Dissecting
HORMONAL PATHWAYS
in **NITROGEN-FIXING**
RHIZOBIUM SYMBIOSES

Arjan van Zeijl

Propositions

1. Gene expression studies are poor predictors of whether a gene commits an important function.
(this thesis)
2. Pleiotropic effects hinder detailed functional analysis of hormonal pathways in plant development.
(this thesis)
3. The need of 473 genes to produce a minimal synthetic *Mycoplasma genitalium* genome, twice that of the common core genes of *Haemophilus influenza* and *M. genitalium*, shows that 'the' minimal genome does not exist (Hutchison III et al., 2016, Science 351:aad6253).
4. Scientific breakthroughs, like CRISPR/Cas9 genome engineering, demonstrate the importance of unbiased fundamental science (Lander, 2016, Cell 164(1):18-28).
5. The strive for longevity of human life is in conflict with efforts to mitigate climate change.
6. The stories written by Indonesian students in the 'typical Dutch' section of 'Resource' demonstrate that, despite a shared history, Dutch culture is difficult to understand.

Propositions belonging to the thesis, entitled:
"Dissecting hormonal pathways in nitrogen-fixing rhizobium symbioses"

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Dissecting hormonal pathways in nitrogen-fixing rhizobium symbioses

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Chapter 1

General Introduction

INTRODUCTION

Nitrogen is a key nutrient for plant growth and development. However, in many ecosystems, availability of fixed forms of nitrogen, such as nitrates or ammonium, is limiting. To overcome nitrogen limitation, some plants evolved the ability to utilize atmospheric nitrogen through a nodular endosymbiosis with nitrogen-fixing soil bacteria [1,2]. In this interaction, the bacteria are intracellularly accommodated in a specialized organ formed on the root of the plant, named the nodule. Inside nodules, the bacteria convert atmospheric nitrogen into ammonium, which they provide to the plant in exchange for photosynthates.

A major research objective of the scientific community is to unravel the evolutionary trajectory towards such nitrogen-fixing endosymbioses. Understanding the genetic basis of such symbioses, provides insight in the processes that lead to the evolution of a major ecological trait. Additionally, it could provide tools to engineer nitrogen-fixation in important crop plants, e.g. rice [3,4].

Nitrogen-fixing root nodule symbioses are restricted to a single clade of flowering plants, known as the nitrogen-fixation clade [5–7]. This clade consists of four orders, namely Fabales, Fagales, Cucurbitales and Rosales. Within the nitrogen-fixation clade, nodulating plant species can be found among ten separate lineages that are scattered among mostly non-nodulating plant species [8]. Among the different nodulating lineages, two types of associations can be distinguished based on the microbial symbiont. Legumes (Fabaceae) and *Parasponia* (Cannabaceae) are nodulated by different genera of gram negative bacteria belonging to the α -, β - and γ -proteobacteria that are collectively called rhizobia [9]. Actinorhizal plants, representing eight lineages in the Fagales, Cucurbitales and Rosales orders, are nodulated by gram positive filamentous actinomycetes of the genus *Frankia* [10]. Based on the phylogenetic distribution of nodulating lineages within the nitrogen-fixing clade, it was hypothesized that nodulation evolved multiple times in parallel [5–7]. Additionally, the fact that nodulation is restricted to a single clade suggested that at least the ancestral species were genetically predisposed to evolve nodulation [5–7]. An alternative explanation for the observed phylogenetic distribution is that nodulation evolved only once in the ancestor of the nitrogen-fixation clade and was subsequently lost in most of its descendants.

Several decades of research on nitrogen-fixing nodule symbioses resulted in two major findings. Firstly, it was found that legumes, *Parasponia* and actinorhizal plants use a conserved set of genes to establish a nitrogen-fixing nodule symbiosis [2]. Secondly, it was

shown that several of these genes also function in a much older and far more widespread endosymbiosis; the symbiosis between plants and arbuscular mycorrhizal fungi [11–18]. These obligate biotrophic fungi colonize roots of most land plants and form highly branched hyphal networks inside existing root cortical cells, known as arbuscules [19,20]. This raises the question of how these existing pathways were rewired to support a nitrogen-fixing nodule symbiosis. Here, I will summarize current knowledge on symbiotic signaling and describe the strategy used in this thesis to provide novel insight in the evolution of nitrogen-fixing nodule symbioses.

Legume Root Nodule Formation and Intracellular Infection

Most insight in the genetic basis of nitrogen-fixing symbioses has been obtained from studies on the interaction between rhizobium bacteria and a limited number of legume species. This showed that the legume-rhizobium interaction is initiated upon rhizospheric signal exchange between plant and microbe. In response to root exudates, containing flavonoids, rhizobium bacteria synthesize and secrete lipo-chitooligosaccharides (LCOs) [21]. These LCOs, also known as Nod factors, consist of four to five β -1,4-linked *N*-acetyl glucosamine residues coupled to a fatty acid chain [22] and are structurally related to LCOs produced by mycorrhizal fungi [23]. Rhizobium LCOs act as symbiotic signals that are perceived by specific receptors at the plant root epidermis [24–27]. LCO perception mitotically activates cells in the root cortex and in some species endodermis and pericycle [28,29]. This results in the formation of a nodule primordium of which some cells are susceptible to infection by rhizobium bacteria. However, to allow intracellular infection to occur, nodule organogenesis and bacterial entry into the root need to be tightly coordinated. Most legumes make use of a root hair-based infection mechanism to allow bacterial entry [30]. This infection mechanism is also driven by perception of rhizobium LCOs and involves a redirection of growth of the root hair cell that perceives the LCO signal [31]. This process, known as root hair curling, entraps the rhizobium bacteria inside an infection pocket. From these infection pockets, cell wall-bound infection threads are formed that guide the rhizobia to the newly formed nodule primordium. Once the nodule primordium is reached, the rhizobium bacteria are released into the host cell as transient nitrogen-fixing organelles. These so-called symbiosomes consist of 1-3 individual bacteria surrounded by a plant-derived membrane [32]. This membrane forms a symbiotic interphase for the transfer of ammonium from the bacteria to the host cell in exchange of photosynthates.

The signaling network involved in the perception of rhizobium LCOs has mainly been identified through genetic studies on the model legumes *Medicago truncatula* (medicago) and *Lotus japonicus* (lotus). Rhizobium LCOs are perceived by a heteromeric complex of

plasma membrane-localized LysM-domain receptor kinases [24–27,33]. Rhizobium LCO signaling requires a genetic network that is largely shared with the mycorrhizal symbiosis. This network, also known as the common symbiosis signaling cascade, consists of an LRR-type receptor kinase (LjSYMRK/MtDMI2), an enzyme involved in mevalonate biosynthesis (MtHMGR1), components of the nuclear pore complex, a nuclear-localized calcium-dependent adenosine triphosphatase (MtMCA8), a nuclear-localized potassium channel (LjCASTOR/MtDMI1, LjPOLLUX) and nuclear-localized calcium channels (MtCNGC15a-c) [11–13,34–36]. A key response activated by this signaling network is the induction of regular oscillations of the nuclear calcium concentration, a response known as calcium spiking [37]. This calcium signal is decoded by a calcium- and calmodulin-dependent protein kinase (CCaMK) [18]. CCaMK interacts with and phosphorylates the transcription factor LjCYCLOPS/MtIPD3 [14,38,39]. Phosphorylation of CYCLOPS by CCaMK activates separate transcriptional cascades for either the mycorrhizal or rhizobium symbiosis. In case of rhizobium symbiosis, a network of closely interacting transcription factors is activated, which includes NSP1, NSP2, NF-YA1, ERN1, ERN2 and NIN [39–48]. The readout of this network is not very well understood but involves massive transcriptional reprogramming [49–51]. Ultimately, the readout of LCO signaling affects the fate of cells in the root cortex, resulting in mitotic activation and subsequent nodule formation [2].

The Role of Plant Hormones in Legume Symbioses

In plant development, lateral organ formation is commonly associated with the formation of a local auxin maximum [52]. Also during nodule formation, a local auxin response maximum is observed in the cells that form the nodule primordium [53–58]. Genetic studies, however, mainly point at an important role for cytokinin signaling in legume nodule formation [59,60]. These studies indicated that a specific cytokinin receptor is not only required but also sufficient to induce nodule organogenesis [59,60]. This receptor, named LjLHK1 in lotus and MtCRE1 in medicago, is orthologous to the AtAHK4/AtCRE1 cytokinin receptor from *Arabidopsis thaliana* (arabidopsis) [61–64]. Genetic analyses positioned this receptor downstream of CCaMK but upstream of NIN in the symbiosis signaling network [59,65]. Gain-of-function mutants of *LjLHK1/MtCRE1* can form nodules in the absence of rhizobium bacteria [59,66]. Similarly, exogenous application of cytokinin induces the formation of nodule-like structures on the roots of several legume plants [67–70]. On the other hand, loss-of-function mutants in *LjLHK1/MtCRE1* develop only limited number of nodules and the formation of these nodules is severely delayed [60,61]. The formation of a few nodules in *Ljlhk1/Mtcre1* mutants is probably due to redundancy, as nodulation is completely abolished in a triple mutant in lotus in which two additional cytokinin receptors have been mutated or after RNAi-mediated silencing of additional cytokinin receptors in the *Mtcre1-1*

1 mutant [62,64]. A role for cytokinin signaling in nodule formation is also supported by the rapid transcriptional activation of several type-A response regulators [61,63,71,72]. These genes are negative regulators of cytokinin signaling and are among the first responding genes following cytokinin perception [73]. Furthermore, exogenous application of cytokinin induces the expression of several symbiotic genes, including the transcription factors *NSP1*, *NSP2*, *ERN1* and *NIN* [61,69]. Analysis of the cytokinin responsive reporter TCS in lotus suggests that cytokinin signaling is first induced in the dividing cortical cells that form the nodule primordium [62]. This is consistent with the expression pattern of *MtRR4* in medicago, which is expressed in inner cortical cells already at six hours after rhizobium inoculation [61,74]. However, in a recent study in medicago an improved version of the TCS reporter was used, named TCSn [75], which revealed an initial activation of cytokinin signaling in the epidermis and outer cortex at four hours post LCO exposure [51]. These authors also performed laser-capture microdissection coupled to RNAseq, which confirmed the activation of cytokinin signaling genes in the epidermis. This analysis also revealed that cytokinin biosynthesis genes are induced in epidermal cells following LCO exposure [51]. Consistently, I show in **Chapter 3** that cytokinin concentrations are increased within three hours of LCO exposure [76].

Despite the important role of cytokinin in nodule organogenesis, the mechanism by which cytokinin controls this process is not well understood. Recent evidence suggests that one of the main functions of cytokinin signaling is the induction of *NIN* expression in the root cortex [74]. *NIN* encodes a transcription factor that by itself is required but also sufficient to induce root nodule formation [40,44,46,74]. *NIN* expression is first induced by CYCLOPS in the epidermis, where it is required for root hair-based infection [39,40,65,74]. Additionally, *NIN* function in the root epidermis appears required for the activation of cytokinin signaling in the root cortex. Inoculation of wild-type medicago plants with rhizobium bacteria induces *MtRR4* expression in inner root cortical cells, a response not observed in the *Mtnin* mutant [74]. Epidermal expression of *NIN* is sufficient to induce the formation of nodule-like structures; however, this requires MtCRE1. In contrast, expression of *NIN* in the root cortex induces nodule-like structures independent of MtCRE1 [74]. This suggests that *NIN* and cytokinin might form a positive feedback loop, in which epidermal expression of *NIN* activates cytokinin signaling in the root cortex. In turn, cytokinin is required to induce cortical expression of *NIN* and subsequent nodule formation.

Cytokinin signaling also seems important for the regulation of auxin transport during nodule formation. Measurements of auxin transport in wild-type medicago showed that acropetal (towards the root tip) auxin transport is reduced just below the inoculation site at 24 hours

post rhizobial inoculation [61,77,78]. In contrast, basipetal (from the root tip upwards) auxin transport was increased just above the inoculation site [78]. Both responses are dependent on cytokinin signaling, as they were absent in the *Mtcre1* mutant [61,78]. A mathematical modeling study tested through which mechanism an auxin maximum could be created during root nodule symbiosis. This showed that a local reduction in auxin transport in the root cortex creates an auxin accumulation pattern that is most comparable to that observed during nodule formation [79]. How this fits with the auxin transport measurements is not completely clear at this point. However, the fact that application of auxin transport inhibitors to the *Mtcre1* mutant rescues its nodulation phenotype, suggests that the regulation of auxin transport is an important function of cytokinin signaling during nodule initiation. Whether cytokinins regulate auxin transport directly, as shown in arabidopsis, or indirectly through for example flavonoids remains to be demonstrated [78,80–84].

In contrast to the positive role of auxin and cytokinin in nodule formation, the gaseous hormone ethylene negatively regulates rhizobial infection and nodule formation. Application of ethylene, or its biosynthetic precursor 1-amino-cyclopropane carboxylic acid (ACC), strongly inhibits nodule formation in several legume species [85–88]. On the other hand, application of the ethylene synthesis inhibitor aminoethoxyvinylglycine (AVG) promotes nodule formation and rhizobial infection [87–90]. Ethylene is perceived by a family of ER-localized receptors. These receptors control activity of the EIN2 protein, a central component of the ethylene signaling pathway [91]. Perception of ethylene by the ethylene receptors results in cleavage and translocation of the C-terminal domain of EIN2 to the nucleus, where it activates downstream gene expression [91–93]. The *Mtein2* mutant in medicago is hyperinfected by rhizobium and forms clusters of often fused nodule primordia [29,86,94]. Such nodule clusters are also observed on lotus plants in which the expression of both *EIN2* orthologous copies is reduced through RNAi-mediated knockdown [95]. *Mtein2* mutant nodules are radially distributed along the root, whereas in wild-type plants nodules often form opposite protoxylem poles [94]. This radial distribution might be regulated by local production of ethylene, as in pea (*Pisum sativum*) ethylene biosynthesis genes were shown to be expressed opposite phloem poles [96]. Ethylene affects nodule formation already at an early step in the LCO signaling cascade, probably upstream or at the point of calcium spiking. In the presence of ethylene, an increased concentration of LCO molecules is needed to induce calcium spiking [88]. Ethylene also affects the duration and frequency of calcium spiking, with a decrease of spiking frequency observed in the *Mtein2* mutant [88]. Like the effect of ethylene, jasmonic acid and abscisic acid (ABA) have also been shown to negatively regulate calcium spiking [97,98]. Expression of a dominant-negative allele of arabidopsis *Atabi1* in medicago roots induces ABA insensitivity and hyper-nodulation [98].

1 The GRAS-type transcriptional regulators NSP1 and NSP2 are essential for rhizobial infection and root nodule formation [41,42,99]. Both proteins are required for the transcriptional activation of several LCO-responsive genes, including *NIN* and *ERN1* [100,101]. NSP1 can directly bind the promoters of these genes *in vitro*, however *in planta* this requires NSP2. NSP1 and NSP2 interact to form heterodimers and this interaction is required for root nodule formation [100]. NSP1 and NSP2 also promote mycorrhizal colonization, though are not essential [1,23,102–105]. Under non-symbiotic conditions, NSP1 and NSP2 are required to produce strigolactones [104]. In medicago *nsp* mutants as well as rice (*Oryza sativa*) lines in which both *NSP1* and *NSP2* are silenced, strigolactone levels are reduced and this correlates with reduced expression of *D27* [104]. Preliminary binding studies suggest that NSP1 can bind to specific elements in the *D27* promoter, thereby regulating its expression [104]. *D27* encodes a carotenoid isomerase that commits the first enzymatic step in strigolactone biosynthesis, which is the conversion of all-*trans*- β -carotene into 9-*cis*- β -carotene [106]. Subsequent activity of the carotenoid cleavage dioxygenases CCD7 and CCD8 results in the formation of carlactone, a strigolactone precursor [106,107]. The conversion of carlactone into strigolactones involves a cytochrome P450 encoded by *MAX1* [108–110]. Strigolactone biosynthesis and *D27* expression is increased under nutrient limitation, especially phosphate starvation [111–113]. Under these conditions, part of the strigolactone pool is exuded into the rhizosphere to attract mycorrhizal fungi [112,114,115]. Strigolactones induce germination of mycorrhizal spores and stimulate hyphal branching, thereby, promoting plant root colonization [116,117]. Several lines of evidence suggest that strigolactones also promote root nodule formation. Exogenous application of a low concentration of the synthetic strigolactone analog GR24 promoted nodule formation in medicago and alfalfa (*Medicago sativa*) [118,119]. Furthermore, strigolactone-deficient mutants in pea (*Pisum sativum*) and lotus produce less nodules, a phenotype that can be rescued by external application of GR24 [120–122]. Additionally, expression of *MtD27* and *MtCCD8* is induced during rhizobium infection in medicago root hairs [49]. In **Chapter 4**, I show that symbiotic expression of *MtD27* is dependent on NSP1-NSP2 and that *MtD27* is expressed throughout nodule formation [123].

DELLA proteins are mostly known for their role as negative regulators of gibberellin (GA) signaling. GA perception by the receptor GID1 targets DELLA proteins for degradation by the 26S proteasome [124]. DELLAs are emerging as transcriptional integrators of various signaling networks, due to their ability to interact with a range of different transcription factors, including those involved in jasmonic acid as well as ethylene signaling [125,126]. DELLAs also play a central role in rhizobium- as well as mycorrhizae-induced signaling [127–131]. This is based on the finding that DELLAs can interact with CCaMK and CYCLOPS. This

interaction stabilizes the CCaMK-CYCLOPS complex and results in increased phosphorylation of CYCLOPS by CCaMK [129,130]. Additionally, DELLAs were shown to interact with NSP2, NF-YA1 and RAD1, a GRAS-type transcription factor required for arbuscule formation [127,130,132,133]. This suggests that DELLAs might form a bridge between CCaMK-CYCLOPS and other symbiotic transcription factors, including the NSP2-NSP1 heterodimer, NF-YA1 and RAD1. In medicago, MtDELLA1 was shown to bind to the promoter of *MtERN1*, which activates *MtERN1* expression [127]. *ERN1* encodes an AP2/ERF transcription factor that is involved in rhizobial infection and nodule formation [45,48]. Besides MtDELLA1, also MtNSP2-MtNSP1 and MtNF-YA1 were shown to bind the *MtERN1* promoter and transactivate *MtERN1* expression [47,100]. This suggests that the interaction between DELLAs, NSP2-NSP1 and NF-YA1 is relevant for the transcriptional regulation of at least *MtERN1* but probably also other symbiotic genes. Consistent with the central position of DELLAs in symbiotic transcriptional networks, medicago mutants in either a single or multiple *DELLA* gene(s) are severely affected in rhizobial infection, nodule formation as well as arbuscule formation by mycorrhizal fungi [127,128,130]. Similarly, application of GA to roots hampers infection thread formation, nodule organogenesis and arbuscule formation [127–130]. This effect can be rescued by expression of a GA-resistant form of *MtDELLA1*, named *della1-Δ18* [128]. In medicago, ectopic expression of *della1-Δ18* is also sufficient to rescue arbuscule formation in the *Mtcyclops* mutant [128]. Consistently, ectopic expression in wild-type medicago induces the expression of multiple genes normally induced by infection with arbuscular mycorrhizal fungi [132,134]. Additionally, epidermal expression of *della1-Δ18* in non-inoculated medicago roots is sufficient to induce expression of *MtERN1* and *MtENOD11*, a marker for rhizobial infection [127]. Several transcriptome studies indicate that the GA biosynthetic pathway is activated 12-48 hours post rhizobial inoculation [49,50,135,136]. This suggests that GA might be produced following successful infection to attenuate LCO-induced signaling. Interestingly, several rhizobium bacteria are also found to produce GA and this reduces the number of nodules formed by the host plant [137]. It is proposed that rhizobium bacteria secrete GA to prevent infection by other bacteria [137].

Symbiotic susceptibility is affected by nutrient concentrations in the soil. In most legume species, nodule formation is suppressed when moderate concentrations of fixed nitrogen are available [138,139]. Experiments using exogenous application of nitrogen revealed that nitrogen affects symbiotic functioning at several levels [138]. This includes a reduction in the number of rhizobial infections, a decrease in nitrogen fixation per unit of nodule mass and a reduction in nodule size, such that in some cases nodule formation is completely blocked [138]. Several studies indicate that exogenous nitrogen also affects the induction of LCO-responsive genes, including *NIN* [140–142]. Mutants in the ‘autoregulation of nodulation’

pathway are unable to control nodule number and as a result are hyper-nodulated. These mutants, however, are also less sensitive to the effect of exogenous nitrate on nodulation [143,144]. Rhizobial inoculation as well as the addition of fixed nitrogen induces the expression of genes encoding small secreted peptides, known as CLE peptides [145–147]. These CLE peptides are transported upwards to the shoot, where they are perceived by a CLAVATA1-like receptor, named LjHAR1 in lotus and MtSUNN in medicago [148–150]. This results in the production of a signal that is transported to the roots to suppress root nodule formation. Analysis of lotus mutants affected in CLE-HAR1 signaling, suggested that this downwards signal could be cytokinin [151]. Ethylene has also been hypothesized to be involved in the negative regulation of nodule formation by fixed nitrogen. Increased rates of ethylene evolution have been measured after nitrate treatment of alfalfa as well as soybean (*Glycine max*) roots [152,153]. Additionally, application of the ethylene synthesis inhibitor AVG rescued nodule formation in nitrate-treated alfalfa roots [154]. In case of mycorrhization, mainly the concentration of inorganic phosphate is regulating the degree of colonization [155]. Interestingly, in arabidopsis, phosphate starvation reduces the concentration of GA in the root, resulting in DELLA stabilization [156]. This suggests that mycorrhizal colonization in response to soil phosphate concentration might be regulated, in part, through an effect on the GA-DELLA balance [128].

Evolutionary Trajectory Towards a Nitrogen-Fixing Endosymbiosis

In legumes, *Parasponia* and actinorhizas activation of the common symbiosis signaling cascade mitotically activates cells leading to root nodule formation [2]. This is different from the mycorrhizal symbiosis where arbuscules are formed in existing cortical cells. This implies that symbiotic signaling or its readout has been adapted in legumes, *Parasponia* and actinorhizas to support root nodule formation.

A role for the common symbiotic signaling cascade in *Parasponia* and actinorhizal nodule formation is based on reverse genetic studies. These studies showed that *PanNFP1*, *CgSYMRK/DgSYMRK*, *CgCCaMK* and *CgNIN* are essential for nodule formation in *Parasponia andersonii*, *Casuarina glauca* and *Datisca glomerata* [157–161]. Additionally, measurements of the nuclear calcium concentration showed that calcium spiking, a hallmark of symbiotic signaling, is induced in roots of *P. andersonii* and the actinorhizal plants *Alnus glutinosa* and *C. glauca* in response to rhizobial or *Frankia* symbiotic signals [162,163].

In *Parasponia* and most legume species, the common symbiosis signaling network is activated by rhizobium LCOs [160,164]. The *Frankia* symbiotic signals that activate actinorhizal nodule formation are currently unknown. Analysis of the genomes of two basal *Frankia* strains

revealed that these strains encode LCO biosynthesis genes [165,166]. These genes are expressed inside nodules of *D. glomerata*, suggesting a role for *Frankia* LCOs in symbiosis with their actinorhizal hosts [165,166]. However, in most *Frankia* genomes LCO biosynthesis genes cannot be found [167]. Instead, characterization of the signaling molecules produced by *Frankia* sp. strain CcI3 - a symbiont of *C. glauca* - suggests that these are biochemically distinct from LCOs [163]. LCO-independent nodulation mechanisms have also been described for several legume species belonging to the genus *Aeschynomene* as well as an LCO receptor mutant in soybean [168–170]. In the latter case, nodulation is dependent on the injection of rhizobial effectors via the type III secretion system, whereas LCO-independent nodulation of *Aeschynomene* legumes does not require effector delivery [168,171]. Despite differences in microbial signals, these LCO-independent nodulation mechanisms also involve activation of the common symbiosis signaling pathway. This because loss of the most upstream component, the LRR-type receptor SYMRK, abolishes nodule formation on *Aeschynomene evenia* as well as *C. glauca* [158,172].

To evolve susceptibility to rhizobial or *Frankia* signals, nodulating lineages might have obtained specific receptors. In medicago and lotus, both the LCO receptors *MtLYK3/LjNFR1* and *MtNFP/LjNFR5* resulted from gene duplications [160,173,174]. In case of *MtLYK3/LjNFR1*, evidence supports that this duplication predated the evolution of rhizobium symbiosis in the legume family [174], suggesting that these receptors specifically evolved to function in rhizobium symbiosis. In *Parasponia*, two close homologs of *MtNFP/LjNFR5* are present, of which *PanNFP2* appears most closely related [160,175]. Orthologues of *PanNFP2* are absent from the genomes of closely related non-nodulating *Trema* species [175], suggesting that *PanNFP2* specifically evolved to recognize rhizobial LCOs. In line with this hypothesis is that calcium spiking cannot be observed in roots of *Trema tomentosa* after addition of LCOs from the broad host-range strain *Sinorhizobium fredii* NGR234 [162]. However, specific LCO receptors might not be sufficient to confer LCO-responsiveness. Ectopic expression of *MtNFP* and *MtLYK3* under their native medicago promoters in tomato (*Solanum lycopersicum*), tobacco (*Nicotiana tabacum*), poplar (*Populus x canescens*) and strawberry (*Fragaria x ananassa*) proved insufficient to induce symbiotic responses [176]. Therefore, besides specific receptors, nodulating lineages might contain physiological adaptations that confer symbiotic susceptibility. This might include changes in nutritional status or in the hormonal balance, e.g. in ethylene, jasmonate or abscisic acid signaling.

Among nodulating lineages, activation of the common symbiosis signaling cascade appears sufficient to induce the onset of root nodule formation. In lotus or medicago, ectopic expression of wild-type or dominant active alleles of the nod factor receptors or SYMRK,

CCaMK, CYCLOPS, LjLHK1/MtCRE1 or NIN is sufficient to induce nodule-like structures in the absence of rhizobium bacteria [39,46,59,66,74,177–179]. These so-called spontaneous nodules were also observed on roots of the actinorhizal plants *C. glauca* and *Discaria trinervis* as well as on roots of *P. andersonii* after ectopic expression of *CgCCaMK*, *DtCCaMK* and *MtCCaMK*, respectively [160,161]. However, activation of the common symbiosis signaling pathway by itself appears insufficient to explain the nodulation phenotype of legumes, *Parasponia* or actinorhizas. Genes encoding components of this pathway have been found in the genomes of all land plants examined, except for those that have lost the mycorrhizal symbiosis [180,181]. Also for legume symbiotic genes not part of the common symbiosis signaling pathway, putative orthologues have been found in non-nodulating plant species [182,183]. *Trans*-complementation assays of legume symbiotic mutants with orthologues from species outside the nitrogen-fixation clade suggest that these genes do not contain specific adaptations. These studies were mainly done using genes from rice, which showed that *OsSYMRK*, *OsCASTOR*, *OsPOLLUX*, *OsCCaMK*, *OsCYCLOPS*, *OsNSP1* and *OsNSP2* encompass all necessary functions to support root nodule formation [38,159,184–186]. Additionally, transfer of the nodulation trait to non-nodulating species based on expression of legume symbiosis genes proved unsuccessful [176]. Therefore, legumes, *Parasponia* and actinorhizas most likely possess specific adaptations to the readout of LCO signaling that are essential for nodule organogenesis.

A possible adaptation to the readout of LCO signaling is the activation of cytokinin signaling. As discussed above, activation of cytokinin signaling in response to the perception of rhizobial LCOs is essential for root nodule formation in lotus and medicago [60,61]. In contrast, cytokinin signaling appears not to be required for mycorrhization [187]. This suggests that in legumes, the cytokinin signaling pathway was specifically recruited to support root nodule formation. Mutagenesis of *PanHK4*, the orthologue of *MtCRE1/LjLHK1* in *P. andersonii*, showed that this gene is not essential for *Parasponia* nodule organogenesis (Chapter 5). However, it should be noted that redundant functioning of additional cytokinin receptors cannot be excluded. Therefore, a better understanding of cytokinin functioning during nodule formation is required to determine how cytokinin signaling was recruited to support nodule formation in legumes and potentially other nodulating lineages.

A Strategy to Uncover Symbiotic Adaptations

The main objective of this thesis was to identify evolutionary adaptations that underlie the emergence of a nitrogen-fixing root nodule symbiosis. One way to achieve this is to deepen our knowledge on the readout of rhizobium-induced signaling in legumes. Legumes are very powerful in this respect as many experimental tools are already available. To determine

whether candidates that come from such studies might represent lineage-specific adaptations or are also required for nitrogen-fixing symbioses in other lineages, studies on *Parasponia* that represents an ‘evolutionary replicate’ of the symbiosis are essential. This already proved to be successful, with respect to the role of the common symbiosis signaling cascade [2]. Another way to gain insight is to perform comparative studies on closely related species that differ in their ability to form nitrogen-fixing root nodules. Such studies could include genome as well as transcriptome comparisons (see van Velzen et al. [175]; **Chapter 6**). The success of these comparisons highly depends on the genetic distance between species. In this respect, *Parasponia* could provide an ideal model system, as it is closely related to non-nodulating *Trema* (see below). Therefore, I propose to adopt *Parasponia* as an experimental model system, which could be used alongside legumes, to decipher the genetic basis of nitrogen-fixing root nodule symbioses.

The *Parasponia*-*Trema* Experimental Model System

Parasponia represents a phylogenetic clade consisting of five tropical tree species indigenous to the Malay Archipelago [188]. Phylogenetic reconstruction indicates that the *Parasponia* lineage is embedded in the non-nodulating genus *Trema* [175,189]. This close phylogenetic relationship is underlined by recent analysis of the genome sequences of several *Parasponia* and *Trema* species that showed that these species are genetically very similar [175]. In fact, these species are so closely related that in rare cases inter-specific crosses are possible [175]. This suggests that the *Parasponia* lineage, including its symbiotic capacity, only recently evolved from an ancestral *Trema* species [190]. However, a different scenario is suggested by evidence for recent parallel pseudogenization of orthologues of *PanNFP2*, *PanNIN* and *PanRPG* in *Trema* spp. [175]. These genes perform essential symbiotic functions in legumes [24,26,40,191]. Also in the genomes of other Rosales species evidence for pseudogenization of *NFP2*, *NIN* and *RPG* was found [175]. This suggests the possibility that *Trema* and related Rosales species once possessed the capacity to nodulate. Irrespective of which evolutionary scenario is correct, studies on *Parasponia* in comparison to legumes or non-nodulating *Trema* spp. can still provide valuable insights into the genetic architecture of nitrogen-fixing endosymbioses.

Research on the nitrogen-fixing symbiosis of *Parasponia* already resulted in the development of several experimental procedures that aid this research. Efficient protocols for *in vitro* propagation of *P. andersonii*, *Parasponia rigida* and *T. tomentosa* are available [192–194]. Additionally, protocols for seed propagation and germination have been developed [175,195]. Also, reference quality genomes for *P. andersonii* and *Trema orientalis* have been generated as well as several draft genomes for additional *Parasponia* and *Trema* species

1 [175]. These provide a platform for genome as well as transcriptome studies. However, to analyze candidate genes coming from such analyses, reverse genetic tools are essential. To this end, a protocol for transient root transformation of *P. andersonii* and *T. tomentosa* has been developed [193]. This protocol is based on the formation of transgenic roots after transfer of the *root inducing locus (rol)* by *Agrobacterium rhizogenes*. Unfortunately, this affects the root's hormonal balance [196] and is therefore not well suited for the analysis of genes involved in hormone homeostasis. Additionally, this system is based on transient transformation of roots meaning that material cannot be maintained and each root results from an individual transformation event, resulting in considerable variation. To overcome this, I developed a protocol for stable transformation of *Parasponia*. Using this protocol, stable transgenic lines can be obtained in a period of four months. In combination with CRISPR/Cas9 mutagenesis, stable knockout mutants can be generated. This makes *Parasponia* well-suited for reverse genetic analysis (**Chapter 5**).

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Chapter 2

**Lipo-chitooligosaccharides
modulate plant host immunity
to enable endosymbioses**

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ABSTRACT

Symbiotic nitrogen fixing rhizobium bacteria and arbuscular mycorrhizal fungi use lipochitooligosaccharide (LCO) signals to communicate with potential host plants. Upon a compatible match an intimate relation is established during which the microsymbiont is allowed to enter root(-derived) cells. Plants perceive microbial LCO molecules by specific LysM-domain containing receptor-like kinases. These do not only activate a 'common symbiosis signaling pathway' that is shared in both symbioses, but also modulate innate immune responses. Recent studies revealed that symbiotic LCO receptors are closely related to chitin innate immune receptors, and some of these receptors even function in symbiosis as well as immunity. This raises questions about how plants manage to translate structurally very similar microbial signals into different outputs. Here, we describe the current view on chitin and LCO perception in innate immunity and endosymbiosis and question how LCOs might modulate the immune system. Furthermore, we discuss what it takes to become an endosymbiont.

INTRODUCTION

Plants are continuously exposed to microbes that range from beneficial to pathogenic. Plant roots, in particular, encounter massive numbers of microbes, known as the soil-root microbiome [1,2]. Two of the most extensively studied examples of beneficial microbes are arbuscular mycorrhizal (AM) fungi and nitrogen-fixing rhizobium bacteria. These microbes can live in intimate contact with their plant hosts and are intracellularly accommodated to establish an endosymbiotic relation. AM fungi are hosted inside the root inner cortex cells of the vast majority of land plants. Their highly branched hyphae are contained within specialized host membrane compartments to form host-microbe interface structures called arbuscules. The resulting symbiotic interface facilitates exchange of nutrients, such as phosphorus and nitrogen, for photosynthates [3]. Rhizobium bacteria establish an endosymbiosis with leguminous plants, which results in formation of a novel organ, the root nodule. Inside nodule cells, rhizobium bacteria are hosted as transient nitrogen-fixing organelles called symbiosomes. Symbiosomes contain a plant-derived outer membrane that forms a symbiotic interface between plant cytoplasm and bacterium facilitating exchange of nutrients between both partners. At the other end of the spectrum, pathogenic microbes can invade plant roots and extract nutrients at the expense of the plant. Biotrophic pathogens colonize living plant cells, where they form feeding structures, such as haustoria, that function as interfaces between plants and microbes, which are analogous to symbiotic interfaces.

Given the plethora of microbes that roots are exposed to, plants must be able to distinguish between friend and foe. Like pathogens, symbiotic microbes are initially recognized as foreign organisms, which triggers the plant innate immune system [4]. Microbes typically express a range of microbe/pathogen-associated molecular patterns (MAMPs/PAMPs) that are perceived by plant pattern recognition receptors to activate immune responses. This forms a first, basal level of defense to block microbial penetration of plant cells [5–7]. Therefore, endosymbionts like rhizobium and AM fungi have to modulate the innate immune system.

To establish symbiosis and evade innate immune responses, symbiotic microbes engage in a molecular dialog with potential plant hosts. Microbial lipochitooligosaccharides (LCOs) have been identified as the key signals to allow entry of the microbes into plant root cells. The use of LCOs appears to have been invented at least two times independently in evolution to establish an endosymbiosis with plants, first by AM fungi and later by rhizobium bacteria and possibly also by actinorhizal *Frankia* bacteria [8,9]. LCOs are structurally related to MAMPs

such as chitin-oligosaccharides and peptidoglycans. In recent years, it has become clear that perception of all these chitin-like molecules involves related LysM-domain-containing receptor-like proteins. Furthermore, recent data indicate that perception of symbiotic LCOs is much more intertwined with innate immune signaling than previously imagined. This raises questions about how plants manage to integrate very similar microbial signals to distinguish friend from foe. Here, we review our current understanding of the perception of chitin-like molecules in endosymbioses and innate immune signaling and discuss how LCOs might modulate the immune system. Furthermore, we discuss what it takes to become an endosymbiont.

The Ancient Arbuscular Mycorrhizal Symbiosis

AM symbiosis between plants and fungi of the Glomeromycota phylum is thought to have originated ~450 million years ago in the Devonian era [10,11]. Fossil records from that period suggest that since then AM symbiosis has remained largely unchanged [12]. Fungal colonization of the root generally starts with formation of a hyphal hyphopodium at the epidermis. Subsequently, the plant actively facilitates the entry of fungal hyphae by formation of a pre-penetration apparatus through remodeling of its cytoskeleton [13]. After crossing the epidermis, fungal hyphae mostly spread intercellularly until they reach root inner cortex cells, where the hyphae branch and form intracellular arbuscules. During arbuscule formation, the hyphae become surrounded by a specialized host membrane to form an optimized symbiotic interface that facilitates the efficient exchange of nutrients [3]. This interface lacks a structured cell wall, which implies that extensive modulation of the host cell wall occurs [14]. Recently, unravelling of the genome sequence of the model AM fungus *Rhizophagus irregularis* revealed that this species lacks cell wall-degrading enzymes [15,16]. This indicates that AM fungi mostly rely on their plant hosts to deliver cell wall-remodeling enzymes to facilitate infection. Such enzymes might be delivered through a symbiosis-dedicated exocytosis pathway. Two homologous v-SNARE proteins, part of a symbiotic clade, have been identified in the legume *Medicago truncatula*, which are essential for the formation of the cell wall-free interface in both AM and rhizobial symbioses [17]. Because fungal cell wall-degrading enzymes can release so-called damage-associated molecular patterns that trigger defense responses [18,19], the absence of cell wall-degrading enzymes in AM fungi may therefore be a key adaptation to avoid a strong defense response.

Signals in Arbuscular Mycorrhizal Symbiosis

AM fungi sense the presence of potential host plants by plant secreted signals such as strigolactones, 2-hydroxy fatty acids, and flavonoids [20]. Perception of these molecules stimulates growth and branching of fungal hyphae, which increase the chance to contact

the roots of a host plant. In turn, AM fungi release diffusible signals that are perceived by the host and induce symbiosis-specific responses. Typical responses include the induction of calcium oscillations in and around the nucleus (known as calcium spiking), stimulation of lateral root formation, activation of symbiosis-specific genes and branching of root hairs [21–24]. The nature of such AM signals was recently revealed by purifying exudate fractions of *in vitro* root cultures infected with *R. irregularis* and from germinated fungal spores [25,26]. By testing these fractions in various bioassays for symbiotic responses, it was found that *R. irregularis* makes acylated chitin-like molecules called myc-LCOs. Myc-LCOs consist of β -1-4-linked *N*-acetyl glucosamine (GlcNAc) residues with an acyl chain at the non-reducing residue [26]. *R. irregularis* produces a mix of sulfated and non-sulfated tetrameric and pentameric LCOs mostly acylated with either an oleic acid (C18:1) or palmitic acid (C16:0) [26]. Application of such myc-LCO mixtures at sub-nanomolar concentrations stimulates mycorrhization in a variety of plant species. This indicates that myc-LCOs are efficiently perceived by a broad range of plants.

A key response that is triggered by myc-LCOs is the activation of calcium spiking, which forms a central response in a genetically identified signaling network. Initially, this network has been characterized in two legume species: *M. truncatula* and *Lotus japonicus*. Because the identified genes are essential for both AM and rhizobial symbioses, the genetic network is referred to as the common symbiosis signaling pathway [11,27]. This pathway consists of an LRR (leucine-rich repeat)-receptor kinase (named LjSYMRK in *L. japonicus* and MtDMI2 in *M. truncatula*) [28,29], a putative cation channel located at the nuclear envelope (LjCASTOR, LjPOLLUX, and MtDMI1) [30–33], components of the nuclear pore [34–36], and a calcium channel (MtMCA8) [37]. These components are all required to induce calcium spiking [38,39]. These regular oscillations in calcium concentration induced upon LCO perception are decoded by a nuclear-localized calcium-calmodulin-dependent kinase (CCaMK; in *M. truncatula* also called MtDMI3) that interacts directly with the transcription factor LjCYCLOPS/MtIPD3 [40–42]. Phylogenetic studies revealed that the core components of the common symbiosis signaling pathway are conserved in the earliest land plants [43,44]. This is in line with the ancient origin of the AM symbiosis and the fact that AM fungi have an extremely wide host range.

In addition to LCOs, short-chain chitooligosaccharides consisting of four or five GlcNAc residues that lack an acyl chain have been proposed to play a role in AM symbiosis [25]. Tetrameric and pentameric chitooligosaccharides were identified in germinated spore exudates at markedly higher concentration than LCOs and their secretion was induced by the application of strigolactones. Such short-chain chitooligosaccharides are also able to

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trigger calcium oscillations in epidermal cells of legume as well as non-legume (cultured) roots [24,25]. Like myc-LCO signaling, chitooligosaccharide-induced signaling is dependent on the common symbiosis signaling pathway. However, in contrast to myc-LCOs short-chain chitooligosaccharides fail to stimulate formation of lateral roots [26]. Further, the calcium signatures that are induced by chitin oligomers differ from the calcium spikes induced upon mycorrhizal (or rhizobial) infection or LCO application. Both mycorrhizal infection (cell entry) and LCO perception are correlated with high frequency and regular calcium spiking, whereas short-chain chitin oligomers induce irregular calcium spikes that are less frequent [25,45]. Low frequency calcium spiking has been correlated with pre-infection stages [45]. Therefore, the output of these calcium spikes may be different. These studies also indicate that perception of LCOs and chitin oligomers in root organ cultures differs markedly from that observed in intact plants. In contrast to intact plants, root organ cultures appear to be more than 1,000 times less sensitive to LCOs. This might be explained by an altered hormone balance in the root cultures. Therefore, it remains to be determined what role short-chain chitin oligomers play in mycorrhization in intact plants, and how they cross talk with myc-LCO signaling.

Rhizobium Co-Opted Lipochitooligosaccharide Biosynthesis Through Convergent Evolution

Myc-LCOs are structurally similar to LCOs produced by symbiotic nitrogen-fixing rhizobium bacteria (also known as Nod factors) [26,46]. Rhizobium bacteria secrete LCOs in response to a specific blend of amongst others flavonoids released by plant roots. Flavonoids activate the transcriptional activator NodD, which in turn induces rhizobial nodulation (*nod*) genes to produce LCOs [47]. Rhizobium LCOs control several key processes in symbiosis. They are essential, and even sufficient, to induce formation of root nodules. Nodule organogenesis starts with the re-programming of root cortical cells that re-enter the cell cycle to form a nodule primordium [48]. These primordium cells acquire the ability to take up rhizobium bacteria intracellularly. However, to ensure their uptake the bacteria need to reach the primordium cells at the right time. Therefore, the process of nodule organogenesis needs to be tightly coordinated with the entry of the bacteria into the roots, i.e., the infection process. This infection process is also controlled by rhizobium LCO signaling and can be genetically uncoupled from the organogenesis program [49]. Depending on the legume species, different modes of root infection have been identified [50]. The best studied and most common mechanism involves the formation of so-called infection threads that initiate in the root hairs [51]. Rhizobium bacteria attach to the root hairs and upon LCO signaling induce continuous reorientation of the growth direction of the root hair to entrap the bacteria in a closed cavity. From there, a cell wall-bound tube-like structure, the infection thread, is formed that guides the bacteria to the nodule primordium cells. At this point, the

bacteria are first able to release from the infection threads to be taken up into the nodule cells and form symbiosomes. Also, this process depends on rhizobium LCO signaling [52]. The formation of infection threads in the root hairs is most sensitive to structural variations in the LCO molecules, which thereby play a major role in determining host specificity [53–57].

Nitrogen-fixing rhizobium symbionts form a polyphyletic group representing 13 genera within the α -proteobacteria and 2 genera within the β -proteobacteria (**Table 1**) [58,59]. Symbiotic rhizobium bacteria have in common the fact that they combine two genetic traits, namely a set of nitrogen fixation (*nif*) genes that encode the nitrogenase enzyme complex and a set of (*nod*) genes that allow LCO biosynthesis. The nitrogen fixation trait has an ancient origin that dates back 1.5-2.2 billion years [60]. This trait was repeatedly horizontally transmitted to a diverse range of microbial species, including those that have given birth to current rhizobium symbionts [61,62]. An ancestral rhizobium symbiont managed, most probably as a unique evolutionary event, to combine the nitrogen fixation trait with its ability to produce LCOs. This event must have occurred at least 60 million years ago, prior to, or coinciding with, the birth of nitrogen fixing root nodules in legumes [63]. Once established, the unique combination of nitrogen fixation and LCO biosynthesis spread via lateral gene transfer [64]. Such spreading is especially prominent within the α -proteobacteria (**Table 1**). These rhizobium symbionts have organized the nitrogen fixation and LCO biosynthesis genes on symbiotic plasmids or as symbiotic islands in their genomes.

Symbiotic rhizobium species have in common a set of only five core *nod* genes, but generally contain many more lineage-specific genes that allow variation in LCO structure. The core *nod* genes encode an N-acetylglucosaminyltransferase (NodC) that synthesizes the chitin tetramer or pentamer backbone, a chitooligosaccharide deacetylase (NodB) that removes the acetyl group of the non-reducing glucosamine, and an N-acyltransferase (NodA) that subsequently attaches a C16-C20 lipid tail to this position. Two additional core genes, *nodI* and *nodJ*, encode an ATP binding cassette (ABC) transporter that facilitates LCO secretion [65–67]. As biosynthesis of chitin oligomers is not a common feature of prokaryotes, it raises questions about the evolutionary origin of these core *nod* genes. A survey of the *R. irregularis* genome sequence failed to identify homologs of these genes. Therefore, it is unlikely that an ancestral rhizobium acquired the ability to synthesize LCOs via lateral gene transfer from AM fungi. In search for paralogs of core *nod* genes, it was found that *Burkholderia* species (β -proteobacteriales) harbor paralogs of *nodI* and *nodJ*. The genus *Burkholderia* is made up of many symbiotic rhizobia, and their symbiotic capacity is considered to be more than 50 million years old [68,69]. Interestingly, *nodI-nodJ* paralogous genes are absent in non-

Table 1: Overview of symbiotic rhizobia in the orders Rhizobiales and Burkholderiales.

α-Proteobacteria			
Rhizobiales	Genera	Number of symbiotic genera	Symbiotic genera
Aurantimonadaceae	4	-	
Bartonellaceae	1	-	
Beijerinckiaceae	8	-	
Bradyrhizobiaceae	12	2	<i>Bosea</i> , <i>Bradyrhizobium</i>
Brucellaceae	6	1	<i>Ochrobactrum</i>
Cohaesibacteraceae	2	-	
Hyphomicrobiaceae	21	1	<i>Devosia</i>
Methylobacteriaceae	3	2	<i>Methylobacterium</i> , <i>Microvirga</i>
Methylocystaceae	8	-	
Phyllobacteriaceae	12	3	<i>Aminobacter</i> , <i>Mesorhizobium</i> , <i>Phyllobacterium</i>
Rhizobiaceae	6	3	<i>Rhizobium</i> / <i>Agrobacterium</i> , <i>Sinella</i> , <i>Sinorhizobium</i>
Rhodobiaceae	9	-	
Xanthobacteraceae	7	1	<i>Azorhizobium</i>
β-Proteobacteria			
Burkholderiales	Genera	Number of symbiotic genera	Symbiotic genera
Alcaligenaceae	20	-	
Burkholderiaceae	12	2	<i>Burkholderia</i> , <i>Cupriavidus</i>
Oxalobacteraceae	15	-	
Sutterellaceae	2	-	

symbiotic α -proteobacteria, and phylogenetic analyses suggest that at least the rhizobial LCO transport system originates from ancestral *Burkholderia* [70]. However, the origin of *nodI* and *nodJ* is not necessarily indicative for the origin of LCO biosynthesis in symbiotic rhizobia. Similar studies with *nodA*, *nodB*, and *nodC* did not identify putative paralogous genes, despite massive (meta) genome sequencing programs. Therefore, an alternative scenario in which LCO biosynthesis genes evolved first in a different, yet unknown, species cannot

be ruled out. Only upon emergence of the *nodABC* operon in *Burkholderia* via lateral gene transfer were the *nodI* and *nodJ* genes recruited to facilitate LCO secretion. As symbiotic α -proteobacteria display greater sequence diversity in *nodABC* compared to *Burkholderia*, it is a likely scenario that this operon evolved first in an ancestral α -proteobacteria species [69,70].

Lipochitooligosaccharide Perception in Symbiosis Intertwines with Innate Immune Signaling

Rhizobium and Myc-LCOs are structurally very similar and both are perceived by related LysM-domain-containing receptor-like kinases (LysM-RKs). The LysM (lysine motif) domain is a widely distributed protein domain that generally is thought to bind GlcNAc molecules [71]. Unique to plants is the presence of LysM domains in the extracellular part of transmembrane receptor kinases [72]. LysM-RKs are characterized by an extracellular part with one to three LysM domains that are distinct in sequence, suggesting a single evolutionary origin of this type of receptors [72].

