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This research was conducted under the auspices of the Graduate School of Experimental Plant Sciences.

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Thesis
submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. dr. M.J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Tuesday 17 April 2012
at 4:00 p.m. in the Aula.

Rik H.M. Op den Camp
Evolution of rhizobium symbiosis
136 pages.

Thesis Wageningen University, Wageningen, NL (2012)
With references, with summaries in Dutch and English

ISBN 978-94-6137-198-2

The research for this thesis was funded by the Netherlands Organisation for Scientific Research (NWO; VIDI grant 864.06.007, to René Geurts and Equipment Grant 834.08.001 to Harro J. Bouwmeester) and Russian Federation for Basic Research (grant for Centre of Excellence 047.018.001 to Elena Fedorova)

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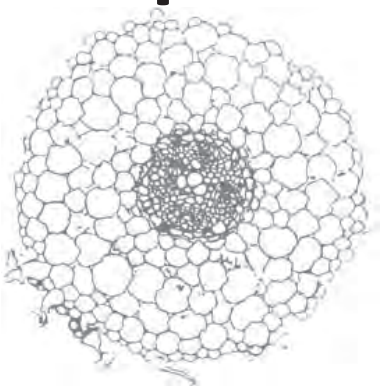
Chapter 1



Introduction

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A focus on evolution

Plants are sessile organisms and therefore restricted in their ability to acquire nutrients. In order to gain better access to nutrients, plants potentially can profit from several endosymbioses. The oldest and most widespread endosymbiosis is the arbuscular mycorrhizal (AM) symbiosis. This symbiosis is estimated to be 400 million years old, based on fossil and phylogenetic data and is present in over 80% of all plant species today (Remy et al., 1994; Wang et al., 2010). This implicates that the AM symbiosis is as old as the rise of land plants and may have been a key adaptation of plants for their initial colonization of land. AM fungi provide their host mainly with phosphate and water, for which in return the plant provides them with carbohydrates (Bonfante and Genre, 2010). Since this symbiosis is maintained millions of years after its birth, it still provides current plants with a way to overcome water and nutrient shortages on land (Wang et al., 2010). A much smaller group of plant species has evolved an endosymbiosis with nitrogen fixing soil bacteria. These plants are part of a single lineage of angiosperms also referred to as the N_2-fixing clade (Soltis et al., 1995; Soltis et al., 1999). Nitrogen fixing soil bacteria can fix atmospheric nitrogen, using an enzyme complex termed nitrogenase, into ammonium and provide this to their hosts in return for carbohydrates (Kouchi et al., 2010; Perrine-Walker et al., 2011). Host plants of the N_2-fixing clade are all part of the rosids I (Fabidae) subclass in the orders; Fabales, Rosales, Fagales and Curcubitales (Fig. 1). Species that can engage an endosymbiosis with nitrogen fixing gram positive soil bacteria of the genus Frankia are found in the Rosales, Fagales and Curcubitales orders. All species that have evolved an endosymbiosis with gram negative

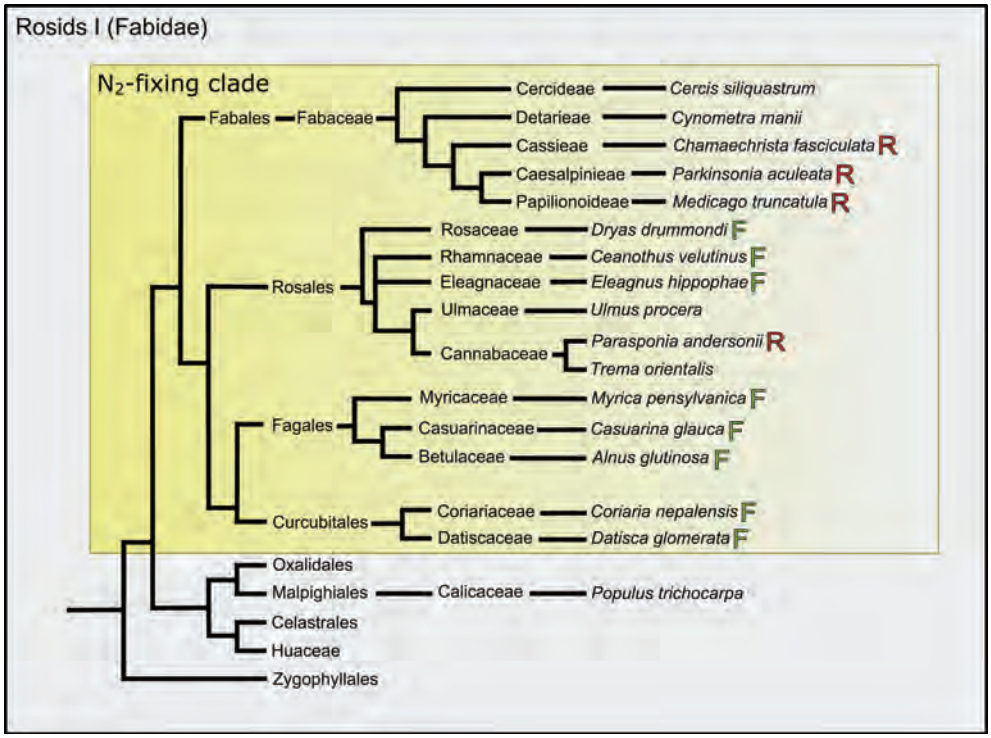

Figure 1. Phylogenetic representation of the N_2-fixing clade. R: rhizobium symbiosis, F: frankia symbiosis. Note that several tribes and (sub-)families are not shown for sake of simplicity (this is an unscaled adapted tree).

nitrogen fixing soil bacteria called rhizobia are all part of the legume family (*Fabaceae*) in the Fabales order, with a single exception; a small group of tropical tree species of the *Cannabaceae* family in the Rosales order called *Parasponia* (Fig. 1). Since all plants with a nitrogen fixing endosymbiosis are found in one N_2-fixing clade, it is hypothesized that there has been a single predisposed ancestor at the root of this clade (Soltis et al., 1995).

In this thesis a broad range of topics will be covered, but the main aim is focused on unravelling the evolution of rhizobium symbiosis. I started with the following research questions, which are used as a theme throughout the thesis; '*Why, from the whole plant kingdom, only plant families from the rosids I subclass have evolved the beneficial rhizobium symbiosis? What makes these species unique? Has a determinative evolutionary event occurred at the origin of the Fabaceae?*' Sub questions of these all-embracing research questions will be addressed.

Economic and social significance of endosymbiotic N_2-fixation

Besides these rather fundamental questions, scientists have always aimed to get non-leguminous plants to engage a symbiosis with rhizobium (Gewin, 2010). By practice of co-cultivation or crop rotation farmers recognized already for centuries that it was beneficial to mix N_2-fixing crops with crops incapable of fixing nitrogen, although the basis of the enhanced growth was not apparent (Burris and Roberts, 1993; Karlen et al., 1994; Lee et al., 2007). More than 120 years ago, it has been demonstrated that legume nodule associated microorganisms fix atmospheric nitrogen (Hellriegel, 1886; Hellriegel and Wilfarth, 1888). Later the Dutch scientist Martinus Willem Beijerinck proved that these microorganisms (rhizobium bacteria) were responsible to induce nodules on legume roots (Beijerinck, 1890). Nowadays legumes are still widely used to be plowed under to improve the fertility of the soil in order to reduce fertilizer nitrogen use (Cocking, 2009). It has been estimated that the annual energy requirement for fertilizer nitrogen production is approximately 1.1% of global energy use in 2008 (Dawson and Hilton, 2011). In the laborious production process of artificial fertilizer roughly 5% of the worlds annual natural gas production is required (Cocking, 2009; Dawson and Hilton, 2011). There is much debate on whether biological nitrogen fixation can yield sufficient nitrogen to feed the current global population. Still, biological nitrogen fixation (mainly of legume-rhizobium symbiosis origin) provides a major contribution to the total nitrogen used in agriculture (Dawson and Hilton, 2011). To further expand the part of biological nitrogen fixation use in agriculture is highly desirable. This can partly be achieved by optimizing the current use of leguminous crops, mainly in developing countries (Ken Giller, proceedings of the N2 AFRIKA meeting 2010). Without the input of industrial produced fertilizer nitrogen it is estimated that only about half of the current global population can be fed (Erisman et al., 2008). Although extrapolation of these figures is difficult, it is inevitable that with the increase of the world population and the depletion of natural resources nitrogen availability remains a topic of interest (Dawson and Hilton, 2011). In the end, scientist see it as a major challenge for future agriculture to transfer the rhizobium symbiosis to non-legumes (Gewin, 2010). In this thesis we bring legumes on non-legumes closer to each other as previously thought and which may provide a blueprint for a future transfer of the rhizobium symbiosis to the major non-legume crops (Chapter 4 and 5). Before getting to this conclusion I will first introduce the molecular genetic aspects of the rhizobium and the arbuscular mycorrhiza symbiosis.

Root endosymbiosis

Bacterial species that are able to engage a nitrogen fixing symbiosis with legumes are all referred to as rhizobia species. There are over 70 species of rhizobia and these are spread among the α- and β-proteobacteria (Masson-Boivin et al., 2009). The common characteristic of rhizobia is the set of genes required for legume-rhizobium symbiosis, for example *nod* (nodulation) and *nif* (nitrogen fixation) genes. These genes are generally localized on separate symbiotic plasmids or occasionally as chromosomal symbiotic islands (Sullivan et al., 1995). The molecular dialog between host and microsymbiont starts with signal exchange in the rhizosphere. Legumes secrete a mixture of flavonoids to attract rhizobia to their rhizosphere. In return rhizobia secrete signal molecules called Nod factors. Nod factors are synthesized by the *nod* gene encoded proteins and can determine the host range of a rhizobium species (Masson-Boivin et al., 2009). Upon perception of these Nod factors by the host, rhizobial infection starts and a developmental program is initiated to give rise to the growth of a new organ; the root nodule. Nodules provide rhizobia optimized conditions for nitrogen fixation (Downie, 2009). Generally, the rhizobia enter a legume host plant by means of so called infection threads. Rhizobia attach to the surface of a root hair and form a microcolony. In response to rhizobial excreted Nod factors, the root hair curls around the microcolony and subsequently an infection thread is formed. This infection thread will grow from the microcolony trough the root hair cell up to cells in the root cortex (Oldroyd and Downie, 2008). Nodule organogenesis is initiated from these cortical cells, parallel to rhizobium infection upon perception of Nod factors in the epidermis (Crespi and Frugier, 2008). Cells that will make up the central zone of the root nodule are infected by infection threads that penetrate host cells and release rhizobia intracellularly (Oldroyd and Downie, 2008). The microsymbiont is maintained in individual plant derived membrane compartments called symbiosomes (Ivanov et al., 2010).

The genetic network driving the establishment of this symbiosis has been largely unravelled using mainly two legumes as model species, namely *Medicago truncatula* and *Lotus japonicus*. Among the key Nod factor signalling genes are three genes encoding receptor kinases, LjNFR5/MtNFP, LjNFR1/MtLYK3 and LjSYMRK/MtDMI2 that are located at the plasma membrane (Madsen et al., 2011). Both receptors harbor LysM motifs in the extracellular domain and are involved specifically in perception of Nod factors (Limpens et al., 2003; Radutoiu et al., 2003; Arrighi et al., 2006; Radutoiu et al., 2007). Upon perception of the Nod factor by these receptors a signalling network is triggered that involves among others a third plasma membrane localized receptor kinase (LjSYMRK/MtDMI2), a cation channel LjCASTOR/LjPOLLUX/MtDMI1 and two nucleoporins LjNUP85/LjNUP133 (Kouchi et al., 2010). The output of this part of the signalling pathway is a root epidermal Ca^{2+} spiking signal, which is likely to be interpreted by the nuclear localized Calcium Calmodulin dependent Kinase (CCaMK; MtDMI3 and LjCCaMK) and the CCaMK interacting protein MtIPD3/LjCYCLOPS (Yano et al., 2008; Capoen et al., 2011; Limpens et al., 2011). In this way kinase activity of CCaMK activates downstream pathways, such as the cytokinin signalling pathway and various transcription factors; e.g. NSP1, NSP2, ERN1 and NIN (Kouchi et al., 2010). Removal of the auto inhibitory domain of CCaMK results in spontaneous root nodule formation in the absence of rhizobia, underlining the importance of this protein in establishment of rhizobium symbiosis in legumes (Gleason et al., 2006).

Several lines of evidence suggest that in evolution, legumes have recruited Nod factor signalling genes from the more ancient AM symbiosis to function in rhizobium symbiosis (Bishopp et al., 2009; Bonfante and Genre, 2010; Liu et al., 2011). The arbuscular mycorrhiza (AM) symbiosis is over 400 million years old and is the oldest known root endosymbiosis (Remy et al., 1994; Wang et al., 2010). This is much older compared to 50-60 million year old rhizobium symbiosis (Cannon et al., 2010). First evidence for a shared genetic pathway or rhizobium and AM symbiosis came from mutagenesis screens in *Pisum sativum* and *Vicia faba* in the late 1980s. In these screens the first mutants were characterized affected in both symbioses (Duc et al., 1989). Further research revealed that most of the key elements of the Nod factor signalling pathway also had a function in AM symbiosis, like for example LjSYMRK/MtDMI2, CCaMK, LjNUP85/LjNUP133 and LjCYCLOPS/MtIPD3. Therefore these genes are part of the so called common symbiosis pathway (Bonfante and Genre, 2010). Evolutionary studies indicate that in genes of the Nod factor signalling pathway only a limited number of legume specific changes have occurred, rather then that legumes have evolved new specific genes (Godfroy et al., 2006; Heckmann et al., 2006; Liu et al., 2011). Examples of such evolutionary changes are alterations on the protein level or in cis-regulatory elements in promoter regions of the genes. That these changes were only limited is underlined by the finding that orthologs of Nod factor signalling genes from non-legume species can at least partially complement legume plants with a mutation in the corresponding orthologous locus (Godfroy et al., 2006; Heckmann et al., 2006; Liu et al., 2011). A putative ancestral function in the mycorrhizal symbiosis has not been revealed for all genes of the Nod factor signalling network. However the recent finding of the ancestral function of the, aforethought nodulation specific, NSP1/NSP2 transcription factors has been a major step forward in understanding to what extent the Nod factor signalling pathway has been adopted from the AM signalling pathway (Liu et al., 2011).

AM fungi are obligate biotrophs, which can not be cultured without their host and can not take up nutrients outside of a plant cell. AM spores are therefore highly responsive to plant root secreted signals in order to localize a potential host. These signals are strigolactons, a class of carotenoid derived molecules that also act as plant hormones (Kohlen et al., 2011). Strigolactones cause AM spores to germinate and stimulate branching of fungal hyphae to explore the host's rhizosphere (Akiyama et al., 2005). NSP1 and NSP2 are indispensable for strigolactone biosynthesis and in line with this function the *nsp1/nsp2* double mutant was found to be less colonized by AM fungi (Liu et al., 2011).

AM fungi produce signal molecules, called Myc factors, that trigger a signalling pathway which is largely shared with the rhizobium symbiosis and which also results in a Ca^{2+} signal in epidermal cells in proximity of the fungal hyphae (Kosuta et al., 2003; Kosuta et al., 2008). The contact between the plant and fungus is followed by the attachment of a hyphopodium to the root surface. From this hyphopodium, hyphae will migrate intracellulary to finally reach the root cortical cell layers. Root cortical cells are then colonized by hyphae. Once a hypha enters inside a cortical cell it branches into a fine network called the arbuscles, which is lined with a plant derived membrane (Bonfante and Genre, 2010). Like in case of rhizobia, the fungal microsymbiont is maintained in a plant derived membrane and this structure, also referred to as the symbiotic interface or intracellular perimicrobial compartment, facilitates the nutrient exchange between host and microsymbiont (Ivanov et al., 2010). It is noteworthy that also the intracellular colonization by *Frankia* species and for the

intracellular colonization of rhizobium in the non-legume *Parasponia* a similar symbiotic interface is created, suggesting a common (convergent) evolutionary route towards the formation of intracellular perimicrobial compartments (Ivanov et al., 2010; Op den Camp et al., 2010; Perrine-Walker et al., 2011).

For several genes of the aforementioned common symbiosis pathway, also involvement in the *Frankia* symbiosis has been demonstrated (Kouchi et al., 2010; Perrine-Walker et al., 2011). This may indicate that a common set of genes is responsible for the establishment of a symbiotic interface. It has been suggested that by intracellular entrance of a microsymbiont exocytosis drives the growth of the perimicrobial compartment (Ivanov et al., 2010). In the light of root endosymbiosis evolution, this implicates that part of the common symbiotic network has been recruited from the exocytosis cellular machinery. Recent identified genes involved in both AM and rhizobium intracellular progression and accommodation further support this theory (Ivanov et al., unpublished results) (Murray et al., 2011).

After this introduction to the rhizobium and AM symbioses I will describe the approach used in this thesis to tackle the research questions to study the evolution of the rhizobium symbiosis.

Evolution of rhizobium symbiosis in the Fabaceae

To tackle these research questions I approached the rhizobium symbiosis from two different evolutionary perspectives; (I) a focus on evolutionary events in the Papillionoid lineage of the *Fabaceae* and (II) convergent evolution in legumes and of the *Parasponia* genus. First I tried to trace back evolution of the *Fabaceae* making use of legume genome sequences. With the technical advances made in DNA sequencing techniques and due to large scale use of these techniques in the past decade, the genomes of *Medicago truncatula*, *Lotus japonicus* and *Glycine max* (soybean) have been (largely) sequenced. Comparing these genomes with the genome sequences of non-legumes (e.g. *Arabidopsis thaliana*, *Populus trichocarpa* and *Vitis vinifera*) may provide insight in legume specific evolution. Rhizobium symbiosis is hypothesized to have evolved several times independently within the legume family, including once in the Papilionoid subfamily (Doyle, 1994; 2011). Based on genome comparisons, a whole genome duplication (WGD) has been estimated to have occurred at the root of the legume subfamily *Papilionoideae* (Fig. 1) (Cannon et al., 2010). Gene duplications are a powerful mechanism to evolve new molecular functions, with maximal creation potential upon a WGD (Osborn et al., 2003; Paterson, 2005; Cui et al., 2006; Shoemaker et al., 2006; Van de Peer et al., 2009). This *Papilionoideae* specific WGD could have led to extensive neo- and sub-functionalization allowing genes to gain a (specific) function in the rhizobium symbiosis (Lynch and Force, 2000). In this thesis it is hypothesized the *Papilionoideae* specific WGD contributed to the evolution of rhizobium symbiosis in that subfamily. To trace back the importance of this WGD we perform a phylogenetic analysis to identify duplicated genes that were maintained in different legume lineages. We analyzed the sequenced genomes of three Papilionoid legumes to identify duplicated genes and compared them to three non-legume genomes. Only gene pairs maintained in all three legumes were counted. The analysis yielded 261 of such gene pairs (De Mita, unpublished results). In this thesis we describe a case in order to test whether genes found with this approach indeed function in rhizobium symbiosis (Chapter 2). For this test case we selected genes from the cytokinin

phosphorelay pathway to investigate their putative role in rhizobium symbiosis. In chapter 2 we provide a proof of principle for our phylogenetic strategy to identify genes originating from the Papilionoid specific WGD that have gained a function in rhizobium symbiosis. The two genes identified in this way encode type-A Response Regulator genes and we show that these are involved in rhizobium symbiosis, but also have a role in root development. These findings provided further support for the hypothesis that legumes have recruited key Nod factor signalling genes from existing signalling networks, like in this instance lateral root development (Bishopp et al., 2009).

It has been long known that cytokinin signalling fulfilled a role as an integral part of Nod factor signalling (Torrey, 1961; Frugier et al., 2008). From the cytokinin phosphorelay cascade the histidine kinase cytokinin receptor, type-B and type-A cytokinin response regulators have been shown to play a role in rhizobium symbiosis (Gonzalez-Rizzo et al., 2006; Murray et al., 2007; Vernie et al., 2008). Cytokinin signalling is positioned in the Nod factor signalling pathway downstream of CCaMK (Gonzalez-Rizzo et al., 2006), which we further underline in chapter 2, where we show that transcriptional activation type-A RRs by nod factor does not occur in the *Mtccamk/Mtdmi3* mutant (Op den Camp et al., 2011). Legumes respond to externally applied cytokinin by initiation nodule organogenesis, visible as the mitotic activation of cortical cells (Fig. 1, discussion)(Cooper and Long, 1994; Mathesius et al., 2000; Heckmann et al., 2011). In contrary, non-legumes do not respond to cytokinin in this way. In line with this, it can be hypothesized that the legume specific response to cytokinin represents an evolutionary adaptation to gain rhizobium symbiosis.

Besides legume response to cytokinin, we focus further on cytokinin signalling as integrated part of Nod factor signalling. Although always hypothesized that cytokinin accumulation plays a role downstream of Nod factor perception, its actual abundance in roots upon symbiotic signalling has never been investigated. We tackled this outstanding question by setting up a method to quantify the endogenous abundance of cytokinins in roots of *M. truncatula*. We demonstrate that cytokinins accumulate as a secondary signal upon Nod factor perception (Chapter 3).

Convergent evolution of rhizobium symbiosis in the Cannabaceae

The rhizobium symbiosis found in the non-legume *Parasponia* is interesting to study because of two main characters; it is an independent evolutionary event from legumes and it gained rhizobium symbiosis only recently (Streng et al., 2011). In order to investigate the genetic constraints of the rhizobium symbiosis we tested whether the independently evolved symbiosis in *Parasponia* makes use of the same Nod factor signalling pathway components as legumes. In this thesis we demonstrate that this indeed is the case (chapter 4). One exception to the aforementioned common symbiotic pathway genes are the Nod factor receptors (Bonfante and Genre, 2010). It is generally thought that legumes have evolved specific set of LysM-type receptors to perceive rhizobial Nod factors (Bonfante and Genre, 2010; Kouchi et al., 2010). In legumes the Nod factor receptors MtLYK3/LjNFR1 and MtNFP1/LjNFR5 are part of largely diverged LysM-type receptor kinase family that underwent several rounds of legume specific duplications. Therefore this may represent a possible determinative evolutionary event in the evolution of rhizobium symbiosis. This thesis provides the first experimental data that also Nod factor receptors have been co-

opted from AM-fungi induced signalling and therefore are part of the common symbiotic pathway. We show that in *Parasponia* the Nod factor receptor PaNFP has a double role in both rhizobium as AM symbiosis. These findings provide strong support for the hypothesis that during evolution a Myc factor receptor, as part of the common signalling cascade, has been recruited to serve as Nod factor receptor in the rhizobial plant symbiosis. Also it suggests that the Myc factor will have similar structural characteristics as Nod factors. Indeed it appeared that the Myc factor structure is very similar to the Nod factor (Maillet et al., 2011). The implications of these findings are set out in a review, which is included in this thesis as chapter 5. Our results suggest that non-legumes that can engage AM symbiosis, can possibly recognize Nod factor-like molecules as well.

Symbiotic promiscuity

Specificity for rhizobium microsymbionts is generally thought to have emerged upon coevolution between host and microbe (Provorov and Vorobyov, 2008; Martinez-Romero, 2009; Masson-Boivin et al., 2009). This implies that the ground state of a plant host in the rhizobium symbiosis is a high level of promiscuity (Sprent, 1994). In line with that we hypothesize that the more recent evolved non-legume rhizobium host *Parasponia* is highly promiscuous (chapter 6).

We demonstrate that rhizobia from a broad phylogenetic range could nodulate *Parasponia*. Besides, these species appeared to produce an even broader range of Nod factor structures. Interestingly, the effectiveness of the symbiosis varied greatly. The strains tested varied in nodule number and nitrogen fixation rate. Some stains appeared to behave rather as a biotrophic pathogen than as a symbiont. Is this the drawback of *Parasponia* being highly promiscuous, or are we looking at evolution in progress? Legumes can overcome non-performing rhizobia by imposing sanctions (Kiers et al., 2003). To what extent can other non-nodulating species outside the *Fabaceae* recognize Nod factors? It is clear that Nod factor recognition only is not the key to an efficient symbiosis (Downie, 2009; Masson-Boivin et al., 2009). Ultimately further comparative studies on *Parasponia* and legumes as well as *Parasponia* and its non-nodulating sister genus *Trema* (Fig. 1) will provide a blueprint for a future transfer of the rhizobium symbiosis to the major non-legume crops.

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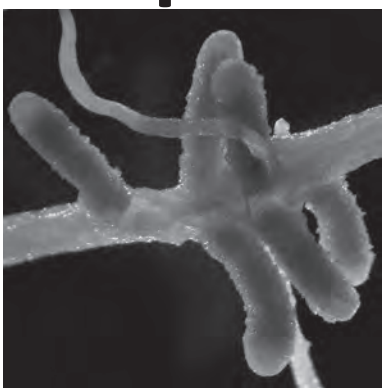
Chapter 2



A phylogenetic strategy based on a legume-specific whole genome duplication yields symbiotic cytokinin type-A Response Regulators

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Adapted version from: Op den Camp, R.H.M. et al. (2011) *Plant Physiol* **157** doi:pp.111.187526

Legumes host their rhizobium symbiont in novel root organs, called nodules. Nodules originate from differentiated root cortical cells that de-differentiate and subsequently form nodule primordia, a process controlled by cytokinin. A whole genome duplication (WGD) has occurred at the root of the legume *Papilionoideae* subfamily. We hypothesize that gene pairs originating from this duplication event and are conserved in distinct *Papilionoideae* lineages have evolved symbiotic functions. A phylogenetic strategy was applied to search for such gene pairs in order to identify novel regulators of nodulation, using the cytokinin phosphorelay pathway as a test case. In this way two paralogous type-A cytokinin Response Regulators were identified that are involved in root nodule symbiosis. *MtRR9* and *MtRR11* in *Medicago truncatula*, and an ortholog in *Lotus japonicus*, are rapidly induced upon rhizobium Nod factor signaling. Constitutive expression of *MtRR9* results in arrested primordia that have emerged from cortical, endodermal and pericycle cells. In legumes lateral root primordia are not exclusively formed from pericycle cells, but also involves the root cortical cell layer. Therefore, the MtRR9 induced foci of cell divisions show a strong resemblance to lateral root primordia, suggesting an ancestral function of *MtRR9* in this process. Together, these findings provide a proof of principle for the applied phylogenetic strategy to identify genes with a symbiotic function in legumes.

Introduction

Most legumes (*Fabaceae*) can establish a unique endosymbiosis with nitrogen fixing soil bacteria, collectively named rhizobium. Rhizobium bacteria grant their hosts access to combined nitrogen. To achieve this, root nodules are formed, which are unique plant organs that provide optimal conditions for rhizobium to fix nitrogen. The rhizobium-legume symbiosis is set in motion by bacterial signal molecules named Nod factors. Nod factors are perceived by plant-specific LysM domain trans-membrane receptors, which in turn activate downstream signaling networks essential for nodule organogenesis (Kouchi et al., 2010). Among the downstream signaling networks is the cytokinin phosphorelay pathway (Frugier et al., 2008). How legumes have recruited such genes to function in symbiosis remains largely unknown. Recently it was shown that legumes of the large *Papilionoideae* subfamily (Papilionoids) underwent a whole genome duplication (WGD) (Cannon et al., 2006). This duplication event occurred early in Papilionoid evolution, as it is estimated to have occurred 56-65 million years ago (Fawcett et al., 2009; Cannon et al., 2010). Papilionoids represent all major legume crops and rhizobium symbiosis is common to most of the ~13,000 species (Gepts et al., 2005). We hypothesize that the Papilionoid-specific WGD has contributed substantially to the makeup of root nodules in this subfamily, even though rhizobium symbiosis itself possibly evolved already at an earlier time point (Fawcett et al., 2009; Cannon et al., 2010). To test this hypothesis we focused on the cytokinin phosphorelay signaling pathway.

The role of cytokinin signaling in root nodule symbiosis is demonstrated by physiological and molecular genetic studies. Early studies showed that in some legume species initiation of nodule organogenesis could be mimicked by external cytokinin application. For example in alfalfa (*Medicago sativa*), lotus (*Lotus japonicus*) and white cover (*Trifolium repens*) the formation of nodule-like structures can be triggered with an architecture similar to Nod factor induced nodules (Cooper and Long, 1994; Mathesius at al., 2000a; Heckmann et al., 2011). Additionally, in many legume species it is shown that externally applied

cytokinin leads to induction of symbiotic genes, which can also be activated by Nod factors (Frugier et al., 2008). Genetic integration of the cytokinin phosphorelay pathway in Nod factor signaling is best demonstrated by gain-of-function and loss-of-function mutants of the histidine kinase cytokinin receptor *LjLHK1/MtCRE1* in lotus and medicago (*Medicago truncatula*). A functional *LjLHK1/MtCRE1* gene is indispensable for nodule formation and a dominant positive mutation in the receiver domain even leads to spontaneous nodule formation (Gonzalez-Rizzo et al., 2006; Murray et al., 2007; Tirichine et al., 2007; Limpens et al. 2011, Plet et al., 2011). Spontaneous nodulation driven by the gain-of-function HK mutant requires other components of the Nod factor induced signaling pathway, e.g. NSP2 and NIN, which underlines the intertwining of both networks. *LjLHK1/MtCRE1* also functions in lateral root formation, indicating that the symbiotic activity of these HKs is derived from this non-symbiotic process (Gonzalez-Rizzo et al., 2006; Murray et al., 2007; Tirichine et al., 2007; Plet et al. 2011). Examples of cytokinin signaling related to root development are the control meristem size, cell differentiation, vasculature development and lateral root primordium initiation (Bishopp et al., 2009). The latter process generally is considered to occur in the root pericycle, whereas in legumes root nodule primordia are largely formed from root cortical cells (Laplaze et al., 2007; Crespi and Frugier 2008; Peret et al., 2009).

The cytokinin phosphorelay pathway consists of four signaling components; histidine kinase cytokinin receptors (HK), phosphotransfer proteins (HP) and two types of Response Regulators (RRs). Upon activation, HK phosphorylates an HP. Subsequently, HP migrates to the nucleus and transfers the phosphate to a type-B RR, which in turn acts as a transcriptional activator. Among the primary targets of type-B RRs are so-called type-A RRs. Both RR types are homologous in sequence, although type-A RRs lack a putative DNA-binding domain. It is generally assumed that type-A RRs act as negative regulators of cytokinin signaling (Muller and Sheen, 2007). In line with the symbiotic role of *LjHK1/MtCRE1*, it can be anticipated that also other components of the cytokinin phosphorelay pathway have evolved to they function in symbiotic signaling. One such gene is the A-type response regulator *MtRR4*, which functions downstream of *MtCRE1* (Plet et al. 2011).

In the study presented here the cytokinin phosphorelay components from three Papilionoid legume species for which substantial genome information is available; namely medicago, lotus and soybean (*Glycine max*) were analyzed in order to find gene pairs that were maintained from the Papilionoid specific WGD. We used as criterion that both gene copies should be maintained in all three legume species and the timing of the duplication should match the WGD event. One such gene pair, encoding type-A RRs, was found. Functional studies revealed that these genes are transcriptionally induced upon Nod factor signaling in both medicago and lotus. For the medicago genes *MtRR9* and *MtRR11* we show that their induction depends on the nuclear localized Calcium Calmodulin Kinase (CCaMK); a key element in Nod factor signaling (Levy et al., 2004; Mitra et al., 2004a; Smit et al., 2005). Ectopic expression of *MtRR*9 results in arrested lateral primordia that are associated with multiple cortical and pericycle cell divisions. These data provide a proof of principle for the phylogenetic strategy based on a legume specific WGD to identify genes involved in rhizobial symbiosis.

Results

One gene pair of type-A Response Regulators is maintained upon the Papilionoid specific WGD

The genes encoding components of the cytokinin phosphorelay pathway are well characterized in *Arabidopsis thaliana* (arabidopsis), which facilitated the identification of legume genes of this pathway (Supplement file S1). To test whether some of these genes are specifically duplicated in Papilionoid legumes, we performed a phylogenetic analysis to identify gene pairs that originate from the Papilionoid specific WGD (Fig. S1). The genomes of three legumes; medicago, lotus and soybean and three non-legumes; arabidopsis, black cottonwood poplar (*Populus trichocarpa*) and grapevine (*Vitis vinifera*) were analyzed.

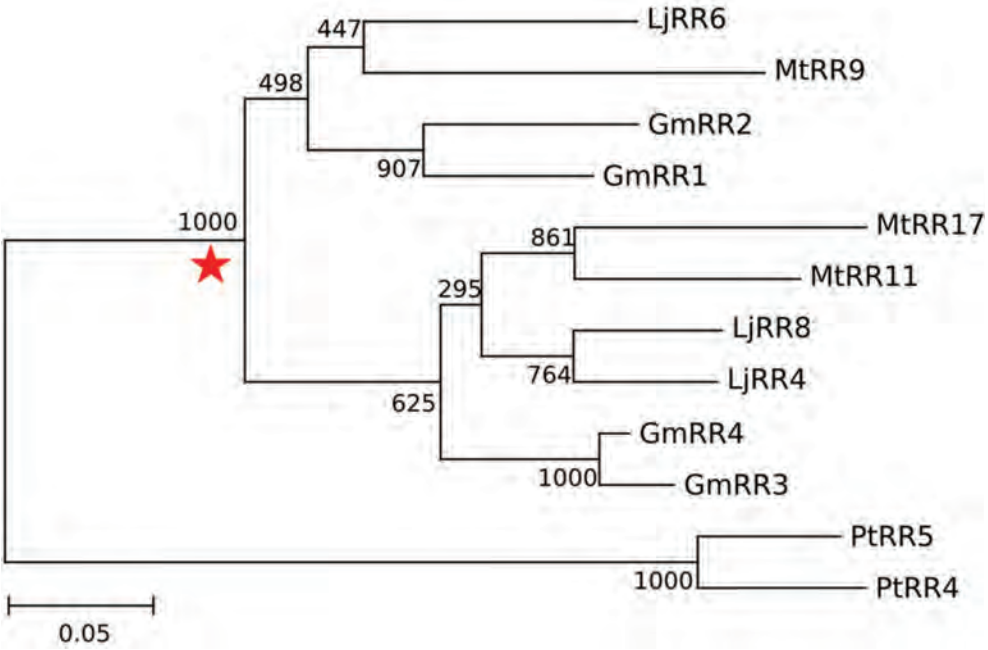

Figure 1. Maintained duplication of type-A RR genes in legumes. Red star marks legume specific duplication in this selection of orthology group 2.4 (complete tree of type-A RRs is shown in Fig. S1). Tree is constructed using maximum likelihood phylogeny (PHYML v3 (Anisimova and Gascuel, 2006) and branch support test from 1,000 bootstrap repetitions. Mt = M. truncatula, Lj = L. japonicus, Gm = G. max and Pt = P. trichocarpa.

Only one clade displayed a legume specific duplication maintained in all three legume species (Fig. 1). This clade belongs to the type-A RR gene family and is referred to as orthology group 2.4 in Figure S1. To date this duplication, we used a maximum likelihood estimation based on the molecular clock hypothesis (Kimura, 1969). The duplication was estimated to have occurred 61 million years ago, by which it falls within the confidence interval for the Papilionoid specific WGD (Fawcett et al., 2009). Besides this duplication event, lineage specific duplications occurred in all species investigated. In case of soybean and black cottonwood poplar this is likely the result of more recent WGDs (Shoemaker et al., 2006; Fawcett et al., 2009; Schmutz et al., 2010). The medicago type-A RR genes in orthology group 2.4 were named *MtRR9*, *MtRR11* and *MtRR17*, of which the latter represents a pseudogene

due to a frame-shift mutation. The soybean genes were named *GmRR1* to *GmRR4*, whereas nomenclature for lotus and black cottonwood poplar was adopted from literature; *LjRR4*, *LjRR6*, *LjRR8*, *PtRR4* and *PtRR5* (Ramirez-Carvajal et al., 2008; Ishida et al., 2009) (Fig. 1).

Nod factor induced expression of duplicated type-A RR genes

Transcriptional regulation upon Nod factor application was tested in order to investigate whether the identified paralogous pair of RR genes could play a role in rhizobium symbiosis. To investigate the extent of Nod factor induction of type-A RR genes we included all 12 medicago type-A RRs in this analysis. For 6 genes expression in roots could be detected, of which three were transcriptionally activated 3 hours after application of *Sinorhizobium meliloti* Nod factors, including *MtRR9* and *MtRR11* (Fig. 2A; Fig. S2). This suggests that both genes could have a function early in symbiotic signaling. Besides *MtRR9* and *MtRR11*, also *MtRR8* was strongly induced, whereas MtRR5 was downregulated (Fig. S2). *MtRR8* is the putative ortholog of *AtARR5* (Fig. S1); a gene widely used as cytokinin responsive marker in a diverse range of species, including legumes (D'Agostino et al., 2000; Lohar et al., 2004). Noteworthy, we did not find Nod factor induced transcriptional activation of *MtRR4*; a type-A RR that is transcriptional activated upon rhizobium inoculation within 24 hours (Gonzalez-Rizzo et al., 2006; Plet et al., 2011).

