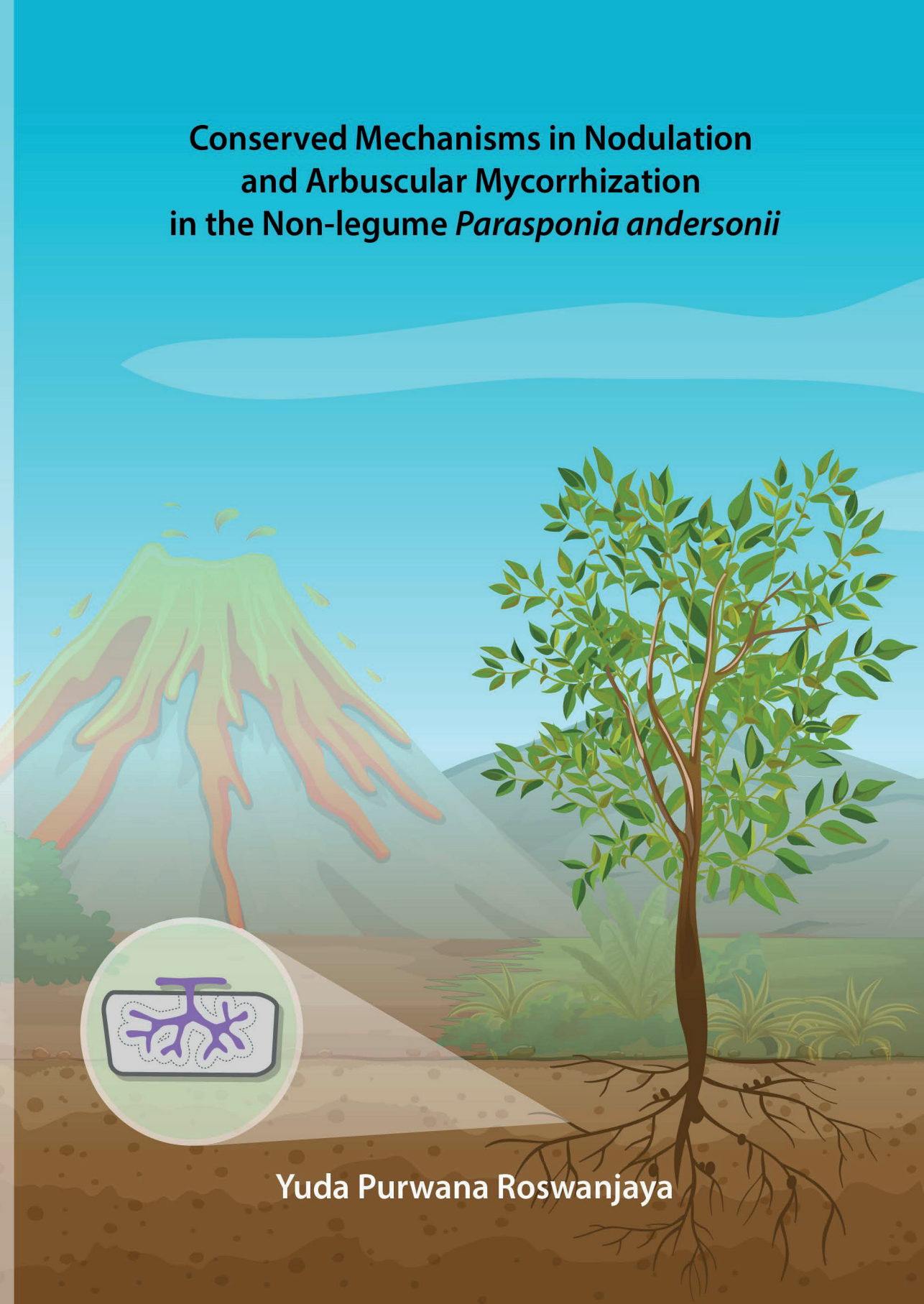
Conserved Mechanisms in Nodulation and Arbuscular Mycorrhization in the Non-legume *Parasponia andersonii*



Yuda Purwana Roswanjaya

Propositions

1. In *Parasponia*, GAT1 is not needed for nodulation, however nodulation is needed for maintenance of GAT1 in *Parasponia*.
   (this thesis)
2. Ignoring rare non-canonical splice sites in gene annotation projects is not scientifically justified.
   (this thesis)
3. The conclusion that plants display “cognitive behaviour” requires a stricter definition of the term cognitive behaviour.
4. Studying seed dispersal without analyzing seedling survival has no meaning in the context of plant conservation.
5. Lifestyle is the key to the success of COVID-19 vaccination campaigns.
6. Trauma changes a personality, so does the healing process.

