatula by targeting NSP2." The Plant Journal **72**(3): 512-522.

Lin, K., et al. (2014). "Single nucleus genome sequencing reveals high similarity among nuclei of an endomycorrhizal fungus." PLoS Genet **10**(1): e1004078.

Liu, W., et al. (2011). "Strigolactone biosynthesis in Medicago truncatula and rice requires the symbiotic GRAS-type transcription factors NSP1 and NSP2." The Plant cell **23**(10): 3853-3865.

Maillet, F., et al. (2011). "Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza." Nature **469**(7328): 58-63.

Marsh, J. F., et al. (2007). "Medicago truncatula NIN is essential for rhizobial-independent nodule organogenesis induced by autoactive calcium/calmodulin-dependent protein kinase." Plant Physiology **144**(1): 324-335.

Marvel, D. J., et al. (1987). "Rhizobium symbiotic genes required for nodulation of legume and nonlegume hosts." Proceedings of the National Academy of Sciences **84**(5): 1319-1323.

Murakami, Y., et al. (2007). "Positional cloning identifies Lotus japonicus NSP2, a putative transcription factor of the GRAS family, required for NIN and ENOD40 gene expression in nodule initiation." DNA research **13**(6): 255-265.

Murakami, Y., et al. (2013). "Down-regulation of NSP2 expression in developmentally young regions of Lotus japonicus roots in response to rhizobial inoculation." Plant and Cell Physiology **54**(4): 518-527.

Parniske, M. (2008). "Arbuscular mycorrhiza: the mother of plant root endosymbioses." Nature Reviews Microbiology **6**(10): 763-775.

Roberts, N. J., et al. (2013). "Rhizobial and mycorrhizal symbioses in Lotus japonicus require lectin nucleotide phosphohydrolase, which acts upstream of calcium signaling." Plant Physiology **161**(1): 556-567.

Schauser, L., et al. (1999). "A plant regulator controlling development of symbiotic root nodules." Nature **402**(6758): 191-195.

Shtark, O. Y., et al. (2016). "Arbuscular mycorrhiza development in pea (Pisum sativum L.) mutants impaired in five early nodulation genes including putative orthologs of NSP1 and NSP2." Symbiosis **68**(1-3): 129-144.

Singh, S., et al. (2014). "CYCLOPS, a DNA-binding transcriptional activator, orchestrates symbiotic root nodule development." Cell Host & Microbe **15**(2): 139-152.

Smit, P., et al. (2007). "Medicago LYK3, an entry receptor in rhizobial nodulation factor signaling." Plant Physiology **145**(1): 183-191.

Smit, P., et al. (2005). "NSP1 of the GRAS protein family is essential for rhizobial Nod factor-induced transcription." Science **308**(5729): 1789-1791.

Soyano, T., et al. (2013). "Nodule inception directly targets NF-Y subunit genes to regulate essential processes of root nodule development in Lotus japonicus." PLoS Genet **9**(3): e1003352.

Stracke, S., et al. (2002). "A plant receptor-like kinase required for both bacterial and fungal symbiosis." Nature **417**(6892): 959-962.

Streng, A., et al. (2011). "Evolutionary origin of rhizobium Nod factor signaling." Plant Signaling & Behavior **6**(10): 1510-1514.

Sun, J., et al. (2015). "Activation of symbiosis signaling by arbuscular mycorrhizal fungi in legumes and rice." The Plant cell **27**(3): 823-838.

Takeda, N., et al. (2013). "CERBERUS and NSP1 of Lotus japonicus are common symbiosis genes that modulate arbuscular mycorrhiza development." Plant and Cell Physiology **54**(10): 1711-1723.

Tisserant, B., et al. (1993). "In planta histochemical staining of fungal alkaline phosphatase activity for analysis of efficient arbuscular mycorrhizal infections." Mycological Research **97**(2): 245-250.

van Zeijl, A., et al. (2015). "Rhizobium lipo-chitooligosaccharide signaling triggers accumulation of cytokinins in Medicago truncatula roots." Molecular Plant **8**(8): 1213-1226.

Vernié, T., et al. (2015). "The NIN Transcription Factor Coordinates Diverse Nodulation Programs in Different Tissues of the Medicago truncatula Root." The Plant cell **27**(12): 3410-3424.

Wang, C., et al. (2013). "Splice variants of the SIP1 transcripts play a role in nodule organogenesis in Lotus japonicus." Plant Molecular Biology **82**(1-2): 97-111.

Yano, K., et al. (2008). "CYCLOPS, a mediator of symbiotic intracellular accommodation." Proceedings of the National Academy of Sciences **105**(51): 20540-20545.

Yokota, K., et al. (2010). "Function of GRAS proteins in root nodule symbiosis is retained in homologs of a non-legume, rice." Plant and Cell Physiology **51**(9): 1436-1442.

Yoneyama, K., et al. (2012). "How do nitrogen and phosphorus deficiencies affect strigolactone production and exudation?" Planta **235**(6): 1197-1207.

Zhang, X.-C., et al. (2015). "Jasmonate signalling in Arabidopsis involves SGT1b–HSP70–HSP90 chaperone complexes." Nature Plants **1**(5).

Chapter 6

Dual Effect of Ethylene on Root Nodulation of *Parasponia andersonii*

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Abstract

Legumes and *Parasponia* species engage in a mutualistic symbiosis with nitrogen fixing soil bacteria collectively referred to as rhizobia. The consequence of such engagement is the formation of nodules on the plant root system. Although the interaction is beneficial to the plant, the number of nodules is strictly regulated by different mechanisms. In legumes, one of the components which have been discovered to control nodule number is ethylene. It has been shown that inhibitors of ethylene biosynthesis or ethylene perception enhance rhizobium nodulation. Additionally, genetic studies in *Medicago truncatula* and *Lotus japonicus* revealed that interference in the ethylene signaling pathway can lead to an uncontrolled number of root nodules. It remains unclear however, whether similar ethylene inhibitory mechanisms are also functional in *Parasponia*. Here we showed the negative effect of ethylene on root nodulation of *Parasponia andersonii*. Ethylene insensitivity was induced through RNAi of *PanEIN2*, a key regulator of ethylene signaling pathway. The *PanEIN2* gene expression was reduced up to 80% resulting in a 20-fold increase in nodule number compared to control plants, similar as reported for *Lotus japonicus*. In addition, a novel phenotype was observed, as nodules on *PanEIN2* RNAi roots were defected in intracellular infection**.** Taken together these data support the hypothesis that similar to legumes, ethylene–mediated inhibition of root nodule formation evolved in *Parasponia*. The occurrence of different phenotypes in *Parasponia* and legumes nodules also suggests that in *Parasponia*, ethylene signaling plays a dual role in root nodule formation.

Keywords: *Parasponia,* Ethylene insensitivity, *PanEIN2,* intracellular infection.

Introduction

The nitrogen fixing rhizobium symbiosis is a biotrophic interaction in which both partners benefit. The plant supplies the rhizobium bacteria with carbon enabling them to use this energy to fix atmospheric nitrogen into ammonia. This newly fixed ammonia can be exploited by the plant, providing it a selective advantage, especially under nitrogen limiting growth conditions. The nitrogen fixing rhizobium symbiosis is a well-known character of many species of the Fabaceae (~18.000 species of which the vast majority can establish a nitrogen fixing rhizobium symbiosis), but also occurs in the *Parasponia* genus of the Cannabaceae. Most probable the symbiosis evolved in parallel in both lineages and emerged at different moments in time. Genetic studies in legumes revealed that bacterial induced symbiotic signaling triggered by secreted lipo-chitooligosaccharide (LCOs) signals intertwine with the plant hormonal networks to establish a novel organ –the root nodule- to host the bacterial symbionts. Among others, ethylene is found to act as a negative regulator of root nodule formation. Here, we focused whether this character is constrained in *Parasponia andersonii*.

Although, the nitrogen fixing rhizobium symbiosis in legumes evolved about 60 million years ago, this symbiosis in *Parasponia* is considered to be significantly younger (Behm *et al.*, 2014). The genus *Parasponia* represents only 5 tropical tree species that are closely related to species of the genus *Trema*. The 5 *Pasasponia* species are exclusively found in the Malay Archipellago, were they grow on the nitrogen poor volcanic mountain sloops. Although the age of the *Parasponia* genus could not be determined due to the lack of fossil data records, several lines of evidence suggest that it is much younger than the legume lineage. First, *Parasponia* is phenotypically and molecularly very similar to *Trema*, suggesting it has emerged from a basal *Trema* species. Second, its root nodules are more basal when compared to legume root nodules. Consequently, *Parasponia*-rhizobium symbiosis is less advanced and fix nitrogen less effective than in case of legumes (Op den Camp *et al.*, 2012; Behm *et al.*, 2014).

Comparative studies between legumes and *Parasponia* showed that genetic constraints at least in part have guided the evolution of the rhizobium symbiosis trait in both lineages. For example like legumes rhizobium, the LCO induced signaling network is essential also in case of *Parasponia* root nodule formation. However, since the rhizobium symbiosis in *Parasponia* is considered to be much younger that in legumes, it can be hypothesized that only networks have

been recruited that are essential for nodule formation, whereas genetic adaptations that further enhance the symbiotic engagement may not (yet) have occurred in *Parasponia*.

Rhizobium secreted LCOs are perceived by specific LysM-type receptor kinases (Untergasser *et al.*, 2008). Upon perception, active cytokinins accumulate in the differentiation zone of the root prior first cell divisions that will give birth to a nodule primordium (van Zeijl *et al.*, 2015). A nodule primordium is associated with formation of a local auxin maximum (Takanashi *et al.*, 2011; Imanishi *et al.*, 2014). Formation of such maximum requires cytokinin signaling (Plet *et al.*, 2011). Among other responses, the cytokinin induced signaling may interfere with auxin transport. Such local inhibition of auxin efflux may result in an formation of a local auxin maximum (Plet *et al.*, 2011).

Abscisic acid, jasmonate and ethylene are negative regulators of rhizobium LCO signaling (Sun *et al.*, 2006; Ding *et al.*, 2008). It has been revealed that ethylene is transiently induced by rhizobia during nodule initiation (Ligero *et al.*, 1986; Caba *et al.*, 1998) and negatively affects the process of nodule development. Ethylene also inhibits bacterial infection and determines the radial positioning of the nodule primoridum in legume roots (Prayitno *et al.*, 2006; Penmetsa *et al.*, 2003; Prayitno *et al.*, 2006a). The inhibitory effect of ethylene on nodulation has been pharmacologically studied by exogenous application of ethylene (Goodlass and Smith, 1979; Lee and LaRue, 1992), or through application of the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC), and/or ACC synthase inhibitor aminoethoxyvinylglycine (AVG) (Yu *et al.*, 1979). In legumes, application of ACC or AVG resulted in different effects on root nodule formation. Whereas ACC inhibits formation of this new organ, AVG results in increased nodulation efficiency in legumes (Lee and LaRue, 1992; Peters and Crist-Estes, 1989; Lohar *et al.*, 2009; Gresshoff *et al.*, 2009; Guinel and LaRue, 1992).

Besides pharmacological studies, also genetics revealed a regulatory role of ethylene signaling in nodule formation. Especially ETHYLENE INSENSITIVE 2 (*EIN2*) commits an important role in regulating nodule numbers. Studies in the non-legume *Arabidopsis thaliana* (Arabidopsis) revealed that EIN2 acts as a transcriptional modulator upon proteolytic activation. It interacts with specific transcription factors, thereby increasing their stability (Merchante *et al.*, 2015). Mutating the *EIN2* orthologous gene in *Medicago* (named *MtSKL*) or knocking it down by RNA interference (RNAi) in *Lotus japonicus* results in an increase in number of root nodules (Miyata

et al., 2013). Furthermore, in *Medicago Mtein2/Mtskl* knockout mutant roots display hypernodulating character in the root that are interspersed with zones that lack nodules.

Here we investigated the role of *EIN2* in *Parasponia* root nodule formation. We show that when the *P. andersonii EIN2* (*PanEIN2*) gene is supressed, nodule formation is enhanced. In line with this we conclude that *PanEIN2* has negative effect on nodule formation in *P. andersonii*. This suggests that the negative effect of ethylene signaling on root nodule formation is a generic function, rather than a genetic adaptation in the legume lineage.

Results

Increased nodulation efficiency in Parasponia upon interference with the ethylene pathway

In legumes the inhibitory effect of ethylene on nodulation can be modulated by manipulating the ACC (1-aminocyclopropane-1-carboxylic acid) concentration. For example, applying exogenous ACC results in decreased nodulation efficiencies in *Medicago*, whereas the reverse effect is achieved by exogenous application of the ACC synthase inhibitor AVG (Aminoethoxyvinylglycine) (Penmetsa and Cook, 1997). To test whether this response also occurs in *Parasponia*, we grew *Bradyrhizobium elkanii* (strain WUR3) inoculated *Parasponia* plantlets *in vitro*, and supplemented the medium either with 10 µm AVG or 50 µm ACC. This revealed that addition of AVG results in increased nodulation, whereas plants grown on ACC had reduced nodulation efficiency (Figure 1). These results indicated that nodule formation in *Parasponia* is also controlled, at least in part, by ethylene.

PanEIN2 is a negative regulator of rhizobium root nodule formation

Using BLAST tools we identified a *Parasponia EIN2* homologous gene (*PanEIN2*) in the draft *Parasponia* genome sequence (data not shown). *PanEIN2* consist of 7 exons and encodes a protein of 1,294 amino acids. The N-terminal (450 amino acids) of PanEIN2 contains a NRAMP domain, including a hydrophobic core of 13 transmembrane domains, whereas the C-terminal region contains a CEND transcriptional activator domain and a putative nuclear localization signal (1,262-1,288 amino acids). Phylogenetic reconstruction using the *EIN2* gene of *Medicago*, *Lotus*, *Soybean* and *Arabidopsis* revealed that *PanEIN2* represents a putative *EIN2* orthologous gene (Figure 2). Analysing available RNAseq data sets for *Parasponia* revealed that *PanEIN2* is

ubiquitously expressed in bellow and above grown tissues (Figure 3), which further supports that *PanEIN2* represents a functional gene.

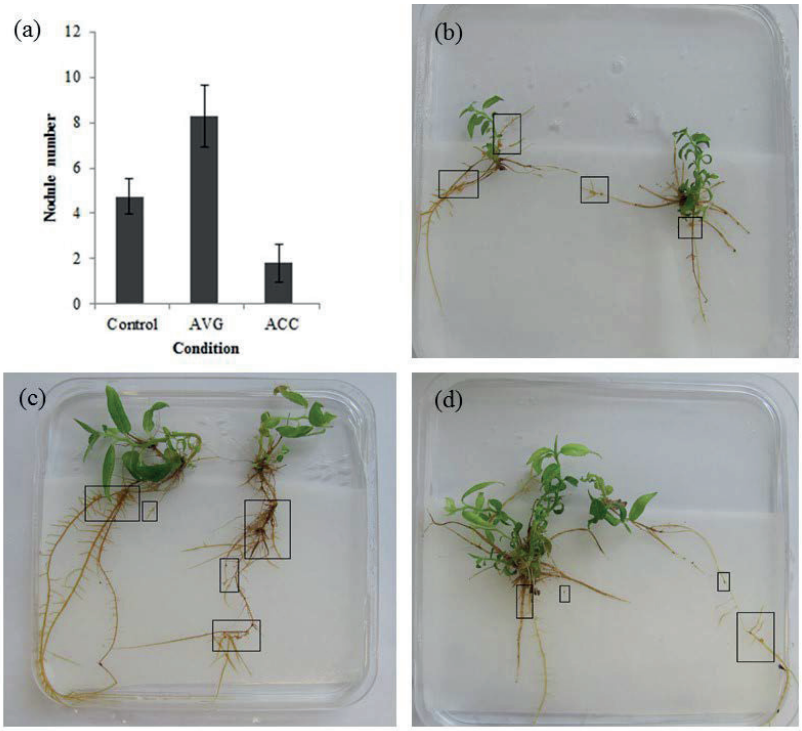

Figure 1. Effects of AVG and ACC on root nodulation of *Pararasponia andersonii*. Nodule numbers increased by adding AVG and reduced after applying ACC (a). Plants were inoculated with *Bradyrhizobium elkanii* (WUR3) and grown for 8 weeks at medium supplemented with 0.2 mM Potassium nitrate. Nodules grown on roots incubated with water as Control (b), 10 µm AVG (c) and 50 µm ACC (d). Boxes represent the nodules formed on the roots.

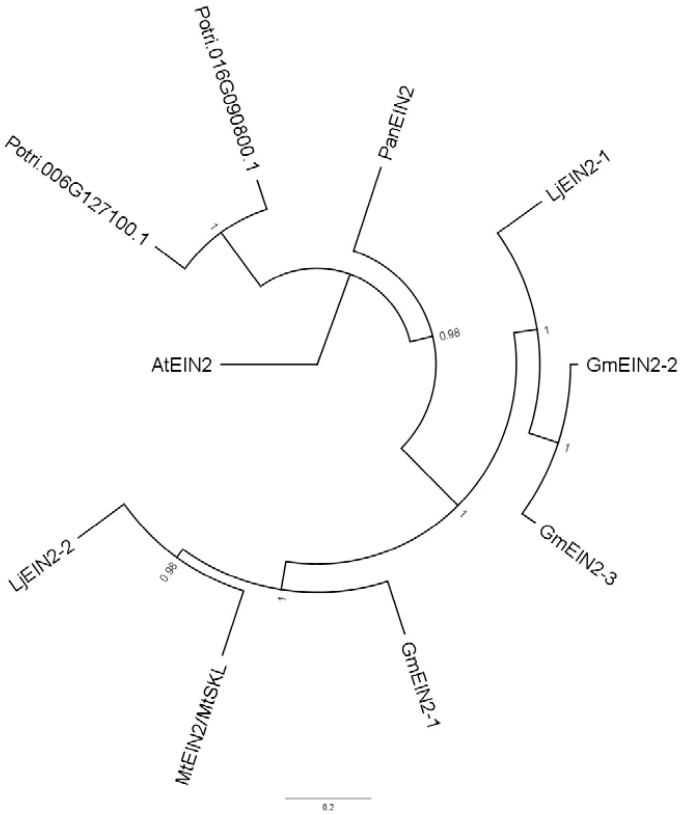

Figure 2. Phylogeny analysis of *PanEIN2*. Phylogenetic tree showing the relation of *PanEIN2* to *EIN2* orthologous genes of *Medicago*, *Lotus*, *soybean* and *Arabidopsis*.