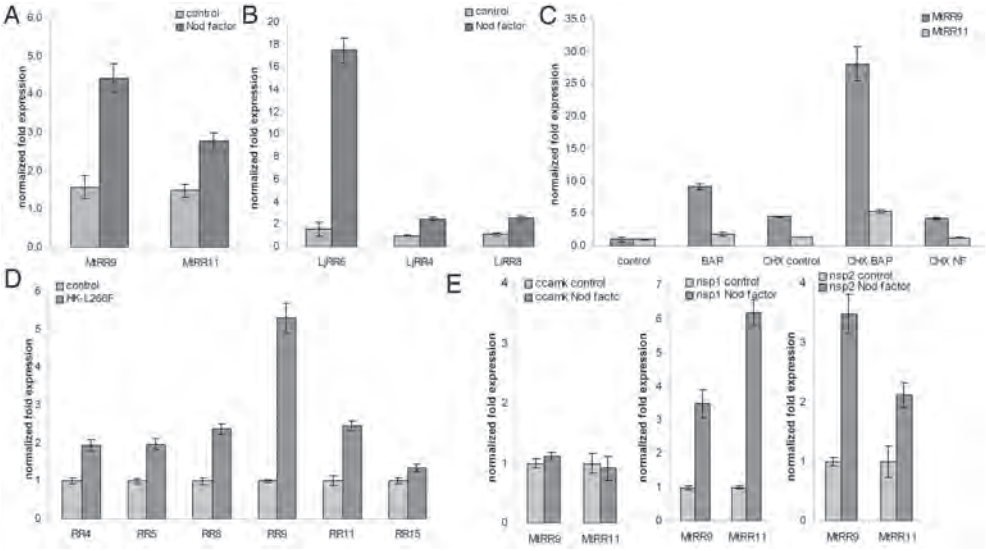

Figure 2. A-B, Relative expression of RR genes was determined using quantitative RT-PCR after 3 hours application of Nod factors (10^{-9}M), for medicago (A) and lotus (B). C, Relative expression levels in medicago roots of *MtRR9* and *MtRR11* in absence or presence of cycloheximide (CHX) during exposure to Nod factors (3 h) or 6-benzylaminopurine (BAP 10^{-8}M, 1 h). D, Relative expression levels in medicago control roots (empty vector) versus roots harboring the Mt35S:CRE1*[L267F] construct. E, Relative expression levels of *MtRR9* and *MtRR11* in medicago mutant ccamk, nsp1 and nsp2 roots. Quantification was normalized using stable expressed reference genes *Mt-GAPDH*, *MtPTB*, *LjATPS* and *LjUBQ*. Bars represent SD of three technical repeats.

To determine whether the Nod factor induced expression of the duplicated gene pair in orthology group 2.4 is conserved in legumes we studied the expression of the orthologous lotus genes *LjRR4*, *LjRR6* and *LjRR8* (Fig. 1). To this end Nod factors of *Sinorhizobium* sp. NGR234, a symbiont of lotus, were applied to lotus roots for 3 hours. This revealed that in lotus mainly *LjRR6* is activated, which is in line with the findings in medicago where the

orthologous gene, *MtRR9*, also is most strongly induced (Fig. 2A,B).

Since type-A RRs are primary targets of cytokinin signaling in arabidopsis (To and Kieber, 2008), we also studied the regulation of *MtRR9* and *MtRR11* upon cytokinin and Nod factor application in the presence of the protein synthesis blocker cycloheximide. Both genes were induced by cytokinin (BAP 10^{-8}M), also in the presence of cycloheximide (Fig. 2C). This is in contrast to Nod factor induced expression, where protein synthesis was essential for transcriptional activation of both RRs (Fig. 2C). To further support that medicago type-A RR genes are targets of the cytokinin phosphorelay pathway we isolated RNA from medicago roots transformed with the gain-of-function MtCRE1 construct (35S:MtCRE1*[L267F]), which causes spontaneous nodule formation (Limpens et al., 2011). Quantitative RT-PCR on root RNA showed that from all type-A RR genes MtRR9 was most strongly induced, but also *MtRR4*, *MtRR5*, *MtRR8* and *MtRR11* were activated (Fig. 2D). These results show that these five genes are indeed primary targets of cytokinin signaling downstream of MtCRE1 and suggests that their expression is under direct control of a type-B RR.

In legumes, Nod factor signaling is achieved by a conserved signaling pathway that contains several key proteins, including a nuclear localized Calcium Calmodulin dependent kinase (CCaMK) and two GRAS-type transcription factors NSP1 and NSP2 (Mitra et al., 2004a; Kalo et al., 2005; Smit et al., 2005). CCaMK, NSP1 and NSP2 are reported to be essential for the induction of nearly all symbiotic genes by Nod factors (Mitra et al., 2004b). We studied the transcriptional regulation of *MtRR9* and *MtRR11* upon Nod factor application in the medicago Nod factor signaling knockout mutants Mtdmi3 (ccamk), *Mtnsp1* and *Mtnsp2* to determine whether induction depends on these key symbiotic genes. This revealed that the induction of *MtRR9* and *MtRR11* was dependent on CCaMK, but could be triggered in both *nsp* mutants (Fig. 2E). This suggests that Nod factor activation of the cytokinin phosphorelay pathway can occur independently from both GRAS-type regulators resulting in bifurcation of Nod factor induced signaling downstream of CCaMK. A similar bifurcation of Nod factor signaling downstream of CCaMK also has been shown in lotus (Madsen et al. 2010). All together these studies suggest that legume type-A RR genes have gained a function in Nod factor induced root nodule formation.

MtRR9 is induced in the Nod factor susceptible zone

To study the symbiotic regulation of *MtRR9* and *MtRR11* in more detail the spatial expression pattern of both genes was determined using β-glucuronidase (GUS) reporter constructs. For both genes ~2,500 bp upstream of the transcriptional start site was used as putative promoter. In medicago roots the non-symbiotic expression pattern of pMtRR9::GUS was found exclusively in the root meristematic zone (Fig, 3A,B). pMtRR11::GUS was found not to be expressed in the meristem, but in the epidermis, cortex and endodermis of the differentiation zone, including the zone susceptible to Nod factors (Fig. 3E-G). Upon local application of Nod factors to the susceptible zone the *MtRR9* promoter activity was induced in all cell layers within 3 hours (Fig. 3C,D). Such elevated expression in the epidermis and cortex was less obvious for pMtRR11::GUS, since non-symbiotic expression was already present and GUS is not very suitable for quantitative interpretations. However, upon application of Nod factors the *MtRR11* promoter was found to be elevated in the pericycle (Fig 3 G,H). In root nodules both genes were found to be expressed in the apical region of

differentiated nodules, a region similar to that observed for *MtCRE1* expression (Fig. 3I,J) (Plet et al., 2011).

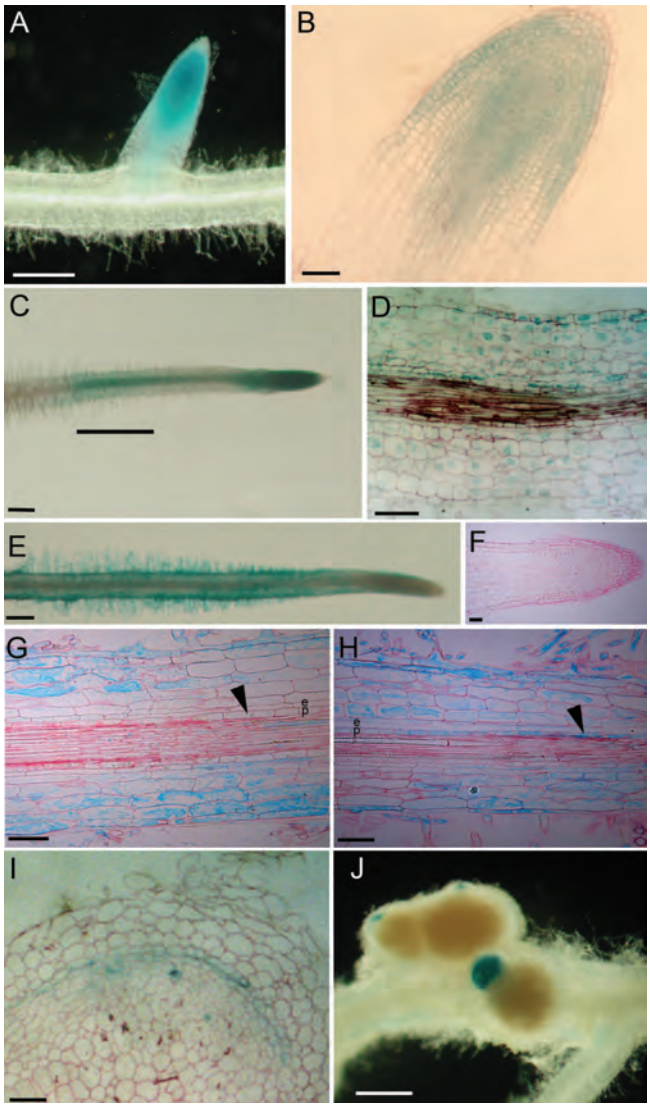

Figure 3. Spatial expression pattern of *MtRR9* (A-D,I) and *MtRR11* (E-H,J). pMtRR9::GUS/pMtRR11::GUS transformed and histochemical stained roots. A, Untreated root meristem. B, Microsection of untreated root meristem. C, Root locally treated with Nod factors (10^{-9}M). Large bar indicates location of Nod factor containing agarose slice. D, Microsection of root at location of Nod factor (10^{-9}M) exposure (3 h). E, Untreated root. F, Microsection of untreated root meristem. G, Microsection of zone susceptible to Nod factors of untreated root. H, Microsection of the root after Nod factor (10^{-9}M) exposure (3 h). I, Expression of pMtRR9::GUS in nodule, microsection. J, Expression of pMtRR11::GUS in nodule. p: pericycle and e: endodermis. Arrowheads point to the pericycle. Bars: A,C,E,J 400µm; B,D,F-H,I 100µm.

Based on this study we conclude that upon symbiotic signaling the spatial expression patterns of both genes largely overlap. The induction of *MtRR9* upon Nod factor signaling in the epidermis, cortex, endodermis and pericycle, and of *MtRR11* in the pericycle, of the susceptible zone prior the occurrence of symbiotic cell divisions, suggests that both genes function in root nodule primordium formation. Since *MtRR9* and its lotus ortholog *LjRR6* are most strongly induced by Nod factors, *MtRR9* is strongest induced in 35S:MtCRE1*[L267F] roots and *MtRR9* is activated in the zone susceptible to Nod factors, we focused on *MtRR9* for further functional studies.

Ectopic expression of MtRR9 results in arrested primordia

To investigate the role of *MtRR9* in root nodule primordium formation we conducted ectopic expression as well as RNAi experiments. First we made an RNAi construct to target *MtRR9* and introduced it into medicago roots by Agrobacterium rhizogenes-mediated transformation. As *MtRR9* is highly homologous to *MtRR11* as well as several other type-A RRs, we determined the specificity of this targeting construct. Therefore, the expression of all six root expressed type-A RRs was quantified by RT-PCR. Analysis showed that this RNAi construct affects *MtRR9* and *MtRR11*, but also *MtRR5*. The latter is the closest homolog of MtRR9 and *MtRR11* though showed opposite regulation by Nod factors when compared to *MtRR9/MtRR11* (Fig. S1; Fig. S2). mRNA levels of all three genes show a knock down level of ~50% in medicago RNAi roots (Fig 4A). We searched for a primordium formation phenotype in the RNAi plants and noted that the RNAi roots had ~33% fewer emerged lateral roots when compared to wild type plants (n=46, Mann and Whitney test P<0.05; Fig. 4B,C). Inoculation of these RNAi roots resulted also in decreased nodulation efficiency of ~33% of the average number of nodules per transgenic root (n=37, Mann and Whitney test P<0.05; Fig. 4D). These findings indicate that type-A RR genes *MtRR9* and *MtRR11*, and possibly *MtRR5*, are required both for nodule organogenesis and lateral root formation.

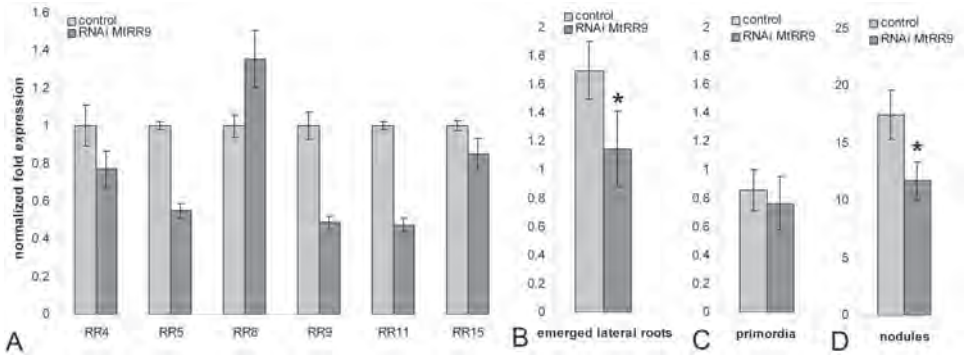

Figure 4. A, Quantification of root expressed type-A RRs. Relative expression levels in medicago pooled control roots (empty vector) versus roots harboring the MtRR9-RNAi construct. Quantification was normalized using stable expressed reference genes *MtGAPDH* and *MtPTB*. Bars represent SD of three technical repeats. B-C, Number of emerged lateral roots (B) and lateral root primordia (C) per transgenic root of medicago plants harboring either a control (empty vector) or the MtRR9-RNAi construct. Asterisk indicates that the difference in number of emerged lateral roots between control and MtRR9-RNAi is statistically significant (Mann whitney Test, P<0.05). Error bars represent SE (n=46). D, Number of nodules per transgenic root of medicago plants harboring either a control (empty vector) or the MtRR9-RNAi construct. Asterisk indicates that the difference in nodule number between control and MtRR9-RNAi is statistically significant (Mann whitney Test, P<0.05). Error bars represent SE (n=37). Two independent biological replicates were performed for all experiments (A-D).

Next, we ectopically expressed *MtRR9* in medicago roots using the constitutive CaMV 35S promoter. For arabidopsis it is reported that ectopic expression of different type-A RRs results in the formation of more lateral roots (Ren et al., 2009). Similarly, pCaMV35S::MtRR9 expressing medicago roots showed an increased number of emerged lateral roots (Fig. S3). Furthermore, such transgenic roots also contained primordia-like structures that where positioned in between emerged lateral roots (Fig. 5A, Fig. S4, Fig. S5). These could either represent arrested lateral root primordia or mimic *de novo* induced root nodule primordia. Microscopic analysis of sections of these MtRR9 induced primordia showed that cell divisions had occurred in the pericycle, endodermis as well as in the root cortex (Fig. 5B). In medicago

such cell divisions can also be triggered by Nod factors (Timmers et al., 1999), which might suggest that the MtRR9 induced primordia have a symbiotic nature.
To determine whether the capacity to induce such primordia is specific for legume type-A RR genes of orthology group 2.4, we conducted the same experiment with *PtRR5*, the putative ortholog of black cottonwood poplar. Transgenic medicago roots ectopically expressing *PtRR5* also formed such primordia, although to a lesser extent (Fig. S4). This indicates that the *MtRR9* encoded protein has not specifically evolved to fulfill such function.

In contrast to medicago, lotus root nodule primordia originate from the middle and outer cortical layers (Szczyglowski et al., 1998; van Spronsen et al., 2001). To determine whether ectopic expression of *MtRR9* can mitotically activate outer cortical cells, we introduced CaMV35Sp::MtRR9 into lotus roots. Also lotus primordia are induced, similar as in medicago (Fig. 5D). Sectioning revealed that in lotus not only pericycle and inner cortical cells divided, but also cells in the middle and outer cortical layers (Fig. 5E). This shows that the location of MtRR9 induced cell divisions coincides with the spatial position of symbiotic divisions in the cortex. Further it indicates that there is not a specific function of MtRR9 dedicated to indeterminate-type nodulation.

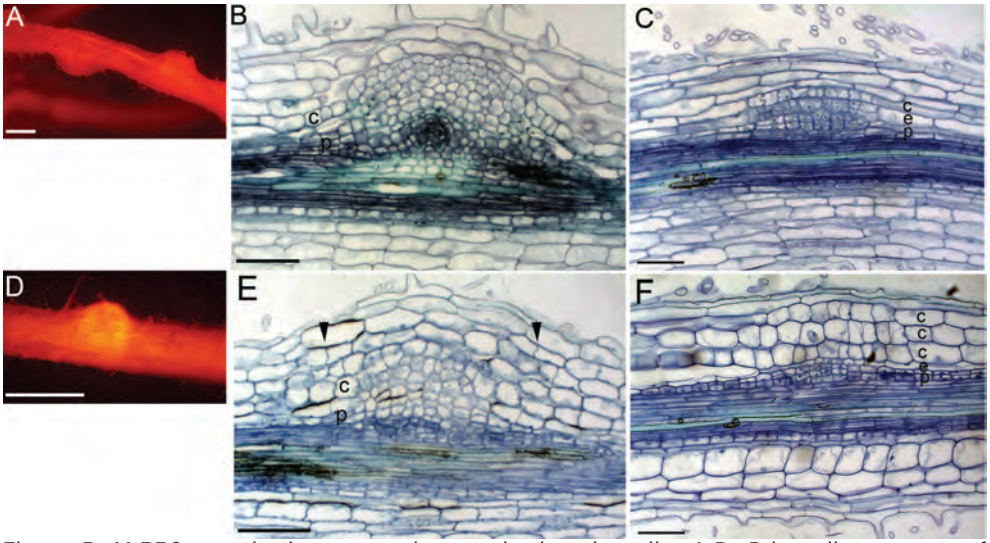

Figure 5. MtRR9 constitutive expression results in primordia. A,D, Primordia on roots of medicago (A) and lotus (D) as result of constitutive expression MtRR9 (pCaMV35S::MtRR9). Transgenic roots were selected based on DsRed fluorescence. B, Microsection of a medicago primordium shows cell divisions in the inner cortical cell layers and pericycle. E, Microsection of a lotus primordium with cell divisions in inner and outer cortical cell layers and pericycle. Microsection of young lateral root primordia; E, medicago. F, lotus. c: cortex, e: endodermis and p: pericycle. Bars: 100µm. Divisions in the outer cortex are marked with arrowheads. c: cortex, en: endodermis and p: pericycle. Bars: A,C 400µm; B,D-F 100µm.

The general view is that lateral root primordia develop from the pericycle and endodermal cell layers (Peret et al., 2009). However, for many species, including some legumes, it has been reported that also cortical cell divisions can accompany lateral root development (Tchermak-Woess and Dolezal, 1953; Mallory et al., 1970; McCully, 1975; Bryne et al., 1977; Casero et al., 1996; Mathesius et al., 2000b). To better compare the MtRR9 induced primordia we studied the involvement of cortical cells during lateral root primordium formation in lotus

and medicago. In both species we observed that the formation of a lateral root primordium in the pericycle cell layer is associated with cell divisions in the endodermal and cortical cell layers (Fig. 5C,F). Interestingly, in lotus the cell divisions in the cortex were extended more to the outer cortical cell layers when compared to medicago, similar as observed upon Nod factor induced nodule primordium formation (Szczyglowski et al., 1998; van Spronsen et al., 2001). Since both species display root cortical cell divisions during lateral root formation it suggests that ectopic expression of *MtRR9* results in arrested primordia that are the result of activation of shared developmental programs essential for nodule as well as lateral root formation.

Discussion

In this study we present a phylogenetic strategy to identify genes originating from the Papilionoid specific WGD that have gained a function in Rhizobium symbiosis. To test the strategy we focused on the cytokinin phosphorelay pathway, since it is presumed to be an integrative part of Rhizobium induced signaling (Frugier et al., 2008). A total of 22 orthology groups were investigated and resulted in the identification of a single conserved gene pair originating from this WGD. We demonstrate that the encoded type-A response regulators are part of the Nod factor induced symbiotic signaling cascade. This shows that despite massive gene loss upon the WGD event that occurred early in the evolution of the Papilionoid sub-family, duplicated gene pairs can be identified that have contributed to the evolution of the rhizobium symbiosis in this subfamily. Therefore, the presented phylogenetic approach can be a useful tool to identify novel genes that function in Rhizobium symbiosis.

Type-A RRs are generally considered to be negative regulators of cytokinin phosphorelay signaling (Hwang and Sheen, 2001; Osakabe et al., 2002; Kiba et al., 2003; Kiba et al., 2004; To et al., 2004; Hirose et al., 2007). In accordance with this view we hypothesize that also *MtRR9* and *MtRR11* are part of a negative feedback mechanism on the cytokinin phosphorelay signaling. Cytokinin plays a negative role in lateral root initiation and it has been shown that arabidopsis type-A RR mutants may fulfill a key function to control this inhibition (To et al., 2004; Ren et al., 2009). The function of *MtRR9* as a negative regulator is based on the finding that ectopic *MtRR9* expression results in more lateral roots, which is in line with similar findings for ectopic expression of type-A RRs in arabidopsis and with the fact that lowered endogenous levels of cytokinin lead to a higher lateral root density (Laplaze et al. 2007; Nibau et al. 2008; Ren et al. 2009). Furthermore, we observed an increased number of arrested lateral primordia. Besides its negative role in the initiation of lateral roots, cytokinin is known to regulate, antagonistically to auxin, the proper patterning of the embryonic root meristem (Müller and Sheen, 2008). Whether similar genetic mechanisms regulate lateral root meristem development is unknown. We anticipate that ectopic expression of a negative regulator of cytokinin signaling may disturb proper lateral root meristem patterning, resulting in arrested lateral primordia.

In contrast to ectopic expression of type-A RR genes, type-A RR arabidopsis mutants have fewer lateral roots, which reflect the negative role of cytokinin in lateral root initiation. Type-A RRs function redundantly, since inhibitory effects were only observed when multiple members were knocked out (To et al., 2004). Our RNAi construct was designed with the intention only to target *MtRR9*, but due to high homology also other type-A RRs were

knocked down. Therefore the observed lowered amount of emerged lateral roots on the RNAi roots are probably due to the combined knockdown of multiple type-A RRs, suggesting redundant functioning of these genes. Notably also a decrease in nodule number is observed in knockdown roots. This seems a paradox, as cytokinin is promoting root nodule formation one would anticipate that down-regulation of cytokinin inhibitor genes would promote root nodule formation. So far we do not have a mechanistical explanation for this finding, though we anticipate that the positive effect of cytokinin on root nodule formation acts only transiently, subsequently resulting in a new auxin maximum in the developing root nodule primordium (Plet et al., 2011). The three genes targeted by RNAi show opposite regulation by Nod factors; *MtRR9* and *MtRR11* were transcriptionally activated, whereas *MtRR5* was downregulated. This may provide an explanation for the observed phenotype as precise cytokinin signaling may be crucial for nodule development. Constitutive knockdown of *MtRR9*, *MtRR11* and *MtRR5* therefore may act negatively on root nodule formation as well.

Strikingly, the arrested primordia that are obtained in *MtRR9* over-expression roots are composed of cells that originate from the cortex, endodermis and pericycle. This observation made us to investigate the ontogeny of lateral root primordia in lotus and medicago. It was found that in both legumes also the cortical ground tissue contributes substantially to this developmental process. Furthermore, we noticed that -to some extent- the spatial position of mitotically active cortical cells in lateral root primordia coincides with the spatial position of nodule primordia. This suggests that the potential to mitotically reactivate cortical cells is not an exclusive characteristic of Rhizobium Nod factor induced signaling, but an intrinsic feature of these cells. Though, the fact that root cortical cells are mitotically activated during lateral root development is not a legume specific character, as it is reported for several non-legume species as well (Tchermak-Woess and Dolezal, 1953; Mallory et al., 1970; McCully, 1975; Casero et al., 1996). The precise function of the dividing cortical cells during lateral root primordium formation remains unknown. Two possible functions can be hypothesized. Either the re-differentiated cortical cells become an integrative part of the primordium, or alternatively these divisions facilitate lateral root emergence through the cortex. The latter hypothesis is proposed for plants that have multiple cortex layers (Peret et al., 2009), which applies to medicago and lotus that have at least five cortical cell layers.

Besides *MtRR9*, other type-A RRs were shown to function in Rhizobium symbiosis (Gonzalez-Rizzo et al., 2006; Vernie et al., 2008). Although their exact molecular functioning remains elusive, we can now position *MtRR9* and *MtRR11* in the Nod factor signaling network. We demonstrate that these genes are transcriptionally activated upon Rhizobium Nod factor signaling. This Nod factor induced expression is not dependent on the GRAS-type transcription factor complex MtNSP1-MtNSP2, whereas it requires MtCCaMK/MtDMI3; a nuclear localized and calcium regulated kinase that functions upstream of the MtNSP1-MtNSP2 transcription factor complex (Kouchi et al., 2010). Furthermore, we found that Nod factor induced *MtRR9* and *MtRR11* expression requires de novo protein synthesis, indicating that Nod factor regulated gene products have a positive effect on the cytokinin signaling pathway. These could be newly synthesized enzymes involved in re-allocation or metabolism of bioactive cytokinin. Such presumed cytokinin signal is then likely perceived by the HK receptor MtCRE1 that has several type-A RRs among its downstream targets; including MtRR4, 5, 8, 9 and 11 (Plet et al., 2011).

The identification of a novel gene pair involved in the rhizobium-legume symbiosis by using a phylogenetic approach based on the Papilionoid specific WGD provides a proof-of-principle for the feasibility of this approach. Therefore we propose that this phylogenetic strategy can be used on a genome-wide scale to identify new (candidate) genes involved in rhizobium symbiosis, even when such gene pairs share redundant functions, which hampers their identification by forward genetic screens.

Materials and Methods

Vectors and constructs

MtRR9 and *PtRR5* full length genomic sequence and MtRR9 RNAi target sequence were derived by PCR amplification using the primers listed in table S1. The genes were cloned into a pENTR-D-Topo vector (Invitrogen) creating pENTR1-2_MtRR9 and pENTR1-2_PtRR5. The CaMV35S promoter and terminator were cloned into a pENTR4-1 and pENTR2-3 (Invitrogen) thereby creating two modified pENTR clones: pENTR4-1_p35S and pENTR2-3_T35S. All three pENTR vectors were combined into the binary destination vector pKGW-RR-MGW by a multisite gateway reaction (Invitrogen). pKGW-RR-MGW contains pAtUBQ10::DsRED1 of pRedRoot as selection marker (Limpens et al., 2004). The *MtRR9* RNAi target sequence was cloned into the DsRed modified gateway vector pK7GWIWG2(II) driven by the CaMV35S promoter as described in Limpens et al. (2005). 35S:MtCRE1*[L267F] was used as described in Limpens et al. (2011).

The putative promoter region of *MtRR9* and *MtRR11*, ~2,500 bp upstream of the translational start site, was PCR amplified using primers listed in table S1. The putative promoters were cloned into a pENTR-D-Topo thereby creating pENTR1-2_pMtRR9 and pENTR1-2_pMtRR11. Subsequently each promoter was recombined into pKGWFS7-RR containing a GUS-GFP fusion reporter as well as pAtUBQ10::DsRed1 as selectable marker (Karimi et al., 2002). All cloning vectors and constructs are available upon request from our laboratory or via the Functional Genomics unit of the Department of Plant Systems Biology (VIB-Ghent University).

Plant materials and treatments

For the Quantitative RT-PCR on type-A RR genes, medicago and lotus germinated seedlings were grown vertically on modified Fåhraeus medium agar plates with low nitrate (0.2 mM $Ca_2(NO_3)_2$) on top of filter paper for 48 hour (Fåhraeus, 1957). Then water dissolved Nod factors (~10^{-9} M) (*Sinorhizobium sp.* NGR234 Nod factors for lotus and *Sinorhizobium meliloti* Nod factors for medicago) or water as a control was pipetted on top of every root (Hussain et al., 1998). Roots were exposed for 3h and subsequently 1 cm root pieces were cut just above the root-tip and were snap-frozen (n=15). For Cycloheximide (CHX) experiments, plants were grown in modified Fåhraeus slides using modified liquid Fåhraeus medium (Heidstra et al., 1994) with low nitrate (0.2 mM $Ca(NO^3)_2$). A single germinated seedling was placed in each slide and medium was exchanged every 24 h. Experiments were done with plants grown for 48 h in Fåhraeus slides. Plants in the slides were treated either with 6-benzylaminopurine (BAP, 10^{-8}M) purified Nod factors (~10^{-9} M), 50µM CHX, 50µM CHX + Nod factors (~10^{-9} M), 50µM CHX + 10^{-8}M BAP for 3 hours or Fåhraeus-medium as a control.

Subsequently root pieces were snap-frozen, as described above. For all experiments plants were grown in an environmentally controlled growth chamber at 20°C with a 16h-light/8h-dark cycle and 70% relative humidity.

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 1 µg 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). All primers including the genes used for normalization (*MtGAPDH*, *MtPTB*, *LjATPS* and *LjUBQ*) are given in table S1. As control for the experimental set up of each Nod factor induced sample, the induction of *NIN* in both medicago and lotus were checked and confirmed (not shown). Data analysis was performed using BioRad iQ5 software (BioRad). Baselines were set at 100 RFU to calculate the Ct-values, Ct values of 31 and higher were excluded from the analysis, though still checked for transcriptional induction (see table S1). A representative sample out of three independent biological replicates is shown in all figures.

Plant transformation and nodulation assay

Agrobacterium rhizogenes-mediated hairy roots transformation was used to transform medicago (Jemalong A17) as described in (Limpens et al., 2004) with the adaptation that 0.2mM $Ca_2(NO_3)_2$ was used in Fåhraeus medium instead. Transgenic roots were selected based on *DsRED1* expression. Three weeks after transformation transgenic roots from promoter studies and ectopic expression studies were transferred to low nitrate Fåhraeus plates (0.2mM $Ca_2(NO_3)_2$). MtRR9 RNAi and empty vector control plants were investigated for lateral roots and primordia 10 days after transfer to Fåhraeus plates. After transformation, MtRR9 RNAi and empty vector control plants were grown and inoculated for three weeks in perlite as described in Limpens et al. (2004). pMtRR9::GUS and pMtRR11::GUS transformed plants were inoculated and grown in perlite in the same way. The differentiation zone (at ~0.7 cm above the tip) of pMtRR9::GUS and pMtRR11::GUS transgenic roots were exposed on Fåhraeus plates for 3 hours to 2-3 mm thin slices of respectively Nod factor (10^{-9}M) or deionized water dissolved in low melting point water-agarose. Afterwards these roots were fixed and sectioned as described in (Limpens et al., 2005). Histochemical GUS staining was performed as described in supplemental protocols. pCaMV35S::MtRR9 and pCaMV35S::PtRR5 roots were investigated for lateral roots and primordia 10 days after transfer to Fåhraeus plates. Primordia were fixed and sectioned as described in the supplemental protocols. All statistical tests were executed using SigmaStat software v3.5 (Systat Software, San Jose, California).

Histochemical analysis and microscopy

Fixation of roots 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. The completely dehydrated roots were embedded in technovit 7100 (Heraeus-Kulzer, Wehrheim, Germany) according to the manufacturers protocol. GUS staining was performed up to 4 hours in 0.1 M phosphate buffer pH7.0 containing, 3% sucrose, 0.5 mM EDTA, 0.1 M $K_4Fe(CN)_6$, 0.1 M $K_3Fe(CN)_6$ and 1 mM x-gluc (first dissolved in DMFO). GUS stained roots were cleared in 70% ethanol for 48 hours before microscopic analysis and subsequent embedding in technovit 7100. Microtome sections of 5 µm were stained with toluidine blue or ruthedium red and photographed using a Leica DM5500B microscope equipped with a Leica DFC425C camera (Leica microsystems, Wetzlar, Germany). Transgenic roots were photographed using a Leica MZIII fluorescence stereomacroscope for DsRED1 marker gene detection (filter settings; excitation 565/30 and emission 620/60). Images from GUS staining were taken using NIKON SMZ-U binocular equipped with a NIKON coolpix 990 camera (Nikon Corporation, Tokyo, Japan). Images were digitally processed using Photoshop CS3 (Adobe Systems, San Jose, California)

Phylogeny

The phylogenetic trees were reconstructed using the maximum likelihood method implemented in the software PhyML version 3.0 (Guindon and Gascuel, 2003). Manually cured alignment of full length nucleotide coding sequences was used for tree building. The software was set to use a BIONJ start tree, the nearest neighbor interchange tree searching method, the general time-reversible model of sequence evolution, gamma-distributed rates of evolution with four discrete categories and empirical nucleotide frequencies. Non-parametric bootstrap repetitions were used to evaluate statistical support to branches. Rooting was performed using the midpoint method. There were in total 38 RR genes found in medicago of A-type (4,5,8,9,10,11,15,17,19,22,31 and 37), B-type (1,2,3,6,12,13,14,16,18and 38) and P-type (7,20,21,23,24,25,26,27,28,29,30,32,33,34,35 and 36) they are listed in supplemental figure 1 (A+B type only) and in the supplemental file "sequences.fas". Sequences of all genes used in this phylogenetic analysis can be found in supplemental file "sequences.fas". Used annotations:

Medicago truncatula (www.medicago.org)
Lotus japonicus (http://www.kazusa.or.jp/lotus/index.html)
Glycine max (www.phytozome.net)
Populus trichocarpa (http://genome.jgi-psf.org/)
Vitis vinefera (www.genoscope.cns.fr)
Arabidopsis thaliana (www.ncbi.nlm.nih.gov/Genbank)

Acknowledgements

Special thanks to Eva Deinum for support with the statistical analyses and undergraduate students Hans van Kessel, Frank Leavis and Ruben Higler who contributed to this project. This work was supported by the Dutch Science Foundation (NWO) (VIDI 864.06.007 to R.G.).