Genetic and biochemical studies in *L. japonicus* and *M. truncatula* have led to the identification of two different LysM-RKs that are essential for rhizobium LCO perception and signaling: LjNFR1/MtLYK3 and LjNFR5/MtNFP [73–77]. Strikingly, LjNFR5 and MtNFP lack critical amino acids in the kinase domain and do not show autophosphorylation, indicating that they have a non-functional kinase [73,76,78]. However, deletion of the intracellular kinase domain abolishes the activity of MtNFP [79,80], which suggests that specific binding partners confer the signaling role of these receptors. LjNFR5 and MtNFP have been shown to form a complex with LjNFR1 and MtLYK3, respectively, in addition to both proteins forming homomers [52,78]. LjNFR1 and MtLYK3 have a functional kinase domain, and heteromerization with LjNFR5/MtNFP is therefore proposed to constitute a functional rhizobium LCO receptor complex [78,80]. In addition, LjNFR5 was found to interact with LjSYMRK as well as with a small Rho-like GTPase LjROP6 [81,82]. LjSYMRK is cleaved *in vivo*, releasing a large part of its extracellular domain, allowing the remaining protein to interact specifically with LjNFR5 [82]. Both proteins, LjSYMRK/MtDMI2 and LjROP6, possibly in conjunction with LjNFR5/MtNFP, play a role in rhizobial infection.

Interestingly, myc-LCOs are also perceived by MtNFP, as the ability of myc-LCOs to induce lateral root formation in *M. truncatula* is dependent on *MtNFP* [26]. Furthermore, early transcriptional changes induced by myc-LCOs are largely dependent on MtNFP [83]. Rhizobium and myc-LCOs each trigger their own set of transcriptional changes, and differences were even observed between sulfated and non-sulfated myc-LCOs. This suggests that additional

(co)receptors are involved. The involvement of additional myc-LCO (co)receptors is also supported by the fact that *Ljnfr5/Mtnfp* knockout mutants are not impaired in mycorrhizal colonization [75,84]. In *M. truncatula*, a paralog of *MtNFP*, *MtLYR1*, is upregulated upon mycorrhization [85–87]. Therefore, it has been proposed that duplication of an ancient LjNFR5/MtNFP-like myc-LCO receptor allowed one copy to obtain a new function in Nod factor signaling while the other maintained its role in mycorrhization [88]. This hypothesis is supported by the observation that in *Parasponia*, the only non-legume species able to establish a rhizobium symbiosis, only a single *LjNFR5/MtNFP* ortholog is present and is required for the intracellular accommodation of both rhizobia and AM fungi [88]. As *Parasponia* obtained the ability to nodulate relatively recently and independently from legumes [89], this strongly suggests that perception of rhizobium LCOs evolved from the ancient myc-LCO perception mechanism [8]. A recent origin of nodulation in *Parasponia* also fits with the primitive nature of rhizobium infection of this species. Whereas most legumes guide their rhizobial symbionts via intracellular infection threads toward the newly formed root nodule, *Parasponia* allow rhizobium to enter first apoplastically via a mechanism known as crack entry [90]. Only when it reaches the nodule is rhizobium hosted intracellularly. However, it is not hosted as a symbiosome but rather in so-called fixation threads that have some resemblance to mycorrhizal arbuscules. Knockdown of the *Parasponia andersonii* NFP ortholog (*PaNFP*) specifically blocks the formation of fixation threads as well as arbuscules, suggesting that this process may in fact be the generic function of symbiotic LCO signaling. In legumes, this mechanism evolved to allow more strict control over rhizobium infection and in some legumes co-evolved into a highly specific plant-rhizobium partner selection mechanism [56]. Whether *MtLYR1* is indeed involved in myc-LCO perception remains to be demonstrated, but additional LCO (co)receptors may also be involved. Recently, it was shown that *MtLYR3* has a high affinity for LCOs and is expressed in roots and nodules [91]. *MtLYR3* and *MtNFP* are the result of an ancient tandem duplication, and their genomic location also shows synteny with the *MtLYR1* genomic region [76]. Therefore, *MtLYR3* may represent an additional coreceptor for LCO perception.

Analogous to LjNFR5/NFP-type LysM-RKs, it was found that LjNFR1 and MtLYK3 also play a role in mycorrhization. *Mtlyk3* and *Ljnfr1* mutants are impaired in mycorrhizal infection [92]. However, infection is not completely blocked, again suggesting that additional LysM-RKs are involved. These data further suggest evolution of the rhizobium LCO perception mechanism from the ancient mycorrhizal signaling machinery. Intriguingly, it was found in rice (*Oryza sativa*) that a single receptor, OsCERK1, facilitates infection of AM fungi but is also essential for chitin-triggered defense responses [92,93]. OsCERK1 is a close homolog, if not an ortholog, of the chitin innate immune receptor AtCERK1 of *Arabidopsis thaliana*

(*Arabidopsis*) and the legume rhizobium LCO receptors LjNFR1 and MtLYK3. The ability of LjNFR1/MtLYK3 and OsCERK1 to function in symbiotic signaling correlates with the presence of a YAQ/YAR motif in the kinase domain [93–95]. Defense-related chitin signaling does not require this motif, as *Arabidopsis* AtCERK1 appears to have lost it [94,95]. Furthermore, the kinase domain of OsCERK1 when fused to the extracellular part of LjNFR1 was able to fully complement the nodulation defect of the *Ljnr1* mutant [93]. This indicates that no additional evolution of the kinase domain is required to function in nodulation and underlines that symbiotic LCO perception and innate immune signaling intertwine.

Chitin-Induced Innate Immune Signaling Shows Analogies to Symbiotic Lipochitooligosaccharide Signaling

In contrast to tetrameric and pentameric chitin oligomers, longer chain chitin oligomers (hexa- to octamers) or peptidoglycans are typically perceived as MAMPs that trigger innate immunity [96,97]. Perception of these chitin-like molecules also involves LysM-RKs. The functioning of these receptors has been best studied in *Arabidopsis*, a species that lost the AM symbiosis, and in rice, which does form an AM symbiosis.

In *Arabidopsis*, at least three LysM-RKs are involved in chitin-induced innate immune signaling: AtCERK1, AtLYK4, and AtLYK5 [98–104]. As mentioned above, AtCERK1 is a close homolog, or even an ortholog, of LjNFR1/MtLYK3 and was originally identified as the key chitin receptor on the basis of its mutant phenotype and its ability to bind chitin oligomers. Octameric chitin oligomers cause dimerization of AtCERK1, leading to activation of its kinase domain [105]. Short-chain chitin oligomers also bind to the extracellular part of AtCERK1 but fail to induce dimerization. Interestingly, such short-chain chitin oligomers could attenuate AtCERK1 signaling in a dose-dependent manner [105]. Therefore, it is possible that the short-chain chitin oligomers found in the exudates of AM fungi [25] actually interfere with defense activation by longer chain oligomers. The relative abundance of the different chitin oligomers may in such cases play an important role in determining the outcome of the responses. AtCERK1 can form a heteromer complex with AtLYK5 and AtLYK4 [100,103]. AtLYK5 and AtLYK4 belong to the same LysM-RK subfamily as LjNFR5/MtNFP and likewise do not have an active kinase domain. AtLYK5 binds chitin oligomers with much higher affinity than AtCERK1, indicating that AtLYK5 is in fact the major chitin receptor in *Arabidopsis*. AtLYK5 functions partly redundantly with AtLYK4, as both bind chitin and only a double mutant completely blocks chitin signaling, similar to *Atcerk1* mutants. Binding of chitin oligomers by AtLYK5 induces heteromerization with AtCERK1, resulting in activation of its kinase domain [103]. This indicates the involvement of a receptor complex analogous to the LjNFR5-LjNFR1 and MtNFP-MtLYK3 receptor complexes in symbiosis.

In contrast to AtCERK1, OsCERK1 was not found to bind chitin oligomers, indicating differences in affinity for chitin-like molecules [102,106]. To perceive myc-LCOs, it is likely that OsCERK1 pairs with LysM coreceptors, for which homologs of LjNFR5/MtNFP in rice, such as OsLYR1, are key candidates. To perceive chitooligosaccharides, rice relies on the LysM-domain-containing CHITIN ELICITOR BINDING PROTEIN (OsCEBiP), which lacks an intracellular kinase domain [106]. OsCEBiP binds chitin and forms a heteromer complex with OsCERK1 to activate chitin-triggered defense responses [107,108]. Notably, homologs of OsCEBiP in Arabidopsis, AtLYM1, AtLYM2, and AtLYM3 are not involved in AtCERK1-mediated innate immunity [100,102]. Nevertheless, AtLYM2 was shown to bind chitin and contributed to resistance against the fungal pathogen *Alternaria brassicicola* in an AtCERK1-independent manner [101,109]. Additionally, AtLYM2 was shown to control the transport ability of plasmodesmata in a chitin-dependent manner, which plays a role in plant immunity to pathogenic fungi such as *Botrytis cinerea* [110]. This suggests that at least two different pathways (AtCERK1 dependent and AtCERK1 independent) are operational in chitin-based defense. It is currently not known with which coreceptor(s) AtLYM2 interacts to relay signaling. In contrast, AtLYM1 and AtLYM3 were shown to form a complex with AtCERK1 to trigger immunity in response to peptidoglycans [111]. In rice, the LYM homologs OsLYP4 and OsLYP6 play a role in peptidoglycan perception as well as chitin perception [105]. Overall, these data indicate that CERK1 homologs function as a coreceptor in diverse signaling complexes related to perception of chitin-like molecules, including LCOs.

Two Models for Dual Functioning of Lipochitooligosaccharide LysM-RKs

Specific gene duplication events early in legume evolution provided the freedom to evolve symbiosis specific functions of LjNFR1/MtLYK3 and LjNFR5/MtNFP [72,88,95]. However, three lines of evidence also indicate that LjNFR1/MtLYK3 and LjNFR5/MtNFP still have a dual function in symbiosis and defense signaling, in analogy to the dual function of OsCERK1. (I.) Rhizobium LCOs transiently trigger defense-related gene expression in an LjNFR1-dependent manner [94]. (II.) MtNFP acts, independent of MtLYK3, in defense against fungal pathogens, including *Verticillium alboatrum* and *Colletotrichum trifolii*, or the oomycete *Aphanomyces euteiches* [112,113]. A lack of MtNFP makes plants more susceptible to such pathogens, whereas ectopic expression of MtNFP increases resistance [112]. (III.) A strong coexpression of both receptors –LjNFR5-LjNFR1 or MtNFP-MtLYK3– in *Nicotiana benthamiana* leaves triggered a spontaneous cell death response [77,80]. This response requires an active LjNFR1/MtLYK3 kinase domain. An early cell death response was also observed in *M. truncatula* nodules when *MtNFP* was overexpressed [52]. This work further showed that both receptors are under tight posttranslational control in legume roots and nodules, likely to prevent premature induction of defense responses. Taken together, these data suggest

that dual functioning of LCO receptors in defense and symbiosis is not unique for rice (and other non-legumes) but has also been maintained in legumes to function in rhizobium symbiosis. This implies that genetic constraints preserve this dual functioning [93]. The biological meaning of this dual functioning can be explained in two ways. A first model is based on competition for CERK1/NFR1/LYK3-type proteins by different receptor complexes. High affinity for LCOs (and/or short-chain chitin oligomers) leads to the preferential formation of symbiotic receptor complexes at the expense of the formation of complexes that act in defense signaling [93] (**Figure 1A**). Variations in affinities for different chitin-like molecules encoded by the extracellular receptor domains determine which complexes are formed. This would automatically block chitin-induced defense responses, without the need to independently suppress chitin-triggered immunity. This model is in line with the finding that LCOs can repress innate immunity [114]. As an alternative model, the MAMP-triggered responses may actually be recruited to facilitate/regulate the infection process by symbiotic microbes (**Figure 1B**). For example, several typical early MAMP-triggered responses, such as calcium influx, production of reactive oxygen species (ROS), and focal exocytosis, have been implicated in the formation and growth of infection threads during the rhizobium interaction [115]. Infection threads are tip-growing structures, and ROS production is thought to facilitate the oxidative cross-linking of the infection thread matrix to allow the formation of a tube-like infection thread. The spatiotemporal regulation of receptor complexes may then be important to fine-tune the responses to prevent too-strong defense responses [52]. This hypothesis is also in line with the essential role for symbiotic LCO receptors in infection thread formation [53,54,57,74,76], which is independent from their role in activating the common symbiosis signaling pathway [116]. Several studies suggest that symbiotic LCO receptors are located in lipid-raft-like membrane domains in the plasma membrane, which is thought to play an important role in controlling their turnover and their ability to form specific complexes [117–119]. Localization of MtNFP and MtLYK3 in the nodule showed that these receptors accumulate in a very narrow zone at the nodule apex and are rapidly removed from the infection thread membrane to allow the release of the bacteria from the infection threads [52].

Lipochitooligosaccharide Perception Is Not Sufficient to Evade Innate Immunity

LCO perception may play an even broader role in suppression of PAMP-triggered immune responses, as LCOs were shown to suppress flg22 (a 22 amino acid peptide fragment of flagellin)-induced immune responses in various plant species. In *Arabidopsis* this response depends specifically on AtLYK3 but not on AtCERK1 [114]. *Atlyk3* mutants appear to be more resistant to fungal pathogens such as *B. cinerea* and *Pectobacterium carotovorum*, suggesting that these pathogens exploit this receptor kinase to infect plants [120]. These

data led to the speculation that suppression of innate immunity may have been the original/first function of LCOs [121]. However, it is difficult to imagine why non-symbiotic plants such as *Arabidopsis* would have maintained a LCO perception system to repress innate immunity. It is also difficult to reconcile with the observation that LCOs do trigger defense responses [94]. Clearly, a better understanding of the cross talk between the various receptor complexes, potentially via shared downstream partners, involved in LCO and MAMP perception is required to resolve this apparent paradox.

Hijacking the LysM-RK activated common symbiosis signaling pathway, which is operational in the vast majority of land plants, would seem to be an attractive strategy for biotrophic pathogens to colonize root cells [122,123]. However, studies that indicate that pathogens make use of this strategy are exceptionally scarce. Root knot nematodes have been suggested to secrete signal molecules, which trigger similar root hair deformations as LCOs [124]. This ability is (partially) dependent on LjNFR1-LjNFR5 and LjSYMRK, and correspondently absent in root hairs of *Arabidopsis*. This led to the suggestion that root knot nematodes may produce LCO-like molecules to make use of the common symbiosis signaling pathway [124]. However, a clear involvement of the common symbiosis signaling pathway in root knot nematode infection has not been shown, and the nature of the nematode signals remains to be determined.

In *M. truncatula*, the *Mtccamk* mutant fails to show cytoplasmic aggregation at attempted penetration sites of *C. trifolii* and *Phytophthora medicaginis* [125], a response that is typically triggered upon fungal contact (including AM fungi) or touch [126]. However, this defect does not affect overall fungal colonization, suggesting that MtCCaMK is not required for infection by these pathogens. In fact, most studies on (hemi)biotrophic pathogenic fungi or oomycetes suggest that such pathogens do not hijack the common symbiosis signaling pathway. In *L. japonicus* leaf cells, haustorium formation by rust fungi is not dependent on the common symbiosis signaling pathway [127]. Similarly, intracellular colonization of rice roots by the biotrophic rice blast fungus *Magnaporthe oryzae* does not require OsCCaMK [128]. Also, the oomycete *Phytophthora palmivora* does not require any components of the common symbiosis signaling pathway to infect and form haustoria in *M. truncatula* roots [129]. Taken together, it appears that pathogenic fungi and oomycetes do not exploit the common symbiosis signaling pathway by producing LCOs, or short chain-chitin oligosaccharides, to facilitate plant colonization. Likewise, no bacterial pathogens have been identified that gained the core *nod* genes via horizontal gene transfer, allowing them to exploit these to colonize/parasitize potential host plants. This suggests that there may be as

yet unknown penalties associated with LCO-induced signaling that have been overcome by Glomeromycota and symbiotic rhizobia. It suggests that additional adaptations are required to benefit from LCO signaling. Such adaptations may include the evasion of recognition by the innate immune system. For example, in the case of Glomeromycota, the lack of cell wall-degrading enzymes is thought to minimize their perception as potential pathogens [15], and secreted effector proteins have evolved to modulate defense [130]. In case of rhizobium (*Sinorhizobium meliloti* and *Mesorhizobium loti*), it was suggested that flagellin, has been adapted so that it does not trigger defense responses [131,132]. Furthermore, rhizobial surface polysaccharides play important roles in the suppression of defense responses in legume roots [133]. Additionally, adaptations in the rhizobial type III secretion system (T3SS) and its associated effector proteins appear to play a key role, which is further discussed below.

From Pathogen to Symbiont

As lateral gene transfer is a common mechanism in prokaryotes [134], it can be envisioned that new potential symbionts may arise. However, the restricted number of actual rhizobium symbionts suggests that evolution of *de novo* symbionts is hampered by genetic barriers that affect the relation with potential host plants. To get insights into such barriers, comparative studies of a relatively young symbiont with its nonsymbiotic relatives have been conducted. For this, *Cupriavidus taiwanensis* is used, as it likely represents a recently evolved nitrogen-fixing symbiont. *C. taiwanensis* is the only symbiotic species in the genus *Cupriavidus* and sister genus *Ralstonia*. *Cupriavidus* and *Ralstonia* are diverged from *Burkholderia* and form a distinct lineage in the β -proteobacteriales [69,135]. *C. taiwanensis* contains a 35-kb symbiotic region on a self-transferrable plasmid. This region contains 31 symbiosis genes organized in operons interspersed with retro-elements, which may be indicative for recent structural rearrangements [136]. *C. taiwanensis* contains only a single operon of 10 *nod* genes, *nodBCIJHASUQ*, that are essential for LCO biosynthesis. This enables *C. taiwanensis* to produce pentameric LCOs with either a vaccenic acid (C18:1) or palmitic acid (C16:0) as lipid tail. Additionally, an O-carbamoyl (determined by *nodU*), an N-methyl (determined by *nodS*), and/or 6-O-sulfate group (determined by *nodH* and *nodQ*) can be present [136]. This pallet of LCOs is similar to that produced by a broad range of different symbiotic rhizobia in the α -proteobacteriales [137,138]. But in contrast to several of these species, *C. taiwanensis* has a restricted host range and nodulates only the legume *Mimosa pudica*. This suggests that the host range of *C. taiwanensis* is constrained by other factors. In search for these factors, it was found that the host-range of *C. taiwanensis* can be extended by mutating its T3SS [139]. Wild-type *C. taiwanensis* forms only infective nodules with degenerative symbiosomes on

A

B

Leucaena leucocephala, whereas the T3SS knockout mutant forms fully functional nodules. This suggests that wild-type *C. taiwanensis* secretes components during its symbiotic lifestyle that hamper symbiosome development in *L. leucocephala*. Interestingly, the organization of the T3SS operon in *C. taiwanensis* is atypical for symbiotic rhizobia, but more similar to what is found in the opportunistic pathogen *Burkholderia cenocepacia* [139]. The biological process in which the T3SS of *C. taiwanensis* functions remains unknown. However, these studies make apparent that the symbiotic lifestyle of *C. taiwanensis* is hampered by a not yet adapted T3SS, thereby restricting its symbiotic potential.

A similar finding was obtained in an experimental evolutionary approach in which the symbiotic plasmid of *C. taiwanensis* was introduced into its relative, *Ralstonia solanacearum* [140]. *R. solanacearum* is a broad host range plant pathogen that invades roots intercellularly and causes wilting upon colonization of the vascular system [141]. The chimeric *R. solanacearum* produced LCOs but was not able to trigger root nodule formation on *M. punica*. However, this dramatically changed upon inactivation of its T3SS and the master virulence regulator hrpG. Now the mutated chimeric *R. solanacearum* strain could trigger infected root nodules, although the bacteria did not yet fix nitrogen and triggered

Figure 1. Two hypothetical models to explain the evolutionary conserved dual function of lipochitooligosaccharide (LCO) receptors in symbiosis and immune signaling.

(A) Chitooligosaccharides (COs) and LCOs are perceived by LysM-domain-containing receptors CERK1/NFR1/LYK3. These LysM-RKs (receptor-like kinases) function as coreceptors in various complexes. Different affinities for the various chitin-like molecules determine which complexes are formed. In rice, chitin perception involves a complex between OsCERK1 and the membrane-anchored CEBIP. Additionally, the GPI-anchored OsLYP4 and OsLYP6 are involved in chitin and peptidoglycan perception. LCOs preferentially recruit the coreceptor CERK1/NFR1/LYK3 into the symbiotic complex at the expense of the complexes involved in MAMP-triggered immune responses. LCO perception involves a complex between CERK1/NFR1/LYK3 and NFR5/NFP-homologs, which have a nonfunctional kinase. This leads to the activation of the common symbiosis signaling pathway, which in turn may affect MAMP-triggered immunity.

(B) Symbiotic LCO receptor complexes activate early immune responses to facilitate symbiont infection. Several early MAMP-triggered immune responses, such as ion changes (including calcium influx), ROS production via respiratory burst oxidases (RBOH), and focal exocytosis, are triggered upon LCO perception and have been implicated in rhizobial infection [188]. LCO receptor heteromer complexes may be formed especially at lipid-raft-like membrane domains (marked in red). Such domains can be marked by the symbiotic remorin SYMREM1 [117] or flotillins [189], which controls their signaling potential and endocytotic turnover to prevent too-strong defense responses. In addition to forming a heteromer complex with the CERK1-orthologous LysM-RK LjNFR1, the kinase-dead LjNFR5 has been found to interact with the processed form of the common symbiosis signaling pathway LRR receptor kinase SYMRK (SYMRK-ΔMLD) [82] as well as with the small GTPase ROP6 [81], which control rhizobial infection. Additionally, a U-box E3-ubiquitin ligase (PUB1) has been found to interact with MtLYK3, which may control its ubiquitination-dependent turnover, similar to the role of PUB homologs in immune receptor complexes [188,173]. Activation of the common symbiosis signaling pathway, marked by calcium spiking, may further influence the posttranslational turnover of the receptor complexes.

defense responses in the nodules [140]. Again, this finding illustrates that adaptive changes in the secretome are essential for a successful transfer of the nitrogen-fixing symbiosis trait. Subsequent selection (coculturing) cycles in *M. pudica* further increased the symbiotic capacity of the chimeric *R. solanacearum*, indicating that plants can select mutations in the bacteria that further reduce the activation of host defenses [140,142].

Besides potential negative effects, T3SS-dependent effector proteins can also be used by rhizobium to facilitate symbiosis, and as such play a role as host range determinants in addition to LCOs [143]. The broad host range strain *Sinorhizobium fredii* NGR234 has been shown to use its T3SS to inject so-called nodulation outer proteins (Nops) into plant cells to suppress defense responses [144,145]. Transcriptional activation of the T3SS-encoding genes was shown to be induced by flavonoids in a NodD-dependent manner, indicating a common regulatory mechanism with LCO biosynthesis genes [146,147]. Strikingly, some rhizobia have even evolved T3SS effector proteins that allow them to bypass LCO signaling to enable nodulation. The LCO-deficient *Bradyrhizobium elkanii* *nodC* mutant BEnodC was shown to induce nodulation in soybean by making use of its T3SS [148]. This suggests that this strain evolved effectors that when injected into the plant cells can bypass the LCO receptors. It is currently not known how such effectors trigger nodulation. It would be interesting to see whether they signal via the common symbiosis signaling pathway or activate nodulation downstream of this pathway. The LCO-deficient *B. elkanii* strain failed to form infection threads in roots hairs but instead invaded roots via crack entry [148]. This is in line with a specific role for rhizobium LCO receptors in infection thread formation, as discussed above. Most *Bradyrhizobium* species rely on LCOs to nodulate, which suggests that this LCO-independent nodulation arose later in evolution. Furthermore, although the LCO-deficient strain could nodulate, its effectiveness was much improved upon addition of LCOs, indicating that LCO-induced signaling is a more efficient strategy.

Perspective: Hormonal Responses May Be Key to Symbiotic Lipochitooligosaccharide Signaling

The studies described above make clear that symbiotic rhizobia have not only gained the capacity to produce LCOs but also need to reset their T3SS secretome to overcome pathogenicity. Furthermore, we have presented two models that show how perception of LCOs can modulate the balance between different LysM-RK receptor complexes and/or use the readout of innate immune signaling to benefit rhizobial infection [Figure 1]. It remains unclear, however, to what extent symbiotic LCO signals can also actively repress innate immunity. In this last section, we focus on the potential role of plant hormones in such active repression of innate immunity. On the basis of recent studies, we speculate

on a central role for DELLA proteins in hormonal cross talk to repress defense responses in symbiosis. However, it should be noted that regulation and cross talk between various hormones, especially in roots, is far from understood.

Symbiotic LCO signaling causes changes in the hormonal landscape of the host plant. This is most obvious in the case of rhizobium-induced nodule organogenesis, but myc-LCOs also induce changes in root architecture. In fact, all classical plant hormones have been implicated in nodulation as well as mycorrhization [149–151], including the major defense hormones salicylic acid (SA), jasmonic acid (JA), and ethylene [152]. Therefore, cross talk between the LCO-induced common symbiosis signaling pathway and various hormonal networks is likely to influence innate immune signaling.

SA is an important regulator of resistance to biotrophic pathogens [153], and SA has also been shown to have a negative effect on both rhizobial and mycorrhizal symbioses [154–158]. AM fungi and rhizobia seem to employ LCO signaling to suppress SA-dependent defense responses. Inoculation of alfalfa (*Medicago sativa*) plants with LCO-deficient rhizobia or an incompatible rhizobium strain induces accumulation of SA, whereas a slight reduction in SA levels is observed upon inoculation with compatible rhizobia [156]. Likewise, inoculation of tomato with the rather incompatible AM fungal species *Glomus mosseae* elevates SA concentrations in the root and expression of the SA-responsive gene *PR1a*, whereas this is not observed upon inoculation with the very compatible fungal species *R. irregularis* [159]. Studies in pea (*Pisum sativum*) revealed that mycorrhization induces a transient rise in SA levels, which is repressed during prolonged colonization. In contrast, a constant rise in SA levels is induced in pea plants mutated in CCaMK, suggesting that repression of SA biosynthesis is based on activation of the common symbiosis signalling network [154].

Whereas SA is a major signal in resistance to biotrophic pathogens, defense against necrotrophic pathogens requires JA as signal [152]. Both hormones antagonize each other, such that activation of JA signaling can compromise SA-dependent defense. Several biotrophic pathogens exploit this antagonism to suppress SA-mediated defense [160]. For example, several *Pseudomonas syringae* strains produce the effector coronatine (COR), which acts as an analog of jasmonate-isoleucine (JA-Ile), the bioactive form of JA [5]. By binding the JA receptor COI1, COR induces proteasomal degradation of the JAZ proteins that function as negative regulators (transcriptional repressors) of JA signaling [152]. COR-deficient strains of *P. syringae* have been shown to produce other effectors to activate JA responses; e.g. HopX1. *HopX1* encodes a cysteine protease that degrades JAZ, thereby activating JA signaling in order to antagonize SA-mediated defense [161].

Recently, DELLA proteins have been implicated as important regulators of the JA-SA balance [162]. DELLAs belong to the GRAS family of transcriptional regulators and were originally identified as gibberellin (GA)-sensitive repressors of plant growth [163,164]. However, DELLA proteins appear to form signaling hubs involved in various hormone signaling pathways, including in auxin, ethylene, and JA signaling [165]. DELLAs promote JA signaling by competing for JAZ binding with MYC2, a key transcriptional activator of JA signaling [166,167]. Activation of JA signaling by MYC2 promotes DELLA accumulation, further attenuating MYC2 repression by JAZ proteins [168,169]. Recent data suggest that AM fungi may exploit this DELLA function to reduce SA-mediated defense responses. Experiments in rice and *M. truncatula* revealed that DELLA mutants in rice (*Osslr1*) and *M. truncatula* (*Mtdella1/Mtdella2* double mutant) are severely impaired in root infection and/or arbuscule formation [170,171], indicating that DELLA proteins are essential transcriptional regulators of this endosymbiosis. In line with this, GA application at high concentrations was shown to block colonization, whereas lower concentrations were already sufficient to block arbuscule formation [172]. It is therefore tempting to speculate that DELLAs might promote colonization and arbuscule formation through immune modulation by sequestering JAZ proteins, affecting the JA-SA signalling balance.

Ectopic expression in *M. truncatula* of a non-degradable *MtDELLA1* allele (*Mtdella1-Δ18*) in the vasculature and endodermis is sufficient to sustain arbuscule formation in the root cortex of the *Mtdella1/Mtdella2* double mutant or upon GA application [170]. Additionally, ectopic expression of *Mtdella1-Δ18* enables arbuscule formation in an *L. japonicus* *Ljcydlops* mutant. The CCaMK-interacting protein *LjCYCLOPS/MtIDP3* is a key protein of the common symbiotic signaling pathway [42], suggesting that DELLA proteins intersect at some point with LCO signaling but do so in a non-cell-autonomous fashion [170]. Intriguingly, expression of the dominant active *Mtdella1-Δ18* allele in an *M. truncatula* *Mtccamk* mutant did not rescue the mycorrhizal colonization defect, which indicates that additional input of CCaMK is required. In rice, the DELLA *OsSLR1* was shown to interact with the GRAS family *OsDIP1* (DELLA interacting protein 1), which is induced upon mycorrhization [171]. *DIP1* was also shown to interact with the GRAS protein *RAM1* (required for arbuscular mycorrhization 1), which is essential for root colonization by AM fungi [174]. This led to the suggestion that DELLA, *DIP1*, and *RAM1* are part of a larger GRAS protein regulatory complex [175]. Other GRAS family proteins that play a role in both mycorrhization and nodulation include the transcriptional regulators *NSP1* and *NSP2* [176–179]. Both proteins are required for the production of strigolactones [178], which act as plant signals that promote hyphal branching of AM fungi as well as root and shoot architecture [180–183]. Both *NSP1* and *NSP2* have been shown to control mycorrhizal colonization [26,178,179,184,185]. However, the relative

mild effects on mycorrhizal colonization contrasts with the essential role for NSP1 and NSP2 during nodulation [176,177,186]. This suggests that different GRAS complexes are involved in the control of the two symbioses. However, our understanding of the functioning of these GRAS complexes in relation to symbiotic LCO signalling remains scant [187] and will remain a major objective in future research.

As has become apparent, the mechanisms behind LCO-mediated modulation of innate immunity are far from clear. However, recent studies not only provided novel insight but also extended the studies to other, nonlegume plant species, such as rice and *Arabidopsis*. This will open new frontiers to unravel the fundament of symbiotic signaling.

SUMMARY POINTS

1. Combining the nitrogen fixation trait with the capacity to synthesize LCOs marked the origin of nitrogen-fixing rhizobia. The ability to make LCOs enabled rhizobia to activate the signaling pathway that is used by host plants to establish an endosymbiosis with AM fungi. This common symbiosis signaling pathway is essential for the formation of a symbiotic host-microbe interface. In legumes, this signaling pathway evolved to allow formation of root nodules and a more strict control over rhizobium infection.
2. CERK1-type LysM-RKs have dual functions in immune signaling and symbiosis, indicating that LCO signaling and chitin-based immune signaling are intertwined.
3. Two putative models have been put forward to explain this evolutionarily conserved dual function. (I.) Perception of LCOs modulates the balance between different LysM-RK receptor complexes, favoring a symbiotic complex at the expense of complexes required for immune responses. (II.) Early immune responses are co-opted to facilitate symbiont infection. Tight regulation of the receptor complexes at the posttranslational level, involving endocytotic turnover, subsequently prevents activation of defense responses.
4. Microbial activation of the common symbiosis signaling pathway is not sufficient to evade innate immunity. Therefore, microbes need to evolve additional genetic adaptations to benefit from LCO signaling. In rhizobia, the T3SS especially needs to be adapted to evade immune responses.
5. Microbial activation of the common symbiosis signaling pathway can modulate innate immune responses through its effect on the hormonal landscape. On the basis of recent studies, we speculate on a central role for DELLA proteins in hormonal cross talk to repress defense responses in symbiosis, in part by influencing the SA-JA balance.

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Chapter 3

Rhizobium lipochitooligosaccharide signaling triggers accumulation of cytokinins in *Medicago truncatula* roots

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ABSTRACT

The legume rhizobium symbiosis is initiated upon perception of bacterial secreted lipochitooligosaccharides (LCOs). Perception of these signals by the plant initiates a signalling cascade that leads to nodule formation. Several studies have implicated a function for cytokinin in this process. However, whether cytokinin accumulation and subsequent signalling are an integral part of rhizobium LCO signalling remains elusive. Here, we show that cytokinin signalling is required for the majority of transcriptional changes induced by rhizobium LCOs. In addition, we demonstrate that several cytokinins accumulate in the root susceptible zone three hours post rhizobium LCO application, including the biologically most active cytokinins *trans*-zeatin and isopentenyl adenine. These responses are dependent on calcium- and calmodulin-dependent protein kinase (CCaMK), a key protein in rhizobial LCO induced signalling. Analysis of the ethylene insensitive *Mtein2/Mtsickle* mutant showed that LCO-induced cytokinin accumulation is negatively regulated by ethylene. Together with transcriptional induction of ethylene biosynthesis genes, it suggests a feedback loop negatively regulating LCO signalling and subsequent cytokinin accumulation. We argue that cytokinin accumulation is a key step in the pathway leading to nodule organogenesis and that this is tightly controlled by feedback loops.

INTRODUCTION

Legumes can engage endosymbiotically with different nitrogen-fixing rhizobial species. Upon signal exchange between host and microsymbiont, a developmental program is initiated that gives rise to a new organ, the root nodule [1-4]. These nodules are colonized intracellularly, providing a niche to the bacteria optimal for the fixation of atmospheric nitrogen. The key signal molecules that initiate nodule organogenesis are bacterial secreted lipochitooligosaccharides (LCOs), also known as Nodulation (Nod) factors [5]. In some legumes, these bacterial signals are even sufficient to trigger the complete developmental program resulting in formation of (empty) nodules [6-9]. Rhizobium LCOs are perceived by LysM domain receptor kinases at the root epidermis [10-13]. This activates a signalling cascade consisting of a plasma membrane localized LRR-type receptor kinase (named MtDMI2 in *Medicago truncatula*), a cation ion channel (named MtDMI1 in *M. truncatula*) and several components of the nuclear pore complex [14]. Activation of this signalling cascade results in nuclear calcium oscillations, which are interpreted by a nuclear localized calcium- and calmodulin-dependent protein kinase (CCaMK, named MtDMI3 in *M. truncatula*) [15, 16]. CCaMK activates the transcriptional regulator CYCLOPS, which subsequently activates transcription of downstream targets, including the transcription factor NODULE INCEPTION (NIN) [17]. The rhizobium LCO signalling pathway interacts with plant hormone homeostasis at multiple levels. For example, activation of cytokinin phosphorelay signalling is essential for nodule formation, whereas ethylene signalling is known to inhibit this process [18-20]. Unravelling how these hormonal pathways are integrated in symbiotic LCO signalling is key to understand how legume plants establish symbiosis with rhizobium.

Ethylene negatively regulates rhizobium LCO-induced signalling and subsequent nodule formation. The first and rate-limiting step in ethylene biosynthesis is conversion of S-adenosyl-L-methionine (S-AdoMet) into 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthases (ACS) [21]. Subsequently, ACC is converted into ethylene by ACC oxidase (ACO) activity [21]. Interestingly, in pea (*Pisum sativum*) roots, ACO genes are expressed in pericycle, endodermal and inner cortical cells opposite phloem poles, limiting nodule initiation to opposite protoxylem poles [22]. Ethylene is perceived by a set of ER-localized receptors that control activity of the ER-localized EIN2 protein. EIN2 forms a central component of the ethylene signalling pathway [21, 23, 24]. Upon activation, EIN2 is cleaved, by which its C-terminal domain is released and can move to the nucleus to modulate transcription [25]. The *Mtein2* mutant in *M. truncatula*, named *Mtsickle*, is ethylene insensitive and displays hyper nodulation upon rhizobium inoculation, forming up to ten times more nodules in a distinct zone [26, 27]. Ethylene has been shown to affect LCO signalling at an early step in

the signalling cascade, most likely upstream or at the point of calcium oscillation. This is based on the observation that, in presence of ethylene, an increased LCO concentration is required to initiate this cellular calcium response. Furthermore, a decrease in oscillation frequency is observed in *Mtein2/Mtsickle* compared to wild type [19].

In contrast to ethylene, cytokinin acts as an important positive regulator of nodule organogenesis. This was first illustrated by physiological studies, which showed that external application of cytokinin triggers the formation of nodule-like structures on the roots of several legume plants [28-31]. Genetic studies revealed involvement of a specific cytokinin receptor in nodulation: MtCRE1 and LjLHK1 in *M. truncatula* and *Lotus japonicas*, respectively. Both receptors are putatively orthologous to the arabidopsis (*Arabidopsis thaliana*) AtAHK4/AtCRE1 cytokinin receptor, which functions redundantly to cytokinin receptors AtAHK2 and AtAHK3 [32-37]. Not only are MtCRE1 and LjLHK1 essential for nodule organogenesis; gain-of-function mutations that make these receptors hypersensitive to cytokinin lead to spontaneous nodule formation [20, 38]. Several symbiotic genes have been reported to be responsive to cytokinin signalling, including the transcriptional regulators *NSP1*, *NSP2*, *ERN1* and *NIN* [37]. Rhizobium LCO signalling also induces expression of typical cytokinin responsive genes such as type-A Response Regulators [35-37, 39]. This further underlines integration of cytokinin phosphorelay signalling in the LCO signalling pathway.

Naturally occurring cytokinins are *N*⁶-substituted adenine derivatives that can be classified as either aromatic or isoprenoid, depending on their side chain. Of these, the isoprenoid-type cytokinins are most abundant in plants and their biosynthesis is well studied [40, 41]. The rate-limiting step in the biosynthesis of isoprenoid-type cytokinins is catalysed by the isopentenyl transferases (IPTs), which can be classified into two groups depending on their substrate. Adenylate-IPTs use adenosine 5'-phosphate (AMP, ADP or ATP) as substrate and are involved in the production of isopentenyl adenine (iP), *trans*-zeatin (tZ) and dihydrozeatin (DZ), whereas tRNA-IPTs use tRNAs as substrate and are involved in biosynthesis of *cis*-zeatin (cZ). Biosynthesis of tZ-type cytokinins involves hydroxylation of iP-nucleotides by a group of cytochrome P450 monooxygenases, which in arabidopsis are named AtCYP735A1 and AtCYP735A2 [42]. Release of bioactive cytokinins from their nucleotide precursors is catalysed by a group of 5'-monophosphate phosphoribohydrolases, referred to as LONELY GUYS (LOGs) [43, 44]. Cytokinin oxidases/dehydrogenases (CKXs) are involved in cytokinin degradation [45], whereas adenine phosphoribosyl transferases (APTs) inactivate cytokinin by converting them back to their nucleotide form [46]. Recently, several studies reported a link between cytokinin biosynthesis genes and nodule formation [47, 48]. In *L. japonicus*, *LjIPT3* expression increases within three hours after rhizobial inoculation, and

in *M. truncatula* both *MtLOG1* and *MtLOG2* are up-regulated during later stages of nodule formation. Moreover, RNA interference-mediated knock-down of *LjIPT3* and *MtLOG1* leads to a reduction in the total number of nodules formed per plant [47, 48]. However, what remains unknown is whether cytokinins are produced as a signalling intermediate upon rhizobium LCO perception and to what extent this contributes to rhizobium LCO-induced signalling.

In this study, we used RNA sequencing to determine the role of cytokinin in rhizobium LCO-induced signalling in *M. truncatula*. This revealed that the majority of transcriptional changes induced three hours post rhizobium LCO application are dependent on the cytokinin receptor, MtCRE1. Furthermore, we show that LCO treatment induces expression of cytokinin as well as ethylene biosynthesis genes. Measurements of the cytokinin concentration in *M. truncatula* roots showed an accumulation of bioactive cytokinins three hours after rhizobium LCO application. The latter response is inhibited by ethylene, suggesting the presence of a negative feedback loop.

RESULTS

The Majority of Rhizobium LCO-Induced Transcriptional Changes Are MtCRE1-Dependent

Activation of the cytokinin receptor MtCRE1/LjLHK1 is both sufficient and essential to trigger nodule organogenesis [18, 20]. However, the temporal and mechanistic regulation of the cytokinin signalling pathway upon rhizobium LCO signalling remains unknown. To gain insight in the regulation of this signalling pathway, we studied the role of MtCRE1 in rhizobium LCO-induced gene expression by RNA sequencing (RNA-seq). Three biological replicates of *M. truncatula* wild type and *Mtcre1* mutant were grown vertically on agar-solidified Fåhræus medium (0.2 mM $\text{Ca}(\text{NO}_3)_2$) supplemented with 1 μM amino ethoxyvinylglycine (AVG), commonly used in *in vitro* nodulation assays to increase nodulation ability [e.g. 49, 50-53]. Seedling roots were treated with *Sinorhizobium meliloti* LCOs ($\sim 10^{-9}$ M) for three hours, and RNA was isolated from the zone of the root susceptible to rhizobium infection. This zone, hereafter referred to as the susceptible zone, encompasses a region of ~ 5 mm and is analogous to the distal region of the elongation zone and the entire differentiation zone of the root. We chose to sample after three hours, as this time point represents a stage in the interaction at which cytokinin-dependent transcriptional changes are reported but mitotic activation of pericycle and cortical cells is not yet occurring [36, 37, 54]. As cytokinin signalling is important for root development [55], we first compared the transcriptomes of mock treated wild-type and *Mtcre1* mutant plants. This revealed 69 genes that display a significant transcriptional difference using a minimal fold change (FC) larger than two and

Figure 1. The majority of transcriptional changes induced three hours after rhizobium LCO treatment depend on MtCRE1.

A Venn diagram showing the number of genes differentially expressed ($FC > 2$, $p < 0.001$) in the root susceptible zone of wild-type *M. truncatula* and *Mtcre1* mutant upon rhizobium LCO treatment (10^{-9} M, 3 h). Gene expression was determined by RNA-seq of three biological replicates per treatment/genotype combination. Yellow, orange and red indicate the number of genes differentially expressed in wild type only, both wild type and *Mtcre1* and *Mtcre1* only, respectively.

a Bonferroni-corrected p -value smaller than 0.001 (**Supplemental Table 1**). This limited number of differentially expressed genes between wild type and *Mtcre1* is consistent with the absence of any obvious root phenotype in *Mtcre1* [37]. Using the same criteria, we identified 814 genes in *M. truncatula* wild-type roots that display differential expression upon rhizobium LCO signalling. Of these genes, 609 were up-regulated and 205 were down-regulated (**Supplemental Table 2**). Among the up-regulated genes are *MtENOD11* and *MtERN1* as well as the cytokinin responsive genes *MtNIN*, *MtCLE13*, *MtRR4*, *MtRR5*, *MtRR8*, *MtRR9* and *MtRR11* that were found in previous studies (**Supplemental Figure 1**) [35, 36, 56-58]. In case of the *Mtcre1* mutant, only 243 genes were differentially expressed upon LCO application (233 up-regulated and 10 down-regulated). Comparing the transcriptional changes observed in wild type and *Mtcre1* mutant after rhizobium LCO application demonstrates that a large set of 596 genes, which corresponds to ~73% of the genes differentially expressed in wild type, are dependent on MtCRE1-mediated signalling ($FC > 2$, $p < 0.001$) (**Figure 1**). Twenty-five genes showed significant differential expression upon LCO treatment in the *Mtcre1* mutant specifically ($FC > 2$, $p < 0.001$) (**Supplemental Table 3**).

To validate our data, the expression of the set of differentially regulated genes is compared to that on the Medicago Gene Expression Atlas [59]. To this end, an averaged expression was calculated over several studies comparable to ours, consisting of samples taken at six and 24

hours after LCO treatment and one, three and five days after rhizobium inoculation [59-61]. Of the 814 differentially regulated genes identified in wild-type *M. truncatula*, 623 genes are represented by at least one unique probe on the array. Of these, ~82% (508 genes) behave similar in the reported microarray studies (**Supplemental Figures 2-3**). Approximately 10% (63 genes) of the genes responded exclusively in our experiment, whereas ~8% (52 genes) responds differently between both sets (**Supplemental Figure 2** and **Supplemental Table 5**). Overall, this comparison suggests that the RNA-seq data are in line with previous studies.

Gene Ontology (GO) enrichment analysis identified a significant overrepresentation of 48 GO terms associated with genes differentially regulated in wild-type *M. truncatula* specifically ($p < 0.05$). These included phosphorelay response regulator activity, adenine phosphoribosyl transferase activity and 1-aminocyclopropane-1-carboxylate synthase activity (**Supplemental Table 6**). This points to an induction of cytokinin signalling, cytokinin turnover and ethylene biosynthesis, respectively. The same analysis on genes differentially expressed in both wild type and *Mtcre1* mutant after rhizobium LCO exposure revealed 27 significantly enriched GO terms ($p < 0.05$) (**Supplemental Table 7**). Among these are GO terms associated with chitin breakdown and phosphorylation of inositol phosphates. These data show that rhizobium LCO signalling induces rapid transcriptional reprogramming, which to a large extent is dependent on a functional MtCRE1 cytokinin receptor. This implies that cytokinin has a key function at the preinfection stage of the rhizobium-legume interaction, three hours after rhizobium LCO perception.

Spatiotemporal Localization of the Cytokinin Response

Since cytokinin signalling is an essential step in nodule formation, we questioned in which cell layers cytokinin signalling is induced upon rhizobium LCO perception. To this end, we used the synthetic cytokinin reporter TCS driving β -glucuronidase (*TCS:GUS*) [62]. This chimeric cytokinin reporter construct was introduced in *M. truncatula* roots using *Agrobacterium rhizogenes*-mediated transformation. Spatiotemporal expression analysis revealed a moderate level of variation between individual transgenic roots carrying *TCS:GUS*. Despite this level of variation, faint expression of the *TCS:GUS* reporter is observed in some, but not all, pericycle cells in mock-treated roots. This is consistent with observations in arabidopsis, where expression of the TCS reporter was observed in xylem pole pericycle cells specifically [63]. Application of rhizobium LCOs ($\sim 10^{-9}$ M) increased expression of the *TCS:GUS* reporter in the susceptible zone specifically in ~30% of the transgenic roots ($n = 12/38$). Sectioning of these root segments revealed induction of *TCS:GUS* in the cortex, endodermis and pericycle (**Figure 2**). This suggests that cytokinin acts as a non-cell-autonomous signal, during the early stages of the legume-rhizobium interaction.

Figure 2. Expression of the synthetic cytokinin responsive reporter *TCS:GUS* in *M. truncatula* transgenic roots upon application of rhizobium LCOs.

(A-B) Whole mount images of mock-treated **(A)** or rhizobium LCOs-treated (10^{-9} M, 3 h) **(B)** transgenic roots stained for GUS activity. Transgenic roots were generated by *Agrobacterium rhizogenes*-mediated transformation.

(C-D) Sections through the susceptible zone of roots shown in **(A)** and **(B)**, respectively. Sections are counter stained with ruthenium red. Scale bars represent 50 μ m.

Rhizobium LCO Signalling Induces a Change in the Bioactive Cytokinin Pool

Mining the transcriptome data, we noted differential expression of genes involved in cytokinin biosynthesis and/or activation. A putative *IPT* (Medtr2g022140), *CYP735A* (Medtr6g017325) and *LOG* (Medtr1g057020) homolog were induced three hours after LCO application (**Figure 3A**). We refer to these genes as *MtIPT1*, *MtCYP735A1* and *MtLOG3* based on consecutive numbering in relation to a previous study [48]. Expression of *MtCYP735A1* is induced ~6-fold in wild type and ~2.5-fold in *Mtcre1* after LCO application (**Figure 3A**), although induction in *Mtcre1* was not found to be significant when using the stringent correction for multiple testing. This suggests that LCO-induced expression of *MtCYP735A1* may be partly dependent on MtCRE1. By contrast, *MtIPT1* expression is induced ~4-fold in both wild-type *M. truncatula* and *Mtcre1* mutant, indicating that induction of this gene by rhizobium LCOs is MtCRE1 independent. This suggests that rhizobium LCO-induced signalling leads to (local) cytokinin biosynthesis. Besides cytokinin synthesis genes, genes involved in cytokinin metabolism are also induced by rhizobium LCOs (**Figure 3B**). These include two putative *APT* (Medtr3g106780, Medtr5g012210) and two putative *CKX* (Medtr4g126150, Medtr2g039410) homologs, referred to as *MtAPT1*, *MtAPT2* and *MtCKX2*, *MtCKX3*, respectively [58]. These genes are induced in wild-type *M. truncatula* but not *Mtcre1*. As *MtLOG3* is also induced in an MtCRE1-dependent manner, this suggests a set of feedback loops tightly regulating LCO-induced cytokinin levels. Therefore, these data indicate that LCO signalling may induce a rapid change in the cytokinin concentration in the *M. truncatula* root susceptible zone.