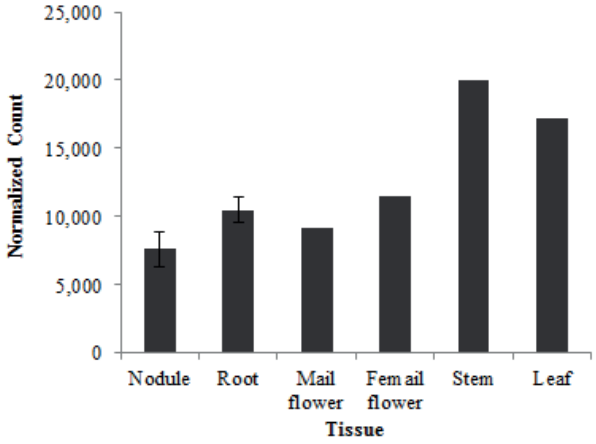

Figure 3. Expression patterns of *PanEIN2* in different tissues. Expression of *PanEIN2* based on *Parasponia* RNAseq expression atlas. *PanEIN2* expression was detected in nodules, roots, female flowers, male flowers, stem and leaf tissues. Expression counted based on average of 3 biological replicates for nodule and root, and single library for flowers, stem and leaf.

To assess the effect of ethylene on nodulation we performed a RNAi *PanEIN2* knockdown study using *A. rhizogenes* mediated root transformation of *Parasponia* transgenic roots which showed on average >50% reduction in *PanEIN2* transcripts (Figure 4). To determine whether reduced expression of *PanEIN2* affects nodulation efficiency, *P. andersonii* plantlets containing transgenic *PanEIN2* RNAi roots were grown *in vitro* and inoculated with *B. elkanii* WUR3. Eight weeks post inoculation we observed a 20-fold increase in nodule number on *PanEIN2* RNAi roots when compared to empty vector control roots (Figure 5a). This shows that the inhibitory role of ethylene signaling in root nodule formation is conserved in *Parasponia* and legumes.

***PanEIN2* knocked down inhibits intracellular infection in *Parasponia* root nodules**

We noted that nodules formed on *PanEIN2* RNAi roots are relatively small. Control plants (transformed with empty vector) harboured big and multi lobed nodules that were present on older parts of the root. In contrast, in the ethylene-insensitive transgenic *PanEIN2* RNAi roots hundreds of small, white, single lobed nodules were distributed all over the root (Figure 6). Determining nodule fresh weights revealed that average of *PanEIN2* RNAi nodules were only

~20% of the size of nodules in wild type plants (Figure 5b). Therefore, we planned to study ontology of *PanEIN2* nodules. To do so, plastic imbedded nodules were sectioned and toluidine blue stained. Nodules formed in control plants were fully colonized, containing many cells harbouring fixation threads (Figure 7e-f). In contrast, nodules formed on *PanEIN2* knocked down roots did not contain infected cells. Only intercellular infections were observed (Figure 7a-d). This shows that a functional ethylene signaling pathway is essential for intracellular infection of *Parasponia* nodules.

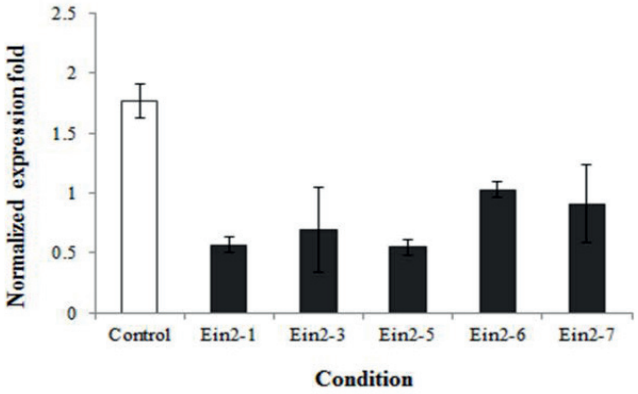

Figure 4. *PanEIN2* expression in control (white bar) and *PaEIN2* RNAi knockdown roots (black bars, individual EIN2 Knocked down plants). Shown are expression analysis of independently transformed roots containing *PanEIN2* RNAi construct.

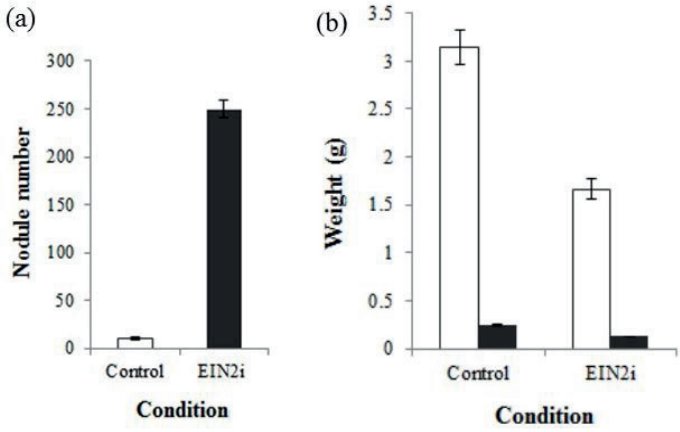

Figure 5. Average number of nodules per transgenic root in control (white bar) and *PanEIN2* knocked down (black bar) in *Parasponia* plants (a). Nodule fresh weight (white bar) and dry weight (black bar) in control (transformed with empty vector) and *PanEIN2* knocked down plants (b).

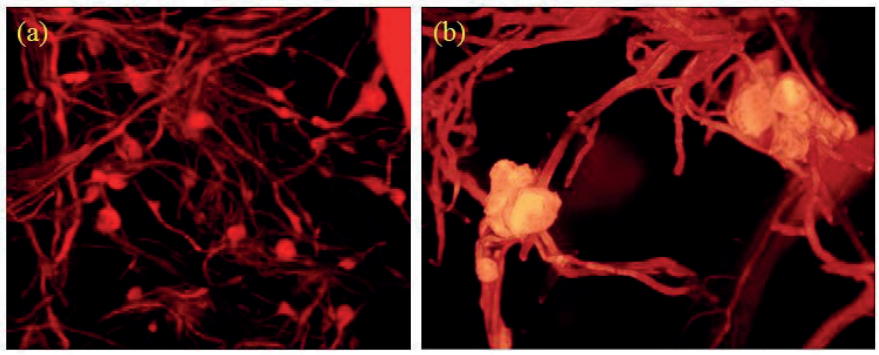

Figure 6. Nodulation phenotype of the *Parasponia andersonii PanEIN2* RNAi mutant (a) and Control (b) (transformed with empty vector). Nodules are monitored 8 weeks after inoculation with *Bradyrhizobium elkanii* (WUR3) and selected based on red florescence due to DsRED1 expression.

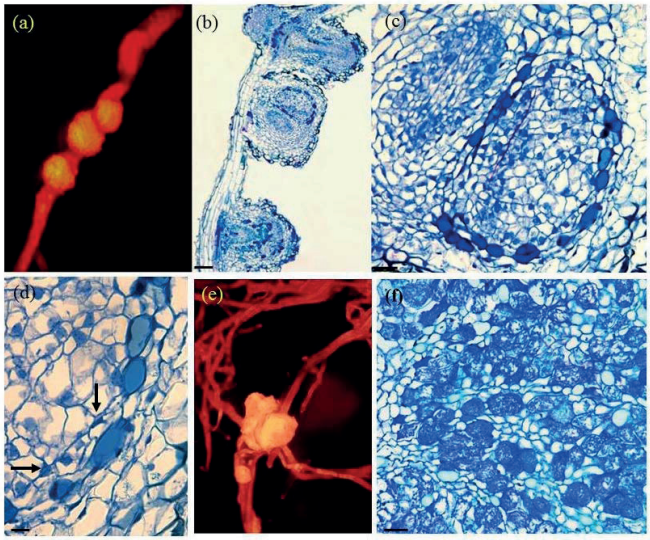

Figure 7. Nodulation and infection phenotype of the *Parasponia anadersonii EIN2* mutants. Longitudinal section of control and *EIN2* RNAi knock down in *Parasponia* plants. (a) Nodule formed on transgenic *Parasponia PanEIN2* knocked down mutants (selected based on red florescence due to DsRED1 expression). Scale bar: 0.5 mm. (b) Cross section of nodule on mutant plants. Scale bar 75µm. (c) Longitudinal section of nodule on *PanEIN2* knocked down plants. Scale bars 50 µm. (d) detail of panel c, only intercellular colonization occurred in *PanEIN2* RNAi nodules (arrows) and no intracellular infection was observed .Scale bar: 25µm. (e) Nodule formed on transgenic *Parasponia transformed* with empty vector (selected based on red florescence due to DsRED1 expression). Scale bar: 0.5 mm. (f) Cross section of nodule infected cells on control plants. Scale bars 50µm.

Discussion

In a diverse range of legume species it is reported that ethylene has a negative role in nodulation initiated by rhizobia (Oldroyd *et al.*, 2001; Nukui *et al.*, 2000; Goodlass and Smith, 1979; Lee and LaRue, 1992; Penmetsa and Cook, 1997). Here we provide evidence that ethylene has similar effect on *Parasponia* root nodule formation. Nodule number dramatically increased upon knockdown of the ethylene signaling gene *PanEIN2*. In addition, we found that *PanEIN2* RNAi nodules are hampered in intracellular infection, a phenotype unknown in legumes. This indicates that in *Parasponia* ethylene signaling plays a dual role in root nodule formation.

The gaseous hormone ethylene is synthesized via a methionine dependent pathway, in where methionine is enzymatically converted to ethylene in 3 subsequent steps in which methionine is converted in to ethylene with 1-aminocyclopropane-1- carboxylate (ACC) as intermediate. In this biosynthetic pathway production of ACC by ACC synthase is considered the rate limiting step. Application of the ACC synthase inhibitor (AVG) was resulted in an increase in the number of nodules in legumes, whereas the opposite effect was obtained upon application of exogenous ACC (Peters and Crist-Estes, 1989; Schmidt *et al.*, 1999; Penmetsa and Cook, 1997; Nukui *et al.*, 2000). In accordance, in current study similar results were obtained in *Parasponia*, which shows that ethylene acts as a generic inhibitor of rhizobium root nodule formation.

Although the response of legume species to ethylene can vary, exogenous ethylene generally affects the frequency of nodule primordia formation (Nukui *et al.*, 2000). The underlying molecular mechanisms remain unknown. Studies in pea suggest that transcriptional regulation of the biosynthetic pathway may function as a regulatory mechanism. In situ hybridization studies indicated that ACC oxidase expression is spatially regulated, and in this way restricting the positioning of nodule primordia. Interfering with this spatial regulation –or alternatively the ethylene signaling pathway- affects not only number of root nodules, but also their positioning in respect to the xylem axis in legume root (Heidstra *et al.*, 1997; Penmetsa and Cook, 1997). It remains to be studied whether a similar positioning also in *Parasponia* correlates with spatial regulation of ethylene biosynthesis.

Studies in *Medicago* and *Lotus* revealed different phenotypes when mutating or knocking down *EIN2* genes. In *Medicago*, zones of hyper-nodulating clusters are formed that are interspaced with non-nodulating root segments (Penmetsa and Cook, 1997). In contrast to *Medicago* that *MtEIN2* is a single gene, two paralogous *LjEIN2* genes have been identified in *Lotus* (Miyata *et al.*, 2013). Knocking down both these genes resulted in significantly more nodules when compared to wild type roots, though hypernodulating root segments interspaced with non-nodualting segments have not been reported (Miyata *et al.*, 2013). Similarly, in *Parasponia* we did not observe such hypernodualting clusters. This indicates that the hypernodulating clusters are a phenotype unique to *Medicago.* Alternatively, the studies in *Lotus* (Miyata *et al.*, 2013) and ours in *Parasponia* are based on RNAi, which may result in some functional EIN2 protein. This may explain the difference in nodulation phenotype with *Medicago*, which is an E.M.S. knock out mutant.

Our results showed that in *Parasponia PanEIN2* signaling is essential for intracellular infection of nodule cells. Such role for ethylene signaling has not been found in legumes, suggesting a novel function of this highly conserved pathway in *Parasponia*. However, it remains open whether *Parasponia* adapted its ethylene signaling cascade, or more specifically, the functioning of *EIN2* to form nitrogen fixing root nodules. Studies in *Arabidopsis* have shown that the C-terminal part of AtEIN2 can act as a transcriptional modulator, there by stabilizing transcription factors AtEIN3 and AtEIL1 (Tsuda and Somssich, 2015). Binding of ethylene to ER-localized ETR-type receptors modulates their phosphorylation activity, although precise regulation remains elusive (Merchante *et al.*, 2013). Ethylene binding results in inactivation of the negative regulatory kinase CTR1, which is associated with the ETR–type receptors. Deactivation of CTR1 triggers proteolytic cleavage of EIN2, allowing the C-terminal domain to commits its function. Identification of the interacting transcription factor complex in *Parasponia* (and/or legumes) may provide insight to what extend evolution of the ethylene-*EIN2* signaling network is constraint in higher plants.

Materials and Methods

Construction of Parasponia EIN2RNAi vector

Degenerate primers were designed based on the sequences of *AtEIN2* (*Arabidopsis thaliana*), *PtEIN2* (*Populus trichocarpa*), *VvEIN2* (*Vitis vinifera*), *LjEIN2* (*Lotus japonicas*) and *MtEIN2* (*Medicago truncatula*) in order to amplify *EIN2*-like genes from *P. andersonii*: (forward (GATGGRGYTGATGAGGATCT) and reverse (CCCCTGGCTGGTTTDGMAGC)). Subsequent PCR on root cDNA of *P. andersonii* resulted in 226 bp amplicon. Partial sequence of *Parasponia EIN2* gene was cloned into a pENTR-D-Topo plasmid (forward (CACCACAGTGTTTGTATCAGAG), reverse (TTTCCATGGCAGCTGAGAATATT)). Subsequent RNAi construct was made by recombination of the amplified region into the binary vector pK7GWIWG2 (II) driven by the CaMV35S promoter as described in Limpens et al. (2005) (Limpens *et al.*, 2004). pK7GWIWG2(II) contains pAtUBQ10::DsRED1 of pRedRoot as selection marker (Limpens et al., 2004).

Plant transformation and nodulation assay

Agrobacterium rhizogenes-mediated hairy roots transformation was used to transform *Parasponia andersonii* as described in (Cao *et al.*, 2012). Transgenic roots were selected based on *DsRED1* expression. Transgenic roots were transferred to low nutrient EKM [pH 6.6; 100ml Macro elements 10X (g/liter): KH_2PO_4:1.2, K_2HPO_4:3.6, $MgSO_4.7H_2PO_4$:2.5, Na_2SO_4:1.0; 1ml Micro elements (mg/100ml): $MnSO_4$:100, $ZnSO_4.7H_2O$:25, $CuSO_4.5H_2O$: 25, H_3BO_3: 25; 1ml Fe-citrate (15mM); CaSO4.2H2O: 0.25; NH4NO3: 0.02] medium. Plant transformed with empty vector was investigated as control plants. After two weeks, *PaEIN2* RNAi and empty vector control plants were transferred to the pots and inoculated with *Bradyrhizobium elkanii* WUR3 at a final optical density of 0.15 by directly irrigating after planting in the root. The cultured plants were irrigated three times per week; once with EKM medium (supplemented with 0.2 mM potassium nitrate) and two times with water.

Analysing of RNA seq data

Primary analysis was done based on 100 bp paired-end read sequencing of the *Parasponia* transcriptome. Reads were mapped with HiSat2 and mapped to the *PanEIN2* gene model and counted. Finally raw reads were normalised with DeSeq2 which resulted in normalized counts.

Quantitative RT-PCR

RNA was isolated from snap-frozen roots samples using the plant RNA kit (E.Z.N.A, Omega Biotek, Norcross, USA) as described in the manufacturer protocol. cDNA was synthesized from 1µg total RNA using i-script cDNA synthesis kit (Bio-Rad, Hercules, USA) as described in the manufacturer protocol. Quantitative RT-PCR has been performed using SYBR green based detection (Eurogentec, Maastricht, the Netherlands). Experimental setup and execution have been conducted using a MyIQ optical cycler, according to protocol provided by manufacturer (Biorad, Hercules, USA). Data analysis was performed using BioRad iQ5 software (BioRad). Baselines were set at 100 RFU to calculate the Ctvalues, Ct values of 31 and higher were excluded from the analysis.

Primers used:

PaACT-qF CCTCATTGGAATGGAAGCAC

PaACT-qR TTCCAGGAAACATGGTGGAC
PaEIN2-qF ATGAATCGGCTAAGTCAAGT
PaEIN2-qR GGCTTTCCATAAGTGAGAGG

Microscopy analysis

Fixation of nodules was performed in 5% glutaraldehyde (v/v) and 3% sucrose (w/v) dissolved in phosphate buffer (pH7.0). Vacuum infiltration of this solution was applied for at least 1 hour. Subsequently an ethanol dehydration series was carried out. Plastic infiltration of samples was done in four steps, included solution A (100ml Technovit7100, 1pack HrdnerI, 2.5ml PEG400):100% ETOH in 1:3, 1:1, 3:1 ratio respectively for 30-60min in room temperature and finally treated with 100% solution A for overnight at the 4°C. All material was transferred into cupules and nodules located in the middle, solution A was removed and polymerization solution (15ml Solution A, 1ml Hardener II) was added immediately. To remove air from the samples, cupules were covered with parafilm and left for overnight at room temperature. After polymerization, holders were put on the blocks and holding solution (technovit3040: 2part powder, 1 part liquid) was added from the hole located in the centre of holder and kept for 15 min at room temperature.