Supplemental figures

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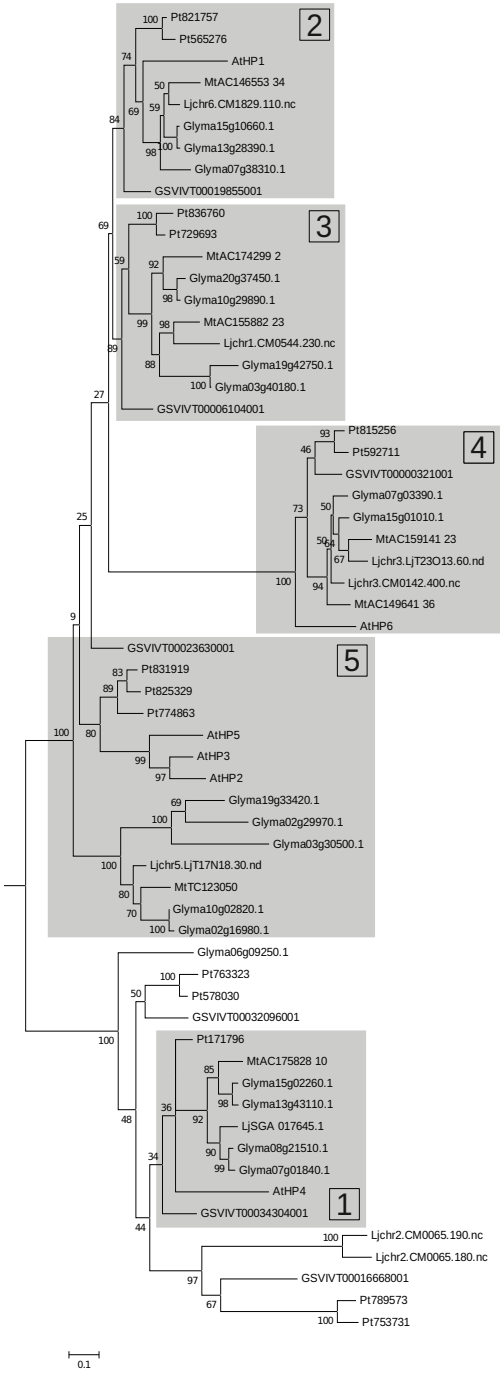

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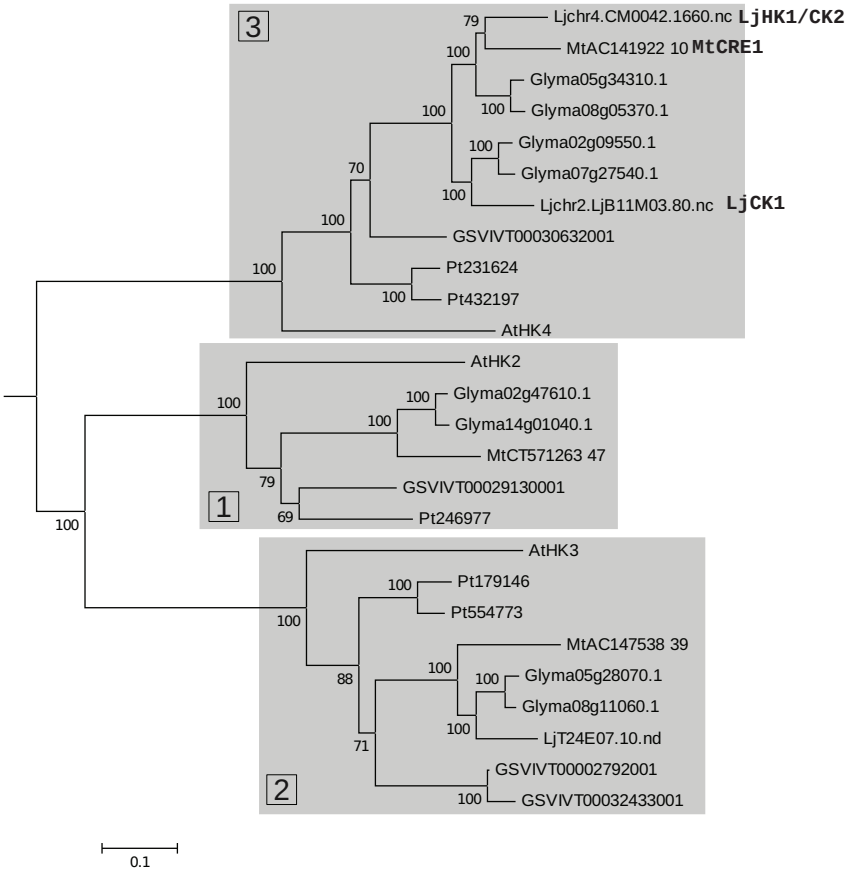
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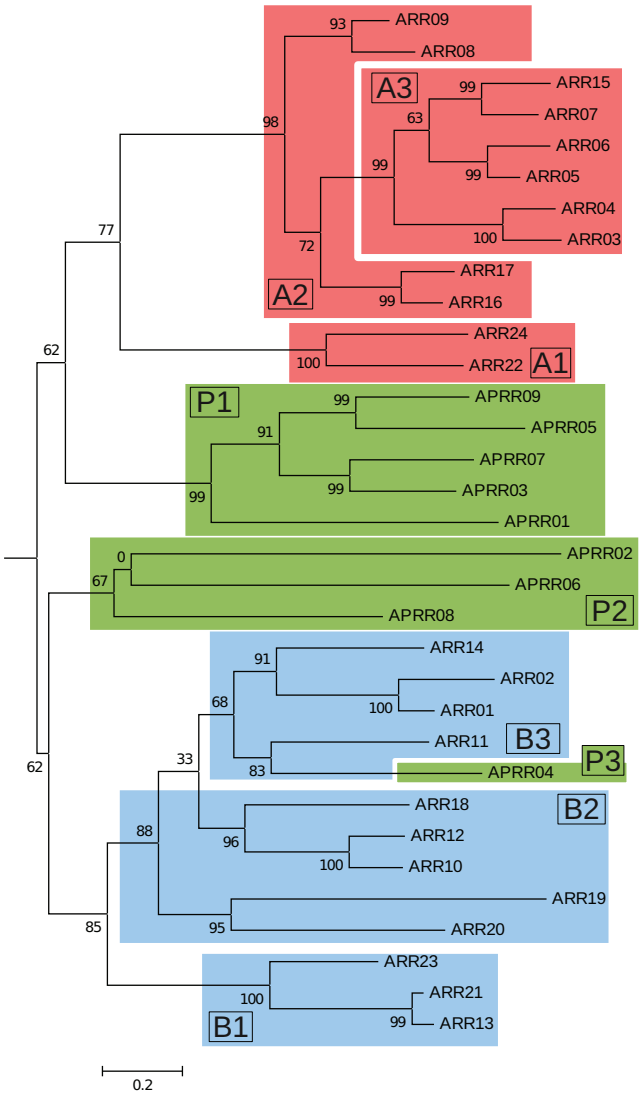
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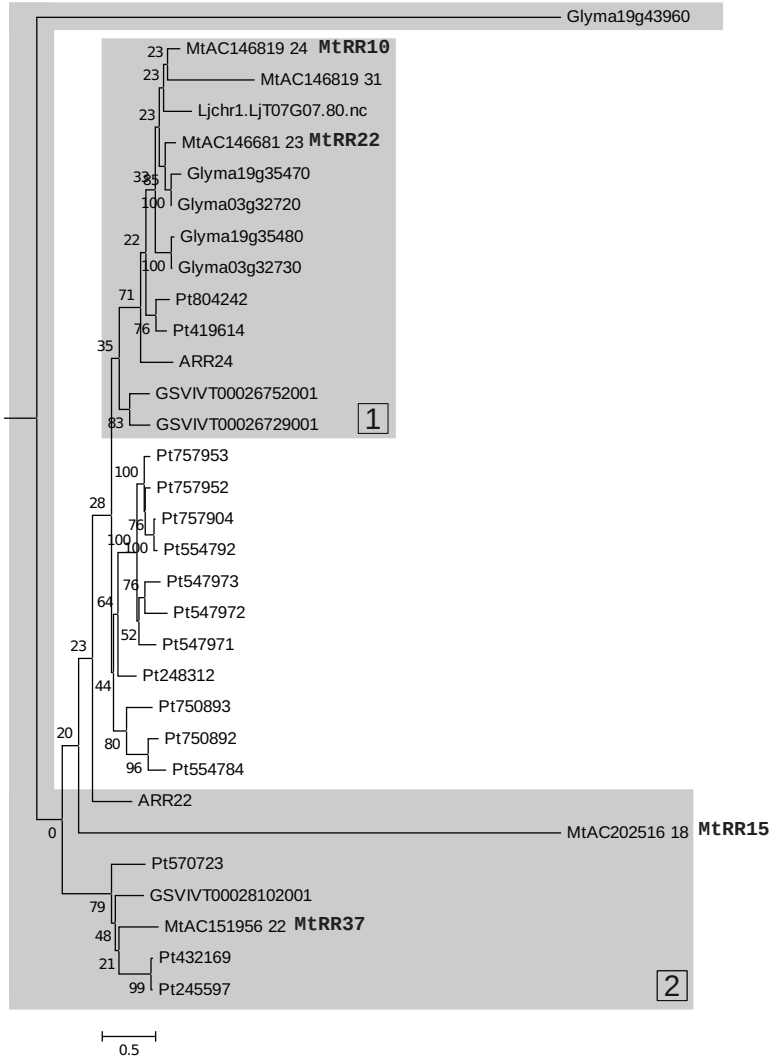
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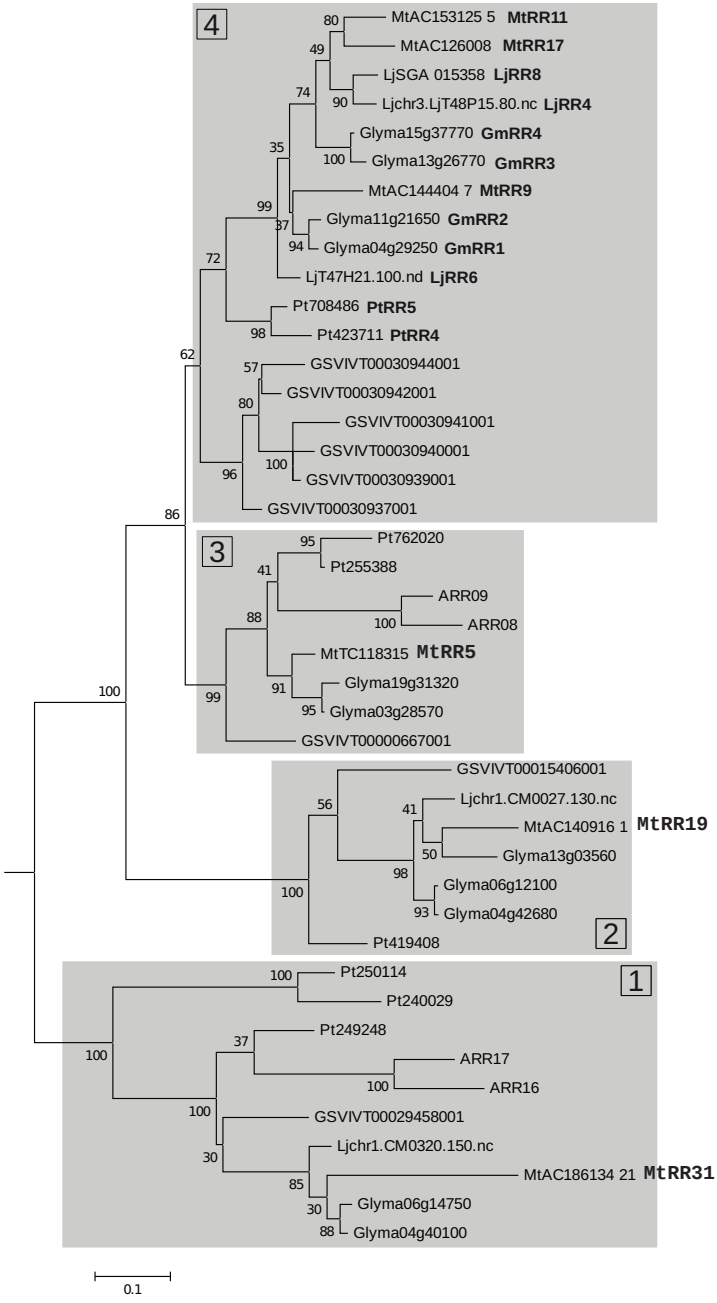
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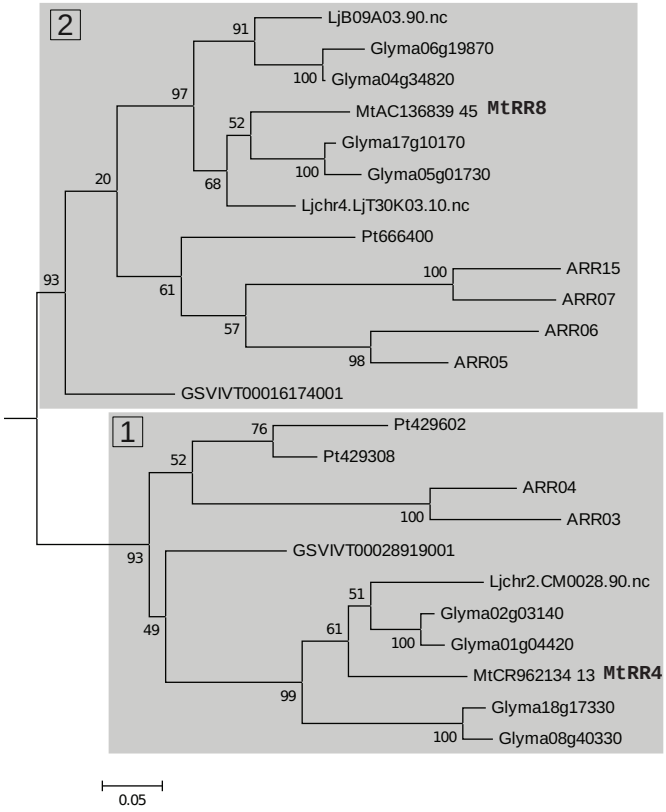
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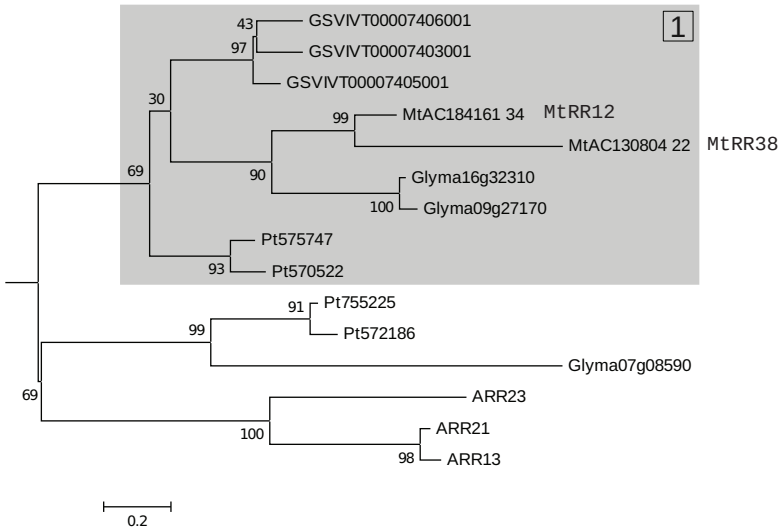
G. RRB1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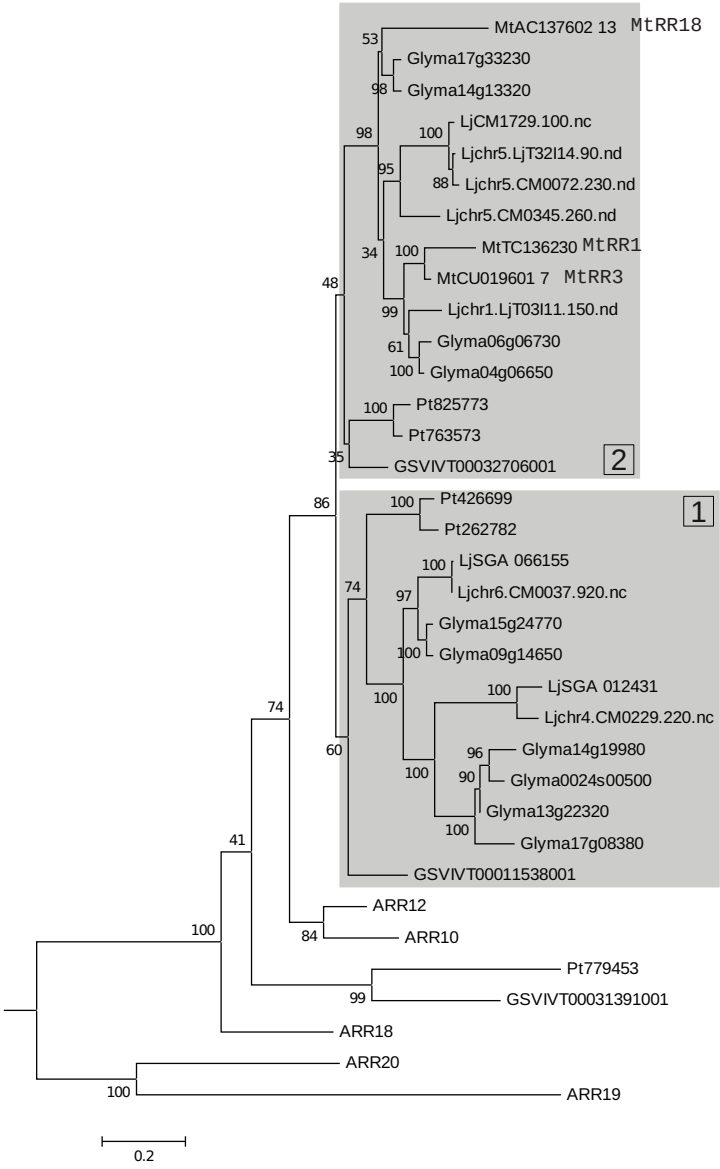
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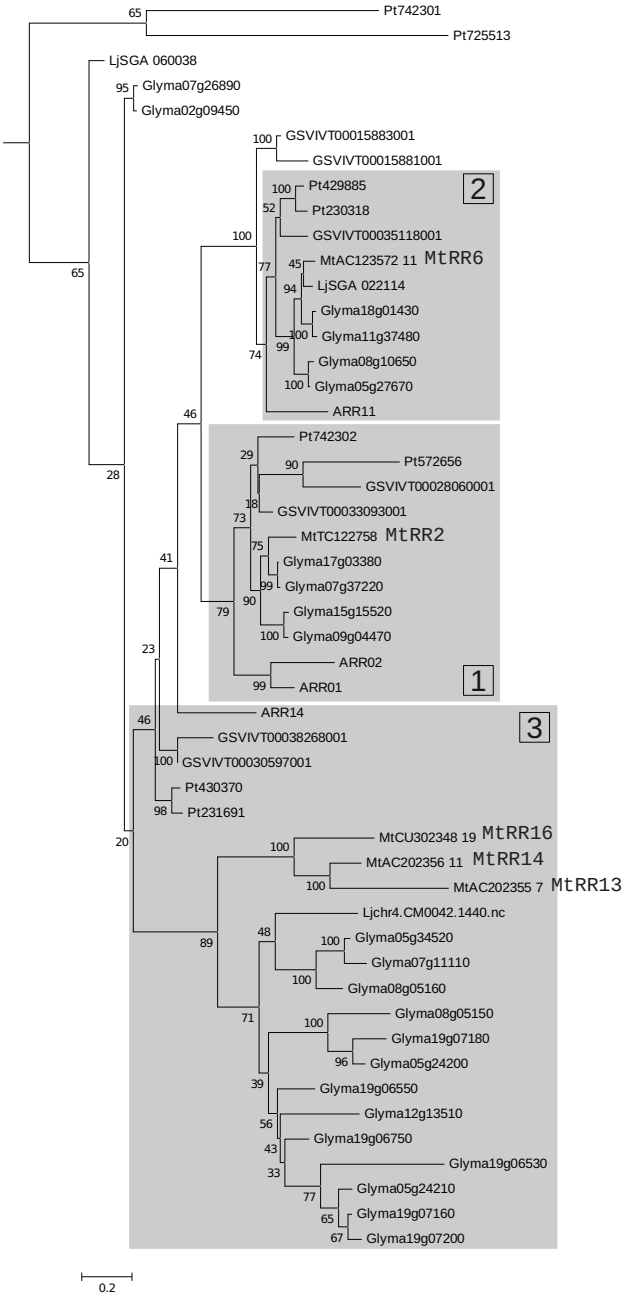
I. RRB3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MtCU302348 19 MtRR16
MtAC202356 11 MtRR14
MtAC202355 7 MtRR13
Ljchr4.CM0042.1440.nc
Glyma05g34520
Glyma07g11110
Glyma08g05160
Glyma08g05150
Glyma19g07180
Glyma05g24200
Glyma19g06550
Glyma12g13510
Glyma19g06750
Glyma19g06530
Glyma05g24210
Glyma19g07160
Glyma19g07200
0.2

Figure S1 (pp 36-44). Phylogenetic analysis of genes encoding components of the cytokinin signaling pathway. Phylogenetic-based definition of legume orthology groups (OGs) for the Phosphotransfer (HP), Histidine Kinase (HK) and Response Regulators (RR) families. Trees A-B and D-U represent the phylogenetic relationships of source arabidopsis genes and their homologues in other species. In each of these trees, numbered gray frames indicate the empirically defined OG. Trees were reconstructed as described in the supplemental protocols. When given, branch support is obtained from 100 non-parametric bootstrap repetitions. The scale of branch length is expressed in number of substitutions per nucleotide site. Tree C includes only RR genes from arabidopsis, incorporating type-A and type-B as well as pseudo RRs (named type-P). Groups were defined empirically from this tree and constrained to be consistent with different types. These groups are indicated by colored frames (red for type-A, blue for type-B and green for type-P). These groups were used for homology search and phylogenetic reconstruction as displayed in trees D-U. Tree C was reconstructed as described in the supplemental protocols, except that no variation of substitution rates was allowed and branch support was evaluated by the approximate likelihood ratio test (SH-like option). The scale of branch length is expressed in number of substitutions per nucleotide site.

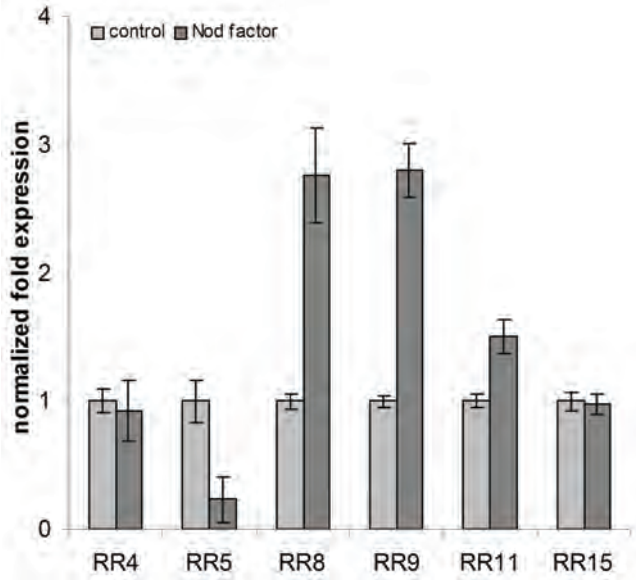

Figure S2 (upper left). Quantitative RT-PCR of medicago type-A RR genes upon Nod factor application. Relative expression was determined after 3 hours application of Nod factors versus control. *MtRR10*, *17* ,*19* ,*22* ,*31* and *37* were found not to be expressed in roots and are not shown. Quantification was normalized using stable expressed reference genes *MtGAPDH* and *MtPTB*. Bars represent SD of three technical repeats.

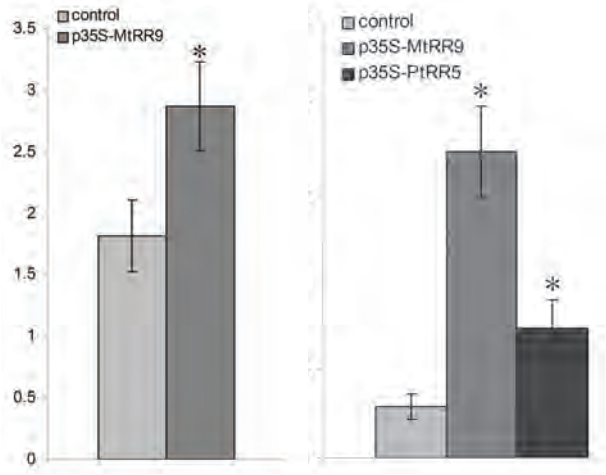

Figure S3 (left). Number of emerged lateral roots per transgenic medicago root, harboring either a control (empty vector) or the pCaMV35S::MtRR9 construct. Asterisk indicates that the difference in emerged lateral root number between control and pCaMV35S::MtRR9 is statistically significant (Mann whitney Test, $P<0.05$). Error bars represent SE ($n>55$).

Figure S4 (right). Number of primordia per transgenic medicago root, harboring either a control (empty vector), pCaMV35S::MtRR9 or pCaMV35S::PtRR5. Asterisk indicates that both pCaMV35S::MtRR9 and pCaMV35S::PtRR5 is statistically significantly compared to the control (ANOVA analysis $P<0.001$). Error bars represent SE ($n>46$).

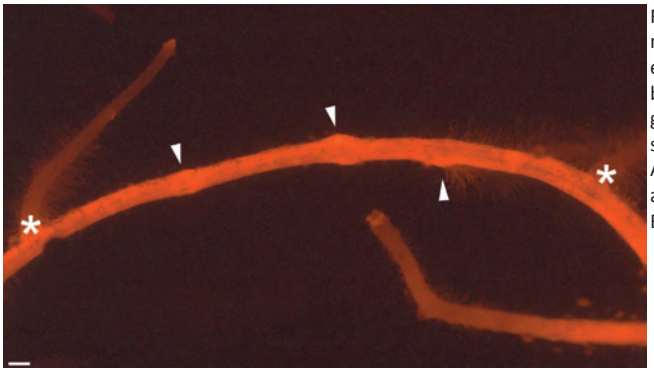

Figure S5. Primordia on roots of medicago as result of constitutive expression MtRR9 appear mainly in between emerged lateral roots. Transgenic pCaMV35S::MtRR9 roots were selected based on DsRed fluorescence. Arrowheads mark primordia and asterisks mark emerged lateral roots. Bar: 100µm.

Supplemental data (online available at plantphysiol.org)

Supplemental table S1. List of primers.
Supplemental file S1. File with sequences in fasta format “sequences.fas”.

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Chapter 3

Nod factor signaling triggers cytokinin accumulation

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As submitted to *Proc Natl Acad Sci U S A* (under review)

The legume rhizobium symbiosis is initiated by signal exchange in the rhizosphere. Rhizobium produces signal molecules called Nod factors, which are sufficient to trigger nodule formation. External application of the phytohormone cytokinin can also result in nodule formation. It has been a longstanding question whether cytokinin accumulates upon symbiotic signaling [Torrey, J.G. (1961) Exp. Cell Res. 23: 281-299]. We demonstrate now for the first time that this is indeed the case. By quantifying cytokinin and their derivatives in *Medicago truncatula* root segments that are susceptible to rhizobium interaction we provide evidence that the amounts of trans-zeatin, isopentenyl adenine and isopentenyl adenine riboside increase upon Nod factor signaling. This accumulation occurs prior to the onset of mitotic activation of cortical cells and accumulation of auxin. This response is dependent on CCaMK; a key regulator of root nodule organogenesis. Furthermore, it was found that cytokinin accumulation in the *Mtein2/Mtsickle* mutant was higher than in wild type roots. This suggests a cross talk between ethylene and symbiotic cytokinin biosynthesis, in which ethylene has a negative effect on Nod factor induced cytokinin accumulation. The method used to measure cytokinin as well as auxin provides a tool to further study hormone interactions in rhizobium symbiosis.

Introduction

Legumes can engage in a nitrogen-fixing symbiosis with several soil bacterial species from the order Rhizobiales, hereafter called rhizobia. Upon signal exchange between host and microsymbiont, a developmental program is initiated that gives rise to the formation of a new organ; the root nodule (Crespi and Frugier, 2008; Kouchi et al., 2010; Murray, 2011; Yokota and Hayashi, 2011). These nodules provide a niche that is optimal for the fixation of atmospheric nitrogen by rhizobia (Downie, 2009).

The key signal molecules that initiate the nodule organogenesis program are Nod factors (Lerouge et al., 1990). These are specific lipochito-oligosaccharides secreted by rhizobia. Nod factors are essential for most symbiotic responses including nodule organogenesis. They mitotically activate root cortical cells to form a nodule primordium from which a mature nodule develops. In some plant species Nod factors are even sufficient to trigger the complete developmental program resulting in formation of (empty) nodules (Truchet et al., 1991; Mergaert et al., 1993; Relić et al., 1994; Stokkermans and Peters, 1994). Root cortical cell divisions or even nodule formation can also be induced by external application of cytokinin (Torrey, 1961; Cooper and Long, 1994; Mathesius et al., 2000; Heckmann et al., 2011). However, whether Nod factor signaling results in the accumulation of cytokinin as secondary signal remains elusive.

Nod factors are perceived by LysM domain receptor kinases (Limpens et al., 2003; Radutoiu et al., 2003; Arrighi et al., 2006; Radutoiu et al., 2007). These activate a signaling cascade that includes a nuclear localized Calcium Calmodulin Kinase (CCaMK/DMI3) (Levy et al., 2004; Mitra et al., 2004a). A dominant active form of CCaMK can trigger the onset of root nodule development. This leads to spontaneous nodule formation in absence of rhizobium (Gleason et al., 2006; Tirichine et al., 2006). However, such response is dependent on the presence of the cytokinin receptor CRE1 in *Medicago truncatula* and LHK1 in *Lotus japonicus* (Gonzalez-Rizzo et al., 2006; Murray et al., 2007; Plet et al., 2011). Not only is this histidine kinase receptor essential for nodule organogenesis, also a gain of function

mutation, making the receptor hypersensitive to cytokinin, leads to spontaneous nodule formation (Tirichine et al., 2007; Ovchinnikova et al., 2011). CCaMK and MtCRE1/LjLHK1 are both essential to trigger downstream symbiotic responses including expression of symbiotic genes like *NIN*, which by it self is indispensable for nodule formation (Schauser et al., 1999; Gonzalez-Rizzo et al., 2006; Marsh et al., 2007). Nod factor signaling also induces expression of typical cytokinin responsive genes like type-A response regulators (Gonzalez-Rizzo et al., 2006; Vernie et al., 2008; Op den Camp et al 2011; Plet et al., 2011). This further underlines integration of cytokinin phosphorelay signaling in the Nod factor signaling pathway.

Growth and developmental signaling in plants is generally controlled by integrative pathways triggered by different hormones (Bishopp et al., 2011a; Depuydt and Hardtke, 2011). For example, in legumes ethylene negatively affects root nodulation (Shaharoona et al., 2011) and for *Arabidopsis thaliana* (arabidopsis) it has been shown that in the root meristem cytokinin can modulate auxin signaling and transport to determine meristem size and root elongation (Dello Ioio et al., 2008). For the latter process, cytokinin modulates polar auxin transport, and this modulation is negatively regulated by ethylene (Ruzicka et al., 2009).

Here we aim to study the hypothesis that Nod factors induce cytokinin accumulation that subsequently triggers root cortical cell divisions. Therefore we studied cytokinin accumulation prior to the first Nod factor induced cortical cell divisions that ultimately give rise to the nodule primordium. These divisions occur 18-24 h post inoculation (Mathesius et al., 1998; Timmers et al., 1999). We quantified different cytokinin derivatives and their conjugates in *M. truncatula* in the root zone susceptible to symbiotic interaction within 3 hours after Nod factor application. In order to investigate a putative role for ethylene in the regulation of cytokinin accumulation, we exploited the M. truncatula ethylene insensitive *Mtein2/Mtsickle* mutant (Penmetsa et al., 2008). Taken together, these studies show that Nod factor signaling indeed triggers the accumulation of cytokinin prior to changes in auxin.

Results

Cytokinin and auxin extraction from M. truncatula roots

In the past few years several new methods have been developed to quantify plant hormones in nano- to picomolar range using high-performance liquid chromatography–mass spectrometry (Novak et al., 2008; Kojima et al., 2009; Pan et al., 2010). Natural occurring cytokinins comprise a large group of molecules with a range of structural variations. They are classified into four basic groups. The basic structures in these groups are isopentenyl adenine (iP), trans-zeatin (tZ), cis-zeatin (cZ) and dihydro-zeatin (DHZ). In addition, conjugates do occur formed by modifications such as glycosylation and ribosilation (Kamada-Nobusada and Sakakibara, 2009). Since not only cytokinin, but also auxin is involved in nodulation, we searched for a method suitable to detect cytokinin as well as auxin (Huo et al., 2006; Prayitno et al., 2006; van Noorden et al., 2006; Plet et al., 2011; Rightmyer and Long, 2011). To do so, two methods commonly used in our laboratory to extract auxin were compared for the extraction efficiency of both cytokinin and auxin (for details see Materials and Methods). Extraction buffer with eight internal standards (IS) (see Table 1) was added to 25 snap frozen *M. truncatula* root segments of ~2 cm (total about 100 mg fresh weight) starting about 1 cm above the root tip. This segment encompasses the zone that is highly

susceptible to rhizobium Nod factors. The added IS in principle allow identification of 21 different endogenous cytokinin derivatives and indole-3-acetic acid (IAA); the most prominent auxin in plants (Table S1). We evaluated the two methods by analyzing extracts using ultra-performance liquid chromatography coupled to a tandem mass spectrometer (UPLC-MS/MS).

	pmol/ml and recovery (%)	
	Method 1 (MeOH)	Method 2 (EtOAc-MeOH)
[$^{13}C_6$]-IAA	9.51 ± 2.40 (95%)	7.26 ± 1.22 (73%)
[$^{2}H_6$]-iP	35.68 ± 1.01 (36%)	33.75 ± 1.58 (34%)
[$^{2}H_6$]-iPR	45.82 ± 3.27 (46%)	33.07 ± 1.06 (33%)
[$^{2}H_5$]-tZ	20.83 ± 2.52 (21%)	17.75 ± 1.69 (18%)
[$^{2}H_5$]-tZR	18.66 ± 2.47 (19%)	13.41 ± 2.66 (13%)
[$^{2}H_3$]-DHZR	35.97 ± 4.85 (36%)	20.65 ± 2.40 (21%)
[$^{2}H_5$]-tZ9G	14.06 ± 1.92 (14%)	12.95 ± 3.23 (13%)
[$^{2}H_3$]-DHZ	21.53 ± 2.65 (22%)	20.14 ± 3.46 (20%)

Table 1. Hormone extraction method comparison. Shown are the recovered amounts (±SD, N=3, in pmol/ml) and the recovery percentages of the internal standards.

We determined which method gave the highest IS recovery. With both methods auxin, IAA, and 9 endogenous cytokinin components, iP, iP-riboside (iPR), cZ, tZ, tZ-riboside (tZR), tZ-9-glycosylated (tZ9G), cZ-riboside (cZR), meta-topolin (mT) and DHZ-riboside (DHZR) could be detected in the root extracts, but were not quantified in this test. Method 1 resulted in the highest IS recovery percentages and recovery rates where in the range as previously reported (Kojima et al., 2008; Novak et al., 2008) (Table 1). The results were reproducible and the experimental set up is sensitive enough to measure accurately in the pico-molar range. Therefore, we decided to use method 1 for the quantification of endogenous cytokinins and auxins in *M. truncatula* roots.

Nod factor induced responses associated to cytokinin signaling can be detected within 3 hours

To study Nod factor induced cytokinin signaling, we created a reporter construct that can be induced by Nod factors as well as cytokinin. The best candidate seemed to be the promoter region of *MtNIN*, since this gene can be activated by cytokinin as well as Nod factors and the cytokinin receptor MtCRE1 is required for this induction (Gonzalez-Rizzo et al., 2006; Plet et al., 2011). First we confirmed that a hypersensitive mutation in *MtCRE1* (MtCRE1^{L267F}) is sufficient to induce *MtNIN* expression, as has been shown before in L. japonicus (Fig. S1) (Tirichine et al., 2007). Quantitative RT-PCR of MtNIN on RNA isolated from Agrobacterium rhizogenes transformed M. truncatula roots containing CaMV35S::MtCRE1^{L267F} showed a 6-fold increased expression of MtNIN compared to control roots. This shows that also in *M. truncatula*, *NIN* expression is controlled by cytokinin phosphorelay signaling.

To get insight in the timing of cytokinin signaling after Nod factor application we used a *MtNIN* promoter GUS reporter construct (MtNINp::GUS). In L. japonicus it has been shown that *LjNIN* expression can be observed 2 to 5 h post Nod factor application (Schauser et al., 1999). We tested whether MtNINp::GUS can be detected in a similar time frame. *M.*

truncatula transgenic roots carrying MtNINp::GUS were treated either with *Sinorhizobium meliloti* Nod factors (~10^{-9} M) or cytokinin (10^{-7} M BAP) and roots were GUS stained 3 h after application. As shown in Figure 1, both cytokinin and Nod factors activate the *MtNIN* promoter in epidermis, cortex and other inner layers in the susceptible root zone. Based on these results we decided to use this 3 h time point to test whether Nod factors induce the accumulation of cytokinin in the susceptible zone of the root.

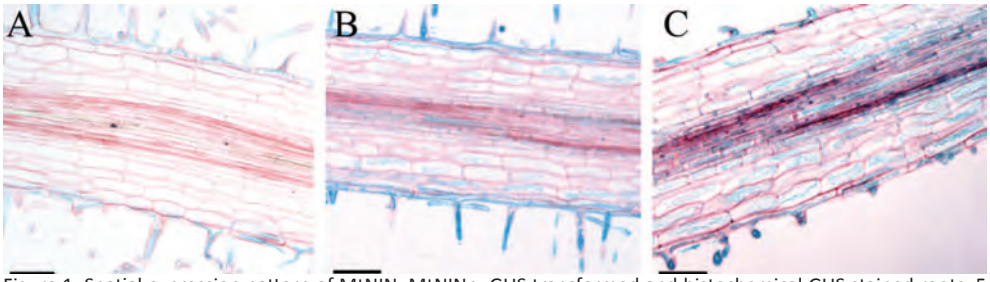

Figure 1. Spatial expression pattern of MtNIN. MtNINp::GUS transformed and histochemical GUS stained roots, 5 µm microsections. A, Untreated root. B, Root treated with cytokinin for 3 h (BAP 10^{-7}M). C, Root treated with Nod factor for 3 h (~10^{-9}M). Bar: A-C 100µm.

Nod factor signaling induces cytokinin accumulation

M. truncatula wild type seedlings were grown vertically on agar plates and the susceptible zone of the root treated with 100 µl *S. meliloti* Nod factors (~10^{-9} M). Three hours after treatment the susceptible zones were dissected, and were subsequently extracted using Method 1 and analyzed by UPLC-MS/MS. As anticipated no change in IAA amount in treated and untreated root segments could be observed (Fig. S2).

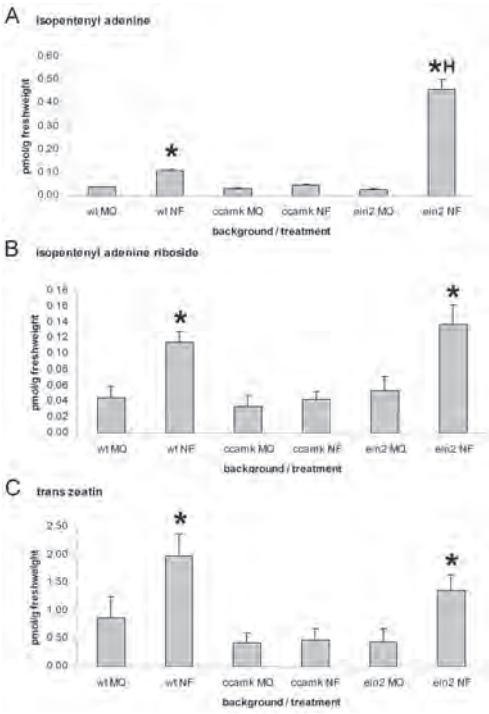

Figure 2. Cytokinin quantification in M. truncatula roots. Amounts of isopentenyl adenine (A), isopentenyl adenine riboside (B) and trans-zeatin (C) in M. truncatula water-treated control roots (MQ) and 3 h Nod factors treated roots (NF). Plants tested are wild type (wt), Mtdmi3/Mtccamk (ccamk) and Mtein2/Mtsickle (ein2). Asterisk indicates a statistical difference in cytokinin amount between treated and untreated samples (t-test P<0.001 for iP, t-test P<0.05 for tZ and iPR). "H" indicates that the increased amount of iP in Nod factor treated Mtein2/Mtsickle (ein2) roots is statistically significant compared to wild type (wt) Nod factor treated roots (t-test, P<0.001). Error bars represent SEM of 6 biological replicates (N=6).

The endogenous cytokinins iP, tZ, iPR and tZ9G were detected in sufficient amounts for quantification in relation to the IS. Other cytokinins could be detected, but their concentration was too low to allow reliable quantification. The amounts of iP, iPR and tZ were 2-3 times higher after exposure to Nod factors (Fig. 2A-C). The amount of the 9-glycosylated conjugate of trans-zeatin (tZ9G) did not change upon Nod factor exposure (Fig. S3). These results

demonstrate that a number of cytokinins accumulate upon Nod factor exposure, including the biologically most active cytokinins iP and tZ.

CCaMK is essential for Nod factor induced transcriptional changes and subsequent nodule formation (Mitra et al., 2004b). To test whether Nod factor induced cytokinin accumulation is CCaMK dependent, we quantified cytokinins in roots of the *M. truncatula Mtdmi3/Mtccamk* mutant (Levy et al., 2004; Mitra et al., 2004a). *Mtdmi3/Mtccam*k roots contained similar amounts of cytokinins as wild type roots, but they were not increased upon Nod factor exposure (Fig. 2A-C, Fig. S3). This shows that Nod factor induced cytokinin accumulation is dependent on CCaMK; a key regulator of root nodule symbiosis.