Figure 3. Rhizobium LCO signalling induces expression of genes involved in cytokinin and ethylene biosynthesis.

(A) Expression of genes involved in cytokinin biosynthesis in wild-type *M. truncatula* and *Mtcre1* mutant.

(B) Expression of genes involved in cytokinin turnover in wild-type *M. truncatula* and *Mtcre1* mutant.

(C) Expression of ACC synthases (ACS) in wild-type *M. truncatula* and *Mtcre1* mutant.

Expression was determined at three hours after rhizobium LCO treatment (10^{-9} M) (or mock) using RNA-seq and normalized to that in the mock-treated wild type. Expression is shown for genes that were found to be differentially expressed ($FC > 2$, $p < 0.001$) in wild type after LCO treatment. Data shown represent the means of three biological replicates $\pm$ SEM. Different letters above the bars indicate a significant difference ($p < 0.001$).

Figure 4. Time course experiment of LCO-responsive genes.

(A) Expression of *MtNIN* and cytokinin response regulators *MtRR4* and *MtRR9*.

(B) Expression of cytokinin biosynthesis genes *MtIPT1*, *MtCYP735A1* and *MtLOG3*.

(C) Expression of cytokinin oxidase/dehydrogenases *MtCKX2* and *MtCKX3*.

(D) Expression of ethylene biosynthesis genes *MtACS1-3*.

Expression was determined at 30 minutes (0.5h), one hour (1h) and three hours (3h) after rhizobium LCO treatment (10^{-9} M) (or mock) using qRT-PCR and normalized to the 3-h mock sample. ne indicates genes not expressed under the conditions specified. Data shown represent the means of three biological replicates $\pm$ SEM. Different letters above the bars indicate a significant difference ($p < 0.01$).

To obtain better insight in the regulation of cytokinin biosynthesis genes by rhizobium LCOs, we studied their expression at different time points (30 minutes, one hour and three hours after LCO exposure), and in absence or presence of the ethylene synthesis inhibitor AVG, using quantitative RT-PCR (qRT-PCR). LCO responsiveness of these samples was confirmed by strong induction of *MtNIN* (Figures 4A and 5A). Analysis of *MtIPT1* expression showed this gene is first induced at one hour after LCO treatment, whereas expression of *MtCYP735A1* and *MtLOG3* is only induced at three hours after LCO exposure (Figure 4B). Expression of *MtIPT1* and *MtCYP735A1* is induced in both the presence and absence of AVG (Figure 5B), which excludes that induction of both cytokinin biosynthesis genes by rhizobium LCOs results from low ethylene levels. Analysis of the expression of the cytokinin oxidases *MtCKX2* and *MtCKX3* and the cytokinin response regulators *MtRR4* and *MtRR9* shows that cytokinin responses are first induced at three hours after LCO exposure (Figure

4A, C). Furthermore, our data indicate that induction of cytokinin signalling by rhizobium LCOs might be inhibited by ethylene, because in the absence of AVG only *MtRR9* but not *MtRR4* expression is induced, and this response is less in the absence of AVG (~3.5- vs 10-fold, respectively) (**Figure 5A**). These data suggest rapid activation of cytokinin biosynthesis upon rhizobium LCO perception.

To test whether rhizobium LCO perception induces a change in the cytokinin concentration, we measured cytokinin levels in the *M. truncatula* root susceptible zone of wild type and the *Mtccamk/Mtdmi3* mutant at three hours after LCO exposure using ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS). For this, we focused on isoprenoid-type cytokinins because these are the predominant form found in plants [40]. Of all cytokinins measured, isopentenyl adenine (iP), iP-riboside (iPR), *trans*-zeatin (tZ), *cis*-zeatin (cZ), tZ-9-glycosylated (tZ9G) and cZ-riboside (cZR) could be detected and quantified. No change in the levels of cZ, tZ9G and cZR were detected after LCO exposure (**Supplemental Figure 4**). By contrast, the amounts of iP, iPR and tZ did increase significantly ~15, 2.5 and 8 times, respectively, in wild type upon LCO application ($p < 0.05$, **Figure 6A-C**). This response is dependent on a functional LCO signaling cascade as it was not observed in the *Mtccamk/Mtdmi3* mutant (**Figure 6A-C**). These results demonstrate that a number of cytokinins accumulate within three hours after rhizobium LCO perception, including the biologically most active cytokinins iP and tZ [41].

To position the cytokinin accumulation in the rhizobium LCO signalling pathway, we analysed this response in the *Mtnsp1*, *Mtnsp2* and *Mtnsp1Mtnsp2* double mutant. In contrast to *MtNSP2*, expression of *MtNSP1* in the *M. truncatula* wild-type root is induced ~5-fold after exposure to rhizobium LCOs, whereas only a ~2.5-fold induction is observed in the *Mtcre1* mutant (**Supplemental Figure 5**). This indicates that LCO-induced *MtNSP1* expression is partly dependent on cytokinin signalling. Next, we measured the cytokinin levels in the *Mtnsp1* and *Mtnsp2* mutants and compared them with wild type. Again we observed accumulation of iP, tZ and iPR in wild-type *M. truncatula* (**Figure 6**). Measurements in the *Mtnsp1*, *Mtnsp2* and *Mtnsp1Mtnsp2* mutant roots showed that basal cytokinin levels were similar to those in wild type (**Figure 6D-F**). As in wild type, LCO treatment induced an accumulation of cytokinin in *Mtnsp1*, *Mtnsp2* and *Mtnsp1Mtnsp2*. However, in these mutants, accumulation of iP and tZ is approximately 60-75% and 30-40% of that in the wild type, respectively (**Figure 6D-F**). Taken together, we demonstrated that rhizobium LCO signalling triggers rapid accumulation of bioactive cytokinin in a CCaMK-dependent manner. This response is controlled only partially by the GRAS-type regulators NSP1 and/or NSP2, possibly in a positive feedback loop.

Figure 5. Expression of LCO-responsive genes in the absence or presence of the ethylene synthesis inhibitor AVG.

(A) Expression of *MtNIN* and cytokinin response regulators *MtRR4* and *MtRR9*.

(B) Expression of cytokinin biosynthesis genes *MtIPT1*, *MtCYP735A1* and *MtLOG3*.

(C) Expression of ethylene biosynthesis genes *MtACS1-3*.

M. truncatula wild-type and *Mtcre1* mutant plants were grown on agar-solidified Fåhræus medium with or without 1 μ M AVG. Expression was determined at three hours after rhizobium LCO treatment (10^{-9} M) (or mock) using qRT-PCR and normalized to that in the mock-treated wild type grown in the absence of AVG. Data shown represent the means of three biological replicates $\pm$ SEM. Different letters above the bars indicate a significant difference ($p < 0.01$).

Figure 6. Cytokinins accumulate in the *M. truncatula* root susceptible zone upon *S. meliloti* LCO signalling.

(A-C) Concentrations of the cytokinins isopentenyl adenine (iP) **(A)**, isopentenyl adenine riboside (iPR) **(B)** and *trans*-zeatin (tZ) **(C)** in root susceptible zones of the *M. truncatula* wild type and *Mtccamk/Mtdmi3* mutant treated with mock or rhizobium LCOs (10^{-9} M, 3 h). Data represent the means of four biological replicates (two for *Mtccamk/Mtdmi3* mock *trans*-zeatin) $\pm$ SEM.

(D-F) Concentrations of the cytokinins isopentenyl adenine (iP) **(D)**, isopentenyl adenine riboside (iPR) **(E)** and *trans*-zeatin (tZ) **(F)** in root susceptible zones of the *M. truncatula* wild type and *Mtnsp1*, *Mtnsp2* and *Mtnsp1Mtnsp2* mutants treated with mock or rhizobium LCOs (10^{-9} M, 3 h). Plants were grown on agar-solidified Fåhræus medium containing 1 μ M AVG. Data represent the means of 5-6 biological replicates $\pm$ SEM.

Different letters above the bars indicate a statistical difference ($p < 0.05$). Statistical significance was assessed based on ANOVA.

Ethylene Inhibits Symbiotic Cytokinin Accumulation

Previous work has shown that cytokinin can induce ethylene production [64-66]. Based on this, we hypothesized that LCO-induced cytokinin accumulation might also induce ethylene production. To test this hypothesis, we checked for ethylene biosynthesis genes among

Figure 7. Ethylene negatively regulates LCO-induced cytokinin accumulation.

(A–C) Concentrations of the cytokinins isopentenyl adenine (iP) (A), isopentenyl adenine riboside (iPR) (B) and *trans*-zeatin (tZ) (C) in root susceptible zones of the *M. truncatula* wild type and *Mtein2/Mtsickle* mutant treated with mock or rhizobium LCOs (10⁻⁹ M, 3 h). Plants were grown on agar-solidified Fåhræus medium without AVG. Data represent the means of six biological replicates ± SEM. Different letters above the bars indicate a statistical difference ($p < 0.05$). Statistical significance was assessed based on ANOVA.

the differentially expressed genes identified through RNA-seq. This revealed an MtCRE1-dependent transcriptional induction of three ACS genes (Medtr8g101750, Medtr8g101820, Medtr4g097540) by rhizobium LCOs. However, close inspection of Medtr8g101820 revealed that this gene model either represents a pseudogene, or an error in the current genome assembly (Mt4.0; see **Supplemental Figure 6**). For the other two ACS genes, Medtr8g101750 (*MtACS1*) and Medtr4g097540 (*MtACS2*), transcriptional induction of by rhizobium LCOs is strongly supported (**Figure 3C**). A detailed look at the expression of all putative ethylene biosynthesis genes revealed induction of an additional ACS gene (Medtr6g091760, *MtACS3*) just below the 2-fold threshold ($p < 0.001$, FC = 1.95) (**Figure 3C**).

qRT-PCR reactions on the root samples taken at different time points after LCO treatment, showed that all three ACS genes are induced already at 30 minutes after LCO exposure (**Figure 4D**). At three hours after LCO exposure, expression of all three ACS genes is induced at > 7-fold; however, we also observed dampening of expression at one hour after LCO treatment (**Figure 4D**). This may suggest a fast induction of ACS genes, which may occur in a cytokinin-independent fashion, followed by a strong MtCRE1-dependent induction at three hours after LCO exposure. Induction of *MtACS1* occurs in both the presence and absence of AVG, although induction is much stronger if AVG is added to the growth medium (~3.5- vs ~40-fold) (**Figure 5C**). Expression of *MtACS2* and *MtACS3* is induced only in the presence of AVG, suggesting that induction of these genes requires low ethylene levels (**Figure 5C**). These results indicate an MtCRE1-dependent transcriptional induction of at least one ethylene biosynthesis gene by rhizobium LCOs. This suggests that LCO-induced cytokinin accumulation promotes ethylene biosynthesis.

Ethylene is a known inhibitor of rhizobium LCO signalling and subsequent nodule formation [19, 27]. Therefore, cytokinin-dependent induction of ethylene biosynthesis genes by rhizobium LCOs may restrict LCO-induced cytokinin accumulation. The latter is supported by the effect of AVG on the responsiveness of cytokinin response regulators to rhizobium LCOs (**Figure 5A**). To determine the effect of ethylene on cytokinin accumulation, we exploited the *M. truncatula* ethylene insensitive mutant *Mtein2/Mtsickle*. To this end, *M. truncatula* wild type and *Mtein2/Mtsickle* plants were grown on medium without AVG and treated with rhizobium LCOs for three hours. Under these conditions, wild type accumulated 2- to 3-fold higher concentrations of iP, iPR and tZ after LCO treatment (**Figure 7**). This response seems weaker than compared with the accumulation detected in plants grown on regular plant growth medium that contains 1 μ M AVG (**Figure 6A-C**), indicating an inhibitory effect of ethylene. Cytokinin measurements in *Mtein2/Mtsickle* mutant also indicate negative regulation by ethylene. In this mutant, LCO treatment induced a 15-fold increase in iP concentration, a response five times stronger compared with wild type (**Figure 7A**). Taken together, we conclude that rhizobium LCO-induced cytokinin accumulation is negatively affected by ethylene.

DISCUSSION

Cytokinin signalling is an integral part of the signalling pathway leading to nodule organogenesis. However, whether cytokinin accumulates as a signalling intermediate and to what extent this is required for rhizobium LCO-induced signalling remained elusive. Here, we show that in *M. truncatula*, cytokinin rapidly accumulates in the root susceptible zone upon application of rhizobium LCOs. The accumulating cytokinins include iP and tZ, which are among the biologically most active cytokinins [41]. Furthermore, we show that the majority of transcriptional changes induced three hours after rhizobium LCO treatment are dependent on the cytokinin receptor, MtCRE1. Therefore, we conclude that cytokinin accumulation is a key step in the pathway leading to rhizobium root nodule organogenesis.

Using RNA-seq, we determined the extent of LCO-induced signalling dependent on MtCRE1. This revealed that the vast majority (~73%) of the transcriptional changes induced three hours after LCO application are dependent on this symbiotic cytokinin receptor. Genes that are differentially expressed in both wild type and *Mtcre1* probably function upstream of MtCRE1 or in a cytokinin-independent pathway involved in rhizobial infection [67]. Examples of these are the cytokinin biosynthesis gene *MtIPT1* and genes involved in chitin breakdown. The latter could be involved in removing excess rhizobium LCO signals or could be part of a general defence response induced by these bacterial signal molecules [68, 69]. In addition,

some level of redundancy may contribute to the transcriptional changes observed in *Mtcre1* upon rhizobium LCO exposure. The *Mtcre1-1* mutant used in this study is considered a full knock-out as it has a mutation that creates a premature stop codon in the middle of the kinase domain [37]. It has been reported that in rare cases this mutant can develop a few nodules [37], even though we did not observe nodules on *Mtcre1-1* mutant plants in any of our experiments (data not shown). In case of *L. japonicus*, a similar phenotype of the *Ljlhk1-1* mutant could be explained by redundant functioning of the paralogous genes, *LjLHK1A* and *LjLHK3*, indicating the existence of partial redundancy for symbiotic cytokinin perception [70]. However, in the *M. truncatula* *Mtcre1-1* mutant redundancy by additional histidine receptors in symbiotic cytokinin signalling is most likely limited, as the expression of typical cytokinin responsive genes (e.g. the A-type Response Regulators) is not or only moderately induced in this mutant upon LCO application (**Figure 5A** and **Supplemental Figure 1**). Therefore, we conclude that most genes induced three hours after rhizobium LCO application function in the MtCRE1-dependent nodule organogenesis pathway.

Measurements of the cytokinin concentration in *M. truncatula* root susceptible zones showed accumulation of three cytokinins three hours after rhizobium LCO application. This response is dependent on rhizobium LCO signalling, as it was not observed in the *Mtccamk/ Mtdmi3* mutant. Our observation that both iP and its biosynthetic precursor iPR accumulate upon rhizobium LCO exposure suggests that local biosynthesis is the primary source of these cytokinins. This is supported by induction of the cytokinin biosynthesis gene *MtIPT1* at one hour after LCO exposure in both wild type and *Mtcre1* mutant. However, considering the time frame in which cytokinin accumulates upon LCO signalling, we cannot exclude that the increase in cytokinin concentration is regulated primarily at the protein level.

We also determined the role of the GRAS-type transcriptional regulators, NSP1 and NSP2, in LCO-induced cytokinin accumulation and showed that iP and tZ accumulate to intermediate levels in both single and double mutants compared with wild type. Initially, it was argued that these GRAS-type proteins may act as primary rhizobium LCO response factors [71], for which indeed some experimental evidence was found [16, 72]. However, more in-depth genetic dissection of the rhizobium LCO signalling pathway suggests a more complex role for both proteins, downstream of as well as parallel to symbiotic cytokinin signalling [67]. Our finding shows that both NSP1 and NSP2 may function downstream of symbiotic cytokinin biosynthesis in an autoactivation loop. Such hypothesis is supported by the finding that *MtNSP1* expression is induced three hours after LCO treatment and this induction is in part dependent on MtCRE1.

We uncovered a negative feedback relation between cytokinin and ethylene. Cytokinin measurements showed that upon LCO signalling, the *M. truncatula Mtein2/Mtsickle* knock-out mutant accumulates more cytokinin in the root susceptible zone than wild-type plants. This implies that ethylene signalling inhibits symbiotic cytokinin accumulation. This finding is in line with earlier studies that showed that ethylene interferes with LCO-induced calcium oscillations in and around the nuclei of activated cells [19, 73] and with the absence of cytokinin accumulation in the *Mtccamk/Mtdmi3* mutant (**Figure 6A-C**). Our data also indicate a cytokinin-dependent transcriptional induction of (at least) one ACC synthase by rhizobium LCOs. This is in line with results on *L. japonicus*, which show that the ethylene-producing ACC oxidase *LjACO2* is also induced by rhizobium LCOs [74]. Furthermore, previous work has shown that cytokinin can induce ethylene production [64-66]. Therefore, it is conceivable that LCO signalling, through an induction of cytokinin accumulation, might induce ethylene biosynthesis. This response could be part of a negative feedback signal to inhibit further LCO signalling and subsequent cytokinin accumulation (**Figure 8**). Such a feedback mechanism might be important to restrict LCO-induced cytokinin accumulation to a limited number of cells to prevent initiation of multiple nodule primordia, as can be observed in the *Mtein2/Mtsickle* mutant. This response would resemble the mechanism through which nodule positioning is regulated, where ethylene biosynthesis genes are expressed opposite phloem poles, delineating nodule initiation [22]. In addition, cytokinin-induced ethylene production might be required to restrict rhizobial infections. The latter is suggested by the phenotype of the *L. japonicus Ljlhk1* mutant, which displays excessive infection thread formation upon rhizobial inoculation [18]. This phenotype suggests the absence of a negative feedback signal to inhibit rhizobium LCO-induced signalling. It is conceivable that this signal would be ethylene.

Measurements of the cytokinin concentration in wild-type *M. truncatula* showed that both iP and tZ accumulate after LCO exposure. Interestingly, accumulation of iP after LCO treatment was elevated in the *Mtein2/Mtsickle* mutant compared to wild type, whereas accumulation of tZ was comparable for both genotypes. This suggests that LCO-induced accumulation of iP is more sensitive to negative regulation by ethylene, as compared to accumulation of tZ. However, the mechanistic basis for this difference is currently unclear. Sensitivity to different cytokinins differs between individual cytokinin receptors [75, 76]. The arabidopsis AtAHK4/AtCRE1 cytokinin receptor possesses similar sensitivity to both iP and tZ [75, 76]. AtAHK4/MtCRE1 represents that closest orthologue of MtCRE1 and LjLHK1 and was shown to functionally complement the nodulation defect of the *Ljlhk1* mutant [35-37, 70]. Therefore, it seems plausible that MtCRE1 and LjLHK1 also display equal sensitivity to iP and tZ, leaving the individual contributions of these cytokinins to nodulation undetermined.

Figure 8. Proposed model for the position of cytokinin and ethylene in the rhizobium LCO signalling pathway.

Recent work on *L. japonicus* showed that cytokinins might function not only to induce nodulation but also to suppress nodule formation as part of the autoregulation of nodulation (AON) [77]. Activation of CLE-RS1/2-HAR1 signalling increased levels of the iPRP cytokinin precursors in shoots, suggesting a role for cytokinins in AON. Increased iPRP levels in shoots was linked to an induction of *LjIPT3* in shoots at three days after rhizobium inoculation [77]. This response is much slower than the accumulation of cytokinins that we observed, which occurred at three hours after LCO application. Therefore, it is highly unlikely that the increase in cytokinin concentrations that we observed is caused by activation of AON. Furthermore, Sasaki et al. [77] did not report increased levels of cytokinin in roots after activation of AON. Therefore, it remains undetermined whether cytokinins function in roots to repress nodule formation upon activation of AON. Cytokinin as part of AON seems to function upstream of TML, a Kelch-repeat containing F-box protein [77]. TML appears to inhibit nodule formation downstream of cytokinin signalling, as spontaneous nodule formation induced by *snf2*, a

gain-of-function allele of the cytokinin receptor *LjLHK1*, is also suppressed by TML [78]. Identification of the targets of TML might show if and how cytokinins might function to both induce and suppress nodule initiation.

Nodule primordium formation results from mitotic activation of root cortical and pericycle cells and is associated with the formation of a local auxin maximum [79-83]. Mathematical modelling revealed that creation of such an auxin maximum most likely results from a reduction in auxin transport [84]. In arabidopsis, cytokinin has been shown to affect auxin transport by targeting the PIN auxin efflux carriers at both the gene expression and protein level [85-87]. Also in legumes, cytokinin seems to affect auxin transport. In *Mtcre1* mutant roots, expression of several *PIN* genes is affected and the polar auxin transport rate is increased [37]. Furthermore, reduction of the polar auxin transport rate observed upon inoculation with rhizobia does not occur in *Mtcre1* mutant roots [37]. Based on this, it is hypothesized that cytokinin is the signal that induces auxin accumulation in the root cortex and pericycle upon epidermal perception of rhizobium LCOs [88]. Rapid accumulation of cytokinin and induction of cytokinin signalling in the root cortex, endodermis and pericycle, as observed by *TCS:GUS* expression, suggests that cytokinin functions non-cell-autonomously. We did not observe activation of *TCS:GUS* expression in the root epidermis after rhizobium LCO treatment. This would suggest that cytokinin signalling and possibly cytokinin biosynthesis take place in the root cortex. However, studies on cytokinin response regulators did show activation of cytokinin responses in the root epidermis after rhizobium LCO-induced signalling [36, 58, 89]. A recent study on redundant function of cytokinin receptors in *L. japonicus* also suggests a role for epidermal cytokinin signalling in root nodule initiation [70]. The authors show that cytokinin receptors *LjLHK1A* and *LjLHK3* can partially rescue the *Ljlhk1* mutant phenotype. However, in this case nodule formation is only initiated after rhizobium bacteria reach the root cortex. As only *LjLHK1* is expressed in the root epidermis, this suggests that epidermal cytokinin signalling is required for nodule formation [70]. This is consistent with a function for cytokinin as systemic signal and suggests that cytokinin might bridge the gap between epidermal perception of rhizobium LCOs and initiation of nodule primordia in the interior cell layers of the legume root.

Taken together, we have demonstrated that rhizobium LCOs cause an increase in the abundance of various cytokinins prior to the 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 existing developmental modules have been co-opted during the evolution of legume-rhizobium root nodule symbiosis.

METHODS

Plant Materials and Treatments

Medicago truncatula Jemalong A17, *Mtccamk/Mtdmi3* (TRV25) [90], *Mtnsp1-1* (B85) [71, 90], *Mtnsp2-2* (0-4) [91, 92], *Mtnsp1Mtnsp2* [93], *Mtcre1-1* [37] and *Mtein2/Mtsickle-1* mutant [94] seedlings were grown vertically for three days on modified Fåhræus medium agar plates with low nitrate (including 0.2 mM $\text{Ca}(\text{NO}_3)_2$) supplemented with 1 μM AVG, unless stated otherwise [95]. Then, *Sinorhizobium meliloti* 2011 Nod factors ($\sim 10^{-9}$ M) or water as a control was pipetted on top of the root susceptible zone. Roots were exposed for three hours and subsequently 1-cm root pieces were cut just above the root tip and snap-frozen in liquid nitrogen ($\sim n = 50$ for cytokinin extractions, $\sim n = 15$ for RNA isolation). For all experiments, plants were grown in an environmentally controlled growth chamber at 20°C with a 16-h light/8-h dark cycle and 70% relative humidity.

Vectors and Constructs

The synthetic cytokinin reporter element *TCS* was recombined into a pENTR-D-Topo (Invitrogen, Carlsbad, USA) thereby creating pENTR1-2_TCS [62]. Subsequently, this construct was recombined into pKGWFS7-RR containing a GUS-GFP fusion reporter by a gateway reaction (Invitrogen, Carlsbad, USA). All binary destination vectors used in this study contain AtUBQ10_{pro}:DsRed1 as selectable marker [97]. 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 Transformation and Histology

Agrobacterium rhizogenes-mediated root transformation was used to transform *M. truncatula* (Jemalong A17) as described by Limpens et al. [98]. Transgenic roots were selected based on DsRED1 expression. Transgenic roots were transferred to low nitrate Fåhræus plates (including 0.2 mM $\text{Ca}_2(\text{NO}_3)_2$) supplemented with 1 μM AVG three weeks after transformation. After five days on these plates, *Sinorhizobium meliloti* 2011 Nod factors ($\sim 10^{-9}$ M) or water as a control was pipetted on top of the root zone susceptible to rhizobium infection. Roots were GUS stained three hours after treatment and fixed as described in Limpens et al. [99]. Microtome sections of 9 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 CS6 (Adobe Systems, San Jose, USA).

RNA Sequencing

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's protocol. RNA was sequenced at BGI Tech Solutions (Beijing Genomics Institute, Hong Kong, China) using the Illumina Truseq (Transcriptome) protocol utilizing an Illumina Hiseq 2000 instrument. This generated 90-bp paired-end reads with an average insert size of 130-160 bp. In total 24-26 million clean reads were generated for each sample (**Supplemental Table 8**). Sequencing data were analysed by mapping RNA-seq reads against the *M. truncatula* genome (Mt4.0) using the RNA-seq module implemented in CLC Genomics Workbench v7.5 (CLC bio, Aarhus, Denmark) with default settings, with the exception of the similarity fraction, which was changed to 0.95. As such, > 95% of the reads generated for each sample were successfully mapped against the *M. truncatula* genome (Mt4.0), of which > 90% mapped in pairs (**Supplemental Table 8**). Differentially expressed genes were selected based on a minimal fold change of 2 and a Bonferroni-corrected *p*-value < 0.001, as determined using the EdgeR test implemented in CLC [100]. GO enrichment analysis was performed using the Hypergeometric tests on annotations method implemented in CLC.

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's protocol. cDNA was synthesized from 1 µg total RNA using an i-script cDNA synthesis kit (Bio-Rad, Hercules, USA) as described in the manufacturer's protocol. Real time qRT-PCR was set up in a 10-µl reaction system with 2× iQ SYBR Green Super-mix (Bio-Rad, Hercules, USA). The experimental setup and procedure were executed using a CFX Connect optical cycler, according to the protocol provided by the manufacturer (Bio-Rad, Hercules, USA). All primers including the genes used for normalization (*MtUBQ10* and *MtPTB*) are given in **Supplemental Table 9**. Data analysis was performed using CFX Manager 3.0 software (Bio-Rad, Hercules, USA). Cq values of 32 and higher were excluded from the analysis, though still checked for transcriptional induction (see **Supplemental Table 9**). Statistical significance was determined based on Student's *t*-test (*p* < 0.01), as implemented in CFX Manager 3.0 software (Bio-Rad, Hercules, USA).

Cytokinin Extraction

For extraction of cytokinins from *M. truncatula* root material, ~100 mg of snap-frozen plant material (root susceptible zones) was used. Tissue was ground to a fine powder using 3-mm stainless steel beads at 50 Hz for 1 min in a TissueLyser LT (Qiagen, Germantown, USA). Ground root samples were extracted with 1 ml of 100% methanol (MeOH) containing 2.5

mM of diethyldithiocarbamic acid and stable isotope-labelled cytokinin internal standards (IS, **Supplemental Table 10** numbers 1-7) were used at a concentration of 100 pmol per compound per sample. Samples were extracted by short vortexing and ultrasonication during 10 min. Subsequently, samples were centrifuged at 2000 rpm for 10 min in a tabletop centrifuge at room temperature (RT). Supernatants were transferred to 4 ml glass vials. Pellets were re-extracted with 1 ml 100% MeOH for one hour on a shaker at 4°C. After centrifugation as above, both supernatants were pooled and MeOH evaporated in a speed vacuum system (SPD121P, Thermo Savant, Hastings, UK) 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 speed vacuum system at RT and the residue resuspended in 200 µl acetonitrile/water/formic acid (10:90:0.1, v/v/v). The sample was filtered through a 0.45-µm Minisart SRP4 filter (Sartorius, Goettingen, Germany) and stored at -20°C.

Cytokinin Detection and Quantification by Liquid Chromatography-Tandem Mass Spectrometry

Analyses of cytokinins in *M. truncatula* root extracts were performed by comparing retention times and mass transitions with those of cytokinin standards (**Supplemental Table 10**) 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 [101, 102]. 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 water/acetonitrile gradient started from 0.2% (v/v) of acetonitrile for 1.5 min increasing 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 50°C with a flow rate of 0.6 ml min⁻¹. The sample injection volume was 20 µl. The mass spectrometer was operated in positive electrospray ionization mode. Cone and desolvation gas flows were set to 50 and 1000 l h⁻¹, respectively. The capillary voltage was set at 3.0 kV, the source temperature at 150°C and the desolvation temperature at 650°C. 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 different cytokinins and deuterium-labelled cytokinins were set using the IntelliStart MS Console. MRM transitions selected for cytokinin identification and quantification are shown in **Supplemental Table 10**. Cone voltage was set to 18 eV.

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 the corresponding deuterium-labelled cytokinins (**Supplemental Table 10** numbers 1-7). 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.

Accession Numbers

RNA-seq reads have been deposited in the Array-Express (<http://www.ebi.ac.uk/arrayexpress>) database under accession number E-MTAB-3007.

SUPPLEMENTAL DATA

Supplemental data belonging to this chapter are available at *Molecular Plant Online* (<https://doi.org/10.1016/j.molp.2015.03.010>).

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Chapter 4

The strigolactone biosynthesis gene
DWARF27 is co-opted
in rhizobium symbiosis

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ABSTRACT

Background

Strigolactones are a class of plant hormones of which biosynthesis is activated in response to phosphate starvation. This involves several enzymes, including the carotenoid cleavage dioxygenases 7 (CCD7) and CCD8 and the carotenoid isomerase DWARF27 (D27). *D27* expression is known to be responsive to phosphate starvation. In *Medicago truncatula* and rice (*Oryza sativa*), this transcriptional response requires the GRAS-type proteins NSP1 and NSP2; both proteins are essential for rhizobium induced root nodule formation in legumes. In line with this, we questioned whether MtNSP1-MtNSP2 dependent *MtD27* regulation is co-opted in rhizobium symbiosis.

Results

We provide evidence that MtD27 is involved in strigolactone biosynthesis in *M. truncatula* roots upon phosphate stress. Spatiotemporal expression studies revealed that this gene is also highly expressed in nodule primordia and the meristem and distal infection zone of mature nodules. A similar expression pattern was found for *MtCCD7* and *MtCCD8*. Rhizobium lipo-chitooligosaccharide (LCO) application experiments revealed that, of these genes, *MtD27* is most responsive in an MtNSP1- and MtNSP2-dependent manner. Symbiotic expression of *MtD27* requires components of the symbiosis signaling pathway, including MtDMI1, MtDMI2, MtDMI3/MtCCaMK and, in part, MtERN1. This in contrast to *MtD27* expression upon phosphate starvation, which only requires MtNSP1 and MtNSP2.

Conclusion

Our data show that the phosphate starvation-responsive strigolactone biosynthesis gene *MtD27* is also rapidly induced by rhizobium LCO signals in an MtNSP1- and MtNSP2-dependent manner. Additionally, we show that *MtD27* is co-expressed with *MtCCD7* and *MtCCD8* in nodule primordia and in the infection zone of mature nodules.

BACKGROUND

Legumes evolved the capacity to live in an intimate endosymbiosis with nitrogen-fixing rhizobium bacteria. To host rhizobia intracellularly, nodules are formed on the root of the plant. These nodules provide optimal physiological conditions to the bacteria to fix atmospheric nitrogen gas into ammonia. Recent studies have suggested a role for strigolactones in rhizobium symbiosis [1–6]. Here, we focus on expression of the strigolactone biosynthesis gene *DWARF27* during the legume-rhizobium interaction.

Nodule formation is initiated upon perception of lipo-chitoooligosaccharide (LCO) signals excreted by compatible rhizobium bacteria [7]. These signals mitotically activate root cortical and pericycle cells, resulting in the formation of a nodule primordium [7,8]. Rhizobium LCOs (also known as Nod factors) are also required to initiate an infection process to establish intracellular accommodation of the prokaryotic endosymbiont. This infection process starts in curled root hairs where a tube-like structure is formed intracellularly, which guides the rhizobia to the newly formed nodule primordium. There, the rhizobia are released as organelle-like structures (named symbiosomes), which remain surrounded by a plant-derived membrane. These symbiosomes act as nitrogen-fixing units that provide ammonia to the plant cell in exchange of nutrients [7].

Rhizobium LCO signals are perceived by a specific set of LysM-type receptor kinases at the root epidermis. This activates a signaling cascade that is shared with a more ancient endosymbiosis: that between land plants and arbuscular mycorrhizal fungi [7]. This signaling cascade consists of a plasma membrane-localized LRR-type receptor kinase (named MtDMI2 in *Medicago truncatula*), a cation ion channel in the nuclear envelope (MtDMI1 in *M. truncatula*), and a nuclear-localized Ca^{2+} /calmodulin dependent protein kinase (MtCCaMK/MtDMI3 in *M. truncatula*) [9–11]. Downstream of this cascade, CCaMK phosphorylates the transcriptional activator CYCLOPS, which orchestrates symbiotic root nodule development in conjunction with other transcription factors [12]. Among these are the GRAS-type transcriptional regulators NSP1 and NSP2 [13–15]. Experiments in heterologous systems have revealed that NSP2 can form a heterodimer with NSP1, suggesting a regulatory link between both proteins [16]. Both transcriptional regulators are essential for rhizobium symbiosis, but also promote mycorrhizal infection [17–20].

Under non-symbiotic conditions, NSP1 and NSP2 regulate the expression of *DWARF27* (*D27*), which encodes a key enzyme in strigolactone biosynthesis [17,21]. Strigolactones are a class of plant hormones derived from all-trans- β -carotene [22–24]. They are produced

mainly in the root of the plant and the biosynthesis of their basic structure involves at least three plastid-localized enzymes: the carotenoid isomerase D27 and the carotenoid cleavage dioxygenases 7 (CCD7) and CCD8 [22]. The subsequent activity of these three enzymes results in biosynthesis of carlactone, a precursor to the strigolactones. The conversion of carlactone to strigolactones is not completely resolved yet but there is strong evidence to support that this involves a cytochrome P450 enzyme, encoded by *MAX1* in *Arabidopsis* (*Arabidopsis thaliana*) and rice (*Oryza sativa*) [25–28]. This biosynthetic pathway is under the control of a nutrient sensing mechanism and/or the nutrient status of the plant [29–31]. Especially in plants grown under phosphate-limited conditions, *D27* expression and subsequent strigolactone production is markedly increased in an NSP1- and NSP2-dependent manner [17,29].

Under phosphate limitation, strigolactones are exuded into the rhizosphere to attract arbuscular mycorrhizal fungi [31–34]. These obligatory biotrophic (symbiotic) fungi can sense strigolactones and respond with an increased hyphal branching, thereby, promoting colonization of plant roots [32,35,36]. The endomycorrhizal symbiosis facilitates nutrient exchange between fungus and plant. Mycelium that remains in the soil markedly increases the plant root capacity to access nutrients, especially immobile phosphates. The plant receives these nutrients from the fungi at the expense of carbohydrates [37]. Although strigolactones are not essential for establishment of an endomycorrhizal symbiosis, they contribute significantly to increasing root infection levels [33].

Several reports suggest also a role for strigolactones in legume nodule formation. In *Medicago sativa* and *M. truncatula*, application of 0.1 μ M GR24 was shown to promote nodule formation, whereas slightly higher concentrations inhibited nodule formation in *M. truncatula* [1,5]. In pea (*Pisum sativum*) and *Lotus japonicus*, strigolactone-deficient mutants were shown to produce less nodules, which could be rescued by external application of GR24 [2–4]. Furthermore, in *M. truncatula* root hairs, *MtD27* and *MtCCD8* expression is increased at 5 days post rhizobium inoculation [6]. This implies that *MtD27* expression is regulated also in a symbiotic context. However, the precise regulatory network remains unknown.

Here, we show that in *M. truncatula*, *MtD27* expression is induced by rhizobium LCOs in an MtNSP1- and MtNSP2-dependent manner, similar as found for the induction of *MtD27* by phosphate starvation. However, only induction of *MtD27* by rhizobium LCOs requires the symbiosis signaling cascade. Using promoter-reporter constructs, we show that *MtD27* is expressed throughout nodule formation. After early activation in the epidermis, its expression becomes restricted to the nodule primordium and subsequently to the nodule

Figure 1. Bayesian phylogeny of D27 and D27-like proteins.

Phylogeny was reconstructed based on an alignment of D27 and D27-like proteins from *Arabidopsis* (At), soybean (*Glycine max*) (Glyma), *Lotus japonicus* (Lj), *M. truncatula* (Medtr), rice (Os), poplar (*Populus trichocarpa*) (Potri) and grapevine (*Vitis vinifera*) (VIT). Branch support is indicated by posterior probabilities. Terminals are labeled by their gene name or genbank identifier. Proteins identified from *M. truncatula* are highlighted in bold. The D27 orthology group containing rice OsD27 (gi|2549466546) and *Arabidopsis* AtD27 (At1G03055) is highlighted in blue. Mid-point rooting was applied for better tree visualization.

meristem and infection zone. Furthermore, we show that in nodule primordia and mature nodules *MtD27* is co-expressed with *MtCCD7* and *MtCCD8*.

RESULTS

MtD27 Is Involved in Strigolactone Biosynthesis

The *M. truncatula* D27 ortholog (Medtr1g471050) was identified previously [17]. By searching the *M. truncatula* genome annotation Mt4.0, we noted the presence of 3 close homologs of the original *MtD27* gene. To get insight in the relation of these genes to *MtD27*, we conducted a phylogenetic analysis based on an alignment of D27(-like) proteins of

Figure 2. Effect of *MtD27* knock-down on strigolactone biosynthesis.

(A) Relative transcript abundance as determined by qRT-PCR of *MtD27* and *Medtr7g095920* in *M. truncatula* transgenic roots expressing an empty vector control construct (EVi) or *MtD27* RNAi construct (D27i). Relative transcript abundance was normalized against *MtD27* transcript abundance in roots transformed with the empty vector control (EVi).

(B) Relative quantity of the strigolactone dihydro-orobanchol (DDH) (peak area/g FW) in root exudates collected from *M. truncatula* transgenic roots expressing an empty vector control construct (EVi) or *MtD27* RNAi construct (D27i).

(C) Relative quantity of the strigolactone dihydro-orobanchol (DDH) (peak area/g FW) in root extracts collected from *M. truncatula* transgenic roots expressing an empty vector control construct (EVi) or *MtD27* RNAi construct (D27i). Data shown represent the means of 4-5 biological replicates $\pm$ SEM. Different letters above the bars indicate a statistical difference ($p < 0.05$, Student's *t*-test).

different plant species, including rice and *Arabidopsis* (Figure 1). This showed that these sequences grouped in three separate phylogenetic clades (Figure 1), consistent with a previous report [38]. Interestingly, the D27 clade contains 2 proteins from *M. truncatula*: *MtD27* [17] as well as a close homolog *Medtr7g095920*. This shows that *M. truncatula* contains 2 putative *D27* genes that could function redundantly. Expression of *MtD27* and subsequent strigolactone production is known to be induced by phosphate starvation [17]. To get first insight in whether *Medtr7g095920* may also have a function in strigolactone production in *M. truncatula* roots, we examined its expression pattern. Analysis of publically available microarray data showed that *Medtr7g095920* expression is relatively low and has limited overlap with expression of *MtD27* [39]. In roots, *Medtr7g095920* expression is not induced by phosphate starvation. This in contrast to *MtD27* (Supplemental Figure 1) [17]. This suggests that under phosphate limiting conditions only *MtD27* might be involved in strigolactone biosynthesis in *M. truncatula* roots.

Next, we determined whether *MtD27* represents a functional enzyme in the strigolactone biosynthesis pathway. To this end, we generated *M. truncatula* compound plants bearing transgenic roots in which *MtD27* expression was reduced through RNAi-mediated knock-down. This reduced *MtD27* expression by ~65%, though with substantial variation (Figure

Figure 3. Relative transcript abundance of *MtD27* and *Medtr7g095920* after application of rhizobium LCOs.

Relative transcript abundance as determined by qRT-PCR of *MtD27* and *Medtr7g095920* in *M. truncatula* root susceptible zones 3 h after mock (-LCO) or *S. meliloti* LCO (10^{-9} M) (+LCO) treatment. Data shown represent the means of 2 biological replicates that each were analyzed in 3-fold (technical replicates) $\pm$ SEM. For each gene, transcript abundance was normalized against that of the mock-treated wild type. Different letters above the bars indicate a statistical difference ($p < 0.05$, Students' t-test).

2A). In contrast, expression of *Medtr7g095920* was not reduced, but lower than *MtD27* (**Figure 2A**). Measurements of root extracts as well as root exudates collected from the *MtD27* RNAi roots showed a 45-55% reduction in strigolactone concentrations compared with that in the empty vector control (**Figure 2B-C**). This shows that *MtD27* is involved in strigolactone biosynthesis and represents a functional ortholog of *OsD27* from rice.

Expression of *MtD27* Is Increased upon Perception of Rhizobium LCOs in an MtNSP1- and MtNSP2-Dependent Manner

To obtain insight in the symbiotic function of *MtD27*, we first determined whether its expression is responsive to rhizobium LCOs. Quantitative RT-PCR (qRT-PCR) reactions on RNA isolated from *M. truncatula* roots treated with *Sinorhizobium meliloti* LCOs ($\sim 10^{-9}$ M) for 3 h revealed that expression of *MtD27* is strongly induced (**Figure 3**). To determine whether *Medtr7g095920* is also responsive to rhizobium LCOs, we quantified its expression as well. This showed that, unlike *MtD27*, *Medtr7g095920* is not responsive (**Figure 3**). The low expression of *Medtr7g095920* in combination with its non-responsiveness to phosphate starvation and rhizobium LCOs, let us to decide to focus further studies on the symbiotic function of *MtD27*.

To acquire more insight in the symbiotic responsiveness of *MtD27*, we conducted a time series qRT-PCR experiment. To this end, *M. truncatula* seedlings were grown in Fåhræus slides [40], a system optimized to study early responses induced by rhizobium LCOs in the root epidermis. *M. truncatula* roots of wild-type plants were treated with *S. meliloti* LCOs ($\sim 10^{-9}$ M) for 0, 1, 2 and 3 h. Subsequently, total RNA was isolated from the so-called susceptible zone, a region of about 1 cm just above the root meristem. Expression analysis by qRT-PCR showed a slight induction of *MtD27* already at 1 h post LCO application and a strong > 20 -fold induction after 2-3 h compared with mock-treated roots (**Figure 4A**). This timing and induction level is comparable to that of *MtENOD11*, a gene frequently used as marker for rhizobium LCO-induced signaling in *M. truncatula* [16,41] (**Figure 4A**).

Figure 4. Symbiotic *MtD27* expression is under direct control of *S. meliloti* LCO signaling.

(A) Relative transcript abundance as determined by qRT-PCR of *MtD27* and *MtENOD11* in *M. truncatula* root susceptible zones after 0, 1, 2 or 3 h of LCO treatment (10^{-9} M).

(B) Relative transcript abundance as determined by qRT-PCR of *MtD27* at 3 h after mock (-LCO) or rhizobium LCO (10^{-9} M) (+LCO) treatment in wild type, *Mtdmi1*, *Mtdmi2* and *Mtdmi3*.

(C) Relative transcript abundance as determined by qRT-PCR of *MtD27* at 3 h after mock (-LCO) or rhizobium LCO (10^{-9} M) (+LCO) treatment in wild type, *Mtnsp1*, *Mtnsp2* and *Mtnsp1 Mtnsp2*.

(D) Relative transcript abundance as determined by qRT-PCR of *MtENOD11* at 3 h after mock (-LCO) or rhizobium LCO (10^{-9} M) (+LCO) treatment in wild type and *Mtern1*.

(E) Relative transcript abundance as determined by qRT-PCR of *MtD27* at 3 h after mock (-LCO) or rhizobium LCO (10^{-9} M) (+LCO) treatment in wild type and *Mtern1*.

(F) Relative transcript abundance as determined by qRT-PCR of *MtD27* and *MtENOD11* after mock (-LCO) or rhizobium LCO (10^{-9} M) (+LCO) treatment, in the presence or absence of 50 μ M cycloheximide (CHX).

Data shown represent the means of 3 biological replicates that each were analyzed in 3-fold (technical replicates) $\pm$ SEM. For each gene, transcript abundance was normalized against that of the mock-treated wild type. Different letters above the bars indicate a statistical difference ($p < 0.05$, Students' t-test).

Induction of *MtENOD11* expression by rhizobium LCOs requires a signaling module downstream of LCO perception consisting of MtDMI1, MtDMI2 and MtDMI3/MtCCaMK (the common symbiosis signaling module) and the transcriptional regulators MtNSP1, MtNSP2 and MtERN1 [42–44]. To investigate whether induction of *MtD27* expression by rhizobium LCOs is also dependent on this signaling module, we conducted qRT-PCR on LCO-susceptible root zones of these mutant plants, using *MtENOD11* as control. This revealed that *MtD27* expression is not induced in *Mtdmi1*, *Mtdmi2* and *Mtdmi3* mutant roots (**Figure 4B**), indicating that symbiotic induction of *MtD27* requires the common signaling module genes. Previously, we demonstrated that *MtD27* expression in non-inoculated roots is dependent on MtNSP1 and MtNSP2 [17]. Here, we found that *MtD27* expression is still induced by LCOs in an *Mtnsp1*, *Mtnsp2* and *Mtnsp1Mtnsp2* mutant background, albeit significantly lower than in wild-type roots (~2-3-fold vs ~15-fold, respectively) (**Figure 4C**). Next, we monitored *MtD27* expression in the *Mtern1* mutant, in which LCO-induced *MtENOD11* expression is blocked [45] (**Figure 4D**). In this mutant, the induction of *MtD27* by rhizobium LCOs is about half of that in the wild type (**Figure 4E**). These results show that *MtD27* is a rhizobium LCO-responsive gene whose expression in a symbiotic context is largely dependent on the common signaling module and the transcriptional regulators MtNSP1, MtNSP2 and, in part, MtERN1.

Previously, it was shown in *Vicia sativa* that the LCO inducibility of early nodulin genes is indirect, as an inhibition of protein synthesis by cycloheximide (CHX) blocks early nodulin genes expression [46]. As *MtD27* displays a comparable expression pattern as *MtENOD11*, we tested whether this gene is a primary target of rhizobium LCO signaling. To this end, *M. truncatula* seedlings were grown in Fåhræus slides for 3 days and pre-treated with 50 μ M CHX for 30 minutes prior to LCO treatment (10^{-9} M, 3 h). The expression of *MtD27* and *MtENOD11* in the susceptible root zone was monitored by qRT-PCR. This showed that in control plants, *MtD27* expression is elevated by CHX treatment (**Figure 4F**), suggesting that active protein synthesis is required to keep *MtD27* expression at basal levels. In addition, it showed that, like *MtENOD11*, *MtD27* expression is induced by rhizobium LCOs in the absence of CHX, but not in the presence of CHX (**Figure 4F**). This indicates that induction of *MtD27* expression by rhizobium LCOs requires new protein synthesis and therefore suggests that induction of *MtD27* by rhizobium LCOs is indirect.

***MtD27* Is Co-Expressed with *MtCCD7* and *MtCCD8* During Nodule Formation**

To obtain insight in the symbiotic function of *MtD27*, its spatiotemporal expression pattern is analyzed. For this, a ~1 kb fragment representing the 5' region upstream of the translational start site was cloned into a binary transformation vector in front of a β -glucuronidase (GUS)-

Figure 5. Spatial expression pattern of *MtD27* in *M. truncatula* roots and nodules.

(A) Cross-section through a non-inoculated root. Arrowheads indicate casparian strips, which mark the endodermal cell layer.

(B) Mock-treated control root.

(C) Root treated with *S. meliloti* LCOs (10^{-9} M) for 3 h.

(D) Root at four days post inoculation (dpi) with *S. meliloti* strain 2011.

(E) Root at 7 dpi.

(F) Longitudinal section through a root at 2 dpi.

(G) Longitudinal section through a nodule primordium (7 dpi). The infection thread is indicated with an arrowhead.