Finally sectioning of nodules was performed using a microtome machine and the samples were analysed by microscopy (Leica) after staining with toluidine blue (0.5%) buffer and washing with tap water for 5 minutes.

Application of the ethylene biosynthesis activator and inhibitor and plant inoculation

ACC (A–3903; Sigma) and AVG (A–1284; Sigma) were dissolved in sterile distillated water and stored at 4°C. Chemicals were added to 50 mL autoclaved EKM medium in a final concentration of 50 and 10 μM respectively at 45 to 50°C, mixed, and then poured in petri dishes. *Baradyrhizobium elkanii* strain was grown by standard procedures for this genus in YMB medium (pH 6.8 (g/liter: Manitol: 10.0, K_2HPO_4: 0.5, $MgSO_4.7H_2O$: 0.2, NaCl: 0.1, Yeast Extract: 0.5, pH: 6.8) for three days at 28 °C with shaking at 250 rpm. The optical density of cell culture was determined with nanodrop and centrifuged in 4000 rpm for 10 minutes. The final pallet was dissolved in sterile water and adjusted at the final optical density of 0.15. *Parasponia* roots were immerged in this solution for few three seconds and cultured in EKM medium for 8 weeks at 28°C.

References

1Behm, J.E., Geurts, R., and Kiers, E.T. (2014) Parasponia: a novel system for studying mutualism stability. *Trends in plant science* 19, 757-763.

Caba, J., Recalde, L., and Ligero, F. (1998) Nitrate-induced ethylene biosynthesis and the control of nodulation in alfalfa. *Plant, Cell & Environment* 21, 87-93.

Cao, Q., Den Camp, R.O., Kalhor, M.S., Bisseling, T., and Geurts, R. (2012) Efficiency of Agrobacterium rhizogenes–mediated root transformation of Parasponia and Trema is temperature dependent. *Plant Growth Regulation* 68, 459-465.

Ding, Y., Kalo, P., Yendrek, C., Sun, J., Liang, Y., Marsh, J.F., Oldroyd, G.E. (2008) Abscisic acid coordinates nod factor and cytokinin signaling during the regulation of nodulation in Medicago truncatula. *The Plant Cell* 20, 2681-2695.

Goodlass, G. and Smith, K. (1979) Effects of ethylene on root extension and nodulation of pea (<i>Pisum sativum</i> L.) and white clover (<i>Trifolium repens</i> L.). *Plant and Soil* 51, 387-.

Gresshoff, P.M., Lohar, D., Chan, P.-K., Biswas, B., Jiang, Q., Reid, D., . . . Stacey, G. (2009) Genetic analysis of ethylene regulation of legume nodulation. *Plant signaling & behavior* 4, 818-823.

Guinel, F.C. and LaRue, T.A. (1992) Ethylene inhibitors partly restore nodulation to pea mutant E 107 (brz). *Plant Physiology* 99, 515-518.

Heidstra, R., Yang, W.C., Yalcin, Y., Peck, S., Emons, A., and Bisseling, T. (1997) Ethylene provides positional information on cortical cell division but is not involved in Nod factor-induced root hair tip growth in Rhizobium-legume interaction. *Development* 124, 1781-1787.

Imanishi, L., Perrine-Walker, F.M., Ndour, A., Vayssières, A., Conejero, G., Lucas, M., . . . Svistoonoff, S. (2014) Role of auxin during intercellular infection of Discaria trinervis by Frankia. *Frontiers in plant science* 5.

Lee, K.H. and LaRue, T.A. (1992) Exogenous ethylene inhibits nodulation of Pisum sativum L. cv Sparkle. *Plant Physiology* 100, 1759-1763.

Ligero, F., Lluch, C., and Olivares, J. (1986) Evolution of Ethylene from Roots of< i> Medicago sativa</i> Plants Inoculated with< i> Rhizobium meliloti</i>. *Journal of plant physiology* 125, 361-365.

Lohar, D., Stiller, J., Kam, J., Stacey, G., and Gresshoff, P.M. (2009) Ethylene insensitivity conferred by a mutated Arabidopsis ethylene receptor gene alters nodulation in transgenic Lotus japonicus. *Annals of botany* 104, 277-285.

Merchante, C., Alonso, J.M., and Stepanova, A.N. (2013) Ethylene signaling: simple ligand, complex regulation. *Current opinion in plant biology* 16, 554-560.

Merchante, C., Brumos, J., Yun, J., Hu, Q., Spencer, K.R., Enríquez, P., Alonso, J.M. (2015) Gene-specific translation regulation mediated by the hormone-signaling molecule EIN2. *Cell* 163, 684-697.

Miyata, K., Kawaguchi, M., and Nakagawa, T. (2013) Two distinct EIN2 genes cooperatively regulate ethylene signaling in Lotus japonicus. *Plant and cell physiology* 54, 1469-1477.

Nukui, N., Ezura, H., Yuhashi, K.-I., Yasuta, T., and Minamisawa, K. (2000) Effects of ethylene precursor and inhibitors for ethylene biosynthesis and perception on nodulation in Lotus japonicus and Macroptilium atropurpureum. *Plant and Cell Physiology* 41, 893-897.

Oldroyd, G.E., Engstrom, E.M., and Long, S.R. (2001) Ethylene inhibits the Nod factor signal transduction pathway of Medicago truncatula. *The Plant Cell* 13, 1835-1849.

Op den Camp, R.H., Polone, E., Fedorova, E., Roelofsen, W., Squartini, A., Op den Camp, H.J., . . . Geurts, R. (2012) Nonlegume Parasponia andersonii deploys a broad rhizobium host range strategy resulting in largely variable symbiotic effectiveness. *Molecular Plant-Microbe Interactions* 25, 954-963.

Penmetsa, R.V. and Cook, D.R. (1997) A legume ethylene-insensitive mutant hyperinfected by its rhizobial symbiont. *Science* 275, 527-530.

Penmetsa, R.V., Frugoli, J.A., Smith, L.S., Long, S.R., and Cook, D.R. (2003) Dual genetic pathways controlling nodule number inMedicago truncatula. *Plant Physiology* 131, 998-1008.

Peters, N.K. and Crist-Estes, D.K. (1989) Nodule formation is stimulated by the ethylene inhibitor aminoethoxyvinylglycine. *Plant Physiology* 91, 690-693.

Plet, J., Wasson, A., Ariel, F., Le Signor, C., Baker, D., Mathesius, U., . . . Frugier, F. (2011) MtCRE1-dependent cytokinin signaling integrates bacterial and plant cues to coordinate symbiotic nodule organogenesis in Medicago truncatula. *The Plant Journal* 65, 622-633.

Prayitno, J., Rolfe, B., and Mathesius, U. (2006a) The ethylene-insensitive sickle mutant of Medicago truncatula shows altered auxin transport regulation during nodulation. *Plant Physiol* 142, 168 - 180.

Prayitno, J., Rolfe, B.G., and Mathesius, U. (2006) The Ethylene-Insensitive sickle Mutant of Medicago truncatula Shows Altered Auxin Transport Regulation during Nodulation. *Plant Physiology* 142, 168-180.

Schmidt, J.S., Harper, J.E., Hoffman, T.K., and Bent, A.F. (1999) Regulation of soybean nodulation independent of ethylene signaling. *Plant physiology* 119, 951-960.

Sun, J., Cardoza, V., Mitchell, D.M., Bright, L., Oldroyd, G., and Harris, J.M. (2006) Crosstalk between jasmonic acid, ethylene and Nod factor signaling allows integration of diverse inputs for regulation of nodulation.

Takanashi, K., Sugiyama, A., and Yazaki, K. (2011) Involvement of auxin distribution in root nodule development of Lotus japonicus. *Planta* 234, 73-81.

Tsuda, K. and Somssich, I.E. (2015) Transcriptional networks in plant immunity. *New Phytologist* 206, 932-947.

Untergasser, A., Bisseling, T., and Geurts, R. (2008) Making rhizobium-infected root nodules. In *Prokaryotic symbionts in plants*, pp. 45-69, Springer.

van Zeijl, A., den Camp, R.H.O., Deinum, E.E., Charnikhova, T., Franssen, H., den Camp, H.J.O., . . . Geurts, R. (2015) Rhizobium lipo-chitooligosaccharide signaling triggers accumulation of cytokinins in Medicago truncatula roots. *Molecular plant* 8, 1213-1226.

Yu, Y.-B., Adams, D.O., and Yang, S.F. (1979) 1-Aminocyclopropanecarboxylate synthase, a key enzyme in ethylene biosynthesis. *Archives of biochemistry and biophysics* 198, 280-286.

Chapter 7

General Discussion

Parasponia-Trema a key system to transfer symbiosis to other non-legumes

Species of the *Parasponia* genus are the only known non-legumes capable to establish N_2 fixing symbiosis with rhizobium bacteria. As *Parasponia* and legumes are only remotely related, with a last common ancestor ~100 million years ago, it seems most probable that both lineages have gained the symbiotic trait by independent evolutionary events. Although the *Parasponia*-rhizobium symbiosis was discovered early twenty century (Bernard, 1916), it took till 1973 before *Parasponia* plants were introduced in the lab for research (Trinick, 1973). The initial research in that time was to investigate whether rhizobium symbiosis is unique to *Parasponia*, or also occurs with some closely related sister species of the genus *Trema*. Also, the rhizobium-symbiosis of *Parasponia* was compared with legumes to characterize the commonalties and differences.

Parasponia and *Trema* species are fast growing trees that form pioneer vegetation in the tropics. Species of both genera are morphologically very similar, resulting in several incorrect identifications suggesting that the rhizobium root nodule symbiosis also occurs in *Trema sp.* (Trinick, 1973; Coventry *et al.*, 1976; Trinick and Galbraith, 1976). In 1978, extensive examination in New Guinea and Java on *Trema orientalis* failed to find any nodules. Therefore rhizobium symbiotic relationship within the Cannabaceae (that time still part of the Ulmaceae) was restricted to *Parasponia* (Akkermans *et al.*, 1978). This genus encompasses five species -*P. andersonii, P. melastomatifolia, P. parviflora, P. rigida* and *P. rugosa*- that show a restricted geographical distribution in the Malay Archipelago, where they can be found on slopes of volcanic mountains (**Figure 1**). In contrast, *Trema sp*. can be found pan-tropically. Compared to *Parasponia*, *Trema* sp. is broadly distributed, indicating that their ecological niche is less restricted.

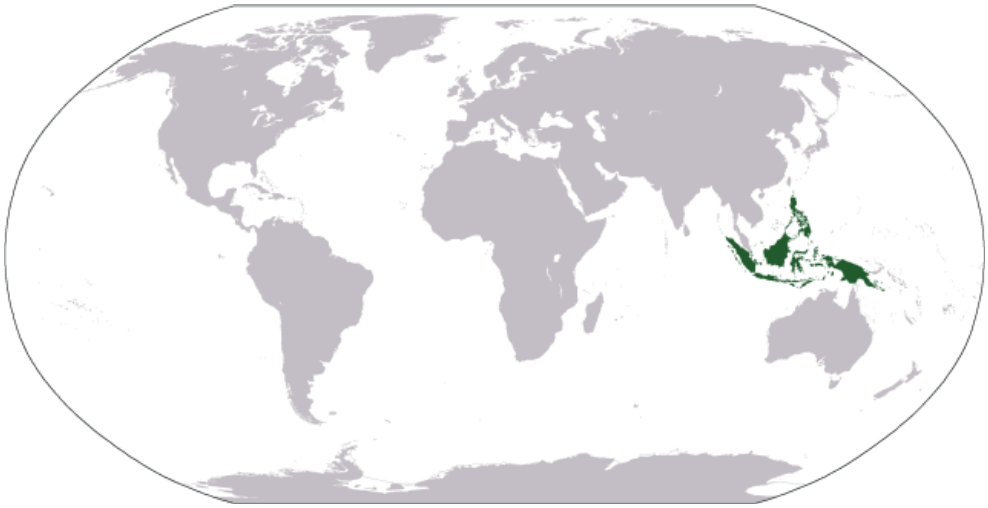

Figure1. Geographical distribution of *Parasponia* sp..

Recent molecular phylogenetic studies based on four chloroplast genes revealed that the *Parasponia* lineage is imbedded in the genus *Trema* (Yang *et al.*, 2013). This further indicates that *Parasponia* and *Trema* are closely related. According to current taxonomic rules, *Parasponia* species may have been included in the genus *Trema*. Since rhizobium nitrogen fixing symbiosis is exclusively occurring on *Parasponia* sp., I argue that *Parasponia* –including it symbiotic trait- evolved relatively recent from an ancestral *Trema* species. As the legume-rhizobium symbiosis is estimated to be ~60 million years old, I assume that this symbiosis with *Parasponia* is significantly younger.

Initial studies of field-collected nodules of 3 *Parasponia* species suggests that these plants are exclusively nodulated by *Bradyrhizobium* sp. (Trinick, 1980). However, experimental work in the laboratory revealed a much broader host range for *P. andersonii* indicating high promiscuity (Op den Camp *et al.*, 2012). Cross-inoculation experiments of these strains with legumes reveal that *Parasponia* and legumes can form root nodules with the same rhizobial strains. This indicates that the underlying mechanisms of nodule formation, intracellular infection, nitrogen fixation and establishing a symbiotic interface to exchange nutrients are largely conserved. Therefore I hypothesize that genetic constraints have guided the evolution of the nitrogen fixing rhizobium symbiosis trait in both lineages. A comparison of *Parasponia* to legumes will provide insights in these constrains. Additionally, as the symbiosis of *Parasponia* is anticipated to be

much younger than the symbiosis in legumes, *Parasponia-Trema* can be used as a comparative system of highly similar plant species that differs in the nitrogen fixing symbiosis trait.
Although the *Paraponia* and *Trema* genera only represent tropical trees, they set seeds within 6 to 9 months after planting (Becking, 1992). Nevertheless, as experimental system for laboratory experiments, it is essential that *in vitro* propagation and transformation protocols are established. For *Parasponia* such methods have been established already in the 90 decade, and these protocols recently have been extended to *Trema tomentosa* (Davey *et al.*, 1993; Cao *et al.*, 2012). Additionally, transformation protocols have been established for both species. Initially these protocols focussed on *Agrobacterium rhizogenes*-based root transformation. This method is commonly used in legumes, as it is relatively fast. Several studies indicated that *A. rhizogenes*-mediated root transformation can be used on *P. andersonnii* and *T. tomentosa* as well (den Camp *et al.*, 2011; Cao *et al.*, 2012; Granqvist *et al.*, 2015) (**Chapter 2, 4, 5** and **6** in this thesis). However, *A. rhizogenes* mediated transformation systems have several disadvantages; including phenotypic variation between individual roots and the presence of the bacterial *rol* genes that affect the plant hormone homeostasis. To overcome this problem *Agrobacterium tumefaciens*-based stable transformation can be applied. I tested whether such method is applicable for *T. tomenotsa*, and found that generating stably transformed plants is relatively straightforward (**Chapter 3**). In combination with the *in vitro* propagation protocols, an unlimited number of clones can be generated from primary transformed plant lines. This reduces the importance of generative propagation, and will allow affective research on this tree species. To establish *Parasponia-Trema* as a comparative research system it will be essential to establish a similar effective transformation protocol for *Parasponia.*

Distinct effect of nitrate on Parasponia-rhizobium interaction

Nitrate retards the development of nodules on all legume species (Streeter and Wong, 1988; Barbulova *et al.*, 2007; Sagan *et al.*, 1995). Nitrate is also responsible for decreasing the level of nitrogen fixation in existing nodules (Carroll and Gresshoff, 1983; Fujikake *et al.*, 2002). In our study on the effect of exogenous nitrate on root nodulation in *Parasponia* (**Chapter 4**) we initially addressed the question whether similar responses as reported for legumes can be also observed in *Parasponia*. We found that nodulation efficiencies of *Parasponia* are reduced in presence of exogenous nitrate, but even at a concentration of 50 mM KNO_3 still a few nodules

were formed. Strikingly, such nodules formed in presence of high nitrate level (>10 mM KNO_3) are defected to fix nitrogen, due to impaired intracellular infection. Although these nitrate grown nodules all contained infected cells, the formation of fixation threads was hampered. Transition from infection thread to fixation thread formation is a developmental switch that controls the adaptation of host cells to accommodate rhizobia with whom it aims to exchange nutrients. Fixation threads form a major expansion inside the cell interior, which requires modulation of the plant vacuole. In *Parasponia* nodule cells that are infected with rhizobium the vacuoles shrink, get fragmented and even can disappear completely, similar as reported for legumes that host rhizobia as transient nitrogen-fixing organelle-like structures (Gavrin *et al.*, 2014). Fixation threads are largely deprived from cell wall structure, allowing a better exchange of nutrients between both partners. Absence of this interface between plant and microbe defects the symbiosis, and may therefore be a very effective way to avoid exploitation by the biotrophic rhizobial microbes.