Symbiotic cytokinin accumulation is affected by the ethylene signaling pathway

Ethylene inhibits Nod factor induced root cortical cell divisions and subsequent nodule formation (Shaharoona et al., 2011). Mechanistically, ethylene could inhibit cytokinin induced cortical cell divisions or ethylene could inhibit Nod factor induced cytokinin accumulation. To investigate this we exploited the *M. truncatula* ethylene insensitive mutant *Mtein2/Mtsickle*. This mutant displays hypernodulation upon rhizobium inoculation, forming up to ten times more nodules in a distinct zone (Penmetsa et al., 2003; Penmetsa et al., 2008). Mtein2/Mtsickle mutant plants were grown and roots were treated in the same way as described for wild type plants. Basal levels of cytokinins in the *Mtein2/Mtsickle* mutant background were similar as compared to wild type roots (Fig. 2A-C, Fig. S2). However, upon Nod factor treatment *Mtein2/Mtsickle* iP levels increased about 15-fold, whereas tZ and iPR increased 2 to 3-fold compared with the mock treated control (Fig. 2A-C). Like in wild type roots, tZ9G did not increase (Fig. S3). The significant higher levels of iP in the *Mtein2/Mtsickle* mutant background suggest that ethylene signaling negatively effects Nod factor induced cytokinin accumulation.

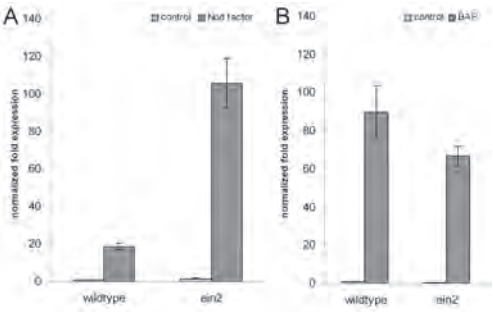

Fig. 3. Relative expression of MtNIN in roots of wild type and Mtein2/Mtsickle (ein2). Expression was determined using quantitative RT-PCR after application of Nod factors (~10-9M, 3 h) (A) or cytokinin (BAP 10-7M, 3 h) (B). Quantification was normalized using stable expressed reference genes MtGAPDH and MtPTB. Bars represent SD of three technical repeats (N=3).

As cytokinin accumulates at higher levels in the *Mtein2/Mtsickle* mutant it seems probable that *MtNIN* is induced to higher levels in this mutant when compared to wild type plants. To test this, we treated M. truncatula seedlings (wild type and Mtein2/Mtsickle) with S. meliloti Nod factors (~10^{-9}M) for 3 h. Quantitative RT-PCR on RNA extracted from the susceptible zones exposed to Nod factors showed a ~20-fold transcriptional upregulation of *MtNIN* in wild type plant roots (Fig. 3A), in the *Mtein2/Mtsickle* mutant however, the transcriptional upregulation upon Nod factor application was 5 to 6-fold higher than in wild type (Fig. 3A). This further supports that ethylene negatively regulates Nod factor triggered cytokinin accumulation, although we can not rule out that ethylene inhibits *MtNIN* expression downstream of Nod factor induced cytokinin signaling. To investigate this, we bypassed Nod factor signaling by treating wild type and *Mtein2/Mtsickle* roots with the cytokinin 6-benzyl-

aminopurine (BAP, 10^{-7} M) for 3 h. Quantitative PCR on RNA extracted from susceptible zones of these BAP treated seedlings showed that *MtNIN* is upregulated to a similar extent in wild type and *Mtein2/Mtsickle* (Fig. 3B). Together, these data indicate that ethylene negatively regulates Nod factor induced cytokinin accumulation, and thereby regulates downstream *MtNIN* expression.

Discussion

We demonstrated that Nod factor signaling leads to a fast accumulation of three structurally different cytokinins in the susceptible zone of *M. truncatula* roots. The accumulating cytokinins include tZ and iP, which are among the biologically most active cytokinins (Kamada-Nobusada and Sakakibara, 2009). Furthermore, the accumulation is dependent on CCaMK, a key regulator of nodule organogenesis. As cytokinin signaling is indispensible for root nodule organogenesis we conclude that Nod factor induced cytokinin accumulation prior to symbiotic cell divisions is a key step in Rhizobium root nodule symbiosis.

Our data show that of the three induced cytokinins, tZ is about 20-fold more abundant in treated as well as control roots than iP and iPR (Fig. 2). What the implications are of this difference remains elusive, since the sensitivity of *M. truncatula* receptors for iP or tZ is unknown.

Basal MtNINp::GUS activity could be detected in the epidermis and was induced by BAP as well as Nod factors in the epidermis and inner cell layers. This uniform activation of the MtNINp::GUS promoter reporter construct suggests that cytokinin also accumulates uniformly upon Nod factor signaling. Since primary Nod factor recognition and signaling take place in the epidermis, we presume that cytokinin accumulates first in this cell layer. This accumulation may spread by cell-to-cell cytokinin signaling or alternatively by a secondary signal of unknown nature. As a result, cytokinin accumulation and downstream cytokinin signaling will reach the cortex to trigger root cortical cell divisions. However, recent studies in *L. japonicus* showed activation of a similar LjNINp::GUS reporter construct exclusively in the root cortex upon cytokinin signaling, whereas Nod factor signaling could activate this construct in the epidermis as well (Heckmann et al., 2011). The reason for this difference remains unclear. It could be the result of differences in experimental setup, since for *L. japonicus LjNIN* expression was studied 48 h post inoculation, whereas we studied *MtNIN* expression 3 h after Nod factor application. Alternatively, variation between both plant species may have occurred due to differences in cis regulatory elements of the *NIN* gene.

We found that upon Nod factor signaling the *M. truncatula Mtein2/Mtsickle* knockout mutant accumulates more cytokinin (iP) in the susceptible root zone than wild type plants. This coincides with an increased *MtNIN* expression in this mutant upon Nod factor application. In contrast, *MtNIN* expression in *Mtein2/Mtsickle* and wild type did not differ upon application of exogenous cytokinin. This suggests that ethylene signaling affects primary Nod factor signaling rather than cytokinin phosphorelay signaling. That ethylene acts upstream of Nod factor induced cytokinin accumulation is also supported by earlier findings that ethylene interferes with Nod factor induced calcium oscillations that occur in and around the nuclei of activated cells (Oldroyd et al., 2001a; Oldroyd et al., 2001b). Besides this direct interference in early Nod factor signaling, ethylene also affects symbiotic cell divisions. In legumes, root

nodules are formed predominantly opposite xylem poles. This coincides with expression of ACC oxidase opposite phloem poles (Heidstra et al., 1997). 1-Aminocyclopropane-1-carboxylate (ACC) is the precursor of ethylene and processing by ACC oxidase is a rate limiting step in ethylene biosynthesis. Inhibition of ethylene biosynthesis affects spatial positioning of nodule primoridia (Heidstra et al., 1997). In the *Mtein2/Mtsickle* mutant cortical cells throughout the whole root circumference in the susceptible zone become mitotically activated, leading to massive formation of root nodule primordia (Penmetsa et al., 2003; Penmetsa et al., 2008). This phenotype therefore is possibly due to lack of ethylene controlled nodule positioning. However, we hypothesize that such mechanism acts independent of the ethylene controlled symbiotic cytokinin accumulation. Most prominent evidence for this hypothesis is the finding that external application of cytokinin, thereby bypassing primary Nod factor signaling, does not mimic a *Mtein2/Mtsickle*-like phenotype (Cooper and Long, 1994; Mathesius et al., 2000; Heckmann et al., 2011; Torrey, 1961).

Cytokinin accumulation upon Nod factor signaling may be due to translocation of existing cytokinins or alternatively to local biosynthesis. We found that both iP and its riboside-derivative, iPR, accumulate upon Nod factor exposure. This indicates activation of cytokinin biosynthesis, as in this pathway iPR is positioned upstream of iP and the latter is more bioactive (Kamada-Nobusada and Sakakibara, 2009). However, the enzyme that catalyzes the conversion of iP to iPR step remains unknown (Kamada-Nobusada and Sakakibara, 2009). Data from arabidopsis suggest that cytokinin biosynthesis occurs in regions where it is active as well (Perilli et al., 2010). Cytokinin biosynthesis is among others controlled by isopentenyl-transferases (IPTs). As IPTs represent rate-limiting enzymes (Takei et al., 2001; Miyawaki et al., 2006; Perilli et al., 2010), we investigated whether any *M. truncatula MtIPT* gene is transcriptionally upregulated in the susceptible zone of the root upon Nod factor signaling. In total we could identify 9 *MtIPT* genes on the basis of homology with arabidopsis (Fig. S4). However, quantitative RT-PCR on RNA extracted from susceptible zones of *M. truncatula* roots exposed to Nod factors (~10^{-9} M/ 3 hours) could not reveal transcriptional regulation of any of the *MtIPT* genes (Fig. S5). This suggests that Nod factor induced cytokinin accumulation either is due to regulation at the protein level or is depending on transcriptional activation of different biosynthetic genes.

Taken together, we demonstrated that Nod factors cause an increase in the abundance of various cytokinins prior to first mitotic divisions. It is now a challenge to unveil the molecular mechanism behind the accumulation of this hormone. This will provide insight into how rhizobia have co-opted existing developmental modules and put them to their use during the evolution of the legume-rhizobium root nodule symbiosis.

Materials and methods

Vectors and constructs

The promoter region of *MtNIN*, 2,500 bp upstream of the translational start site, was PCR amplified using primers listed in table S2. The amplicon was cloned into a pENTR-D-Topo thereby creating pENTR1-2_MtNINp. Subsequently the promoter was recombined into pKGWFS7-RR containing a GUS-GFP fusion reporter as well as pAtUBQ10::DsRed1 as selectable marker creating MtNINp::GUS (Karimi et al., 2002). CaMV35S::MtCRE1$^{[L267F]}$

was used as described in Ovchinnikova et al. (2011). All cloning vectors and constructs are available upon request from our laboratory or via the Functional Genomics unit of the Department of Plant Systems Biology (VIB-Ghent University).

Plant materials and treatments

Medicago truncatula Jemalong A17, *dmi3/Mtccamk* TRV25 (Catoira et al., 2000) and *Mtein2/Mtsickle-1* mutant (Penmetsa and Cook, 1997) seedlings were grown vertically on modified Fåhraeus medium agar plates with low nitrate (0.2 mM Ca(NO3)2) on top of filter paper for 4 days (Fahraeus, 1957). Then, 6-benzylaminopurine (10^{-7}M), *Sinorhizobium meliloti* 2011 Nod factors (~10^{-9} M) or water as a control was pipetted on top of the root susceptible zone. Roots were exposed for 3 h and subsequently 1 cm root pieces were cut just above the root-tip and were snap-frozen (N=25). For all experiments plants were grown in an environmentally controlled growth chamber at 20°C with a 16h-light/8h-dark cycle and 70% relative humidity.

Phylogenetic analysis

The phylogenetic tree was reconstructed using the neighbor-joining method implemented in the software MEGA5 (Tamura et al., 2011). Alignment of full length (predicted) nucleotide coding sequences were used for tree building. Substitution model: Maximum Composite Likelihood, set to use d: Transitions + Transversions and use uniform rates. Branch support was obtained from 1000 bootstrap repetitions.

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 1 µg 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). All primers including the genes used for normalization (*MtGAPDH* and *MtPTB*) are given in table S2. Data analysis was performed using BioRad iQ5 software (BioRad). Baselines were set at 100 RFU to calculate the Ct-values, Ct values of 32 and higher were excluded from the analysis, though still checked for transcriptional induction (see table S2). A representative sample out of two independent biological replicates is shown in all figures.

Plant transformation and histology

Agrobacterium rhizogenes-mediated hairy roots transformation was used to transform *Medicago truncatula* (Jemalong A17) as described in Limpens et al. (2004). Transgenic roots were selected based on *DsRED1* expression. Transgenic roots from promoter studies were transferred to low nitrate Fåhraeus plates (0.2mM $Ca_2(NO_3)_2$) 3 weeks after transformation. After 5 days on these plates, 6-benzylaminopurine (10^{-7}M), *Sinorhizobium meliloti* 2011 Nod factors (~10^{-9} M) or water as a control was pipetted on top of the root zone susceptible to

rhizobium infection of MtNINp::GUS transgenic roots. Roots were GUS stained 3 hours post treatment and fixed as described in Limpens et al. (2005). Microtome sections of 5 µm were stained with ruthenium red and photographed using a Leica DM5500B microscope equipped with a DFC425C camera (Leica Microsystems, Wetzlar, Germany). Images were digitally processed using Photoshop CS3 (Adobe Systems, San Jose, California). CaMV35S::CRE1$^{[L267F]}$ transgenic roots were obtained in the same way and were snap frozen for RNA extraction 5 days after starvation on low nitrate Fåhraeus plates, without any treatment.

Hormone Extraction methods

We used two extraction methods that are based on Ruyter-Spira et al. (2011). For both methods ~100 mg starting material of snap frozen plant material (root susceptible zones) was used, which was ground to a fine powder using 3-mm stainless steal beads at 50 Hz for 2 minutes in a TissueLyser LT (Qiagen, Germantown, USA). The initial method comparison was performed in biological triplicates and the actual measurements using method 1 were performed in six biological replicates. Stable isotope-labeled cytokinin and auxin internal standards (IS) were added to each of the samples (100 pmol of each compound per sample) for recovery assessment and quantification. The following IS were used: [$^{13}C_6$]-IAA, [$^{2}H_6$]-iP, [$^{2}H_6$]-iPR, [$^{2}H_5$]-tZ9G, [$^{2}H_5$]-tZ, [$^{2}H_5$]-tZR, [$^{2}H_3$]-DHZR and [$^{2}H_3$]-DHZ. The details of both methods are as follows:

Method 1). Ground root samples were extracted with 1 ml of 100% methanol (MeOH) containing 2.5 mM of diethyldithiocarbamic acid by short vortexing and ultrasonication during 10 min. Subsequently, samples were centrifuged at 2000 rpm/10 min in a tabletop centrifuge at room temperature (RT). Supernatants were transferred to a glass vial. Pellets were re-extracted with 1 ml 100% MeOH for 1 hour on a shaker at 4°C. After centrifugation as above, both supernatants were pooled and MeOH evaporated in a speedvac at RT. Residues were resuspended in 50 µl MeOH and then diluted in 3 ml of water before loading on a 50 mg GracePure SPE C18-max cartridge (Grace, Columbia, USA). The cartridge was equilibrated with 2 ml of water prior to sample loading. Subsequently the cartridge was washed with 1 ml of water and eluted with 1 ml of 100% acetone. The acetone was evaporated in a speedvac at RT and the residue resuspended in 200 µl acetonitrile:water (25:75). The sample was filtered trough a 0,45 µm Minisart SRP4 filter (Sartorius, Goettingen, Germany) and stored at -20°C.

Method 2). This method is the same as method 1 with the following differences: The first extraction was done with 100% ethyl acetate containing 2.5 mM of diethyldithiocarbamic acid. The ethyl acetate was evaporated in a speedvac at RT. The second extraction was done with MeOH, and both extracts were then pooled and further processed as in method 1. All statistical tests to compare the amounts of hormones were executed using SigmaStat software v3.5 (Systat Software, San Jose, California).

Auxin and cytokinin detection and quantification by liquid chromatography-tandem mass spectrometry

Analyses of auxin (IAA) and cytokinins in M. truncatula root extracts were performed by comparing retention times and mass transitions with those of IAA and cytokinin standards (iP, iPR, iPMP, cZ, tZ, cZR, tZR, tZ7G, tZ9G, tZOG, tZROG, tZRMP, DHZ, DHZR, DHZ7G, DHZ9G,

DHZROG, oT, mT, oTR, mTR) using a Waters Xevo TQ mass spectrometer equipped with an electrospray ionization source coupled to an Acquity UPLC system (Waters, Milford, USA) using settings as previously described (Kohlen et al., 2011; Ruyter-Spira et al., 2011). For auxin and cytokinins two independent chromatographic separations have been conducted on an Acquity UPLC BEH C18 column (100 mm, 2.1 mm, 1.7 mm; Waters, USA) by applying a water/acetonitrile gradient. The gradient used for IAA analysis is identical as described for ABA (65). For cytokinin separation the water/acetonitrile gradient started from 0.2% (v/v) of acetonitrile for 1.5 min and a rise to 20% (v/v) of acetonitrile in 8.5 min. To wash the column the water/acetonitrile gradient was increased to 70% (v/v) acetonitrile in a 1.0 min gradient, which was maintained for 0.7 min before going back to 0.2% acetonitrile using a 0.3 min gradient, prior to the next run. Finally, the column was equilibrated for 2.5 min using this solvent composition. The column was operated at 50ºC with a flow-rate of 0.6 ml min^{-1}. Sample injection volume was 20 µl. The mass spectrometer was operated in positive ESI mode. Cone and desolvation gas flows were set to 50 and 1,000 l h^{-1}, respectively. The capillary voltage was set at 3.0 kV, the source temperature at 150ºC and the desolvation temperature at 650ºC. The cone voltage was optimized for each standard compound using the IntelliStart MS Console (Waters, Milford, USA). Argon was used for fragmentation by collision induced dissociation (CID). Multiple reaction monitoring (MRM) was used for quantification. Parent-daughter transitions for the standards IAA, [phenyl $^{13}C_6$]-IAA, cytokinins and deuterium labeled cytokinins were set using the IntelliStart MS Console. MRM-transitions selected for identification of IAA were mass-to-charge ratio (*m/z*) 176 >103 at a collision energy of 30 eV and 176 >130 at 16 eV; for [phenyl 13C6]-IAA *m/z* 182 >109 at 28 eV and 182 >136 at 16 eV. Cone voltage was set to 18 eV. MRM-transitions selected for cytokinin identification and quantification are shown in table S1.

IAA and cytokinins were quantified using a calibration curve with known amount of standards and based on the ratio of the peak areas of the MRM-transition for standards to the MRM transition for [phenyl $^{13}C_6$]-IAA or corresponding deuterium labeled cytokinins (table S1). Data acquisition and analysis were performed using MassLynx 4.1 (TargetLynx) software (Waters, Milford, USA). The summed area of all corresponding MRM transitions was used for statistical analysis.

Acknowledgements

This work was supported by the Dutch Science Foundation (NWO) (VIDI 864.06.007 to R.G. and Equipment Grant 834.08.001 to H.J.B.).

Supplemental figures

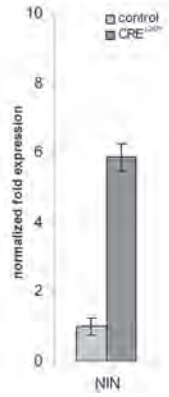

Figure S1 (left). Relative expression of MtNIN in *M. truncatula* 35S::CRE1^{L267F} roots. Quantitative expression of *MtNIN* in control roots (empty vector) versus 35S::CRE1^{L267F} expressing roots. Quantification was normalized using stable expressed reference genes *MtGAPDH* and *MtPTB*. Bars represent SD of three technical repeats.

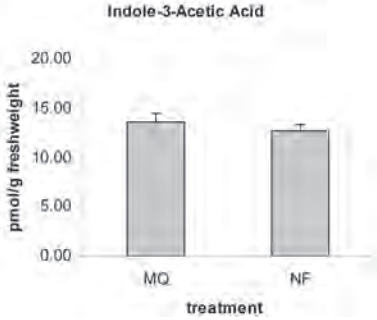

Figure S2 (right). Quantification of Indole-3-Acetic Acid (IAA) in *M. truncatula* wild type (Jemalong A17) roots. Amounts were quantified in water-treated control roots (MQ) and in 3 hours Nod factor treated roots (NF). There is no statistical significant difference (t-test, P=0.388). Error bars represent SEM on 6 biological replicates (N=6).

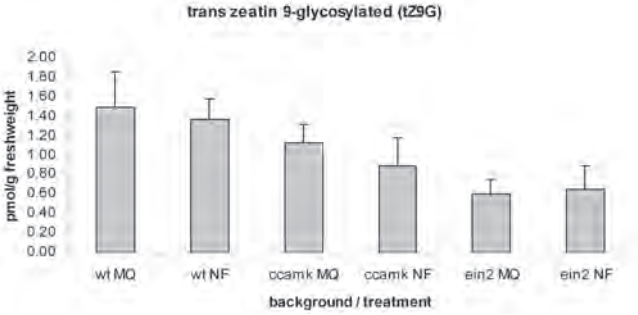

Figure S3 (right). Quantification of trans-zeatin-9-glycosylated (tZ9G) in *M. truncatula* wild type (Jemalong A17), *Mtdmi3/Mtccamk* and *Mtein2/Mtsickle* roots. Amounts were quantified in water-treated control roots (MQ) and in 3 hours Nod factors treated roots (NF) of *M. truncatula* wild type (wt), *Mtdmi3/Mtccamk* (ccamk) or *Mtein2/Mtsickle* (ein2). There is no statistically significant difference among all background/treatments (ANOVA, P = 0.083). Error bars represent SEM of 6 biological replicates (N=6).

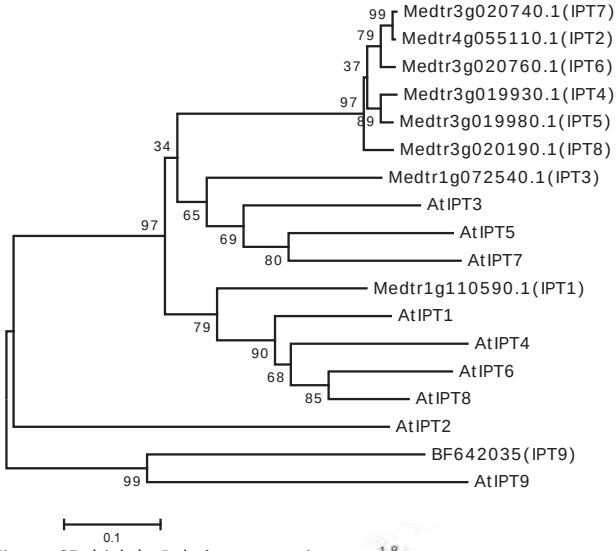

Figure S4 (left). Phylogeny of *M. truncatula* and *A. thaliana IPT* genes. Branch support was obtained from 1000 bootstrap repetitions. Scale bar: 0.1 substitutions per site.

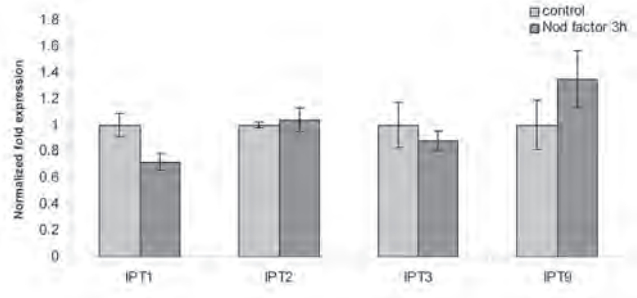

Figure S5 (right). Relative expression of *MtIPT* genes upon Nod factor treatment. A-B, Quantitative RT-PCR of *MtIPT1,2,3* and *9* in *M. truncatula* wild type (Jemalong A17) roots was determined upon Nod factors (~10^{-9}M, 3 h) application. Quantification was normalized using stable expressed reference genes *MtGAPDH* and *MtPTB*. Bars represent SD of three technical repeats.

number	Compound	Mass	MRM-transition	Cone Volt.	Col. Energy
1	*D6-IP*	209	210.16 > 137.06	22	20
2	*D6-IPR*	341	342.29 > 137.07	28	32
3	*D5-tZ*	224	225.16 > 136.90	26	18
4	*D5-tZR*	356	357.22 > 225.18	28	20
5	*D5-Z9G*	386	387.29 > 136.93	34	34
6	*D3-DHZ*	224	225.16 > 149.07	32	22
7	*D3-DHZR*	356	357.22 > 225.18	24	20
8	*13C6-IAA*	181	182.00 > 136.00	18	16
9	*IAA*	175	176.00 > 130.00	18	16
10	*iP*	203	204.10 > 136.05	20	16
11	*iPR*	335	336.10 > 136.09	24	8
12	*iPMP*	415	416.10 > 204.16	24	18
13	*cZ*	219	220.10 > 136.08	40	16
14	*tZ*	219	220.07 > 202.11	22	12
15	*cZR*	351	352.16 > 220.18	26	20
16	*tZR*	351	352.16 > 136.02	26	34
17	*tZ7G*	381	382.17 > 220.16	30	22
18	*tZ9G*	381	382.17 > 220.16	30	22
19	*tZOG*	381	382.17 > 220.16	24	16
20	*tZROG*	513	514.18 > 136.07	30	52
21	*tZRMP*	431	432.17 > 220.17	26	22
22	*DHZ*	221	222.10 > 148.02	30	22
23	*DHZR*	353	354.16 > 222.18	20	20
24	*DHZ7G*	383	384.18 > 222.18	26	22
25	*DHZ9G*	383	384.18 > 222.18	26	22
26	*DHZROG*	515	516.24 > 222.16	30	32
27	*oT*	241	242.04 > 107.06	28	24
28	*mT*	241	242.04 > 136.03	28	18
29	*oTR*	373	374.17 > 136.08	28	34
30	*mTR*	373	374.17 > 242.15	26	20

Table.S1. MRM transitions table for each IS of cytokinin and IAA.

Primers

qPCR primers

Gene Name	Forward primer	Reverse primer	CT >32	Reference (IMGAGv3.5)
IPT1	AAGAGCTGGGTGGGACCTACAAC	AAATTCTTCTCCACCGCCGTTAG		Medtr1g110590.1
IPT2	GGATGTCAAATGGCACTGGG	ATTGCAGGCCTTGCATGTG		Medtr4g055110.1
IPT3	TGGAAGAAGATTGTTGCAGAGCC	AGATCACCCTGAGAAATTGGCAC		Medtr1g072540.1
IPT4	ATTTGGCTCGATGTGTCTTTACC	TGCCGCAACCATTTCACC	X	Medtr3g019930.1
IPT5	AGAGATTCCTAGAGGGGACAAGC	TCGGTGTGTGTTTCCCATTTC	X	Medtr3g019980.1
IPT6	TTTTATTTGGGTCGACGTGTCTC	TCATCTACCATCCCTGCTTCAAC	X	Medtr3g020760.1
IPT7	GATTTCTGGAGGAGACAAGCCC	CACCATGAGGCGTGTGTTTAGC	X	Medtr3g020740.1
IPT8	GTTGATGAAATGGTTCAGGCAG	ATTCCTTTTGTGTTATCCGCACC	X	Medtr3g020190.1
IPT9	AAGTTCAGAACGGTTTCCAGAGG	AAACGACAGTTGACTTGGTTTCG		BF642035
NIN	GGGAGAAAGTCCGGGGACAA	GACACACACCGATGCTCTTTGC		FJ719774
GAPDH	CTTCTGTTGCTTGATGAGGCTGAG	GATTTCGCCGAGACACAGTTTCT		BT052418
PTB	CGCCTTGTCAGCATTGATGTC	AGGATTCCAGGCACTGGTTCA		CT963079

Cloning primers

pNIN	ATATAACATCAGAGGGAGTGTTCAAA	CCTTATAATTAAAGTTGTTTCTCAGATCC	CR936325

Table.S2. List of primers used for qPCR and cloning.

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Chapter 4

LysM-type mycorrhizal receptor recruited for rhizobium symbiosis in non-legume *Parasponia*

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Adapted version from: Op den Camp, R.H.M. et al. (2011) *Science* **331**:909-912

Rhizobium root nodule symbiosis is generally considered to be unique for legumes. However, there is one exception and that is *Parasponia*. In this non-legume the rhizobial nodule symbiosis evolved independently and is, like in legumes, induced by rhizobium Nod factors. We used *Parasponia* to identify genetic constraints underlying evolution of Nod factor signalling. Part of the signalling cascade, downstream of Nod factor perception, has been recruited from the more ancient arbuscular endomycorrhizal symbiosis. However, legume Nod factor receptors that activate this common signalling pathway are not essential for arbuscular endomycorrhizae. Here we show that in *Parasponia* a single Nod factor-like receptor is indispensable for both symbiotic interactions. Therefore we conclude that also the Nod factor perception mechanism is recruited from the widespread endomycorrhizal symbiosis.

Introduction

The rhizobial nodule symbiosis is wide spread in the legume family (*Fabaceae*). Although this nitrogen fixing symbiosis provides the plant with a major advantage, it is in principle restricted to a single family and it is a major challenge for future agriculture to transfer this symbiosis to non-legumes (Gewin et al., 2010). The genus *Parasponia* could provide a key to this, as it encompasses the only non-legume species that acquired also the rhizobium symbiosis (Akkermans et al., 1978; Trinick, 1973). *Parasponia* comprises several tropical tree species and belongs to *Celtidaceae* (Sytsma et al., 2002). *Celtidaceae* (order Rosales) and *Fabaceae* (order Fabales) are only remotely related. Further, not a single species phylogenetically positioned between *Parasponia* and *Fabaceae*, is able to establish such rhizobium symbiosis. Hence, in all probability the common ancestor of present *Parasponia* species has gained the rhizobium nodule symbiosis independent from legumes. Therefore, a legume-*Parasponia* comparison provides a key to identify genetic constraints underlying this symbiosis. In this study we focused on parallel evolution of the recognition of the rhizobial signal that starts the symbiotic interaction, the Nod factor.

Parasponia makes lateral root-like nodules that are associated with cell divisions in the root cortex (Webster et al., 1995). Rhizobium enters the *Parasponia* root intercellularly and becomes imbedded in a dense matrix. Rhizobium obtains an intracellular lifestyle when it reaches a nodule primordium. There, cortical cells are infected via thread-like structures that remain connected to the plasmamembrane. These so-called fixation threads branch and fill up the cells and provide a niche to rhizobium to fix nitrogen (Webster et al., 1995). This is illustrated by the expression, in these threads, of the rhizobium *nifH* gene that encodes one of the subunits of nitrogenase (Fig. S1). In contrast, rhizobium enters most legume roots via root hair-based intracellular infection threads and the bacteria are released in nodule cells as membrane surrounded nitrogen fixing organelle-like structures (symbiosomes) that harbor a single or only a few bacteria. Legume nodules are considered to be genuine organs with a unique ontogeny (Pawlowski et al. 1996). The fact that the rhizobium symbiosis is very common in the 65 million year old *Fabaceae* led to the conclusion that the symbiotic interaction has emerged as early as 60 million years ago (Sprent, 2008). In contrast, the lateral root-like nodule structure and more primitive rhizobium infections in *Parasponia* (Webster et al., 1995), together with the very close relation with the non-nodulating genus Trema (Akkermans et al., 1978; Sytsma et al., 2002; Trinick, 1973), strongly suggests that *Parasponi*a has gained the rhizobium symbiosis more recent than legumes.

A key step in rhizobium symbiosis is the recognition by the host of bacterial Nod factors, which are specific lipochito-oligosaccharides. This holds for (almost) all nodulated legumes, but also for *Parasponia* (Marvell et al., 1987). This implies that a non-legume species has evolved independently from legumes a Nod factor perception mechanism. In legumes, Nod factors are perceived by specific LysM receptor kinases that co-evolved with the Nod factor structure of their host specific Rhizobium species (Arrighi et al., 2006; Limpens et al., 2003; Radutoiu et al., 2003, 2007). Legume Nod factor receptors activate a common signalling cascade that is shared with and recruited from the more common and far more ancient arbuscular mycorrhizal symbiosis (Markmann et al., 2009; Oldroyd et al., 2009). This common signalling pathway comprises an additional plasmamembrane receptor kinase, several components in the nuclear envelope including a cation ion channel and subunits of nuclear pores, and a nuclear localized Calcium Calmodulin dependent Kinase (CCaMK) (Markmann et al., 2009; Oldroyd et al., 2009). Rhizobium and mycorrhizae induced signalling diverges downstream of CCaMK, possibly due to a different nature of the induced calcium spiking (Kosuta et al., 2008; Oldroyd et al., 2009). Since legume Nod factor receptors are not essential for mycorrhization, it is generally assumed that mycorrhizal symbiosis is controlled by other receptors specific for mycorrhizal signals (i.e. Myc factor). Such Myc factor receptors, like Nod factor receptors, are presumed to activate the common symbiotic signalling pathway (Kosuta et al., 2008; Markmann et al., 2009; Oldroyd et al., 2009; Zhang et al., 2007, 2009). Two scenarios can be envisioned how Nod factor receptors could have evolved. The complete mycorrhizal signalling pathway, including the Myc receptor, has been recruited by legumes resulting in a common signalling pathway. In such a case, present Nod factor receptors have emerged upon gene duplication events and subsequently neofunctionalized during co-evolution with specific rhizobium species. In this scenario Myc receptors would be close homologs of known Nod factor receptors as was argued previously (Zhang et al., 2007, 2009). However, such scenario also implies that early in rhizobium symbiosis evolution a single receptor fulfilled a dual function; namely in mycorrhization as well as in rhizobium symbiosis. A second scenario is that only the common signalling pathway devoid of a fungal specific Myc receptor was recruited and a novel receptor obtained the ability to activate this common signalling pathway upon Nod factor recognition. We favor the first hypothesis, because it is more simple and finds support in the fact that the chito-oligosaccharide backbone of Nod factors is a “fungal” characteristic, as chitin is a major component in fungal cell walls. The occurrence of Nod factor signalling in *Parasponia* provides a possibility to investigate this hypothesis.

First, we confirmed and extended the idea that *Parasponia*-rhizobium symbiosis is induced by Nod factors. To this end we used *Parasponia andersonii* (further referred to as Parasponia) a species that can be nodulated by the broad host strain *Sinorhizobium* sp. NGR234 (Pueppke et al., 1999). A mutant of *Sinorhizobium* sp. NGR234 (NGR234ΔnodABC) that does not produce Nod factors was unable to trigger nodule formation nor infect roots of Parasponia plantlets (n=0/30), whereas wild type NGR234 does form nodules on ~40% of the plantlets (n=12/30; 8 weeks post inoculation (wpi)), similar as reported previously (Webster et al., 1995). Furthermore, root cortical cell divisions could be induced by local application of Nod factors (n=16/19; Fig. S2). Next, we obtained first evidence that also in Parasponia the common symbiotic pathway is recruited to facilitate rhizobium symbiosis. A dominant active form of *Medicago truncatula MtCCaMK* was introduced in Parasponia roots

(see Materials and Methods). In legumes CCaMK is a key element in the common symbiotic pathway and dominant active forms of this kinase result in spontaneous nodulation in absence of rhizobium (Gleason et al., 2006; Tirichine et al., 2006). In Parasponia we also observed spontaneous formation of nodule-like structures (n=6/30, Fig. 1), indicating that activation of the common signalling pathway is sufficient to induce nodule organogenesis. These data suggest that also in Parasponia the common signalling pathway is activated upon Nod factor perception.

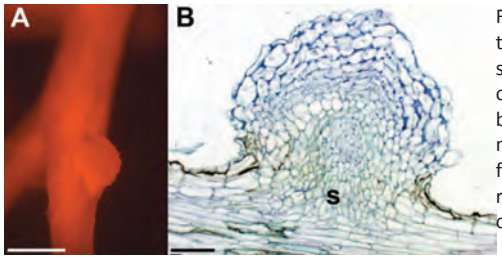


Figure 1. Parasponia spontaneous nodule-like structure triggered by dominant active MtCCaMK. (A) Nodule-like structure on a transgenic Parasponia root (selected based on red fluorescence due to DsRED1 expression). Scale bar: 0.5 mm (B) Longitudinal section of spontaneous nodule-like structure. Nodule-like structure originates from cortical and pericycle cell layers and has a rudimentary stele (s), reflecting the lateral root-like origin of Parasponia nodules. Scale bar: 50 µm.

In legumes two different Nod factor receptor types are involved. One of these, *MtLYK3/LjNFR1* in *M. truncatula*/*Lotus japonicus*, has several paralogous genes that resulted from recent duplication events (Arrighi et al., 2006; Lohmann et al., 2010; Limpens et al., 2003; Zhang et al., 2007, 2009; Zhu et al., 2006). In contrast, the second Nod factor receptor (*MtNFP/LjNFR5*) has only one paralog in *M. truncatula* and *L. japonicus* (Arrighi et al., 2006; Lohmann et al., 2010), and a putative orthologous gene is absent in Arabidopsis; a species that is unable to establish mycorrhizal symbiosis (Arrighi et al., 2006; Zhang et al., 2007, 2009). Interestingly, in *M. truncatula* this paralog, *MtLYR1*, is transcriptionally upregulated during mycorrhization (Gomez et al., 2009). Therefore we focused on the putative *MtNFP/LjNFR5* orthologous gene in Parasponia. To clone Parasponia homologs a BAC library was constructed and screened with *MtNFP* as probe. All eight positive BACs came from a single locus and shared the region containing one *MtNFP/LjNFR5*-like LysM receptor that we named *PaNFP* (*Parasponia andersonii NOD FACTOR PERCEPTION*). Southern blotting as well as sequencing of Parasponia *MtNFP/LjNFR5*-like sequences generated by PCR using degenerated primers and genomic DNA as well as nodule and root cDNA confirmed that *Parasponia* has a single *NFP*-like gene. Next we searched for *MtNFP/LjNFR5*-like genes in available genome sequences of other Fabidae (Rosid I) species (see Materials and Methods).