(H) Longitudinal section through an eighteen-day-old nodule.

The *MtD27* spatial expression pattern was analyzed in *M. truncatula* transgenic roots expressing an *MtD27* promoter-reporter GUS construct. Scale bars are equal to 25 μ m (A, F and G), 0.5 mm (B-E) and 50 μ m (H). Sections were counterstained with Ruthenium Red.

encoding sequence. This construct was used to create *M. truncatula* compound plants carrying transgenic roots. In non-inoculated plants, grown on buffered nodulation medium (BNM) containing no nitrate but a relatively high phosphate concentration (0.5 mM PO_4^{3-}), *MtD27* expression was observed in the vasculature and pericycle (Figure 5A-B). LCO ($\sim 10^{-9}$ M) treatment for 3 h induced *MtD27* expression in the root epidermis (Figure 5C).

Next, we determined the spatial expression of *MtD27* following inoculation with *S. meliloti* strain 2011. This showed that at 4 days post inoculation (dpi), the *pMtD27::GUS* transgenic roots showed a patched GUS staining, which was associated with rhizobium root hair infections (**Figure 5D**). At 7 dpi, the expression in the root ceased but GUS activity accumulated in nodule primordia (**Figure 5E**). Sectioning nodule primordia revealed expression of *pMtD27::GUS* in dividing pericycle and cortical cells (**Figure 5F**). This expression maintains in the developing nodule primordium (**Figure 5G**) but becomes more restricted in the mature nodule, where it is visible only in the meristem and distal infection zone (**Figure 5H**). Taken together, the LCO responsiveness and its spatial expression pattern in nodule primordia, nodule meristem and infection zone strongly support a symbiotic function of *MtD27*.

As strigolactone biosynthesis requires at least three enzymes, we tested whether besides *MtD27*, also *CCD7* and *CCD8* are responsive to rhizobium LCOs in *M. truncatula*. To this end, we first identified the putative orthologs of *CCD7* and *CCD8* from the *M. truncatula* genome based on homology to Arabidopsis genes. This revealed that one copy of *CCD7* (Medt7g045370; *MtCCD7*) and two copies of *CCD8* (Medtr3g109610 and Medtr7g063800) are encoded in the *M. truncatula* genome (**Supplemental Figure 2**). Medtr3g109610 was described previously as *MtCCD8* [6]. Analysis of the Medicago gene atlas showed that *MtCCD8* and Medtr7g063800 show a similar expression pattern, though expression of *MtCCD8* is about 10- to 20-fold higher when compared to Medtr7g063800 [39]. This also applies to expression of both genes during phosphate starvation and rhizobium LCO application (**Supplemental Figure 3**) and therefore we decided to focus on *MtCCD8* for the remainder of this study. Expression analysis by qRT-PCR on samples taken from plants grown in Fåhræus slides showed that *MtCCD7* and *MtCCD8* were not induced at 3 h post rhizobium LCO application (**Figure 6A**). In contrast, in roots grown on agar-solidified Fåhræus medium supplemented with amino ethoxyvinylglycine (AVG), expression of *MtCCD8* is induced ~5-fold 3-h after application of rhizobium LCOs, whereas *MtCCD7* expression was not substantially affected (**Figure 6B**).

To determine the spatial-temporal expression pattern of *MtCCD7* and *MtCCD8*, promoter-reporter GUS constructs were created and introduced in *M. truncatula* roots using *A. rhizogenes*-mediated transformation. We noted a basal expression pattern of both genes in the young root tip, including the susceptible zone and did not observe a discernible change in expression pattern of neither *MtCCD7* nor *MtCCD8* following LCO treatment (**Supplemental Figure 4**). This suggests that of the *MtD27*-*MtCCD7*-*MtCCD8* biosynthesis module, *MtD27* expression is most strictly controlled in a spatial-temporal manner. However,

Figure 6. Expression of *MtD27*, *MtCCD7* and *MtCCD8* upon treatment with rhizobium LCOs.

(A) Relative transcript abundance as determined by qRT-PCR of *MtD27*, *MtCCD7* and *MtCCD8* in *M. truncatula* root susceptible zones 3 h after mock or rhizobium LCO (10^{-9} M) treatment. RNA was isolated from plants grown in Fåhræus slides.

(B) Relative transcript abundance as determined by qRT-PCR of *MtD27*, *MtCCD7* and *MtCCD8* in *M. truncatula* root susceptible zones 3 or 6 h after mock or rhizobium LCO (10^{-9} M) treatment. RNA was isolated from plants grown on agar-solidified Fåhræus medium supplemented with 1 μ M AVG.

Data shown represent the means of 2-3 biological replicates that each were analyzed in 3-fold (technical replicates) $\pm$ SEM. For each gene, transcript abundance was normalized against that of the mock-treated wild type. Different letters above the bars indicate a statistical difference ($p < 0.05$, Students' t-test).

upon inoculation with *S. meliloti*, it showed that in young (two-day-old) nodule primordia, *MtCCD7* and *MtCCD8* are co-expressed with *MtD27* (Figure 5F and 7A-B). Moreover, in mature nodules, *MtD27*, *MtCCD7* and *MtCCD8* are co-expressed in the nodule meristem and distal infection zone (Figure 5H and 7C-D).

Previous studies on strigolactone deficient mutants revealed that strigolactones, at least in part, contribute to root nodule formation and functioning [2–4]. To test whether *MtD27* function is required for rhizobium symbiosis, we determined the nodulation phenotype of *MtD27* RNAi roots. Under these conditions, the *MtD27* RNAi construct reduced *MtD27* expression by $> 90\%$ (Supplemental Figure 5A). Examination of the *MtD27* RNAi roots showed that they can be effectively nodulated (Supplemental Figure 5B). Sectioning of ~40 nodules did not reveal any discernible difference in nodule morphology between nodules formed on control or *MtD27* RNAi roots (Supplemental Figure 5C-D). This suggests that either the reduction in *MtD27* expression is not sufficient to cause a phenotype or that *MtD27* is not essential for root nodule development and functioning.

Figure 7. Spatial expression pattern of *MtCCD7* and *MtCCD8* in *M. truncatula* nodule primordia and mature nodules.

(A) Longitudinal section through a root expressing the *pMtCCD7::GUS* construct 2 days post inoculation (dpi) with *S. meliloti* strain 2011.

(B) Longitudinal section through a root expressing the *pMtCCD8::GUS* construct at 2 dpi. Arrowhead points at an infection thread growing inside the root hair cell.

(C) Longitudinal section through a mature nodule expressing the *pMtCCD7::GUS* construct.

(D) Longitudinal section through a mature nodule expressing the *pMtCCD8::GUS* construct.

Expression patterns were analyzed in *M. truncatula* transgenic roots expressing promoter-reporter GUS constructs. Scale bars are equal to 50 μm . Sections were counterstained with Ruthenium Red.

The Induction of *MtD27* Expression by Phosphate Deprivation Is Independent of the Common Symbiotic Signaling Cascade

Besides rhizobium LCOs, also phosphate starvation elevates *MtD27* expression in an NSP1- and NSP2-dependent manner [17]. Induction of *MtD27* by rhizobium LCOs requires MtDMI3/MtCCaMK and, in part, MtERN1. DMI3/CCaMK is positioned directly upstream of NSP1-NSP2 in the LCO signaling pathway [7,47], whereas ERN1 functions in concert with NSP1-NSP2 to regulate expression of *MtENOD11* [48]. In line with this, we questioned whether MtDMI3/MtCCaMK and MtERN1 are also required to induce *MtD27* expression in response to a low phosphate status. First, we determined to what extent the spatial *MtD27* expression in *M. truncatula* roots is affected by different phosphate regimes. To this end, transgenic *M. truncatula* plants carrying the *MtD27* promoter-GUS reporter construct were grown in perlite for 2 weeks at high phosphate (200 μM PO_4^{3-})-containing medium and

Figure 8. Spatial expression pattern of *MtD27* upon phosphate starvation.

(A) Transgenic *M. truncatula* root expressing *pMtD27::GUS* grown in full nutrient condition (200 μM PO_4^{3-}).

(B) Longitudinal section of the root shown in (A).

(C) Expression of *pMtD27::GUS* in *M. truncatula* transgenic roots after 5 days of phosphate starvation (0 μM PO_4^{3-}).

(D) Longitudinal section of the root shown in (C).

(E) Relative transcript abundance as determined by qRT-PCR of *MtD27* in wild type (WT) and the *Mtdmi3* mutant and *Mtnsp1 Mtnsp2* double mutant after 2 days of phosphate starvation (0 μM PO_4^{3-}).

(F) Relative transcript abundance as determined by qRT-PCR of *MtD27* in WT and the *Mtern1* mutant and *Mtnsp1 Mtnsp2* double mutant after 2 days of phosphate starvation.

Scale bars are equal to 250 μm in (A) and (C) and 50 μm in (B) and (D). Sections were counterstained with Ruthenium Red. Data in (E-F) represent the means of 3 biological replicates that each were analyzed in 3-fold (technical replicates) $\pm$ SEM. For each gene, transcript abundance was normalized against that of the mock-treated wild type. Different letters above the bars indicate a statistical difference ($p < 0.05$, Student's *t*-test).

subsequently transferred to no phosphate (0 μM PO_4^{3-}) medium. Plant roots were stained histochemically for GUS activity 5 days after the transfer and compared to control roots. In all plants, GUS staining could be observed in the stele of the root as well as in the root apical meristem (Figure 8A-B). Phosphate-starved roots displayed a much more intense staining than control roots, which was most clear in the root apical meristem (Figure 8C-D). However, unlike treatment with rhizobium LCOs, phosphate starvation did not change the spatial expression pattern of *MtD27*.

Next, we determined whether MtDMI3/MtCCaMK and MtERN1 are also essential to mediate *MtD27* expression by phosphate starvation. To this end, *M. truncatula* *Mtdmi3* and *Mtern1* knockout mutants were grown in an aeroponic system containing medium with high phosphate (200 μM PO_4^{3-}) and subsequently transferred to a medium containing no phosphate for 2 days. To determine the expression of *MtD27*, qRT-PCR was conducted on RNA isolated from the bottom 2-3 cm of the root. This study reveals that in both mutants induction of *MtD27* in response to phosphate deprivation is similar to the induction found in roots of wild-type plants (**Figure 8E-F**). This indicates that the phosphate response of *MtD27* expression is independent of the symbiotic signaling genes *MtDMI3/MtCCaMK* and *MtERN1*.

DISCUSSION

Several lines of evidence indicate that strigolactones play a role in the legume rhizobium symbiosis [1–6]. Here, we showed that expression of *MtD27*, a gene that is acting in the strigolactone biosynthesis pathway, is strongly elevated by rhizobium LCO-induced signaling. Additionally, we found that this gene is co-expressed with *MtCCD7* and *MtCCD8* in nodule primordia as well as in the infection zone of mature nodules. This suggests a putative function for these strigolactone biosynthesis genes during several stages of the legume-rhizobium interaction.

Studies with the strigolactone analog GR24 revealed a nodulation-enhancing effect when applied exogenously [1,5]. In line with this, a severe reduction in endogenous strigolactone levels due to mutations in *ccd7* or *ccd8* is linked to a moderate decrease in nodulation efficiency [2–4]. Foo and Davies [2] conclude that, although strigolactones influence nodule initiation, they are not essential. We were unable to confirm these results in *MtD27* RNAi-knockdown roots of *M. truncatula*. We cannot rule out that in our *MtD27* RNAi experiments, *MtD27* expression is not sufficiently reduced to cause such moderate nodulation phenotype. Alternatively, phenotypes caused by an altered D27 function may be weaker to that of phenotypes of *ccd7* or *ccd8* mutants. This hypothesis finds support by studies in Arabidopsis and rice, where shoot branching phenotypes were much more severe in *ccd7* and *ccd8* mutants when compared to *d27* [21,38]. Based on this, Waters et al. [38] speculate on residual bioactive compounds present in *Atd27* mutants. If such a residual bioactive compound also exists in *M. truncatula*, it is possible that its activity is sufficient for proper nodule initiation and development.

Figure 9. Schematic model depicting the induction of *MtD27* expression by phosphate starvation and rhizobium LCO-induced signaling.

Rhizobium LCOs activate expression of *MtD27* through the common symbiosis signaling module, consisting of MtDMI1, MtDMI2 and MtDMI3. Downstream of this module MtERN1 is required for full induction of *MtD27*. MtERN1 function is partly redundant, suggesting that MtERN1 might function in conjunction with or redundant to another unknown transcriptional regulator, indicated as X. This unknown factor might be MtERN2, a transcriptional regulator closely related to MtERN1 [48]. The pathway leading to activation of *MtD27* expression by phosphate starvation remains unknown. The GRAS proteins MtNSP1 and MtNSP2 are required for expression of *MtD27* during phosphate starvation and following rhizobium LCO application. We propose that these proteins function in parallel to both signaling pathways, as previously already suggested for the LCO signaling cascade [7,15].

It remains currently unknown how strigolactones promote nodule initiation. One possible mechanism is through promoting the formation of a nodular auxin maximum. Mathematical modelling predicts that such maximum is most likely created through a local reduction in the auxin transport capacity in the root cortex [49]. Such reductions in root auxin transport capacity have been observed following rhizobial inoculation [50,51]. Strigolactone-deficient mutants of *Arabidopsis* show elevated auxin transport in both shoots and roots [52]. It is proposed that strigolactones act by targeting the PIN auxin-efflux carriers at both the gene expression and protein level [53–57]. Therefore, it is possible that rhizobium-induced strigolactone biosynthesis will affect auxin transport and as such contributes to create and/or maintain an auxin maximum during nodule formation. However, it is unlikely that

strigolactones alone are sufficient to reduce the auxin transport capacity upon perception of rhizobium LCOs, as strigolactone-deficient mutants still form nodules, although less than wild-type plants [2,3]. Possibly, strigolactones function redundantly to another signal, like for example cytokinin [51,58–60] or flavonoids [61].

MtD27 expression is elevated within 1–2 h post LCO application, by which it is among the earliest responsive genes. This transcriptional activation is under control of the rhizobium LCO signaling network, which includes MtDMI2, MtDMI1, MtDMI3/MtCCaMK, MtNSP1, MtNSP2 and, in part, MtERN1. We found that also expression of *MtCCD7* and *MtCCD8* was induced following application of rhizobium LCOs. However, these responses were less pronounced when compared to *MtD27*. Expression of *MtCCD7* was only slightly affected by application of rhizobium LCOs, whereas induction of *MtCCD8* was dependent on the growth system. Spatial-temporal expression analysis revealed that, in a symbiotic context, especially *MtD27* expression is strictly controlled in a spatial-temporal manner. Under non-symbiotic conditions, *MtD27* is mainly expressed in the stele of the root, whereas rhizobium LCOs activate expression in the root epidermis. Such clear spatial-temporal regulation was not observed for *MtCCD7* nor *MtCCD8*, as both genes have a much broader expression pattern under non-symbiotic conditions. Why *MtD27* is strictly controlled under symbiotic conditions remains unknown. Expression of *MtCCD7* and *MtCCD8* might not be rate limiting, or alternatively, induction of *MtD27* by rhizobium LCOs might be part of a priming response that prepares epidermal cells for the infection process [6].

Our data hint at a putative role for strigolactones in mature nodules as *MtD27*, *MtCCD7* and *MtCCD8* are co-expressed in the nodule meristem and distal infection zone. This may suggest that strigolactones promote meristem functioning and/or rhizobial infection. Recently, it was shown that *MtD27* and *MtCCD8* are transcriptionally induced in infected root hairs [6]. Our data also indicate expression of *MtD27* and *MtCCD8* in cells that contain growing infection threads in the root nodule, supporting a putative function for *MtD27* and *MtCCD8* in the infection process. Furthermore, induction of *MtD27* expression by rhizobium LCOs is partly dependent on MtERN1, a transcription factor required for infection thread development [45].

MtD27 is also transcriptionally activated by phosphate starvation stress and this induction is dependent on MtNSP1 and MtNSP2 [17]. We studied the spatial regulation of *MtD27* in response to phosphate starvation and found that the spatial expression pattern remains unchanged but *MtD27* expression is increased in the stele and apical root meristem. The transcriptional activation of *MtD27* in response to the phosphate status in the environment

coincides with an increased exudation of strigolactones [29,30,34]. Generally, it is anticipated that this response is contributing to the attraction of endomycorrhizal fungi, which enhance phosphate acquisition from the environment [33,36]. We tested whether the induction of *MtD27* by phosphate starvation is dependent on the common signaling pathway as well as whether this response is (partially) dependent on MtERN1. Neither signaling components were involved in the induction of *MtD27* expression by phosphate starvation. This indicates that the signaling pathways regulating transcriptional activation of *MtD27* by rhizobium LCOs and phosphate starvation only share NSP1 and NSP2 (**Figure 9**). Interestingly, *MtD27* transcript abundance is reduced in an *Mtnsp1Mtnsp2* mutant background when compared to wild type [17]. Furthermore, we found that induction of this gene upon phosphate stress or LCO signaling still occurs in these mutants, although at very moderate levels (**Figure 4E** and **8F**). Taken together, this supports the hypothesis that MtNSP1 and MtNSP2 function in a parallel pathway to facilitate induction of *MtD27* by rhizobium LCOs and phosphate starvation stress, rather than being part of the primary signaling cascades [7,15] (**Figure 9**).

The D27-CCD7-CCD8 biosynthetic module is largely conserved in higher plants. As we showed that *MtD27* is transcriptionally activated in a spatial-temporal manner in response to rhizobium LCOs in *M. truncatula* roots, this gene may represent an excellent marker gene to study rhizobium-induced signaling in a phylogenetic context.

CONCLUSIONS

Here, we showed that in *M. truncatula*, *MtD27* expression is rapidly increased upon perception of rhizobium LCOs. The gene remains expressed in the dividing cells of the nodule primordium and at subsequent stages its expression becomes confined to the nodule meristem and distal infection zone of the mature nodule. Analysis of the expression of *MtCCD7* and *MtCCD8* showed that they are co-expressed with *MtD27* in nodule primordia and mature nodules. Additionally, we show that symbiotic expression of *MtD27* as well as its expression during phosphate starvation is dependent on the GRAS-type regulators MtNSP1 and MtNSP2. This suggests that the NSP1-NSP2-D27 regulatory unit is co-opted to function in rhizobium symbiosis.

METHODS

Plant Materials and Growth Conditions

M. truncatula Jemalong A17, *dmi1-1* (C71) [9,42], *dmi2-1* (TR25) [62], *dmi3-1* (TRV25) [10], *nsp1-1* (B85) [14,42], *nsp2-2* (0-4) [13,43], *nsp1 nsp2* [17] and *ern1 (bit1-1)* [45] were used in this study. Plants were grown in a growth chamber at 20°C under 16/8 photoperiod at 50 $\mu\text{moles of photons m}^{-2} \text{ s}^{-1}$. For gene expression studies, plants were grown in modified Fåhræus slides [40]. A single germinated seedling was placed in each slide and medium was refreshed every 24 h. Seedlings were grown for 3 days, before subjecting them to a 3 h treatment with *Sinorhizobium meliloti* LCOs ($\sim 10^{-9} \text{ M}$), unless stated otherwise. Subsequently, 1-cm root segments were cut just above the root tip and snap-frozen in liquid nitrogen.

For analysis of transcriptional induction of *MtD27* by phosphate starvation, plants were grown as previously described [17]. This time, plants were grown for ~ 2.5 weeks before subjecting them to a two-day phosphate starvation regime. Subsequently, 2 to 3-cm root segments including the root tip were cut and snap-frozen in liquid nitrogen.

Phylogenetic Reconstruction

Predicted proteomes of *Glycine max* (Wm82.a2.v1) [63], *Lotus japonicus* (Lj2.5) [64], *Medicago truncatula* (Mt4.0v1) [65], *Oryza sativa* (v7.0) [66], *Populus trichocarpa* (v3.0) [67] and *Vitis vinifera* (Genoscope.12X) [68] were obtained through Phytozome 10 (<http://phytozome.jgi.doe.gov/>). These proteomes were searched by BLAST using *A. thaliana* proteins (TAIR10, www.arabidopsis.org) as query. For phylogenetic reconstruction, full length (predicted) protein sequences were aligned using MAFFT v7.017 [69] implemented in Geneious R6 (Biomatters, Auckland, New Zealand), using default parameter settings. After manual inspection, alignments were used for tree building using MrBayes 3.2.2 [70] implemented in Geneious R6, using default parameter settings, with the exception of the rate matrix, for which *wag* was used. Midpoint rooting was performed for better tree visualization.

Vectors and Constructs

For promoter-GUS reporter assays, a ~ 1 kb (*MtD27*) or ~ 2 kb (*MtCCD7* and *MtCCD8*) fragment upstream of the translational start site was amplified from *M. truncatula* Jemalong A17 genomic DNA using the primers listed in **Supplemental Table 1**. The p*MtD27* fragment and a β -glucuronidase (GUS)-encoding sequence were recombined into a pDONR-L4L1 and pDONR-L1L2, thereby creating pENTR4-1_p*MtD27* and pENTR1-2_GUS, respectively. These two constructs were combined with a pENTR2-3_t35S and subsequently recombined into

the binary destination vector pKGW-RR-MGW by a multisite gateway reaction (Invitrogen, Carlsbad, USA). The putative promoter fragments of *MtCCD7* and *MtCCD8* were recombined into a pENTR-D-TOPO vector (Invitrogen, Carlsbad, USA), creating pENTR1-2_p*MtCCD7* and pENTR1-2_p*MtCCD8*, respectively. Subsequently, both constructs were recombined into pKGWFS7-RR, containing a GUS-GFP fusion reporter, by a single-site gateway reaction (Invitrogen, Carlsbad, USA), creating pKGWFS7-RR_p*MtCCD7*-GUS and pKGWFS7-RR_p*MtCCD8*-GUS, respectively.

For RNAi-mediated knockdown of *MtD27*, a 268-bp fragment was amplified from *M. truncatula* Jemalong A17 root cDNA, using primer pairs MtD27i-F and MtD27i-R (see **Supplemental Table 1**), and cloned into pENTR-D-TOPO (Invitrogen, Carlsbad, USA). The *MtD27* RNAi fragment was recombined into the DsRed-modified gateway vector pK7GWIWG2(II)-RR driven by the CaMV35S promoter [71] to obtain the binary construct pK7GWIWG2(II)-RR-p35S-MtD27-RNAi. For the empty vector control, a pENTR containing a ~70 bp multiple cloning site was recombined into pK7GWIWG2(II)-RR to obtain the binary plasmid pK7GWIWG2(II)-RR-p35S-RNAi-control. All vectors used in this study contain *pAtUBQ10::DsRED1* as selection marker [72]. All cloning vectors are available upon request from Plant Systems Biology (V.I.B.-Ghent University).

Plant Transformation and Treatments

A. rhizogenes-mediated root transformation of *M. truncatula* was performed as previously described [72]. For treatments with rhizobium LCOs, compound plants were transferred to agar-solidified buffered nodulation medium (BNM, 0.9% Daishin agar (Duchefa, Haarlem, The Netherlands)) [73] containing 1 μ M aminoethoxyvinylglycine (AVG) (Sigma, St. Louis, USA). After 3 days, ~100 μ l of rhizobium LCOs ($\sim 10^{-9}$ M) was pipetted on top of the root susceptible zone. After 3 h, roots were fixed in 90% acetone and subsequently stained for GUS activity. For nodulation assays, compound plants were transferred to perlite and watered with Fåhræus [74] medium without nitrate. One week after transfer, plants were inoculated with *S. meliloti* strain 2011 ($OD_{600} = 0.05$ -0.1). For the phosphate starvation experiment, compound plants were transferred to perlite and watered with half-strength Hoagland medium [75]. After one week, plants were removed from perlite and washed three times with demineralized water to get rid of the nutrient salts. Plants were re-planted in fresh perlite and watered with half-strength Hoagland medium with (200μ M PO_4^{3-}) or without (0μ M PO_4^{3-}) phosphate, respectively. After 5 days, plants were removed from perlite and stained for GUS activity.

Histochemical Staining and Microtome Sectioning

For histochemical GUS staining of *M. truncatula* roots and nodules, samples were first rinsed three times with 100 mM phosphate buffer (PBS, pH = 7.2). Samples were transferred to GUS-staining buffer (contains 2 mM $K_3Fe(CN)_6$, 2 mM $K_4Fe(CN)_6$, 10 mM EDTA, 0.1% Triton X-100 and 1 mg/ml X-Gluc salt (Duchefa, Haarlem, The Netherlands) in 100 mM PBS, pH = 7.2) and placed under vacuum for 30 min. Next, the samples were incubated in the dark at 37°C for 3 h. Stained roots were rinsed with PBS (pH = 7.2) three times to stop the reaction.

For histoiresin embedding, roots and nodule samples were fixed with 5% glutaraldehyde PBS (pH = 7.2) solution overnight. After fixation, the samples were rinsed with PBS (pH = 7.2) three times and dehydrated through ethanol gradients (20%, 40%, 60%, 80% and 100%). Afterwards, the samples were embedded in Technovit 7100 (Heraeus-Kulzer, Wehrheim, Germany), according to the manufacturer's protocol. GUS-stained samples were sectioned to 7 μ m using a microtome (Reichert-Jung, Leica Microsystems, Rijswijk, The Netherlands) and stained with 0.1% Ruthenium Red for 15 min. Images were taken using a Leica DM5500B microscope equipped with a Leica DFC425C camera (Leica Microsystems, Wetzlar, Germany). Images were digitally processed using Photoshop CS6 (Adobe Systems, San Jose, USA).

qRT-PCR Analysis

RNA was isolated from snap-frozen root material using the plant RNA kit (E.Z.N.A. Omega Biotek, Norcross, USA) following the supplier's manual. cDNA was synthesized from 1 μ g total RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, USA). qRT-PCR reactions were set up in a 20 μ l reaction system with 2 $\times$ iQ SYBR Green Super-mix (Bio-Rad, Hercules, USA) and the iQ5 Real-time PCR detection system according to the manufacturer's manuals. All primers used in this study were designed using the qPCR settings of Primer3Plus [76]. Relative expression values were calculated using the $2^{-\Delta\Delta C_t}$ method, using *M. truncatula* ubiquitin (*MtUBQ10*) and polypyrimidine tract-binding protein (*MtPTB*) as reference genes. Statistical significance was determined based on a Students' t-test (unpaired, two tailed, equal variance). All primers used in this study are listed in **Supplemental Table 1**.

Strigolactone Analysis

M. truncatula root exudates and root extracts were purified and concentrated as previously described [17,30] with minor modifications. Compound (*MtD27* RNAi and empty vector control) plants were grown on perlite and watered twice a week with 50 mL half-strength MS medium (Duchefa, Haarlem, The Netherlands). Seven days prior to strigolactone analysis, pots were washed with 3 volumes half-strength MS medium without PO_4^{3-} (Duchefa, Haarlem, The Netherlands) to initiate phosphate starvation. Strigolactone quantification

was performed by comparing retention time and mass transitions with those of an available didehydro-orobanchol standard using ultra-performance LC coupled to MS/MS using [$^2\text{H}_6$]2'-epi-5-deoxystrigol as an internal standard, as previously described [77]. Didehydro-orobanchol MS/MS fragmentation spectra of *M. truncatula* were obtained as previously described [17]. Results were subjected to a Students' t-test (unpaired, two tailed, equal variance).

Medicago Gene Atlas IDs

Probe IDs used for analysis of gene expression using the Medicago gene expression atlas [39] are listed in **Supplemental Table 2**.

SUPPLEMENTAL DATA

Supplemental Figures 1-5 are available as Additional Files 1-5 and Supplemental Tables 1-2 are available as Additional Files 6-7 on the BMC Plant Biology website (<https://doi.org/10.1186/s12870-015-0651-x>).

AUTHOR CONTRIBUTIONS

R.G. conceived of the study; R.G., W.C.Y. and T.B. supervised the study; W.K. and A.v.Z. performed functional characterization of MtD27; W.L. and T.T.X. determined the spatiotemporal expression pattern of MtD27; W.L. and A.v.Z. determined rhizobium LCO-induced expression of MtD27; A.v.Z. determined the MtCCD7 and MtCCD8 spatiotemporal expression patterns; T.T.X. and A.v.Z. determined expression of MtD27 in response to phosphate starvation; A.v.Z. and R.G. wrote the manuscript; W.K., W.L., T.T.X., W.C.Y. and T.B. assisted in editing the manuscript.

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Chapter 5

CRISPR/Cas9-mediated mutagenesis
of four putative symbiosis genes of
the tropical tree *Parasponia andersonii*
reveals novel phenotypes

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ABSTRACT

Parasponia represents five fast growing tropical tree species in the Cannabaceae and is the only plant lineage besides legumes that can establish nitrogen-fixing nodules with rhizobium. Comparative analyses between legumes and *Parasponia* allows identification of conserved genetic networks controlling this symbiosis. However, such analyses are currently hampered due to the absence of powerful reverse genetic tools for *Parasponia*. Here, we present a fast and efficient protocol for CRISPR/Cas9 mutagenesis in *Agrobacterium tumefaciens*-transformed *Parasponia andersonii* plants. Using this protocol, mutants can be obtained within three months. Due to efficient micro-propagation, bi-allelic mutants can be studied in the T₀ generation, allowing phenotypic evaluation within six months after transformation. As proof of principle, we mutated four genes - *PanHK4*, *PanEIN2*, *PanNSP1*, and *PanNSP2* - that control cytokinin, ethylene or strigolactone hormonal networks and that in legumes commit essential symbiotic functions. Knockout mutants in *Panein2* and *Panhk4* displayed developmental phenotypes that are associated with ethylene or cytokinin signaling mutations. Like in legumes, NSP GRAS-type transcriptional regulators showed to be essential for nodule formation in *P. andersonii*. Conversely, symbiotic phenotypes of *Panhk4* and *Panein2* mutants differ from that in legumes. This illustrates the value of *Parasponia* trees as research model for reverse genetic studies.

INTRODUCTION

Parasponia are tropical tree species belonging to the Cannabis family (Cannabaceae) and are known as the only non-legume plants that can establish a nitrogen-fixing endosymbiosis with rhizobium [1-3]. The *Parasponia* genus consists of five species indigenous to the Malay Archipelago and Papua New Guinea, where they grow on the slopes of volcanic mountains [2, 4, 5]. *Parasponia* spp. are typical fast-growing pioneer plants, capable of covering nitrogen-poor eroded soils in a relatively short time span [4]. Under suitable greenhouse conditions, young *Parasponia* trees can grow at speeds exceeding 45 centimeters per month, and fix up to 850 kg N ha⁻¹ year⁻¹ in association with rhizobium [6-8]. Therefore, *Parasponia* could represent a valuable tool for studies aimed at understanding the genetics underlying rhizobium symbiosis as well as tree biology [9-13].

Like legumes, *Parasponia* develops specialized root nodular organs to host the rhizobium partner. Nodules provide the rhizobium bacteria with suitable environmental conditions to convert atmospheric nitrogen into ammonium. The Cannabaceae and legume families (Fabaceae) diverged about a hundred million years ago [14], underlining that the rhizobium symbiosis in legumes and *Parasponia* evolved largely independent [15]. This is reflected in the distinct nodule-types found in both lineages [9]. Legume nodules possess a large central zone of infected cells that is surrounded by peripheral vascular bundles. In contrast, *Parasponia* nodules have a central vascular bundle and infected cells in the peripheral zone, giving these nodules a lateral root-like appearance. Nevertheless, initial comparative studies revealed that both symbioses are founded on conserved signaling networks. In legumes as well as *Parasponia*, root nodule formation is induced upon recognition of rhizobial secreted lipo-chitooligosaccharide (LCO) signals [16-18]. Research on model legumes, like *Medicago truncatula* and *Lotus japonicus*, showed that the perception of these symbiotic signals requires a signaling cascade that has been largely co-opted from the much older endomycorrhizal symbiosis [10, 19]. In legumes, activation of the LCO signaling network results in a massive transcriptional reprogramming, requiring among others the GRAS-type transcriptional regulators NODULATION SIGNALING PATHWAY 1 (NSP1) and NSP2 and the cytokinin receptor MtCRE1/LjLHK1 [20-26]. Subsequent nodule formation is tightly controlled by regulatory feedback loops, including negative regulation by ethylene signaling [27-29].

A reference quality genome sequence for *Parasponia andersonii* and draft genome sequences of two additional *Parasponia* species have been generated [30]. Mining these genomes uncovered ~1,800 putative symbiosis genes, of which hundreds are close homologs

of legume symbiosis genes [30]. Initial reverse genetic studies in *P. andersonii*, using a transient *Agrobacterium rhizogenes*-based root transformation system, revealed that at least two genes - *NOD FACTOR PERCEPTION 1* (*PanNFP1*) and *CALCIUM AND CALMODULIN-DEPENDENT PROTEIN KINASE* (*PanCCaMK*) - commit conserved functions in the *Parasponia* and legume LCO signaling pathways [18]. We argue that a more comprehensive comparative analysis between legumes and *Parasponia* will allow identification of conserved genetic networks that are essential to establish symbiosis with rhizobium. However, to use *Parasponia* as an effective research model - alongside the legume models *M. truncatula* and *L. japonicus* - efficient transformation and genome editing tools are required.

Here, we exploit an efficient *in vitro* micro-propagation system available for *P. andersonii* to establish stable transformation and CRISPR/Cas9-mediated mutagenesis for this species [31-33]. We show that using *Agrobacterium tumefaciens*-mediated transformation, stable transgenic lines of *P. andersonii* can be obtained in ~3-4 months. Additionally, we show that *P. andersonii* is amenable to targeted mutagenesis using the CRISPR/Cas9 system. As ~40% of the resulting T₀ lines harbor bi-allelic mutations, these can be phenotyped upon *in vitro* propagation. As proof of concept, we mutated four genes in *P. andersonii* that in legumes control hormonal pathways as well as commit symbiotic functions. These include, the GRAS-type transcriptional regulators *NSP1* and *NSP2* that both are essential for nodule organogenesis [21, 22, 25] as well as control strigolactone biosynthesis by mediating *DWARF27* (*D27*) expression [34, 35]; *HISTIDINE KINASE 4* (*HK4*), which encodes a cytokinin receptor that in legumes is essential for nodule organogenesis [20, 23, 24]; *ETHYLENE INSENSITIVE 2* (*EIN2*), which is an essential hub in ethylene signaling and a negative regulator of nodulation in legumes [27, 28, 36]. The corresponding *P. andersonii* mutants display hormone-related phenotypes. However, the symbiotic phenotypes of *Panhk4* and *Panein2* mutants deviate from those described for legumes. This illustrates that *P. andersonii* can be used as an effective model for CRISPR/Cas9-mediated reverse genetic studies.

RESULTS

Agrobacterium tumefaciens*-Mediated Transformation of *Parasponia

To establish a protocol for stable transformation of *P. andersonii*, we first determined the most optimal conditions for regeneration of non-transgenic tissue. We compared regeneration efficiencies of nine different tissue explant types in combination with eleven different media, including the propagation and root-inducing media previously used for *P. andersonii* (**Supplemental Tables 1-2**) [18, 31]. This revealed that young stem pieces and petioles placed on original propagation medium regenerate plantlets most efficiently

Figure 1. *P. andersonii* stem and petiole explants can be efficiently transformed using *Agrobacterium tumefaciens*.

(A) Petiole explant at four weeks after transformation using *A. tumefaciens*.

(B) Stem explant at five weeks after transformation using *A. tumefaciens*.

(C) Small transgenic shoots at ten weeks after transformation.

Explants were incubated on root-inducing medium for nine days, prior to transfer to propagation medium. DsRED fluorescence indicates transgenic tissue. Scale bars are equal to 2.5 mm. Shown from top to bottom are bright-field images, overlays of bright-field and DsRED fluorescence and DsRED fluorescence images.

(Supplemental Table 1). Next, we questioned whether stem pieces and petioles could be effectively transformed using *A. tumefaciens*. To this end, we used *A. tumefaciens* AGL1 carrying a binary transformation vector containing in its T-DNA the kanamycin resistance gene *NPTII* and the red fluorescent protein *DsRED1*. Co-cultivation of *A. tumefaciens* and *P. andersonii* stem or petiole explants was conducted in darkness for two days at 21°C to promote T-DNA transfer [31]. Afterwards, tissue explants were placed on selective medium and incubated at 28°C in the light. These latter conditions are most favorable for *P. andersonii* regeneration [31]. From day eight onwards, DsRED1-fluorescent cells could be observed indicating successful transfer of the T-DNA.

Recent research on *Arabidopsis thaliana* showed that acquisition of pluripotency requires activation of a root developmental program [37]. We tested whether an initial culturing period on root-inducing medium further improves the transformation efficiency of *P. andersonii*. This showed to be the case (**Supplemental Table 3**). About half of the explants formed regenerative calli at four weeks post co-cultivation (**Figure 1A**). When 2 mm in size, transgenic calli were separated from tissue explants, which stimulated shoot formation

(**Figure 1B-C**). Two to three months after start of transformation, a single shoot was selected from each explant to ensure that selected transgenic lines represent independent transformation events. These shoots can be genotyped and vegetatively propagated (**Supplemental Figure 1**).

To characterize the resulting transgenic *P. andersonii* lines at the molecular level, we selected - based on red fluorescence - twenty independent transformants for further analyses. PCR reactions using primers amplifying a sequence near the right T-DNA border indicated complete T-DNA integration in 19 out of 20 lines (**Supplemental Table 4**). To determine whether the transformation procedure might affect ploidy level of the regenerated transgenic lines, we estimated genome size based on flow cytometry. This showed no effect of the transformation procedure on genome size of transgenic lines (**Supplemental Table 4**). To estimate the number of T-DNA integrations, we used quantitative RT-PCR (qRT-PCR) as well as Southern blotting. This showed an overall low T-DNA copy number, varying between one and three integrations per line (**Supplemental Table 4**). We selected three transgenic lines with a single T-DNA integration to examine T-DNA stability. In greenhouse-grown trees as well as *in vitro* propagated material, DsRED1 fluorescence could still be observed at one year after transgenic lines were selected (**Supplemental Figure 2**). This indicates that *trans*-genes remain stably integrated in the *P. andersonii* genome and actively transcribed, even after multiple rounds of vegetative propagation. Taken together, the protocol described above allows to generate *A. tumefaciens*-transformed *P. andersonii* plantlets within three months, which upon vegetative propagation can be phenotyped.

***Parasponia* Is Amenable to CRISPR/Cas9-Mediated Mutagenesis**

To test whether CRISPR/Cas9 could be used for targeted mutagenesis in *P. andersonii*, we aimed at mutating the *P. andersonii* putative orthologues of *EIN2*, *MtCRE1/LjLHK1*, *NSP1* and *NSP2*. These genes were selected, because they control legume root nodule formation as well as commit essential non-symbiotic functions in hormone homeostasis. Putative orthologues of all four genes were previously identified from the *P. andersonii* genome and named *PanEIN2*, *PanHK4*, *PanNSP1* and *PanNSP2*, respectively [30]. Phylogenetic reconstruction based on protein sequences confirmed that these represent the most likely orthologues of legume symbiotic genes (**Supplemental Figures 3-6**). To mutate *PanEIN2*, *PanHK4*, *PanNSP1* and *PanNSP2*, three single guide RNAs (sgRNAs) targeting *PanHK4* and *PanNSP2* and single sgRNAs targeting *PanEIN2* and *PanNSP1* were placed under an *A. thaliana* *AtU6* small RNA promoter [38]. These were cloned into a binary transformation vector containing the *NPTII* kanamycin resistance gene as well as a Cas9-encoding sequence

Table 1. Mutation frequency in CRISPR/Cas9 transgenic lines.

Target gene	No. of sgRNA's	No. of lines	Non-mutated ¹	Mutated		
				Bi-allelic	Heterozygous	Unknown ²
<i>NSP1</i>	1	29	15 (51.7%)	11 (37.9%)	0	3 (10.3%)
<i>NSP2</i>	3	29	13 (44.8%)	10 (34.5%)	3 (10.3%)	3 (10.3%)
<i>EIN2</i>	1	9	1 (11.1%)	6 (66.7%)	2 (22.2%)	0
<i>HK4</i>	3	26	13 (50.0%)	12 (46.2%)	1 (3.8%)	0
Total		93	42 (45.2%)	39 (41.9%)	6 (6.5%)	6 (%)

¹ This includes an unknown number of individuals that are not transgenic.

² Sequencing of the PCR product indicates that plants are mutated, but the exact mutation and zygosity were not determined.

fused to an N-terminal nuclear-localization signal and driven by the CaMV 35S promoter [39, 40]. The resulting constructs as well a control construct containing only the NPTII- and Cas9-encoding sequences were transformed to *P. andersonii* using the method described above. For all constructs, transgenic shoots were obtained, although in case of the construct targeting *PanHK4* regeneration took considerably longer (up to six months). Genotyping of regenerated shoots showed that > 85% contained the *Cas9* gene, indicating successful transformation. Potential mutations at any of the target sites were identified through PCR amplification and subsequent sequencing of the PCR product. This revealed mutations at the target site in about half of the transgenic shoots examined, of which the majority were bi-allelic (**Table 1**). Most mutations represent small insertion and deletions but also larger deletions and inversions were identified, some of which occur in between two target sites (**Supplemental Figures 7-10**). In case of *PanHK4*, most mutants contained small in-frame deletions of three or six base pairs that most likely do not disrupt protein function. In fact, only two bi-allelic knockout mutants could be identified (**Supplemental Figure 8**). For the remaining three constructs, multiple bi-allelic knockout mutants were identified of which three individuals were selected for further studies (for an overview of mutant alleles see **Supplemental Figures 7-10**).

For phenotypic evaluation, *P. andersonii* T₀ transgenic lines are propagated vegetatively. Therefore, we first evaluated whether any of the mutant lines might be chimeric. To this end, tissue samples were taken from at least three different positions and genotyped for the corresponding target mutation. For each of the mutant lines, except *Pannsp2-9*, the exact same mutations were retrieved, suggesting that genome editing occurred soon after T-DNA integration. In case of *Pannsp2-9*, chimeric mutations were detected at the first of three target sites (**Supplemental Figure 10C**). However, the nature of the mutations at the second

Figure 2. *Panein2* mutants are insensitive to ethylene treatment.

(A) Percentage of abscised leaves after mock or ethylene treatment. Data represent means of 3-5 biological replicates $\pm$ SEM. Dots represent measurement values of biological repeats. Different letters indicate statistical significance ($p < 0.05$) as determined by ANOVA in combination with Tukey post-hoc test.

(B) Representative images showing abscission of leaves on a transgenic control line, but not on a *Panein2* mutant. Abscission points are indicated by arrowheads.

and third target site prevent that gene function could be restored in this line. Therefore, all eleven mutants are suitable for phenotypic evaluation. This proves that CRISPR/Cas9 can be used to efficiently mutagenize *P. andersonii* in the T_0 generation.

Non-Symbiotic Phenotypes in *Parasponia ein2*, *hk4*, *nsp1* and *nsp2* Mutant Lines

To characterize the resulting *Panein2*, *Panhk4*, *Pannsp1* and *Pannsp2* mutant lines, we studied their non-symbiotic phenotypes. *PanEIN2* putatively encodes a central component of the ethylene signaling pathway and therefore *Panein2* mutants are expected to be ethylene insensitive. One phenotype triggered in response to ethylene treatment is abscission of leaves and flowers [41-43]. We exploited this phenotype to assess ethylene sensitivity of *Panein2* mutants. To this end, the tips of young shoot branches of greenhouse grown trees were exposed to ethylene gas. Within three days, ethylene triggered abscission of ~65% of treated leaves on wild-type *P. andersonii* as well as control transgenic lines (**Figure 2**). In contrast, leaf abscission was not observed on *Panein2* mutant trees (**Figure 2**). This demonstrates that *Panein2* mutants are indeed ethylene insensitive.

Inspection of *Panein2* mutant trees revealed an additional non-symbiotic phenotype. These trees form bisexual flowers containing both male and female reproductive organs (**Figure 3A-C**). In contrast, wild-type *P. andersonii* trees form unisexual flowers that contain either stamens or carpels [4] (**Figure 3D-E**). This suggests that ethylene is involved in the regulation of *Parasponia* sex type.

Figure 3. *Panein2* mutants form bisexual flowers.

(A) Mature *Panein2* flower. Note the presence of both stamen and a carpel inside *Panein2* flowers.

(B) Immature *Panein2* flower. Note the presence of stigmata, indicating presence of a carpel inside the flower.

(C) Immature *Panein2* flower of which sepals have been removed to show the presence of stamen.

(D) Mature wild-type male flower.

(E) Mature wild-type female flowers. Left, young female flower. Right, older female flower.

Scale bars are equal to 1 mm.

Cytokinins are important regulators of cambial activity [44-46]. To determine whether reduced cytokinin sensitivity in *Panhk4* mutant lines affects activity of the procambium, we sectioned the base of young axillary shoot branches. This showed that cambium activity is reduced in *Panhk4* mutant lines compared to transgenic controls (**Figure 4**). Therefore, we conclude that PanHK4-mediated cytokinin signaling is required for regulation of *P. andersonii* secondary growth.

Expression studies in *M. truncatula*, previously identified a set of genes downregulated in roots of *Mtnsp1* and *Mtnsp2* mutants [34]. Among these are *DWARF27* (*MtD27*) (Medtr1g471050) and *MORE AXILLARY BRANCHING 1* (*MtMAX1*) (Medtr3g104560) that are putatively involved in strigolactone biosynthesis [34, 35, 47, 48]. We identified putative *P. andersonii* orthologues of these genes (**Supplemental Figures 11-12**) and compared their expression levels in young root segments of three *Pannsp1*, *Pannsp2* and control plants

Figure 4. *Panhk4* mutants display reduced cambial activity.

Stem cross-sections of control transgenic and *Panhk4* mutant lines. Sections were made of the base of the second youngest branch of four month old trees grown under greenhouse conditions. The location of the procambium is indicated by red lines. Scale bars represent 100 μ m.

by qRT-PCR. This showed that expression of *PanD27* and *PanMAX1* is reduced in roots of *Pannsp1* and *Pannsp2* mutant lines (**Figure 5**). We noted that *Pannsp1* mutant lines differ in the level of *PanD27* and *PanMAX1* expression. Both genes have an intermediate expression level in *Pannsp1-6* and *Pannsp1-13*, compared to *Pannsp1-39* and *Pannsp2* mutants (**Figure 5**). The three *Pannsp1* mutant lines differ from each other in the type of mutations that were created. *Pannsp1-6* and *Pannsp1-13* contain a one base pair insertion and five base pair deletion close to the 5'-end of the coding region, respectively. These mutations are immediately followed by a second in-frame ATG that in wild-type PanNSP1 encodes a methionine at position 16. In contrast, *Pannsp1-39* contains a large 232 base pair deletion that removes this in-frame ATG (see **Supplemental Figure 9**). Together, this suggests that *Pannsp1-6* and *Pannsp1-13* might represent weak alleles. Overall, these data suggest that regulation of *D27* and *MAX1* expression by NSP1 and NSP2 is conserved between *M. truncatula* and *P. andersonii*.

Taken together, we showed that EIN2, HK4, NSP1 and NSP2 in *P. andersonii* commit non-symbiotic functions in hormonal homeostasis. These functions are in line with what is described for other plant species, suggesting that the generated *P. andersonii* lines represent true mutants.

Figure 5. Expression of *PanD27* and *PanMAX1* is reduced in *Pannsp1* and *Pannsp2* mutant roots.

Relative expression of *PanD27* (A) and *PanMAX1* (B) in roots of transgenic control and *Pannsp1* and *Pannsp2* mutant lines. Data represent means of three biological replicates $\pm$ SEM. Dots represent measurement values of biological repeats. Different letters indicate statistical significance ($p < 0.05$) as determined by ANOVA in combination with Tukey post-hoc test.

Nodulation Phenotypes of *Parasponia Panein2* and *Panhk4* Mutants Differ from Their Legume Counterparts

To determine whether *PanEIN2*, *PanHK4*, *PanNSP1* and *PanNSP2* perform similar functions during nodule formation as their legume orthologous, *P. andersonii* mutant plantlets were inoculated with *Mesorhizobium plurifarium* BOR2 [30]. Nodulation phenotypes were examined one month post inoculation.

The strong *Pannsp1-39* mutant allele and all three *Pannsp2* mutant lines are unable to form root nodules (Figure 6A and Supplemental Figure 13). This is similar as described for *M. truncatula*, *L. japonicus* and *Pisum sativum* *nsp1* and *nsp2* mutants [21, 22, 25, 49]. In contrast, the weak *Pannsp1* alleles *Pannsp1-6* and *Pannsp1-13* could be nodulated similar as

Figure 6. Nodule formation on *P. andersonii* CRISPR/Cas9 mutant lines.