Why the mechanism of microbial control at the level of the formation of the symbiotic stage has not been found in legumes may have several reasons. First, this mechanism may be functional only in species that form fixation threads. This mode of infection is not exclusive for *Parasponia*, but also occur in *Andira* and some *Chamaecrista* species (de Faria *et al.*, 1986; Naisbitt *et al.*, 1992). However studies on the effect of exogenous nitrate have been limited to legume crops (Lupin, Chickpea, Soybean and Bean) and the models *Medicago truncatula* and *Lotus japonicus*. Therefore, extending these studies to more basal legumes will be relevant. Secondly, legumes may have evolved novel adaptations to control the rhizobium symbiosis, which are not present in *Parasponia*. For example, exogenous nitrate fully blocks formation of root nodules in the studied legumes (Barbulova *et al.*, 2007; Carroll and Gresshoff, 1983), which makes an additional mechanism to control the microbial partner at a later stage superficial. In contrast to legumes, rhizobium LCO induced signaling is not blocked in *Parasponia*. Studies presented in this thesis show that LCOs trigger for example calcium oscillations in root epidermal cells of *Parasponia* roots grown in presence of exogenous nitrate. An activated LCO signaling machinery will ultimately lead to the onset of root nodule organogenesis.

The rhizobium symbiosis in legumes as well as *Parasponia* is founded on the much older arbuscular mycorrhzal symbiosis (Szczyglowski and Amyot, 2003). Genetic studies identified so-called common symbiosis signaling genes that are essential for rhizobium and arbuscular

mycorrhizal induced signaling (Parniske, 2008). In line with this, is the finding that rhizobium and mycorrhizal fungi produce structurally very similar LCO signaling molecules (Gough and Cullimore, 2011). In model legumes LysM-type receptor kinases have been identified that specially recognize rhizobium LCOs, whereas these receptors are not essential for mycorrhzae. This indicates that perception mechanisms of rhizobium and mycorrhizal signals have been diverged in course of legume evolution. Since the *Parasponia-rhizobium* symbiosis is considered to be much younger, it can be anticipated that neofunctionalization of rhizobium specific features is less advanced when compared to legumes. This hypothesis is supported by the finding from a LysM-type receptor –PaNFP- that commits a dual function in rhizobium and endomycorrhizal symbiosis (den Camp *et al.*, 2011).

Abuscular mycorrhization and nodulation are very different symbioses in the terms of host responses and microbe involvement (Kosuta *et al.*, 2003). Above all, arbuscular mycorrhizal fungi do not trigger cell divisions in the root cortex. However, in both interactions intracellular hosting of the microsymbiont is key to establish a functional symbiosis (Oldroyd *et al.*, 2009). In case of mycorrhizal fungi this leads to highly branched membrane compartments -known as arbuscules- in root cortical cells. These arbuscules facilitate nutrient exchange. The cellular machinery controlling intracellular hosting of arbuscular mycorrhizal fungi or rhizobium bacteria is very similar. For example, in both symbionts the same vascular and membrane target SNARE proteins are used (Limpens *et al.*, 2009).

Arbuscular mycorrhizal symbiosis is established when the plant experience nutrient deficiencies, especially phosphates. Arbuscular mycorrhzal fungi also facilitate the uptake of exogenous nitrates and supply them to the plant host in return for carbohydrates. However, the underlying mechanisms that plants are used to manipulate this flux of nutrients remain largely unknown. Since the rhizobium nitrogen fixing root nodule symbiosis is founded on arbuscular mycorrhizal symbiosis, it is tempting to speculate that the mechanism employed by *Parasponia* to control rhizobium at presence of high exogenous nitrates is derived from arbuscular mycorrhiza.

Shared features of the Parasponia and Legume rhizobium symbioses

To identify shared functional features between legumes and *Parasponia* in respect to rhizobium symbiosis, it was studied whether the transcriptional regulators NODULATION SIGNALING PATHWAY1 (NSP1) and NSP2 commit symbiotic functions in *Parasponia*. NSP1 and NSP2

belong to the GRAS proteins; a conserved family of plant-specific transcriptional regulators that play diverse roles in root and shoot development. In the legumes *Medicago* and *Lotus NSP1* and *NSP2* are essential for rhizobium LCO signaling and nodule formation (Kaló *et al.*, 2005; Hirsch *et al.*, 2009; Heckmann *et al.*, 2006). Furthermore, knockout mutants of *Medicago* or *Lotus nsp1* or *nsp2* display mild mycorrhizal phenotypes in which the *nsp1* and *nsp2* mutants show reduced hyphal colonization (Takeda *et al.*, 2013; Maillet *et al.*, 2011). In addition it was found that both proteins are essential transcriptional regulators of *DWARF27*, a gene encoding a key enzyme in the strigolactone biosynthesis pathway (Liu *et al.*, 2011). As strigolactones are not only exuded signals that are perceived by arbuscular mycorrhizal fungi, but also endogenous hormones, it may explain why *NSP1* and *NSP2* are conserved in higher plants, including *Arabidopsis* (Liu *et al.*, 2011).

In my research I found that *NSP1* and *NSP2* are also essential for *Parasponia* root nodule formation, whereas the mycorrhizal infection is only slightly reduced. This phenotype is very similar as reported for *Medicago*, and underlines that recruitment of both proteins to commit synmbiotic functions are guided by genetic constraints.

The molecular functioning of *NSP1* and *NSP2* in a symbiotic context remains unclear. Early studies suggested that both proteins are primary response factors that activate transcription upon LCO perception (Smit *et al.*, 2005). This hypothesis was further supported by the identification of *NSP1*-NSP2 binding sites in the promoter of the *MtENOD11* LCO responsive gene. However, other studies indicate that *NSP1* and *NSP2* are not essential components of the LCO signaling pathway, but rather may function as facilitators (Limpens and Bisseling, 2014). Mutations in such facilitator proteins may affect the readout of the LCO signaling pathway in such a way that symbiotic responses that are associated with root nodule formation are ceased.

Ethylene-mediated negative control of Parasponia nodulation provides insights in genetic constraints underlying rhizobium symbiosis

In legumes an appropriate level of nitrogen fixing root nodules is beneficial to the host plant, but excessive nodulation diminishes plant growth. Therefore, to control the number of nodules and to maintain proper symbiotic balance, host plants have evolved mechanisms to regulate nodulation. This so-called autoregulation of nodulation (AON) functions in shoots and is based

on a root-to-shoot long-distance signaling (Nutman, 1952; Pierce and Bauer, 1983; van Brussel *et al.*, 2002). Genetic mutations in AON result in a markedly increased number of nodules.

In addition to the systemic AON mechanism, the gaseous phytohormone ethylene plays important roles in the negative regulation of nodulation. The role of ethylene in physiological processes throughout the life cycle of the plant has been intensively studied. Among other processes, ethylene regulates developmental processes like senescence and abscission, but also responses to biotic stresses that include pathogens and in case of legumes rhizobium.

The inhibitory effect of ethylene on nodulation has been studied pharmacologically as well as genetically. Among the strongest evidence that ethylene signaling affects root nodule formation has been obtained by studying the *sickle* mutant in *Medicago trancatula* (Medicago) (Penmetsa and Cook, 1997). *SICKLE* encodes the *Medicago* ortholog of *EIN2* (*ETHYLENE INSENSITIVE2*) (Varma Penmetsa *et al.*, 2008). The *EIN2* gene was initially identified in *A. thaliana* and the encoded protein plays a central role in signal transmission leading to transcriptional responses (Jun *et al.*, 2004; Alonso *et al.*, 1999; Shibuya *et al.*, 2004; Varma Penmetsa *et al.*, 2008). *EIN2* mutants have been generated in several other plant species, including *Rice*, *Petunia, Lotus* and *Medicago* (Jun *et al.*, 2004; Shibuya *et al.*, 2004; Varma Penmetsa *et al.*, 2008). Detailed phenotypic analyses of *Medicago sickle/ein2* mutants revealed that ethylene signaling negatively regulates rhizobium LCO-induced oscillations of the nuclear calcium concentration (also known as calcium spiking), LCO induced gene expression, the number of nodules formed, the positioning of nodule primordia in the root, and growth of infection threads (Oldroyd *et al.*, 2001; Penmetsa and Cook, 1997; Heidstra *et al.*, 1997). Similar studies, though less detailed, have been conducted in *Lotus*. In most plants *EIN2* is a single gene that commits the central role in ethylene signaling. However, *Lotus* harbors 2 paralogous genes of *EIN2*; *LjEIN2*-1 and *LjEIN2-2*, of which the latter is the putative ortholog of *MtSKL/MtEIN2*. Mutating *LjEIN2-2* (also named *ENIGMA*) affects radial positioning of nodules, but did not result in a hypernodulating phenotype as has been reported for *Medicago* (Chan *et al.*, 2013). However, by using RNAi to knockdown both paralogous genes nodulation significantly increased (Miyata *et al.*, 2013). This suggests that in *Lotus EIN2* functioning in nodulation is sub functionalized over two gene paralogs.

Since the regulatory role of ethylene signaling on root nodulation seems to be conserved in legumes, we investigated the symbiotic role of *EIN2* in *Parasponia*. Like most plant species, and

in contrast to *Lotus*, *Parasponia* contains only a single *EIN2* gene. Knocking down of this gene by RNAi resulted in a 20-fold increase of nodules (**Chapter 6**). This underlines that the negative effect of ethylene on nodulation is not legume specific, but also is present in *Parasonia*.

The question remains whether the ethylene signaling pathway has been adapted in legumes and *Parasponia*, and if so, how such adaptations may look like. Till now, knowledge of the molecular functioning of the ethylene signaling pathway has been largely revealed based on studies in *Arabidopsis*. Ethylene is perceived by a family of ER-localized receptors, which are negative regulators of the signaling pathway (Chang *et al.*, 1993; Hua *et al.*, 1995; Hua and Meyerowitz, 1998; Hua *et al.*, 1998; Sakai *et al.*, 1998). Downstream of these receptors acts the CTR1 kinase that –again as a negative regulator- controls the functioning of *EIN2*. The membrane localized EIN2 protein is a central transducer of the ethylene signal (Kieber *et al.*, 1993; Alonso *et al.*, 1999). If CTR1 is inactivated and cannot phosphorylate EIN2, this protein is proteolitically activated resulting in release of its C terminal part. The EIN2 C terminus is translocated to nucleus where it will activate a transcriptional network (Ju *et al.*, 2012; Qiao *et al.*, 2012; Wen *et al.*, 2012). In the absence of ethylene, the active receptors recruit CTR1 to phosphorylate the C-terminal domain of EIN2 to repress the downstream ethylene response.

To commits its regulatory function in root nodule formation the ethylene pathway could have experience adaptations at several levels. However, only three evolutionary scenarios can explain the functioning of the ethylene signaling in nodulation. (I.) The ethylene signaling pathway has not been adapted in legumes and *Parasponia*, but its negative role on nodulation is a pleiotropic effect of its generic functioning in plant growth and development. To my opinion this is the most unlikely scenario, since slight modulation of the ethylene homeostasis already affects the nodulation efficiency of the plant. (II.) Alternatively, the ethylene signaling cascade has been recruited to control root nodule formation. In such scenario the recruitment occurred in parallel in the *Parasponia* and legume lineages and may reflect an adaption that evolved after initial birth of the symbiosis. Recruitment of ethylene as negative regulator for nodulation can be achieved by adaptation of the expression domain of ethylene biosynthesis genes. For example, in pea it has been shown that an ACC oxidase gene is expressed in cortical cells opposite phloem poles, which may be causal for the fact that nodules are generally formed opposite xyleme poles (Heidstra *et al.*, 1997). To find support for the hypothesis that such spatial expression pattern is an evolutionary adaptation a comparative study on the expression profile of ethylene

biosynthesis and signaling genes in roots of legumes and non-legumes will be relevant. Additionally, reverse genetic studies in where expression domains are disturbed will be essential. (III.) A third evolutionary scenario is the adaptation of ethylene signaling pathway in such a way that it affects the readout of the LCO signaling pathway. The LCO signaling pathway is highly conserved in higher plant species as it is also essential for the more ancient and widespread mycorrhizal symbiosis. A central question that remains in rhizobium symbiosis research is which genetic changes have occurred so that upon the activation of this pathway cortical cell divisions are triggered. As ethylene has a negative effect on LCO signaling already at the level of calcium spiking (Oldroyd *et al.*, 2001), it can be envisioned that adaptations in ethylene homeostasis are essential to allow a symbiotic readout of this pathway in such a way that root nodule formation is triggered. To test this hypothesis it will be essential to modulate the ethylene signaling pathway in a non-nodulating plant species and monitor the effect of this modulation on the LCO signaling pathway.

Future Research

In this thesis I present transformation protocols for *T. tomentosa*, a close non-symbiotic relative of *Parasponia* species. Recent studies revealed that *T. tomentosa* does not display calcium oscillation in response to a complex mixture of LCO molecules extracted from the broad host range of strain *Sinorhizobium fredii* NGR234 (Granqvist *et al.*, 2015). This strain can nodulate hundreds of legume species as well as *Parasponia* (den Camp *et al.*, 2011; D'Haeze and Holsters, 2002; Pueppke and Broughton, 1999). In line with this *S. fredii* NGR234 LCOs trigger calcium spiking in *Parasponia* root hair cells (Granqvist *et al.*, 2015). The absence of this early symbiotic response (calcium spiking can be detected 10 min post LCO application), makes *T. tomentosa* an excellent species in a comparative approach to *Parasponia*.

I have conducted first experiments aiming to test the third evolutionary scenario on ethylene signaling as described above. I conducted RNAi of *T. tomentosa EIN2* using *A. rhizogenes* transformation. Of the 20 compound plants that have been generated, two contained nodule-like structures on their roots, 8 weeks post inoculation (Figure 2). Sectioning of these nodule like structures indicate massive cell divisions in pericycle and inner cortical cells, which were deprived from rhizobium intracellular infection. Therefore it can't be ruled out that these

structures were the result of growth deviations (e.g. derived from lateral root primordia). To rule this out the experiment should be reproduced, and additional symbiotic readouts should be monitored; e.g. symbiotic gene expression and/or calcium spiking. Also it is wise to generate stable lines to commit this research, rather than rely on *A. rhizogenes* transformation. A protocol for this has been presented in this thesis.

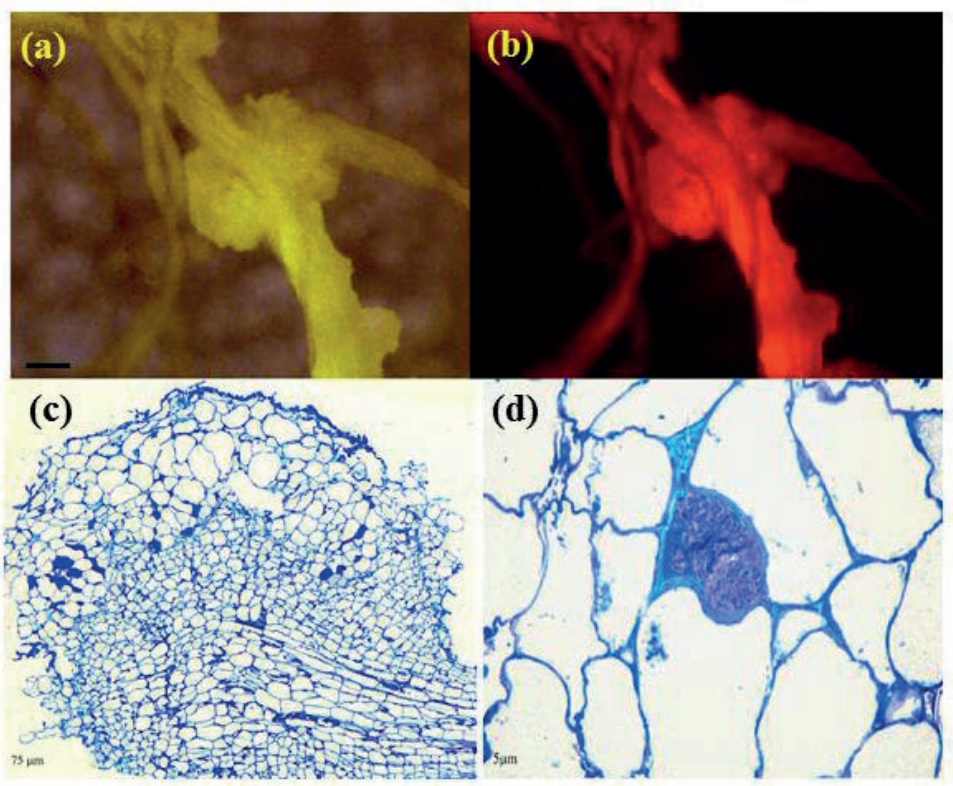

Figure 2. Nodule-like structure on *Trema tomentosa TtoEIN2* RNAi knocked down roots. Transgenic roots are selected based on red florescence due to DsRED1 expression. (a-b) Nodule-like structure phenotype on roots. Scale bar: 0.5 mm. (c) Cross section of nodule-like structures. Scale bar 75µm. (d) Detail of panel c. Scale bar 5µm. Roots monitored 8 weeks after inoculation with *Bradyrhizobium elkanii* (WUR3).

References

Akiyama, K., Matsuzaki, K.-i. & Hayashi, H. (2005). Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. *Nature* 435(7043): 824-827.

Akkermans, A., Abdulkadir, S. & Trinick, M. (1978). N2-fixing root nodules in Ulmaceae: Parasponia or (and) Trema spp.? *Plant and Soil* 49(3): 711-715.

Alonso, J. M., Hirayama, T., Roman, G., Nourizadeh, S. & Ecker, J. R. (1999). EIN2, a bifunctional transducer of ethylene and stress responses in Arabidopsis. *Science* 284(5423): 2148-2152.

Ané, J.-M., Kiss, G. B., Riely, B. K., Penmetsa, R. V., Oldroyd, G. E., Ayax, C., Lévy, J., Debellé, F., Baek, J.-M. & Kalo, P. (2004). Medicago truncatula DMI1 required for bacterial and fungal symbioses in legumes. *Science* 303(5662): 1364-1367.