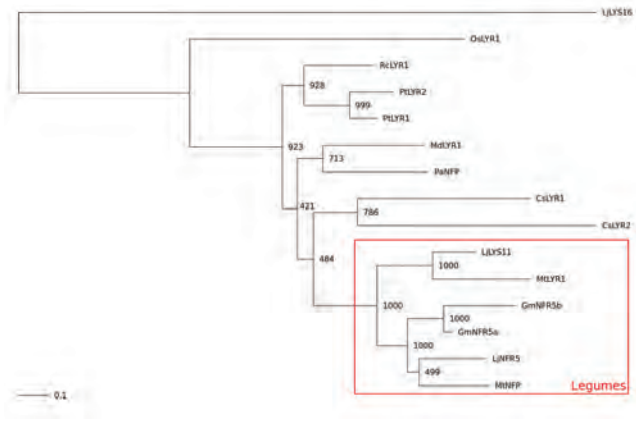


Figure 2: Maximum-likelihood phylogeny of MtNFP/LjNFR5-like genes in the Rosid I (Fabidae) clade. Note that Parasponia (Pa), apple (Md) and castor bean (Rc) contain only a single gene whereas in poplar (Pt), cucumber (Cs) and legumes (Gm/Lj/Mt) lineage specific duplications have occurred. In legumes LjNFR5, MtNFP, GmNFR5a and GmNFR5b are rhizobium Nod factors receptors. Branch lengths are proportional to the number of amino acid substitutions per site. Branch support was obtained from 1,000 bootstrap repetitions. LjLYS16 and the closest MtNFP/LjNFR5 homolog in Oryza sativa (OsLYR1) were used as outgroups.

Apple (*Malus x domestica*), a close relative of Parasponia, also has only a single *MtNFP/LjNFR5*-like gene that we named MdLYR1 (*Malus x domestica LYK-RELATED1* (Arrighi et al., 2006)). Subsequent phylogenetic analysis revealed that *PaNFP* and *MdLYR1* are close homologs of legume *MtNFP/LjNFR5* and *MtLYR1/LjLYS11* (Fig. 2). Based on this we conclude that, in contrast to legumes, Parasponia contains only a single *MtNFP/LjNFR5*-like gene. The legume-specific nature of the gene duplications is supported by the presence of two conserved deletions in the legume genes (Fig. S3). Also a substantial level of microsynteny in paralogous regions as well as a low level of nucleotide substitutions in paralogous gene pairs support the recent nature of the duplication (Lohmann et al., 2010; Zhang et al., 2009). To determine whether this duplication predates the *Fabaceae*, we searched for *MtNFP/LjNFR5*-like sequences in a collection of cDNA clones from the basal legume *Chamaecrista fasciculata* (Cannon et al., 2010). We identified a single clone (named *CfNFP1*) that is phylogenetically ancestral to the duplication observed in *M. truncatula* and *L. japonicus* (Fig. S4). Therefore we conclude that the duplication of *MtNFP/LjNFR5* in the legume lineage was not essential to gain symbiosis with Rhizobium.

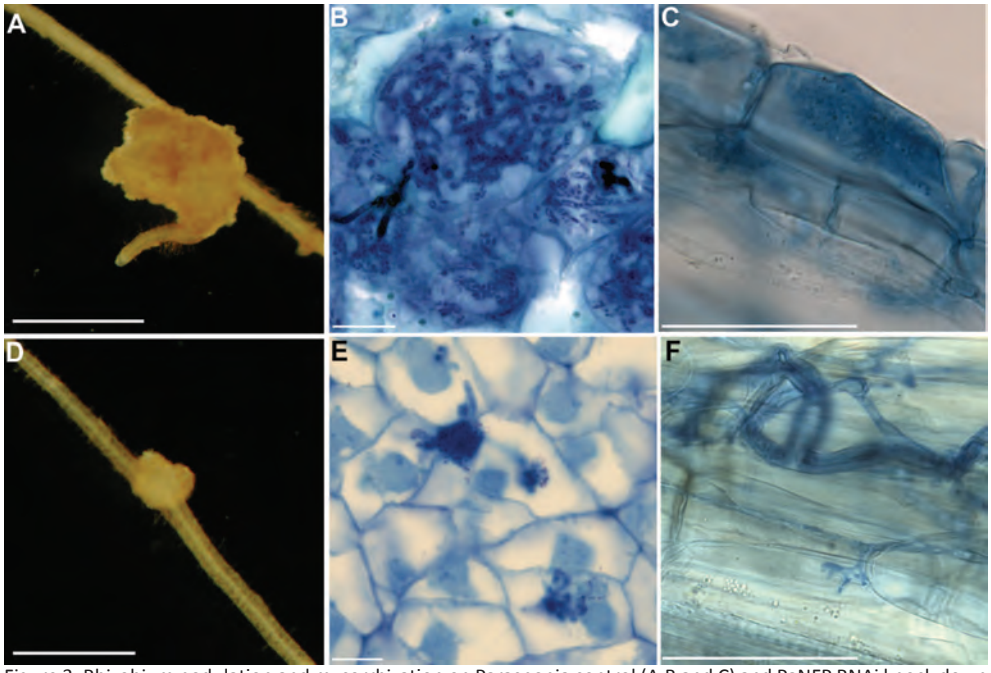


Figure 3: Rhizobium nodulation and mycorrhization on Parasponia control (A,B and C) and PaNFP RNAi knock down (D,E and F) roots. (A) Control nodule. Scale bar: 1.0 mm (B) Rhizobium fixation threads in control nodule. Scale bar: 10 µm. (C) Arbuscle in inner root cortical cell of (slightly squashed) control roots. Scale bar: 50 µm. (D) PaNFP RNAi Nodule. Scale bar: 1.0 mm. (E) Aborted fixation threads in PaNFP RNAi nodule. Scale bar: 10 µm. (F) Aborted intracellular infection of Glomus intraradices in PaNFP RNAi root. Scale bar: 50 µm.

RT-PCR studies revealed that *PaNFP* is expressed in roots (Fig. S5). To study whether *PaNFP* has a symbiotic function we performed RNAi knockdown experiments (see Materials and Methods). Parasponia roots transformed with the empty vector (control roots) could be nodulated effectively with *Sinorhizobium* sp. NGR234 (Fig. 3A; 11 out 30 plants formed nodules and in total 55 nodules were formed 8 wpi). Transgenic Parasponia roots that express a *PaNFP* RNAi construct have markedly reduced *PaNFP* expression levels (often below detection level and in case it is detected ≥50% reduced; Fig. S5). Inoculation of such

RNAi roots with *Sinorhizobium* sp. NGR234 resulted in a reduced number of nodules when compared to control plants (PaNFP RNAi: 13 nodules on 30 plants, 8 wpi). Further, all nodules formed on RNAi roots were much smaller compared to nodules on control roots (Fig. 3A,D). Sectioning of NFP-RNAi nodules showed that they harboured rhizobium intercellularly, but fixation thread formation was completely blocked in all nodules investigated (n=10) (Fig. 3B,E). This demonstrated that PaNFP is involved in nodule formation and is essential for the switch to an intracellular lifestyle of rhizobium. Also in legumes *MtNFP/LjNFR5* is essential for nodule formation as well as intracellular accommodation of rhizobium (Arrighi et al., 2006; Radutoiu et al., 2007). Based on these results we conclude that Parasponia has recruited a gene orthologous to the *MtNFP/LjNFR5* Nod factor receptor in legumes to control rhizobium symbiosis. This points to constraints in evolution of Nod factor perception mechanisms. As hypothesized above, a Nod factor receptor could have been recruited from the mycorrhizal signalling pathway. Since Parasponia has only a single *MtNFP/LjNFR5*-like gene we determined whether PaNFP is also essential for endomycorrhization. *PaNFP* RNAi knockdown and control roots were inoculated with *Glomus intraradices*. This showed that both are equally well infected by fungal hyphae. However, arbuscle formation is blocked in *PaNFP* RNAi roots, whereas in control roots arbuscules were effectively formed (Fig. 3C,F; Fig. S6). PaNFP therefore is also essential for successful intracellular infection during arbuscle formation by mycorrhizal fungi. We conclude that in Parasponia a single *MtNFP/LjNFR5*-like receptor, *PaNFP*, fulfills a dual symbiotic function and controls the intracellular life style of both AM fungi and rhizobium.

Our findings in Parasponia provide strong support for the hypothesis that during evolution a Myc factor receptor, as part of the common signalling cascade, has been recruited to serve as Nod factor receptor in the rhizobial plant symbiosis. Since in Parasponia *PaNFP* fulfills a dual function, it suggests that only a few adaptions -if any at all- will have occurred to enable perception of a new ligand; rhizobium Nod factors. Also it suggests that the Myc factor will have similar structural characteristics as Nod factors. In most legumes *MtNFP/LjNFR5* underwent at least one round of gene duplication (Fig. 2). However, our data suggest that this duplication occurred within the *Papilionoideae* subfamily of the *Fabaceae* (e.g. Medicago, Lotus and Glycine), since *CfNFP* of *Chamaecrista*, as part of the basal *Caesalpinioideae* subfamily, is ancestral to the duplication events (Fig. S4). Therefore it is likely that in *Chamaecrista* mycorrhization and rhizobium symbiosis also is controlled by just a single receptor; CfNFP. In more recent legumes, like *M. truncatula* and *L. japonicus*, a duplication of this receptor has occurred and only one of these has evolved as a Nod factor receptor. It seems very probable that the second copy functions as Myc factor receptor.

The bacterial genera -collectively named rhizobium- that evolved the ability to establish a nodule symbiosis, in general, acquired this by horizontal transfer of *nod* genes (Moulin et al., 2001). This event allowed them to produce fungal-like molecules, namely Nod factors, by which they could use the ancient mechanism by which endomycorrhizal fungi establish an intracellular life style and turned these rhizobia from free living bacteria into nitrogen fixing endosymbionts. However, although the endomycorrhizal symbiosis is wide spread in the plant kingdom only very few plant lineages, namely legumes and Parasponia, have recruited this mechanism for the rhizobial nodule symbiosis. Studies on the constraints underlying this evolutionary event in Parasponia can provide insight whether and how this nitrogen fixing symbiosis can be transferred to other non-legumes.

Materials and methods

Rhizobium strains

Sinorhizobium sp. NGR234 and a nodABC deletion mutant were used to nodulate *Parasponia* (Price et al., 1992; Pueppke and Broughton, 1999). These strains contained either pHC60 constitutive expressing GFP (Genbank: FJ151627) (Cheng and Walker, 1998) or pSm_ nifHp::GFP. This *Sinorhizobium meliloti nifH* promoter GFP construct was made according to Starker et al. (2006).

In vitro micro-propagation of Parasponia

For all studies a single *Parasponia andersonii* plant was selected (named WU1) and clonally propagated. To obtain an in vitro culture axillary buds of greenhouse-grown plants of P. andersonii line WU1 were surface-sterilized in 4% hypochlorite (commercial bleach) for 7 min, followed by six washes with sterile water. Each bud was cut in pieces and placed horizontally on the surface of McCown Woody Plant Medium with vitamins (Duchefa) supplemented with 1 mg/L benzyl amino purine (BAP) and 0.1 mg/L indole-3-butyric acid (IBA). Daichin agar (0.8% w/v, Duchefa) plates were then positioned vertically at 28 °C (16/8 h light/darkness) (Davey et al., 1993). Shoots emerged within 2-4 weeks.

Parasponia BAC library construction and screening

Based on flow cytometry the genome size of *P. andersonii* line WU1 was determined to be ~400 Mbp. Nuclei were isolated from very young leaves. The DNA was partially digested by HindIII and subsequently ligated in the vector pAGIBAC1, resulting in a library of 36,864 clones with an average insert size of 125 kb. This library has a ~10x coverage. This library was named: PA__Ba. DNA was spotted on nylon filter membranes (Hybond N, Amersham) and the filters were screened with a 32P-dATP labelled probe designed on the extracellular part of MtNFP and PaNFP.

Cloning MtNFP/LjNFR5-like genes using PCR and degenerate primers

Degenerate primers were designed based on the sequences of *MtNFP/LjNFR5* and *MtLYR1/LjLYS11* in order to amplify *MtNFP/LjNFR5*-like genes from *P. andersonii*: RosidNFP0-F: ATGSAAATCTKGTGAAACTAATGG and RosidNFP0-R: CATCCAYTTTCTTAIICTCTCYTCTCT. Subsequent PCR on genomic DNA or root cDNA of P. andersonii resulted in 473 bp amplicon. Cloning and sequencing revealed that the amplicon represented only a single gene. RACE PCR was performed on RNA isolated from root tissue, making use of the Smart RACE cDNA Amplification kit (Clontech).

Constructs

A truncated dominant active MtCCaMK construct driven by the CaMV 35S promoter was made according to Gleason et al. (2006) and cloned in the binary vector pHGW-RR-MGW-R4R3 that contains DsRED1 as selectable marker. The PaNFP RNAi construct was made by cloning 422 bp of the kinase domain and cloned in pENTR-D-topo (PaNFP1i-f

CACCTGGACAGAGATGGAAACTGCT and PaNFP1i-r GAAGCAAAAGCAGTTTTGGAA). Subsequent hairpin construct was made by recombination of the amplified region into the binary vector pK7GWIWG2(II)-RedRoot (VIB-Ghent University) containing the fluorescent reporter *DsRED1* as a selectable marker.

Agrobacterium rhizogenes Transformation

Shoots of ~1 cm length were cut from callus producing shoots and transferred in EKM agar medium plates half covered with sterile filter paper (Becking, 1983). *Agrobacterium rhizogenes*, strain MSU440, containing the appropriate binary plasmid (all containing *DsRED1* as selectable marker) was applied on the cut site and two more wounds were produced with a needle dipped in *A. rhizogenes*. Transfected plants were placed in line at agar medium plates half cover with sterile filter paper. The plates were sealed and positioned vertically 21 °C (16/8 h light/darkness). After 5 days the shoots were transferred to Emergence medium (Limpens et al., 2004) 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 5 days 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.

RNA isolation and PaNFP expression levels

P. andersonii root RNA was isolated from snap-frozen root samples using CTAB extraction buffer 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 DNAse I treated and further purified on RNeasy Mini Spin columns (Qiagen). Next, cDNA was synthesized from 1 µg 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 (Eurogentec, Maastricht, the Netherlands). 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 *PaNFP* and *PaLysM-RK* in control and RNAi knockdown roots was normalized using a *P. andersonii* actin gene.

Primers used:
PaACT-qF CCTCATTGGAATGGAAGCAC
PaACT-qR TTCCAGGAAACATGGTGGAC
PaNFP-qF GGCCTTTGGTTCGAGATACA
PaNFP-qR CATCACTTGGTTGCCATACG
PaLysM-RK-qF ATGGCGATGTATCTCCCAAG
PaLysM-RK-qR TTCAGCAACTGAACCGTCTG

Parasponia nodulation assay

(Transgenic) *P. andersonii* plants were grown on agraperlite (Maasmond-Westland, The Netherlands) saturated with Farhaeus medium (without $Ca(NO_3)^2$) and inoculated with *Sinorhizobium* sp. NGR234 (OD600 = 0.1). Plants were grown for 8 weeks in a conditioned greenhouse at 28°C, 85% humidity and 16/8h light/darkness.

Parasponia mycorrhization assay

Transgenic *P. andersonii* plants were planted in a mixture of sand and hydro granules, ratio 1:1 together with onion plants colonized by *Glomus intraradices* (nurse plants). The pots were placed in a growth chamber for 4 weeks at 28°C and 85% humidity and covered with plastic foil for the first 3 days. The plants were watered three times a week, once with Hoagland's solution (Hoagland, 1950) and twice with water. To characterize mycorrhyzation, *P. andersonii* roots were collected, washed thoroughly with water 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 rinsing the roots twice 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 Trouvelot et al. (1986).

Phylogenetic analysis

Sequences were generated in this project in the case of *Parasponia andersonii* and otherwise retrieved from the following resources; Genbank and/or Phytozome (phytozome.net) (Chan et al., 2010; Huang et al., 2009; Sato et al., 2008; Schmutz et al., 20101; Tuskan et al., 2006). In the case of *Malus x domestica* genomic sequences have been retrieved from: Istituto Agrario di San Michele All'Adige (genomics.research.iasma.it) (Velasco et al., 2010) whereas *Chamaecrista fasciculata CfNFP* has been obtained from Cannon et al. (2010). Protein sequences were aligned using MUSCLE version 3.8.31 (Edgar, 2004) with default options. All sites with at least 90% alignment gaps or missing data were removed. Phylogenetic trees were reconstructed by maximum likelihood using PhyML version 3.0 (Guindon et al., 2010). Options were: BIONJ starting tree, nearest-neighbor interchange searching method, LG amino acid substitution matrix (Gascuel, 2008), four gamma-distributed discrete rates of evolution with free shape parameter and no invariant sites. 1000 non-parametric bootstrap repetitions were used to evaluate statistical support to branches. Rooting was performed using the midpoint method. Sequences of all genes used in this phylogenetic analysis using the following annotations (Cf: *Chamaecrista fasciculata*, Cs: *Cucumis sativus*, Gm: *Glycine max*, Lj: *Lotus japonicus*, Md: *Malus x domestica*, Mt: *Medicago truncatula*, Pa: *Parasponia andersonii*, Pt: *Populus trichocarpa*, Rc: *Ricinus communis*).

Protein	Resource/Accession number
CfLYK1	Cf9227d.
CsLYR1	phytozome: Cucsa.366740
CsLYR2	phytozome: Cucsa.101780
GmNFR5a	genbank: ADJ19108, phytozome: glyma11g06740
GmNFR5b	genbank: ABQ59613, phytozome: glyma01g38560

LjNFR5	genbank: CAE02598
LjLYS11	genbank: BAI79285
LjLYS16	genbank: BAI79280
MdLYR1	genomics.research.iasma.it: MDC019637.222 (region 19366-21228)
MtNFP	genbank: ABF50224, phytozome:Medtr5g018990
MtLYR1	genbank:AC148241(region:82400-84169),phytozome: Medtr8g093910
OsNFP	genbank:AC103891.2(region:42264-44132),phytzme:LOC_Os03g13080.1
PaNFP	genbank: HQ705608
PtLYR1	phytozome: scaffold_5 (region: 9805534..9807399), ptv21.034724m.
PtLYR2	genbank: XP_002310198, phytozome: POPTR_0007s12210.
RcLYR1	genbank XP_002533280, phytozome: 29250.t000012

Acknowledgements

We thank Bill Broughton for NGR234, Euan James, Paul Hadobas and TJ Higgens for Parasponia seeds, Peter van Dijk for flow cytometry, Jayson Talag and Wolfgang Golser for BAC library construction. This research is funded by the Dutch Science Organization NWO (VIDI 864.06.007).

Supplemental Figures

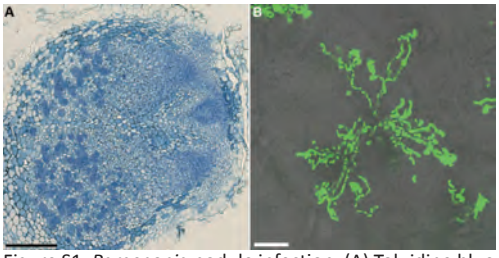


Figure S1. *Parasponia* nodule infection. (A) Toluidine blue stained section of *Parasponia* nodule. Dark blue stained infected cells are located at the base of the nodule. Scale bar: 200 µm. (B) Confocal image (bright field overlay) of intracellular fixation treads. *Sinorhizobium* sp. NGR234 is transformed with nifH::GFP. Scale bar: 10 µm.

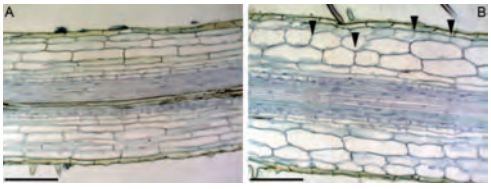
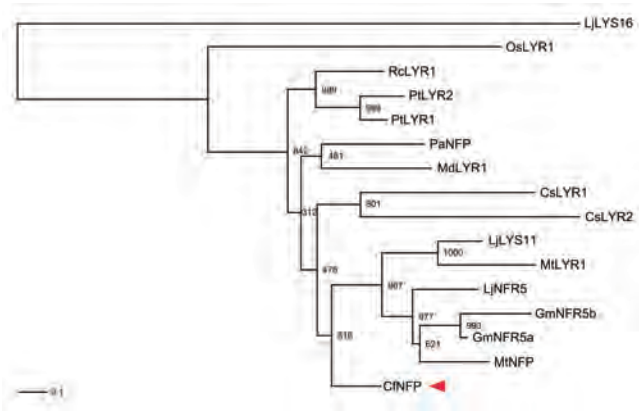


Figure S2. Nod factor induced cortical cell divisions. (A) Longitudinal section of a control root and (B) and a root locally treated with *Sinorhizobium* sp. NGR234 Nod factors (~10-7 M) (48 h after treatment). Cell divisions occurred mainly in the outer cortical cell layers (marked with arrowheads). Scale bars: 100 µm

Figure S4. Maximum-likelihood phylogeny of *MtNFP/LjNFR5*-like genes in Rosid I (*Fabidae*) clade. Gene duplication events in legumes are relatively recent and have occurred after the *Chamaecrista fasciculata* (*CfNFP*, red arrowhead) split. Branch lengths are proportional to the number of amino acid substitutions per site. Branch support was obtained from 1000 bootstrap repetitions. Note that this tree and Fig. 2 present a minor inconsistency regarding the *MtNFP, LjNFR5, GmNFR5a* and GmNFR5b subclade likely due to a lack of phylogenetic resolution resulting in modest branch support. Cf: *Chamaecrista fasciculata*, Cs: *Cucumis sativus*, Gm: *Glycine max*, Lj: *Lotus japonicus*, Md: *Malus x domestica*, Mt: *Medicago truncatula*, Pa: *Parasponia andersonii*, Pt: *Populus trichocarpa*, Rc: *Ricinus communis*.



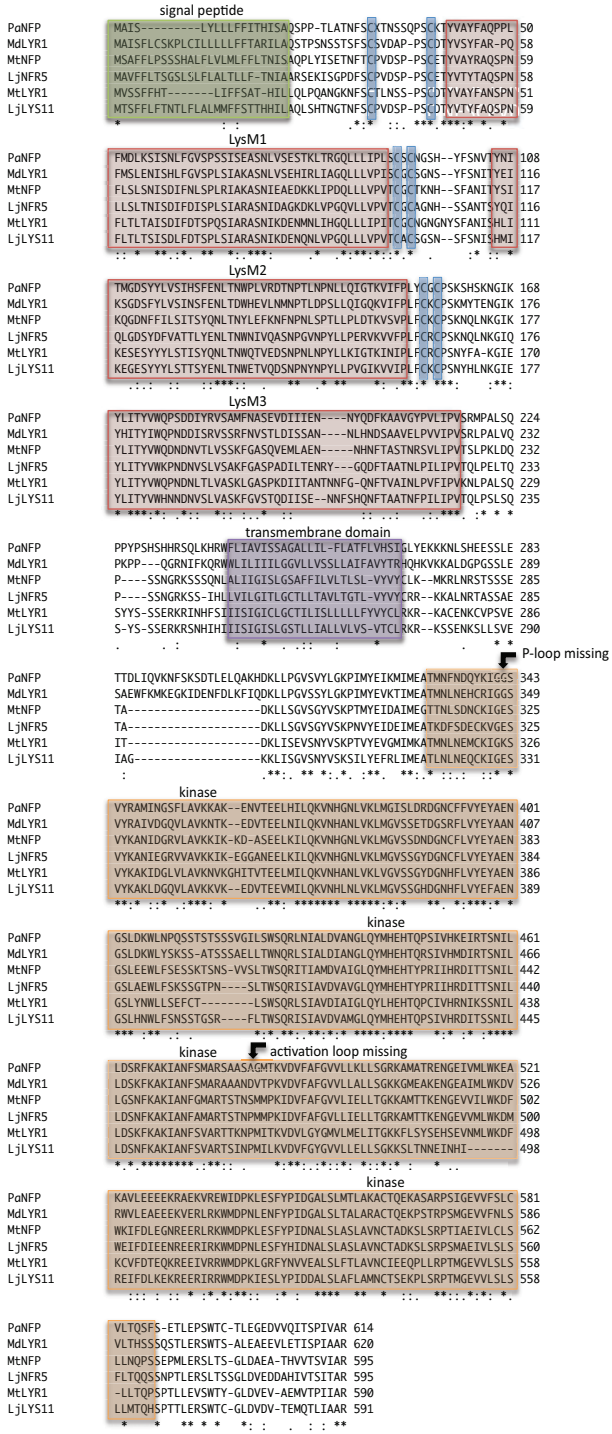
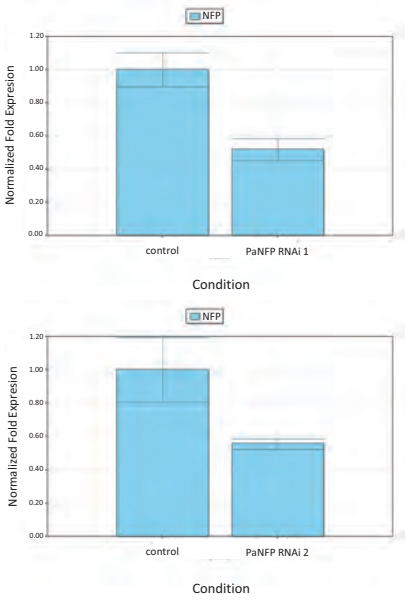


Figure S3. Amino acid alignment of the LysM-type receptor kinases *PaNFP* (*P. andersonii*), *MdLYR1* (apple), *MtNFP*, *MtLYR1* (*M. truncatula*) and *LjNFR5*, *LjLYS11* (*L. japonicus*) showing conserved domain structure.



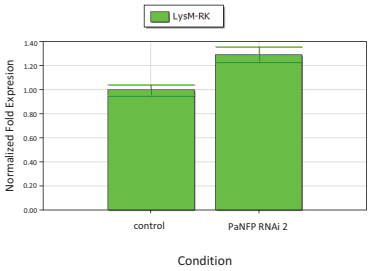


Figure S5. *PaNFP* expression control and *PaNFP* RNAi knockdown roots. Shown are two representative examples of control roots and RNAi knock down roots with ~50 % reduction. As control the expression level of a *Parasponia* LysM-RK homologous to *Arabidopsis CERK* (*AtCERK*) is determined (Wan et al., 2008). Note: *PaNFP* is low expressed in control roots, and knock down levels in RNAi roots are frequently below detection limits (n=8).

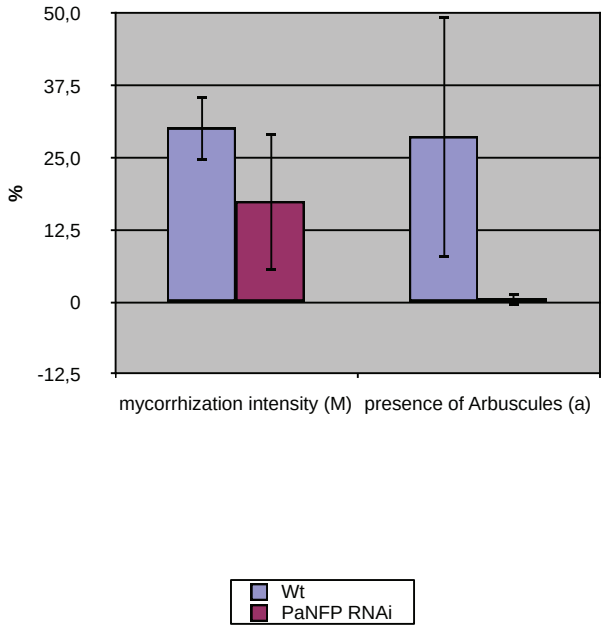


Figure S6. *Glomus intraradices* mycorrhization of wild type (n=6) and *PaNFP* knock down roots (n=8). Mycorrhization intensity (M) and presence of arbuscules in infected segments (a) is determined in whole roots including older regions that have formed prior mycorrhizea inoculation. 'M' and 'a' were quantified according Trouvelot et al. (2008).

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Chapter 5

Evolutionary Origin of Rhizobium Nod factor Signaling

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Adapted version from: Streng et al. (2011) *Plant Sig Beh* **6**:1510 - 1514

For over two decades now, it is known that the nodule symbiosis between legume plants and nitrogen fixing rhizobium bacteria is set in motion by the bacterial signal molecule named nodulation (Nod) factor (Lerouge et al., 1990). Upon Nod factor perception a signalling cascade is activated that is also essential for endomycorrhizal symbiosis (Fig. 1). This suggests that rhizobium co-opted the evolutionary far more ancient mycorrhizal signalling pathway in order to establish an endosymbiotic interaction with legumes (Remy et al., 1994). As arbuscular mycorrhizal fungi of the Glomeromycota phylum can establish a symbiosis with the fast majority of land plants, it is most probable that this signalling cascade is wide spread in plant kingdom (Wang et al., 2010). However, Nod factor perception generally is considered to be unique to legumes. Two recent breakthroughs on the evolutionary origin of Rhizobium Nod factor signalling demonstrate that this is not the case (Maillet et al., 2011; Op den Camp et al., 2011) The purification of Nod factor-like molecules excreted by the mycorrhizal fungus *Glomus intraradices* and the role of the LysM-type Nod factor receptor PaNFP in the non-legume *Parasponia andersonii* provide novel understanding on the evolution of rhizobial Nod factor signalling.

Rhizobium Nod factors and responses in legumes

Elucidation of the Nod factor structure was a major step in the molecular approach to unravel the signalling pathway in legumes that is essential for the establishment of rhizobium symbiosis. Rhizobial Nod factor molecules are lipochito-oligosaccharides (LCOs) consisting of three to five N-acetyl-glucosamines that on the amino group of the non-reducing glucosamine is acylated with a fatty acid of 16 to 20 C-atoms in length (C16 to C20). Furthermore, species specific substitutions can be present on the terminal glucosamines, thereby determining specific recognition of Nod factor structure by the legume host plants (D'Haeze and Holsters, 2002).

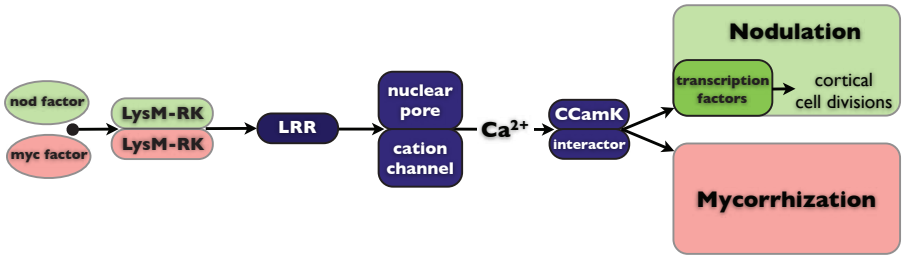

Figure 1. Schematic representation of the genetically dissected symbiosis signalling pathway. In legumes rhizobium Nod factors and mycorrhizal Myc factors are perceived by distinct receptor complexes. In case of Nod factors these are the LysM-RK type receptors MtLYK3/LjNFR1 and MtNFP/LjNFR5, whereas Myc factors remain to be elucidated. In *Parasponia* PaNFP fulfils a dual function and acts in both symbioses. The subsequent common signalling pathway consists of several components including a plasma membrane localized LRR-type receptor (MtDMI2/LjSymRK), a cation channel in the nuclear envelope (MtDMI1/LjCASTOR/LjPOLLUX) and subunits of the nuclear pore (NUP85, NUP133), and a nuclear localized complex of calcium calmodulin dependent kinase (CCaMK) and interactor protein MtIPD3/LjCYCLOPS. Downstream of CCaMK the rhizobium and mycorrhiza induced responses bifurcate.

Examples of such modifications are glycosylation, sulfation, acetylation and methylation, for which the particular rhizobium species harbours specific *nodulation* (*nod*) genes (Downie, 2009; Mergaert et al., 1997). Therefore, it is generally assumed that the perception of Nod factors by legume host plants has co-evolved with their corresponding rhizobial symbionts.

Some rhizobium species however, produce a diverse mixture of Nod factors often resulting in a large range of host plants (Pueppke et al., 1999). Of such broad host range rhizobium species, *Sinorhizobium* sp. NGR234 is iconic, as it is well studied at a molecular and genetic level (Broughton et al., 2000). This species not only nodulates hundreds of legume species, but also *Parasponia*; the only non-legume genus able to establish a similar symbiosis with rhizobium (Morrison et al., 1983; Pueppke et al., 1999).

Nod factors are sufficient to trigger all symbiotic responses essential to develop fully differentiated nodules on legume roots (Mathesius et al., 1998; Truchet et al., 1991). Furthermore, Nod factor signalling is imperative for intracellular bacterial infection (Ardourel et al., 1994). This infection process is set in motion through changes in growing root hairs resulting in root hair curling around Nod factor secreting rhizobia. These captured bacteria can initiate the formation of intracellular infection threads. Such infection threads are surrounded by a plant derived membrane and contain files of clonally propagating rhizobia. Within infection threads the rhizobia remain cell wall bound. Infection threads grow to the base of epidermal cells. Simultaneously, activation of mitosis in cortical cells below the infected root hair cells results in a nodule primordium. As infection threads reach newly formed nodule primordia the rhizobia are released into the nodule cells. Release from infection threads occurs at specific sites that do not contain a cell wall. Since the budding off of rhizobium from the infection thread have a droplet-like appearance, these regions in the infection threads are known as 'unwalled infection droplets'. In this endocytotic-like process, rhizobia become surrounded by a plant membrane. Such unit, of one or a few rhizobia surrounded by a plant membrane, is named symbiosome. Symbiosomes act like transient nitrogen fixing organelles. Thereby the surrounding symbiosome membrane acts as an interface between both partners enabling exchange of nutrients. Along the whole infection process, from early responses in root hairs down to the formation of symbiosomes, Nod factor signalling is important.

In some more basal legume species symbiosome formation does not occur (De Faria et al., 1986; Naisbitt et al., 1992). Instead rhizobia remain in intracellular thread-like structures; so-called fixation threads. These fixation threads differ from ordinary infection threads by a significant thinner cell wall, enabling a more efficient exchange of nutrient across the membrane interfase.

Mycorrhizae and Rhizobium common signalling pathway

It is known for a long time that mycorrhizae and rhizobium symbioses in legumes share some common key signalling genes (Duc et al., 1989). This so-called common symbiotic signalling pathway has been unravelled in pea (*Pisum sativum*) and the legume model species; *Medicago truncatula* (medicago) and *Lotus japonicus* (lotus), respectively. In both species the common symbiotic signalling pathway comprises a rather conserved set of genes encoding a plasma membrane receptor kinase (MtDMI2 and LjSYMRK), several components in the nuclear envelope including a cation ion channel (MtDMI1, LjCASTOR and LjPOLLUX), a nuclear localized Calcium Calmodulin dependent Kinase (CCaMK; MtDMI3 and LjCCaMK) and a CCaMK interacting protein (MtIPD3 and LjCYCLOPS) (Fig. 1) (Kouchi et al., 2010). Mycorrhizae and rhizobium induced signalling bifurcates downstream of CCaMK, possibly due to a different nature of the calcium signal (Kosuta et al., 2008).

In legumes the common signalling pathway is activated by LysM-type Nod factor receptors. Nod factors are perceived by two distinct LysM-type receptor kinases (LysM-RKs) that form a heterodimeric complex to achieve symbiotic signalling (Madsen et al., 2010; Nakagawa et al. 2011). In medicago and lotus these receptors, named MtLYK3/LjNFR1 and MtNFP/LjNFR5, are not essential for mycorrhizal symbiosis, suggesting that in legumes Nod factor receptors have evolved specifically to support rhizobium Nod factor signalling. This raises immediate questions concerning the evolutionary origin of rhizobium Nod factor perception and how mycorrhizae achieve activation of the common signalling pathway. Research on *Parasponia* and the mycorrhizal fungus *G. intraradices* revealed first answers to these questions.

Nod factor signalling in Parasponia

The genus *Parasponia* comprises about 6 species and is part of the *Celtidaceae* (Akkermans et al., 1978; Sujatha et al., 2002; Tjepkema and Cartica, 1982; Trinick, 1979; Yesson et al., 2004). Recent molecular phylogenetic studies combine this family with the *Cannabaceae* (Sytsma et al., 2002). All *Parasponia* species can establish a nitrogen fixing endosymbiosis with rhizobium species that also can nodulate legumes (Becking, 1983, 1992; Trinick, 1973; Trinick and Galbraith, 1976; Webster et al., 1995). Interestingly, the *Parasponia*-rhizobium symbiosis is also Nod factor driven (Op den Camp et al., 2011; Pawlowski and Sprent, 2007). Because the *Celtidaceae/Cannabaceae* and *Fabaceae* are only remotely related, it is most probable that both lineages have gained the symbiotic capacity independently. Therefore, a comparison of Parasponia and legumes will provide insights in genetic constraints underlying rhizobium symbiosis.

The *Parasponia*-rhizobium symbiosis is most likely relatively young as *Parasponia* is very closely related to its non-symbiotic sister genus Trema (Sytsma et al., 2002). This hypothesis is further supported by the rather primitive nature of *Parasponia* root nodules. First, the nodule ontogeny differs with that the ontogeny of legume nodules. *Parasponia* nodules are modified lateral roots with a central vascular bundle and infected cells in the peripheral zone. In contrast, legume nodules have a peripheral vasculature with a central zone of infected cells (Pawlowski and Sprent, 2007). Furthermore, there is a distinct difference in the infection mode. Rhizobium enters the *Parasponia* root intercellularly by crack entry and only when a bacteria reaches a nodule primoridium, intracellular infection occurs. Once inside a nodule cell, fixation threads are formed, similar as found for some basal legumes (Webster et al., 1995).

As the *Parasponia*-rhizobium symbiosis is relatively young when compared to legumes, it can provide additional insights in the evolutionary origin of symbiotic genes. Such comparative evolutionary studies have been conducted on LysM-type Nod factor receptors. Plant LysM-RK genes can be divided into three major clades, two of which contain a legume Nod factor receptor, MtNFP/LjNFR5 and MtLYK3/LjNFR1, respectively (Arrighi et al., 2006; Lohmann et al., 2010; Zhang et al.,2007, 2009; Zhu et al., 2006). The latter clade includes also the chitin innate immune receptor, AtCERK1 of *Arabidopsis thaliana* (arabidopsis) (Iizasa et al., 2010; Miya et al., 2007; Petutschnig et al., 2010; Wan et al., 2008). Legume gene duplication events of different nature have occurred in all three classes resulting in a relative large number of 15 to 17 genes in for example lotus and medicago, whereas in arabidopsis only 5 such genes are found (Lohmann et al., 2010; Zhang et al., 2009). Especially the MtLYK3/

LjNFR1-class expanded significantly in both legumes. As the Nod factor receptor MtLYK3/ LjNFR1 is a close homolog of arabidopsis AtCERK1, it suggests that both genes share a recent common ancestral gene. Comparative studies revealed that in evolution the kinase domain of both proteins underwent specific amino acid substitutions enabling it to trigger specific responses (Nakagawa et al., 2011).