(A) Nodule number per gram root fresh weight on wild type (WT), transgenic control and *Pannsp1* and *Pannsp2* mutant lines. Nodule number was determined at one month after inoculation with *Mesorhizobium plurifarium* BOR2.

(B) Nodule number per gram root fresh weight on wild type (WT), transgenic control and *Panein2* and *Panhk4* mutant lines. Nodule number was determined at one month after inoculation with *Mesorhizobium plurifarium* BOR2.

(C) Representative images of one month-old nodules. Note the dark color of *Panein2* nodules. Scale bars are equal to 0.5 mm.

Data in **(A-B)** represent means of 5-7 biological replicates $\pm$ SEM. Dots represent measurement values of biological repeats. Different letters indicate statistical significance ($p < 0.05$) as determined by ANOVA in combination with Tukey post-hoc test. Data on nodule number and root weight are shown in **Supplemental Figure 13**.

wild-type or control transgenic plants (**Figure 6A** and **Supplemental Figure 13**), suggesting that residual PanNSP1 activity is sufficient to support root nodule formation. Overall, these data show that NSP1 and NSP2 functioning is essential for root nodule formation in *Parasponia*.

Analysis of the nodulation phenotype of *P. andersonii* *Panhk4* mutants showed that PanHK4 is not required for root nodule formation. Both *Panhk4* mutant lines formed a similar amount of nodules as wild type and transgenic controls (**Figure 6B** and **Supplemental Figure 13**). This is different from the corresponding legume mutants - *M. truncatula* *Mtcre1* and *L. japonicus* *Ljlhk1* - that generally are not forming root nodules [23, 24].

The phenotype of *P. andersonii* *Panein2* mutants also differs from that of legume mutants. *M. truncatula* *ein2* mutants - as well as *L. japonicus* plants in which both EIN2-encoding genes have been silenced - form more nodules than wild type, which are clustered in distinct zones along the root [27, 36]. *Panein2* mutants do not form such nodule clusters and nodule number is not higher than wild type (**Figure 6B** and **Supplemental Figure 13**). However, nodules formed on *Panein2* mutant plants are generally smaller and dark colored when compared to nodules of control plants (**Figure 6C**). This suggests impaired nodule development in *P. andersonii* *ein2* mutants.

To determine the cyto-architecture of *Panein2*, *Panhk4* and *Pannsp1-6/Pannsp1-13* mutant nodules, we sectioned ~10 nodules for each mutant line and studied these by light microscopy. Wild-type *P. andersonii* nodules harbor an apical meristem, followed by several cell layers that contain infection threads (**Figure 7A**) [50]. Below this infection zone, 2-3 cell layers are found that display vacuolar fragmentation and increase in size compared to non-infected cells (**Figure 7B**). These cells are immediately followed by cells that are fully filled with fixation threads (**Figure 7B**). The general cyto-architecture of *Panhk4* and *Pannsp1-6/Pannsp1-13* mutant nodules does not differ from that of wild-type or transgenic control nodules (**Figure 7A, C-D**), suggesting that these are fully functional. In contrast, in *Panein2* mutant nodules intracellular infection is hampered (**Figure 7E-H**). Most (> 75%) *Panein2* mutant nodules harbor only infection threads as well as large apoplastic colonies (**Figure 7H**). Some mutant nodules, harbor cells that contain fixation threads. However, even in the best nodules, fixation thread formation is severely delayed and many cells in the fixation zone still show vacuolar fragmentation (**Figure 7H**). This shows that ethylene signaling is required for efficient fixation thread formation in *P. andersonii* nodules.

Taken together, these data reveal symbiotic mutant phenotypes for *nsp1*, *nsp2* and *ein2*, whereas no effect on nodule formation was found by knocking out *hk4* in *P. andersonii*. Interestingly, we uncovered a novel role for the ethylene signaling component EIN2 in intracellular infection of *P. andersonii* nodules.

Figure 7. Cyto-architecture of CRISPR/Cas9 mutant nodules.

Longitudinal nodule sections of one month-old nodules formed on transgenic control line Ctr-9 (A), *Pannsp1-13* (C), *Panhk4-25* (D), *Panein2-15* (E) and *Panein2-17* (F).

(B) Zoom in of cells in the infection (IZ) and fixation zone (FZ) of a transgenic control nodule shown in (A). Note the presence of small fragmented vacuoles in infected cells in the infection zone.

DISCUSSION

Comparative studies between legumes and the Cannabaceae tree *Parasponia* can provide insights in ‘core’ genetic networks underlying rhizobium symbiosis [30]. To facilitate such studies, we aimed to establish a reverse genetics platform for *P. andersonii* based on CRISPR/Cas9 genome editing. We show that using *A. tumefaciens* transformation, *P. andersonii* stable transgenic lines can be obtained in 3-4 months. In combination with CRISPR/Cas9 mutagenesis, this allows efficient generation of bi-allelic knockout mutants. As proof-of-concept, we mutated four genes that commit essential symbiotic functions in legumes as well as control different hormonal networks. Characterization of the resulting lines revealed both symbiotic as well as non-symbiotic mutant phenotypes. Therefore, we conclude that stable *A. tumefaciens*-mediated transformation in combination with CRISPR/Cas9 genome editing can be efficiently used for reverse genetic analysis in *P. andersonii*.

Plant transformation efficiency is the main bottleneck in plant genome editing [51, 52]. Especially regeneration of an entire transgenic plant out of a single transformed cell remains difficult for most plant species. We took advantage of an efficient micro-propagation system available for *Parasponia* spp. to establish a protocol for stable transformation [31-33]. About 8-12 weeks after cocultivation with *A. tumefaciens*, ~50% of explants develop transgenic shoots. This relatively high efficiency is, in part, obtained through an initial nine-day culturing period on root-inducing medium, prior to incubation on standard propagation medium. This adaptation in the protocol was inspired by a recent study that showed that regeneration of plant cells consists of two distinctive steps [37]. Regenerative competence is established through activation of a root developmental program, followed by activation of shoot promoting factors that are required to complete shoot regeneration [37]. The latter explains why transfer to propagation medium is required to regenerate *P. andersonii* transgenic shoots. However, this promoting effect of rooting medium on regeneration of transgenic shoots might differ between different explant types, as noted for *P. andersonii* stems and petioles (**Supplemental Table 3**).

(G) Zoom in on cells in the basal part of the *Panein2-15* nodule shown in **(E)**. Indicated by an arrowhead are cells containing fixation threads.

(H) Zoom in of the *Panein2-17* nodule shown in **(F)**. Indicated by an arrowhead are infection threads. Indicated by an arrow are large apoplastic colonies.

Scale bars in **(A, C-F)** are equal to 150 μm and 25 μm in **(B, G-H)**.

An advantage of the *Parasponia* system is that T_0 transgenic knockout mutants can be clonally propagated through *in vitro* micro-propagation [31-33]. This allows a large number of rooted plantlets to be generated in a relatively short time span. As a result, phenotypic characterization can be initiated already at four months after start of transformation. However, a disadvantage of clonal propagation in combination with CRISPR/Cas9 mutagenesis is the possibility of obtaining chimeric mutants. Among the mutant lines we created, we identified one line (out of twelve) that was chimeric for one out of three CRISPR target sites (**Supplemental Figure 10C**). Most mutant lines were genetically homogeneous, suggesting that mutations are generally induced soon after T-DNA integration. This is consistent with results in poplar, which also revealed a low percentage of chimeric mutants [53]. Since chimaeras are occasionally observed, thorough genotypic analysis will be required when phenotyping is performed in the T_0 generation. Besides vegetative propagation, *Parasponia* trees can also be propagated generatively. Under suitable greenhouse conditions, *Parasponia* trees flower within ~6-9 months and are self-compatible [4]. However, *Parasponia* trees can be monoecious or diecious and female flowers are wind pollinated [5]. This complicates selfing of trees and the production of pure seed badges. Additionally, *Parasponia* trees are fast growing and occupy a substantial amount of space in a tropical greenhouse (28°C, ~100% relative humidity), making generative propagation of multiple mutant lines logistically somewhat challenging. As an alternative for generative propagation, transgenic lines can be maintained *in vitro* and propagated vegetatively. Additionally, the fast and efficient transformation procedure presented here, will allow recreation of a particular mutant in less than six months.

Among the mutants we created, *Panhk4* and *Panein2* showed symbiotic phenotypes that differ from corresponding legume mutants. *P. andersonii* *Panhk4* mutants form nodules with a wild-type cyto-architecture, suggesting that these nodules are fully functional. Analysis of stem cross-sections showed that *Panhk4* mutants possess reduced cambial activity, suggesting that *PanHK4* encodes a functional cytokinin receptor. *M. truncatula* and *L. japonicus* mutants in the cytokinin receptors orthologous to *PanHK4* are characterized as nodulation deficient [23, 24]. However, these mutants occasionally form nodules [24, 54, 55]. This suggests redundant functioning of additional cytokinin receptors in both legume species. The *P. andersonii* genome also encodes two additional cytokinin receptors: *PanHK2* and *PanHK3* [30] (**Supplemental Figure 4**). Therefore, redundant functioning of one of these receptors cannot be excluded. Additional experiments are required to examine this possibility and determine the exact contribution of cytokinin to *Parasponia* nodule formation.

Mutation of *PanEIN2* revealed a different function for ethylene signaling in regulation of rhizobium symbiosis between legumes and *Parasponia*. In legumes, ethylene negatively regulates rhizobial infection and root nodule formation [27, 28, 36]. This is illustrated by the phenotype of the *M. truncatula ein2* mutant (named *sickle*) that forms extensive epidermal infection threads and clusters of small nodules [36, 56]. *P. andersonii ein2* mutants also form smaller nodules than wild type. However, in contrast to the *Mtein2* mutant, these nodules are regularly spaced on the root system. This suggests that in *Parasponia* ethylene signaling is not involved in regulating nodule number. Additionally, also the infection phenotype of *PanEin2* mutants differs from that in legumes. In *M. truncatula* and *L. japonicus*, interference with ethylene signaling increases the number of epidermal infection threads but does not affect intracellular colonization of nodule cells [36, 57, 58]. In contrast, in *P. andersonii PanEin2* mutants, intracellular colonization is hampered. Inside nodules, large apoplastic colonies are observed and fixation thread formation is severely reduced or even completely absent. This suggests that in *Parasponia* a functional ethylene signaling pathway is required for efficient intracellular infection of nodule cells. Since both nodule formation as well as intracellular infection are differentially regulated by ethylene in legumes and *Parasponia*, this suggests independent recruitment of ethylene function during evolution of rhizobium symbiosis in both lineages.

Mutagenesis of the *NSP2* orthologue of *P. andersonii* indicated a conserved symbiotic role for this GRAS-type transcriptional regulator. In legumes, NSP2 works in concert with NSP1 to control root nodule formation [59]. Mutagenesis of the *NSP1* orthologue of *P. andersonii* resulted in contrasting nodulation phenotypes. Two mutant lines, *Pannsp1-6* and *Pannsp1-13*, form nodules with a wild-type cyto-architecture, whereas mutant line *Pannsp1-39* is unable to form nodules (**Figure 6-7**). However, all three mutants are affected in transcriptional regulation of strigolactone biosynthesis genes *PanD27* and *PanMAX1* (**Figure 5**). The three *Pannsp1* mutant lines differ from each other in the type of mutations that were created. *Pannsp1-6* and *Pannsp1-13* contain small deletions that are immediately followed by a second in-frame ATG that in wild-type PanNSP1 encodes a methionine at position 16. In contrast, *Pannsp1-39* contains a larger deletion that removes this in-frame ATG (see **Supplemental Figure 9**). Several reports have shown that alternative start codons are occasionally used to initiate transcription [60-62]. Therefore, *Pannsp1-6* and *Pannsp1-13* most probably represent weak alleles that still possess residual PanNSP1 function. Such residual levels of PanNSP1 are affecting expression of strigolactone biosynthesis genes, but are still sufficient to allow nodule formation. Therefore, we argue that the *P. andersonii Pannsp1-39* line carries a knockout mutation, indicating that in *P. andersonii* both NSP1 and NSP2 are essential for rhizobium root nodule formation.

Taken together, we showed that *P. andersonii* can be efficiently transformed using *A. tumefaciens* and is amenable to targeted mutagenesis using CRISPR/Cas9. This protocol takes only marginally more time than the transient *A. rhizogenes* transformation system that is generally used to study root nodule formation [e.g. 18, 31, 63, 64, 65] but has several advantages. One of these is the absence of the *A. rhizogenes* root inducing locus (*rol*) that interferes with hormone homeostasis [66]. The protocol we developed will allow studies on *P. andersonii* symbiosis genes to determine to what extent legumes and *Parasponia* use similar mechanism to establish a nitrogen-fixing symbiosis with rhizobium. Additionally, *Parasponia* might present an excellent reverse genetics platform to ask basic questions in relation to general tree biology.

METHODS

Plant Materials and Growth Conditions

All experiments were conducted using *Parasponia andersonii* WU1 or offspring thereof [18, 30]. *P. andersonii* trees were grown in a conditioned greenhouse at 28°C, 85% humidity and a 16/8h day/night regime. For *in vitro* culturing, *P. andersonii* was grown in an Elbanton growth cabinet at 28°C, 16/8h day/night. Growth of young *P. andersonii* plantlets for nodulation assays or qRT-PCR analysis was performed in 1 L crystal-clear polypropylene containers equipped with an gas exchange filter (OS140BOX, Duchefa Biochemie, The Netherlands). Pots were half-filled with agraperlite (Maasmond-Westland, The Netherlands) and watered with modified EKM medium (3 mM MES ($C_6H_{13}NO_4$) pH 6.6, 2.08 mM $MgSO_4$, 0.88 mM KH_2PO_4 , 2.07 mM K_2HPO_4 , 1.45 mM $CaCl_2$, 0.70 mM Na_2SO_4 , 0.375 mM NH_4NO_3 , 15 μM Fe-citrate, 6.6 μM $MnSO_4$, 1.5 μM $ZnSO_4$, 1.6 μM $CuSO_4$, 4 μM H_3BO_3 , 4.1 μM Na_2MoO_4) [67] and placed in a climate room set at 28°C, 16/8h day/night. For nodulation assays, EKM medium was inoculated with *Mesorhizobium plurifarium* BOR2 ($OD_{600} = 0.025$) [30].

Vectors and Constructs

For CRISPR/Cas9-mediated mutagenesis, binary transformation constructs were created using Golden Gate assembly [68]. For an overview of all Golden Gate clones used in this study, see **Supplemental Table 5**. sgRNAs were designed based on the principles described in Doench et al. [69] and PCR amplified using specific forward primers and a universal reverse primer (**Supplemental Table 6**), using Addgene plasmid # 46966 as template [38]. These were cloned behind the AtU6p small RNA promoter and inserted behind the neomycin phosphotransferase II gene (*NPTII*) and an *A. thaliana* codon-optimized variant of Cas9 [40] fused to an N-terminal nuclear localization signal and driven by the 35S promoter

(**Supplemental Table 5**). As negative control, a binary vector was created containing only the NPTII- and NLS-Cas9-encoding sequences (**Supplemental Table 5**). To setup *P. andersonii* stable transformation, vector pKGWFS7-RR was used [70].

Phylogenetic Reconstruction

Protein sequences of *Glycine max* (Wm82.a2.v1) [71], *Medicago truncatula* (Mt4.0v1) [72, 73] and *Populus trichocarpa* (v3.0) [74] were obtained through Phytozome 10 (<http://phytozome.jgi.doe.gov/>). Protein sequences of *Parasponia andersonii* (PanWU01x14_asm01_ann01) and *Trema orientalis* (TorRG33x02_asm01_ann01) were obtained from www.parasponia.org [30]. These sequences were mined using sequences from *Arabidopsis thaliana* (TAIR10, www.arabidopsis.org) [75] and *M. truncatula*. Protein sequences were aligned using MAFFT v7.017 [76] implemented in Geneious 8.1.9 (Biomatters, New Zealand), using default parameter settings. Phylogenetic trees were constructed using FastTree [77] implemented in Geneious 8.1.9. Mid-point rooting was applied for better tree visualization using FigTree v1.4.2 (<http://tree.bio.ed.ac.uk/software/figtree/>).

Plant Transformation

Stable transformation of *P. andersonii* was performed using *Agrobacterium tumefaciens* strain AGL1 [78]. *A. tumefaciens* was grown for two days on agar-solidified LB medium containing appropriate antibiotics. For each *P. andersonii* transformation, two Petri dishes (Ø 9 cm) of *A. tumefaciens* were used. Bacteria were scraped from plate and resuspended in 25 ml of infiltration medium (SH10 (**Supplemental Table 7**), 20 mg/l acetosyringone (Sigma, USA), 0.001% (v/v) Silwet L-77 (www.arabidopsis.com)). *P. andersonii* tissue explants used for transformation were harvested from mature trees grown under greenhouse conditions and sterilized in 2% commercial bleach for 15 minutes. Tissue explants were cut at both ends inside the *A. tumefaciens* suspension, creating fresh wound surfaces, and kept inside the suspension for about 20 minutes. Subsequently, excess liquid was removed from tissue explants using sterilized filter paper and explants were placed on co-cultivation medium (Root-inducing medium (**Supplemental Table 7**), 20 mg/l acetosyringone (Sigma, USA)). Plates were incubated for two days at 21°C in darkness. After two days, tissue explants were washed three times using SH10 (**Supplemental Table 7**) and subsequently dried using filter paper. Tissue explants were placed on root-inducing medium containing 50 mg/l kanamycin and 300 mg/l cefotaxime and incubated at 28°C, 16h/8h (day/night). Nine days after transformation, tissue explants were transferred to propagation medium (**Supplemental Table 7**) containing 50 mg/l kanamycin and 300 mg/l cefotaxime. Plates were refreshed every other week. When regenerative calli reached ~2 mm in size they were separated

from tissue explants to stimulate shoot formation. A single shoot was selected per tissue explant. These shoots were propagated on propagation medium (**Supplemental Table 7**). Rooted plantlets were generated by placing individual shoots on root-inducing medium (**Supplemental Table 7**).

Characterization of Transgenic Lines

For T-DNA copy number estimates based on qPCR analysis, genomic DNA was isolated using the DNeasy Plant Mini Kit (Qiagen, Germany). qPCR was set up in a 10 µl reaction system with 2x iQ SYBR Green Super-mix (Bio-Rad, Hercules, USA) and 5 ng template DNA. The experimental setup and procedure were executed on a CFX Connect optical cycler, according to the manufacturer's protocol (Bio-Rad, USA). T-DNA copy number was estimated using two primer pairs amplifying part of the T-DNA and two primer pairs amplifying single copy *P. andersonii* genes (*PanAGT1* and *PanWU01x14_asm01_ann01_338920*) that were selected based on a study by Duarte et al. [79]. Primer sequences are listed in **Supplemental Table 6**. Data analysis was performed using CFX Manager 3.0 software (Bio-Rad, USA). For T-DNA copy number estimates based on Southern blotting, genomic DNA was separately digested with *XbaI*, *HindIII* and *EcoRI*. Blots were hybridized with a 516 bp α-32P-labelled probe corresponding to part of the *nptII* gene that was amplified using primers *nptII_Fw* and *nptII_Rv* listed in **Supplemental Table 6**.

Genotyping of transgenic lines was performed using the Phire Plant Direct PCR Kit (Thermo Scientific, USA) and gene specific primers listed in **Supplemental Table 6**. Ploidy estimates of transgenic lines were determined by FACS as described by van Velzen et al. [30].

To determine ethylene sensitivity of *Panein2* mutants, tips of young branches of four months-old trees were covered with 1 L plastic bags and injected with 1 ml of pure ethylene gas. After three days, bags were removed and leaf abscission examined. Total number of leaves on treated branches varied from 6-18.

Microtome Sectioning

Stem cross-sections were made from the base of the second uppermost axillary branch of three month-old trees. Shoot tissue was fixed in 5 % glutaraldehyde and embedded in Technovit 7100 (Heraeus-Kulzer, Germany), according to the manufacturer's protocol. Semi-thin (8 µm) sections were cut using a microtome (Reichert-Jung, Leica Microsystems, The Netherlands) and stained with 0.05% Toluidine Blue O. Images were taken using a Leica DM5500B microscope equipped with a DFC425C camera (Leica Microsystems, Germany).

Nodule tissue fixation and embedding was performed as previously described [80]. Semi-thin (0.6 µm) sections were cut using a Leica Ultracut microtome (Leica Microsystems, Germany) and photographed as described above.

RNA Isolation and qRT-PCR Analysis

RNA was isolated from snap-frozen root tips (~2-3 cm) as described by van Velzen et al. [30]. cDNA was prepared from 1 µg of total RNA using the i-script cDNA synthesis kit (Bio-Rad, USA), following the manufacturer's instructions. RT-qPCR was set up as described above. Normalization was performed based on two stably expressed reference genes (*PanUNK2*, *PanEF1α*), chosen based on previous study [81, 82]. All primers are listed in **Supplemental Table 6**.

Statistical Analysis

Statistical differences were determined based on One-way ANOVA and Tukey post-hoc tests. Statistical analyses were performed using IBM SPSS Statistics 23.0 (IBM, USA).

Accession Numbers

Gene identifiers for all *P. andersonii* genes used in this study can be found in **Supplemental Table 8**. Sequences can be downloaded from www.parasponia.org

AUTHOR CONTRIBUTIONS

Conceptualization, A.v.Z. and R.G.; Methodology, A.v.Z., M.H., S.L. and W.K.; Investigation, A.v.Z., T.A.K.W., M.S.K., L.R., F.B., M.H., S.L., E.F. and W.K.; Formal Analysis, A.v.Z., T.A.K.W. and E.F.; Visualization, A.v.Z.; Writing – Original Draft, A.v.Z.; Writing – Review & Editing, A.v.Z. and R.G.; Funding Acquisition, T.B. and R.G.; Supervision, R.G.

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SUPPLEMENTAL DATA

Supplemental Table 1. Regeneration efficiency of different explant types. Regeneration was determined for non-transgenic material incubated on propagation medium.

Explant type	Regeneration efficiency
Young leaves	-
Mature leaves	+/-
Midveins	+/-
Stem pieces (5 mm)	++
Stem pieces (1 mm)	+/-
Stem pieces, cut length-wise	+
Petioles	+++
Shoot apical meristems	-
Tissue culture shoots	+

+++ , means that > 90% of explants develop multiple regenerative calli.

-, indicates that no regeneration was observed, tissue turned dark brown in color.

Supplemental Table 2. Hormone composition of tissue culture media used in this study.

Hormone	Propagation medium	Root-inducing medium	Alternative media								
			1	2	3	4	5	6	7	8	9
BAP (mg/L)	1	-	0.33	1	3	0.33	1	3	0.33	1	3
IBA (mg/L)	0.1	1	-	-	-	-	-	-	-	-	-
NAA(mg/L)	-	0.1	0.03	0.03	0.03	0.1	0.1	0.1	0.3	0.3	0.3

Supplemental Table 3. Regeneration of transgenic material after co-cultivation and continuous culturing on propagation medium (Prop) or co-cultivation and culturing for seven additional days on root-inducing medium and subsequent transfer to propagation medium (Root -> prop). Regeneration was scored 6 weeks after start of transformation.

	Prop	Root -> prop
Petioles	+	++
Stem pieces (5 mm)	+/-	++

++ , means that > 50% of explants develop regenerative calli.

-, means that < 20% of explants develop regenerative calli.

Supplemental Table 4. Molecular characterization of transgenic lines.

Line ¹	T-DNA copy number estimate		Ploidy	Presence of right border sequences ⁴
	qRT-PCR ²	Southern blot ³		
1	2.9	na	Diploid	+
3*	1.0	1	Diploid	+
4	1.2	na	Diploid	+
12	0.9	na	Diploid	+
13	1.0	2	Diploid	+
18	1.9	na	Diploid	+
19	2.9	3	Diploid	+
21	1.1	na	Diploid	+
31	1.9	2	Diploid	+
33*	1.0	1	Diploid	+
37	2.1	na	Diploid	+
43*	0.9	1	Diploid	+
47	2.8	na	Diploid	+
49	1.1	na	Diploid	+
50	nd	na	Diploid	-
52	1.0	1	Diploid	+
54	0.5	2	Diploid	+
55	0.8	na	Diploid	+
59	0.4	na	Diploid	+
61	0.9	na	Diploid	+

¹ Asterisks indicate lines selected for further analyses.

² These numbers indicate measurement values from qRT-PCR on genomic DNA. nd indicates that T-DNA was not detected.

³ na indicates samples not analyzed by Southern blot.

⁴ PCR was performed using primers amplifying a sequence close to the T-DNA right border. +/- indicates presence/absence of a PCR amplicon.

Supplemental Table 5. List of Golden Gate¹ constructs used in this study.

Construct	Description	Level	Backbone	Contains ²	Literature
1	<i>nptII</i> resistance cassette	1	pICH47802	pICSL70004: <i>nptII</i>	-
2	35S _{pro} :QNLS-Cas9:35S _{ter}	1	pICH47742	pICH41388:35S _{pro} , pAGM5331:QNLS, pICH41308::aCas9, pICH41414:35S _{ter}	Fauser et al. [42]
3	PanEIN2sgRNA1	1	pICH47751	pICSL01009:AtU6p, corresponding PCR amplicon	Nekrasov et al. [40]
4	PanNSP1sgRNA1	1	pICH47751	pICSL01009:AtU6p, corresponding PCR amplicon	Nekrasov et al. [40]
5	PanNSP2sgRNA1	1	pICH47751	pICSL01009:AtU6p, corresponding PCR amplicon	Nekrasov et al. [40]
6	PanNSP2sgRNA2	1	pICH47761	pICSL01009:AtU6p, corresponding PCR amplicon	Nekrasov et al. [40]
7	PanNSP2sgRNA3	1	pICH47841	pICSL01009:AtU6p, corresponding PCR amplicon	Nekrasov et al. [40]
8	CRISPR_ctrl	2	pICSL4723	1R: construct 1, 2F: construct 2, end-link pICH41744	-
9	CRISPR_PanEIN2	2	pICSL4723	1R: construct 1, 2F: construct 2, 3F: construct 3, end-link pICH41766	-
10	CRISPR_PanNSP1	2	pICSL4723	1R: construct 1, 2F: construct 2, 3F: construct 4, end-link pICH41766	-
11	CRISPR_PanNSP2	2	pICSL4723	1R: construct 1, 2F: construct 2, 3F: construct 5, 4F: construct 6, 5R: construct 7, end-link pICH41800	-

¹ Backbones and standard parts used for Golden Gate assembly are from Engler et al. [41].

² 1F, 2R, etc. indicate position and orientation in level 2 constructs. Construct followed by a number refers to constructs build during this study and described in this table.

Supplemental Table 6. Primers used in this study.

Name	Purpose	Sequence
PanEIN2_sgRNA1	Clone sgRNA	tgtggtctcaattGTGTTAATCCTGGA AAATGGGggttttagagctagaaatagcaag
PanNSP1_sgRNA1	Clone sgRNA	tgtggtctcaattGCCAGTCCATGATG TGATCCGggttttagagctagaaatagcaag
PanNSP2_sgRNA1	Clone sgRNA	tgtggtctcaattGAAGACGACCATGG CTGCGCGggttttagagctagaaatagcaag
PanNSP2_sgRNA2	Clone sgRNA	tgtggtctcaattGACTGGAACGTCC TTACCGGggttttagagctagaaatagcaag
PanNSP2_sgRNA3	Clone sgRNA	tgtggtctcaattGTAGTTCGACACCG CCTACCGggttttagagctagaaatagcaag
sgRNA_Rv	Clone sgRNA	tgtggtctcaAGCGTAATGCCAATTTGTAC
geno_Cas9_Fw	Amplify aCas9	TTCGATCTCGCTGAGGATGC
geno_Cas9_Rv	Amplify aCas9	TAGCGAGAGGTCCCACGTAG
geno_PanEIN2-crispr-Fw	Genotyping CRISPR mutants	CATTGCAACAGCCTGTGGAC
geno_PanEIN2-crispr-Rv	Genotyping CRISPR mutants	CTGAGCAAGACCCCTTCCAG
geno_PanNSP1-crispr-Fw	Genotyping CRISPR mutants	TGGTTTCTCGTGGCCTTTGT
geno_PanNSP1-crispr-Rv	Genotyping CRISPR mutants	CTGTGGCCTTAGCTGAGCTT
geno_PanNSP2-crispr-Fw	Genotyping CRISPR mutants	ACTTCCACTCTGTCCCCGAA
geno_PanNSP2-crispr-Rv	Genotyping CRISPR mutants	GACCGGTGACTGAAGTGAGG
qPanD27_Fw	qRT-PCR	TCGGATCGCCATTCAACATC
qPanD27_Rv	qRT-PCR	GACAAGTTCCTGCTGTTTTG
qPanMAX1_Fw	qRT-PCR	TGATGAGATCGTGGCCAAGAG
qPanMAX1_Rv	qRT-PCR	AAAAACGTTCTCGCAACCG
qPanUNK2_Fw	qRT-PCR	TGCCATTGGTGTAGCTGTG
qPanUNK2_Rv	qRT-PCR	GTGTCTATCACTGCCTCTTTGC
qPanEF1 α _Fw	qRT-PCR	AGACAAGGTTAAGCGTGCAG
qPanEF1 α _Rv	qRT-PCR	TGCAACTGGGCAACAACTC
qTCN-1_Fw	T-DNA copy number	AAGCGCGTTACAAGAAAGCC
qTCN-1_Rv	T-DNA copy number	ACGTTGCCCGCATAATTACG
qTCN-3_Fw	T-DNA copy number	TGCACGACCACGCATTAATG
qTCN-3_Rv	T-DNA copy number	ACGATGCCATGTTCTCTGTC
PanAGT1_Fw	T-DNA copy number	AGCGCTTGATCTCCTTTTCG
PanAGT1_Rv	T-DNA copy number	TGCTTTACCTTGTGGCAGTC
Pan338920_Fw	T-DNA copy number	TGAAGCACAACACAGATGG
Pan338920_Rv	T-DNA copy number	TGCTTTCAGCCAGTTAACCG
nptII_Fw	Southern blotting	CACAACAGACAATCGGCTGC
nptII_Rv	Southern blotting	TGATATTCGGCAAGCAGGCA

Supplemental Table 7. Composition of tissue culture media.

Component	Medium		
	SH10	Propagation	Root-inducing
Schenk & Hildebrandt basal salt medium ^{1,2}	1x	1x	1x
Schenk & Hildebrandt vitamin mixture ^{1,2}	1x	1x	1x
Sucrose ¹	1% (w/v)	1% (w/v)	2% (w/v)
BAP (6-Benzylaminopurine) ³	-	1 mg/l	-
IBA (Indole-3-butyric acid) ³	-	0.1 mg/l	1 mg/l
NAA (1-Naphthaleneacetic acid) ³	-	-	0.1 mg/l
MES (2-ethanesulfonic acid) ³	3 mM, pH = 5.8	3 mM, pH = 5.8	3 mM, pH = 5.8
Daishin agar ¹	-	0.8% (w/v)	0.8% (w/v)

¹ Duchefa Biochemie, Haarlem, The Netherlands² Schenk and Hildebrandt, 1972³ Sigma, St. Louis, USA**Supplemental Table 8. GeneIDs for all *P. andersonii* genes used in this study. GeneIDs refer to *P. andersonii* gene models, which can be searched for on www.parasponia.org.**

Name	GeneID
<i>PanEIN2</i>	PanWU01x14_asm01_ann01_090380
<i>PanHK2</i>	PanWU01x14_asm01_ann01_002510
<i>PanHK3</i>	PanWU01x14_asm01_ann01_222240
<i>PanHK4</i>	PanWU01x14_asm01_ann01_103390
<i>PanNSP1</i>	PanWU01x14_asm01_ann01_334190
<i>PanSCL26</i>	PanWU01x14_asm01_ann01_236490
<i>PanSCL34</i>	PanWU01x14_asm01_ann01_107700
<i>PanSCL16</i>	PanWU01x14_asm01_ann01_130270
<i>PanSHR</i>	PanWU01x14_asm01_ann01_108080
<i>PanNSP2</i>	PanWU01x14_asm01_ann01_157260
<i>PanSCL28</i>	PanWU01x14_asm01_ann01_101430
<i>PanSCL18</i>	PanWU01x14_asm01_ann01_295370
<i>PanSCL17</i>	PanWU01x14_asm01_ann01_245480
<i>PanSCL27</i>	PanWU01x14_asm01_ann01_245490
<i>PanRAM1</i>	PanWU01x14_asm01_ann01_146390
<i>PanD27</i>	PanWU01x14_asm01_ann01_194930
<i>PanD27L1</i>	PanWU01x14_asm01_ann01_057040
<i>PanD27L2</i>	PanWU01x14_asm01_ann01_143920
<i>PanMAX1</i>	PanWU01x14_asm01_ann01_182160
<i>PanUNK2</i>	PanWU01x14_asm01_ann01_211960
<i>PanEF1α</i>	PanWU01x14_asm01_ann01_070680
<i>PanAGT1</i>	PanWU01x14_asm01_ann01_040540
-	PanWU01x14_asm01_ann01_338920

Supplemental Figure 1. *P. andersonii* transgenic shoots can be effectively propagated.

Transgenic *P. andersonii* shoots propagated *in vitro*. Images were taken six months after transformation. Scale bars are equal to 2.5 mm. Shown from left to right are bright-field images, overlays of bright-field and DsRED fluorescence and DsRED fluorescence images.

Supplemental Figure 2. *Trans*-genes remain stably integrated in the *P. andersonii* genome.

Bright field (top) and DsRED1 fluorescence (bottom) images of leaves harvested from mature trees grown under greenhouse conditions (A) or *in vitro* propagated material (B). Images are shown for transgenic lines 3, 33 and 43 and control plants not expressing the DsRED1 marker. Images were taken one year after transgenic lines were selected. Scale bars are equal to 5 mm.

Supplemental Figure 3. Phylogeny of EIN2 proteins.

Phylogeny was reconstructed based on an alignment of EIN2 proteins from *Arabidopsis thaliana* (At), soybean (*Glycine max*, Gm), *Lotus japonicus* (Lj), *Medicago truncatula* (Mt), poplar (*Populus trichocarpa*, Potri), *Parasponia andersonii* (Pan) and *Trema orientalis* (Tor). Branch support is indicated by FastTree support values [78]. Terminals are labeled by their gene name or gene identifier. EIN2 proteins of *P. andersonii* and *T. orientalis* are highlighted in blue and red, respectively. Mid-point rooting was applied for better tree visualization.

Phylogeny was reconstructed based on an alignment of cytokinin receptor proteins from *Arabidopsis thaliana* (At), soybean (*Glycine max*, Gm), *Lotus japonicus* (Lj), *Medicago truncatula* (Mt), poplar (*Populus trichocarpa*, Potri), *Parasponia andersonii* (Pan) and *Trema orientalis* (Tor). Branch support is indicated by FastTree support values [78]. Terminals are labeled by their gene name or gene identifier. The MtCRE1/LjLHK1 putative orthologues of *P. andersonii* and *T. orientalis* are highlighted in blue and red, respectively. Mid-point rooting was applied for better tree visualization.

Supplemental Figure 5. Phylogeny of NSP1 and related GRAS proteins.

Phylogeny was reconstructed based on an alignment of GRAS proteins from *Arabidopsis thaliana* (At), soybean (*Glycine max*, Gm), *Medicago truncatula* (Mt), poplar (*Populus trichocarpa*, Potri), *Parasponia andersonii* (Pan) and *Trema orientalis* (Tor). Branch support is indicated by FastTree support values [78]. Terminals are labeled by their gene name or gene identifier. The MtNSP1 putative orthologues of *P. andersonii* and *T. orientalis* are highlighted in blue and red, respectively. Mid-point rooting was applied for better tree visualization.

Phylogeny was reconstructed based on an alignment of GRAS proteins from *Arabidopsis thaliana* (At), soybean (*Glycine max*, Gm), *Medicago truncatula* (Mt), poplar (*Populus trichocarpa*, Potri), *Parasponia andersonii* (Pan) and *Trema orientalis* (Tor). Branch support is indicated by FastTree support values [78]. Terminals are labeled by their gene name or gene identifier. The MtNSP2 putative orthologues of *P. andersonii* and *T. orientalis* are highlighted in blue and red, respectively. Mid-point rooting was applied for better tree visualization.

Supplemental Figures 7. CRISPR mutant alleles of *Panein2* mutant lines.

(A) Schematic representation of *PanEIN2* gene model. Indicated by a grey arrowhead is the location of the sgRNA target site.

(B) Sequence alignment of the first exon of *PanEIN2* in wild type (WT) and *Panein2* mutant lines. For bi-allelic mutant lines, both alleles are shown (A and B alleles). Highlighted in blue and red are the sgRNA target site and PAM sequence, respectively. Arrow in sequence indicates a large (> 100 bp) insertion in *Panein2-12* allele B.

Supplemental Figures 8. CRISPR mutant alleles of *Panhk4* mutant lines.

(A) Schematic representation of *PanHK4* gene model. Indicated by grey arrowheads are the locations of three sgRNA target sites.

(B) Sequence alignment of the first exon of *PanHK4* in wild type (WT) and *Panhk4* mutant lines. For bi-allelic mutant lines, both alleles are shown (A and B alleles). Highlighted in blue and red are the sgRNA target sites and PAM sequences, respectively.

Supplemental Figures 11. Phylogeny of D27 and D27-like proteins.

Phylogeny was reconstructed based on an alignment of D27 and D27-like proteins from *Arabidopsis thaliana* (At), soybean (*Glycine max*, Gm), *Medicago truncatula* (Mt), poplar (*Populus trichocarpa*, Potri), *Parasponia andersonii* (Pan) and *Trema orientalis* (Tor). Branch support is indicated by FastTree support values [78]. Terminals are labeled by their gene name or gene identifier. The *P. andersonii* and *T. orientalis* putative orthologues of MtD27 are highlighted in blue and red, respectively. Mid-point rooting was applied for better tree visualization.

Supplemental Figures 10. CRISPR mutant alleles of *Pannsp2* mutant lines.

(A) Schematic representation of *PanNSP2* gene model. Indicated by grey arrowheads are the locations of three sgRNA target sites.

(B) Sequence alignment of the first part of the *PanNSP2* gene in wild type (WT) and *Pannsp2* mutant lines. For bi-allelic mutant lines, both alleles are shown (A and B alleles). Highlighted in blue and red are the sgRNA target sites and PAM sequences, respectively.

(C) Sequence alignment of part of the *PanNSP2* gene in wild type (WT) and *Pannsp2-9*. The region depicted corresponds to the first two sgRNA target sites. Sequences from multiple individual *Pannsp2-9* shoots are shown, to indicate that this line is chimeric for the first sgRNA target site. Highlighted in blue and red are the sgRNA target sites and PAM sequences, respectively.

Supplemental Figures 12. Phylogeny of MAX1 proteins.

Phylogeny was reconstructed based on an alignment of MAX1 proteins from *Arabidopsis thaliana* (At), soybean (*Glycine max*, Gm), *Medicago truncatula* (Mt), poplar (*Populus trichocarpa*, Potri), *Parasponia andersonii* (Pan) and *Trema orientalis* (Tor). Branch support is indicated by FastTree support values [78]. Terminals are labeled by their gene name or gene identifier. The *P. andersonii* and *T. orientalis* putative orthologues of MtMAX1 are highlighted in blue and red, respectively. Mid-point rooting was applied for better tree visualization.

Supplemental Figure 13. Nodule formation on *P. andersonii* CRISPR/Cas9 mutant lines.

Nodule number and fresh root weight data belonging to **Figure 7**. Nodule number and root fresh weight was determined at one month post inoculation with *Mesorhizobium plurifarum* BOR2. Data represent means of 5-7 biological replicates $\pm$ SEM. Dots represent measurement values of biological repeats. Different letters indicate statistical significance ($p < 0.05$) as determined by ANOVA in combination with Tukey post-hoc test. WT, wild type.

Chapter 6

The background of the page features a faint, artistic illustration. On the left, a plant with several leaves and a network of roots is depicted. On the right, there is a circular inset showing a microscopic view of plant cells, likely from a root, with distinct cell walls and internal structures.

Nodulating *Parasponia* and non-nodulating *Trema* species harbor distinct root transcriptomes

Arjan van Zeijl, Robin van Velzen, Rens Holmer, Kerstin Guhl, Sidney Linders, Marijke Hartog, Jan Verver, Wouter Kohlen, Ton Bisseling and Rene Geurts

ABSTRACT

Parasponia represents the only non-legume lineage able to establish a nitrogen-fixing nodule symbiosis with rhizobium. Phylogenetic reconstruction showed that the *Parasponia* lineage is embedded in the non-nodulating genus *Trema*. This close genetic relationship suggests that both groups of species only recently diverged in nodulation ability. Here, we exploited this close phylogenetic relationship to determine to what extent differentiation in gene expression is correlated with the nodulation trait. By RNA-seq, we show that *Parasponia* and *Trema* harbor distinct root transcriptomes. This demonstrates that gene expression differentiation occurred between *Parasponia* and two *Trema* lineages. Among genes higher expressed in roots of *Parasponia* are several involved in mycorrhizal symbiosis as well as jasmonic acid biosynthesis. Measurements of hormone concentrations showed that *Parasponia* and *Trema* roots display a different jasmonic acid/salicylic acid balance. It is now a challenge to determine which of these differences are relevant in a symbiotic context, as mutants in jasmonic acid biosynthesis are unaffected in nodule development.

INTRODUCTION

Plants require large amounts of mineral nitrogen for growth and development. However, in soil the amount of mineral nitrogen is often limiting. To cope with nitrogen limitation, some plants evolved an endosymbiotic relationship with nitrogen-fixing actinomycetes of the genus *Frankia* or with rhizobium bacteria [1, 2]. To allow this symbiosis to occur, these plants develop specialized organs on their roots, known as nodules. Nodules provide optimal physiological conditions for the fixation of atmospheric nitrogen by the microbial symbiont. Nitrogen-fixing endosymbioses are found among four taxonomic orders of flowering plants that together form the nitrogen-fixation clade [3-5]. Within this clade, nodulation is restricted to ten separate lineages that are scattered among many non-nodulating plant lineages [6]. The fact that nodulating lineages are phylogenetically related suggests that genetic adaptations that allowed these nitrogen-fixing symbioses have occurred in a common ancestor.

A major aim in symbiosis research is to understand the evolutionary trajectory towards such endosymbiosis. Nitrogen-fixing root nodules are best studied in legumes; especially in *Lotus japonicus* and *Medicago truncatula*, two species that serve as model. Legumes together with *Parasponia* (Cannabaceae) represent the only two lineages that can form nodules with rhizobium bacteria [3, 7-9]. Studies on *M. truncatula* and *L. japonicus* uncovered that the genetic network used to establish symbiosis with rhizobium was partly co-opted from that involved in the arbuscular mycorrhizal (AM) symbiosis [10-17]. To initiate symbiosis, both rhizobia and AM fungi excrete lipo-chitooligosaccharide (LCO) signals [18, 19]. These are perceived by LysM-type receptors at the plant root epidermis, which activates a signaling network largely shared between both symbioses [20-24]. Perception of rhizobium LCOs mitotically activates root cortical cells leading to nodule organogenesis [25, 26]. Rhizobia intracellularly colonize nodule cells, but remain separated from the host cell cytoplasm by a host-derived membrane. Formation of this host-microbe interface membrane involves an exocytotic pathway that has also been co-opted from AM symbiosis [27-29].

To establish symbiosis, rhizobia and mycorrhizal fungi need to suppress plant immunity [30, 31]. In legumes, inoculation with incompatible rhizobium bacteria elevates salicylic acid (SA) levels [32, 33]. SA is an important regulator of basal immunity against biotrophic microbes and acts antagonistically to jasmonic acid (JA)-induced signaling [34-36]. JA is mainly involved in defense against necrotrophs and is produced from α -linolenic acid, which is released from galactolipids in the chloroplast membrane [35, 37]. Consecutive activity of 13S-lipoxygenases (LOX), allene oxide synthases (AOS) and allene oxide cyclases (AOC) converts α -linolenic

acid into the JA precursor *cis*-(+)-12-oxophytodienoic acid (OPDA). OPDA is subsequently converted into JA and its bioactive conjugate jasmonoyl-L-isoleucine (JA-Ile) [37, 38]. In alfalfa (*Medicago sativa*), an increase in LOX activity is correlated with successful infection by rhizobium, suggesting a role for JA in the nodulation process [39]. Similarly, application of 0.1 μ M JA to roots of *L. japonicus* promotes rhizobial infection and root nodule formation, whereas higher concentrations generally inhibit nodule organogenesis [40, 41].

Generally, traits evolve due to a combination of gene duplication and subsequent neofunctionalization, gene loss as well as regulatory evolution. To unravel the evolutionary trajectory of a nitrogen-fixing endosymbiosis, we recently adopted *Parasponia* as a research model [42]. The *Parasponia* lineage is embedded in the non-nodulating *Trema* genus [42, 43]. This close phylogenetic relationship between species that differ in their ability to nodulate provides an excellent model to get insight is the genetic changes that are associated with root nodule formation. Analysis of sequenced genomes for three *Parasponia* and three *Trema* species revealed > 25,000 orthologous gene models and only limited number of copy number variants between *Parasponia* and *Trema* [42]. This analysis also showed that *Trema* spp. independently lost several putative symbiosis genes that are considered to be essential for root nodule formation in *Parasponia* [42]. This suggests that *Trema* spp. no longer possess the potential to nodulate, or even have lost this trait in recent history. Irrespective of the evolutionary trajectory, the close relationship of *Parasponia* and *Trema*, which only recently diverged in nodulation behavior, provides an excellent model to determine to what extent gene expression differentiation is associated with the nodulation trait.

Here, we show that *Parasponia* and *Trema* harbor distinct root transcriptomes. This shows that gene expression differentiation occurred between both groups of species. Among the differentially expressed genes are several involved in mycorrhizal symbiosis and JA homeostasis. Measurements of root hormone content revealed that *Parasponia* roots contain higher OPDA and JA, but lower SA concentrations compared to *Trema*, indicating a difference in JA/SA balance. The biological significance of this remains currently unknown, as mutants in JA biosynthesis are unaffected in nodule development.

RESULTS

Parasponia and *Trema* Root Transcriptomes Are Distinct

Nodulating *Parasponia* recently diverged from closely related non-nodulating *Trema* species. We questioned whether regulatory evolution occurred after the *Parasponia*-*Trema*

Figure 1. *Parasponia* and *Trema* harbor distinct root transcriptomes.

Principal component analysis (PCA) on gene expression values in roots of two *Parasponia* and three *Trema* species. PCA was performed on DESeq2 ‘regularized log’ transformed read counts obtained after mapping of *P. andersonii* and *P. rigida* reads to the *P. andersonii* genome and *T. levigata*, *T. orientalis* and *T. tomentosa* reads against the *T. orientalis* genome. Each dot represents a biological replicate. Plotted are first two principal components (PC1 and PC2) and indicated is the percentage of explained variance by each principal component.

split. Therefore, we determined root transcriptomes of two *Parasponia* species as well as the closely-related sister species *Trema levigata* and the outgroup species *Trema orientalis* and *Trema tomentosa* [42] by RNA sequencing (RNA-seq). To this end, non-inoculated seedlings of all five species were grown in perlite watered with EKM medium (5 mM KNO₃) [44]. When inoculated, this condition is conducive for *Parasponia* root nodule formation (**Supplemental Figure 1**). After four weeks of growth, RNA was isolated from a ~5-10 mm region just above the root tip of young freshly growing roots. For analysis of RNA-seq data, we focused on 24,246 high-confidence orthologous gene pairs identified between the genomes of *Parasponia andersonii* and *T. orientalis* [42]. This allows direct comparison of gene expression values between *Parasponia* and *Trema* species. Principal component analysis (PCA) on DESeq2 ‘regularized log’ transformed read counts separates *Parasponia* from *Trema* samples along the first principal component (PC1; **Figure 1**). This separation is not affected by cross-mapping of reads to either the *P. andersonii* or *T. orientalis* genome (**Supplemental Figure 2**). The separation along PC1 explains $\geq 75\%$ of the observed variance, indicating substantial variation between *Parasponia* and *Trema* root transcriptomes (**Figure 1** and **Supplemental Figure 2**). Including samples from *P. andersonii* and *T. tomentosa* plants obtained through *in vitro* propagation and grown under a different nitrogen regime (0.375 mM NH₄NO₃) does not affect this separation, indicating robust differences (**Supplemental**

Table 1. Classification of differentially expressed (fold change > 2, $p < 0.05$) genes in functional classes.