Arrighi, J.-F., Barre, A., Amor, B. B., Bersoult, A., Soriano, L. C., Mirabella, R., de Carvalho-Niebel, F., Journet, E.-P., Ghérardi, M. & Huguet, T. (2006). The Medicago truncatula lysine motif-receptor-like kinase gene family includes NFP and new nodule-expressed genes. *Plant Physiology* 142(1): 265-279.

Barbulova, A., Rogato, A., D'Apuzzo, E., Omrane, S. & Chiurazzi, M. (2007). Differential effects of combined N sources on early steps of the Nod factor-dependent transduction pathway in Lotus japonicus. *Molecular Plant-Microbe Interactions* 20(8): 994-1003.

Beatty, P. H. & Good, A. G. (2011). Future prospects for cereals that fix nitrogen. *Science* 333(6041): 416-417.

Becking, J. (1992).The Rhizobium symbiosis of the nonlegume Parasponia. 497-559: Routledge: Chapman and Hall, NY, USA.

Behm, J. E., Geurts, R. & Kiers, E. T. (2014). Parasponia: a novel system for studying mutualism stability. *Trends in Plant Science* 19(12): 757-763.

Benková, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertová, D., Jürgens, G. & Friml, J. (2003). Local, efflux-dependent auxin gradients as a common module for plant organ formation. *Cell* 115(5): 591-602.

Bernard, C. (1916). *Groene bemesting.* Ruijgrok.

Besserer, A., Puech-Pagès, V., Kiefer, P., Gomez-Roldan, V., Jauneau, A., Roy, S., Portais, J.-C., Roux, C., Bécard, G. & Séjalon-Delmas, N. (2006). Strigolactones stimulate arbuscular mycorrhizal fungi by activating mitochondria. *PLoS Biol* 4(7): e226.

Boisson-Dernier, A., Chabaud, M., Garcia, F., Bécard, G., Rosenberg, C. & Barker, D. G. (2001). Agrobacterium rhizogenes-transformed roots of Medicago truncatula for the study of nitrogen-fixing and endomycorrhizal symbiotic associations. *Molecular Plant-Microbe Interactions* 14(6): 695-700.

Bravo, A., York, T., Pumplin, N., Mueller, L. A. & Harrison, M. J. (2016). Genes conserved for arbuscular mycorrhizal symbiosis identified through phylogenomics. *Nature Plants* 2: 15208.

Broghammer, A., Krusell, L., Blaise, M., Sauer, J., Sullivan, J. T., Maolanon, N., Vinther, M., Lorentzen, A., Madsen, E. B. & Jensen, K. J. (2012). Legume receptors perceive the rhizobial lipochitin oligosaccharide signal molecules by direct binding. *Proceedings of the National Academy of Sciences* 109(34): 13859-13864.

Buendia, L., Wang, T., Girardin, A. & Lefebvre, B. (2015). The LysM receptor-like kinase SlLYK10 regulates the arbuscular mycorrhizal symbiosis in tomato. *New Phytologist*.

Burrill, T. J. & Hansen, R. (1917). Is symbiosis possible between legume bacteria and non-legume plants? *Bulletin (University of Illinois (Urbana-Champaign campus). Agricultural Experiment Station); no. 202*.

Caba, J., Recalde, L. & Ligero, F. (1998). Nitrate-induced ethylene biosynthesis and the control of nodulation in alfalfa. *Plant, Cell & Environment* 21(1): 87-93.

Cao, Q., Den Camp, R. O., Kalhor, M. S., Bisseling, T. & Geurts, R. (2012). Efficiency of Agrobacterium rhizogenes–mediated root transformation of Parasponia and Trema is temperature dependent. *Plant Growth Regulation* 68(3): 459-465.

Carroll, B. J. & Gresshoff, P. M. (1983). Nitrate inhibition of nodulation and nitrogen fixation in white clover. *Zeitschrift für Pflanzenphysiologie* 110(1): 77-88.

Carroll, B. J., McNeil, D. L. & Gresshoff, P. M. (1985). A supernodulation and nitrate-tolerant symbiotic (nts) soybean mutant. *Plant Physiology* 78(1): 34-40.

Casimiro, I., Marchant, A., Bhalerao, R. P., Beeckman, T., Dhooge, S., Swarup, R., Graham, N., Inzé, D., Sandberg, G. & Casero, P. J. (2001). Auxin transport promotes Arabidopsis lateral root initiation. *The Plant cell* 13(4): 843-852.

Cerri, M. R., Frances, L., Kelner, A., Fournier, J., Middleton, P. H., Auriac, M.-C., Mysore, K. S., Wen, J., Erard, M. & Barker, D. G. (2016). The Symbiosis-Related ERN Transcription Factors Act in Concert to Coordinate Rhizobial Host Root Infection. *Plant Physiology*: pp. 00230.02016.

Cerri, M. R., Frances, L., Laloum, T., Auriac, M.-C., Niebel, A., Oldroyd, G. E., Barker, D. G., Fournier, J. & de Carvalho-Niebel, F. (2012). Medicago truncatula ERN transcription factors: regulatory interplay with NSP1/NSP2 GRAS factors and expression dynamics throughout rhizobial infection. *Plant Physiology* 160(4): 2155-2172.

Chabaud, M., Gherbi, H., Pirolles, E., Vaissayre, V., Fournier, J., Moukouanga, D., Franche, C., Bogusz, D., Tisa, L. S. & Barker, D. G. (2016). Chitinase-resistant hydrophilic symbiotic factors secreted by Frankia activate both Ca2+ spiking and NIN gene expression in the actinorhizal plant Casuarina glauca. *New Phytologist* 209(1): 86-93.

Chan, P. K., Biswas, B. & Gresshoff, P. M. (2013). Classical Ethylene Insensitive Mutants of the Arabidopsis EIN2 Orthologue Lack the Expected 'hypernodulation'Response in Lotus japonicusF. *Journal of Integrative Plant Biology* 55(4): 395-408.

Chang, C., Kwok, S. F., Bleecker, A. B. & Meyerowitz, E. M. (1993). Arabidopsis ethylene-response gene ETR1: similarity of product to two-component regulators. *SCIENCE-NEW YORK THEN WASHINGTON*- 262: 539-539.

Chen, C., Ané, J. M. & Zhu, H. (2008). OsIPD3, an ortholog of the Medicago truncatula DMI3 interacting protein IPD3, is required for mycorrhizal symbiosis in rice. *New Phytologist* 180(2): 311-315.

Chen, T., Zhu, H., Ke, D., Cai, K., Wang, C., Gou, H., Hong, Z. & Zhang, Z. (2012). A MAP kinase kinase interacts with SymRK and regulates nodule organogenesis in Lotus japonicus. *The Plant cell* 24(2): 823-838.

Chiu, W.-L., Peters, G. A., Levieille, G., Still, P. C., Cousins, S., Osborne, B. & Elhai, J. (2005). Nitrogen deprivation stimulates symbiotic gland development in Gunnera manicata. *Plant Physiology* 139(1): 224-230.

Clavijo, F., Diedhiou, I., Vaissayre, V., Brottier, L., Acolatse, J., Moukouanga, D., Crabos, A., Auguy, F., Franche, C. & Gherbi, H. (2015). The Casuarina NIN gene is transcriptionally activated throughout Frankia root infection as well as in response to bacterial diffusible signals. *New Phytologist* 208(3): 887-903.

Colpaert, N., Tilleman, S., Van Montagu, M., Gheysen, G. & Terryn, N. (2008). Composite Phaseolus vulgaris plants with transgenic roots as research tool. *African Journal of Biotechnology* 7(4).

Cooper, J. B. & Long, S. R. (1994). Morphogenetic rescue of Rhizobium meliloti nodulation mutants by trans-zeatin secretion. *The Plant cell* 6(2): 215-225.

Coventry, D., Trinick, M. & Appleby, C. (1976). A search for a leghaemoglobin-like compound in root nodules ofTrema cannabina lour. *Biochimica et Biophysica Acta (BBA)-Protein Structure* 420(1): 105-111.

D'Haeze, W. & Holsters, M. (2002). Nod factor structures, responses, and perception during initiation of nodule development. *Glycobiology* 12(6): 79R-105R.

Davey, M., Webster, G., Manders, G., Ringrose, F., Power, J. & Cocking, E. (1993). Effective nodulation of micro-propagated shoots of the non-legume Parasponia andersonii by Bradyrhizobium. *Journal of Experimental Botany* 44(5): 863-867.

Dawson, C. J. & Hilton, J. (2011). Fertiliser availability in a resource-limited world: Production and recycling of nitrogen and phosphorus. *Food Policy* 36: S14-S22.

de Faria, S. M., Sutherland, J. M. & Sprent, J. I. (1986). A new type of infected cell in root nodules of Andira spp.(Leguminosae). *Plant Science* 45(2): 143-147.

Deinum, E. E., Geurts, R., Bisseling, T. & Mulder, B. M. (2012). Modeling a cortical auxin maximum for nodulation: different signatures of potential strategies. *Front Plant Sci* 3: 96.

Delaux, P.-M., Varala, K., Edger, P. P., Coruzzi, G. M., Pires, J. C. & Ané, J.-M. (2014). Comparative phylogenomics uncovers the impact of symbiotic associations on host genome evolution. *PLoS Genet* 10(7): e1004487.

Delaux, P. M., Bécard, G. & Combier, J. P. (2013). NSP1 is a component of the Myc signaling pathway. *New Phytologist* 199(1): 59-65.

den Camp, R. O., Streng, A., De Mita, S., Cao, Q., Polone, E., Liu, W., Ammiraju, J. S., Kudrna, D., Wing, R. & Untergasser, A. (2011). LysM-type mycorrhizal receptor recruited for rhizobium symbiosis in nonlegume Parasponia. *Science* 331(6019): 909-912.

Diédhiou, I., Tromas, A., Cissoko, M., Gray, K., Parizot, B., Crabos, A., Alloisio, N., Fournier, P., Carro, L. & Svistoonoff, S. (2014). Identification of potential transcriptional regulators of actinorhizal symbioses in Casuarina glauca and Alnus glutinosa. *BMC plant biology* 14(1): 1.

Dillen, W., Clercq, J., Kapila, J., Zambre, M., Montagu, M. & Angenon, G. (1997). The effect of temperature on Agrobacterium tumefaciens-mediated gene transfer to plants. *The Plant Journal* 12(6): 1459-1463.

Ding, Y., Kalo, P., Yendrek, C., Sun, J., Liang, Y., Marsh, J. F., Harris, J. M. & Oldroyd, G. E. (2008). Abscisic acid coordinates nod factor and cytokinin signaling during the regulation of nodulation in Medicago truncatula. *The Plant cell* 20(10): 2681-2695.

Doyle, J. J. (1998). Phylogenetic perspectives on nodulation: evolving views of plants and symbiotic bacteria. *Trends in Plant Science* 3(12): 473-478.

Doyle, J. J. (2011). Phylogenetic perspectives on the origins of nodulation. *Molecular Plant-Microbe Interactions* 24(11): 1289-1295.

Drennan, D. & Norton, C. (1972). The effect of ethrel on nodulation inPisum sativum L. *Plant and Soil* 36(1-3): 53-57.

Elise, S., Etienne-Pascal, J., de Fernanda, C.-N., Gérard, D. & Julia, F. (2005). The Medicago truncatula SUNN gene encodes a CLV1-like leucine-rich repeat receptor kinase that regulates nodule number and root length. *Plant Molecular Biology* 58(6): 809-822.

Endre, G., Kereszt, A., Kevei, Z., Mihacea, S., Kaló, P. & Kiss, G. B. (2002). A receptor kinase gene regulating symbiotic nodule development. *Nature* 417(6892): 962-966.

Engstrom, E. M. (2011). Phylogenetic analysis of GRAS proteins from moss, lycophyte and vascular plant lineages reveals that GRAS genes arose and underwent substantial diversification in the ancestral lineage common to bryophytes and vascular plants. *Plant Signaling & Behavior* 6(6): 850-854.

Estrada-Navarrete, G., Alvarado-Affantranger, X., Olivares, J.-E., Díaz-Camino, C., Santana, O., Murillo, E., Guillén, G., Sánchez-Guevara, N., Acosta, J. & Quinto, C. (2006). Agrobacterium rhizogenes transformation of the Phaseolus spp.: a tool for functional genomics. *Molecular Plant-Microbe Interactions* 19(12): 1385-1393.

Faria, S. M. d., McInroy, S. G. & Sprent, J. I. (1987). The occurrence of infected cells, with persistent infection threads, in legume root nodules. *Canadian Journal of Botany* 65(3): 553-558.

Ferguson, B. J. & Mathesius, U. (2003). Signaling interactions during nodule development. *Journal of Plant Growth Regulation* 22(1): 47-72.

Frugier, F., Kosuta, S., Murray, J. D., Crespi, M. & Szczyglowski, K. (2008). Cytokinin: secret agent of symbiosis. *Trends in Plant Science* 13(3): 115-120.

Fujikake, H., Yashima, H., Sato, T., Ohtake, N., Sueyoshi, K. & Ohyama, T. (2002). Rapid and reversible nitrate inhibition of nodule growth and N2 fixation activity in soybean (Glycine max (L.) Merr.). *Soil Science and Plant Nutrition* 48(2): 211-217.

Gavrin, A., Kaiser, B. N., Geiger, D., Tyerman, S. D., Wen, Z., Bisseling, T. & Fedorova, E. E. (2014). Adjustment of host cells for accommodation of symbiotic bacteria: vacuole defunctionalization, HOPS suppression, and TIP1g retargeting in Medicago. *The Plant cell* 26(9): 3809-3822.

Geurts, R., Fedorova, E. & Bisseling, T. (2005). Nod factor signaling genes and their function in the early stages of Rhizobium infection. *Current Opinion in Plant Biology* 8(4): 346-352.

Geurts, R. & Vleeshouwers, V. G. (2012). Mycorrhizal symbiosis: ancient signalling mechanisms co-opted. *Current Biology* 22(23): R997-R999.

Geurts, R., Xiao, T. T. & Reinhold-Hurek, B. (2016). What Does It Take to Evolve A Nitrogen-Fixing Endosymbiosis? *Trends in Plant Science* 21(3): 199-208.

Gherbi, H., Markmann, K., Svistoonoff, S., Estevan, J., Autran, D., Giczey, G., Auguy, F., Péret, B., Laplaze, L. & Franche, C. (2008). SymRK defines a common genetic basis for plant root endosymbioses with arbuscular mycorrhiza fungi, rhizobia, and Frankiabacteria. *Proceedings of the National Academy of Sciences* 105(12): 4928-4932.

Gobbato, E. (2015). Recent developments in arbuscular mycorrhizal signaling. *Current Opinion in Plant Biology* 26: 1-7.

Gobbato, E., Marsh, J. F., Vernié, T., Wang, E., Maillet, F., Kim, J., Miller, J. B., Sun, J., Bano, S. A. & Ratet, P. (2012). A GRAS-type transcription factor with a specific function in mycorrhizal signaling. *Current Biology* 22(23): 2236-2241.

Gonzalez-Rizzo, S., Crespi, M. & Frugier, F. (2006). The Medicago truncatula CRE1 cytokinin receptor regulates lateral root development and early symbiotic interaction with Sinorhizobium meliloti. *The Plant cell* 18(10): 2680-2693.

Goodlass, G. & Smith, K. (1979). Effects of ethylene on root extension and nodulation of pea (<i>Pisum sativum</i> L.) and white clover (<i>Trifolium repens</i> L.). *Plant and Soil* 51(3): 387-395.

Gough, C. & Cullimore, J. (2011). Lipo-chitooligosaccharide signaling in endosymbiotic plant-microbe interactions. *Molecular Plant-Microbe Interactions* 24(8): 867-878.

Granqvist, E., Sun, J., Op den Camp, R., Pujic, P., Hill, L., Normand, P., Morris, R. J., Downie, J. A., Geurts, R. & Oldroyd, G. E. (2015). Bacterial-induced calcium oscillations are common to nitrogen-fixing associations of nodulating legumes and non-legumes. *New Phytologist* 207(3): 551-558.

Gresshoff, P. M., Lohar, D., Chan, P.-K., Biswas, B., Jiang, Q., Reid, D., Ferguson, B. & Stacey, G. (2009). Genetic analysis of ethylene regulation of legume nodulation. *Plant Signaling & Behavior* 4(9): 818-823.

Grobbelaar, N., Clarke, B. & Hough, M. (1971). The nodulation and nitrogen fixation of isolated roots of Phaseolus vulgaris L. *Plant and Soil* 35(1): 203-214.

Groth, M., Takeda, N., Perry, J., Uchida, H., Dräxl, S., Brachmann, A., Sato, S., Tabata, S., Kawaguchi, M. & Wang, T. L. (2010). NENA, a Lotus japonicus homolog of Sec13, is required for rhizodermal infection by arbuscular mycorrhiza fungi and rhizobia but dispensable for cortical endosymbiotic development. *The Plant cell* 22(7): 2509-2526.

Guinel, F. C. & LaRue, T. A. (1992). Ethylene inhibitors partly restore nodulation to pea mutant E 107 (brz). *Plant Physiology* 99(2): 515-518.

Guo, J., Wang, Y., Song, C., Zhou, J., Qiu, L., Huang, H. & Wang, Y. (2010). A single origin and moderate bottleneck during domestication of soybean (Glycine max): implications from microsatellites and nucleotide sequences. *Annals of Botany* 106(3): 505-514.

Gutjahr, C. & Parniske, M. (2013). Cell and developmental biology of arbuscular mycorrhiza symbiosis.

Heckmann, A. B., Lombardo, F., Miwa, H., Perry, J. A., Bunnewell, S., Parniske, M., Wang, T. L. & Downie, J. A. (2006). Lotus japonicus nodulation requires two GRAS domain regulators, one of which is functionally conserved in a non-legume. *Plant Physiology* 142(4): 1739-1750.