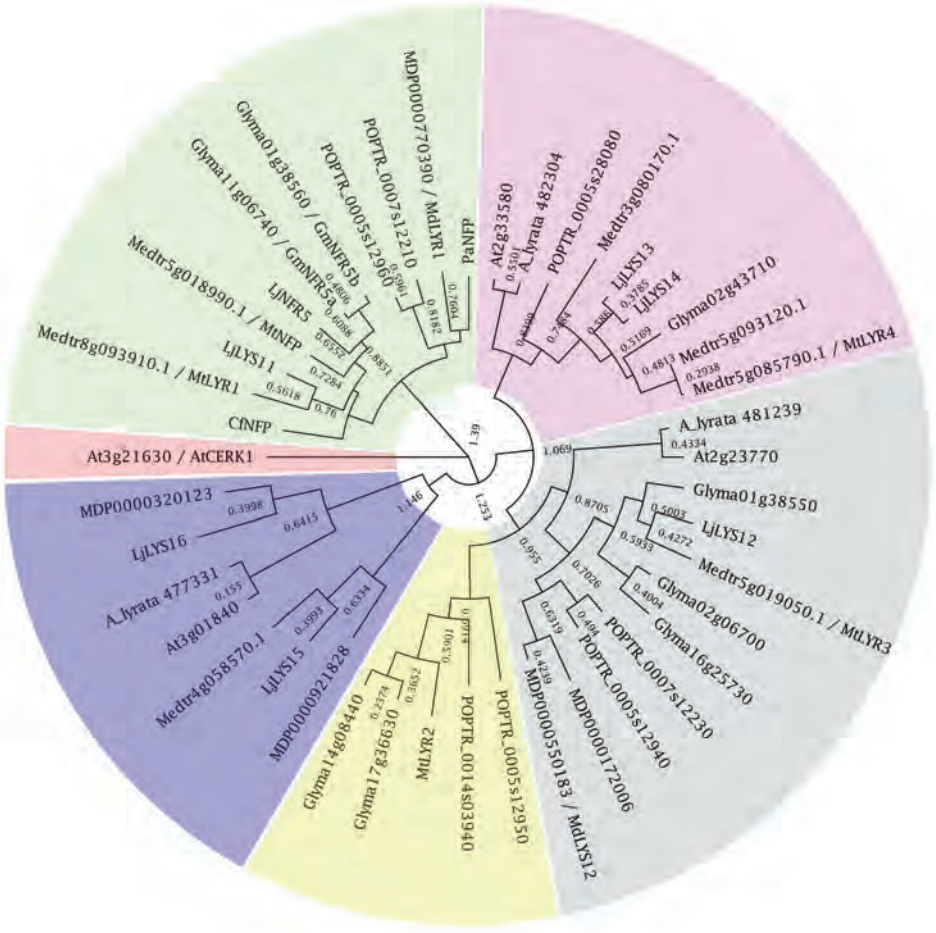

Figure 2. Bayesian phylogenic tree of MtNFP/LjNFR5-class of LysM-RKs using *Arabidopsis thaliana* AtCERK1 as outgroup. Five orthology groups can be recognized indicated in different colours. *Parasponia andersonii* (Pa), *Malus domesticus* (Md), *Populus trichocarpa* (POPTR), *Lotus japonicus* (Lj), *Glycine max* (Glyma), *Medicago truncatula* (Mt/Medtr) and *Chamaecrista fasciculata* (Cf). Branch lengths are proportional to the number of amino acid substitutions per site. The analysis was run for 290,000 generations, sampling every 200 generations using Geneious software with default settings.

Interestingly, the MtNFP/LjNFR5-class of LysM-type receptors is less expanded in legumes, and MtNFP/LjNFR5 underwent only a single legume specific duplication event in the subfamiliy of medicago, lotus and soybean (*Glycine max*) (Op den Camp et al., 2011; Zhang et al., 2007). MtNFP/LjNFR5-homologs can be found in many non-legume species, including *Parasponia* (green orthology group Fig. 2). Functional analysis of this gene in *Parasponia andersonii* revealed a dual symbiotic function. PaNFP controls intracellular infection of rhizobium and mycorrhizal fungi (Op den Camp et al., 2011). *PaNFP* knockdown lines are

blocked specifically in the formation of symbiotic interfaces. In case of rhizobium this is the switch from initial intercellular infection to the formation of fixation threads. In case of mycorrhizae, root cortical cells get infected by cell wall bound fungal hyphae (known as trunks), but arbuscules are not formed. Arbuscles represent the symbiotic interface that supports nutrient exchange in this ancient symbiosis. As *Parasponia* has only a single MtNFP/LjNFR5-homolog, as is the case in other non-legume species, it suggests that in *Parasponia* the mycorrhizal signalling perception mechanism has been co-opted to achieve rhizobium Nod factor signalling (green orthology group Figure 2) (Op den Camp et al., 2011). Interestingly, arabidopsis lacks a clear MtNFP/LjNFR5-homologous gene (green orthology group Figure 2) (Zhang et al., 2009), which is in line with the inability of this species to establish a mycorrhizal symbiosis (Smith and Read, 2008).

Mycorrhizae secrete Nod factor-like signal molecules

The finding that in *Parasponia* a single LysM-type Nod factor receptor is essential for mycorrhizal symbiosis implies that mycorrhizae produce Nod factor-like LCOs. This is indeed the case (Maillet et al., 2011). *G. intraradices* secretes symbiotic LCOs that are a mixture of Nod factor-like molecules. These LCOs stimulate the mycorrhizal symbiosis in legumes as well as non-legumes. Like Nod factors, such mycorrhizal signalling factors, or Myc factors, also contain a tetrameric or pentameric N-acetyl glucosamine backbone that is acylated at the non-reducing end with either a C16:0 or C18:1 acyl chain. Such lipids are common in microbes and also found on Nod factors of several rhizobium species (D'Haeze and Holsters, 2002). Furthermore, a sulphate group can be present at the reducing end of the Myc factor, similar as can be found on Nod factors of certain rhizobium species; e.g. *Sinorhizobium meliloti* (Lerouge et al., 1990; Maillet et al., 2011). Based on these structural similarities we can conclude that Myc factors and Nod factors are very related, with the notion that in case of Nod factors more structural variation in side-groups and/or acyl chains are known. This finding, together with the knowledge that a MtNFP/LjNFR5-type Nod factor receptor can control two symbioses, discloses the evolutionary origin of rhizobium Nod factor signalling. In evolution, an ancestral free-living nitrogen fixing rhizobium species has gained a biosynthetic pathway of LCOs. This could be the result of horizontal gene transfer of fungal genes, a hypothesis that is supported by the finding of rhizobial endosymbionts in *Glomeromycota* species (Bonfante et al., 2009). Alternatively, such ancestral rhizobium species has phenocopied the Myc factor by synthesizing LCOs along an different pathway, an hypothesis that find support by the unique nature of the rhizobial N-acetylglucosaminyl transferase nodC that is most homologous to animal hyaluronan synthases (Merzendorfer, 2006). Either way, the net result is that an LCO producing rhizobium can activate the mycorrhizal signalling cascade of higher plants. In some plant species, like ancestral legumes and *Parasponia*, it has gained intracellular access, which would have lead to the formation of nodular organs to host the microsymbionts. Ultimately, this resulted in the nitrogen fixing symbioses as we see today.

Perspectives for the near Future

The finding that in *Parasponia* an orthologous gene of a legume Nod factor receptor has been recruited to support rhizobium symbiosis strongly suggests that there are genetic constraints underlying evolution of this symbiotic association. A further comparison of the

rhizobium symbioses in legumes and *Parasponia* can reveal an in depth characterization of these genetic constraints.

Interestingly, the PaNFP receptor also controls mycorrhization in *Parasponia*, which provides leads for future research on the evolution of Nod factor signalling. First of all this finding, together with the identification of Nod factor-like molecules secreted by the fungus *G. intraradices*, points to the evolutionary origin of Rhizobium Nod factor signalling; namely the ancient mycorrhizal symbiosis. Simultaneously, these findings underline that the shared common signalling cascade extends from initial signal perception down to CCaMK controlled gene expression. Possibly the shared elements extend even further as the intracellular infection structures of mycorrhiza and Rhizobium display quite some similarities in *Parasponia*. Therefore it is tempting to speculate that both symbioses also share cellular processes.

The fact that mycorrhizal symbiosis can occur with the vast majority of land plants it underlines that LCO signalling is wide spread. Identification of the underlying genes in non-legumes will provide insight in the evolutionary events that have occurred in legumes to exploit the Nod factor-based rhizobium symbiosis. The fact that some species have lost the ability to establish a mycorrhizal symbiosis, e.g. species of the genus *Arabidopsis*, can be exploited to identify the LCO signalling cascade in non-legumes. For example, *Arabidopsis* species have lost most genes of the common signalling pathway as well as MtNFP/LjNFR5 (green orthology group Figure 2) (Wang et al., 2010). Following this argumentation, one could postulate that also root expressed genes of the orthology group of *MtLYR2* have a symbiotic function as *Arabidopsis* species have lost this gene (yellow orthology group Figure 2).

In legumes MtNFP/LjNFR5 interacts with MtLYK3/LjNFR1, which is essential for symbiotic signalling. Such heterodimerisation is essential as MtNFP/LjNFR5 does not contain a functional kinase domain, and therefore is dependent on a interacting partner for downstream signalling (Arrighi et al., 2006). Identification of the interacting counterpart of MtNFP/LjNFR5-type receptors in non-legumes is therefore important to understand the functioning of the LCO perception mechanism in non-legumes; including *Parasponia*. Ultimately such comparative studies on *Parasponia* and legumes as well as *Parasponia* and it non-nodulating sister *Trema* could provide a blueprint for a future transfer of the rhizobium symbiosis to the major non-legume crops.

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Chapter 6

The non-legume *Parasponia* deploys a broad rhizobium host range strategy resulting in largely variable symbiotic effectiveness

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As submitted to *Mol Plant Microbe Interact* (Accepted with revision)

The non-legume *Parasponia* has evolved the rhizobium symbiosis independent from legumes and has done so only recently. We aim to study the promiscuity of such newly evolved symbiotic engagement and determine the symbiotic effectiveness of infecting rhizobium species. It was found that *Parasponia andersonii* can be nodulated by a broad range of rhizobia belonging to 4 different genera, and therefore we conclude that this non-legume is highly promiscuous for rhizobial engagement. A drawback of this high promiscuity is the possibility that low efficient strains can infect nodules as well. The strains identified displayed a range in nitrogen fixation effectiveness including a very inefficient rhizobium species, *Rhizobium tropici* WUR1. As this species is able to make effective nodules on two different legume species it suggests that the ineffectiveness of *P. andersonii* nodules is the result of the incompatibility between both partners. In *P. andersonii* nodules rhizobia of this strain become embedded in a dense matrix, but remain vital. This suggests that sanctions or genetic control against underperforming microsymbionts may not be effective in *Parasponia*. Therefore we argue that the *Parasponia*-Rhizobium symbiosis is a delicate balance between mutual benefits and parasitic colonization.

Introduction

Most legume species can engage a symbiosis with nitrogen fixing soil bacteria collectively referred to as rhizobium. Besides the common occurrence within the legume family (*Fabaceae*), this nitrogen-fixing rhizobium symbiosis has evolved only once in another plant species (Cannon et al., 2010; Trinick, 1973). This independent evolutionary event occurred in a small genus of tropical trees found in the *Cannabaceae* called *Parasponia*. The *Parasponia*-rhizobium symbiosis is considered to have arisen only recently when compared to legumes (Op den Camp et al., 2011). We investigated two aspects of the *Parasponia*-rhizobium symbiosis; namely its promiscuity and the effectiveness of the nodules formed.

Symbiotic rhizobium bacteria form a diverse group of more than ten genera within the phylum of the Proteobacteria that have gained capacity to live in symbiosis with legumes. Many legume species display a restricted host range and can only be nodulated by a limited number of bacterial species or even strains. On the other hand also highly promiscuous legumes are known. Legumes that display a restricted host range or are highly promiscuous do not form unified taxonomic groups; especially promiscuous species are dispersed within the *Fabaceae* (Perret et al., 2000). Specificity for rhizobium microsymbionts is generally thought to have emerged upon co-evolution between host and microbe (Martinez-Romero, 2009; Masson-Boivin et al., 2009; Provorov and Vorobyov 2008). This implies that high promiscuity for nitrogen fixing rhizobia was the ground state of ancestral host plants (Sprent, 1994). We aimed to test this hypothesis by studying the promiscuity of the more recently evolved non-legume rhizobium host *Parasponia*.

The evolutionary recent origin of the *Parasponia* genus is best supported by its very close phylogenetic relation with the genus Trema (Sytsma et al., 2002). Furthermore, the symbiotic engagement with rhizobium displays several basal characteristics suggesting a recent emergence. A characteristic of *Parasponia* being a relatively young host plant for rhizobium is the infection mechanism by which the endosymbiont enters the plant. In *Parasponia*, rhizobia enter by means of so called crack-entry, which is considered to be a basal mode of entry and only found in a very limited number of legume species (Charpentier and Oldroyd

2010; Goormachtig et al., 2004; Madsen et al., 2010; Sprent, 2007). In contrast, in most legumes rhizobia enter by the stringent host controlled mechanism of epidermal root hair entry (Charpentier and Oldroyd 2010). In this entry mode, a single rhizobium attaches to a root hair and forms a microcolony, which is subsequently enclosed by the curling root hair. From the enclosed colony an infection thread is formed, by which the rhizobia can progress into the root cortex. At the same time a nodule primordium is initiated in the cortex, which will be reached by an infection thread that susbsequently releases the rhizobia intracellularly to become nitrogen fixing symbiosomes (Charpentier and Oldroyd 2010; Kouchi et al., 2010). Crack entry starts with colonization of the root surface, which coincides with cortical cell divisions that subsequently lead to rupturing of the epidermis. Rhizobia can also enter through cracks in the epidermis, for example at the base of a lateral root (Charpentier and Oldroyd 2010; Webster et al., 1995). Next, rhizobia colonize intercellular spaces before forming infection threads, which will infect the nodule cortical cells (Capoen et al. 2010). Both epidermal root hair entry as well as crack entry are dependent on a genetic network triggered by a signal molecule excreted by rhizobium species; the Nod factor (Ardourel et al., 1994; Charpentier and Oldroyd 2010; Goormachtig et al., 2004; Op den Camp et al., 2011; Smit et al., 2007).

Among other factors, recognition of the bacterial Nod factor signaling molecules determines the host range of rhizobia. Nod factors are lipochitooligosaccharides consisting of three to five N-acetyl-glucosamines and a lipid moiety. Rhizobium species specific additions can be present on the terminal glucosamines, thereby determining host specific recognition (D'Haeze and Holsters, 2002). Examples of such modifications are glycosylation, sulfation, acetylation and methylation, for which the particular rhizobium species harbor specific nodulation (*nod*, *nol* and *noe*) genes (Mergaert et al., 1997). The standing hypothesis is that that recognition of Nod factors by legume host plants is a driving force in co-evolution of both symbiotic partners and will result in host specificity (Arrighi et al., 2006; Downie 2009; Heath and Tiffin 2007; Limpens et al., 2003; Masson-Boivin et al., 2009; Radutoiu et al., 2003; Radutoiu et al., 2007). Former experiments showed that *Parasponia* could be nodulated by a variety of rhizobium species (Becking, 1983; Becking, 1992; Trinick and Galbraith 1980). However, at that time the Nod factor structure was not yet resolved and rhizobium phylogeny was still based on cross-inoculation groups. Since Parasponia has evolved the rhizobium symbiosis only recently, we hypothesize that at least for the Nod factor recognition less host specificity has evolved compared to legumes. To test this we identified a diverse range of rhizobium species that nodulate *P. andersonii*.

Here we show that *P. andersonii* can form nodules with 4 rhizobia strains from 4 different genera. Based on the genome sequences of these 4 strains we determined the core set of Nod factor biosynthesis genes essential to nodulate *P. andersonii*. We conclude that *P. andersonii* is highly promiscuous, but whether a host and its microsymbiont have a successful interaction resulting in effective nitrogen fixation, goes beyond Nod factor recognition (Downie, 2009; Masson-Boivin et al., 2009). We found that the nitrogen fixation rate varied greatly among the 4 strains tested. The least efficient nitrogen fixing rhizobium strain also resulted in an aberrant nodule structure; host cells died, while the rhizobia persisted in these dead cells. This suggests that the *Parasponia*-rhizobium symbiosis is a delicate balance between mutual benefits and parasitic colonization.

Results

Parasponia is highly promiscuous for rhizobial endosymbionts

Previous studies have shown that *Parasponia* species can be nodulated by *Sinorhizobium* sp. strain NGR234, though with a low nodulation efficiency (Op den Camp et al., 2011; Trinick 1980; Trinick and Galbraith 1980; Webster et al. 1995). We aimed to find a more efficient rhizobium for future genetic studies on *Parasponia*. In the past various rhizobium strains have been isolated from *P. andersonii* nodules during field expeditions in Papua New Guinea (Trinick, 1980b). The two most efficient nodulating strains, CP279 and CP283, belong to the *Bradyrhizobium* genus (Trinick, 1980b; Trinick and Hadobas 1988; Webster et al. 1995). We obtained stocks of these two strains, but unfortunately these could not be revived. In stead, two other rhizobium species were tested for nodulation of *P. andersonii* plantlets; *Bradyrhizobium elkanii* strain WUR3 isolated from *Chamaecrista fasiculata* nodules and *Rhizobium sullae* strain IS123T isolated from *Hedysarum coronarium* (Squartini et al., 2002). Both species were found to nodulate *P. andersonii* plants highly efficient (n=10/10). This 100% nodulation efficiency is much higher when compared to *Sinorhizobium* sp. NGR234, which nodulates only ~40% of the inoculated plants (Webster et al. 1995; Op den Camp et al., 2011).

plant species	bacterial strain	fraction nodulated plants	number of nodules per plant	ARA (µmol C_2H_4/h/g fw)
Parasponia andersonii	*Rhizobium tropici* WUR1	12 / 14	5.7 ± 4.6	0.29 ± 0.17
	Sinorhizobium sp. NGR234	2 / 9	5.4 ± 4.3	0.52 ± 0.30
	Mesorhizobium plurifarium WUR2	2 / 15	10.7 ± 1.2	0.84 ± 0.75
	Bradyrhizobium elkanii WUR3	14 / 14	9.8 ± 6.2	2.88 ± 1.39 *
Vigna unguiculata	*Rhizobium tropici* WUR1	15 / 15	21.3 ± 5.3	0.91 ± 0.51
	Sinorhizobium sp. NGR234	15 / 15	27.7 ± 10.0	1.29 ± 0.66
Lotus japonicus	*Rhizobium tropici* WUR1	10 / 15	1.6 ± 1.7	ND
	Sinorhizobium sp. NGR234	13 / 15	6.0 ± 4.1	0.01 ± 0.02
Arachis hypogaea	*Rhizobium tropici* WUR1	0 / 15	ND	ND
	Sinorhizobium sp. NGR234	10 / 12	18.0 ± 13.2	ND

Table 1. Symbiotic efficiency of rhizobia with the hosts: *P. andersonii*, cowpea, lotus and groundnut. Data from four weeks after inoculation for each strain. Nitrogen fixation was measured with the acetylene reduction assay (ARA), with output in µmol C2H4/hour/gram nodule fresh weight. Errors represent ± SD (for the sample size see fraction of nodulated plants). ND = Not Detected. Asterisk marks statistical significant difference between B. elkanii WUR3 and the other 3 strains (ANOVA, P = <0.001).

Interestingly, *P. andersonii* plantlets grown for propagation under greenhouse conditions in commercial soil of mixed European origin were also frequently nodulated. From these nodules several rhizobium isolates could be cultured that were genotyped using 16S rRNA gene sequencing. With these sequences a 16S rRNA phylogenetic tree was constructed (Fig. 1). Based on this study we identified two additional species as Wageningen University Rhizobium (WUR) strains; *Rhizobium tropici* strain WUR1 and *Mesorhizobium plurifarium* strain WUR2. Taken together, these results show that *P. andersonii* can be nodulated by a diverse range of rhizobium species and therefore we conclude that *Parasponia* is highly promiscuous for rhizobial endosymbionts. Four strains from the genera *Rhizobium*, *Mesorhizobium*, *Sinorhizobium* and *Bradyrhizobium* were selected for further studies; namely *R. tropici* WUR1, *M. plurifarium* WUR2, *Sinorhizobium* sp. NGR234 and *B. elkanii* WUR3, respectively.

Nod factor synthesis genes of Parasponia compatible rhizobia are highly diverse

The rhizobia that can nodulate *P. andersonii* comprise a broad spectrum within the rhizobial phylogeny. In case of legumes the prokaryotic host range is frequently determined by

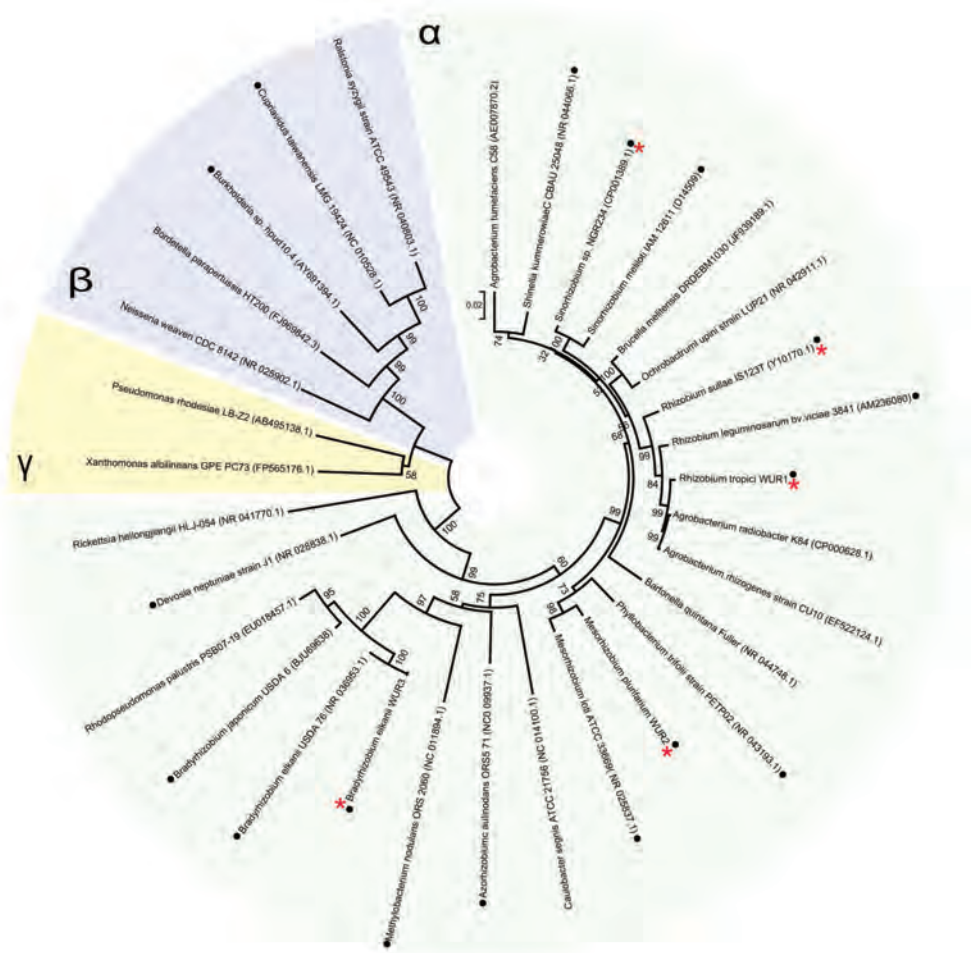

Figure 1. Unrooted phylogenetic tree of 16S rRNA gene sequences from selected α-, β- and γ-proteobacteria. Reconstructed with the neighbor-joining method implemented in the software package MEGA5. Branch support is obtained from 1000 bootstrap repetitions. Genera marked with a black dot contain rhizobia. Red asterisk mark the strains confirmed to nodulate *P. andersonii*.

the structure of the rhizobium secreted Nod factors (Masson-Boivin et al., 2009). We questioned whether the spectrum of Nod factor biosynthesis genes differs between the selected *P. andersonii* compatible rhizobium species. Therefore we sequenced the genomes of *R. tropici* WUR1, *M. plurifarium* WUR2 and *B. elkanii* WUR3 and compared *nod*, *nol* and *noe* genes involved in Nod factor biosynthesis with the *Sinorhizobium* sp. NGR234 orthologs (Table 2). This broad host strain NGR234 is known to produce a large mixture of decorated Nod factors and therefore we anticipate that most *nod*, *nol* and *noe* genes involved in Nod factor biosynthesis are present in this species (Perret et al., 2000; Schmeisser et al., 2009). In total we investigated 19 genes, 17 of which are present in *Sinorhizobium* sp. NGR234 plus two additional genes found in other species; namely *nodX* and *noeC* (Table 2). Of these, 9 are in common in all 4 investigated species. These include the *nodABC* operon encoding enzymes essential for the biosynthesis of the lipochitooligosaccharide core as well as the transcriptional regulator *NodD*. *B. elkanii* WUR3 does not have genes encoding for *NodE* and *NodF*. This is in line with other *Bradyrhizobium* species, e.g. *B. japonicum* USDA110

Gene	Function	*Sinorhizobium sp.* NGR234	*R. tropici* WUR1	*M. plurifarium* WUR2	*B. elkanii* WUR3
NodA	N-Acyltransferase	X	X	X	X
NodB	Deacetylase	X	X	X	X
NodC	N-acetyl-glucosaminyltransferase	X	X	X	X
NodD	Transcription factor	X	X	X	X
NodE	B-acetoacetylsynthase	X	X	X	
NodF	Acyl carrier protein	X	X	X	
NodH	Sulfotransferase	X	X	X	
NodL	Acetyl transferase	X	X	X	X
NodP	Sulfate adenylyltransferase	X	X	X	X
NodQ	Sulfate adenylyltransferase/adenylylsulfate kinase	X	X	X	X
NodS	Methyltransferase	X	X	X	X
NodU	Carbamoyl transferase	X	X	X	X
NodX	Acyltransferase				X
NodZ	Fucosyltransferase	X			X
NolL	Acyltransferase	X			X
NolO	Carbamoyltransferase	X			X
NoeC	UbiA prenyltransferase				
NoeE	Sulfotransferase	X			X
NoeI	Methyltransferase	X			X

Table 2. Nod factor synthesis genes per strain. Colors: Purple, basic lipochitooligosaccharide backbone synthesis. Light blue: transcriptional regulation. Other colors: functional groups of Nod factor decoration genes.

that also lack *NodE* and *NodF* homologs (Kaneko et al., 2002). Furthermore, *B. elkanii* WUR3 lacks NodH, a protein that transfers the NodPQ synthesized 3'-phosphoadenosine-5'-phosphosulfate to the reducing glucosamine residue of the Nod factor (Perret et al., 2000; Schwedock et al., 1990; Schwedock et al., 1994). But *B. elkanii* WUR3 has both *nodPQ* as well as *noeE*, which is also able to transfer the 3'-phosphoadenosine-5'-phosphosulfate to a fucosylated reducing glucosamine residue (Perret et al., 2000). In line with this, *B. elkanii* WUR3 harbors *NodZ* that fucosylates this residue (Perret et al., 2000). As *R. tropici* WUR1 and *M. plurifarium* WUR2 both have *nodH*, *nodP* and *nodQ*, it suggests that all three strains can produce sulphated Nod factors. *B. elkanii* WUR3 has, like *Sinorhizobium* sp. NGR234, two more fucosyl dependent genes that can further decorate a fucosylated reducing glucosamine residue; namely *NoeI* and *NolL* (Downie, 2009). Alternatively, the reducing glucosamine residue can also be acetylated as this strain also harbors the *nodX* gene (Downie, 2009). In summary, these data suggest that *B. elkanii* WUR3 has the capacity to produce the most diverse and most baroque decorated Nod factors, whereas *R. tropici* WUR1 and *M. plurifarium* WUR2 lack many of the genes essential to decorate Nod factors. Since *P. andersonii* can engage a root nodule symbiosis with all four tested strains, we postulate that *P. andersonii* has a low specificity for Nod factor structure.

Parasponia nodules infected with different rhizobia vary in symbiotic efficiency

Nod factor recognition and subsequent nodule formation does not always lead to an effective nitrogen fixing symbiosis. It is known that some rhizobia can colonize nodules, but lack sufficient nitrogenase activity resulting in poor fixation (Den Herder and Parniske, 2009). To identify the most efficient strain for *P. andersonii* we conducted a comparative study between the 4 selected rhizobia. Their symbiotic efficiency was studied by comparing nodule number and rate of nitrogen fixation. We inoculated *P. andersonii* plantlets either with *R. tropici* WUR1, *M. plurifarium* WUR2, *Sinorhizobium* sp. NGR234 or *B. elkanii* WUR3. Four weeks after inoculation the number of nodulated plants, nodule number per plant and nitrogen fixation rate were determined (Table 1). The latter was done by using the acetylene reduction assay (Bergersen, 1970). *B. elkanii* WUR3 was found to have the highest nitrogen fixation rate compared to the other three species. This difference was found to be statistically significant (ANOVA test ($P<0.001$)). However, nodulation efficiency varied largely between

the various rhizobium species (Table 1). *Sinorhizobium* sp. NGR234 only nodulated 2 out of 9 plantlets, confirming earlier nodulation studies with this strain (Webster et al., 1995; Op den Camp et al., 2011). A comparable efficiency was found for *M. plurifarium* WUR2, whereas both other strains nodulated with almost 100% efficiency (Table 1). The number of nodules varied among the strains tested, but was found not to be statistically significant (ANOVA test P=0.102) (Table 1). Next, we questioned whether *P. andersonii* profits from the higher nitrogen fixation rate of *B. elkanii* WUR3. Therefore we compared the shoot weight of *B. elkanii* WUR3 inoculated plantlets with the shoots weight plantlets inoculated with

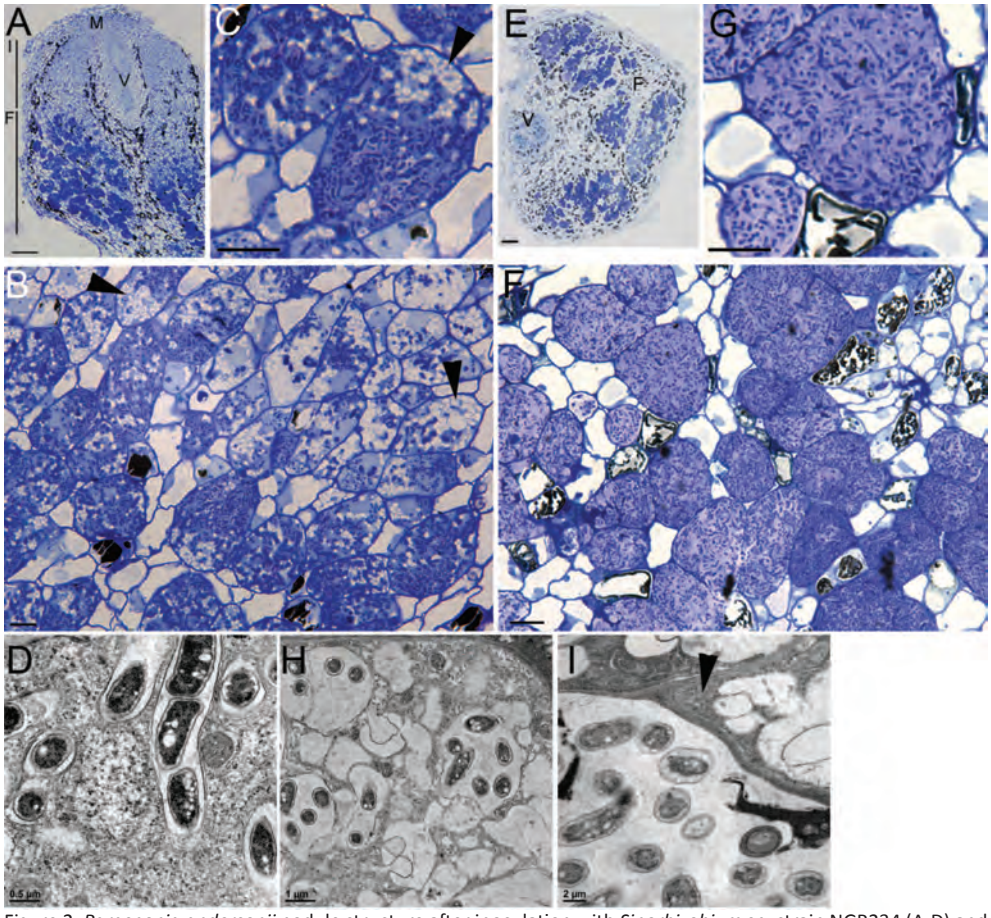

Figure 2. *Parasponia andersonii* nodule structure after inoculation with *Sinorhizobium* sp. strain NGR234 (A-D) and *Rhizobium tropici* strain WUR1 (E-I). ~1 µm thin resin embedded sections of a *Sinorhizobium* sp. NGR234 nodule (A-C). A, single lobe; central vascular bundle (V), nodule meristem (M), infection zone (I) and fixation zone (F). B, cells on the border of infection and fixation zone, examples of shattered vacuoles are marked with arrowheads. C, detail of panel B, cell almost completely filled with fixation threads, shattered vacuole is marked with arrowhead. D, Transmission Electron Microscope (TEM) image of fixation threads filled with rhizobia. ~1µm thin resin embedded sections of a *R. tropici* WUR1 nodule (E-F). E, cross section through the vascular bundle (V) and fixation zone (F) of a nodule. F, cells of fixation zone, with rhizobia in light purple stained dense matrix inside dead nodule cells. G, detail of panel F, large dead infected cell with rhizobia in light purple stained dense matrix surrounded by non-infected living cells. H, TEM image of fusing and disintegrating fixation threads with >4 fila of rhizobia. I, TEM image of a dead fixation zone cell like shown in panel G, filled with living rhizobia. Surrounding two cells have normal cytoplasm as marked with the arrow head. Bars: A,E 100 µm; B,C 10 µm; F,G 25 µm; D 0.5 µm; H 1 µm; I 2 µm.

the least efficient nitrogen fixing strain, *R. tropici* WUR1. Plants inoculated with the best fixing strain had generated a significant higher shoot weight four weeks post inoculation; *B. elkanii* WUR3 with 0.122 ± 0.055 g versus *R. tropici* WUR1 with 0.040 ± 0.018 g (t-test, P<0.001). Therefore we conclude that *B. elkanii* WUR3 is an efficient symbiotic partner for *P. andersonii*.

Rhizobium tropici WUR1 triggers cell death in Parasponia root nodules

To investigate whether there are cytological differences in effective and ineffective *Parasponia* nodules we studied nodule morphology in more detail using light and scanning electron microscopy. Four week old nodules infected with either *M. plurifarium* WUR2, *Sinorhizobium* sp. NGR234 or *B. elkanii* WUR3 showed a canonical organization, similar as described before (Fig. 2A-D, Fig. S1, Fig. S2) (Trinick and Galbraith, 1976; Webster et al., 1995). In short, nodules resemble modified lateral roots with a central vascular bundle and a peripheral zone of infected cortical cells. From the nodule meristem, cells progress to the infection zone, where rhizobium infection threads penetrate the host cell and persist intracellularly (Fig. 2A). Upon intracellular infection, the vacuole of an infected cortical cell shatters into multiple small vacuoles (Fig. 2B-C). Infection threads undergo a transition to fixation threads, which have a much thinner cell wall, but still contain a plant derived membrane and harbor at first a single phylum of rhizobia (Op den Camp et al., 2011). In older cells, towards to the base of the nodule these fixation threads completely fill the host cell and encompass up to four phyla of rhizobia (Fig. 2D). This zone of filled cells stretches out for over twenty cell layers and is the zone where actual nitrogen fixation is occurring (Fig. 2A).

R. tropici WUR1 infected nodules differed from nodules infected with any of the other three species as the number of cells that contained fixation threads stretch only for 1-3 layers close to the meristem of the nodule. More basal cells are completely filled with bacteria embedded in a dense matrix (Fig. 2E-G). These structures are possibly the result of continuous growth and fusion of infection threads, thereby pushing aside the host cytoplasm (Fig. 2H). Ultimately this would lead to the death of the host cell, whereas the rhizobia persist encapsulated in a matrix inside the dead plant cells (Fig. 2G,I). These fully colonized cells were surrounded by non-infected cells with a normal cytoarchitecture (Fig. 2I). Occasionally, similar dead cell have been observed in functional nodules colonized by *Sinorhizobium* sp. NGR234, *M. plurifarium* WUR2 and *B. elkanii* WUR3. Still, these cells were never as abundant and in such young layers of the fixation zone, as found for *R. tropici* WUR1.

We questioned whether *R. tropici* WUR1 could still fix nitrogen in the encapsulated state. Therefore we transformed *R. tropici* WUR1 with a *nifH* promoter GFP reporter construct (pSm_nifHp::GFP) to monitor transcriptional regulation of nitrogenase. By monitoring GFP fluorescence in *P. andersonii* nodules induced by this tagged strain it was found that *nifH* expression continued in the matrix encapsulated form (Fig. S3). The processing of GFP also implies that these encapsulated rhizobia are still metabolically active.