Functional class	Higher expressed in <i>Parasponia</i>	Lower expressed in <i>Parasponia</i>
Transcription factors	9	2
Symbiosis	3	0
Hormone homeostasis	8	5
Immunity	14	7
Enzymes	39	65
Transporters	8	21
Signaling	12	17
Cell wall	3	3
No annotation	33	23
Other	50	68
Total	179	211

Figure 3). Therefore, we conclude that the root transcriptomes of two *Parasponia* species are distinct from that of the *Trema* sister species *T. levigata*, as well as the outgroup species *T. orientalis* and *T. tomentosa*.

Genes Lower Expressed in *Parasponia* Roots Are Enriched for Genes with a Nodule-Enhanced Expression Profile

We questioned whether regulatory evolution between *Parasponia* and *Trema* is relevant in a symbiotic context. To this end, we first identified genes that show consistent differential expression between both groups of species. For this, we made use of both RNA-seq datasets described above. Based on a minimal fold change larger than two and an FDR-corrected p -value smaller than 0.05, we identified 179 and 211 genes that are consistently higher or lower expressed in roots of *Parasponia* spp., respectively (**Supplemental Tables 1-2**). These differentially expressed genes were manually divided into nine functional classes, including symbiosis, transcription factors and hormone homeostasis (**Table 1**). This showed that the majority of differentially expressed genes encode enzymes involved in primary and secondary metabolism (**Table 1**).

The differentially expressed genes theoretically represent three, partly overlapping classes: I. genes that perform a symbiotic function, II. genes that exclusively perform non-symbiotic functions and III. genes that commit pleiotropic functions. To get first insight in which genes

Figure 2. Expression of mycorrhizal marker genes is higher in roots of *Parasponia*.

Expression of the mycorrhizal marker genes AMT4, AMT5, PT4, STR2, RAD1 and VPY in roots of *P. andersonii* (Pan), *P. rigida* (Pri), *T. levigata* (Tle), *T. orientalis* (Tor) and *T. tomentosa* (Tto). Expression levels were calculated after mapping of *P. andersonii* and *P. rigida* reads to the *P. andersonii* genome and *T. levigata*, *T. orientalis* and *T. tomentosa* reads against the *T. orientalis* genome. Expression levels are presented as DESeq2 normalized read counts. Data represent means of 3 biological replicates $\pm$ SEM. Dots represent expression values in each of the biological replicates. Grey panel highlighting of expression plots indicates differential expression (fold change > 2, $p < 0.05$), consistent across all comparisons.

may commit a symbiotic function (class I and III), we compared the list of differentially expressed genes to a published set of genes that show nodule-enhanced expression in *P. andersonii* [42]. This revealed that 18 of the genes that are higher expressed in *Parasponia* roots display a nodule-enhanced expression pattern (see **Supplemental Table 1**). This includes *Vapyrin* (VPY), which encodes a putative membrane trafficking protein required for AM fungal and rhizobium symbiosis in *M. truncatula* [45, 46]. Among genes that are lower expressed in roots of *Parasponia* are 35 genes that are nodule-enhanced (see **Supplemental Table 2**). This represents significant enrichment ($p < 0.001$, hypergeometric test), suggesting that reduced expression in the root might potentiate genes to function in symbiosis.

α -linolenic acid

13S-lipoxygenases

13-HPOT

Allene oxide synthases

12,13-EOT

Allene oxide cyclases

cis-(+)-12-OPDA

OPDA Reductase 3

OPC-8:0

(+)-7-*iso*-jasmonic acid

JA-amino synthetase

jasmonoyl-L-isoleucine

AM Marker Genes Are Higher Expressed in Roots of *Parasponia*

The genetic networks used by legumes and *Parasponia* to establish symbiosis with rhizobium have been partly co-opted from that involved in AM symbiosis [2, 24]. Interestingly, among the genes differentially expressed between *Parasponia* and *Trema* roots are three genes of which orthologs are shown to be required for AM symbiosis in other species (Table 1). These genes, *VPY*, the GRAS-type transcriptional regulator *RAD1* and the phosphate transporter *PT4* [47, 48], are all higher expressed in roots of *Parasponia* (Figure 2 and Supplemental Figure 4). As mentioned above, *VPY* also functions during legume-rhizobium symbiosis and displays a nodule-enhanced expression pattern in *P. andersonii* (Supplemental Figure 5). Conversely, *PanRAD1* and *PanPT4* are not expressed in nodules (Supplemental Figure 5), in line with specific functions in mycorrhization. *VPY* and *PT4* are often used as marker genes for AM symbiosis in legumes, together with the GRAS-type transcriptional regulator *RAM1*, the glycerol-3-phosphate acyl transferase *RAM2*, the subtilase *SbtM1*, *BCP1* encoding a blue copper-binding protein, the ammonium transporter *LjAMT2;2* and the half-size ABC transporters *STR* and *STR2* [49-55]. We questioned whether any of these additional AM marker genes are also higher expressed in *Parasponia* roots. The *Parasponia-Trema* orthologues of *RAM1* and *STR* are not or very lowly expressed in non-inoculated roots, whereas a *RAM2* orthologue shows equal expression between both species groups (data not shown). For *SbtM1* and *BCP1* no clear orthologues could be identified in *Parasponia-Trema* genomes. However, homologs of *LjAMT2;2* (*AMT4* and *AMT5*) and *STR2* showed to be higher expressed in roots of *Parasponia* (Figure 2 and Supplemental Figure 4). Together, these data suggest that at least six AM marker genes, among which three transporters of phosphates or ammonium, are higher expressed in roots of *Parasponia*, when compared to *Trema* species.

Parasponia and *Trema* Roots Contain a Different JA/SA Balance

Changes to the hormone balance are known to cause pleiotropic effects and as such might alter symbiotic susceptibility as well as cause profound transcriptional changes [40, 56, 57]. Among differentially expressed hormone-related genes, the 13S-lipoxygenase *LOX2* is

Figure 3. JA biosynthesis genes *LOX2*, *LOX3* and *AOC2* are higher expressed in roots of *Parasponia*.

On the left, an overview of the JA biosynthesis pathway. On the right, expression of the corresponding JA biosynthesis genes in roots of *P. andersonii* (Pan), *P. rigida* (Pri), *T. levigata* (Tle), *T. orientalis* (Tor) and *T. tomentosa* (Tto). Expression levels were calculated after mapping of *P. andersonii* and *P. rigida* reads to the *P. andersonii* genome and *T. levigata*, *T. orientalis* and *T. tomentosa* reads against the *T. orientalis* genome. Expression levels are presented as DESeq2 normalized read counts. Data represent means of 3 biological replicates $\pm$ SEM. Dots represent expression values in each of the biological replicates. Grey panel highlighting of expression plots indicates differential expression (fold change > 2, $p < 0.05$), consistent across all comparisons.

Figure 4. *Parasponia* and *Trema* roots contain different concentrations of OPDA, JA and SA

Concentrations of OPDA (A), JA (B), JA-Ile (C), IAA (D), ABA (E) and SA (F) in roots of *P. andersonii* (Pan) and *T. tomentosa* (Tto). Data represent means of 6 biological replicates $\pm$ SEM. Dots represent expression values in each of the biological replicates. Grey panel highlighting indicates statistical significance ($p < 0.05$, Student's t -test). FW, fresh weight.

induced in nodules, suggesting a potential symbiotic function (Supplemental Figure 6). *LOX2* is putatively orthologous to *AtLOX2* from *Arabidopsis*, which is involved in JA biosynthesis [58]. Besides *LOX2*, also its close paralogue, *LOX3*, and the allene oxide cyclase *AOC2* are higher expressed in roots of *Parasponia* (Figure 3). Close inspection of the JA biosynthesis pathway, suggests that additional genes involved in production of the JA-precursor OPDA are also higher expressed in *Parasponia* roots, though not deemed significant across comparisons (Figure 3).

Higher expression of JA biosynthesis genes in roots of *Parasponia* could affect the root's hormone balance. Therefore, we measured concentrations of OPDA, JA, JA-Ile, salicylic acid (SA), abscisic acid (ABA) and indoleacetic acid (IAA) in roots of *P. andersonii* and *T. tomentosa*. Hormones were extracted from the same root zone as used for RNA-seq. In line with RNA-seq results, concentrations of OPDA and JA are 2.5- and 5-fold higher in roots of *P. andersonii*, respectively (Figure 4A-B). However, concentrations of the bio-active conjugate JA-Ile as well as ABA and IAA do not differ between both species (Figure 4C-E). Interestingly, SA concentrations are 2.5-fold lower in roots of *P. andersonii* (Figure 4F). This shows that young root segments of *Parasponia* and *Trema* harbor a distinct JA/SA balance.

Figure 5. JA deficiency does not affect nodulation ability.

(A) Nodule formation on JA biosynthesis mutants at 6 weeks after inoculation with *M. plurifarium* BOR2. Data represent means of 9-29 biological replicates $\pm$ SEM. Dots represent individual data points. For JA mutants, nodulation data was combined from 3-4 individual transgenic lines, each containing 6-8 biological replicates. Different letters on bars indicate statistical significance ($p < 0.05$, ANOVA with Tukey post-hoc test). Data for each of the individual lines can be found in **Supplemental Figure 10**, which also contains data on nodule number per plant and root fresh weight. This data was used to calculate the data presented here. WT, wild-type *P. andersonii*; Ctr-44, transgenic control line expressing the Cas9 gene only [Chapter 5].

(B-E) Longitudinal nodule sections through a transgenic control nodule (B), nodule formed on *Panlox2-lox3* mutant line 3 (C), nodule formed on *Panaoc1-aoc2* mutant line 32 (D) and nodule formed on *Panjar1* mutant line 10 (E). Bars represent 150 μm.

JA Deficiency Does Not Affect Rhizobium Symbiosis in *Parasponia*

To determine the role of JA in *Parasponia*-rhizobium symbiosis, we generated *PanLOX2-PanLOX3* and *PanAOC1-PanAOC2* double mutant lines and *PanJAR1* single mutant lines, using CRISPR/Cas9 [Chapter 5]. Among these, *Panaoc1-Panaoc2* double mutants are expected to be OPDA and JA deficient and *Panjar1* mutants to be unable to convert JA into JA-Ile. An overview of mutant lines can be found in **Supplemental Figure 7-9**. To determine symbiotic mutant phenotypes, mutant lines were inoculated with *Mesorhizobium plurifarium* BOR2 [42]. After six weeks, nodule number was examined, which revealed no difference from wild type (**Figure 5A** and **Supplemental Figure 10**). To determine whether mutant nodules might be affected in intracellular colonization by rhizobium, we sectioned > 10 nodules of *Panlox2-Panlox3-3*, *Panaoc1-Panaoc2-32* and *Panjar1-10*. This showed that mutant nodules contain a wild-type cyto-architecture and are fully infected by rhizobium bacteria (**Figure 5B-E**). Therefore, mutant analysis indicates that JA deficiency does not affect *Parasponia*-rhizobium symbiosis.

DISCUSSION

Parasponia and *Trema* represent closely related species with opposing symbiotic phenotypes. Genome comparisons revealed only limited number of copy number variants between both groups of species [42]. Here, we performed comparative transcriptome analysis between two *Parasponia* and three *Trema* species. This showed that *Parasponia* and *Trema* harbor distinct root transcriptomes, suggesting extensive regulatory evolution. Amongst others, this affected root hormone homeostasis. Expression analysis as well as hormone quantification showed that *Parasponia* and *Trema* roots harbor a different JA/SA balance. However, this difference does not appear to be essential for *Parasponia* root nodule formation, as JA-deficient mutants are unaffected in nodule development. Therefore, it is now a challenge to determine whether the transcriptional differences between *Parasponia* and *Trema* are relevant in a symbiotic context

PCA analysis on root expression values separated *Parasponia* from *Trema* samples, indicating that both groups of species harbor distinct root transcriptomes. The difference in transcriptomes is not correlated to phylogenetic distance, as *T. levigata* is more closely related to *Parasponia* than to *T. orientalis* or *T. tomentosa* [42]. This suggests that the 'core' *Trema* root transcriptome might better represent the ancestral state. Therefore, it is most parsimonious that regulatory evolution occurred in the most recent common ancestor of all *Parasponia* species, resulting in transcriptional divergence from *Trema*. However, *Trema* species independently lost genes that are putatively essential for rhizobium symbiosis in *Parasponia*, which has led to the hypothesis that *Trema* once possessed the ability to nodulate [42]. Therefore, it cannot be excluded that the transcriptional differences between *Parasponia* and *Trema* are due to parallel regulatory evolution in independent *Trema* lineages. However, this assumes that the transcriptional differences between both groups of species are directly or indirectly correlated to nodulation capacity. An overrepresentation of nodule-enhanced genes among differentially expressed genes between *Parasponia* and *Trema* roots, provides some support for this assumption.

Hormone measurements showed a higher concentration of OPDA and JA in roots of *P. andersonii* compared to *T. tomentosa*. In contrast, SA concentrations were higher in roots of *T. tomentosa*, indicating a shift in JA/SA balance. Higher expression of JA biosynthesis genes in roots of *P. andersonii* and *P. rigida* is consistent with higher OPDA and JA concentrations in *P. andersonii* roots, and suggests that the difference in OPDA and JA concentrations is consistent between *Parasponia* and *Trema* species. Whether the same applies to SA is difficult to predict. JA and SA function largely antagonistically and extensive cross-talk occurs

between both hormone signaling pathways [34-36]. In *Arabidopsis*, basal SA concentrations are increased in the JA-deficient *dde2* mutant, mutated in allene oxide synthase [59]. This implies that in wild-type *Arabidopsis*, JA signaling reduces SA concentrations. Therefore, it is possible that reduced basal SA levels in roots of *Parasponia* result from increased JA biosynthesis. Conversely, reduced SA concentrations could relieve inhibition of JA signaling, promoting JA biosynthesis through positive feedback [60, 61].

A reduction in root SA concentrations could be relevant in a symbiotic context. SA functions in defense against biotrophic microbes [35] and therefore likely affects infection by rhizobia. In legumes, infection by incompatible rhizobium bacteria triggers an increase in SA concentration, whereas during a compatible interaction SA levels remain unaffected or are even slightly reduced [32, 33, 62]. We analyzed *Parasponia* mutants affected in JA biosynthesis and were unable to detect a nodulation phenotype. Whether in these mutants SA concentrations were increased to similar concentration as detected in *T. tomentosa* roots remains to be examined. Therefore, the ecological significance of a change in JA/SA balance between *Parasponia* and *Trema* roots remains unclear.

The absence of a nodulation phenotype for *Parasponia* JA mutants is consistent with studies in legumes. RNAi-mediated silencing of multiple LOX-encoding genes in soybean (*Glycine max*) or *MtAOC1* in *Medicago truncatula* did not affect nodule development [63, 64]. Studies in *L. japonicus*, however, suggest a role for JA in controlling nodule number under shaded conditions. On wild-type *L. japonicus* grown under low red/far-red light conditions or in a phytochrome B (*phyB*) mutant, nodule number is reduced [41]. Analysis of the *phyB* mutant showed that it contained reduced concentrations of JA-Ile. Addition of low JA concentrations to the *phyB* mutant or wild-type plants grown under low red/far-red rescued the nodulation phenotype [41]. This suggest that under specific environmental conditions, JA is involved in regulation of nodulation, though is not essential for nodule development.

In roots of *Parasponia*, we detected higher expression of several genes commonly used as markers for mycorrhizal symbiosis. This included *RAD1*, *AMT4*, *AMT5*, *PT4*, *STR2* and *VPY*. *RAD1* is closely related to *RAM1* and both GRAS proteins form a protein complex together with DELLA proteins [48, 65, 66]. Over-expression of *RAM1* or a stabilized version of *M. truncatula* *DELLA2* (*della2-Δ18*) can induce expression of most, if not all, of the AM marker genes [51, 65, 66]. In non-inoculated *Parasponia* or *Trema* roots *RAM1* is not expressed. However, it might be possible that *RAD1*, either alone or in combination with DELLAs, is responsible for the induction of *AMT4*, *AMT5*, *PT4*, *STR2* and *VPY* expression in roots of *Parasponia*. Besides induced by mycorrhizal signaling, *RAD1* expression is induced by

phosphate starvation in *L. japonicus* root tips [48]. Phosphate starvation also stimulates accumulation of DELLA proteins in roots, through a reduction in gibberellic acid content [67]. Although highly speculative, it could be hypothesized that higher expression of AM marker genes in *Parasponia* roots, results from constitutive phosphate starvation signaling. In support of this hypothesis, phosphate starvation has been shown to increase JA levels in *Arabidopsis* roots and leaves [68]. Additionally, phosphate stress signaling has been shown to shift the JA/SA signaling balance in favor of JA response [69].

Trema are successional pioneering tree species that occur pantropically. They grow in areas where vegetation has been cleared due to natural or man-made disturbances, such as landslides, volcanic ash deposits and forest gaps [70-72]. *Parasponia* grows in similar habitats but its distribution is restricted to the Malay Archipelago and Papua New Guinea [73]. *Parasponia* seems to prefer nitrogen-poor eroded soils and is capable of forming dense stands on fresh deposits of volcanic ash [72, 73]. Besides scarce in nitrogen, *Parasponia* habitats are often low in phosphorus as well as other essential nutrients [74, 75]. To support plant growth in nutrient-limited environments, plants interact with soil microbes, including mycorrhizal fungi and rhizobium bacteria. Phosphate limitation is an important regulator of AM symbiosis [76]. Therefore, constitutive phosphate starvation and/or higher expression of AM marker genes could promote mycorrhizal colonization of *Parasponia* roots. Experiments to compare mycorrhization efficiency between *Parasponia* and *Trema* species have failed to detect differences (Roswanjaya et al., personal communication). However, these experiments were done under relatively high inoculation density, which could mask mild differences in mycorrhization efficiency. In *Arabidopsis*, salicylic acid and phosphate stress signaling have been shown to function as important regulators of root microbiome composition [69, 77]. Therefore, it is possible that root transcriptome differences between *Parasponia* and *Trema* are correlated to different microbiome dependencies. Additionally, in *L. japonicus*, nitrogen-fixation has been shown to drastically affect root microbiome composition [78]. However, whether this directs evolution in pathways regulating microbiome assembly is unknown.

Taken together, we have shown that *Parasponia* and *Trema* roots harbor distinct transcriptomes and display a difference in root hormone balance. These differences could represent essential adaptations required for symbiosis establishment or indirectly result from differences in niche specialization. Therefore, unveiling the evolutionary drivers underlying the differences between *Parasponia* and *Trema* roots could provide insight in the ecological consequences of nitrogen-fixation.

METHODS

Plant Materials and Growth Conditions

For RNA-seq, seeds of *Parasponia andersonii* WU1, *Parasponia rigida* WU20, *Trema levigata* WU50, *Trema orientalis* RG33 and *Trema tomentosa* WU10 were germinated as previously described [42, 79]. After approximately two weeks, seedlings were transferred to sterile 1 L crystal-clear polypropylene containers (OS140BOX, Duchefa Biochemie, The Netherlands) half-filled with agraperlite (Maasmond-Westland, The Netherlands) and watered with modified EKM medium (3 mM MES ($C_6H_{13}NO_4$) pH 6.6, 2.08 mM $MgSO_4$, 0.88 mM KH_2PO_4 , 2.07 mM K_2HPO_4 , 1.45 mM $CaCl_2$, 0.70 mM Na_2SO_4 , 5 mM KNO_3 , 15 μM Fe-citrate, 6.6 μM $MnSO_4$, 1.5 μM $ZnSO_4$, 1.6 μM $CuSO_4$, 4 μM H_3BO_3 , 4.1 μM Na_2MoO_4) [44]. Plants were grown for 3-4 weeks in a climate room set at 28°C, 16 h/8 h day/night, prior to tissue isolation.

For the second RNA-seq experiment, as well as hormone quantification and mutant analysis, *in vitro* propagated plantlets were used. *In vitro* propagation was performed as previously described [80]. Plants were grown under similar conditions as described above, with the modification that the nitrogen concentration in EKM medium was changed to 0.375 mM NH_4NO_3 . For nodulation assays, EKM was inoculated with *Mesorhizobium plurifarium* BOR2 ($OD_{600} = 0.025$) [42]. Nodulation was assessed six weeks post inoculation.

RNA Sequencing

For RNA isolation, tissue was harvested from a ~5-10 mm region just above the root tip of young freshly growing roots and snap-frozen in liquid nitrogen. Material from ~5 plants was combined to form a single biological replicate. RNA was isolated as previously described [42]. Library preparation and RNA sequencing was conducted by B.G.I. (Shenzhen, China). Paired-end 100 bp reads were mapped against the *P. andersonii* WU1.14 and *T. orientalis* RG33.2 reference genomes [42] using HISAT2 (version 2.02) [81] using an index that includes exon and splice site information in the RNA-seq alignments. Mapped reads were assigned to transcripts with featureCounts (version 1.5.0) [82]. Principal component analysis, normalization and differential gene expression analysis were performed with DESeq2 (version 1.14.1) [83]. Analysis was based on 24,246 high-confidence 1-on-1 orthologue pairs identified between the genomes of *P. andersonii* and *T. orientalis* [42]. Differentially expressed genes were selected based on a fold-change > 2 and an FDR-corrected *p*-value < 0.05 across pair-wise comparisons. Pair-wise comparisons were conducted between each *Parasponia* and *Trema* species (six comparisons in total) and between the *P. andersonii* and *T. tomentosa* RNA-seq samples obtained from *in vitro* propagated plantlets. To account for artefacts resulting from mapping to different genomes, reads of all species were mapped

to both the *P. andersonii* and *T. orientalis* genomes. Differential expression was calculated after mapping of *Parasponia* reads to the *P. andersonii* genome and *Trema* reads to the *T. orientalis* genome, as well as after mapping of reads from both *Parasponia* and *Trema* to the *P. andersonii* genome or the *T. orientalis* genome. Genes were considered differentially expressed when they passed selection criteria after each of these three mapping strategies. For plotting of expression graphs, DESeq2 normalized count data obtained after mapping of *Parasponia* reads to the *P. andersonii* genome and *Trema* reads to the *T. orientalis* genome were used. Expression levels in *P. andersonii* nodules are obtained from van Velzen et al. [42]. Significant overrepresentation of nodule-enhanced genes among differentially expressed genes is calculated based on the hypergeometric probability.

Hormone Extraction

Hormone purification was performed using an adapted protocol based on Dobrev and Kaminek [84]. For extraction, ~20 mg of snap-frozen root material was used. Tissue was ground to a fine powder using 3-mm stainless steel beads at 50 Hz for 1 min in a TissueLyser LT (Qiagen, Germantown, USA). Ground root samples were extracted with 1.5 ml of 100% methanol containing 1 M formic acid and 1 μ M of stable-isotope labelled internal standards (see **Supplemental Table 3**). Samples were vortexed and sonicated for 10 min in a Branson 3510 ultrasonic bath (Branson Ultrasonics, USA) and left rotating over night at 4°C for extraction. Samples were centrifuged for 10 min at 2500 g and the liquid phase was carefully transferred to a 4 ml amber glass vial. Samples were re-extracted by rotating for 1h at 4°C with 1.0 ml of 80% methanol containing 1 M formic acid. Both fractions were combined and dried in a speedvac concentrator (Savant SC210A) coupled tot a refrigerated vapor trap (RVT5105, Thermo Fisher Scientific). The residue was dissolved in 1 ml 1 M formic acid. Undissolved particles were pelleted before loading the samples on MCX columns (Oasis®, Waters, 30 mg 1 cc), which were previously equilibrated with 1 ml 100% methanol (UPC/MS grade) followed by 1 ml of 1 M formic acid. Columns were washed with 1 ml 1 M formic acid and eluted with 1 ml methanol. Samples were dried in a speedvac and stored dry at 4°C. Samples where re-suspended in 100 μ l 50% acetonitrile containing 1% formic acid and filtered through a 0.45 μ m Minisart SRP4 filter (Sartorius, Germany) prior to analysis.

Hormone Quantification by Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS/MS)

Hormone quantification was performed on a Waters TQS Xevo tandem quadrupole mass spectrometer equipped with an electrospray ionization source and coupled to an Acquity UPLC system (Waters, USA). Chromatographic separation was achieved using an Acquity

UPLC BEH C18 column (100 x 2.1 mm, 1.7 μ m) (Waters, USA) applying a 0.1% formic acid / acetonitrile gradient, starting at 15% acetonitrile for 3.0 min and subsequently increasing to 50% (v/v) acetonitrile in 16 min. After that the column was rinsed with 100% acetonitrile for 2 min, before equilibrating the column with 15% acetonitrile for 1 min before subsequent injection. Total run time was 20 min. The column was operated at 40°C with a flow-rate of 0.4 ml/min and sample injection volume was 5 μ l.

The mass spectrometer was operated in positive electrospray ionization (ESI) mode for OPDA, JA-Ile and IAA. SA, JA and ABA measurements were performed in negative electrospray ionization mode. The cone and desolvation gas flows were 150 and 800 L h⁻¹, respectively. Argon was used for fragmentation by collision-induced dissociation in the ScanWave collision cell. The capillary voltage was set at 3 kV in positive mode and 1 kV in negative mode. The source temperature at 150°C and the desolvation gas temperature at 550°C. Cone voltage (CV), collision energy (CE) and parent and daughter transitions were optimized for each component. Multiple reaction monitoring (MRM) transitions were selected based on the most abundant and specific fragment ions. The optimized parameters for OPDA, JA, JA-Ile, SA, ABA and IAA can be found in **Supplemental Table 3**.

Quantification was performed 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 the corresponding deuterium-labelled internal standards (Supplemental Table 10 numbers 1–7). Data acquisition and analysis were performed using MassLynx 4.1 (TargetLynx) software (Waters, USA).

CRISPR/Cas9 Mutagenesis and Histology

Stable transformation and CRISPR/Cas9 mutagenesis is done as described in **Chapter 5**. Single-guide RNAs (sgRNAs) were PCR amplified using primers listed in **Supplemental Table 4**. Three sgRNAs were designed for each gene using the CRISPR design tool implemented in Geneious R9 (Biomatters, Auckland, New Zealand). For double knockouts, six sgRNAs were expressed using a single binary construct. Genotyping of candidate mutants was performed using gene-specific primers listed in **Supplemental Table 4**.

Nodule tissue was fixed in 5 % glutaraldehyde PBS (pH = 7.2) solution overnight. After fixation, samples were dehydrated in an ethanol series and subsequently embedded in Technovit 7100 (Heraeus-Kulzer, Wehrheim, Germany), according to the manufacturer's protocol. Semi-thin (3.5 μ m) sections were cut using a microtome (Reichert-Jung, Leica

Microsystems, Rijswijk, The Netherlands) and stained with 0.05% Toluidine Blue-O, before being photographed using a Leica DM5500B microscope equipped with a DFC425C camera (Leica Microsystems, Wetzlar, Germany).

Accession Numbers

GeneIDs of all genes mentioned in this chapter can be found in **Supplemental Table 5**. Sequences can be downloaded from www.parasponia.org.

AUTHOR CONTRIBUTIONS

R.G. conceived of and supervised the study together with T.B.; A.v.Z. designed and planned experiments, with input from R.G., R.v.V. and W.K.; A.v.Z., R.v.V., M.H., S.L. and W.K. prepared samples for RNA isolation and hormone measurements; A.v.Z. and J.V. isolated RNA and R.v.V. and R.H. performed RNA-seq read mapping; A.v.Z. performed differential expression analysis; K.G. performed hormone quantification; A.v.Z., M.H. and S.L. generated CRISPR/Cas9 mutants and determined mutant phenotypes; A.v.Z. and R.G. analyzed the data; A.v.Z. wrote the text and R.G. assisted in editing the chapter.

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SUPPLEMENTAL DATA

Supplemental Figure 1. Experimental conditions are conducive for *Parasponia* root nodule formation. Root nodules are formed on roots of *P. rigida* grown from seed in perlite. Perlite was watered with EKM (5mM KNO₃) and inoculated with *Mesorhizobium plurifarium* BOR2. Arrowheads indicate nodules.

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Supplemental Figure 2. Principal component analysis is not affected by mapping to different genomes.

Principal component analysis (PCA) on gene expression values in roots of two *Parasponia* and three *Trema* species.

(A) PCA was performed on DESeq2 ‘regularized log’ transformed read counts obtained after mapping of *P. andersonii* and *P. rigida* reads to the *P. andersonii* genome and *T. levigata*, *T. orientalis* and *T. tomentosa* reads against the *T. orientalis* genome. Note: this plot is identical to the plot shown in Fig. 1.

(B) PCA was performed on DESeq2 ‘regularized log’ transformed read counts obtained after mapping of reads from all five species to the *P. andersonii* genome.

(C) PCA was performed on DESeq2 ‘regularized log’ transformed read counts obtained after mapping reads from all five species to the *T. orientalis* genome.

Plotted are first two principal components (PC1 and PC2) and indicated is the percentage of explained variance by each principal component.

Supplemental Figure 3. Principal component analysis including expression values from plants obtained through *in vitro* micro-propagation.

Principal component analysis (PCA) on gene expression values in roots of two *Parasponia* and three *Trema* species grown from seed and roots of *P. andersonii* and *T. tomentosa* plants obtained through *in vitro* micro-propagation. PCA was performed on DESeq2 'regularized log' transformed read counts obtained after mapping of *P. andersonii* and *P. rigida* reads to

the *P. andersonii* genome and *T. levigata*, *T. orientalis* and *T. tomentosa* reads against the *T. orientalis* genome. Plotted are first two principal components (PC1 and PC2) and indicated is the percentage of explained variance by each principal component.

Supplemental Figure 4. Expression of mycorrhizal marker genes in roots of *P. andersonii* and *T. tomentosa* plants obtained through *in vitro* micro-propagation.

Expression of the mycorrhizal marker genes AMT4, AMT5, PT4, STR2, RAD1 and VPY in roots of *P. andersonii* (Pan) and *T. tomentosa* (Tto) plants obtained through *in vitro* micro-propagation. Expression levels were calculated after mapping of *P. andersonii* reads to the *P. andersonii* genome and *T. tomentosa* reads against the *T. orientalis* genome. Expression levels are presented as DESeq2 normalized read counts. Data represent means of 3 biological replicates ± SEM. Dots represent expression values in each of the biological replicates. Grey panel highlighting of expression plots indicates differential expression (fold change > 2, $p < 0.05$), consistent across all comparisons.

Supplemental Figure 5. Expression of *VPY*, *RAD1* and *PT4* in *P. andersonii* roots and nodules.

Expression of *PanVPY*, *PanRAD1* and *PanPT4* in roots and during three stages of nodule development (S1-S3). Expression levels are presented as DESeq2 normalized read counts. Data represent means of 3 biological replicates $\pm$ SEM. Dots represent expression values in each of the biological replicates. Grey panel highlighting indicates significant nodule-enhanced expression (fold change > 2 , $p < 0.05$), according to van Velzen et al. [42].

Supplemental Figure 6. Expression of *LOX2*, *LOX3* and *AOC2* in *P. andersonii* roots and nodules.

Expression of *PanLOX2*, *PanLOX3* and *PanAOC2* in roots and during three stages of nodule development (S1-S3). Expression levels are presented as DESeq2 normalized read counts. Data represent means of 3 biological replicates $\pm$ SEM. Dots represent expression values in each of the biological replicates. Grey panel highlighting indicates significant nodule-enhanced expression (fold change > 2 , $p < 0.05$), according to van Velzen et al. [42].

Supplemental Figure 8. CRISPR mutant alleles of *Panjar1* mutants.

- (A) Schematic representation of *PanJAR1* gene model, indicated are the locations of three sgRNA target sites.
- (B) Alignment of *PanJAR1* sequences in wild type (WT) and *Panaoc1-Panaoc2* double mutant lines. Indicated in blue and red highlighting are the sgRNA target sites and PAM sequences, respectively.

Supplemental Figure 7. CRISPR mutant alleles of *Panlox2-Panlox3* double mutants.

- (A) Schematic representation of *PanLOX2* gene model, indicated are the locations of three sgRNA target sites.
- (B) Alignment of *PanLOX2* sequences in wild type (WT) and *Panaoc1-Panaoc2* double mutant lines. Indicated in blue and red highlighting are the sgRNA target site and PAM sequences, respectively.
- (C) Schematic representation of *PanLOX3* gene model, indicated are the locations of three sgRNA target sites.
- (D) Alignment of *PanLOX3* sequences in wild type (WT) and *Panaoc1-Panaoc2* double mutant lines. Indicated in blue and red highlighting are the sgRNA target site and PAM sequences, respectively.

Supplemental Figure 10. Nodulation data of JA mutants.

Nodule formation on JA biosynthesis mutants at 6 weeks after inoculation with *M. plurifarium* BOR2. Data represent means of 6-9 biological replicates $\pm$ SEM. Dots represent individual data points. Different letters on bars indicate statistical significance ($p < 0.05$, ANOVA with Tukey post-hoc test). This data corresponds to the data shown in **Figure 5**. WT, wild-type *P. andersonii*; Ctr-44, transgenic control line expressing the Cas9 gene only [**Chapter 5**].

Supplemental Figure 9. CRISPR mutant alleles of *Panaoc1-Panaoc2* double mutants.

- (A) Schematic representation of *PanaOC1* gene model, indicated are the locations of three sgRNA target sites.
- (B) Alignment of *PanaOC1* sequences in wild type (WT) and *Panaoc1-Panaoc2* double mutant lines. Indicated in blue and red highlighting are the sgRNA target site and PAM sequences, respectively. Shown are the first exon and beginning of the third exon.
- (C) Schematic representation of *PanaOC2* gene model, indicated are the locations of three sgRNA target sites.
- (D) Alignment of *PanaOC2* sequences in wild type (WT) and *Panaoc1-Panaoc2* double mutant lines. Indicated in blue and red highlighting are the sgRNA target site and PAM sequences, respectively. Shown are exon 1 and the beginning of exon 2.

Supplemental Table 1. Genes consistently higher expressed in roots of *Parasponia* compared to *Trema*.

GeneID	Gene name	Functional annotation	Nodule enhanced
PanWU01x14_asm01_ann01_147580	<i>PanBHLH2</i>	Basic helix-loop-helix transcription factor	TRUE
PanWU01x14_asm01_ann01_284910	<i>PanCRK1</i>	Cysteine rich receptor like kinase	TRUE
PanWU01x14_asm01_ann01_085160	<i>PanLOX2</i>	Lipoxygenase	TRUE
PanWU01x14_asm01_ann01_341680	<i>PanVPY</i>	Ankyrin repeat containing protein	TRUE
PanWU01x14_asm01_ann01_227750	-	Patatin-related protein	TRUE
PanWU01x14_asm01_ann01_282200	-	Acid phosphatase	TRUE
PanWU01x14_asm01_ann01_065850	-	Dienelactone hydrolase	TRUE
PanWU01x14_asm01_ann01_223330	-	Glycoside hydrolase	TRUE
PanWU01x14_asm01_ann01_027160	-	Bet v I/Major latex protein	TRUE
PanWU01x14_asm01_ann01_272840	-	Tyrosine-protein kinase	TRUE
PanWU01x14_asm01_ann01_304770	-	Serine/threonine protein kinase	TRUE
PanWU01x14_asm01_ann01_255510	-	Laccase	TRUE
PanWU01x14_asm01_ann01_190770	-	Cysteine-rich secretory protein, allergen V5/Tpx-1-related	TRUE
PanWU01x14_asm01_ann01_072810	-	Spastin	TRUE
PanWU01x14_asm01_ann01_255030	-	Germin	TRUE
PanWU01x14_asm01_ann01_292340	-	NB-ARC domain, LRR domain containing protein	TRUE
PanWU01x14_asm01_ann01_011890	-	Succinylglutamate-semialdehyde dehydrogenase	TRUE
PanWU01x14_asm01_ann01_274070	-	ATP-binding cassette containing protein	TRUE
PanWU01x14_asm01_ann01_158610	<i>PanAOC2</i>	Allene oxide cyclase	FALSE
PanWU01x14_asm01_ann01_262200	<i>PanBHLH85</i>	Basic helix-loop-helix transcription factor	FALSE
PanWU01x14_asm01_ann01_364320	<i>PanCRK20</i>	Cysteine rich receptor like kinase	FALSE
PanWU01x14_asm01_ann01_085170	<i>PanLOX3</i>	Lipoxygenase	FALSE
PanWU01x14_asm01_ann01_197540	<i>PanMES7</i>	Methyl esterase	FALSE
PanWU01x14_asm01_ann01_215630	<i>PanMYB71</i>	MYB transcription factor	FALSE
PanWU01x14_asm01_ann01_171710	<i>PanNFB10</i>	Nuclear transcription factor Y subunit B	FALSE
PanWU01x14_asm01_ann01_341930	<i>PanNPF27</i>	Proton-dependent oligopeptide transporter	FALSE
PanWU01x14_asm01_ann01_280620	<i>PanPAL3</i>	Phenylalanine ammonia-lyase	FALSE
PanWU01x14_asm01_ann01_258770	<i>PanPIN5</i>	Auxin efflux carrier	FALSE
PanWU01x14_asm01_ann01_225280	<i>PanPT4</i>	Phosphate transporter	FALSE
PanWU01x14_asm01_ann01_257240	<i>PanRAD1</i>	GRAS transcription factor	FALSE
PanWU01x14_asm01_ann01_134900	<i>PanTLP25</i>	Thaumatococin	FALSE
PanWU01x14_asm01_ann01_024300	<i>PanWAK9</i>	Wall-associated receptor kinase	FALSE
PanWU01x14_asm01_ann01_073270	<i>PanWAT27</i>	Plant-drug/metabolite exporter	FALSE
PanWU01x14_asm01_ann01_143830	<i>PanWRKY16</i>	WRKY domain containing protein	FALSE
PanWU01x14_asm01_ann01_065890	-	NB-ARC domain, LRR domain containing protein	FALSE
PanWU01x14_asm01_ann01_072570	-	Cytochrome P450, E-class, group I	FALSE
PanWU01x14_asm01_ann01_202680	-	Transferase	FALSE
PanWU01x14_asm01_ann01_210240	-	None	FALSE
PanWU01x14_asm01_ann01_338770	-	HAD-like domain containing protein	FALSE
PanWU01x14_asm01_ann01_370590	-	None	FALSE
PanWU01x14_asm01_ann01_364540	-	Major intrinsic protein	FALSE
PanWU01x14_asm01_ann01_020190	-	LRR domain containing protein	FALSE
PanWU01x14_asm01_ann01_353370	-	NB-ARC domain, LRR domain containing protein	FALSE
PanWU01x14_asm01_ann01_018530	-	Very-long-chain 3-ketoacyl-CoA synthase	FALSE
PanWU01x14_asm01_ann01_156760	-	Nucleoside phosphorylase	FALSE
PanWU01x14_asm01_ann01_334870	-	None	FALSE
PanWU01x14_asm01_ann01_087710	-	None	FALSE
PanWU01x14_asm01_ann01_095210	-	Oxoglutarate/iron-dependent dioxygenase	FALSE
PanWU01x14_asm01_ann01_343310	-	None	FALSE
PanWU01x14_asm01_ann01_050940	-	NAD(P)-binding domain containing protein	FALSE
PanWU01x14_asm01_ann01_015620	-	None	FALSE
PanWU01x14_asm01_ann01_249830	-	Tyrosine-protein kinase	FALSE
PanWU01x14_asm01_ann01_193420	-	E3 ubiquitin-protein ligase SIN-like	FALSE
PanWU01x14_asm01_ann01_320570	-	Erythronate-4-phosphate dehydrogenase	FALSE
PanWU01x14_asm01_ann01_170190	-	Expansin	FALSE
PanWU01x14_asm01_ann01_297550	-	Serine/threonine protein kinase	FALSE
PanWU01x14_asm01_ann01_044440	-	DOMON domain containing protein	FALSE
PanWU01x14_asm01_ann01_337840	-	None	FALSE

PanWU01x14_asm01_ann01_121030	-	None	FALSE
PanWU01x14_asm01_ann01_027420	-	F-box domain containing protein	FALSE
PanWU01x14_asm01_ann01_348670	-	Organ specific protein	FALSE
PanWU01x14_asm01_ann01_037440	-	Transferase	FALSE
PanWU01x14_asm01_ann01_153370	-	None	FALSE
PanWU01x14_asm01_ann01_221020	-	None	FALSE
PanWU01x14_asm01_ann01_059410	-	None	FALSE
PanWU01x14_asm01_ann01_072790	-	Spastin	FALSE
PanWU01x14_asm01_ann01_320590	-	D-isomer specific 2-hydroxyacid dehydrogenase, catalytic domain containing protein	FALSE
PanWU01x14_asm01_ann01_086360	-	GPCR kinase	FALSE
PanWU01x14_asm01_ann01_199170	-	UDP-glucuronosyl/UDP-glucosyltransferase	FALSE
PanWU01x14_asm01_ann01_248980	-	None	FALSE
PanWU01x14_asm01_ann01_336310	-	Transferase	FALSE
PanWU01x14_asm01_ann01_221040	-	None	FALSE
PanWU01x14_asm01_ann01_037190	-	NTF2-like domain containing protein	FALSE
PanWU01x14_asm01_ann01_215550	-	Glyoxalase I	FALSE
PanWU01x14_asm01_ann01_365110	-	None	FALSE
PanWU01x14_asm01_ann01_006880	-	None	FALSE
PanWU01x14_asm01_ann01_165880	-	Mlo-related protein	FALSE
PanWU01x14_asm01_ann01_203150	-	NADPH-dependent FMN reductase-like	FALSE
PanWU01x14_asm01_ann01_257980	-	Peroxidase	FALSE
PanWU01x14_asm01_ann01_182570	-	Pectinesterase inhibitor domain containing protein	FALSE
PanWU01x14_asm01_ann01_022780	-	None	FALSE
PanWU01x14_asm01_ann01_000730	-	S-receptor-like serine/threonine-protein kinase	FALSE
PanWU01x14_asm01_ann01_097450	-	None	FALSE
PanWU01x14_asm01_ann01_028980	-	Thylakoid luminal 17.9 kDa protein	FALSE
PanWU01x14_asm01_ann01_049450	-	Polyketide cyclase SnoaI-like domain containing protein	FALSE
PanWU01x14_asm01_ann01_105700	-	Very-long-chain 3-ketoacyl-CoA synthase	FALSE
PanWU01x14_asm01_ann01_086300	-	Seipin family	FALSE
PanWU01x14_asm01_ann01_300310	-	None	FALSE
PanWU01x14_asm01_ann01_243090	-	Transferase	FALSE
PanWU01x14_asm01_ann01_338780	-	B3 DNA binding domain containing protein	FALSE
PanWU01x14_asm01_ann01_198170	-	Photosystem II PsbO, manganese-stabilising	FALSE
PanWU01x14_asm01_ann01_266880	-	Octamer-binding transcription factor	FALSE
PanWU01x14_asm01_ann01_271560	-	None	FALSE
PanWU01x14_asm01_ann01_298520	-	Parvalbumin	FALSE
PanWU01x14_asm01_ann01_032210	-	None	FALSE
PanWU01x14_asm01_ann01_266220	-	43kDa postsynaptic protein	FALSE
PanWU01x14_asm01_ann01_339430	-	Bifunctional P-protein, chorismate mutase/prephenate dehydratase	FALSE
PanWU01x14_asm01_ann01_071410	-	Gnk2-like domain containing protein	FALSE
PanWU01x14_asm01_ann01_111660	-	Cation transporter	FALSE
PanWU01x14_asm01_ann01_011250	-	None	FALSE
PanWU01x14_asm01_ann01_059970	-	Ionotropic glutamate receptor	FALSE
PanWU01x14_asm01_ann01_216490	-	None	FALSE
PanWU01x14_asm01_ann01_199230	-	UDP-glucuronosyl/UDP-glucosyltransferase	FALSE
PanWU01x14_asm01_ann01_370000	-	UDP-glucuronosyl/UDP-glucosyltransferase	FALSE
PanWU01x14_asm01_ann01_148180	-	Homeodomain-like	FALSE
PanWU01x14_asm01_ann01_270290	-	None	FALSE
PanWU01x14_asm01_ann01_066330	-	Glycosyl hydrolase	FALSE
PanWU01x14_asm01_ann01_149700	-	None	FALSE
PanWU01x14_asm01_ann01_190510	-	Small auxin-up RNA	FALSE
PanWU01x14_asm01_ann01_124800	-	The fantastic four family	FALSE
PanWU01x14_asm01_ann01_354340	-	Cytochrome P450, E-class, group I	FALSE
PanWU01x14_asm01_ann01_267840	-	UDP-glucuronosyl/UDP-glucosyltransferase	FALSE
PanWU01x14_asm01_ann01_223830	-	Germin	FALSE
PanWU01x14_asm01_ann01_284440	-	O-methyltransferase	FALSE
PanWU01x14_asm01_ann01_177610	-	CBS domain containing protein	FALSE
PanWU01x14_asm01_ann01_004800	-	FkbH domain containing protein	FALSE
PanWU01x14_asm01_ann01_127880	-	GUN4-like	FALSE
PanWU01x14_asm01_ann01_182200	-	None	FALSE
PanWU01x14_asm01_ann01_182190	-	None	FALSE

PanWU01x14_asm01_ann01_351880	-	None	FALSE
PanWU01x14_asm01_ann01_340500	-	Patatin-related protein	FALSE
PanWU01x14_asm01_ann01_089360	-	Self-incompatibility protein	FALSE
PanWU01x14_asm01_ann01_100950	-	late embryogenesis abundant protein	FALSE
PanWU01x14_asm01_ann01_174880	-	Glutathione S-transferase, C-terminal-like	FALSE
PanWU01x14_asm01_ann01_216470	-	Lateral organ boundaries domain containing protein	FALSE
PanWU01x14_asm01_ann01_339400	-	Proton-dependent oligopeptide transporter family	FALSE
PanWU01x14_asm01_ann01_099000	-	Major intrinsic protein	FALSE
PanWU01x14_asm01_ann01_233810	-	Octamer-binding transcription factor	FALSE
PanWU01x14_asm01_ann01_014470	-	DNA polymerase	FALSE
PanWU01x14_asm01_ann01_214300	-	ATP-binding protein	FALSE
PanWU01x14_asm01_ann01_345090	-	Sugar/inositol transporter	FALSE
PanWU01x14_asm01_ann01_040390	-	Acid phosphatase	FALSE
PanWU01x14_asm01_ann01_047780	-	Methylthioribose kinase	FALSE
PanWU01x14_asm01_ann01_283100	-	Small heat shock protein HSP	FALSE
PanWU01x14_asm01_ann01_076090	-	PGG domain containing protein	FALSE
PanWU01x14_asm01_ann01_102650	-	S-crystallin	FALSE
PanWU01x14_asm01_ann01_203810	-	Cellulose synthase	FALSE
PanWU01x14_asm01_ann01_367460	-	Proteinase inhibitor	FALSE
PanWU01x14_asm01_ann01_007980	-	Heavy metal-associated domain containing protein	FALSE
PanWU01x14_asm01_ann01_253150	-	Transferase	FALSE
PanWU01x14_asm01_ann01_307320	-	Plastocyanin	FALSE
PanWU01x14_asm01_ann01_062840	-	Probable membrane-associated kinase regulator	FALSE
PanWU01x14_asm01_ann01_216200	-	Mitogen-activated protein kinase kinase kinase	FALSE
PanWU01x14_asm01_ann01_194640	-	None	FALSE
PanWU01x14_asm01_ann01_308800	-	None	FALSE
PanWU01x14_asm01_ann01_296480	-	EVE domain containing protein	FALSE
PanWU01x14_asm01_ann01_010230	-	UDP-glucuronosyl/UDP-glucosyltransferase	FALSE
PanWU01x14_asm01_ann01_231770	-	Lupus La protein	FALSE
PanWU01x14_asm01_ann01_072170	-	Hopanoid-associated sugar epimerase	FALSE
PanWU01x14_asm01_ann01_168000	-	Glycoside hydrolase	FALSE
PanWU01x14_asm01_ann01_080530	-	Transferase	FALSE
PanWU01x14_asm01_ann01_169010	-	Small GTP-binding domain containing protein	FALSE
PanWU01x14_asm01_ann01_161450	-	L-ascorbate oxidase	FALSE
PanWU01x14_asm01_ann01_176520	-	Zinc finger, LSD1-type	FALSE
PanWU01x14_asm01_ann01_322930	-	SOUL haem-binding protein	FALSE
PanWU01x14_asm01_ann01_322710	-	Glutathione S-transferase, C-terminal-like	FALSE
PanWU01x14_asm01_ann01_282030	-	Protein YLS	FALSE
PanWU01x14_asm01_ann01_282460	-	Parvalbumin	FALSE
PanWU01x14_asm01_ann01_067620	-	None	FALSE
PanWU01x14_asm01_ann01_135110	-	Lipopolysaccharide-modifying protein	FALSE
PanWU01x14_asm01_ann01_262250	-	None	FALSE
PanWU01x14_asm01_ann01_009780	-	Cytochrome P450, E-class, group I	FALSE
PanWU01x14_asm01_ann01_214840	-	DYW domain containing protein	FALSE
PanWU01x14_asm01_ann01_061220	-	Xanthine/uracil/vitamin C permease	FALSE
PanWU01x14_asm01_ann01_071810	-	Peptidase M1, alanine aminopeptidase/leukotriene A4 hydrolase	FALSE
PanWU01x14_asm01_ann01_109850	-	Tyrosine-protein kinase	FALSE
PanWU01x14_asm01_ann01_042310	-	Sugar/inositol transporter	FALSE
PanWU01x14_asm01_ann01_371260	-	Gnk2-like domain containing protein	FALSE
PanWU01x14_asm01_ann01_094540	-	Beta-hexosaminidase	FALSE
PanWU01x14_asm01_ann01_258060	-	S-adenosyl-L-methionine-dependent methyltransferase	FALSE
PanWU01x14_asm01_ann01_167320	-	CASP-like protein 4A	FALSE
PanWU01x14_asm01_ann01_298980	-	None	FALSE
PanWU01x14_asm01_ann01_119670	-	Transferase	FALSE
PanWU01x14_asm01_ann01_334410	-	Splicing factor-like protein	FALSE
PanWU01x14_asm01_ann01_330480	-	None	FALSE
PanWU01x14_asm01_ann01_044040	-	P-loop containing nucleoside triphosphate hydrolase	FALSE
PanWU01x14_asm01_ann01_299370	-	Coatomer beta subunit	FALSE
PanWU01x14_asm01_ann01_202790	-	Protein RETICULATA-related	FALSE
PanWU01x14_asm01_ann01_074110	-	Mitochondrial carrier domain containing protein	FALSE

Supplemental Table 2. Genes consistently lower expressed in roots of *Parasponia* compared to *Trema*.