Heckmann, A. B., Sandal, N., Bek, A. S., Madsen, L. H., Jurkiewicz, A., Nielsen, M. W., Tirichine, L. & Stougaard, J. (2011). Cytokinin induction of root nodule primordia in Lotus japonicus is regulated by a mechanism operating in the root cortex. *Molecular Plant-Microbe Interactions* 24(11): 1385-1395.

Heidstra, R., Yang, W. C., Yalcin, Y., Peck, S., Emons, A. & Bisseling, T. (1997). Ethylene provides positional information on cortical cell division but is not involved in Nod factor-induced root hair tip growth in Rhizobium-legume interaction. *Development* 124(9): 1781-1787.

Hirakawa, Y., Shinohara, H., Kondo, Y., Inoue, A., Nakanomyo, I., Ogawa, M., Sawa, S., Ohashi-Ito, K., Matsubayashi, Y. & Fukuda, H. (2008). Non-cell-autonomous control of vascular stem cell fate by a CLE peptide/receptor system. *Proceedings of the National Academy of Sciences* 105(39): 15208-15213.

Hirel, B., Le Gouis, J., Ney, B. & Gallais, A. (2007). The challenge of improving nitrogen use efficiency in crop plants: towards a more central role for genetic variability and quantitative genetics within integrated approaches. *Journal of Experimental Botany* 58(9): 2369-2387.

Hirsch, S., Kim, J., Muñoz, A., Heckmann, A. B., Downie, J. A. & Oldroyd, G. E. (2009). GRAS proteins form a DNA binding complex to induce gene expression during nodulation signaling in Medicago truncatula. *The Plant cell* 21(2): 545-557.

Hogekamp, C., Arndt, D., Pereira, P. A., Becker, J. D., Hohnjec, N. & Küster, H. (2011). Laser microdissection unravels cell-type-specific transcription in arbuscular mycorrhizal roots, including CAAT-box transcription factor gene expression correlating with fungal contact and spread. *Plant Physiology* 157(4): 2023-2043.

Horváth, B., Yeun, L. H., Domonkos, Á., Halász, G., Gobbato, E., Ayaydin, F., Miró, K., Hirsch, S., Sun, J. & Tadege, M. (2011). Medicago truncatula IPD3 is a member of the common symbiotic signaling pathway required for rhizobial and mycorrhizal symbioses. *Molecular Plant-Microbe Interactions* 24(11): 1345-1358.

Hua, J., Chang, C., Sun, Q. & Meyerowitz, E. M. (1995). Ethylene insensitivity conferred by Arabidopsis ERS gene. *Science* 269(5231): 1712.

Hua, J. & Meyerowitz, E. M. (1998). Ethylene responses are negatively regulated by a receptor gene family in Arabidopsis thaliana. *Cell* 94(2): 261-271.

Hua, J., Sakai, H., Nourizadeh, S., Chen, Q. G., Bleecker, A. B., Ecker, J. R. & Meyerowitz, E. M. (1998). EIN4 and ERS2 are members of the putative ethylene receptor gene family in Arabidopsis. *The Plant cell* 10(8): 1321-1332.

Huault, E., Laffont, C., Wen, J., Mysore, K. S., Ratet, P., Duc, G. & Frugier, F. (2014). Local and systemic regulation of plant root system architecture and symbiotic nodulation by a receptor-like kinase. *PLoS Genet* 10(12): e1004891.

Huo, X., Schnabel, E., Hughes, K. & Frugoli, J. (2006). RNAi phenotypes and the localization of a protein:: GUS fusion imply a role for Medicago truncatula PIN genes in nodulation. *Journal of Plant Growth Regulation* 25(2): 156-165.

II, A. (2003). An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG II. *Botanical Journal of the Linnean Society* 141(4): 399-436.

Imaizumi-Anraku, H., Takeda, N., Charpentier, M., Perry, J., Miwa, H., Umehara, Y., Kouchi, H., Murakami, Y., Mulder, L. & Vickers, K. (2005). Plastid proteins crucial for symbiotic fungal and bacterial entry into plant roots. *Nature* 433(7025): 527-531.

Imanishi, L., Perrine-Walker, F. M., Ndour, A., Vayssières, A., Conejero, G., Lucas, M., Champion, A., Laplaze, L., Wall, L. & Svistoonoff, S. (2014). Role of auxin during intercellular infection of Discaria trinervis by Frankia. *Frontiers in plant science* 5.

Ivanov, S., Fedorova, E. E., Limpens, E., De Mita, S., Genre, A., Bonfante, P. & Bisseling, T. (2012). Rhizobium–legume symbiosis shares an exocytotic pathway required for arbuscule formation. *Proceedings of the National Academy of Sciences* 109(21): 8316-8321.

Ju, C., Yoon, G. M., Shemansky, J. M., Lin, D. Y., Ying, Z. I., Chang, J., Garrett, W. M., Kessenbrock, M., Groth, G. & Tucker, M. L. (2012). CTR1 phosphorylates the central regulator EIN2 to control ethylene hormone signaling from the ER membrane to the nucleus in Arabidopsis. *Proceedings of the National Academy of Sciences* 109(47): 19486-19491.

Jun, S.-H., Han, M.-J., Lee, S., Seo, Y. S., Kim, W. T. & An, G. (2004). OsEIN2 is a positive component in ethylene signaling in rice. *Plant and Cell Physiology* 45(3): 281-289.

Kahle, J., Baake, M., Doenecke, D. & Albig, W. (2005). Subunits of the heterotrimeric transcription factor NF-Y are imported into the nucleus by distinct pathways involving importin β and importin 13. *Molecular and Cellular Biology* 25(13): 5339-5354.

Kaló, P., Gleason, C., Edwards, A., Marsh, J., Mitra, R. M., Hirsch, S., Jakab, J., Sims, S., Long, S. R. & Rogers, J. (2005). Nodulation signaling in legumes requires NSP2, a member of the GRAS family of transcriptional regulators. *Science* 308(5729): 1786-1789.

Kanamori, N., Madsen, L. H., Radutoiu, S., Frantescu, M., Quistgaard, E. M., Miwa, H., Downie, J. A., James, E. K., Felle, H. H. & Haaning, L. L. (2006). A nucleoporin is required for induction of Ca2+ spiking in legume nodule development and essential for rhizobial and fungal symbiosis. *Proceedings of the National Academy of Sciences of the United States of America* 103(2): 359-364.

Karimi, M., Inzé, D. & Depicker, A. (2002). GATEWAY™ vectors for< i> Agrobacterium</i>-mediated plant transformation. *Trends in Plant Science* 7(5): 193-195.

Kawaguchi, M., Imaizumi-Anraku, H., Koiwa, H., Niwa, S., Ikuta, A., Syono, K. & Akao, S. (2002). Root, root hair, and symbiotic mutants of the model legume Lotus japonicus. *Molecular Plant-Microbe Interactions* 15(1): 17-26.

Kerk, N. M., Jiang, K. & Feldman, L. J. (2000). Auxin metabolism in the root apical meristem. *Plant Physiology* 122(3): 925-932.

Kieber, J. J., Rothenberg, M., Roman, G., Feldmann, K. A. & Ecker, J. R. (1993). CTR1, a negative regulator of the ethylene response pathway in Arabidopsis, encodes a member of the raf family of protein kinases. *Cell* 72(3): 427-441.

Kistner, C. & Parniske, M. (2002). Evolution of signal transduction in intracellular symbiosis. *Trends in Plant Science* 7(11): 511-518.

Kistner, C., Winzer, T., Pitzschke, A., Mulder, L., Sato, S., Kaneko, T., Tabata, S., Sandal, N., Stougaard, J. & Webb, K. J. (2005). Seven Lotus japonicus genes required for transcriptional reprogramming of the root during fungal and bacterial symbiosis. *The Plant cell* 17(8): 2217-2229.

Kondo, T., Hasegawa, H. & Suzuki, M. (2000). Transformation and regeneration of garlic (Allium sativum L.) by Agrobacterium-mediated gene transfer. *Plant Cell Reports* 19(10): 989-993.

Kosuta, S., Chabaud, M., Lougnon, G., Gough, C., Dénarié, J., Barker, D. G. & Bécard, G. (2003). A diffusible factor from arbuscular mycorrhizal fungi induces symbiosis-specific MtENOD11 expression in roots ofMedicago truncatula. *Plant Physiology* 131(3): 952-962.

Larkin, P., Gibson, J., Mathesius, U., Weinman, J., Gartner, E., Hall, E., Tanner, G., Rolfe, B. & Djordjevic, M. (1996). Transgenic white clover. Studies with the auxin-responsive promoter, GH3, in root gravitropism and lateral root development. *Transgenic research* 5(5): 325-335.

Lauressergues, D., Delaux, P. M., Formey, D., Lelandais-Brière, C., Fort, S., Cottaz, S., Bécard, G., Niebel, A., Roux, C. & Combier, J. P. (2012). The microRNA miR171h modulates arbuscular mycorrhizal colonization of Medicago truncatula by targeting NSP2. *The Plant Journal* 72(3): 512-522.

Lavin, M., Herendeen, P. S. & Wojciechowski, M. F. (2005). Evolutionary rates analysis of Leguminosae implicates a rapid diversification of lineages during the Tertiary. *Systematic biology* 54(4): 575-594.

Lee, G.-A., Crawford, G. W., Liu, L. & Chen, X. (2007). Plants and people from the Early Neolithic to Shang periods in North China. *Proceedings of the National Academy of Sciences* 104(3): 1087-1092.

Lee, K. H. & LaRue, T. A. (1992). Exogenous ethylene inhibits nodulation of Pisum sativum L. cv Sparkle. *Plant Physiology* 100(4): 1759-1763.

Lévy, J., Bres, C., Geurts, R., Chalhoub, B., Kulikova, O., Duc, G., Journet, E.-P., Ané, J.-M., Lauber, E. & Bisseling, T. (2004). A putative Ca2+ and calmodulin-dependent protein kinase required for bacterial and fungal symbioses. *Science* 303(5662): 1361-1364.

Ligero, F., Lluch, C. & Olivares, J. (1986). Evolution of Ethylene from Roots of< i> Medicago sativa</i> Plants Inoculated with< i> Rhizobium meliloti</i>. *Journal of plant physiology* 125(3): 361-365.

Limpens, E. & Bisseling, T. (2014). CYCLOPS: a new vision on Rhizobium-induced nodule organogenesis. *Cell Host & Microbe* 15(2): 127-129.

Limpens, E., Ivanov, S., van Esse, W., Voets, G., Fedorova, E. & Bisseling, T. (2009). Medicago N2-fixing symbiosomes acquire the endocytic identity marker Rab7 but delay the acquisition of vacuolar identity. *The Plant cell* 21(9): 2811-2828.

Limpens, E., Ramos, J., Franken, C., Raz, V., Compaan, B., Franssen, H., Bisseling, T. & Geurts, R. (2004). RNA interference in Agrobacterium rhizogenes-transformed roots of Arabidopsis and Medicago truncatula. *Journal of Experimental Botany* 55(399): 983-992.

Lin, K., Limpens, E., Zhang, Z., Ivanov, S., Saunders, D. G., Mu, D., Pang, E., Cao, H., Cha, H. & Lin, T. (2014). Single nucleus genome sequencing reveals high similarity among nuclei of an endomycorrhizal fungus. *PLoS Genet* 10(1): e1004078.

Liu, W., Kohlen, W., Lillo, A., den Camp, R. O., Ivanov, S., Hartog, M., Limpens, E., Jamil, M., Smaczniak, C. & Kaufmann, K. (2011). Strigolactone biosynthesis in Medicago truncatula and rice requires the symbiotic GRAS-type transcription factors NSP1 and NSP2. *The Plant Cell Online* 23(10): 3853-3865.

Liu, X. & Widmer, A. (2014). Genome-wide comparative analysis of the GRAS gene family in Populus, Arabidopsis and rice. *Plant Molecular Biology Reporter* 32(6): 1129-1145.

Lloyd, G. (1980).Commercially-feasible micropropagation of mountain laurel, Kalmia latifolia, by use of shoot-tip culture. In *Int. Plant. Prop. Soc.*, Vol. 30, 421-427.

Lohar, D., Stiller, J., Kam, J., Stacey, G. & Gresshoff, P. M. (2009). Ethylene insensitivity conferred by a mutated Arabidopsis ethylene receptor gene alters nodulation in transgenic Lotus japonicus. *Annals of Botany* 104(2): 277-285.

Lohar, D. P., Sharopova, N., Endre, G., Penuela, S., Samac, D., Town, C., Silverstein, K. A. & VandenBosch, K. A. (2006). Transcript analysis of early nodulation events in Medicago truncatula. *Plant Physiology* 140(1): 221-234.

Lum, M. R. & Hirsch, A. M. (2002). Roots and their symbiotic microbes: strategies to obtain nitrogen and phosphorus in a nutrient-limiting environment. *Journal of Plant Growth Regulation* 21(4): 368-382.

Madsen, E. B., Antolín-Llovera, M., Grossmann, C., Ye, J., Vieweg, S., Broghammer, A., Krusell, L., Radutoiu, S., Jensen, O. N. & Stougaard, J. (2011). Autophosphorylation is essential for the in vivo function of the Lotus japonicus Nod factor receptor 1 and receptor-mediated signalling in cooperation with Nod factor receptor 5. *The Plant Journal* 65(3): 404-417.

Madsen, E. B., Madsen, L. H., Radutoiu, S., Olbryt, M., Rakwalska, M., Szczyglowski, K., Sato, S., Kaneko, T., Tabata, S. & Sandal, N. (2003). A receptor kinase gene of the LysM type is involved in legumeperception of rhizobial signals. *Nature* 425(6958): 637-640.

Magori, S., Oka-Kira, E., Shibata, S., Umehara, Y., Kouchi, H., Hase, Y., Tanaka, A., Sato, S., Tabata, S. & Kawaguchi, M. (2009). TOO MUCH LOVE, a root regulator associated with the long-distance control of nodulation in Lotus japonicus. *Molecular Plant-Microbe Interactions* 22(3): 259-268.

Maillet, F., Poinsot, V., André, O., Puech-Pagès, V., Haouy, A., Gueunier, M., Cromer, L., Giraudet, D., Formey, D. & Niebel, A. (2011). Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza. *Nature* 469(7328): 58-63.

Malkov, N., Fliegmann, J., Rosenberg, C., Gasciolli, V., Timmers, A. C., Nurisso, A., Cullimore, J. & Bono, J.-J. (2016). Molecular basis of lipo-chitooligosaccharide recognition by the lysin motif receptor-like kinase LYR3 in legumes. *Biochemical Journal* 473(10): 1369-1378.

Marhavý, P., Vanstraelen, M., De Rybel, B., Zhaojun, D., Bennett, M. J., Beeckman, T. & Benková, E. (2013). Auxin reflux between the endodermis and pericycle promotes lateral root initiation. *The EMBO journal* 32(1): 149-158.

Markmann, K., Giczey, G. & Parniske, M. (2008). Functional adaptation of a plant receptor-kinase paved the way for the evolution of intracellular root symbioses with bacteria. *PLoS Biol* 6(3): e68.

Marsh, J. F., Rakocevic, A., Mitra, R. M., Brocard, L., Sun, J., Eschstruth, A., Long, S. R., Schultze, M., Ratet, P. & Oldroyd, G. E. (2007). Medicago truncatula NIN is essential for rhizobial-independent nodule organogenesis induced by autoactive calcium/calmodulin-dependent protein kinase. *Plant Physiology* 144(1): 324-335.

Martín, A. C., Del Pozo, J. C., Iglesias, J., Rubio, V., Solano, R., De La Peña, A., Leyva, A. & Paz-Ares, J. (2000). Influence of cytokinins on the expression of phosphate starvation responsive genes in Arabidopsis. *The Plant Journal* 24(5): 559-567.

Martínez-Romero, E. (2009). Coevolution in Rhizobium-legume symbiosis? *DNA and cell biology* 28(8): 361-370.

Marvel, D. J., Torrey, J. G. & Ausubel, F. M. (1987). Rhizobium symbiotic genes required for nodulation of legume and nonlegume hosts. *Proceedings of the National Academy of Sciences* 84(5): 1319-1323.

Mathesius, U., Schlaman, H. R., Spaink, H. P., Of Sautter, C., Rolfe, B. G. & Djordjevic, M. A. (1998). Auxin transport inhibition precedes root nodule formation in white clover roots and is regulated by flavonoids and derivatives of chitin oligosaccharides. *The Plant Journal* 14(1): 23-34.

Mattsson, J., Ckurshumova, W. & Berleth, T. (2003). Auxin signaling in Arabidopsis leaf vascular development. *Plant Physiology* 131(3): 1327-1339.

Merchante, C., Alonso, J. M. & Stepanova, A. N. (2013). Ethylene signaling: simple ligand, complex regulation. *Current Opinion in Plant Biology* 16(5): 554-560.

Merchante, C., Brumos, J., Yun, J., Hu, Q., Spencer, K. R., Enríquez, P., Binder, B. M., Heber, S., Stepanova, A. N. & Alonso, J. M. (2015). Gene-specific translation regulation mediated by the hormone-signaling molecule EIN2. *Cell* 163(3): 684-697.

Messinese, E., Mun, J.-H., Yeun, L. H., Jayaraman, D., Rougé, P., Barre, A., Lougnon, G., Schornack, S., Bono, J.-J. & Cook, D. R. (2007). A novel nuclear protein interacts with the symbiotic DMI3 calcium-and calmodulin-dependent protein kinase of Medicago truncatula. *Molecular Plant-Microbe Interactions* 20(8): 912-921.

Mitra, R. M., Gleason, C. A., Edwards, A., Hadfield, J., Downie, J. A., Oldroyd, G. E. & Long, S. R. (2004). A Ca2+/calmodulin-dependent protein kinase required for symbiotic nodule development: gene identification by transcript-based cloning. *Proceedings of the National Academy of Sciences of the United States of America* 101(13): 4701-4705.