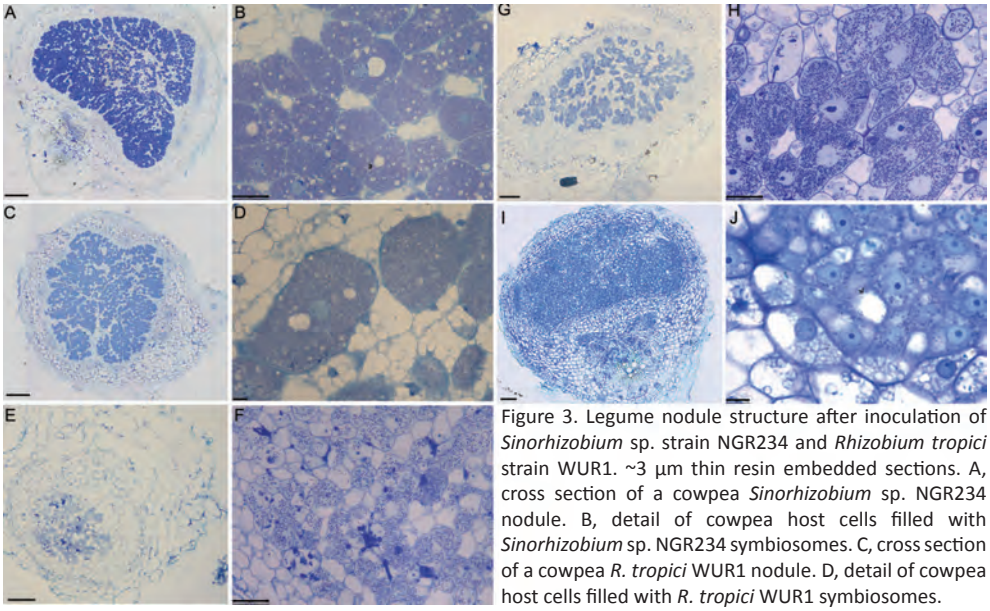

Figure 3. Legume nodule structure after inoculation of *Sinorhizobium* sp. strain NGR234 and *Rhizobium tropici* strain WUR1. ~3 µm thin resin embedded sections. A, cross section of a cowpea *Sinorhizobium* sp. NGR234 nodule. B, detail of cowpea host cells filled with *Sinorhizobium* sp. NGR234 symbiosomes. C, cross section of a cowpea *R. tropici* WUR1 nodule. D, detail of cowpea host cells filled with *R. tropici* WUR1 symbiosomes. E, cross section of a lotus *Sinorhizobium* sp. NGR234 nodule. F, detail of lotus host cells filled with *Sinorhizobium* sp. NGR234 symbiosomes. G, cross section of a lotus *R. tropici* WUR1 nodule. H, detail of lotus host cells filled with *R. tropici* WUR1 symbiosomes. I, cross section of a groundnut *Sinorhizobium* sp. NGR234 nodule. J, detail of groundnut host cells filled with *Sinorhizobium* sp. NGR234 symbiosomes.

Rhizobium tropici WUR1 can establish an efficient symbiosis with cowpea and lotus

The results obtained with *R. tropici* WUR1 on *P. andersonii* made us question whether the parasitic nature of the interaction is an intrinsic character of this strain, or the result of incompatibly with this specific host plant. To discriminate between these two hypotheses we tested the symbiotic capacity of *R. tropici* WUR1 on different legume host plants. To this end we selected *Vigna unguiculata* (cowpea) as it is generally be considered as highly promiscuous, *Arachis hypogaea* (groundnut) as it is infected by crack entry and the model legume *Lotus japonicus* (lotus) (Boogerd and van Rossum, 1997; Lewin et al., 1987; Pajuelo and Stougaard 2005; Perret et al., 2000; Witzany, 2011). As positive control we inoculated these legumes with *Sinorhizobium* sp. NGR234, which is known to nodulate all three species (Pueppke and Broughton 1999).

Sinorhizobium sp. NGR234 nodulated all three legumes, although with different efficiencies (Table 1). *Sinorhizobium* sp. NGR234 infected cowpea nodules displayed a canonical cytoarchitecture and fixed nitrogen most efficient (Table 1, Fig. 3A,B). In contrast nodules on lotus and groundnut were less effective, which is in line with previous reports (Fig. 3E,F,I,J Table 1) (Schumpp et al., 2009; Wong and Patchamuthu, 1988; Pueppke and Broughton, 1999). *R. tropici* WUR1 was found to nodulate both cowpea and lotus, but not groundnut (Table 1). On lotus nodulation was less efficient, though the few nodules formed had a normal cytoarchitecture including well developed symbiosomes in infected cells (Table 1; Fig. 3G,H). Due to the very low nodule number per plant, the nitrogen fixation rate was beyond the detection limit of our experimental setup. In case of cowpea, nodules were found to fix nitrogen at a similar rate compared to *Sinorhizobium* sp. NGR234 infected nodules. Sections

of *R. tropici* WUR1 infected nodules revealed a very similar structure compared to infected *Sinorhizobium* sp. NGR234 nodules (Fig. 3C,D). Taken together, these results show that *R. tropici* WUR1 can establish an effective endosymbiosis with legumes. Therefore we conclude that the aberrant termination of symbiosis as observed after intracellular colonization by *R. tropici* WUR1 in *P. andersonii* is the result of host incompatibility.

Discussion

We showed that *P. andersonii* is highly promiscuous for rhizobia, as it can be nodulated by bacteria from 4 different genera. As rhizobial engagement in the non-leguminous genus *Parasponia* is most likely a far more recent evolutionary event compared to legumes, we argue that high promiscuity is the basal state of rhizobial host plants. A drawback of this high promiscuity is that low efficient strains can infect nodules as well, which is underlined by the finding that *P. andersonii* host strains display a range in nitrogen fixation effectiveness.

The rhizobial strains studied here display diversity in nodulation genes suggesting that *P. andersonii* can recognize Nod factors with a range of decorations. *R. tropici* WUR1 and *M. plurifarium* WUR2 harbor a similar set of nod genes. The presence of the sulfotransferase *nodH* as well as the sulfate adenylyltransferase encoded by *nodPQ* suggests both species produce sulfated Nod factors. Likewise, these genes are present in *Sinorhizobium* sp. NGR234 (Schmeisser et al., 2009). However *nodH* is lacking in *B. elkanii* WUR3 suggesting that Nod factor sulphation is not essential to infect P. andersonii. Alternatively, the lack of *nodH* in *B. elkanii* WUR3 is functionally complemented by *noeE*, which encodes a similar (fucose-specific) sulfotransferase (Perret et al., 2000). Considering that *B. elkanii* WUR3 does not posses *nodEF*, one could speculate that the minimum nod gene set essential to nodulate *Parasponia* consists only of *nodA*, *nodB*, *nodC*, *nodS* and *nodU*. The Nod factor produced by such minimal gene set would be very basic and structurally closely related to Myc Factors (Maillet et al., 2011). Therefore it is conceivable that the line between recognizing Nod factors or Myc factors in *Parasponia* species is very fine. We hypothesize that due to the young age of its rhizobium symbiosis, *Parasponia* Nod factor receptors did not coevolve yet with rhizobia and therefore did not diverge from mycorrhizal recognition to develop specificity for the Nod factor. This hypothesis is in line with our previous finding that a single Nod factor receptor, PaNFP, controls both symbioses in *P. andersonii* (Op den Camp et al., 2011). A consequence of this hypothesis is that the read out of Nod/Myc factor signaling pathway may be determined by the physiological condition of the host plant; e.g. N and/or P-status.

A drawback for *Parasponia* sp. of being very promiscuous can be that it grants access to inefficient rhizobium strains. We showed that the nitrogen fixation rate varies greatly among the different microsymbionts. Legumes have an additional strategy to prevent underperforming microsymbionts, namely premature nodule senescence. In legumes this host plant controlled process actively ends the symbiosis due to fusions of lytic vesicles to symbiosomes (Limpens et al., 2009). As a result the microsymbionts are actively degraded, and ultimately the host cell dies as well (Van de Velde et al., 2006). Senescence normally only occurs when nodules mature, which is also reported for Parasponia (Puppo et al., 2005; Trinick 1979). However, when rhizobia are underperforming either by host incompatibility or by loss-of-function mutations, this mechanism can be triggered prematurely in legumes

(Hirsch and Smith, 1987; Van de Velde et al., 2006). We did not observe premature nodule senescence in case of *P. andersonii* nodules that are colonized by the ineffective *R. tropici* strain WUR1. In contrast, the rhizobia are not lysed by the plant and even remained metabolically active. A similar phenotype was reported previously (Trinick et al., 1989). Taken together, this suggests that *Parasponia* did not evolve a mechanism to control underperforming host strains yet. This implies that *Parasponia* plants may not be entirely in control over the situation inside a nodule. In contrast, for legumes it has been shown that they can impose sanctions against underperforming strains (Kiers et al., 2003). We hypothesize that due to the young age of the *Parasponia* symbiosis, sanctions against underperforming microsymbionts are not yet effective. Therefore, there may be a delicate balance between mutual benefits and parasitic colonization in case of such underperforming rhizobia that colonize *Parasponia*.

Materials and methods

Rhizobium strains

Wageningen University Rhizobium (WUR) strains isolated from nodules on *Parasponia andersonii* trees grown in potting soil of mixed European origin are: *Rhizobium tropici* strain WUR1 and *Mesorhizobium plurifarium* strain WUR2 and from nodules on *Chamaecrista fasiculata*, *Bradyrhizobium elkanii* strain WUR3 was isolated. Further we used *Sinorhizobium* sp. strain NGR234 and Rhizobium sullae strain IS123T (Price et al., 1992; Squartini et al., 2002). *R. tropici* WUR1 was transformed with pHC60 harboring pSm_nifHp::GFP (Op den Camp et al, 2011).

Sequencing

DNA was isolated from rhizobium liquid cultures. Cultures were lysed, washed, chloroform/phenol extracted and the ethanol precipitated DNA was purified using a Qiagen DNeasy Blood & Tissue Kit according to the manufactures protocol (Qiagen, Hilden, Germany). DNA was sequenced with a paired end run on an Illumina Genome Analyzer II (Illumina Inc., San Diego, USA) platform. Quality trimmed reads were assembled using CLC Genomics Workbench software (CLC bio, Aarhus, Denmark). This resulted for *R. tropici* WUR1 in 21 x 106 paired reads with an average length of 223 nt, for *M. plurifarium* WUR2 in 16 x 106 paired reads with an average length of 218 nt and for *B. elkanii* WUR3 in 33 x 106 paired reads with an average length of 244 nt. The approximate total genome sizes based on the sum of all assembled contigs were for *R. tropici* WUR1; 6.6MB (116 contigs, average lenght 58kb), for *M. plurifarium* WUR2; 7.2MB (82 contigs, average lenght 87kb) and for *B. elkanii* WUR3; 8.3MB (184 contigs, average lenght 45kb). Genes were predicted using FGENESB (Softberry, Mount Kisco, USA), BASys (Van Domselaar et al., 2005) and manual annotation. Estimated full lenght 16S rDNA and genes encoding proteins involved in Nod factor synthesis were identified by BLAST searches. Their sequences were submitted to GenBank. The Artemis genome browser was used to view and edit the data (Rutherford et al., 2000). Sequences used from *Sinorhizobium* sp. NGR234 were obtained from the NCBI database under BioProject PRJNA59081.

Plant Materials

Clonally propagated *Parasponia andersonii* WU1 plantlets were used in all nodulation assays (Op den Camp et al. 2011). *Lotus japonicus* gifu seeds were sterilized by soaking them subsequently in sulfuric acid for 3 min followed by 6 washes with water and 7 min in 4% commercial bleach, again followed by 6 washing steps with sterile water. Then seeds were put on agar plates to allow germination. After germination seedlings were grown on Fåhraeus (Fåhraeus, 1957) medium for one week before transfer to the greenhouse. Seeds from *Vigna unguiculata* or *Arachis hypogaea* were used for nodulation assays without pre-treatment.

Nodulation assay

All plants were grown on an autoclaved 1:1 mixture of ø 1-2 mm hydrogranules and fine sand, weekly watered with EKM medium (Becking , 1983). *P. andersonii* plantlets were inoculated directly after transfer from tissue culture whereas *V. unguiculata* and *A. hypogaea* seeds were first germinated and grown for 3 days in the greenhouse before inoculation. L. japonicus seeds were germinated in vitro and 1 week old plants were transferred to the greenhouse and inoculated. All plants were inoculated with 2 ml of liquid culture of a rhizobium strain (OD600 = 0.1). Inoculated plants were grown for 4 weeks in a conditioned greenhouse at 28°C, >85% humidity and 16/8 h artificial light/darkness. Only *L. japonicus* plants were grown for four weeks in a conditioned greenhouse at 20°C, <70% humidity and 16/8 h artificial light/darkness. The four rhizobium strains tested were re-isolated as described below from several independent nodules for all inoculations and identity of the isolates was confirmed by sequencing their 16S rRNA genes.

Isolation of rhizobium strains from nodules

The selected nodules were surface sterilized in 96% EtOH for 20 s followed by 4% sodium hypochlorite for 6 min (reduced to 4 min in case of small nodules). Finally the nodules were washed seven times with sterile distilled water. Each nodule was squashed in 20-100 µl 0.9% NaCl solution, the volume varying in proportion to the nodule size. The suspension was serially diluted and streaked on yeast-mannitol agar plates and these were incubated at 28 °C.

Strain characterization

Cells were lysed by resuspending a loopful of plate-grown isolated colonies in 50 µl of lysis buffer (0.25% SDS, 0.05M NaOH) followed by stirring for 60 s on a vortex and heating at 95 °C for 15 min. The lysate was centrifuged for 15 min and 10 µl of the supernatant were mixed with 90 µl of sterile water. One µl of the lysate was used for PCR amplification of the 16S rRNA gene region using the universal bacterial primers 63F (5'CAGGCCTAACACATGCAAGTC) (Marchesi et al., 1998) and 1389R (5'ACGGGCGGTGTGTACAAG) (Osborn et al., 2000). PCR products were Sanger sequenced and analyzed using the DNASTAR software package (DNASTAR, Madison, USA).

Acetylene reduction assay

Nitrogen fixation was measured by the acetylene reduction assay (Bergersen, 1970). The root system (n=15) was washed free of sand, separated from the shoot and put in a 10 ml vial for *P. andersonii* and *L. japonicus*. For *V. unguiculata* and *A. hypogaea* a 35 ml vial was used instead. The humidity was preserved during the test adding in the vial some wet tissue paper. The vials were sealed with rubber stoppers and ten percent of air was withdrawn from each vial and replaced with acetylene. After 1 h of incubation 0.2 ml of headspace gas was drawn from each sample and injected in a ChromPack gas chromatograph equipped with a Porapak R column (80-100 mesh; 2 m x 2 mm ID) and a flame ionization detector (Varian ChromPack, Bergen Op Zoom, the Netherlands). Nitrogen was used as carrier gas at 20 ml/min. Statistical analysis was performed using SigmaStat software package (Systat Software, San Jose, USA).

Histology and microscopy

Fixation of roots was performed for 24 h at 40°C 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. The completely dehydrated roots were embedded in Technovit 7100 (Heraeus-Kulzer, Wehrheim, Germany) according to the manufacturers protocol. Microtome sections of 3-5 µm were stained with toluidine blue and photographed using a Leica DM5500B microscope equipped with a DFC425C camera (Leica Microsystems B.V., Wetzlar, Germany). Images were digitally processed using Photoshop CS3 (Adobe Systems, San Jose, California). For transmission electron microscopy and for the images in Figures 2, 3FH, S1 and S2, nodules were processed as described by Wang et al. (Wang et al., 2010). For confocal microscopy, fresh hand-sectioned nodule cuttings were stained with FM-64 and imaged with a Zeiss LSM 510 confocal laser scanning microscope (Carl-Zeiss, Germany); excitation 488 (GFP), 543 nm (FM-64); GFP emission was selectively detected by using a 505 ± 530 nm band pass filter, FM-64 emission was detected in another channel using a 560 ± 615 nm band pass.

Phylogenetic analysis

The phylogenetic trees were reconstructed using the neighbor-joining method implemented in the software MEGA5 (Tamura et al., 2011). Alignment of >1200 bp long 16S rRNA gene sequences was used for tree building. Reference sequences were retrieved from the Ribosomal Database Project (Cole et al., 2009). Default settings were used and branch support was obtained from 1000 bootstrap repetitions.

Acknowlegdements

This work was supported by the Dutch Science Foundation (NWO) VIDI 864.06.007 to R.G. and the Russian Federation Basic Research grant for Centre of Excellence 047.018.001 to E.F. We thank prof. E. C. Cocking for sending the CP strains.

Supplemental Figures

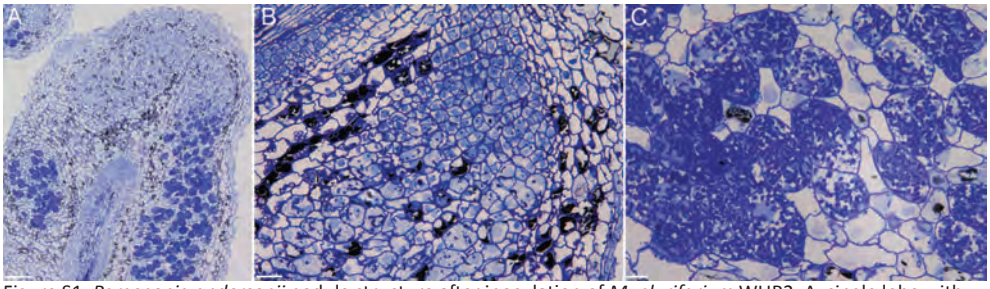

Figure S1. *Parasponia andersonii* nodule structure after inoculation of *M. plurifarium* WUR2. A, single lobe with central vascular bundle and infected cells. B, detail of host cells in the infection zone. C, detail of completely infected host cells, filled with fixation threads. Bars: A 100 µm; B 25 µm, C 10 µm.

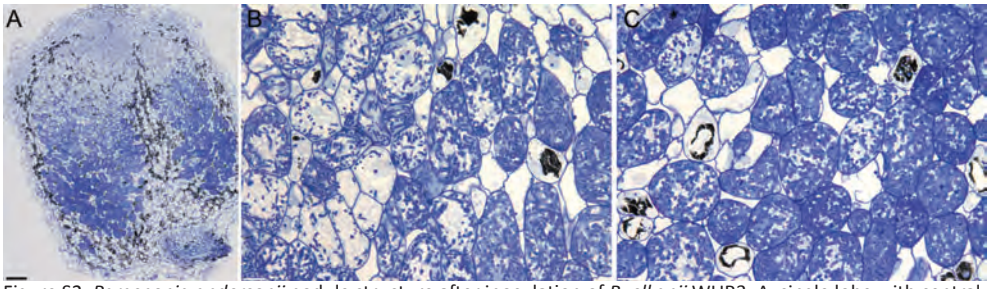

Figure S2. *Parasponia andersonii* nodule structure after inoculation of *B. elkanii* WUR3. A, single lobe with central vascular bundle and infected cells. B, detail of host cells in the infection zone. C, detail of completely infected host cells, filled with fixation threads. Bars: A 100 µm; B,C 10 µm.

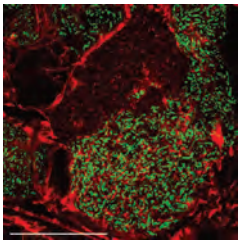

Figure S3. Confocal image of *Parasponia andersonii* FM-64 stained *R. tropici* WUR1 nodule infected cells. *R tropici* WUR1 is transformed with pSm_nifHp::GFP. Merged image from the red (FM-64) and the green (GFP) channel. Bar 100µm.

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Chapter 7

General discussion

Rik H.M. Op den Camp

This thesis describes my study on the evolution of rhizobium symbiosis. I started with a mechanistic approach by studying the role of cytokinin in root nodule formation. Next I implemented a genetic approach; I wanted to determine to what extent the cytokinin signaling pathway has been recruited to function in rhizobium symbiosis. In order to answer this genetic question I used evolutionary phylogenetics. The study of legume phylogeny provided a novel view on the evolution of rhizobium symbiosis. Detailed phylogenetic analysis, combined with the availability of several legume genome sequences allowed tracing of the origin of a whole genome duplication (WGD) at the root of the Papilionoid subfamily (Cannon et al., 2010). This WGD was found not to be essential to evolve rhizobium symbiosis, though contributed significantly to the nodule functioning as we know today (Young et al., 2011). I exploited this duplication event to search for paralogous gene pairs that were maintained in three legumes species; *Medicago truncatula*, *Lotus japonicus* and soybean (*Glycine max*). In this way we were able to provide insight to what extent gene duplications were essential to recruit the cytokinin signaling pathway into rhizobium symbiosis and point to novel genes potentially involved in rhizobium symbiosis. When applied more generic this approach may uncover a large set of symbiotic genes, which even could function redundantly. Therefore, genes found by this method include genes that will not be discovered by more conventional mutant screens.

Aiming to answer the initial research question: *what makes legumes unique that they could evolve the rhizobium symbiosis?* I focused on genetic constraints to evolve this symbiosis. I studied the convergently evolved rhizobium symbiosis in the non-legume genus *Parasponia*. I provide first evidence that *Parasponia* has recruited a genetic network for the rhizobium symbiosis similar as identified in legumes. Further, I found that also the initial signaling receptor was adopted from an older root endosymbiosis; namely arbuscular mycorrhiza (AM). In the next paragraphs the similarities between rhizobium and AM symbioses will be worked out.

Similarities between endosymbiosis signaling pathways

The last decade it has become apparent that the rhizobium symbiosis signaling pathway has been co-opted from the far more ancient AM symbiosis. A set of signaling genes has been found to be essential for both symbioses as well as it has been shown that the lipo-chitooligosaccharide (LCOs) signaling molecules secreted by both microsymbionts are structurally very similar (Bonfante and Genre, 2010; Maillet et al., 2011). The final output of both the rhizobium as the mycorrhiza signaling pathways is the establishment of a perimicrobial compartment; the interface between the cytoplasm of the host and the microbe for nutrient exchange (Ivanov et al., 2010). In legumes these membranes of rhizobium and mycorrhiza have similar properties. For example the VAMP721 vSNARE proteins are required for the establishment of such membranes in both symbioses (Ivanov et al, unpublished results). Altogether these data suggest that certain genetic constraints exist that facilitate evolution of rhizobium symbiosis.

In chapter 4 we demonstrate that *Parasponia* has recruited the same signaling pathway to enable rhizobium symbiosis, including a LysM-type receptor kinase that in legumes recognizes rhizobium LCO signal molecules. Interestingly, in the younger and convergently evolved *Parasponia*-rhizobium symbiosis this receptor can recognize both mycorrhizal and

rhizobial LCOs (named Myc factors and Nod factors, respectively). This in contrast to legumes; their Nod factor receptors are highly specific, whereas a receptor for Myc factors has not been characterized yet. This suggests neofunctionalization in legumes of LCO receptors that has occurred after gene duplication events (Radutoiu et al., 2007; Nakagawa et al., 2011) (Chapter 5). Furthermore, I found two additional constraints in rhizobium symbioses. External application of cytokinin can trigger root cortical cell divisions in *Parasponia* and legumes, whereas Nod factors can trigger a calcium spiking responses to Nod factor exposure (Fig. 1)(Granqvist and Op den Camp, unpublished results). This further underlines that genetic constraints largely guided the evolutionary trajectory to a symbiosis with rhizobium.

Evolutionary predispositions

Besides Rhizobium symbioses in legumes and Parasponia, several other species can form N_2-fixing endosymbiosis with filamentous actinomycete bacteria of the genus *Frankia*. Such plants are collectively known as actinorhizae, and form several independent lineages in the

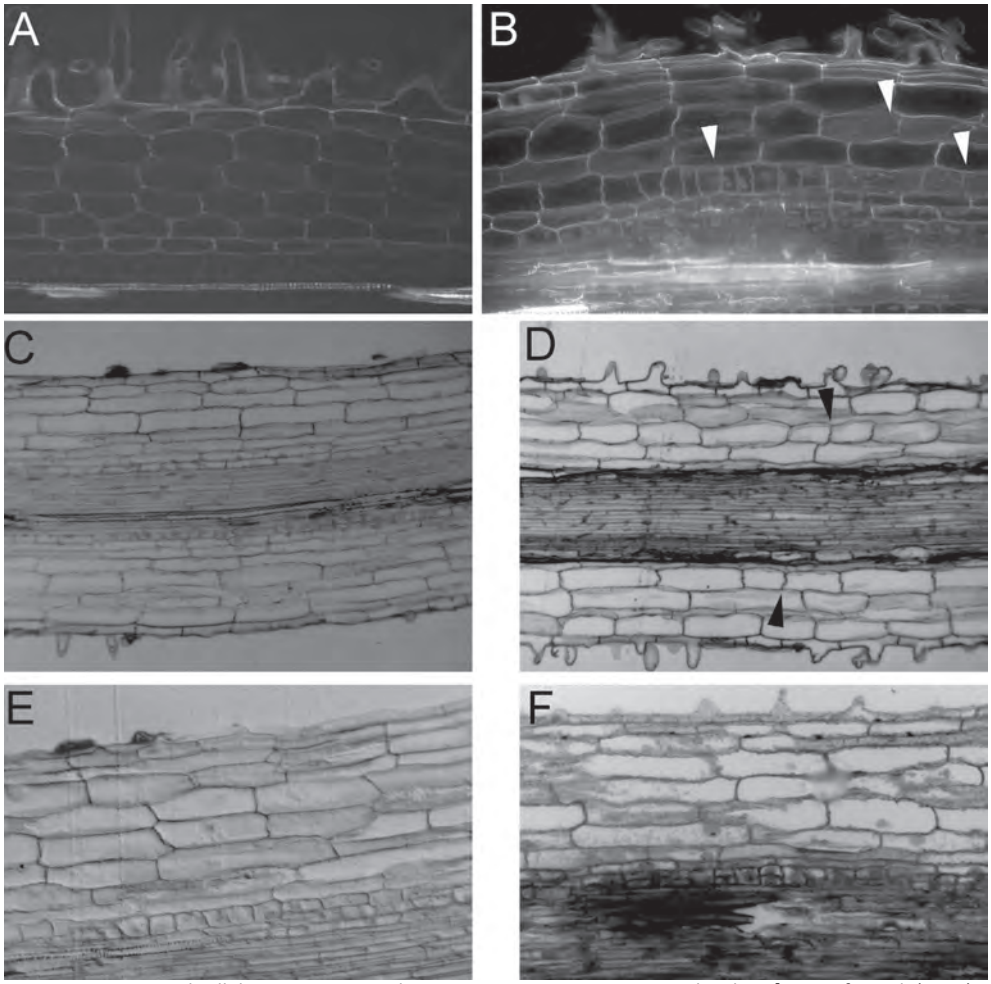

Figure 1. Root cortical cell divisions upon cytokinin exposure. Roots were treated with 10^{-8}M BAP for 48 h (B,D,F) or mock treated with water (A,C,E). A,B *Medicago truncatula*, C,D, *Parasponia andersonii*. E,F *Cercis siliquastrum*.

orders Rosales, Cucurbitalis and Fagales (Fig. 1, introduction). As these orders, together with the Fabales (containing legumes), have a single origin in the Fabidae clade, an evolutionary predisposition is hypothesized to have occurred at the root of this so-called N_2-fixation clade (Soltis et al., 1995). This predisposition would provide these species the potential to evolve root nodules to host symbiotic N_2-fixing endosymbionts. However, not all species in this lineage exploited this potential to evolve such symbiosis.

Hormone signaling networks are complex and cytokinin signaling is intertwined with other plant hormones such as auxin, ethylene and gibberellin (Moubayidin et al., 2009; Ubeda-Tomas and Bennett, 2010). Probably the most studied hormonal cross-talk in plant roots is the antagonistic interaction between auxin and cytokinin, which is a key determinant controlling root meristem activity (Ubeda-Tomas and Bennett, 2010). In legumes, cytokinin is also an integrated part of Nod factor signaling (Gonzalez-Rizzo et al., 2006; Tirichine et al., 2007; Frugier et al., 2008; Murray, 2011; Plet et al., 2011). It is presumed that legumes did not massively evolve new genes, but rather adopted existing signaling modules to be integrated in Nod factor signaling (Godfroy et al., 2006; Heckmann et al., 2006; Liu et al., 2011). Cytokinin is known to trigger root cortical cell divisions in legumes as well as *Parasponia* species (Fig. 1)(Cooper and Long, 1994; Mathesius et al., 2000; Heckmann et al., 2011). I tested whether a similar response can be triggered in the non-nodulating legume *Cercis siliquastrum* as well as tomato (*Solanum lycopersicum*); the latter is not part of the N_2-fixation clade. In neither species root cortical cell divisions could be observed upon application of external cytokinin (Op den Camp, unpublished results; Fig. 1). This indicates that predisposition is not the result of an adaptation in the cytokinin phosphorelay signalling network. Alternatively, *C. siliquastrum* has lost the predisposition. To rule out this latter hypothesis the cytokinin response of additional plant species with the N_2-fixation clade have to be tested.

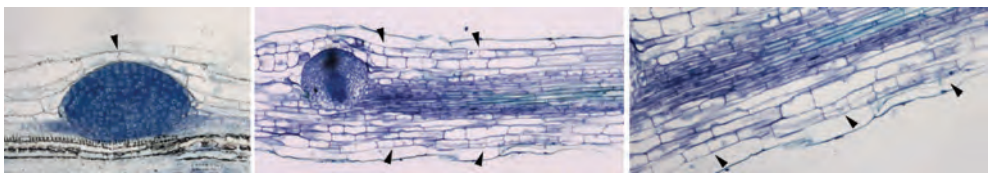

Figure 2. Left panel shows a control transformed tomato root, in which cortical cell divisions may occur in cortical cell layers adjacent to an emerging lateral root primorida (arrowhead). Center and right panel show CaMV35S::MtCRE1^{L267F} transformed tomato roots. Periclinal and anticlinal cortical cell divisions are marked by arrowheads. These divisoins are not only occuring next to emerging lateral roots but along the whole root fragment.

What evolutionary mechanism could have resulted in the recruitment of cytokinin phosphorelay signaling in rhizobium symbiosis? It is presumed that legumes and Parasponia did not massively evolved new genes, but rather adopted ancestral signaling modules to be integrated in Nod factor signaling. Regulatory evolution or changes in coding sequences are driving forces for phenotypic evolution (Tirosh et al., 2009). I hypothesize that slight modifications in the cytokinin receptor sensitivity or in the cis regulatory elements of the corresponding genes account for the specific response to cytokinin in nodulating species. Support for this hypothesis comes from a gain of function mutant in the legume *L. japonicus*. A point mutant in the receiving domain of a cytokinin receptor, likely making is hypersensitive to cytokinin, is sufficient to trigger spontaneous nodule formation in the absence of rhizobium (Tirichine et al., 2007). I cloned this cytokinin receptor from the legume *Medicago truncatula* and introduced the same mutation. *M. truncatula* roots transformed

with this construct spontaneously developed similar empty nodules (Limpens et al., 2011). The same *M. truncatula* construct was able to trigger cell divisions in *Parasponia*, but no nodule organogenesis was triggered (Op den Camp and Cao, unpublished results). Surprisingly, when we transferred this CaMV35S promoter driven construct to tomato we observed similar cortical cell divisions as found in Parasponia (Fig. 2). These data suggest that marginal changes in the hormone homeostasis can initiate cell division in normally quiescent cells.

The methodology described in chapter 3 to quantify minute amounts of cytokinin and auxin in *M. truncatula* roots will further contribute to the understanding of hormone action in root nodule initiation and development. In this chapter it is demonstrated that cytokinin levels accumulate upon Nod factor exposure in the root zone susceptible to symbiotic interaction. We now know which cytokinin derivates can be detected that are involved in the symbiotic interaction. When tracing known components we can further increase the sensitivity of the UPLC-MS\MS equipment and thereby decrease the amount of starting material (Tatsiana Charnikhova, personal communication). This will ease the set up of hormone profile developmental time series and perhaps in the near future allow measuring cell type specific profiles.

Implications of the Papilionoid specific whole genome duplication

In chapter 2 we describe an approach to exploit an ancestral genome duplication in the legume family to search for maintained genes during evolution with a function in rhizobium symbiosis. Such approach has been used before to study the evolution of yeast species (Kellis et al., 2004). One other theoretic example of such approach has been published for *Physcomitrella patens*, in which metabolic genes have been maintained in excess. Although experimental evidence for the importance of these duplicates is lacking, it can be hypothesized that these additional metabolites allowed *Physcomitrella* sp. mosses to grow in extreme conditions (Rensing et al., 2007). Comparing the genome sequences of *M. truncatula*, *L. japonicus* and soybean yielded 261 paralogous gene pairs with the stringency that each of the pairs should be maintained in all these three Papilionoid legume species. Chapter 2 provides a proof-of-principle that at one of these gene pairs encoding a type-A Response Regulators (named *MtRR9* and *MtR11* in *M. truncatula*) indeed evolved functions in rhizobium symbiosis. It will be a future challenge to investigate which fraction of the other 260 gene pairs also has evolved a function in rhizobium symbiosis. An initial closer look to this dataset reveals that among these gene pairs we find some genes already known to be involved in nodulation. For example, MtERN1, which is an Ethylene Response Factor (ERF) transcription factor essential for root nodule symbiosis that controls nodule primordium development as well as rhizobium infection (Middleton et al., 2007).

In additional experiments, I identified a direct or indirect regulatory feedback loop of MtRR9 on MtERN1. Upon ectopic expression of *MtRR9*, *MtERN* is transcriptionally induced (Fig. 3). RNAi mediated knockdown of three A-type RR genes, amongst which is *MtRR9*, leads to downregulation of *MtERN* (Fig. 3). Remarkably, type-A RRs do not have a DNA binding domain and have never been shown to act as transcriptional regulators. A putative model for type-A RR involvement in transcriptional regulation is to function in conjunction with type-B RRs. Type-B RRs do have a DNA binding domain and can regulate downstream

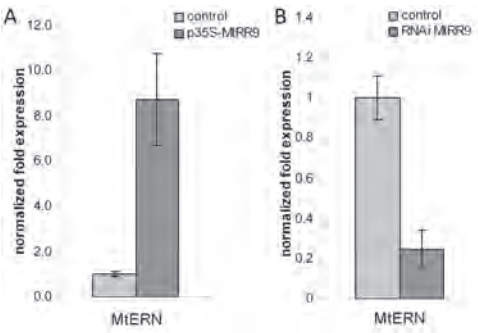

Figure 3. Quantitative RT-PCR of *M. truncatula ERN*. A, pCaMV35S::MtRR9 transformed root RNA versus control transformed root (empty vector). B, pCaMV35S::RNAi-MtRR5,9,11 (MtRR9) transformed root RNA versus control transformed root (empty vector).

gene expression (Argueso et al., 2010). This example provides evidence for the theory that maintaining (parts of a) transcriptional networks after a WGD, may result in the evolution of a semi-independent daughter network (Conant and Wolfe, 2006; Van de Peer et al., 2009).

Two other genes that were found back in the list of 261 maintained gene pairs are the vSNARE proteins *MtVAMP721d/ MtVAMP721e* and the NADPH oxidases *NAPDH-e/ NADPH-f.* Both gene pairs were shown to be involved in rhizobium symbiosis in our laboratory (Ivanov et al., 2010)(Lillo et al., unpublished data). This further underlines the dataset of maintained paralogous gene pairs we provided will be a valuable resource for future research on the evolution of rhizobium symbiosis in the Papilionoid legumes.

Is recognition the key?

The most legumes studied, including the models *M. truncatul*a and *L. japonicus*, recognize only a limited range of Nod factors. In line with this these species have a narrow range of rhizobium they can host. Experimental evidence revealed indeed that the corresponding Nod factor receptors are highly specific (Radutoiu et al., 2007). The extracellular domain of a Nod factor receptor was shown to be involved in rhizobium strain specific recognition (Radutoiu et al., 2007). These highly specific interactions in model plants may have lead to a rather biased hypothesis that Nod factor structure and subsequent recognition may be the key to the rhizobium symbiosis (Perret et al., 2000). Many other legume species are more promiscuous and potentially can recognize a broad range of Nod factors (Perret et al., 2000). In chapter 6 it is demonstrated that *Parasponia* can recognize a broad structural range of Nod factors and is highly promiscuous. Further it is shown in chapter 4 that *Parasponia* has a single receptor, which can recognize both Myc factors as Nod factors. This implies that the ground state of a plant host in the rhizobium symbiosis is a high level of promiscuity (Sprent, 1994). Recently it has been demonstrated that Myc factors and Nod factors are structurally highly related (Maillet et al., 2011). Taken together, I hypothesize that non-legume species that are able to engage an AM symbiosis can recognize both AM as rhizobial LCOs. Perception of a signal molecule, whether this is a Nod factor or a Myc factor, will initiate a downstream signaling pathway in order to establish the symbiosis. The AM and rhizobium pathway may have functionally diverged in legumes. At least it has been shown that certain amino acid substitutions in legume Nod factor receptor kinase domains enables these receptors to trigger specific rhizobium symbiosis responses (Nakagawa et al., 2011). Therefore I conclude that recognition is not the key to the rhizobium symbiosis. The ability to engage an AM symbiosis is more likely to be a constraint to evolve the rhizobium symbiosis. The true key to the rhizobium symbiosis still needs to be unveiled.

How to get to the core of root endosymbioses?