GeneID	Gene name	Functional annotation	Nodule enhanced
PanWU01x14_asm01_ann01_176030	<i>PanYUCCA4</i>	Flavin monooxygenase-like enzyme	TRUE
PanWU01x14_asm01_ann01_057360	<i>PanYSL6</i>	Oligopeptide transporter	TRUE
PanWU01x14_asm01_ann01_045060	<i>PanUGT7</i>	UDP-glucuronosyl/UDP-glucosyltransferase	TRUE
PanWU01x14_asm01_ann01_005340	<i>PanSURE3</i>	Survival protein SurE-like phosphatase/nucleotidase	TRUE
PanWU01x14_asm01_ann01_243640	<i>PanDXS3</i>	Deoxyxylulose-5-phosphate synthase	TRUE
PanWU01x14_asm01_ann01_208890	<i>PanBIN5</i>	Glycogen synthase kinase	TRUE
PanWU01x14_asm01_ann01_183830	-	NADH:flavin oxidoreductase/NADH oxidase, N-terminal	TRUE
PanWU01x14_asm01_ann01_184920	-	Epoxide hydrolase-like	TRUE
PanWU01x14_asm01_ann01_085860	-	Isopenicillin N synthase	TRUE
PanWU01x14_asm01_ann01_105840	-	Cytochrome P450, E-class, group I	TRUE
PanWU01x14_asm01_ann01_323110	-	Laccase	TRUE
PanWU01x14_asm01_ann01_193920	-	Cellulose synthase	TRUE
PanWU01x14_asm01_ann01_215750	-	Aconitase/Iron-responsive element-binding protein	TRUE
PanWU01x14_asm01_ann01_181710	-	Cytochrome P450, E-class, group I	TRUE
PanWU01x14_asm01_ann01_114750	-	PGAP2-interacting protein	TRUE
PanWU01x14_asm01_ann01_171320	-	ATP-binding cassette containing protein	TRUE
PanWU01x14_asm01_ann01_267940	-	Glycerol-3-phosphate dehydrogenase, NAD-dependent	TRUE
PanWU01x14_asm01_ann01_167690	-	Multi antimicrobial extrusion protein	TRUE
PanWU01x14_asm01_ann01_194480	-	Acid phosphatase	TRUE
PanWU01x14_asm01_ann01_177490	-	CRAL-TRIO lipid binding domain containing protein	TRUE
PanWU01x14_asm01_ann01_137540	-	Cdk-activating kinase assembly factor	TRUE
PanWU01x14_asm01_ann01_003680	-	Xyloglucan endotransglucosylase/hydrolase	TRUE
PanWU01x14_asm01_ann01_248020	-	Helicase, C-terminal	TRUE
PanWU01x14_asm01_ann01_359310	-	Xyloglucan endotransglucosylase/hydrolase	TRUE
PanWU01x14_asm01_ann01_275440	-	protein rolling protein	TRUE
PanWU01x14_asm01_ann01_229670	-	Transglycosylase SLT domain	TRUE
PanWU01x14_asm01_ann01_311600	-	Protein rough sheath 2/ASYMMETRIC LEAVES	TRUE
PanWU01x14_asm01_ann01_146560	-	regulator of nonsense transcript protein	TRUE
PanWU01x14_asm01_ann01_084630	-	TPX	TRUE
PanWU01x14_asm01_ann01_268730	-	Exostosin-like	TRUE
PanWU01x14_asm01_ann01_367950	-	Thioredoxin-like fold containing protein	TRUE
PanWU01x14_asm01_ann01_070150	-	Fructose-1,6-bisphosphatase	TRUE
PanWU01x14_asm01_ann01_278960	-	Solute carrier	TRUE
PanWU01x14_asm01_ann01_150310	-	UDP-glucuronosyl/UDP-glucosyltransferase	TRUE
PanWU01x14_asm01_ann01_322230	-	Tricorn protease	TRUE
PanWU01x14_asm01_ann01_036350	<i>PanWAT38</i>	Plant-drug/metabolite exporter	FALSE
PanWU01x14_asm01_ann01_194430	<i>PanVSP7</i>	Acid phosphatase	FALSE
PanWU01x14_asm01_ann01_267470	<i>PanVSP4</i>	Acid phosphatase	FALSE
PanWU01x14_asm01_ann01_078390	<i>PanPGP5</i>	ABC transporter	FALSE
PanWU01x14_asm01_ann01_123470	<i>PanPGP24</i>	ABC transporter	FALSE
PanWU01x14_asm01_ann01_183880	<i>PanOPR1</i>	NADH:flavin oxidoreductase/NADH oxidase	FALSE
PanWU01x14_asm01_ann01_260150	<i>PanOPCL9</i>	4-coumarate-CoA ligase	FALSE
PanWU01x14_asm01_ann01_335420	<i>PanNPF41</i>	Proton-dependent oligopeptide transporter	FALSE
PanWU01x14_asm01_ann01_197500	<i>PanMES5</i>	Methyl esterase	FALSE
PanWU01x14_asm01_ann01_292640	<i>PanCRK5</i>	Cysteine rich receptor like kinase	FALSE
PanWU01x14_asm01_ann01_313040	<i>PanBHLH19</i>	Basic helix-loop-helix transcription factor	FALSE
PanWU01x14_asm01_ann01_227120	-	S-locus glycoprotein	FALSE
PanWU01x14_asm01_ann01_079720	-	NB-ARC domain, LRR domain containing protein	FALSE
PanWU01x14_asm01_ann01_141780	-	Bet v I type allergen	FALSE
PanWU01x14_asm01_ann01_118770	-	Disease resistance response protein	FALSE
PanWU01x14_asm01_ann01_197810	-	B3 DNA binding domain containing protein	FALSE
PanWU01x14_asm01_ann01_180630	-	Small heat shock protein HSP	FALSE
PanWU01x14_asm01_ann01_313510	-	UDP-glucuronosyl/UDP-glucosyltransferase	FALSE
PanWU01x14_asm01_ann01_250540	-	Tyrosine-protein kinase	FALSE
PanWU01x14_asm01_ann01_202580	-	None	FALSE
PanWU01x14_asm01_ann01_328800	-	Small auxin-up RNA	FALSE
PanWU01x14_asm01_ann01_183760	-	UDP-glucuronosyl/UDP-glucosyltransferase	FALSE
PanWU01x14_asm01_ann01_104470	-	Transferase	FALSE

PanWU01x14_asm01_ann01_077580	-	Gibberellin regulated protein	FALSE
PanWU01x14_asm01_ann01_272960	-	Phospholipase-like	FALSE
PanWU01x14_asm01_ann01_081980	-	F-box domain containing protein	FALSE
PanWU01x14_asm01_ann01_082000	-	None	FALSE
PanWU01x14_asm01_ann01_177330	-	Isofenicillin N synthase	FALSE
PanWU01x14_asm01_ann01_036920	-	S-receptor-like serine/threonine-protein kinase	FALSE
PanWU01x14_asm01_ann01_054610	-	Cysteine synthase	FALSE
PanWU01x14_asm01_ann01_303090	-	Transferase	FALSE
PanWU01x14_asm01_ann01_194000	-	NB-ARC domain, LRR domain containing protein	FALSE
PanWU01x14_asm01_ann01_293230	-	UDP-glucuronosyl/UDP-glucosyltransferase	FALSE
PanWU01x14_asm01_ann01_282100	-	Alpha/beta hydrolase fold	FALSE
PanWU01x14_asm01_ann01_078060	-	1,4-alpha-glucan-branching enzyme	FALSE
PanWU01x14_asm01_ann01_163500	-	Transmembrane protein	FALSE
PanWU01x14_asm01_ann01_010260	-	UDP-glucuronosyl/UDP-glucosyltransferase	FALSE
PanWU01x14_asm01_ann01_043100	-	Very-long-chain 3-ketoacyl-CoA synthase	FALSE
PanWU01x14_asm01_ann01_034830	-	Voltage dependent potassium channel	FALSE
PanWU01x14_asm01_ann01_029260	-	Acid phosphatase	FALSE
PanWU01x14_asm01_ann01_115130	-	Calcium-dependent channel	FALSE
PanWU01x14_asm01_ann01_099690	-	Carotenoid oxygenase	FALSE
PanWU01x14_asm01_ann01_080220	-	S-crystallin	FALSE
PanWU01x14_asm01_ann01_276120	-	LRR domain containing protein	FALSE
PanWU01x14_asm01_ann01_093390	-	Multi antimicrobial extrusion protein	FALSE
PanWU01x14_asm01_ann01_168510	-	LRR domain containing protein	FALSE
PanWU01x14_asm01_ann01_031700	-	BURP domain containing protein	FALSE
PanWU01x14_asm01_ann01_031750	-	BURP domain containing protein	FALSE
PanWU01x14_asm01_ann01_068150	-	Avr9/Cf-9 rapidly elicited protein	FALSE
PanWU01x14_asm01_ann01_031690	-	Organ specific protein	FALSE
PanWU01x14_asm01_ann01_238590	-	None	FALSE
PanWU01x14_asm01_ann01_252760	-	Amino acid/polyamine transporter	FALSE
PanWU01x14_asm01_ann01_345560	-	Mono-functional heme-containing catalase	FALSE
PanWU01x14_asm01_ann01_216870	-	GAMYB transcription factor	FALSE
PanWU01x14_asm01_ann01_270840	-	Spastin	FALSE
PanWU01x14_asm01_ann01_226680	-	Protein phosphatase	FALSE
PanWU01x14_asm01_ann01_197430	-	CRIB domain containing protein	FALSE
PanWU01x14_asm01_ann01_326690	-	Alcohol dehydrogenase superfamily, zinc-type	FALSE
PanWU01x14_asm01_ann01_087890	-	Serine/threonine protein kinase	FALSE
PanWU01x14_asm01_ann01_168580	-	Pyruvate kinase	FALSE
PanWU01x14_asm01_ann01_312720	-	Glycoside hydrolase	FALSE
PanWU01x14_asm01_ann01_258360	-	UDP-glucuronosyl/UDP-glucosyltransferase	FALSE
PanWU01x14_asm01_ann01_309070	-	Serine hydroxymethyltransferase	FALSE
PanWU01x14_asm01_ann01_227350	-	Glycoside hydrolase	FALSE
PanWU01x14_asm01_ann01_166560	-	O-methyltransferase COMT-type	FALSE
PanWU01x14_asm01_ann01_018930	-	Universal stress protein	FALSE
PanWU01x14_asm01_ann01_001620	-	Mpv17/PMP	FALSE
PanWU01x14_asm01_ann01_128500	-	Peroxidase	FALSE
PanWU01x14_asm01_ann01_142560	-	Chloride channel, voltage gated	FALSE
PanWU01x14_asm01_ann01_182300	-	S-adenosyl-L-methionine-dependent methyltransferase	FALSE
PanWU01x14_asm01_ann01_271740	-	Amidohydrolase	FALSE
PanWU01x14_asm01_ann01_067000	-	Cellulose synthase	FALSE
PanWU01x14_asm01_ann01_085420	-	Six-hairpin glycosidase	FALSE
PanWU01x14_asm01_ann01_133570	-	Amino acid transporter, transmembrane domain containing protein	FALSE
PanWU01x14_asm01_ann01_256680	-	Parvalbumin	FALSE
PanWU01x14_asm01_ann01_219030	-	Sugar/inositol transporter	FALSE
PanWU01x14_asm01_ann01_006390	-	Alpha/Beta hydrolase fold containing protein	FALSE
PanWU01x14_asm01_ann01_235980	-	Major latex protein domain containing protein	FALSE
PanWU01x14_asm01_ann01_235990	-	Bet v I type allergen	FALSE
PanWU01x14_asm01_ann01_107640	-	Voltage dependent potassium channel	FALSE
PanWU01x14_asm01_ann01_305640	-	Mitochondrial pyruvate carrier	FALSE
PanWU01x14_asm01_ann01_148530	-	D-ribose-binding periplasmic protein	FALSE
PanWU01x14_asm01_ann01_306440	-	Aspartate/other aminotransferase	FALSE
PanWU01x14_asm01_ann01_249190	-	Diacylglycerol O-acyltransferase	FALSE
PanWU01x14_asm01_ann01_228960	-	Lipase	FALSE

PanWU01x14_asm01_ann01_045710	-	N-acyl-L-amino-acid amidohydrolase	FALSE
PanWU01x14_asm01_ann01_177520	-	None	FALSE
PanWU01x14_asm01_ann01_034460	-	Glycosyl transferase	FALSE
PanWU01x14_asm01_ann01_014760	-	Serine/threonine protein kinase	FALSE
PanWU01x14_asm01_ann01_281660	-	Carbonic anhydrase, alpha-class	FALSE
PanWU01x14_asm01_ann01_317730	-	Ternary complex factor MIP1, leucine-zipper	FALSE
PanWU01x14_asm01_ann01_167890	-	Splicing factor-like protein	FALSE
PanWU01x14_asm01_ann01_108930	-	43kDa postsynaptic protein	FALSE
PanWU01x14_asm01_ann01_084470	-	Glycoside hydrolase	FALSE
PanWU01x14_asm01_ann01_084440	-	Very-long-chain 3-ketoacyl-CoA synthase	FALSE
PanWU01x14_asm01_ann01_099650	-	Guanine nucleotide-binding protein, beta subunit	FALSE
PanWU01x14_asm01_ann01_133790	-	Pectin lyase fold/virulence factor	FALSE
PanWU01x14_asm01_ann01_331600	-	ER lumen protein retaining receptor	FALSE
PanWU01x14_asm01_ann01_112690	-	Peroxioredoxin Q	FALSE
PanWU01x14_asm01_ann01_038900	-	Sm-like protein Lsm6/SmF	FALSE
PanWU01x14_asm01_ann01_133560	-	Amino acid transporter, transmembrane domain containing protein	FALSE
PanWU01x14_asm01_ann01_112410	-	None	FALSE
PanWU01x14_asm01_ann01_012030	-	Universal stress protein	FALSE
PanWU01x14_asm01_ann01_079000	-	Glycogen/starch/alpha-glucan phosphorylase	FALSE
PanWU01x14_asm01_ann01_048650	-	Glycoside hydrolase	FALSE
PanWU01x14_asm01_ann01_104000	-	Magnesium-protoporphyrin IX methyltransferase	FALSE
PanWU01x14_asm01_ann01_222720	-	Short-chain dehydrogenase/reductase	FALSE
PanWU01x14_asm01_ann01_274170	-	Ubiquitin-activating enzyme E	FALSE
PanWU01x14_asm01_ann01_200550	-	Pentatricopeptide repeat	FALSE
PanWU01x14_asm01_ann01_038010	-	Chlorophyllase	FALSE
PanWU01x14_asm01_ann01_251780	-	18S pre-ribosomal assembly protein gar2-like protein	FALSE
PanWU01x14_asm01_ann01_188030	-	Protein prenyltransferase, alpha subunit	FALSE
PanWU01x14_asm01_ann01_156170	-	PsbP family	FALSE
PanWU01x14_asm01_ann01_121420	-	None	FALSE
PanWU01x14_asm01_ann01_274960	-	Tyrosine-protein kinase	FALSE
PanWU01x14_asm01_ann01_352370	-	Serine/threonine protein kinase	FALSE
PanWU01x14_asm01_ann01_055290	-	3-dehydroquinase dehydratase type I	FALSE
PanWU01x14_asm01_ann01_261960	-	Protein phosphatase	FALSE
PanWU01x14_asm01_ann01_286000	-	None	FALSE
PanWU01x14_asm01_ann01_329750	-	Tetratricopeptide-like helical domain containing protein	FALSE
PanWU01x14_asm01_ann01_078300	-	DedA family	FALSE
PanWU01x14_asm01_ann01_343020	-	WEB family	FALSE
PanWU01x14_asm01_ann01_274780	-	Armadiillo-type fold containing protein	FALSE
PanWU01x14_asm01_ann01_194960	-	EMSY N-terminal	FALSE
PanWU01x14_asm01_ann01_157910	-	None	FALSE
PanWU01x14_asm01_ann01_312640	-	None	FALSE
PanWU01x14_asm01_ann01_225140	-	ATP-binding cassette containing protein	FALSE
PanWU01x14_asm01_ann01_332850	-	None	FALSE
PanWU01x14_asm01_ann01_249880	-	Non-haem dioxygenase N-terminal domain containing protein	FALSE
PanWU01x14_asm01_ann01_021790	-	AWPM-19-like	FALSE
PanWU01x14_asm01_ann01_190080	-	Amidase	FALSE
PanWU01x14_asm01_ann01_108820	-	Tricorn protease	FALSE
PanWU01x14_asm01_ann01_356640	-	F-box domain containing protein	FALSE
PanWU01x14_asm01_ann01_371870	-	F-box domain containing protein	FALSE
PanWU01x14_asm01_ann01_086200	-	Glycolipid transfer protein domain containing protein	FALSE
PanWU01x14_asm01_ann01_153820	-	Voltage dependent potassium channel	FALSE
PanWU01x14_asm01_ann01_235350	-	Serine/threonine protein kinase	FALSE
PanWU01x14_asm01_ann01_227480	-	Lung seven transmembrane receptor-like	FALSE
PanWU01x14_asm01_ann01_299310	-	Peptidase S9, prolyl oligopeptidase, catalytic domain containing protein	FALSE
PanWU01x14_asm01_ann01_227340	-	Glycoside hydrolase	FALSE
PanWU01x14_asm01_ann01_200710	-	Cytochrome P450, E-class, group II	FALSE
PanWU01x14_asm01_ann01_331960	-	F-box associated domain, type	FALSE
PanWU01x14_asm01_ann01_269470	-	Glyceraldehyde/Erythrose phosphate dehydrogenase family	FALSE
PanWU01x14_asm01_ann01_336400	-	Stomatin family	FALSE
PanWU01x14_asm01_ann01_095570	-	LRR domain containing protein	FALSE
PanWU01x14_asm01_ann01_203500	-	Sieve element occlusion, C-terminal	FALSE

PanWU01x14_asm01_ann01_203490	-	Sieve element occlusion	FALSE
PanWU01x14_asm01_ann01_198660	-	WD40/YVTN repeat-like-containing domain containing protein	FALSE
PanWU01x14_asm01_ann01_169890	-	SGNH hydrolase-type esterase domain containing protein	FALSE
PanWU01x14_asm01_ann01_357800	-	LRR domain containing protein	FALSE
PanWU01x14_asm01_ann01_142010	-	None	FALSE
PanWU01x14_asm01_ann01_332760	-	Pectin lyase fold containing protein	FALSE
PanWU01x14_asm01_ann01_215770	-	Aspartate decarboxylase	FALSE
PanWU01x14_asm01_ann01_312620	-	None	FALSE
PanWU01x14_asm01_ann01_177510	-	None	FALSE
PanWU01x14_asm01_ann01_243880	-	WAT1-related protein	FALSE
PanWU01x14_asm01_ann01_230560	-	Sugar/inositol transporter	FALSE
PanWU01x14_asm01_ann01_230940	-	None	FALSE
PanWU01x14_asm01_ann01_292790	-	None	FALSE
PanWU01x14_asm01_ann01_263290	-	None	FALSE
PanWU01x14_asm01_ann01_310030	-	14-3-3 protein	FALSE
PanWU01x14_asm01_ann01_310320	-	UDP-glucuronosyl/UDP-glucosyltransferase	FALSE
PanWU01x14_asm01_ann01_108830	-	None	FALSE
PanWU01x14_asm01_ann01_314470	-	Caleosin-related	FALSE
PanWU01x14_asm01_ann01_319930	-	Small GTPase superfamily, Ras type	FALSE
PanWU01x14_asm01_ann01_163320	-	Cytochrome P	FALSE
PanWU01x14_asm01_ann01_158460	-	None	FALSE
PanWU01x14_asm01_ann01_157170	-	None	FALSE
PanWU01x14_asm01_ann01_157940	-	None	FALSE
PanWU01x14_asm01_ann01_157930	-	Major facilitator	FALSE
PanWU01x14_asm01_ann01_220370	-	None	FALSE
PanWU01x14_asm01_ann01_241640	-	Serine/threonine protein kinase	FALSE
PanWU01x14_asm01_ann01_067750	-	Transferase	FALSE
PanWU01x14_asm01_ann01_072200	-	None	FALSE
PanWU01x14_asm01_ann01_070210	-	None	FALSE
PanWU01x14_asm01_ann01_271750	-	Amidohydrolase	FALSE

Supplemental Table 3. Multiple reactions monitoring (MRM) transitions table for all hormones and corresponding internal standards used in this study.

Compound	Retention time (min)	Scan mode	MRM transition m/z	Cone voltage (V)	Collision energy (eV)
JA	8.63	Negative	208.94>58.95	22	15
[² H ₆]-JA	8.52	Negative	214.74>58.82	22	20
SA	4.54	Negative	136.8>92.95	36	15
[² H ₄]-SA	4.44	Negative	141.1>96.8	36	15
JA-Ile	11.87	Positive	324.33>278.24	36	15
[² H ₂]-JA-Ile	11.84	Positive	326.16>280.06	15	15
OPDA	16.31	Positive	293.3>275.3	36	10
[² H ₃]-OPDA	16.26	Positive	298.22>279.3	36	10
ABA	6.86	Negative	263.06>153.07	36	15
[² H ₆]-ABA	6.78	Negative	269.2>159.1	27	15
IAA	4.72	Positive	176.28>130.2	32	15
[² H ₃]-IAA	4.72	Positive	182.1>109.08	34	25

Supplemental Table 4. Primers used in this study.

Name	Purpose	Sequence
PanLOX2_sgRNA1	Clone sgRNA	tgtggtctcaattGACAAACCTTAATTTACAGCG gttttagagctagaatagcaag
PanLOX2_sgRNA2	Clone sgRNA	tgtggtctcaattGTTGTAAGGGAACATAATAGA gttttagagctagaatagcaag
PanLOX2_sgRNA3	Clone sgRNA	tgtggtctcaattGTATAACCGTCAAGCGTACTG gttttagagctagaatagcaag
PanLOX3_sgRNA1	Clone sgRNA	tgtggtctcaattGAAACGATGTCGTTTCGGCA gttttagagctagaatagcaag
PanLOX3_sgRNA2	Clone sgRNA	tgtggtctcaattGTGTCTTACCGACCCTCGGAG gttttagagctagaatagcaag
PanLOX3_sgRNA3	Clone sgRNA	tgtggtctcaattGTCGCCTCTCCAACATCCAGC gttttagagctagaatagcaag
PanAOC1_sgRNA1	Clone sgRNA	tgtggtctcaattGAAGAAGATAACGGTGATCTG gttttagagctagaatagcaag
PanAOC1_sgRNA2	Clone sgRNA	tgtggtctcaattGGTTGGGATCTTACTAAAG gttttagagctagaatagcaag
PanAOC1_sgRNA3	Clone sgRNA	tgtggtctcaattGGAGACTTGCAAAAAAGGAT gttttagagctagaatagcaag
PanAOC2_sgRNA1	Clone sgRNA	tgtggtctcaattGAGCGATGTCGTTTTGAGAG gttttagagctagaatagcaag
PanAOC2_sgRNA2	Clone sgRNA	tgtggtctcaattGTATTGATGAGAGTTTAAGGG gttttagagctagaatagcaag
PanAOC2_sgRNA3	Clone sgRNA	tgtggtctcaattGCGAGACAGGGGAAGTCCGG gttttagagctagaatagcaag
PanJAR1_sgRNA1	Clone sgRNA	tgtggtctcaattGAGAAGTCTATATATCTGCA gttttagagctagaatagcaag
PanJAR1_sgRNA2	Clone sgRNA	tgtggtctcaattGGAATTTGAAGCTTTAACAG gttttagagctagaatagcaag
PanJAR1_sgRNA3	Clone sgRNA	tgtggtctcaattGAAAAGATGGAAGATTCAA gttttagagctagaatagcaag
sgRNA_Rv	Clone sgRNA	tgtggtctcaAGCGTAATGCCAACTTTGTAC
geno_PanLOX2-crispr-Fw	Genotyping	GGGATTGAGCTGGCTCTGAA
geno_PanLOX2-crispr-Rv	Genotyping	ACTTACCAGGGACGAGGTCA
geno_PanLOX3-crispr-Fw	Genotyping	TGTCGCTGCTACGCATATG
geno_PanLOX3-crispr-Rv	Genotyping	TTGGGAATTGTCGGACTTGG
geno_PanAOC1-crispr-Fw	Genotyping	ACCACGTGATGAAGTCCACC
geno_PanAOC1-crispr-Rv	Genotyping	GATCCCTGGACCGCTATGTG
geno_PanAOC2-crispr-Fw	Genotyping	ACACAGCAAAGGGGGAAAA
geno_PanAOC2-crispr-Rv	Genotyping	GGTCGCCGAGTAGATCTTG
geno_PanJAR1-crispr-Fw	Genotyping	TTGGCGTCGTAGTTTTTGGC
geno_PanJAR1-crispr-Rv	Genotyping	TCCGCAGAGAAAATGGCAGT

Supplemental Table 5. GeneIDs for all genes mentioned in this chapter. GeneIDs refer to *P. andersonii* gene models, which can be searched for on www.parasponia.org.

Gene name	GeneID
<i>PanACX1</i>	PanWU01x14_asm01_ann01_276320
<i>PanACX2</i>	PanWU01x14_asm01_ann01_191150
<i>PanACX4</i>	PanWU01x14_asm01_ann01_218440
<i>PanAMT4</i>	PanWU01x14_asm01_ann01_281510
<i>PanAMT5</i>	PanWU01x14_asm01_ann01_211750
<i>PanAOC1</i>	PanWU01x14_asm01_ann01_021690
<i>PanAOC2</i>	PanWU01x14_asm01_ann01_158610
<i>PanAOS1</i>	PanWU01x14_asm01_ann01_083380
<i>PanAOS3</i>	PanWU01x14_asm01_ann01_081200
<i>PanAOS4</i>	PanWU01x14_asm01_ann01_032510
<i>PanHCE7/JAR1</i>	PanWU01x14_asm01_ann01_283440
<i>PanKAT1</i>	PanWU01x14_asm01_ann01_129370
<i>PanKAT2</i>	PanWU01x14_asm01_ann01_216730
<i>PanLOX2</i>	PanWU01x14_asm01_ann01_085160
<i>PanLOX3</i>	PanWU01x14_asm01_ann01_085170
<i>PanLOX4</i>	PanWU01x14_asm01_ann01_345980
<i>PanLOX6</i>	PanWU01x14_asm01_ann01_208600
<i>PanMFP2</i>	PanWU01x14_asm01_ann01_142250
<i>PanOPCL1</i>	PanWU01x14_asm01_ann01_151930
<i>PanOPR3</i>	PanWU01x14_asm01_ann01_200770
<i>PanPT4</i>	PanWU01x14_asm01_ann01_225280
<i>PanRAD1</i>	PanWU01x14_asm01_ann01_257240
<i>PanSTR2</i>	PanWU01x14_asm01_ann01_309120
<i>PanVPY</i>	PanWU01x14_asm01_ann01_341680

Chapter 7

**General Discussion:
Recruitment of hormonal networks in
nitrogen-fixing nodule symbioses**

ABSTRACT

Plants require vast amounts of nitrogen for growth and development. To ensure ample nitrogen supply, approximately ten distantly related plant lineages engage endosymbiotically with nitrogen-fixing bacterial species. These associations occur in specialized organs formed on the host root, named nodules. Research on the legume-rhizobium symbiosis showed that perception of rhizobial signals affects plant hormone homeostasis and that multiple hormones are involved in nodule organogenesis. Here, I question whether different nodulating lineages recruited the same hormonal networks to function in nodule formation. Furthermore, I examine whether nodulating species harbor genetic adaptations in hormonal pathways that correlate with nodulation capacity.

INTRODUCTION

In natural ecosystems, nutrient availability is a major determinant of plant growth potential. To increase nutrient uptake, plant roots can associate with a diverse array of soil microbes [1]. A well-known example of this is the nodular endosymbiosis between nitrogen-fixing bacterial species and legume plants [2]. This symbiosis is not unique to legumes but is found among ten separate lineages within the orders Fabales, Fagales, Cucurbitales and Rosales that together form the so-called ‘nitrogen-fixation clade’ [3–6]. Within this clade, legumes (Fabaceae, order Fabales) and *Parasponia* (Cannabaceae, order Rosales) associate with gram negative α -, β - and γ -proteobacteria, collectively named rhizobia [7]. On the other hand, actinorhizal plants, which represent a paraphyletic group of ~220 species belonging to 25 genera in the Fagales, Cucurbitales and Rosales, interact with gram positive actinomycetes of the genus *Frankia* [4,8,9].

Despite differences in microbial partner, the nitrogen-fixing symbioses of legumes, *Parasponia* and actinorhizas are founded on conserved genetic networks. These networks have been largely co-opted from the symbiosis with arbuscular mycorrhizal fungi [10,11]. These obligate biotrophic fungi colonize roots of most land plants and form dense hyphal networks inside existing root cortical cells, known as arbuscules [12,13]. Additionally, all nitrogen-fixing symbioses have in common that the microbial symbionts are accommodated intracellularly inside specialized organs that are formed on the host root [14,15]. These so-called ‘nodules’ results from cell divisions that are induced upon perception of microbial signals. In plants, development is regulated by interacting hormonal networks [16,17]. This implies that microbe-induced signaling affects hormone homeostasis of the host root, subsequently leading to nodule organogenesis. Indeed, several studies have shown that in legumes perception of rhizobial lipo-chitooligosaccharide (LCO) signals transcriptionally activates biosynthesis and signaling pathways of multiple plant hormones, among which are cytokinins, auxins, strigolactones and gibberellins [18–20] [Chapter 3-4]. Additionally, molecular genetic studies have uncovered essential roles of cytokinin and ethylene in nodule organogenesis and microbial infection [21,22]. Most of these studies have been performed on a handful of model legumes. However, data on species from distantly-related branches of the legume family as well as on *Parasponia* and actinorhizal species are emerging.

Here, I will discuss the role of hormonal networks in nitrogen-fixing nodule symbioses and focus on two main aspects, namely: I. whether different nodulating lineages convergently recruited the same hormonal networks, and II. whether nodulating species harbor genetic adaptations in hormonal pathways that correlate with nodulation capacity.

PARALLEL USE OF HORMONES IN SYMBIOSIS

Auxin

A prerequisite for plant organ formation is the formation of a local auxin maximum [23]. These maxima are formed through asymmetric distribution of auxin efflux carriers of the PIN protein family [23,24]. Not surprisingly, analysis of auxin-responsive reporters in the model legumes *Medicago truncatula* (medicago), *Lotus japonicus* (lotus) and *Glycine max* (soybean) as well as the actinorhizal species *Discaria trinervis* (Rhamnaceae, order Rosales) showed that nodule primordia are associated with local auxin response maxima [25–29]. These maxima are often broader and appear more diffuse than those observed during lateral root formation [25,29,30]. The mechanistic basis of the formation of such maxima in response to microbial recognition is not well understood. In most legumes as well as some actinorhizal species, nodule organogenesis is induced in the root cortex and/or pericycle in response to epidermal recognition of microbial signals [9,14,15,31]. Computer simulations predict that the auxin maxima associated with nodule initiation can be generated through local reduction of auxin transport capacity in the root cortex [32]. These simulations also indicate that a diffusible signal originating from a single epidermal cell is sufficient to cause auxin accumulation through reduction of cortical auxin transport [33]. Experimental observations support these predictions. In medicago, auxin transport capacity is reduced below the nodule initiation site at 24 hours after inoculation [27,34]. Additionally, application of auxin transport inhibitors triggers the formation of nodule-like structures on roots of several legume species [35–39]. Similar structures are observed on medicago roots after treatment with flavonoids [40], which function as natural auxin transport inhibitors [41]. Silencing of a flavonoid biosynthesis gene in the actinorhizal tree *Casuarina glauca* (Casuarinaceae, order Fagales) reduced nodule number [42]. However, since flavonoids also function as chemo-attractants for *Frankia* bacteria [43], it cannot be concluded that this results from a direct effect on auxin transport.

Besides auxin export, also activity of auxin import carriers of the AUX/LAX family regulates auxin distribution [44]. The inhibitor 1-naphthoxyacetic acid (1-NOA) interferes with AUX/LAX auxin import activity [45]. Application of 1-NOA to roots of medicago as well as the actinorhizas *D. trinervis* and *C. glauca* negatively affects nodule formation [29,46,47]. Consistently, *Mtlax2* mutants in medicago develop less nodules than the corresponding wild type [46]. Analysis of *MtLAX2* promoter activity showed that this gene is expressed in incipient nodule primordia and at later stages in the nodule meristem but is absent from infected root hairs [46]. A similar expression pattern was observed for *DtAUX1*, a close homolog in *D. trinervis* [29]. In contrast, in *C. glauca* *CgAUX1* promoter activity is observed

in infected root hairs and cortical cells but is absent from uninfected primordial cells [47]. At later stages, promoter activity is observed in fully-infected cells of the mature nodule [47]. Surprisingly, expression of the *CgAUX1* promoter reporter construct in *D. trinervis* restricts promoter activity to the nodule meristem, as is observed for the promoter of *DtAUX1* [29]. This suggests potential differences in regulation and functioning of AUX/LAX-mediated auxin import between different nodulating lineages.

In addition to an important role in nodule organogenesis, recent studies suggest that auxin is also involved in intracellular infection by rhizobium and *Frankia* bacteria. Transcriptomic studies on epidermal cells treated with rhizobial LCOs or infected by rhizobium bacteria indicated transcriptional activation of auxin biosynthesis and signaling genes [19,48]. Additionally, rhizobial inoculation elevated expression of auxin-responsive promoter reporter constructs in medicago root hairs [40,48]. The auxin response factor *MtARF16a* is induced at rhizobial infection sites. Mutation of this gene reduced the number of infection threads, strongly suggesting that auxin signaling is required for rhizobial infection in medicago [48,49]. As described above, the expression pattern of *CgAUX1* also suggests a role for auxin in *Frankia* infection of *C. glauca* root hairs and nodules [47]. Immunolocalization detected accumulation of the auxin phenyl-acetic acid (PAA) in infected cells of mature nodules in *C. glauca* and *D. trinervis* [29,47]. However, the source and subcellular localization of PAA inside these cells is not entirely clear. A source of PAA could be phenyl-acetic hopanetetrol, a specific lipid of *Frankia* vesicles [50,51]. Therefore, it cannot be excluded that PAA is present in the apoplastic space surrounding the *Frankia* hyphae, rather than the host cell cytoplasm. The latter is supported by no detectable signal from the auxin-responsive *DR5* reporter in *D. trinervis* infected cells [29]. However, during mycorrhizal symbiosis, *DR5* signal is detected in arbusculated cells of medicago, tomato (*Solanum lycopersicum*) and rice (*Oryza sativa*) [52]. Overexpression of microRNA393 that results in downregulation of auxin receptor genes blocked arbuscule formation [52]. This indicates that auxin perception is required for intracellular infection by mycorrhizal fungi. Additional experimentation is required to determine, whether intracellular colonization by rhizobia or *Frankia* is equally dependent on auxin signaling.

Cytokinin

In legumes, cytokinin signaling forms an integral part of the nodulation signaling network. Rhizobium inoculation or treatment with rhizobium LCOs rapidly activates expression of typical cytokinin-responsive genes, such as type-A Response Regulators [19,53–57]. Additionally, LCO treatment or rhizobial inoculation induces accumulation of cytokinin in medicago or lotus roots, respectively [20,58] [Chapter 3]. This represents a crucial step in

LCO signaling, as most of the transcriptional changes induced by rhizobium LCOs at three hours post treatment are dependent on cytokinin perception [20] [Chapter 3]. Mutants in the orthologous cytokinin receptors MtCRE1 in medicago and LjLHK1 in lotus are severely affected in nodule organogenesis [55,59]. These mutants only occasionally form nodules, due to redundant function of additional cytokinin receptors [60,61].

Laser dissection-RNAseq revealed transcriptional activation of cytokinin biosynthesis genes as well as genes encoding putative cytokinin transport proteins in medicago root epidermal cells following rhizobium LCO treatment [19]. This suggests that cytokinins might be released from epidermal cells in response to rhizobial recognition to induce nodule initiation in the root cortex. However, cytokinin function during nodule organogenesis appears not to be restricted to legume species that depend on root hair-based infection. Rhizobia infect roots of the tropical legume *Aeschynomene evenia* intercellularly through cracks in the epidermis and induce cortical cell divisions upon direct contact [62]. Silencing of *AeHK1*, the *A. evenia* ortholog of *LjLHK1/MtCRE1*, substantially reduced the proportion of nodulated roots [63]. This suggests that activation of cytokinin signaling represents a universal step during legume root nodule organogenesis.

In contrast to legumes, cytokinin involvement in root nodule formation in actinorhizas and *Parasponia* is not well resolved, yet. Mutagenesis of the *Parasponia andersonii* orthologue of *LjLHK1/MtCRE1*, named *PanHK4*, did not affect root nodule formation [Chapter 5]. However, it cannot be excluded that this results from redundant functioning of PanHK2 and PanHK3, two additional cytokinin receptors encoded in the *P. andersonii* genome [64] [Chapter 5]. Preliminary results suggest that absence of a nodulation phenotype for *Panhk4* mutants could indeed result from redundant functioning of PanHK2 and PanHK3 during *Parasponia* nodule formation. On roots of *P. andersonii* RNAi lines in which *PanHK2*, *PanHK3* and *PanHK4* are co-silenced, reduced nodule formation is observed (Wardhani et al., personal communication). Therefore, the contribution of cytokinin to *Parasponia* nodule formation remains unresolved.

Exogenous cytokinin treatment can induce nodule-like structures on the roots of several legume species [65–68]. However, substantial genetic variation exists in this response, even between different ecotypes of lotus [67]. Cytokinin application has been reported to also induce nodule-like structures on roots of the actinorhizal tree alder (*Alnus glutinosa*, Betulaceae, order Fagales) [69]. This suggests that cytokinin might also be involved in the formation of *Frankia* nodules on alder roots. However, it should be noted that the structure of these pseudonodules differs from that of genuine alder nodules [69]. The same applies to

legume nodule-like structures induced by cytokinin application, questioning the relevance of these results. Therefore, it remains to be determined whether cytokinin is equally important to actinorhizal and *Parasponia* nodule organogenesis as it is to legume nodule development.

Ethylene

Ethylene is generally considered to be a negative regulator of legume nodule formation and rhizobial infection [70]. This was first shown by pharmacological studies, which showed that application of ethylene or its precursor 1-amino-cyclopropane-1-carboxylic acid (ACC) inhibits nodule formation on roots of several legume species [71–73]. Conversely, application of the ACC synthase inhibitor aminoethoxyvinylglycine (AVG) promotes nodule formation and rhizobial infection [71–77]. Strong support for a negative role of ethylene during the legume-rhizobium symbiosis came from analysis of the medicago *Mtein2/Mtskl* mutant. This mutant is ethylene insensitive due to a mutation in *EIN2*, encoding a central component of the ethylene signaling pathway [71,78,79]. The *Mtein2/Mtskl* mutant is hyper-infected, forming a high number of infection threads. Additionally, this mutant forms clusters of small nodules in distinct sickle-shaped zones along the root [31,71]. A similar phenotype is observed in lotus after RNAi-mediated knockdown of both *EIN2*-encoding genes or overexpressing of mutant ethylene receptors, causing ethylene insensitivity [80–82]. The hyper-infection phenotype might result from the absence of negative feedback on LCO-induced signaling. In medicago, several ethylene biosynthesis genes are induced following rhizobial inoculation or LCO treatment [18,20] [Chapter 3]. Likewise, an increase in ethylene release has been measured on alfalfa, *Vicia sativa* and soybean roots after inoculation with rhizobium [83–85]. Ethylene has been shown to affect symbiotic signaling already at an early point in the LCO signaling cascade [73] and therefore LCO-induced ethylene production might directly inhibit subsequent rhizobial infection. In medicago, ethylene treatment increased the concentration of rhizobium LCOs required to initiate calcium spiking, a response observed within minutes of LCO perception [73]. Additionally, a change in spiking frequency was observed in the ethylene-insensitive *Mtein2/Mtskl* mutant compared to wild type [73]. Consistent with a role for ethylene in regulation of early LCO signaling, transcriptome sequencing revealed a massive de-repression of early symbiotic signaling in inoculated *Mtein2/Mtskl* roots [18].

Despite an overall negative effect of ethylene on legume nodule formation, differences in the response to ethylene are observed within the legume family. In soybean, genetic variation in response to ethylene treatment exists between cultivars. Whereas some cultivars are highly sensitive and respond with almost complete inhibition of nodule formation, others are unresponsive to ethylene treatment with regard to nodule development [72,76,86].

In the semiaquatic legume *Sesbania rostrata*, the effect of ethylene on nodule formation depends on the mode of rhizobial infection [87]. Under aerated conditions, *S. rostrata* is infected through a root hair-based infection mechanism. Under these conditions, rhizobial infection is negatively controlled by ethylene [88]. In contrast, under flooded conditions rhizobia infect *S. rostrata* intercellularly in an ethylene-dependent manner [89]. Under these conditions, rhizobia enter the root through epidermal cracks at the site of emerging lateral roots [90,91]. Once inside, the rhizobia proliferate in infection pockets that result from LCO-dependent localized cell death. The formation of these infection pockets involves the production of hydrogen peroxide, ethylene and gibberellic acid [89,92,93]. The switch between both infection modes is likely also regulated by ethylene, which accumulates under anaerobic conditions [87]. Epidermal cells at the lateral root base generally respond to LCO application with a calcium spiking response that is faster and more symmetrical than observed in susceptible root hairs. Addition of AVG induced asymmetric calcium spikes with a reduced frequency [94]. Additionally, this allowed intracellular infection, even under flooded conditions [88,94]. Ethylene-dependent intercellular infection is also observed in the lotus *nena* mutant, which is compromised in root hair-based intracellular infection [95]. Therefore, already within legumes the effect of ethylene on nodule formation and rhizobial infection might differ depending on environmental conditions and rhizobial infection strategy.

Ethylene has little effect on root nodule formation by the actinorhizal species *D. trinervis* [96]. Treatment with the ethylene precursor ACC or the ethylene releasing agent 2-chloroethylphosphonic acid (CEPA) only marginally reduced *D. trinervis* nodule number. On the other hand, addition of AVG or silver ions, which block ethylene perception, resulted in slightly more nodules in response to *Frankia* inoculation [96]. Despite a similar trend, these effects are much less pronounced than observed in legumes. Additionally, *D. trinervis* root growth is strongly affected by interference with ethylene homeostasis [96]. Thus, it cannot be excluded that a small change in nodule number results from an indirect effect of ethylene on root morphology. Therefore, a role for ethylene in regulation of the *D. trinervis*-*Frankia* symbiosis is not supported.

In *Parasponia*, ethylene signaling is required for efficient intracellular infection by rhizobium [Chapter 5]. Rhizobia infect *Parasponia* intercellularly and form small apoplastic colonies in between nodule cells [97,98]. From there, cell wall-bound infection threads are launched. Once inside the cell, these threads change morphology and ramify to form fixation threads [97,99]. *P. andersonii* plants mutated in *EIN2* are ethylene insensitive and develop a wild-type number of nodules [Chapter 5], indicating that in *Parasponia* ethylene does not

regulate nodule number. However, *Panein2* mutant nodules are smaller than wild type and are affected in fixation thread formation [Chapter 5]. In wild-type nodules, several cell layers containing infection threads are found just below the nodule meristem. These are followed by one or two cell layers in which infected cells increase in size and fragmentation of the vacuole occurs, which coincides with the start of fixation thread formation [97]. Immediately below these cell layers, infected cells are fully filled with fixation threads. Some *Panein2* mutant nodules contain only infection threads as well as enlarged apoplastic colonies, but no fixation threads [Chapter 5]. Others contain fixation threads but fixation thread formation appears less efficient compared to wild type. Multiple cell layers containing infection threads are present and infected cells are interspersed with cells that contain only few fixation threads and still display vacuole fragmentation [Chapter 5]. In legumes, intracellular infection also involves cell enlargement, which results from endoreduplication, a prerequisite for intracellular infection [100]. Research on arabidopsis has revealed that ethylene inhibits cell proliferation in the root apical meristem through induction of endoreduplication [101]. Consistently, ethylene has been shown to promote endoreduplication in arabidopsis and cucumber (*Cucumis sativus*) hypocotyl cells [102,103]. Therefore, it could be hypothesized that inefficient formation of fixation threads in *Panein2* mutant nodules is due to the absence of ethylene-induced endoreduplication. In legumes, ethylene is likely not required to perform such function, as *Mtein2* nodules are properly infected by rhizobium bacteria [31]. This indicates a major difference between legumes and *Parasponia* and suggests that ethylene function was independently recruited to regulate rhizobium symbiosis [Chapter 5]. Overall, it can be concluded that ethylene performs distinct functions during the nitrogen-fixing symbioses of legumes, actinorhizas and *Parasponia*.

Strigolactones

The GRAS-type transcriptional regulators NSP1 and NSP2 are essential for nodule formation by legumes and *Parasponia* [67,104,105] [Chapter 5]. In addition, both proteins are required for basal expression of the strigolactone biosynthesis gene *D27* in medicago, rice and *Parasponia* [106] [Chapter 5]. In medicago, *MtD27* expression is strongly induced following rhizobial LCO perception in an MtNSP1- and MtNSP2-dependent manner [107] [Chapter 4]. During rhizobial infection, *MtD27* expression is associated with growing infection threads and dividing cells of the nodule primordium. At later stages, *MtD27* expression is restricted to the meristem and distal infection zone of mature nodules [107] [Chapter 4]. A similar expression pattern is observed for the carotenoid cleavage dioxygenases *MtCCD7* and *MtCCD8* that function downstream of *MtD27* [48,107] [Chapter 4]. This suggests a role for strigolactones in the legume-rhizobium symbiosis. Strigolactones, however, are not essential for legume root nodule formation. Pea (*Pisum sativum*) and lotus strigolactone-deficient mutants

revealed only marginal reduction in nodule numbers [108–110]. Similarly, RNAi-mediated knockdown of *MtD27* expression did not affect nodule number nor intracellular infection by rhizobium bacteria [107] [Chapter 4]. In *Parasponia*, strigolactone function has not been studied. However, expression patterns of *PanD27*, *PanCCD7* and *PanCCD8* suggest that also for *Parasponia* nodule formation strigolactones are not essential. Whereas expression of *PanD27* and *PanCCD7* is enhanced in *Parasponia* nodules, expression of *PanCCD8* is strongly reduced (Figure 1). Therefore, NSP1 and NSP2 are likely required to regulate genes other than *D27* during nitrogen-fixing symbioses.