Miyata, K., Kawaguchi, M. & Nakagawa, T. (2013). Two distinct EIN2 genes cooperatively regulate ethylene signaling in Lotus japonicus. *Plant and Cell Physiology* 54(9): 1469-1477.

Mok, D. W. & Mok, M. C. (2001). Cytokinin metabolism and action. *Annual review of plant biology* 52(1): 89-118.

Mortier, V., Den Herder, G., Whitford, R., Van de Velde, W., Rombauts, S., D'haeseleer, K., Holsters, M. & Goormachtig, S. (2010). CLE peptides control Medicago truncatula nodulation locally and systemically. *Plant Physiology* 153(1): 222-237.

Murakami, Y., Miwa, H., Imaizumi-Anraku, H., Kouchi, H., Downie, J. A., Kawaguchi, M. & Kawasaki, S. (2007). Positional cloning identifies Lotus japonicus NSP2, a putative transcription factor of the GRAS family, required for NIN and ENOD40 gene expression in nodule initiation. *DNA research* 13(6): 255-265.

Murakami, Y., Yokoyama, H., Fukui, R. & Kawaguchi, M. (2013). Down-regulation of NSP2 expression in developmentally young regions of Lotus japonicus roots in response to rhizobial inoculation. *Plant and Cell Physiology* 54(4): 518-527.

Murray, J. D., Karas, B. J., Sato, S., Tabata, S., Amyot, L. & Szczyglowski, K. (2007). A cytokinin perception mutant colonized by Rhizobium in the absence of nodule organogenesis. *Science* 315(5808): 101-104.

Naisbitt, T., James, E. & Sprent, J. (1992). The evolutionary significance of the legume genus Chamaecrista, as determined by nodule structure. *New Phytologist* 122(3): 487-492.

Nap, J.-P. & Bisseling, T. (1990). Developmental biology of a plant-prokaryote symbiosis: the legume root nodule. *Science(Washington)* 250(4983): 948-954.

Ng, J. L. P., Hassan, S., Truong, T. T., Hocart, C. H., Laffont, C., Frugier, F. & Mathesius, U. (2015). Flavonoids and auxin transport inhibitors rescue symbiotic nodulation in the Medicago truncatula cytokinin perception mutant cre1. *The Plant cell* 27(8): 2210-2226.

Nukui, N., Ezura, H., Yuhashi, K.-I., Yasuta, T. & Minamisawa, K. (2000). Effects of ethylene precursor and inhibitors for ethylene biosynthesis and perception on nodulation in Lotus japonicus and Macroptilium atropurpureum. *Plant and Cell Physiology* 41(7): 893-897.

Nutman, P. (1952). Studies on the physiology of nodule formation III. Experiments on the excision of root-tips and nodules. *Annals of Botany* 16(1): 79-103.

Okamoto, S. & Kawaguchi, M. (2015). Shoot HAR1 mediates nitrate inhibition of nodulation in Lotus japonicus. *Plant Signaling & Behavior* 10(5): e1000138.

Okamoto, S., Ohnishi, E., Sato, S., Takahashi, H., Nakazono, M., Tabata, S. & Kawaguchi, M. (2009). Nod factor/nitrate-induced CLE genes that drive HAR1-mediated systemic regulation of nodulation. *Plant and Cell Physiology* 50(1): 67-77.

Okamoto, S., Shinohara, H., Mori, T., Matsubayashi, Y. & Kawaguchi, M. (2013). Root-derived CLE glycopeptides control nodulation by direct binding to HAR1 receptor kinase. *Nature communications* 4.

Oldroyd, G. E., Engstrom, E. M. & Long, S. R. (2001). Ethylene inhibits the Nod factor signal transduction pathway of Medicago truncatula. *The Plant Cell Online* 13(8): 1835-1849.

Oldroyd, G. E., Harrison, M. J. & Paszkowski, U. (2009). Reprogramming plant cells for endosymbiosis. *Science* 324(5928): 753-754.

Olsson, J. E., Nakao, P., Bohlool, B. B. & Gresshoff, P. M. (1989). Lack of systemic suppression of nodulation in split root systems of supernodulating soybean (Glycine max [L.] Merr.) mutants. *Plant Physiology* 90(4): 1347-1352.

Op den Camp, R. H., Polone, E., Fedorova, E., Roelofsen, W., Squartini, A., Op den Camp, H. J., Bisseling, T. & Geurts, R. (2012). Nonlegume Parasponia andersonii deploys a broad rhizobium host range strategy resulting in largely variable symbiotic effectiveness. *Molecular Plant-Microbe Interactions* 25(7): 954-963.

Ovchinnikova, E., Journet, E.-P., Chabaud, M., Cosson, V., Ratet, P., Duc, G., Fedorova, E., Liu, W., den Camp, R. O. & Zhukov, V. (2011). IPD3 controls the formation of nitrogen-fixing symbiosomes in pea and Medicago Spp. *Molecular Plant-Microbe Interactions* 24(11): 1333-1344.

Pacios-Bras, C., Schlaman, H. R., Boot, K., Admiraal, P., Langerak, J. M., Stougaard, J. & Spaink, H. P. (2003). Auxin distribution in Lotus japonicus during root nodule development. *Plant Molecular Biology* 52(6): 1169-1180.

Parniske, M. (2008). Arbuscular mycorrhiza: the mother of plant root endosymbioses. *Nature Reviews Microbiology* 6(10): 763-775.

Penmetsa, R. V. & Cook, D. R. (1997). A legume ethylene-insensitive mutant hyperinfected by its rhizobial symbiont. *Science* 275(5299): 527-530.

Penmetsa, R. V., Frugoli, J. A., Smith, L. S., Long, S. R. & Cook, D. R. (2003). Dual genetic pathways controlling nodule number inMedicago truncatula. *Plant Physiology* 131(3): 998-1008.

Perrine-Walker, F., Doumas, P., Lucas, M., Vaissayre, V., Beauchemin, N. J., Band, L. R., Chopard, J., Crabos, A., Conejero, G. & Péret, B. (2010). Auxin carriers localization drives auxin accumulation in plant cells infected by Frankia in Casuarina glauca actinorhizal nodules. *Plant Physiology* 154(3): 1372-1380.

Peters, N. K. & Crist-Estes, D. K. (1989). Nodule formation is stimulated by the ethylene inhibitor aminoethoxyvinylglycine. *Plant Physiology* 91(2): 690-693.

Pierce, M. & Bauer, W. D. (1983). A rapid regulatory response governing nodulation in soybean. *Plant Physiology* 73(2): 286-290.

Pietraszewska-Bogiel, A., Lefebvre, B., Koini, M. A., Klaus-Heisen, D., Takken, F. L., Geurts, R., Cullimore, J. V. & Gadella, T. W. (2013). Interaction of Medicago truncatula lysin motif receptor-like kinases, NFP and LYK3, produced in Nicotiana benthamiana induces defence-like responses. *PLoS ONE* 8(6): e65055.

Plet, J., Wasson, A., Ariel, F., Le Signor, C., Baker, D., Mathesius, U., Crespi, M. & Frugier, F. (2011). MtCRE1-dependent cytokinin signaling integrates bacterial and plant cues to coordinate symbiotic nodule organogenesis in Medicago truncatula. *The Plant Journal* 65(4): 622-633.

Prayitno, J., Rolfe, B. & Mathesius, U. (2006a). The ethylene-insensitive sickle mutant of Medicago truncatula shows altered auxin transport regulation during nodulation. *Plant Physiol* 142: 168 - 180.

Prayitno, J., Rolfe, B. G. & Mathesius, U. (2006). The Ethylene-Insensitive sickle Mutant of Medicago truncatula Shows Altered Auxin Transport Regulation during Nodulation. *Plant Physiology* 142(1): 168-180.

Pueppke, S. G. & Broughton, W. J. (1999). Rhizobium sp. strain NGR234 and R. fredii USDA257 share exceptionally broad, nested host ranges. *Molecular Plant-Microbe Interactions* 12(4): 293-318.

Qiao, H., Shen, Z., Huang, S.-s. C., Schmitz, R. J., Urich, M. A., Briggs, S. P. & Ecker, J. R. (2012). Processing and subcellular trafficking of ER-tethered EIN2 control response to ethylene gas. *Science* 338(6105): 390-393.

Read, D., Duckett, J., Francis, R., Ligrone, R. & Russell, A. (2000). Symbiotic fungal associations in 'lower'land plants. *Philosophical Transactions of the Royal Society of London B: Biological Sciences* 355(1398): 815-831.

Reid, D. E., Ferguson, B. J. & Gresshoff, P. M. (2011). Inoculation-and nitrate-induced CLE peptides of soybean control NARK-dependent nodule formation. *Molecular Plant-Microbe Interactions* 24(5): 606-618.

Roberts, N. J., Morieri, G., Kalsi, G., Rose, A., Stiller, J., Edwards, A., Xie, F., Gresshoff, P. M., Oldroyd, G. E. & Downie, J. A. (2013). Rhizobial and mycorrhizal symbioses in Lotus japonicus require lectin nucleotide phosphohydrolase, which acts upstream of calcium signaling. *Plant Physiology* 161(1): 556-567.

Roth, L. & Stacey, G. (1989). Bacterium release into host cells of nitrogen-fixing soybean nodules: the symbiosome membrane comes from three sources. *European journal of cell biology* 49(1): 13-23.

Sagan, M., Morandi, D., Tarenghi, E. & Duc, G. (1995). Selection of nodulation and mycorrhizal mutants in the model plant Medicago truncatula (Gaertn.) after γ-ray mutagenesis. *Plant Science* 111(1): 63-71.

Saito, K., Yoshikawa, M., Yano, K., Miwa, H., Uchida, H., Asamizu, E., Sato, S., Tabata, S., Imaizumi-Anraku, H. & Umehara, Y. (2007). NUCLEOPORIN85 is required for calcium spiking, fungal and bacterial symbioses, and seed production in Lotus japonicus. *The Plant cell* 19(2): 610-624.

Sakai, H., Hua, J., Chen, Q. G., Chang, C., Medrano, L. J., Bleecker, A. B. & Meyerowitz, E. M. (1998). ETR2 is an ETR1-like gene involved in ethylene signaling in Arabidopsis. *Proceedings of the National Academy of Sciences* 95(10): 5812-5817.

Salas, M., Park, S., Srivatanakul, M. & Smith, R. (2001). Temperature influence on stable T-DNA integration in plant cells. *Plant Cell Reports* 20(8): 701-705.

Schauser, L., Roussis, A., Stiller, J. & Stougaard, J. (1999). A plant regulator controlling development of symbiotic root nodules. *Nature* 402(6758): 191-195.

Schmidt, J. S., Harper, J. E., Hoffman, T. K. & Bent, A. F. (1999). Regulation of soybean nodulation independent of ethylene signaling. *Plant Physiology* 119(3): 951-960.

Schnabel, E., Kassaw, T., Smith, L., Marsh, J., Oldroyd, G. E., Long, S. R. & Frugoli, J. (2011). ROOT DETERMINED NODULATION 1 regulates nodule number in M. truncatula and defines a highly conserved, uncharacterized plant gene family. *Plant Physiology*: pp. 111.178756.

Shibuya, K., Barry, K. G., Ciardi, J. A., Loucas, H. M., Underwood, B. A., Nourizadeh, S., Ecker, J. R., Klee, H. J. & Clark, D. G. (2004). The central role of PhEIN2 in ethylene responses throughout plant development in petunia. *Plant Physiology* 136(2): 2900-2912.

Shtark, O. Y., Sulima, A. S., Zhernakov, A. I., Kliukova, M. S., Fedorina, J. V., Pinaev, A. G., Kryukov, A. A., Akhtemova, G. A., Tikhonovich, I. A. & Zhukov, V. A. (2016). Arbuscular mycorrhiza development in pea (Pisum sativum L.) mutants impaired in five early nodulation genes including putative orthologs of NSP1 and NSP2. *Symbiosis* 68(1-3): 129-144.

Singh, S., Katzer, K., Lambert, J., Cerri, M. & Parniske, M. (2014). CYCLOPS, a DNA-binding transcriptional activator, orchestrates symbiotic root nodule development. *Cell Host & Microbe* 15(2): 139-152.

Smit, P., Limpens, E., Geurts, R., Fedorova, E., Dolgikh, E., Gough, C. & Bisseling, T. (2007). Medicago LYK3, an entry receptor in rhizobial nodulation factor signaling. *Plant Physiology* 145(1): 183-191.

Smit, P., Raedts, J., Portyanko, V., Debellé, F., Gough, C., Bisseling, T. & Geurts, R. (2005). NSP1 of the GRAS protein family is essential for rhizobial Nod factor-induced transcription. *Science* 308(5729): 1789-1791.

Soyano, T., Hirakawa, H., Sato, S., Hayashi, M. & Kawaguchi, M. (2014). NODULE INCEPTION creates a long-distance negative feedback loop involved in homeostatic regulation of nodule organ production. *Proceedings of the National Academy of Sciences* 111(40): 14607-14612.

Soyano, T., Kouchi, H., Hirota, A. & Hayashi, M. (2013). Nodule inception directly targets NF-Y subunit genes to regulate essential processes of root nodule development in Lotus japonicus. *PLoS Genet* 9(3): e1003352.

Sprent, J. I. (2008). 60Ma of legume nodulation. What's new? What's changing? *Journal of Experimental Botany* 59(5): 1081-1084.

Steen, A., Buist, G., Horsburgh, G. J., Venema, G., Kuipers, O. P., Foster, S. J. & Kok, J. (2005). AcmA of Lactococcus lactis is an N-acetylglucosaminidase with an optimal number of LysM domains for proper functioning. *FEBS Journal* 272(11): 2854-2868.

Stracke, S., Kistner, C., Yoshida, S., Mulder, L., Sato, S., Kaneko, T., Tabata, S., Sandal, N., Stougaard, J. & Szczyglowski, K. (2002). A plant receptor-like kinase required for both bacterial and fungal symbiosis. *Nature* 417(6892): 959-962.

Streeter, J. & Wong, P. P. (1988). Inhibition of legume nodule formation and N2 fixation by nitrate. *Critical Reviews in Plant Sciences* 7(1): 1-23.

Streng, A., op den Camp, R., Bisseling, T. & Geurts, R. (2011). Evolutionary origin of rhizobium Nod factor signaling. *Plant Signaling & Behavior* 6(10): 1510-1514.

Sun, J., Cardoza, V., Mitchell, D. M., Bright, L., Oldroyd, G. & Harris, J. M. (2006). Crosstalk between jasmonic acid, ethylene and Nod factor signaling allows integration of diverse inputs for regulation of nodulation. *The Plant Journal* 46(6): 961-970.

Sun, J., Miller, J. B., Granqvist, E., Wiley-Kalil, A., Gobbato, E., Maillet, F., Cottaz, S., Samain, E., Venkateshwaran, M. & Fort, S. (2015). Activation of symbiosis signaling by arbuscular mycorrhizal fungi in legumes and rice. *The Plant cell* 27(3): 823-838.

Suzaki, T., Yano, K., Ito, M., Umehara, Y., Suganuma, N. & Kawaguchi, M. (2012). Positive and negative regulation of cortical cell division during root nodule development in Lotus japonicus is accompanied by auxin response. *Development* 139(21): 3997-4006.

Sytsma, K. J., Morawetz, J., Pires, J. C., Nepokroeff, M., Conti, E., Zjhra, M., Hall, J. C. & Chase, M. W. (2002). Urticalean rosids: circumscription, rosid ancestry, and phylogenetics based on rbcL, trnL-F, and ndhF sequences. *American Journal of Botany* 89(9): 1531-1546.

Szczyglowski, K. & Amyot, L. (2003). Symbiosis, inventiveness by recruitment? *Plant Physiology* 131(3): 935-940.

Takanashi, K., Sugiyama, A. & Yazaki, K. (2011). Involvement of auxin distribution in root nodule development of Lotus japonicus. *Planta* 234(1): 73-81.

Takeda, N., Tsuzuki, S., Suzaki, T., Parniske, M. & Kawaguchi, M. (2013). CERBERUS and NSP1 of Lotus japonicus are common symbiosis genes that modulate arbuscular mycorrhiza development. *Plant and Cell Physiology* 54(10): 1711-1723.

Tirichine, L., Imaizumi-Anraku, H., Yoshida, S., Murakami, Y., Madsen, L. H., Miwa, H., Nakagawa, T., Sandal, N., Albrektsen, A. S. & Kawaguchi, M. (2006). Deregulation of a Ca2+/calmodulin-dependent kinase leads to spontaneous nodule development. *Nature* 441(7097): 1153-1156.

Tirichine, L., Sandal, N., Madsen, L. H., Radutoiu, S., Albrektsen, A. S., Sato, S., Asamizu, E., Tabata, S. & Stougaard, J. (2007). A gain-of-function mutation in a cytokinin receptor triggers spontaneous root nodule organogenesis. *Science* 315(5808): 104-107.

Tisserant, B., Gianinazzi-Pearson, V., Gianinazzi, S. & Gollotte, A. (1993). In planta histochemical staining of fungal alkaline phosphatase activity for analysis of efficient arbuscular mycorrhizal infections. *Mycological Research* 97(2): 245-250.

Trinick, M. (1973). Symbiosis between Rhizobium and the non-legume, Trema aspera. *Nature* 244: 459-460.

Trinick, M. (1979). Structure of nitrogen-fixing nodules formed by Rhizobium on roots of Parasponia andersonii Planch. *Canadian Journal of Microbiology* 25(5): 565-578.

Trinick, M. (1980). Growth of Parasponia in agar tube culture and symbiotic effectiveness of isolates from Parasponia spp. *New Phytologist* 85(1): 37-45.

Trinick, M. & Galbraith, J. (1976). Structure of root nodules formed by Rhizobium on the non-legume Trema cannabina var. scabra. *Archives of Microbiology* 108(2): 159-166.