Studies on *Parasponia* may provide some vital answers to the question what are the genetic constraints of rhizobium symbiosis. In *Parasponia* the rhizobium symbiosis evolved only recently. This recent evolutionary path will have left very few changes compared to its closest non-nodulating sister species of the *Trema* genus as one would expect to have occurred in legumes compared to their non-nodulating sister species. We postulate that a comparison of *Parasponia* to *Trema* may yield the minimum genetic constraints to evolve the rhizobium symbiosis. Possible changes that *Parasponia* underwent are for example gene duplications and changes in coding or regulatory sequences. The genome sequencing project of *Parasponia andersonii* WU1 has been initiated and the sequencing of *Trema* species and other *Parasponia* species will begin soon. With these genome sequences at hand, comparative genetics will provide insight in the genetic constraints to evolve rhizobium symbiosis. Subsequent transcriptome analysis of *Parasponia/Trema* roots exposed rhizobia and mycorrhizal fungi will illustrate the overlap of both symbiotic pathways. Altogether, fundament of this approach was worked out as part of this thesis and possibly it will become the keystone of future research on root endosymbioses.

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Summary

Summary

The evolution of rhizobium symbiosis is studied from several points of view in this thesis. The ultimate goal of the combined approaches is to unravel the genetic constraints of the symbiotic interaction. To this end the legume rhizobium symbiosis is studied in model plant species from the *Papilionoideae* subfamily such as *Medicago truncatula* and *Lotus japonicus*. In these model plants the genetic signaling cascade used for rhizobium symbiosis has been largely unraveled. The cascade is triggered by lipo-chitooligosaccharade-based signal molecules excreted by rhizobia, called Nod factors.

In chapter 2 we make use of a whole genome duplication that has occurred at the root of the legume *Papilionoideae* subfamily to identify maintained paralogous gene pairs. We hypothesized that a substantial fraction of gene pairs which are maintained in distinct *Papilionoideae* lineages that split roughly 54 million years ago fulfill legume specific functions, among which is rhizobium symbiosis. Furthermore we argue that such approach could identify novel genes as it can also identify genes pairs that are (partially) redundant in function. With applying this approach specifically to the cytokinin phosphorelay pathway we identified a pair of type-A cytokinin Response Regulators that are involved in rhizobium symbiosis. This study provides a proof-of-principle for this strategy.

It is known for over fifty years that cytokinin plays an important role in the symbiotic interaction between rhizobia and legume hosts. External application of cytokinin can even result in nodule formation. Only, never had cytokinin levels been quantified in legume root extracts upon symbiotic interaction. In chapter 3 we describe a method for extraction of both cytokinins and auxin from *Medicago truncatula* roots. We show that cytokinins accumulate in the root zone susceptible to symbiotic interaction upon Nod factor exposure and that this response is dependent on CCaMK; a key gene of the Nod factor signaling cascade. Furthermore, it was found that ethylene signaling has a negative effect on Nod factor induced cytokinin accumulation. The method set up to measure cytokinin as well as auxin provides a tool to further study hormone interactions in rhizobium symbiosis.

Parasponia, the only non-legume that can engage the rhizobium symbiosis is also subject of study in this thesis. The genetics of the *Parasponia*-rhizobium symbiosis had not been studied before. It was therefore unknown whether this independently evolved rhizobium symbiosis makes use of the same symbiotic signaling cascade as legumes. In chapter 4 we provide first evidence that *Parasponia* indeed makes use of the same signaling cascade as found in legumes. Furthermore, we show that in *Parasponia* a single Nod factor-like receptor is indispensable for two symbiotic interactions; rhizobium and mycorrhiza, respectively. Therefore we conclude that the rhizobium Nod factor perception mechanism is recruited from the widespread endomycorrhizal symbiosis.

Parallel to our studies in *Parasponia* (Chapter 4), the research team of Jean Dénarié of the French National Institute for Agricultural Research (INRA) published the structure of the signal molecule of the arbuscular endomycorrhizae; the Myc factor (Maillet et al. 2011, Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza. *Nature* **469**:58-63). It appeared that Myc factors and Nod factors are structurally very similar. In chapter 5 we discuss these findings and present a more thorough phylogenetic analysis of the NFP-like LysM-type receptor kinases. Together, these results suggest that non-legumes that can engage an arbuscular endomycorrhizae symbiosis can recognize Nod factor-like molecules as well.

The last chapter is about a study on the promiscuity and effectiveness of the Parasponia-rhizobium symbiosis. Parasponia uses a single receptor to control entry of rhizobium as well as arbuscular endomycorrhizal fungi and has evolved the rhizobium symbiosis only recently. This made us to hypothesize that Parasponia Nod factor receptors did not coevolve yet with rhizobia and therefore did not diverge from mycorrhizal recognition to develop specificity for the Nod factor. This implies that Parasponia could be a very promiscuous host for rhizobium species. In chapter 6 we describe that Parasponia andersonii can be nodulated by a broad range of rhizobia belonging to 4 different genera, and therefore it is concluded that Parasponia is highly promiscuous for rhizobial engagement. There is a drawback to this high symbiotic promiscuity. Among the strains identified to nodulate Parasponia, a very inefficient rhizobium species, Rhizobium tropici WUR1, was characterized. As this species is able to make effective nodules on two different legume species it suggests that the ineffectiveness of Parasponia andersonii nodules is the result of the incompatibility between both partners. In Parasponia andersonii nodules rhizobia of the ineffective strain become embedded in a dense matrix, but remain vital. This suggests that sanctions or genetic control against underperforming microsymbionts may not be effective in Parasponia. Therefore we argue that the Parasponia-Rhizobium symbiosis is a delicate balance between mutual benefits and parasitic colonization.

Parasponia has been given little attention in the rhizobium symbiosis field over the past two decades but with our efforts renewed interest has been established. We believe that in the end, the comparison of Parasponia to its closest related non-symbiotic sister species Trema, will result in the determination of the genetic constraints of rhizobium symbiosis.

Samenvatting

Summary in Dutch

Rik H.M. Op den Camp and Dorien Meijerink

De evolutie van de rhizobium symbiose

Inleiding

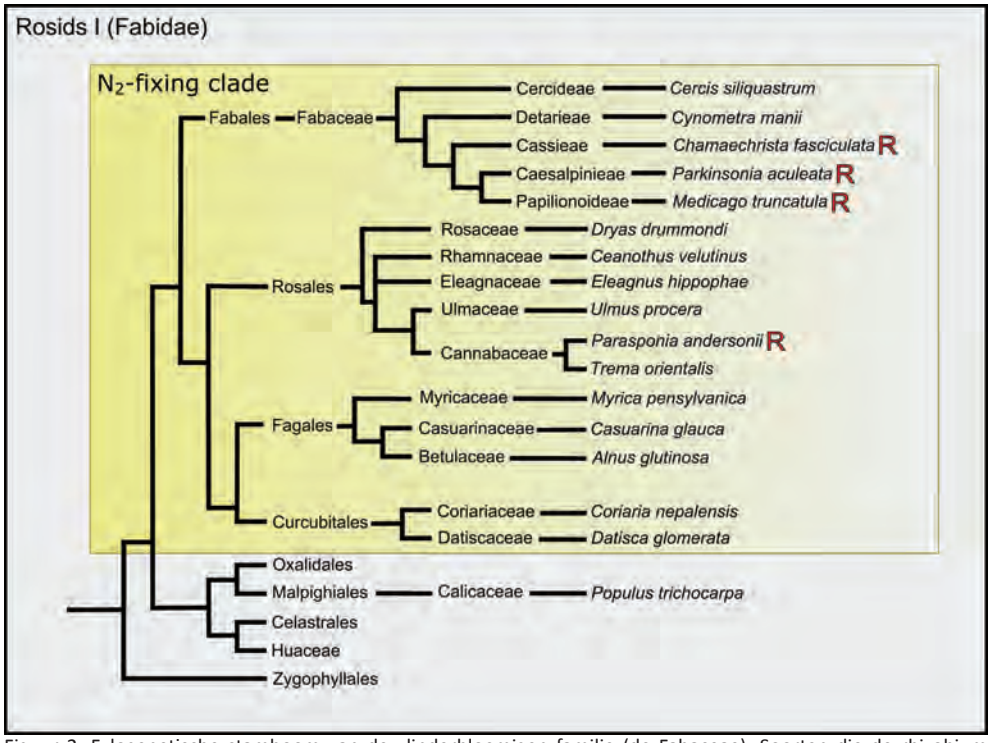
Evolutie is de rode draad in dit proefschift. We kijken op verschillende manieren naar hoe de evolutie heeft kunnen leiden tot een wel heel bijzonderde interactie tussen bacteriën en planten: de rhizobium symbiose. Rhizobium bacteriën zijn in de bodem levende organismen die in staat zijn stikstofgas uit de lucht om te zetten in ammonium, een gebonden vorm van stikstof ('gefixeerd stikstof'). Dit proces wordt stikstof fixatie genoemd en wordt uitgevoerd door het enzym-complex genaamd 'nitrogenase'. Alleen rhizobium bacteriën hebben dit enzym-complex en planten niet. Planten zijn zelf dus niet in staat om op deze manier stikstof te fixeren. Ook heeft een plant het nadeel dat het een immobiel organisme is en dus volledig afhankelijk is van haar directe omgeving voor het opnemen van voedingsstoffen. Planten kunnen normaal dus alleen gebonden vormen stikstof uit de bodem opnemen. De meeste Een groep planten uit de plantenfamilie Fabaceae, ook wel de vlinderbloemigen familie genoemd, is er in geslaagd om hulp te verkrijgen bij het opnemen van stikstof. Ze hebben gedurende de evolutie de eigenschap ontwikkeld om een symbiose aan te kunnen gaan met stikstof fixerende rhizobium bacteriën. Vlinderbloemige planten, zoals de erwt of de sojaboon, laten het toe dat de rhizobia bacteriën hun wortels binnen dringen. Als de rhizobia eenmaal binnen in de wortel zijn aangekomen, begint de plantenwortel daar met het vormen van een nieuw orgaan; de wortelknol (Figuur 1). Uiteindelijk komen de rhizobia terecht binnenin de cellen van de wortelknol en daar voorzien zij de plant van gefixeerd stikstof. Het nitrogenase enzym-complex is zeer gevoelig voor zuurstof. In de wortelknol houdt de plant het zuurstof niveau daarom laag, zodat de stikstof fixatie optimaal kan verlopen. In ruil voor stikstof voorziet de plant de rhizobia van suikers, waarmee de rhizobia goed kunnen groeien en zich verder vermenigvuldigen. Deze samenwerking is uiterst gunstig voor zowel de plant als voor de bacteriën. Vlinderbloemigen kunnen door deze symbiose op voedingsstoffen (stikstof) arme grond groeien en hebben minder kunstmest nodig. Want gebonden stikstof (ammonium) is een belangrijk onderdeel van kunstmest. Helaas zijn maar weinig planten in staat om deze symbiose aan te gaan. In dit proefschrift staan dan ook deze vragen centraal:

Figuur 1. Wortelknollen gevuld met rhizobium bacteriën aan wortels van de Parasponia boom

1. Hoe kan het, dat van het hele plantenrijk, slechts één planten familie - de vlinderbloemigen - deze gunstige rhizobium symbiose heeft verworven?

2. Wat maakt deze vlinderbloemigen familie uniek?

We bekijken deze vragen vanuit twee verschillende evolutionaire invalshoeken. De vlinderbloemigen komen later aan bod want eerst kijken we naar een bijzondere uitzondering. Er is buiten de vlinderbloemigen familie namelijk nog een klein planten geslacht (genus) dat ook deze rhizobium symbiose heeft verworven. Dit is een groep tropische bomen met



Figuur 2. Fylogenetische stamboom van de vlinderbloemigen familie (de Fabaceae). Soorten die de rhizobium symbiose kunnen aangaan worden gemarkeerd door een rode hoofdletter "R". Parasponia behoord tot de Cannabaceae familie. Tussen de Fabaceae en de Cannabaceae vinden we een heel aantal soorten die geen rhizobium symbiose hebben, hier kun je uit afleiden dat ze ieder de rhizobium symbiose apart hebben verworven. Bovendien staan lang niet alle soorten die er tussen liggen vermeld, want dit is een beknopte weergave.

de naam Parasponia. Deze bomen maken deel uit van de Cannabaceae familie en staan evolutionair gezien vrij ver van de vlinderbloemigen familie af. Ze hebben echter wel een gemeenschappelijke voorouder. Dit is duidelijk te zien in de fylogenetische stamboom, er zijn verschillende planten soorten die tussen de vlinderbloemigen en Parasponia instaan die geen rhizobium symbiose kunnen aangaan (Figuur 2). In deze figuur kun je de afstammings lijn van de vlinderbloemigen en Parasponia terugvolgen tot een gezamelijk knooppunt (clade), wat aangeeft dat ze een gemeenschappelijke voorouder hebben. Dat we wortelknollen vinden op wortels van zowel Parasponia als vlinderbloemigen is een voorbeeld van convergente evolutie. Dit betekent dat de twee verschillende soorten onafhankelijk van elkaar dezelfde eigenschap hebben verworven. Een ander bekend voorbeeld van convergente evolutie is de vleugel. Vleugels met een vergelijkbare structuur zijn geëvoleerd in zowel vogels, zoogdieren als insecten (Figuur 3). Terwijl dit soorten zijn die tot verschillende families behoren, hebben ze onafhankelijk van elkaar dezelfde eigenschap – vleugels – ontwikkeld. We kunnen afleiden van de op DNA sequenties

Figuur 3. Voorbeeld van convergente evolutie: de vleugel. vlnr: sprinkhaan, vleermuis en vogel.
Foto's: R. Op den Camp, D. Longhorn en M. Salverda

gebaseerde fylogenetische stamboom, dat Parasponia vergeleken met de vlinderbloemigen nog niet zo lang geleden de rhizobium symbiose heeft verworven. Samen met het feit dat Parasponia onafhankelijk van de vlinderbloemigen familie de rhizobium symbiose heeft verworven, vormt deze plantensoort een erg interessante soort waarmee de rhizobium symbiose verder onderzocht kan worden. Een veelgebruikte techniek om eigenschappen (genen) van planten te bestuderen is het genetisch manipuleren van deze genen. Een gen is een onderdeel van het DNA dat wordt gekopiëerd en vertaald in een aminozuur keten, deze keten vouwt samen tot een eiwit. Door bepaalde genen, en dus de eiwitten waarvoor deze genen coderen, uit te schakelen kun je achterhalen of deze genen belangrijk zijn voor bijvoorbeeld de rhizobium symbiose. De resultaten van ons onderzoek naar Parasponia hebben we in het toonaangevende tijdschrift 'Science' gepubliceerd (hoofdstuk 4 van dit proefschrift). Hieronder volgt een samenvatting van de onderzoeksresultaten.

Het succes van een relatie: een goed gesprek

Rhizobium bacterie stelt zich voor als schimmel.

De sleutel tot een succesvolle samenwerking zit hem in een 'goed gesprek'. Als de rhizobium bacterie er niet in slaagt aan de plant te vertellen dat hij met goede bedoelingen komt, zal de plant er alles aan doen om de bacterie buiten de deur te houden. Wetenschappers denken dat de rhizobium bacterie gedurende de evolutie de kunst om als een goede partij te worden herkend, heeft 'afgekeken' van een schimmel. Dit is echter nog nooit experimenteel bewezen. Deze schimmels heten mycorrhiza. Deze schimmels hebben al sinds 400 miljoen jaar een mutualistische symbiose met bijna alle plantensoorten. Mycorrhiza helpen onder andere binnen in de plantenwortels met het opnemen van water uit de grond. Planten "voelen" wat er om hen heen gebeurt via receptor-eiwitten, die uitsteken vanuit de buitenste cellagen van bijvoorbeeld hun wortels. Receptor-eiwitten kunnen signaalmoleculen herkennen die bijvoorbeeld door rhizobium bacteriën worden afgegeven.

Multitasken door eiwit

Eerder hebben we uitgelegd dat je genen (en dus eiwitten) kunt uitschakelen om de functie van een gen te bestuderen. Het uitschakelen van het receptor-eiwit van de rhizobia symbiose in vlinderbloemigen zorgt ervoor dat de bacteriën niet herkend worden door de plant en dus dat er geen wortelknolletjes worden gemaakt. Echter het uitschakelen van dit eiwit heeft geen invloed op de symbiose met de mycorrhiza schimmels. Dit betekent dat de mycorrhiza schimmels door een ander receptor-eiwit worden herkend. Echter wanneer we hetzelfde receptor-eiwit uitschakelden in Parasponia, kon de plant ineens geen symbiose meer aangaan met zowel de schimmel als met de bacterie. We concludeerden dat Parasponia met één enkel eiwit zowel de schimmel als de bacterie herkent en dat zeer waarschijnlijk in vlinderbloemigen planten gedurende de evolutie één eiwit voor rhizobium bacteriën herkenning en een ander eiwit voor mycorrhiza schimmels herkenning is gaan functioneren.

Weg met kunstmest?

De mycorrhiza schimmel symbiose bestaat vele miljoenen jaren langer en daarom denken de wetenschappers dat gedurende de evolutie de bacterie de schimmel is gaan na-apen door dezelfde moleculen te maken als de schimmel. Daardoor herkent de plant de rhizobium bacterie niet als een vijand maar laat de plant hem binnen en legt hem in de watten als een oude bekende. In vlinderbloemigen is deze herkenning uiteindelijk gescheiden van de schimmel herkenning. Dit kan worden verklaard doordat in een verre voorouder van de plant het schimmel receptor-eiwit is verdubbeld. Dit is een bekend fenomeen voor evolutie biologen. Volgens ons is het verschil tussen vlinderbloemigen en andere plantensoorten kleiner dan gedacht, omdat bijna alle planten een symbiose kunnen hebben met mycorrhiza schimmels. Het is daardoor waarschijnlijk dat de meeste andere plantensoorten de stikstof fixerende bacteriën wel kunnen herkennen als een goede partij. Met deze vinding hebben we een grote stap genomen in het ontrafelen van de eiwitten die betrokken zijn bij de rhizobium symbiose. Wat het verschil is tussen vlinderbloemigen en andere plantensoorten waardoor deze laatsten geen wortelknolletjes kunnen maken, blijft echter wel een onbeantwoorde vraag. Wellicht is het in de toekomst mogelijk om andere plantensoorten genetisch aan te passen, zodat ook zij een symbiose aan kunnen gaan met stikstof fixerende rhizobium bacterien: dat zou een hoop kunstmest schelen!

Kunstmest strooien. Foto: D. Norman

Evolutie van de rhizobium symbiose in de vlinderbloemigen familie

We gaan verder met het bestuderen van de evolutie van de vlinderbloemigen familie, gebruik makend van de drager van erfelijke informatie: DNA. Evolutie kan zich op verschillende manieren afspelen maar heeft bijna altijd te maken met het veranderen van het DNA. Door deze opeenstapeling van DNA-veranderingen kunnnen uiteindelijk nieuwe soorten ontstaan. Het dupliceren van een gen is een belangrijk mechanisme om nieuwe eigenschappen te evolueren. Onder meer door een fout bij het vermenigvuldigen van het DNA tijdens de celdeling die de voortplantings cellen voortbrengt (meiose), kan een gen gedupliceerd worden. Een extreem voorbeeld van gen duplicaties is een duplicatie van het gehele genoom (al het DNA in een cel). Omdat op dat moment alle genen twee keer vertegenwoordigd zijn is het veel waarschijnlijker dat sommige genen een andere functie zullen krijgen, of een bestaande functie zullen verdelen. Deze laatste twee processen worden neo-functionalisatie of sub-functionalisatie genoemd. Men kan achterhalen of een genoom duplicatie ooit heeft plaatsgevonden, door de genomen van verschillende gerelateerde organismen met elkaar te vergelijken. Hiervoor moet eerst wel de hele DNA volgorde (sequentie) bekend zijn. In de afgelopen jaren hebben de technieken om DNA sequenties te bepalen een enorme vlucht genomen. Hierdoor konden wij gebruikmaken van verschillende genoom sequenties van vlinderbloemigen soorten en niet-vlinderbloemigen soorten. Als hypothese stelden we dat er in de gemeenschappelijke voorouder van de vlinderbloemigen subfamilie genaamd Papilioniodeae (zie figuur 2), een genoom duplicatie moest hebben

plaatsgevonden waardoor de evolutie van de rhizobium symbiose mogelijk werd. Door deze genomen met elkaar te vergelijken ontdekte de Amerikaanse wetenschapper Steven Cannon dat er zo'n 50 miljoen jaar geleden inderdaad een genoom duplicatie had plaatsgevonden in de vlinderbloemigen subfamilie Papilioniodeae. Deze subfamilie is zeer groot, ze omvat meer dan 13.000 soorten waaronder belangrijke landbouw gewassen zoals Soja. In deze familie gaan we op zoek naar genen die bewaard zijn gebleven in de evolutie.

Op genen jacht

We gingen van start met het vergelijken van drie vlinderbloemigen genoom sequenties uit de Papilioniodeae subfamilie met drie niet-vlinderbloemigen genoom sequenties. Na een genoom duplicatie gaan de meeste kopieën meteen verloren, het is immers energetisch zeer ongunstig voor de plant om alles twee keer te hebben. Wij stelden de hypothese dat genen die gedurende 50 miljoen jaar aan evolutie behouden zijn, wellicht een belangrijke functie vervullen in de rhizobium symbiose. Daarom gingen we op zoek naar gedupliceerde genen die zijn behouden in alle drie de vlinderbloemige genomen. Door gedetailleerde stambomen te maken van individuele sets van genen van de verschillende plantensoorten konden we deze gedupliceerde genen opsporen. We vonden in totaal 261 paren van genen die gedurende de 50 miljoen jaar aan evolutie behouden waren in de vlinderbloemigen subfamilie Papilioniodeae. Om te testen of deze genen werkelijk een rol spelen in de rhizobium symbiose, namen we er één paar uit en hebben we de functie van deze genen in detail geanalyseerd. De bevindingen van die analyse zijn te lezen in hoofdstuk 2 van dit proefschrift.

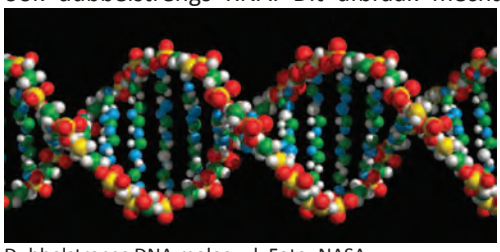
Plantenhormonen regelen groei

Het gedupliceerde genpaar wat onder de loep werd genomen codeert voor eiwitten uit de signaaltransductie-keten van een plantenhormoon genaamd cytokinine (een gen is een onderdeel van het DNA dat wordt gekopiëerd en vertaald in een aminozuur keten, deze keten vouwt samen tot een eiwit). We hebben deze eiwitten MtRR9 en MtRR11 genoemd. Een signaaltransductie-keten wordt geactiveerd in een cel als er een hormoon wordt herkend door zogenaamde receptor-eiwitten. Na herkenning van het hormoon wordt er een keten van signalen geactiveerd in die cel, wat meestal resulteert in het activeren (kopiëren) van verschillende genen. De eiwitten waar deze genen voor coderen zorgen er dan voor dat een bepaald proces in die cel wordt opgestart, zoals celdeling of afweer tegen een ziekteverwekker. Hormonen spelen een sleutelrol bij de groei en ontwikkeling van planten. Zo zijn hormonen betrokken bij het vormen van bloemen of wortels, maar ook bij het vormen van wortelknollen. In gemuteerde planten waar de cytokinine signaaltransductie keten is verstoord kunnen de rhizobium bacteriën geen knolvorming induceren. Om de functie van MtRR9 en MtRR11 te onderzoeken, hebben we een experiment uitgevoerd waarbij we deze genen uitschakelden. De techniek die we daarvoor hebben gebruikt heet RNA interferentie (RNAi).

Uitschakelen van genen en hun eiwitten

Genen op het dubbelstrengs DNA worden gekopiëerd door een enzym-complex genaamd RNA polymerase (transcriptie). Deze losse enkelstrengs keten van gekopieerd DNA heet

een RNA molecuul. RNA kan vervolgens door een ander enzym-complex worden vertaald in een keten van aminozuren (translatie). Deze keten vormt de basis van een nieuw eiwit wat vervolgens een functie in de cel aanneemt. Met de RNAi-techniek maak je in plaats van enkelstrengs een *dubbelstrengs* RNA van het gen dat je wilt uitschakelen in de plant. Dubbelstrengs RNA komt in de plant normaliter niet voor. Het wordt dan ook door de plant als gevaarlijk aangezien en wordt zeer snel afgebroken. Plant-virussen hebben bijvoorbeeld ook dubbelstrengs RNA. Dit afbraak mechanisme is dan ook een onderdeel van het planten immuun systeem. Omdat de plant in het geval van zo'n verdedigingsactie ook het eigen enkelstrengs RNA afbreekt – wat identiek is aan het ingebrachte dubbelstrengs RNA - resulteert dit dus in het uitschakelen van het gekozen gen. Het betreffende eiwit waar het gen voor codeert kan dan niet gemaakt worden.



Dubbelstrengs DNA molecuul. Foto: NASA

Gehavende planten met slechte symbiose

De planten waarin de eiwitten MtRR9 en MtRR11 met RNAi waren uitgeschakeld, waren niet meer in staat de rhizobium symbiose voor 100% te ondersteunen. Ze maakten minder wortelknollen dan planten uit de controle groep. Bovendien ontdekten we dat deze planten ook minder zijwortels maakten dan de controle groep. MtRR9 en MtRR11 zijn dus betrokken bij de vorming van wortelknollen, maar ook bij de vorming van zijwortels. Deze resultaten bevestigen het nut van onze onderzoeksstrategie, die gebruikt maakt van behouden genparen uit de hele genoom duplicatie, om zo nieuwe eigenschappen te vinden die een rol hebben verworven in de evolutie van de rhizobium symbiose. Ook kunnen we uit deze data afleiden dat de genen die betrokken zijn bij de rhizobium symbiose niet alleen zijn geëvolueerd om een rol te krijgen in de symbiose. Deze genen zijn overgenomen uit processen die 50 miljoen jaar geleden al plaatsvonden, zoals bijvoorbeeld zijwortelvorming in het geval van MtRR9 en MtRR11.

Rijst en mais met wortelknollen, toekomstmuziek?

We kunnen concluderen dat vlinderbloemigen wellicht helemaal niet zo uniek zijn als we eerst voor ogen hadden. De genen die betrokken zijn bij wortelknol vorming vinden we ook in niet vlinderbloemigen soorten, zoals Parasponia. Het achterhalen van de complete set genen die betrokken is bij de knolvorming is een toekomstig doel. Het overzetten en het op het juiste moment activeren van deze genen in niet-vlinderbloemigen soorten zal er uiteindelijk toe kunnen leiden dat andere plantensoorten ook wortelknollen met stikstof bindende bacteriën kunnen vormen. Hierdoor kunnen deze planten, zoals rijst of mais, op stikstof arme grond of met veel minder kunstmest groeien. Dit heeft twee belangrijke positieve gevolgen voor de natuur. Wanneer er minder kunstmest nodig is, komen er minder voedingsstoffen in de bodem die schadelijke gevolgen hebben. Bovendien kan er veel energie bespaard worden wanneer er minder kunstmest geproduceerd hoeft te worden. Sommige wetenschappers stellen dat met de huidige voortgang van het onderzoek het overzetten van wortelknol vorming naar belangrijke landbouwgewassen in minder dan 10 jaar zou kunnen worden bereikt.

Acknowlegdements

This thesis could not have been realized without the contribution of and collaboration with many people. I look back on these last four years as a very instructive and cheerful time. I have been allowed to grow and develop myself both personally and professionally. I am most grateful for all the chances and opportunities that were given to me. Therefore I would like to express my deepest feelings of gratitude to my family, friends and colleagues, for supporting, inspiring and motivating me throughout my PhD research. There are a few people that I would like to thank personally:

Promoter prof. dr. Ton Bisseling; for giving me the opportunity to start working on the wonderful topic of rhizobium symbiosis. Your view on the work I produced always elevated it to the next level. Also, you are a very dedicated teacher and I enjoyed the teaching we were involved in together.

Co-promoter dr. René Geurts; without your day to day support and many scientific discussions we had, this thesis would not have reached the quality as it has. You were ever-positive and a great motivator. Although the collective workload appeared to be astronomical in the last year, you managed to guide things successfully through and I am very grateful for this.

Staff of the chair Molecular Biology: Maria, Elena, Henk, Carolien, Pim, Marijke, Jan, Marie-Jose, Olga, Ludmilla, Erik, Jan and Joan. You have introduced me into life at “molbi”, at first in the transitorium. Later, we “survived” together the moving to the Radix, a great achievement of all and I think I can say that after the first years we have now truly settled into our new building. I will remember the good mood, pleasant working atmosphere and the birthday traditions! Thanks for all your personal and scientific support.

My fellow PhD-students: Stefan, Silvester, Gerben, Arend, Wei, Sergey, Alessandra, Chunting, Natalia, Sjef, Aleksandr, Eva, Evgenia, Karin, Yewubdar, Adam, Rik, Vid, Timofey, Juliane, Ting Ting, Maryam, Trupti and Arjan. With you I had a lot of fun in the lab and after work. We traveled together to many places and had a good time anywhere we went. It has been a great time together and I hope to be seeing all of you again, regardless of where our future careers will take us.

Stephane: I look back to two nice years we spent as office neighbors in the transitorium. I have learned a great deal about molecular evolution from you. Your specialist knowledge greatly contributed to this thesis.

Elisa: You came from Italy to the Netherlands to settle with your boyfriend, not a small thing. You brought your microbiology experience to our lab and I enjoyed working together with you. You fitted nicely into the team and I would like to thank you for all your efforts.

Qingqin: You came as a visiting scientist from China to our lab and your first year in the Netherlands you spent without your son. I greatly respected that courage of you. In your work you were very efficient and I learned a great deal from you about growing plants and

tissue culture. But also about Chinese cooking! You greatly contributed to this thesis and I am most grateful to you for that. You returned for a second time to the Netherlands, this time with your son. I hope your scientific career will continue to flourish. I am sure we will meet again and keep in touch, in the Netherlands or in China.

Emma and Giles: It has been a pleasure cooperating with you. I felt very welcome in Norwich and enjoyed my stays at the JIC a lot. I hope that the scientific cooperation between our groups can be continued in the future.

The undergraduate students: Hans, Max, Ruben, Van and Frank. My dutiful helping hands in the lab. I enjoyed teaching you and wish you all the best in your future careers.

Mama, Papa, Laura, Tim, Guus, Evelien, Gerard, Hennie, Annemarie, Simon, Addy, Wouter, Ellen, Frederieke, Opa's en Oma's: Ik wil jullie bedanken voor alle ondersteuning en interesse in mijn werk gedurende de afgelopen jaren. Samen met jullie heb ik me door goede en minder goede tijden weten te slaan. Ook wil ik Frederieke bedanken voor de hulp bij het ontwerpen van de omslag. Wetenschap heb ik niet van een vreemde, als kind stond ik al naast mijn vader in het laboratorium. Zijn ervaring en advies waren zeer belangrijk voor mij tijdens mijn promotie. Onze gedeelde passie heeft uiteindelijk geresulteerd in het eerste Op den Camp – Op den Camp artikel. Weinig mensen kunnen zeggen dat ze een publicatie samen met hun vader hebben en ik ben er heel erg trots op.

Dorien: Samen met jou kan ik alles aan, dus ook een promotie onderzoek en een carriere die ons tijdelijk tot een weekendrelatie dwong. Op jou kon ik altijd steunen, ook als het even tegenzat. Je kon mij altijd zaken van een andere invalshoek laten zien en door jou heb ik altijd positieve energie. En super bedankt voor alle inzichten en schrijfhulp bij de Nederlandse samenvatting van dit proefschrift.

Publications

Op den Camp RHM, Streng A, De Mita S, Cao Q, Polone E, Liu W, Ammiraju JS, Kudrna D, Wing R, Untergasser A, Bisseling T and Geurts R (2011) LysM-type mycorrhizal receptor recruited for rhizobium symbiosis in nonlegume *Parasponia*. *Science* **331**:909-912.

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Curriculum vitae

Rik Hubertus Martinus Op den Camp was born on the 11th of February 1983 in Oostrum, a small town in the province Limburg, The Netherlands. In 1995 he started as a student at the secondary school “Raayland College” in Venray and obtained his athenaeum diploma in 2001. The majors in biology and chemistry have sparked a keen interest in beta science. The scientific career of his father had furthermore set an inspiring example and therefore he continued his studies at Wageningen University. After being enrolled in Molecular life sciences for one year, he decided to switch to Biology for which he received his Bsc-degree in 2006. During his Bsc his favourite topics were developmental and molecular biology, which motivated him to start a research Msc in Cell Biology. He completed his first thesis in the laboratory of molecular biology at Wageningen University on rhizobium symbiosis, supervised by dr. René Geurts. His second thesis was supervised by dr. Geert Smant at the laboratory of Nematology, also at Wageningen University, where he studied plant-nematode interactions. In the last year of his Msc he went abroad for an internship at the Max Planck Institute for Developmental Biology in Tübingen, Germany. In the group of Nobel Prize for physiology or medicin awarded prof. Christiane Nüsslein-Volhard, he studied zebrafish tailfin regeneration. In June 2007 he obtained his Msc degree. In the end, he returned to his first research project on rhizobium symbiosis. Dr. René Geurts had obtained funding for his VIDI project proposal and he offered Rik a job as a PhD student on this project. Four years of work started In the lab of promoter prof. Ton Bisseling and results were presented on several national and international conferences. Within his PhD project he set up various research lines and initiated collaborations with the groups of Plant Physiology and Microbiology of the Wageningen University. Collaboration was set up as well with the group of dr. Giles Oldroyd of the John Innes Centre in Norwich, United Kingdom. He published the work of one of his research projects about the non-legume *Parasponia*-rhizobium symbiosis in the leading journal “Science”. This article crowns the combined effort of all people involved.

Education Statement of the Graduate School

Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: **Rik H.M. Op den Camp**
Date: **17 April 2012**
Group: **Molecuar Biology, Wageningen University & Research Centre**

1) Start-up phase	*date*
► **First presentation of your project**	
Evolution of Nod-factor signaling: a hormonal point of view	Nov 09, 2007
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
Evolutionary Origin of Rhizobium Nod Factor Signaling, a review in Plant Signaling & Behaviour 2011 (6:10, 1510-1514)	Oct 2011
► **Laboratory use of isotopes**	
Course 'Safe Handling with Radioactive Materials and Sources'	Mar 2007
Subtotal Start-up Phase	*9.0 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD student day, Wageningen University	Sep 13, 2007
EPS PhD student day, Leiden University	Feb 26, 2009
EPS PhD retreat, MPI, Cologne (Germany)	Apr 15-17, 2010
EPS PhD student day, Utrecht University	Jun 01, 2010
EPS PhD retreat, Orsay (France)	Jul 05-07, 2011
► **EPS theme symposia**	
EPS theme 1 'Developmental Biology of Plants', Wageningen University	Oct 11, 2007
EPS theme 1 'Developmental Biology of Plants', Leiden University	Jan 30, 2009
EPS theme 1 'Developmental Biology of Plants', Leiden University	Jan 20, 2011
► **NWO Lunteren days and other National Platforms**	
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 07-08, 2008
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 06-07, 2009
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 05-06, 2010
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 04-05, 2011
► **Seminars (series), workshops and symposia**	
Flying seminars (3 per year, 2007 and 2008)	2007-2010
Evolution group I (3 per year)	2007-2010
Mini-symposium 'Plant Breeding in the Genomics Era'	Nov 25, 2011
► **Seminar plus**	
One attended	Apr 14, 2008
► **International symposia and congresses**	
Plant cell elongation: integration of hormonal and environmental signals, Gent Belgium	Dec 09, 2007
IPMB 2009, St Louis, USA: international plant molecular biology congress	Nov 26-30, 2009
Model legume meeting (MLM), St Maxime France	May 16-19, 201
HortiCongress 2011	Nov 04, 2011
► **Presentations**	
Presentation at Leiden University EPS day	Jan 30, 2009
Presentation at the wageningen evolution group	Sep 21, 2009
Presentation at ALW meeting	Apr 06, 2010
Poster at PhD retreat Colone	Apr 15-17, 2010
Poster at legume-rhizobium course	Oct 18-22, 2010
Presentation at JIC	feb 21, 2011
Presentation at ALW meeting	Apr 04, 2011
Poster at MLM	May 16, 2011
Presentation PhD retreat Orsay	Jul 07, 2011
► **IAB interview**	Dec 04, 2009
► **Excursion**	
Subtotal Scientific Exposure	*18.8 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
"Legume-rhizobium symbiosis" course (N2Afrika project)	Oct 18-22, 2010
► **Journal club**	
Participation in a literature discussion group	2007-2011
► **Individual research training**	
Learn to work and analyze data with openarray Biotrove (PRI)	2008
Learn to work and analyze data with microarray (Human Nutrition, WUR)	2009
Learn to work and analyze data with MALDI-TOV (Microbiology, RUN)	2008-2009
Microinjection experiments (John Innes Centre, UK)	Feb 21-23, 2011
Subtotal In-Depth Studies	*7.5 credits**

4) Personal development	*date*
► **Skill training courses**	
Course: Communication in Interdisciplinary Research	May 06, 13, 20 & 27, 2008
DJA on wheels (KNAW project)	Nov-Dec, 2008
PlantingScience project	May 17, 2010
BCF event (bio carreer day)	May 20, 2010
Course: Communication with the media and the general public	Oct 07-08, Nov 02, 2010
Course: Interpersonal Communication for PhD Students	Oct 26-27, 2010
ExPectationS Day	Nov 19, 2010
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*4.8 credits**

TOTAL NUMBER OF CREDIT POINTS*	**40.1**

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: Rik H.M. Op den Camp and Frederieke Meijerink
On the Cover: *Parasponia andersonii* WU1 (front) and *Parasponia andersonii* WU1 nodulated root system (back)
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