GENETIC ADAPTATIONS IN HORMONAL NETWORKS

As outlined above, plant hormones perform essential symbiotic functions. Therefore, nodulating lineages might have acquired genetic adaptations in hormonal networks to support nodule formation. However, little evidence for such adaptations currently exists in literature. One study reports identification of a legume-specific duplication in type-A Response Regulators [56]. These were identified based on a phylogenetic strategy to identify duplicate gene pairs that were retained in the legume Papilionoideae subfamily. This subfamily experienced a whole-genome duplication approximately 58 million years ago, shortly after emergence of the legume-rhizobium symbiosis [56,111,112]. One orthology group containing retained gene duplicates harbors the medicago type-A Response Regulators *MtRR9* and *MtRR11*. Both genes are induced within hours of rhizobium LCO application [56]. This induction is dependent on the cytokinin receptor MtCRE1, suggesting it results from LCO-induced cytokinin signaling [20] [Chapter 3]. RNAi-mediated knockdown of *MtRR9* expression equally affected both lateral root and nodule development [56]. Therefore, it is questionable whether the *MtRR9-MtRR11* duplication was specifically retained to support papilionoid nodule formation.

Despite performing an essential role in nodule initiation, the cytokinin receptors LjLHK1 and MtCRE1 most likely do not contain genetic adaptations. *Trans*-complementation using an orthologous receptor from arabidopsis (*Arabidopsis thaliana*) fully restored nodule organogenesis on *Ljlhk1* and *Mtcre1* mutant roots [60,61]. These experiments were performed using an *AtAHK4/AtCRE1* genomic clone, suggesting that neither the regulatory sequences nor the protein sequences of LjLHK1 and MtCRE1 contain specific adaptations that are essential for nodule formation. In this respect, it is interesting that PanHK4 is not required for root nodule formation by *P. andersonii* [Chapter 5]. As *P. andersonii* is more closely related to legumes than arabidopsis, it is likely that ectopic expression of *PanHK4* would also restore nodulation on *Mtcre1* or *Ljlhk1* mutant roots. This suggests that legumes

Figure 1. Expression of *PanD27*, *PanCCD7* and *PanCCD8* in *P. andersonii* roots and nodules.

Expression of *PanD27*, *PanCCD7* and *PanCCD8* in roots and during three stages of nodule development (S1-S3). Expression levels are presented as DESeq2 normalized read counts. Data represent means of three biological replicates $\pm$ SEM. Dots represent expression values in each of the biological replicates. Data were obtained from van Velzen et al. [64].

and *Parasponia* have different dependencies on cytokinin signaling or specifically MtCRE1/LjLHK1/PanHK4 function during nodule initiation.

In medicago and lotus, *MtCRE1* and *LjLHK1* are the most highly expressed cytokinin receptor genes in roots, respectively [60,61]. Consistently, only *Ljlhk1* mutants are resistant to the effect of exogenous cytokinin on root growth, indicating that LjLHK1 is the main sensor of exogenous cytokinin in lotus roots [60]. In contrast, in *P. andersonii*, *PanHK3* is highest expressed cytokinin receptor gene in roots (**Figure 2**). Overexpression of the lotus orthologue of *PanHK3* is sufficient to rescue the nodulation defect of the *Ljlhk1* mutant [60]. This suggests that a difference in relative expression of cytokinin receptor genes in roots could explain the difference in symbiotic dependencies on *MtCRE1/LjLHK1/PanHK4* between legumes and *Parasponia*. Alternatively, *Parasponia* root nodule formation might not require cytokinin signaling. An important regulator of root nodule formation in legumes, and most probably also *Parasponia*, is the transcription factor NIN [64,113–115]. In legumes, *NIN* expression is first induced in the root epidermis in response to perception of rhizobium LCOs [53,114,116]. At subsequent stages, *NIN* is expressed in the root cortex at sites corresponding to incipient nodule primordia [53,67,114]. Induction of *NIN* in the root cortex requires MtCRE1/LjLHK1-dependent cytokinin signaling [53,55,59]. In medicago, ectopic expression of *MtNIN* in the root cortex can induce nodule organogenesis in an MtCRE1-independent manner [53]. This suggests, that in legumes, an important function of cytokinin signaling is cortical activation of *NIN* expression. In *Parasponia*, cell divisions associated with nodule organogenesis are first induced in the root epidermis [15,30,98]. This might result from direct activation of *PanNIN* by rhizobium LCO-induced signaling. Therefore, it is possible that *Parasponia* root nodule formation is independent of cytokinin signaling.

To identify genetic adaptations that correlate with nodulation capacity, studies on closely related species that differ in symbiotic capacity are most helpful. The *Parasponia* lineage is embedded in the non-nodulating *Trema* genus [64,117]. Genome comparisons between both groups of species identified eleven copy number variants among ~1,800 putative symbiotic genes [64]. This included one gene involved in hormone homeostasis: *IPT4*, an isopentenyl transferase involved in cytokinin biosynthesis that has been lost from the genomes of *Parasponia* spp. [64]. Cytokinin is a positive regulator of legume nodule formation [118] and therefore it is difficult to envision how loss of this gene might promote *Parasponia*-rhizobium symbiosis.

Transcriptome comparisons between non-inoculated roots of two *Parasponia* and three *Trema* species indicated transcriptional divergence between *Parasponia* and *Trema* species. Among the genes differentially expressed between *Parasponia* and *Trema* roots are several involved in jasmonic acid biosynthesis [Chapter 6]. The jasmonic acid precursor *cis*-(+)-12-oxophytodienoic acid (OPDA) is synthesized from α -linolenic acid through consecutive activity of 13S-lipoxygenases (LOX), allene oxide synthases (AOS) and allene oxide cyclases (AOC) [119,120]. Two LOX- and one AOC-encoding gene(s) are consistently higher expressed in roots of *Parasponia* compared to *Trema* [Chapter 6]. This coincides with increased concentrations of OPDA and jasmonic acid, but not its bio-active conjugate JA-Ile, in roots of *Parasponia*. Conversely salicylic acid concentrations are higher in roots of *Trema*, indicating a shift in the jasmonic acid/salicylic acid balance between *Parasponia* and *Trema* roots [Chapter 6]. However, this balance shift might not be correlated to nodulation capacity as *P. andersonii* mutants in jasmonic acid biosynthesis are not affected in nodule development [Chapter 6]. How this affected salicylic acid concentrations remains undetermined.

In the legumes alfalfa and lotus, expression of jasmonic acid biosynthesis genes is induced during the first twelve hours after inoculation with compatible rhizobium bacteria [121,122]. Additionally, several studies report an accumulation of LOX transcripts and LOX protein in legume root nodules [123–125]. However, measurements in medicago, *C. glauca* and *D. glomerata* roots and nodules indicate similar jasmonic acid concentrations between both organs [126]. Interference with jasmonic acid biosynthesis through RNAi-mediated silencing of multiple LOX genes in soybean or *MtAOC1* in medicago did neither disturb nodule development nor nodule functioning [126,127], in line with results in *Parasponia* [Chapter 6]. Similarly, enzymatic jasmonic acid biosynthesis also appears not to be required for mycorrhizal symbiosis in rice (*Oryza sativa*), tomato (*Solanum lycopersicum*) and *M. truncatula* [128–130].

Figure 2. Expression of *PanHK2*, *PanHK3* and *PanHK4* in roots of *P. andersonii*.

Expression of *PanHK2*, *PanHK3* and *PanHK4* in young *P. andersonii* root zones, excluding the root tip. Expression levels are presented as DESeq2 normalized read counts. Data represent means of three biological replicates $\pm$ SEM. Dots represent expression values in each of the biological replicates. Data were obtained from **Chapter 6**.

In the near future, efforts of large international consortia might uncover genetic adaptations that correlate with nodulation capacity. These consortia aim to sequence the genomes of multiple pairs of closely related nodulating and non-nodulating species, including actinorhizas. This facilitates comparisons, as done for *Parasponia-Trema*, to be performed on a much larger scale. However, the success of these studies highly depends on the evolutionary history of the nodulation trait. Data suggest the possibility that *Trema* and related Rosales species lost nodulation ability in recent history [64]. If this scenario would apply to the entire nitrogen-fixation clade, identification of genetic adaptations that correlate with nodulation ability would be challenging. Therefore, it remains to be seen whether adaptations in hormonal networks that are essential for nitrogen-fixing nodule symbioses will be found.

CONCLUDING REMARKS

It is evident that hormones are important regulators of nitrogen-fixing nodule symbioses. However, to what extent hormone function during nodule formation is conserved between different nodulating lineages is still far from clear. To increase insight, comparative studies are essential. These can include reverse genetic studies to determine the role of certain hormonal pathways in nodule formation in legumes, *Parasponia* and actinorhiza or *trans*-complementation assays to determine whether symbiotic genes contain specific adaptations. In this respect, the stable transformation and CRISPR/Cas9 genome-editing protocol developed for *P. andersonii* provides a valuable tool [**Chapter 5**]. Development of similar protocols for one or several actinorhizal species would allow comparative analyses to be performed. This will greatly enhance our understanding of hormone function during nodule development in different nitrogen-fixing lineages.

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SUMMARY

Nitrogen is a key element for plant growth. To meet nitrogen demands, some plants establish an endosymbiotic relationship with nitrogen-fixing rhizobium or *Frankia* bacteria. This involves formation of specialized root lateral organs, named nodules. These nodules are colonized intracellularly, which creates optimal physiological conditions for the fixation of atmospheric nitrogen by the microbial symbiont. Nitrogen-fixing endosymbioses are found among four related taxonomic orders that together form the nitrogen-fixation clade. Within this clade, nodulation is restricted to ten separate lineages that are scattered among mostly non-nodulating plant species. This limited distribution suggests that genetic adaptations that allowed nodulation to evolve occurred in a common ancestor.

A major aim of the scientific community is to unravel the evolutionary trajectory towards a nitrogen-fixing nodule symbiosis. The formation of nitrogen-fixing root nodules is best studied in legumes (Fabaceae, order Fabales); especially in *Lotus japonicus* and *Medicago truncatula*, two species that serve as model. Legumes and *Parasponia* (Cannabaceae, order Rosales) represent the only two lineages that can form nodules with rhizobium bacteria. Studies on *M. truncatula*, *L. japonicus* and *Parasponia* showed, amongst others, that nodule formation is initiated upon perception of rhizobial secreted lipo-chitooligosaccharide (LCO) signals. These signals are structurally related to the symbiotic signals produced by arbuscular mycorrhizal fungi. These obligate biotrophic fungi colonize roots of most land plants and form dense hyphal structures inside existing root cortical cells.

Rhizobial and mycorrhizal LCOs are perceived by LysM-domain-containing receptor-like kinases. These activate a signaling pathway that is largely shared between both symbioses. Symbiotic LCO receptors are closely related to chitin innate immune receptors, and some receptors even function in symbiotic as well as innate immune signaling. In **Chapter 2**, I review the intertwining of symbiotic LCO perception and chitin-triggered immunity. Furthermore, I discuss how rhizobia and mycorrhiza might employ LCO signaling to modulate plant immunity. In a perspective, I speculate on a role for plant hormones in immune modulation, besides an important function in nodule organogenesis.

In legumes, nodule organogenesis requires activation of cytokinin signaling. Mutants in the orthologous cytokinin receptor genes *MtCRE1* and *LjLHK1* in *M. truncatula* and *L. japonicus*, respectively, are severely affected in nodule formation. However, how cytokinin signaling is activated in response to rhizobium LCO perception and to what extent this contributes to rhizobium LCO-induced signaling remained elusive. In **Chapter 3**, I show that the majority of

transcriptional changes induced in wild-type *M. truncatula*, upon application of rhizobium LCOs, are dependent on activation of MtCRE1-mediated cytokinin signaling. Among the genes induced in wild type are several involved in cytokinin biosynthesis. Consistently, cytokinin measurements indicate that cytokinins rapidly accumulate in *M. truncatula* roots upon treatment with rhizobium LCOs. This includes the bioactive cytokinins isopentenyl adenine and *trans*-zeatin. Therefore, I argue that cytokinin accumulation represents a key step in the pathway leading to legume root nodule organogenesis.

Strigolactones are plant hormones of which biosynthesis is increased in response to nutrient limitation. In rice (*Oryza sativa*) and *M. truncatula*, this response requires the GRAS-type transcriptional regulators NSP1 and NSP2. Both proteins regulate expression of *DWARF27* (*D27*), which encodes an enzyme that performs the first committed step in strigolactone biosynthesis. NSP1 and NSP2 are also essential components of the signaling cascade that controls legume root nodule formation. In line with this, I questioned whether the NSP1-NSP2-D27 regulatory module functions in rhizobium symbiosis. In **Chapter 4**, I show that in *M. truncatula* *MtD27* expression is induced within hours after treatment with rhizobium LCOs. Spatiotemporal expression studies revealed that *MtD27* is expressed in the dividing cells of the nodule primordium. At later stages, its expression becomes confined to the meristem and distal infection zone of the mature nodule. Analysis of the expression pattern of *MtCCD7* and *MtCCD8*, two additional strigolactone biosynthesis genes, showed that these genes are co-expressed with *MtD27* in nodule primordia and mature nodules. Additionally, I show that symbiotic expression of *MtD27* requires MtNSP1 and MtNSP2. This suggests that the NSP1-NSP2-D27 regulatory module is co-opted in rhizobium symbiosis.

Comparative studies between legumes and nodulating non-legumes could identify shared genetic networks required for nodule formation. We recently adopted *Parasponia*, the only non-legume lineage able to engage in rhizobium symbiosis. However, to perform functional studies, powerful reverse genetic tools for *Parasponia* are essential. In **Chapter 5**, I describe the development of a fast and efficient protocol for CRISPR/Cas9-mediated mutagenesis in *Agrobacterium tumefaciens*-transformed *Parasponia andersonii* plants. Using this protocol, stable mutants can be obtained in a period of three months. These mutants can be effectively propagated *in vitro*, which allows phenotypic evaluation already in the T₀ generation. As such, phenotypes can be obtained within six months after transformation. As proof-of-principle, we mutated *PanHK4*, *PanEIN2*, *PanNSP1* and *PanNSP2*. These genes are putatively involved in cytokinin and ethylene signaling and regulation of strigolactone biosynthesis, respectively. Additionally, orthologues of these genes perform essential symbiotic functions in legumes. *Panhk4* and *Panein2* knockout mutants display developmental phenotypes

associated with reduced cytokinin and ethylene signaling. Analysis of *Pannsp1* and *Pannsp2* mutants revealed a conserved role for NSP1 and NSP2 in regulation of the strigolactone biosynthesis genes *D27* and *MAX1* and root nodule organogenesis. In contrast, symbiotic mutant phenotypes of *Panhk4* and *Panein2* mutants are different from their legume counterparts. This illustrates the value of *Parasponia* as comparative model - besides legumes - to study the genetics underlying rhizobium symbiosis.

Phylogenetic reconstruction showed that the *Parasponia* lineage is embedded in the non-nodulating *Trema* genus. This close relationship suggests that *Parasponia* and *Trema* only recently diverged in nodulation ability. In **Chapter 6**, I exploited this close relationship to question whether the nodulation trait is associated with gene expression differentiation. To this end, I sequenced root transcriptomes of two *Parasponia* and three *Trema* species. Principal component analysis separated all *Parasponia* samples from those of *Trema* along the first principal component. This component explains more than half of the observed variance, indicating that the root transcriptomes of two *Parasponia* species are distinct from that of the *Trema* sister species *T. levigata*, as well as the outgroup species *T. orientalis* and *T. tomentosa*. To determine, whether the transcriptional differences between *Parasponia* and *Trema* are relevant in a symbiotic context, I compared the list of differentially expressed genes to a list of genes that show nodule-enhanced expression in *P. andersonii*. This revealed significant enrichment of nodule-enhanced genes among genes that are lower expressed in roots of *Parasponia* compared to *Trema*. Among the genes differentially expressed between *Parasponia* and *Trema* roots are several involved in mycorrhizal symbiosis as well as jasmonic acid biosynthesis. Measurements of hormone concentrations, showed that *Parasponia* and *Trema* roots harbor a difference in jasmonic acid/salicylic acid balance. However, mutants in jasmonic acid biosynthesis are unaffected in nodule development. Therefore, it remains a challenge to determine whether the difference in root transcriptomes between *Parasponia* and *Trema* are relevant in a symbiotic context.

In **Chapter 7**, I review hormone function in nitrogen-fixing nodule symbioses in legumes, *Parasponia* and actinorhizal species. In this chapter, I question whether different nodulating lineages recruited the same hormonal networks to function in nodule formation. Additionally, I discuss whether nodulating species harbor genetic adaptations in hormonal pathways that correlate with nodulation capacity.

SAMENVATTING

Voor hun groei en ontwikkeling zijn planten afhankelijk van de beschikbaarheid van voedingsstoffen in de bodem. Een belangrijke voedingsstof voor de groei van planten is gebonden stikstof. Regelmatig is, onder natuurlijke omstandigheden, de hoeveelheid gebonden stikstof in de bodem limiterend, wat leidt tot een reductie in groei. Echter, sommige planten hebben zich gedurende de evolutie aangepast, waardoor de planten goed kunnen overleven op stikstof-arme bodems. Een bekend voorbeeld hiervan zijn planten uit de vlinderbloemigen familie. Bekende gewassen die tot deze familie behoren zijn erwten, bonen en soja. Wanneer vlinderbloemigen groeien op bodems die weinig stikstof bevatten vormen deze planten speciale orgaantjes op hun wortels, die wortelknolletjes genoemd worden. Een deel van de cellen in deze wortelknolletjes zijn volledig gevuld met stikstofbindende rhizobium bacteriën. Rhizobium bacteriën zijn in staat om stikstof gas uit de lucht op te nemen en dit om te zetten in ammonium, een gebonden vorm van stikstof. Het gevormde ammonium wordt door de bacteriën uitgescheiden waardoor dit beschikbaar komt voor de plant. Op deze manier zijn vlinderbloemigen onafhankelijk van de beschikbare hoeveelheid stikstof in de bodem en kunnen deze planten groeien op plekken waar de meeste planten niet kunnen overleven.

De relatie, ook wel symbiose genoemde, met stikstofbindende rhizobium bacteriën is niet uniek voor vlinderbloemigen. Tropische bomen uit het genus *Parasponia* zijn ook in staat om een symbiose aan te gaan met rhizobium, waarbij wortelknolletjes worden gevormd. *Parasponia* is een relatief klein genus dat bestaat uit vijf soorten en behoort tot de hennepfamilie. Daarnaast kunnen nog ongeveer acht groepen van planten, afkomstig uit verschillende families, een stikstofbindende wortelknol symbiose aangaan met bacteriën uit het genus *Frankia*. Deze planten worden gezamenlijk ook wel actinorhiza planten genoemd. De gezamenlijke voorouder van zowel de vlinderbloemigen, actinorhizas en *Parasponia* leefde zo'n honderd miljoen jaar geleden, wat suggereert dat de verschillende wortelknol symbioses grotendeels onafhankelijk geëvolueerd zijn.

Het ontrafelen van de ontstaansgeschiedenis van stikstofbindende symbioses kan belangrijk inzicht bieden in de evolutionaire processen die ten grondslag liggen aan het ontstaan van nieuwe kenmerken. Daarnaast kan dit mogelijk een ingang bieden om dit kenmerk ooit in te bouwen in belangrijke landbouwgewassen, zoals rijst of aardappel. Tijdens mijn promotieonderzoek heb ik een bijdrage geleverd aan het begrijpen van de rhizobium symbiose in zowel vlinderbloemigen en *Parasponia*.

De meeste kennis van stikstofbindende symbioses is opgedaan door onderzoek aan twee vlinderbloemige soorten die gebruikt worden als model: *Lotus japonicus* en *Medicago truncatula* (hierna genoemd medicago). Dit heeft laten zien dat de vorming van wortelknolletjes begint nadat signaalstoffen van de bacterie herkend worden door receptoren van de plant. Deze signaalstoffen bestaan uit een keten van chitine moleculen gekoppeld aan een vetzuurstaart en worden kortweg LCO's genoemd. Een zelfde soort signaalmoleculen wordt ook geproduceerd door mycorrhiza schimmels. Deze schimmels zijn instaat om een symbiose aan te gaan met ongeveer 80% van alle landplanten. Onderzoek heeft aangetoond dat vlinderbloemigen voor de herkenning van rhizobium bacteriën gebruik maken van een set genen die ook gebruikt wordt voor het aangaan van de mycorrhiza symbiose. Dit suggereert dat tijdens de evolutie van rhizobium symbiose, planten een deel van de signaleringsnetwerken die gebruikt worden voor het aangaan van de mycorrhiza symbiose hebben hergebruikt voor het maken van stikstofbindende wortelknolletjes. Voor het herkennen van de signaalmoleculen van mycorrhiza schimmels en rhizobium bacteriën maken planten gebruik van speciale receptoren. Deze receptoren zijn nauw verwant aan receptoren die betrokken zijn bij de afweer tegen ziekteverwekkende schimmels. In **hoofdstuk 2** van dit proefschrift bediscussieer ik hoe de herkenning van symbiotische schimmels en bacteriën verstrengeld is met de herkenning van ziekteverwekkende schimmels. Daarnaast bediscussieer ik hoe mycorrhizas en rhizobium mogelijk gebruik maken van LCO's om de afweerrespons van planten te onderdrukken. In een vooruitblik, speculeer ik over de mogelijke rol van plantenhormonen bij het omzeilen van de afweerrespons.

Plantenhormonen spelen een belangrijke rol als regulatoren van groei en ontwikkeling. Ook bij de ontwikkeling van wortelknolletjes spelen hormonen een belangrijke rol. Onderzoek aan vlinderbloemigen heeft laten zien dat voor de vorming van wortelknolletjes met name het hormoon cytokinine erg belangrijk is. Planten waarin een gen dat codeert voor een van de receptoren van cytokinine defect is, zijn niet meer instaat om wortelknolletjes te vormen. Dit betekent dat de signaleringscascade die geactiveerd wordt na herkenning van cytokinine een belangrijke rol speelt in het proces van wortelknolvorming. Echter het was niet bekend hoe deze signaleringscascade geactiveerd wordt na herkenning van rhizobium bacteriën en hoe dit precies bijdraagt aan de vorming van stikstofbindende wortelknolletjes. In **hoofdstuk 3** laat ik zien dat binnen drie uur na herkenning van rhizobium LCO moleculen een grote verandering in genexpressie (activiteit van genen) plaatsvindt. Een groot deel van deze genen verandert niet of nauwelijks in expressie in planten die ongevoelig zijn voor cytokinine, wat het belang van cytokinine signalering aangeeft vroeg tijdens het proces van knolvorming. Onder de genen die een verhoogde expressie laten zien in zowel het wild type (natuurlijk voorkomende genotype van een plant) als planten die ongevoelig zijn

voor cytokinine bevinden zich genen die betrokken zijn bij de productie van cytokinine. Dit suggereert dat mogelijk de productie van cytokinine wordt verhoogd na herkenning van rhizobium bacteriën. In lijn met deze waarneming, laten metingen van de cytokinine concentratie zien dat de hoeveelheid cytokinine toeneemt binnen drie uur nadat medicago wortels zijn behandeld met LCO moleculen. Op basis hiervan beargumenteer ik dat de ophoping van cytokinine een belangrijke stap vormt in het proces van knolvorming.

Strigolactonen zijn een klasse van plantenhormonen waarvan de productie wordt verhoogd wanneer de beschikbaarheid van voedingsstoffen in de bodem limiterend is. Voorafgaand onderzoek heeft laten zien dat voor deze verhoging de activiteit van twee eiwitten essentieel is. Deze twee eiwitten, NSP1 en NSP2 genaamd, behoren tot de familie van GRAS-type transcriptie factoren. Dit zijn eiwitten die de expressie van andere genen reguleren. Onderzoek aan rijst en medicago heeft aangetoond dat NSP1 en NSP2 betrokken zijn bij de regulatie van het gen *DWARF27* (*D27*). Dit gen codeert voor een enzym dat verantwoordelijk is voor de eerste stap in de productie van strigolactonen. In vlinderbloemigen zijn NSP1 en NSP2 ook essentieel voor de vorming van wortelknolletjes. Om deze reden heb ik de vraag gesteld of de NSP1-NSP2-D27 module ook een rol speelt tijdens knolvorming. In **hoofdstuk 4** laat ik zien dat in medicago de expressie van het *MtD27* (*D27* gen van medicago) gen verhoogd wordt binnen enkele uren nadat wortels behandeld zijn met rhizobium LCO moleculen. Daarnaast laat ik zien dat de expressie van *MtD27* voornamelijk plaatsvindt in de cellen die zich gaan delen en op deze manier bijdragen aan de vorming van het knolletje. In een later stadium, beperkt de expressie van *MtD27* zich tot de delende cellen aan de bovenkant van het knolletje die er voor zorgen dat het knolletje blijft groeien (meristeem genoemd) en de cellen die geïnfecteerd worden door rhizobium. Analyse van de expressie van twee andere genen die ook betrokken zijn bij de productie van strigolactonen, *MtCCD7* en *MtCCD8*, laat zien dat deze genen op dezelfde plekken tot expressie komen als *MtD27*. Daarnaast laat ik zien dat NSP1 en NSP2 essentieel zijn voor de verhoging van de expressie van *MtD27* na herkenning van LCOs. Dit suggereert dat in de vlinderbloemigen de NSP1-NSP2-D27 module gerekruteerd is voor een functie tijdens knolvorming.

Onderzoek aan vlinderbloemigen heeft veel inzicht opgeleverd in de moleculaire mechanismes die een rol spelen tijdens het proces van knolvorming. Het is echter niet bekend in hoeverre *Parasponia* en actinorhiza planten ook gebruik maken van deze mechanismes voor het vormen van stikstofbindende wortelknolletjes. Om hier inzicht in te verkrijgen is vergelijkend onderzoek nodig. Het laboratorium waar ik mijn onderzoek heb verricht is recent begonnen met onderzoek aan *Parasponia*, de enige niet-vlinderbloemige die instaat is om een wortelknol symbiose met rhizobium aan te gaan. Echter, om de functie van specifieke

genen te kunnen onderzoeken is het belangrijke om snel en efficiënt genen te kunnen uitschakelen. Een techniek die hiervoor gebruikt kan worden is CRISPR/Cas9. Met deze techniek kan op hele specifieke plaatsen in het genoom (volledige DNA volgorde aanwezig in elke cel) een breuk worden aangebracht. Deze breuk wordt vervolgens door de plantencel zelf gerepareerd, maar hierbij treden vaak kleine foutjes op waardoor het gen niet langer functioneel is. In **hoofdstuk 5** beschrijf ik een protocol waarmee de CRISPR/Cas9 methode gebruikt kan worden voor het aanbrengen van mutaties (verandering in de DNA volgorde) in het genoom van *Parasponia*. Met deze methode is het mogelijk om, binnen een periode van drie maanden, plantjes te genereren waarin één of enkele genen niet meer functioneel zijn. Als proof-of-concept, heb ik een aantal genen gemuteerd (uitgeschakeld) waarvan bekend is dat ze in vlinderbloemigen essentieel zijn voor het maken van wortelknolletjes. Deze genen - *PanHK4*, *PanEIN2*, *PanNSP1* en *PanNSP2* - zijn respectievelijk betrokken bij de perceptie van cytokinine, signalering van het plantenhormoon ethyleen en het reguleren van de productie van strigolactonen. Planten die mutaties bevatten in *PanHK4* of *PanEIN2* laten een verschil in ontwikkeling zien ten opzichte van planten die geen mutaties bevatten. Deze verschillen komen overeen met wat is gevonden in andere planten. Echter, het effect van het uitschakelen van deze twee genen op wortelknolvorming verschilt van het effect in vlinderbloemigen. Dit suggereert dat de hormonen cytokinine en ethyleen mogelijk een verschillende rol spelen tijdens wortelknolvorming in vlinderbloemigen en *Parasponia*. Anderzijds leidt het uitschakelen van *PanNSP1* en *PanNSP2* wel tot het zelfde effect als eerder waargenomen in vlinderbloemigen, namelijk de afwezigheid van wortelknolletjes. Dit geeft aan dat NSP1 en NSP2 belangrijk zijn voor het maken van wortelknolletjes in zowel *Parasponia* en vlinderbloemigen, wat duidt op een geconserveerde functie. Tevens illustreert dit de kracht van onze aanpak. Alles bij elkaar laten mijn resultaten zien dat de methode die ik ontwikkeld heb goed gebruikt kan worden voor het onderzoeken van de functie van specifieke genen in de tropische boom *Parasponia*.

Parasponia bomen zijn zeer nauw verwant aan tropische bomen uit het genus *Trema*. In tegenstelling tot *Parasponia*, zijn *Trema* bomen echter niet in staat om stikstofbindende wortelknolletjes te vormen. In **hoofdstuk 6** heb ik gebruikt gemaakt van de nauwe verwantschap tussen *Parasponia* en *Trema* om te onderzoeken of het kunnen vormen van stikstofbindende wortelknolletjes gepaard gaat met een verandering in genexpressie. Om dit te kunnen doen, heb ik de expressie van alle genen in de wortels van twee *Parasponia* soorten en drie *Trema* soorten bepaald. Dit heb ik gedaan met een techniek die RNA-seq genoemd wordt. De uitkomsten van dit experiment lieten zien dat de expressie van genen in de wortels van *Parasponia* verschilt van dat in *Trema* wortels. Onder de genen die anders tot expressie komen in *Parasponia* wortels ten opzichte van *Trema* wortels zijn

genen die een bekende rol vervullen in de symbiose met mycorrhiza schimmels en genen die betrokken zijn bij de productie van het plantenhormoon jasmonzuur. Metingen van de hormoonconcentratie laten zien dat *Parasponia* wortels ook inderdaad meer jasmonzuur bevatten in vergelijking met *Trema* wortels. Echter, planten die gemuteerd zijn zodat ze niet meer in staat zijn om jasmonzuur te produceren kunnen nog steeds functionele wortelknolletjes vormen. Daarom is het op dit moment nog niet duidelijk wat het verschil in genexpressie tussen *Parasponia* en *Trema* precies betekent voor de stikstofbindende symbiose tussen *Parasponia* en rhizobium.

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Wei and Ting Ting, thanks for the collaboration on DWARF27. It was a struggle to get this work published but we managed in the end! I am very proud of our paper in BMC Plant Biology. Also, I would like to thank the both of you for your help with introducing me to the MolBi lab. You both helped me find my way when I just started my PhD research. Ting Ting, I also want to thank you for friendship, especially during the first years of my PhD. Finally, I like to wish you both a lot of success in your future careers!

I would like to thank all members of the *Parasponia* team. Both current members - Robin, Rens, Kerstin, Titis, Yuda, Luuk, Fengjiao, Jan V, Kana, Simon, Sidney, Marijke, Wouter and Rene - as well as past members of the team - Maryam, Trupti, Elena and Cao - I am grateful to you all. Robin and Rens, thanks for your help with the bioinformatics part of my PhD research. This was very important for my efforts to compare *Parasponia* and *Trema* root transcriptomes and would have been impossible without. Kerstin and Wouter, thanks for your help with hormone measurements! Fengjiao, Titis, Luuk and Maryam, thanks for your contributions to chapter 5 of my thesis. Your contributions helped to transform this chapter from a mere method description into a real story. I hope we will be able to get this work published soon. Titis, I enjoyed our evening tea breaks and shared dinners after working late. Jan V, I would like to thank you for all your help with RNA isolation and help with resolving cloning issues. Elena, many thanks for all your help with microscopic analyses of my *Parasponia* nodules. Cao, thank you for teaching me how to work with *Parasponia*.

I am also grateful to all the BSc. and MSc. students that I had the pleasure to supervise during my PhD project. Malaika, Özhan, Jonathan, Susan, Titis, Max, Fleur, Luuk, Roos, Maikel, Sidney, Anneke, Fenne, Willem-Jan, Matthijs and Jasper, each and every one of you contributed to my research. I learned a lot from working with you and I hope that you all enjoyed your time at MolBi as much as I did working with you! I hope your training allows you to have a blooming future career.

I would like to thank Maria and Marie-Jose as well as the MolBi lab managers team - Jan Ho, Jan V, Carolien, Olga, Henk, Marijke and Sidney - for all their work 'behind the scenes' to make research at MolBi possible.

I am also grateful to all students, PhD students and staff at Molbi for a great atmosphere. I know I sometimes made your lives difficult with all the critical question I asked during work discussion. I hope that in the end my questions were helpful to improve your research. Also, I would like to thank our neighbors from Cell Biology and Plant Developmental Biology for the nice coffee breaks, lunch breaks and of course lab trips.

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Tot slot wil ik mijn familie en vrienden bedanken voor alle steun en afleiding. Pap en mam, jullie hebben ons in alles gesteund, dank daarvoor! Tessa, Pieter-Harm en Jos, ook jullie bedankt voor de steun en afleiding tijdens weekenden. Jos, bedankt dat jij mijn paranimf wilt zijn!

LIST OF PUBLICATIONS

Robin van Velzen, Rens Holmer, Fengjiao Bu, Luuk Rutten, [Arjan van Zeijl](#), Wei Liu, Luca Santuari, Qingqin Cao, Trupti Sharma, Defeng Shen, Yuda Roswanjaya, Titis Wardhani, et al. (2017) **Parallel loss of symbiosis genes in relatives of nitrogen-fixing non-legume *Parasponia***. *bioRxiv* DOI: 10.1101/169706

Ahmed Abd-El-Haliem, Jack Vossen, [Arjan van Zeijl](#), Sara Dezhsetan, Christa Testerink, Michael Seidl, Martina Beck, James Strutt, Silke Robatzek and Matthieu Joosten (2016) **Biochemical characterization of the tomato phosphatidylinositol-specific phospholipase C (PI-PLC) family and its role in plant immunity**. *Biochim. Biophys. Acta* **1861**: 1365-1378

Erik Limpens*, [Arjan van Zeijl](#)* and Rene Geurts* (2015) **Lipo-chitooligosaccharides modulate plant host immunity to enable endosymbioses**. *Annu. Rev. Phytopathol.* **53**: 311-334

[Arjan van Zeijl](#), Rik op den Camp, Eva Deinum, Tatsiana Charnikhova, Henk Franssen, Huub op den Camp, Harro Bouwmeester, Wouter Kohlen, Ton Bisseling and Rene Geurts (2015) **Rhizobium lipo-chitooligosaccharide signaling triggers accumulation of cytokinins in *Medicago truncatula* roots**. *Mol. Plant* **8**: 1213-1226

[Arjan van Zeijl](#)*, Wei Liu*, Ting Ting Xiao*, Wouter Kohlen, Wei-Cai Yang, Ton Bisseling and Rene Geurts (2015) **The strigolactone biosynthesis gene *DWARF27* is co-opted in rhizobium symbiosis**. *BMC Plant Biol.* **15**: 260

Carolien Ruyter-Spira*, Wouter Kohlen*, Tatsiana Charnikhova, [Arjan van Zeijl](#), Laura van Bezouwen, Norbert de Ruijter, Catarina Cardoso, Juan Antonio Lopez-Raez, Radoslava Matusova, Ralph Bours, Francel Verstappen, and Harro Bouwmeester (2011) **Physiological effects of the synthetic strigolactone analog GR24 on root system architecture in *Arabidopsis*: another belowground role for strigolactones?** *Plant Physiol.* **155**: 721-734

[Arjan van Zeijl](#), Titis Wardhani, Maryam Seifi-Kalhor, Luuk Rutten, Fengjiao Bu, Marijke Hartog, Sidney Linders, Elena Fedorova, Ton Bisseling, Wouter Kohlen and Rene Geurts **CRISPR/Cas9-mediated mutagenesis of four putative symbiosis genes of the tropical tree *Parasponia andersonii* reveals novel phenotypes**. *Manuscript in preparation*.

* Shared first authorship

CURRICULUM VITAE

Adrianus Leonardus (Arjan) van Zeijl was born on the 27th of September 1986 in the Noordoostpolder, the Netherlands. After obtaining his high school degree from the “Emelwerda college” in Emmeloord, the Netherlands, he moved to Wageningen in 2005 to start his university education at Wageningen University. There, he enrolled in the Plant Science bachelor programme, from which he graduated *cum laude* in 2008. During his bachelor studies, he became interested in the molecular aspects of plant biology. This led him to decide to continue his academic education with an MSc in Plant Biotechnology. His first thesis

project was performed at the Laboratory of Plant Physiology, under supervision of Dr Carolien Ruyter-Spira and Prof. Dr Harro Bouwmeester. During this time, he studied how the plant hormone strigolactone affects auxin homeostasis in *Arabidopsis thaliana*. In 2010, his thesis report was awarded the Rijk Zwaan Plant Science Award for the best plant science-related thesis defended at a Dutch University. He performed his second thesis project at the Laboratory of Phytopathology, under supervision of Ahmed Abd-El-Halim and Dr Matthieu Joosten. His thesis focussed on the biochemical characterization of several phospholipase C isoforms from tomato, which included determining the most optimal pH and calcium concentration for *in vitro* enzyme activity. After completion of his second thesis, he decided to extend his Master programme and move to Cologne, Germany for an internship at the Max Planck Institute for Plant Breeding Research. There, he joined the group of Prof. Dr Maarten Koornneef and worked under supervision of Dr Glenda Willems on validation of candidate loci that were identified from Genome-Wide Association and Quantitative Trait Locus studies for freezing tolerance in *A. thaliana*. In the summer of 2011, he completed his Master degree for which he graduated *cum laude*. Later that year, Arjan joined the Laboratory of Molecular Biology at Wageningen University to perform his PhD research. Under supervision of Dr Rene Geurts and promoter Prof. Dr Ton Bisseling, he studied how legumes and *Parasponia* engage in symbiosis with nitrogen-fixing rhizobium bacteria. The results of his research are presented in this thesis.

Education Statement of the Graduate School Experimental Plant Sciences

The Graduate School
**EXPERIMENTAL
PLANT
SCIENCES**

Issued to: Arjan van Zeijl
Date: 18 October 2017
Group: Laboratory of Molecular Biology
University: Wageningen University & Research

1) Start-up phase	<u>date</u>
▶ First presentation of your project Title: Role of cytokinin in the evolution of Parasponia root nodule formation	25 May 2012
▶ Writing or rewriting a project proposal Title: Parasponia; the key to identify constraints in developmental mechanisms underlying rhizobium nodulation	26 Nov 2013
▶ Writing a review or book chapter Title: Lipochitooligosaccharides modulate plant host immunity to enable endosymbioses. (2015) Annual Review of Phytopathology 53, 311-334.	Oct-Dec 2014
▶ MSc courses Advanced Bioinformatics (BIF-30806)	Feb 2013
Laboratory use of isotopes	

Subtotal Start-up Phase

*13.5 credits**

2) Scientific Exposure	<u>date</u>
▶ EPS PhD student days European Plant Science retreat for PhD students, JIC, Norwich, UK EPS PhD student day, Amsterdam, NL ExPeCtionS day 2013; Inspiration and Creativity in science, Wageningen, NL European Plant Science retreat for PhD students, VIB, Ghent, Belgium EPS Student day, Leiden, NL EPS PhD student day 'Get2Gether', Soest, NL	15-17 Aug 2012 30 Nov 2012 01 Feb 2013 24-26 Aug 2013 29 Nov 2013 29-30 Jan 2015
▶ EPS theme symposia EPS theme 1 'Developmental Biology of Plants', Wageningen, NL EPS theme 1 'Developmental Biology of Plants', Leiden, NL EPS theme 1 'Developmental Biology of Plants', Wageningen, NL EPS theme 1 'Developmental Biology of Plants', Wageningen, NL	19 Jan 2012 17 Jan 2012 24 Jan 2014 21 Jan 2016
▶ National meetings (e.g. Lunteren days) and other national platforms Annual meeting 'Experimental Plant Sciences', Lunteren, NL Annual meeting 'Experimental Plant Sciences', Lunteren, NL Annual meeting 'Experimental Plant Sciences', Lunteren, NL Annual meeting 'Experimental Plant Sciences', Lunteren, NL, co-chair 'Hormones' session Annual meeting 'Experimental Plant Sciences', Lunteren, NL, co-chair 'Comparative biology and evolution' session	02-03 Apr 2012 22-23 Apr 2013 14-15 Apr 2014 13-14 Apr 2015 11-12 Apr 2016
▶ Seminars (series), workshops and symposia Seminar Dr. Veronica Grieneisen Seminar Prof. Dr. Ottoline Leyser; 'Auxin, self-organisation, and the colonial nature of plants' Seminar Prof. Dr. Steffen Abel; 'Phosphate sensing in root development' Seminar Prof. Dr. Hong Ma; 'Molecular genetic, transcriptomic and genomic characterization of meiotic recombination in Arabidopsis' Seminar Dr. Pierre Hilson; 'Peptide hormones regulate plant tropic growth and root development' Seminar Prof. Sir David Baulcombe; 'Plant versus virus: defense, counter defense and counter counter defense' Seminar Prof. Sugimoto; 'Strigolactones, new plant hormones. Importance of their stereochemistry for bioactivity as germination stimulant' Mini-symposium 'Frontiers in plant morphogenesis' Seminar Prof. Ruth Finkelstein; 'ABA signaling networks in Arabidopsis' Seminar Andrew Sugden; 'Writing for high impact journals' Seminar Dr. Andreas Niebel; 'The CCAAT-box binding factors MtNF-YA1&2 are transcriptional regulators required for Nod factor signaling, rhizobial infection and nodule development' Seminar Prof. Rüdiger Simon; 'Plant stem cell systems' Seminar Prof. Eric Kramer; 'Auxin and plasmodesmata: measuring fluxes in the root' Seminar Prof. Hanhui Kuang; 'Using the Nicotiana-TMV system to study resistance gene evolution and plant genome stability' PDB start symposium, Wageningen, NL Seminar 'How to write a world class paper', Wageningen Library Seminar Prof. Jiayang Li; 'Understanding the molecular mechanisms underlying rice tillering' Plant Sciences Seminar: Statistics, genetics and omics in research Seminar Dr. Jos Raaijmakers; 'Back to the roots' Seminar Dr. Andrea Bräutigam; 'From 'Omics' to single gene analyses: "Case studies in C4 photosynthesis using next generation sequencing" WPC lecture; 'The secret of a successful PhD: Making the implicit explicit' Plant Sciences Seminar: Mutualism in action, Prof. Toby Kiers and Dr. Rene Geurts Seminar Dr. Melissa Wong; 'Novel insights into genome evolution of legumes -genome comparison, WGD prediction and reconstruction of ancestral genomes' Lecture Prof. Hans Clevers; 'The future of science' Plant Sciences Seminar: Bioinformatics Prof. Dick de Ridder, Paul Kersey Seminar Prof. Marilyn Roossinck; 'Plant virus ecology and evolution: pathogens to mutualists'	17 Nov 2011 13 Jan 2012 20 Mar 2012 29 May 2012 31 May 2012 10 Oct 2012 16 Oct 2012 13 Nov 2012 14 Nov 2012 08 Feb 2012 28 Mar 2013 15 Apr 2013 02 Jul 2013 11 Sep 2013 14 Oct 2013 17 Oct 2013 15 Nov 15 2013 10 Dec 2013 07 Jan 2014 05 Feb 2014 06 Feb 2014 11 Mar 2014 19 May 2014 12 Jun 2014 04 Nov 2014 05 Nov 2014

EPS flying seminar Prof. Dr. George Coupland; 'Seasonal flowering in annual and perennial plants'	19 Jan 2015
WEES seminar Prof. Kevin Foster; 'The evolution of cooperation and competition in microbes'	22 Jan 2015
Seminar Prof. Dr. Jeff Doyle; 'Polyploidy in wild relatives of soybean and other legumes: systematics, comparative and functional genomics, and nodulation'	12 May 2015
Seminar Dr. Siobhan Brady; 'Regulation of root morphogenesis in tomato species in the face of a changing environment'	09 Sep 2015
WEES seminar Prof. Marc-Andre Selosse; 'Mycoheterotrophy and mixotrophy : plants eating mycorrhizal fungi'	26 Nov 2015
Seminar Dr. Alain Goossens; 'How jasmonates provide the key to harness plant chemistry'	08 Dec 2015
Seminar Prof. Dr. Jane Parker; 'Plant intracellular immunity: evolutionary and molecular underpinnings'	21 Jan 2016
Symposium 'Evolution and maintenance of (belowground) cooperation', VU University, Amsterdam	04 Feb 2016
Seminar Dr. Jan Ruijter; 'Analysis of qPCR data. The use and usefulness of amplification curve analysis'	14 Mar 2016
Farewell Symposium EPS director Ton Bisseling; 'The Undergrond Labyrinth: Roots, Friends and Foes'	08 Feb 2017
Seminar plus	
Seminar Prof. Dr. Jeff Doyle; 'Polyploidy in wild relatives of soybean and other legumes: systematics, comparative and functional genomics, and nodulation'	12 May 2015
Seminar Dr. Siobhan Brady; 'Regulation of root morphogenesis in tomato species in the face of a changing environment'	09 Sep 2015
Seminar Dr. Alain Goossens; 'How jasmonates provide the key to harness plant chemistry'	08 Dec 2015
International symposia and congresses	
Plant Molecular Biology Gordon Research Seminar, Holderness, New Hampshire, USA	19-20 Jul 2014
Plant Molecular Biology Gordon Research Conference, Holderness, New Hampshire, USA	20-25 Jul 2014
EMBL PhD symposium, Heidelberg, Germany; 'Exploring Nature's toolbox'	23-25 Oct 2014
Mini-symposium: 'Evolution of Nodulation', Munich, Germany	07-08 Jan 2015
BNF Non-legume satellite symposium, Budapest, Hungary	23-24 Aug 2016
Genomics of nitrogen-fixing organisms workshop, Budapest, Hungary	25 Aug 2016
12th European nitrogen fixation conference, Budapest, Hungary	25-28 Aug 2016
Presentations	
Poster: Summerschool Natural Variation, Wageningen, NL	21-24 Aug 2012
Poster: Lunteren meeting 2013, Lunteren	22-23 Apr 2013
Poster: EPSR 2013, VIB, Ghent (re-use)	24-26 Aug 2013
Talk: PDB start symposium, Wageningen, NL; 'Hormones and symbiosis'	14 Oct 2013
Invited talk: Max-Planck Institute, Cologne, Germany; 'Medicago nodule formation is preceded by cytokinin biosynthesis'	20 Feb 2014
Talk: NWO Lunteren meeting 2014, Lunteren; 'Medicago nodule formation is preceded by cytokinin biosynthesis'	15 Apr 2014
Poster: Plant Molecular Biology Gordon Research Seminar+Conference, Holderness, New Hampshire	19-25 Jul 2014
Poster: EMBL PhD symposium, Heidelberg, Germany; 'Parasponia; the key to solve the nitrogen problem'	23-25 Oct 2014
Talk: EMBL PhD symposium, Heidelberg, Germany; 'Parasponia; the key to unravel the evolutionary trajectory towards a nitrogen-fixing symbiosis'	23-25 Oct 2014
Poster: Mini-symposium 'Evolution of Nodulation', Munich, Germany (re-use)	07-08 Jan 2015
Talk: EPS Get2Gether 2015; Rhizobium lipo-chitooligosaccharide signaling triggers accumulation of cytokinins in Medicago truncatula roots	29-30 Jan 2015
Talk: 12th European Nitrogen fixation conference, Budapest, Hungary	25-28 Aug 2016
IAB interview	
Meeting with a member of the International Advisory Board of EPS	29 Sep 2014
Excursions	
Excursion to Plant Breeding Company Rijk Zwaan, de Lier	27 Sep 2013
Subtotal Scientific Exposure	28.3 credits*

3) In-Depth Studies	<u>date</u>
EPS courses or other PhD courses	
Summerschool 'Natural variation of plants', Wageningen, NL	21-24 Aug 2012
Course 'Current Trends in Phylogenetics', Wageningen, NL	22-26 Oct 2012
Course 'The power of RNA seq', Wageningen, NL	05-07 Jun 2013
Journal club	
Journal club Molecular Biology, WUR	2011-2015
Individual research training	
Subtotal In-Depth Studies	6.6 credits*

4) Personal development	<u>date</u>
Skill training courses	
PhD Competence Assessment	22 May 2012
Project and Time Management	Nov-Dec 2012
ExPeCtationS; Inspiration and Creativity in science (EPS Career Day), Wageningen, NL	01 Feb 2013
Mobilising your -scientific- network	12&19 Jun 2013
WGS training Career Development for Post Docs	May-Jun 2016
Organisation of PhD students day, course or conference	
Organisation of pubquiz at EPS Get2Gether 2015	29-30 Jan 2015
Membership of Board, Committee or PhD council	
Subtotal Personal Development	4.6 credits*

TOTAL NUMBER OF CREDIT POINTS*	53.0
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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.

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