Tsuda, K. & Somssich, I. E. (2015). Transcriptional networks in plant immunity. *New Phytologist* 206(3): 932-947.

Ulmasov, T., Murfett, J., Hagen, G. & Guilfoyle, T. J. (1997). Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. *The Plant Cell Online* 9(11): 1963-1971.

Untergasser, A., Bisseling, T. & Geurts, R. (2008).Making rhizobium-infected root nodules. In *Prokaryotic symbionts in plants*, 45-69: Springer.

van Brussel, A. A., Tak, T., Boot, K. J. & Kijne, J. W. (2002). Autoregulation of root nodule formation: signals of both symbiotic partners studied in a split-root system of Vicia sativa subsp. nigra. *Molecular Plant-Microbe Interactions* 15(4): 341-349.

van Noorden, G. E., Kerim, T., Goffard, N., Wiblin, R., Pellerone, F. I., Rolfe, B. G. & Mathesius, U. (2007). Overlap of proteome changes in Medicago truncatula in response to auxin and Sinorhizobium meliloti. *Plant Physiology* 144(2): 1115-1131.

van Zeijl, A., den Camp, R. H. O., Deinum, E. E., Charnikhova, T., Franssen, H., den Camp, H. J. O., Bouwmeester, H., Kohlen, W., Bisseling, T. & Geurts, R. (2015). Rhizobium lipo-chitooligosaccharide signaling triggers accumulation of cytokinins in Medicago truncatula roots. *Molecular Plant* 8(8): 1213-1226.

Vance, C. P. (2001). Symbiotic nitrogen fixation and phosphorus acquisition. Plant nutrition in a world of declining renewable resources. *Plant Physiology* 127(2): 390-397.

Varma Penmetsa, R., Uribe, P., Anderson, J., Lichtenzveig, J., Gish, J. C., Nam, Y. W., Engstrom, E., Xu, K., Sckisel, G. & Pereira, M. (2008). The Medicago truncatula ortholog of Arabidopsis EIN2, sickle, is a negative regulator of symbiotic and pathogenic microbial associations. *The Plant Journal* 55(4): 580-595.

Vernié, T., Kim, J., Frances, L., Ding, Y., Sun, J., Guan, D., Niebel, A., Gifford, M. L., de Carvalho-Niebel, F. & Oldroyd, G. E. (2015). The NIN Transcription Factor Coordinates Diverse Nodulation Programs in Different Tissues of the Medicago truncatula Root. *The Plant cell* 27(12): 3410-3424.

Vessey, J. K., Pawlowski, K. & Bergman, B. (2004). Root-based N-2-fixing symbioses: Legumes, actinorhizal plants, Parasponia sp and cycads. *Plant and Soil* 266(1-2): 205-230.

Wang, C., Zhu, H., Jin, L., Chen, T., Wang, L., Kang, H., Hong, Z. & Zhang, Z. (2013). Splice variants of the SIP1 transcripts play a role in nodule organogenesis in Lotus japonicus. *Plant Molecular Biology* 82(1-2): 97-111.

Wang, H., Moore, M. J., Soltis, P. S., Bell, C. D., Brockington, S. F., Alexandre, R., Davis, C. C., Latvis, M., Manchester, S. R. & Soltis, D. E. (2009). Rosid radiation and the rapid rise of angiosperm-dominated forests. *Proceedings of the National Academy of Sciences* 106(10): 3853-3858.

Webster, G., Poulton, P., Cocking, E. & Davey, M. (1995). The nodulation of micro-propagated plants of Parasponia andersonii by tropical legume rhizobia. *Journal of Experimental Botany* 46(9): 1131-1137.

Wen, X., Zhang, C., Ji, Y., Zhao, Q., He, W., An, F., Jiang, L. & Guo, H. (2012). Activation of ethylene signaling is mediated by nuclear translocation of the cleaved EIN2 carboxyl terminus. *Cell Research* 22(11): 1613-1616.

White, F., Taylor, B., Huffman, G., Gordon, M. & Nester, E. (1985). Molecular and genetic analysis of the transferred DNA regions of the root-inducing plasmid of Agrobacterium rhizogenes. *Journal of bacteriology* 164(1): 33-44.

Wopereis, J., Pajuelo, E., Dazzo, F. B., Jiang, Q., Gresshoff, P. M., De Bruijn, F. J., Stougaard, J. & Szczyglowski, K. (2000). Short root mutant of Lotus japonicus with a dramatically altered symbiotic phenotype. *The Plant Journal* 23(1): 97-114.

Xiao, T. T., Schilderink, S., Moling, S., Deinum, E. E., Kondorosi, E., Franssen, H., Kulikova, O., Niebel, A. & Bisseling, T. (2014). Fate map of Medicago truncatula root nodules. *Development* 141(18): 3517-3528.

Yang, D.-L., Yang, Y. & He, Z. (2013). Roles of plant hormones and their interplay in rice immunity. *Molecular Plant* 6(3): 675-685.

Yang, M.-Q., van Velzen, R., Bakker, F. T., Sattarian, A., Li, D.-Z. & Yi, T.-S. (2013). Molecular phylogenetics and character evolution of Cannabaceae. *Taxon* 62(3): 473-485.

Yano, K., Yoshida, S., Müller, J., Singh, S., Banba, M., Vickers, K., Markmann, K., White, C., Schuller, B. & Sato, S. (2008). CYCLOPS, a mediator of symbiotic intracellular accommodation. *Proceedings of the National Academy of Sciences* 105(51): 20540-20545.

Yesson, C., Russell, S., Parrish, T., Dalling, J. & Garwood, N. (2004). Phylogenetic framework for Trema (Celtidaceae). *Plant Systematics and Evolution* 248(1-4): 85-109.

Yokota, K., Soyano, T., Kouchi, H. & Hayashi, M. (2010). Function of GRAS proteins in root nodule symbiosis is retained in homologs of a non-legume, rice. *Plant and Cell Physiology* 51(9): 1436-1442.

Yoneyama, K., Xie, X., Kim, H. I., Kisugi, T., Nomura, T., Sekimoto, H., Yokota, T. & Yoneyama, K. (2012). How do nitrogen and phosphorus deficiencies affect strigolactone production and exudation? *Planta* 235(6): 1197-1207.

Young, N. D., Debellé, F., Oldroyd, G. E., Geurts, R., Cannon, S. B., Udvardi, M. K., Benedito, V. A., Mayer, K. F., Gouzy, J. & Schoof, H. (2011). The Medicago genome provides insight into the evolution of rhizobial symbioses. *Nature* 480(7378): 520-524.

Yu, Y.-B., Adams, D. O. & Yang, S. F. (1979). 1-Aminocyclopropanecarboxylate synthase, a key enzyme in ethylene biosynthesis. *Archives of biochemistry and biophysics* 198(1): 280-286.

Zhang, X.-C., Millet, Y. A., Cheng, Z., Bush, J. & Ausubel, F. M. (2015). Jasmonate signalling in Arabidopsis involves SGT1b–HSP70–HSP90 chaperone complexes. *Nature Plants* 1(5).

SUMMARY

Bacteria of the genus Rhizobium play a very important role in agriculture by inducing nitrogen-fixing nodules on the roots of legumes. Root nodule symbiosis enables nitrogen-fixing bacteria to convert atmospheric nitrogen into a form that is directly available for plant growth. This symbiosis can relieve the requirements for added nitrogenous fertilizer during the growth of leguminous crops. Establishment of the rhizobium–legume symbiosis depends on a molecular dialogue, in which rhizobial nodulation (Nod) factors act as symbiotic signals, playing a key role in the control of specificity of infection and nodule formation. Bacterial and legume genes involved in establishing and maintaining the symbiosis are studying over the decades. The expression of "nodulation" genes in the bacteria is activated by signals from plant roots and as a result the bacteria synthesise signals that induce a nodule meristem and enable the bacteria to enter and engage with the host plant.

Research on legume-rhizobium symbioses has emphasized fitness benefits to plants but in our research, we take a different vantage point, focusing on the *Parasponia-rhizobium* symbiosis. *Parasponia* is the only non-legume plant capable of establishing mutualistic relation with rhizobia. This study will provide background knowledge for use in applied objectives as well as yielding a wealth of fundamental knowledge with wide implications from rhizobium symbiosis evolution. Underpinning the work is a continuing investigation of the genes specifically induced during the symbiosis. The communications that occur between the plant and the rhizobia during nodule formation and maintenance constitutes a novel opportunity to study signal transduction in a plant system.

This thesis describes my research on genetic constrains that determine rhizobium-root nodule formation. To identify these constraints we used *Parasponi anadersnii* as only non-legume capable to establish nitrogen fixing rhizobium symbiosis. Our main attempt in this thesis was to find the genetic constraints using *Parasponia* as a key and reconstruct an auto active symbiotic signaling cascade in the non- legume plants.

To facilitate the identification of symbiotic genes in *Parasponia*, first we developed methods to generate transgenic plants. In line with this, a simple and efficient hairy root transformation method was established in **Chapter 2**. We consider this is an improved protocol of *Agrobacterium rhizogenes*-mediated transformation. In about 1 months of *in vitro* culture, we

could recover a high number of transgenic *Parasponia* plants that were resulted from independent transformation.

To determine the genetic elements that underlie the rhizobium symbiosis, we aimed to compare *Parasponia* with closest non nodulating specious, *Trema tomentosa*. To do so, we developed an efficient genetic transformation method for *Trema* mediated by *Agrobacterium tumefaciens* **in Chapter 3**. With this protocol we could able to produce stable transgenic line in 6 month. The entire procedure for generating transgenic plants achieved a transformation frequency of 15% which was sufficiently efficient to conduct experiments at larger scale.

Negative effect of nitrate on root nodulation of legumes has been commonly reported. When legume plants are supplied with nitrate, nodule formation, nodule development and N_2 fixation activity all inhibited. With support of this finding in legumes, we implemented in a physiological study on symbiotic response of *Parasponia* to nitrate. This research opened a novel view on the *Parasponia-rhizobium* symbiosis by discovering a different mechanism that control root nodule formation in *Parasponia* in compare with legumes. In **Chapter 4** we showed that, although legume root nodule formation is inhibited in moderate to high concentration of the nitrate, *Parasponia* forms nodule even in 50 mM nitrate availability. However intracellular infection is markedly reduced. This suggests that *Parasponai-rhizbium* symbiosis is not evolved to regulate the nodule number in presence of the nitrate. In fact the lack of making balance between the need to fixed nitrogen and energy cost to supply rhizobium requirements cause nodule formation in nitrate availability. These results indicate new evidence that *Parasponia-rhizobium* symbiosis evolved recently.

According to the fact that *Parasponia* and legumes are remotely related, it was hypothesized that, *Parasponia-rhizobium* symbiosis evolved independently. Therefore we put forward our attempt to determine the genes required for nodule formation in *Parasponia.* by extending our research on symbiotic genes which are available in non nodulating plants with different function, namely *NSP1* and *NSP2*. It is known for a decade that *NSP1* and *NSP2* genes are transcription factors essential for rhizobial nod factor induction in legumes. In **Chapter 5** we described the role of *NSP* genes during nodulation and mycorrhization in *Parasponia*. Performing *NSP1* and *NSP2* RNAi knocked down *Parasponia* plants showed that these genes positively regulate Root Nodule (RN) and *Amrbuscule Mycorrizal* (AM) formation in *Parasponia*. Mutation in either

NSP1 or *NSP2* markedly reduced nodule formation. It has been previously shown in legumes that NSP proteins have a function in the interaction of plants with AM symbiosis. In the same fashion, in our research knock down mutation in NSPs displayed reduced mycorrhizal colonization level. This shows that *NSP1* and *NSP2* are involved in both nodulation and mycorrhization. This result highlight the idea that RN and AM symbiosis are conserved in part of the pathway and probably bifurcates into two branches by NSP transcription factor allowing specific activation of nodulation or mycorrhization.

Aiming to know the role of hormones in symbiotic behavior, we focused on ethylene as a negative regulator of nodule formation in legumes in **Chapter 6**. We found the negative effect of ethylene on root nodulation of *Parasponia*. For the first time we reported a hyper nodulation (20 fold nodule number in compare with control plants) phenotype in *Parasponia* by performing knocked down mutant of *EIN2* gene, a key regulator of ethylene signaling pathway. Further, in a focused approach we investigated the functional behaviour of *EIN2* of *Trema*, a close relative of *Parasponia* that does not fix nitrogen. *EIN2* knocked down mutant of *Trema* formed nodule like structures in the roots. This result indicated that at least part of the nodule formation capacity in *Parasponia* has been recruited by ethylene signaling pathway during evolution.

Finally, the results obtained in this study provide new insight into the fact that rhizobium symbiosis are under tight genetic constraints that guide endosymbiosis in remotely evolved host plants, legumes and *Parasponia*. Considering this finding along with comparison of *Parasponia* with closest non nodulating sister species, *Trema*, will result in determination of genetic constraints which underlay rhizobium symbiosis. Finding these constraints will help to uncover the core elements and transfer this important trait to major important non legume plants.

In **Chapter 7** the main achievements of this study are discussed and directions for future experiments are highlighted.

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About the author

Maryam Seifi Kalhor was born on 20nd January 1981 in Qom, Iran. After completing high school in his home town he started his higher education in 1999 and finished his bachelor in Plant breeding in 2004 at University of Ilam. She started her MSc-program in Biotechnology at Tabriz University in 2005 and received her MSc certificate with distinguished degree in 2007. She accomplished several research studies funded by different institutes between 2008 and 2010. One of her researches with the title of "improving tolerance to boron toxicity by using of salicylic acid in peppermint" was selected as best national research plans in 2010. After one year she started her PhD in 2011 in Wageningen University, the Netherlands. During her PhD she worked on the *Parasponia* plants and its symbiotic interaction with rhizobium to find genetic constraints underlying this symbiosis. The result of this study is presented in this thesis.

Education Statement of the Graduate School

Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: Maryam Seifi Kalhor
Date: 5 October 2016
Group: Laboratory of Molecular Biology
University: Wageningen University & Research

1) Start-up phase	*date*
► **First presentation of your project**	
Title: Genetic constraints that determine Rhizobium-root nodule formation in Parasponia	Jan 10, 2011
► **Writing or rewriting a project proposal**	
Title: Genetic contrains in evolution of a Rhizobium symbiosis	Aug 2012
► **Writing a review or book chapter**	
► **MSc courses**	
Bioinformatics	2012
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*13.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD student day, Wageningen University	May 20, 2011
EPS PhD student day, University of Amsterdam	Nov 29, 2012
► **EPS theme symposia**	
Annual EPS Theme 1 symposium 'Developmental Biology of Plants', Leiden University	Jan 20, 2011
Annual EPS Theme 1 symposium 'Developmental Biology of Plants', Wageningen University &	Jan 19, 2012
Annual EPS Theme 1 symposium 'Developmental Biology of Plants', Leiden University	Jan 17, 2013
Annual EPS Theme 4 symposium 'Genome Biology', Wageningen University & Research	Dec 13, 2013
Annual EPS Theme 1 symposium 'Developmental Biology of Plants', Wageningen University &	Jan 24, 2014
► **Lunteren days and other National Platforms**	
Annual Meeting 'Experimental Plant Sciences', Lunteren, NL	Apr 04-05, 2011
Annual Meeting 'Experimental Plant Sciences', Lunteren, NL	Apr 22-23, 2013
Annual Meeting 'Experimental Plant Sciences', Lunteren, NL	Apr 14-15, 2014
► **Seminars (series), workshops and symposia**	
Symposium 'Plant Breeding in the Genomics Era'	Nov 25, 2011
Plant Sciences Seminar on Bioinformatics	Mar 12, 2013
EPS Flying Seminar Prof. Graham Farquhar	Mar 13, 2013
Seminar David M. Weller (Root Disease and Biological Control Research Unit)	Sep 25, 2013
Green Life Sciences seminar Prof. Holger Puchta	Sep 26, 2013
WEES Seminar Eric Schranz (Whole genome duplications as drivers of evolutionary)	Nov 20, 2013
Wageningen PhD Symposium	Dec 10, 2013
Open Symposium: Plant Metabolomics	Dec 12, 2013
Invited seminar Jos Raaijmakers (exploring and exploiting the plant microbiome)	Jan 07, 2014
► **International symposia and congresses**	
MiCom 2012: International Student Conference on Microbial Communication, Jena, Germany	Nov 05-08, 2012
EPSO 2013 : International Conference, Porto Heli, Greece	Sep 01-04, 2013
Plant transformation technolgy III conference, Vienna, Austria	Feb 12-15, 2014
► **Presentations**	
MiCom 2012: International Student Conference on Microbial Communication (Poster)	Nov 05-08, 2012
EPSO 2013 : International Student Conference (Poster)	Sep 01-04, 2013
Plant transformation technolgy III conference (Poster)	Feb 12-15, 2014
► **IAB interview**	
► **Excursions**	
Subtotal Scientific Exposure	*11.9 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
Bioinformatics - a User's Approach	Mar 04-08, 2013
Generalized linear model	Jun 14-15, 2012
► **Journal club**	
Member of literature discussion group at laboratory of Molecular Biology	2010-2014
► **Individual research training**	
Subtotal In-Depth Studies	*5.1 credits**

4) Personal development	*date*
► **Skill training courses**	
How to Write a World Class Paper	Oct 26, 2010
Techniques for Writing and Presenting a Scientific Paper	Jul 03-06, 2102
Scientific publishing	Jun 19, 2012
WGS course 'Interpersonal Communication for PhD Students	Apr 10-11, 2014
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*2.2 credits**

TOTAL NUMBER OF CREDIT POINTS*	**32.7**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study.*

Lay-out: Marym Seifi kalhor
Cover: Designed by Sasan Ali Niaei Fard and Maryam Seifi kalhor
Parasponia andersonii (WU1) transgenic roots (front) and *Parasponia andersonii* (WU1) nodule micro-section photo (back).