Propositions belonging to the thesis, entitled:

“Conserved mechanisms in nodulation and arbuscular mycorrhization in the non-legume *Parasponia andersonii*”

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Conserved Mechanisms in Nodulation and Arbuscular Mycorrhization in the Non-legume *Parasponia andersonii*

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Conserved Mechanisms in Nodulation and Arbuscular Mycorrhization in the Non-legume *Parasponia andersonii*

Yuda Purwana Roswanjaya

Thesis

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This thesis is dedicated to my parents

Table of Contents

CHAPTER 1

General Introduction

Yuda Purwana Roswanjaya

Introduction

Land plants have limited access to nutrients. In order to improve their nutrient status, most plants engage endosymbiotically with microorganisms (Parniske, 2000; Thoms *et al.*, 2021). These interactions are advantages to both partners, as a common feature of these symbiotic association is the ability of the microorganisms to provide nutrients that limit plant growth in exchange for photoassimilates which they cannot produce themselves (Kahn *et al.*, 1985; Brachmann & Parniske, 2006; Walder & van der Heijden, 2015; Wang *et al.*, 2017). Among these, the association between arbuscular mycorrhizal (AM) fungi and land plants is one of the oldest symbiotic associations known to the scientific community (Sawers *et al.*, 2008). Fossil records indicate that this interaction was first established some 450 million years ago and, therefore, likely prone to massive convergent co-evolution (Redecker *et al.*, 2000). This led to numerous adaptations in both host plants and AM fungi that are crucial for their symbiotic effectiveness (Smith *et al.*, 2003). Besides provide the host plants with water, the primary nutrient of which acquisition from the soil is improved by AM fungi is inorganic phosphate (Pi). In most cases, the symbiosis with AM fungi leads to an increase of plant fitness, visualized by improved vigor, elevated plant fertility, and increased tolerance to various biotic and abiotic stresses (Newsham *et al.*, 1995; Liu *et al.*, 2007; Kamel *et al.*, 2017). To date, the mutualistic AM symbiosis ranges from angiosperms, gymnosperm, pteridophytes, and bryophytes, covering more than 80% of all terrestrial plant species (Bonfante & Genre, 2008; Smith & Read, 2010; Harrison, 2012).

Although AM Fungi can associate with almost more than three quarters of all land plants, the effect of mycorrhiza infection on plant growth is highly variable; ranging from highly positive to neutral or even negative. Moreover, the mycorrhiza effect on plants can be influenced by the genotype and/or developmental stages of both partners, environmental conditions and other ecological interactions (Smith, 1980; Johnson *et al.*, 1997; Cavagnaro *et al.*, 2004; Jones & Smith, 2004; Facelli *et al.*, 2010). Additionally, it is becoming more and more clear that both partners, either in concert or individually, can also influence each other's development. On the one hand, the symbiotic interaction will enhance fungal growth and the development of interfaces within the plant root. Whereas plant root characteristics themselves (e.g. lateral

root formation and root hair development) will also be altered. These are morphological modifications that cannot be explained by an improved nutrient status alone (Smith & Smith, 2011).

A much smaller group of plant species has evolved an endosymbiosis with nitrogen-fixing bacteria (Markmann & Parniske, 2009). This symbiosis evolved relatively more recent compare to AM symbiosis (~110 MYA). Additionally, this mutualistic interaction is different from AM symbiosis as it leads to the formation of a new organ on the host plant root; the so called root nodule. In these nodules bacteria can fix atmospheric dinitrogen (N_2) in the form of ammonia, and provide this to their host in return for carbohydrates. Host plants able to interact with these nitrogen-fixing bacteria all belong to a single subclade within the Rosids I, Fabidae. The Fabidae subclade is comprised of the orders: Fabales, Fagales, Cucurbitales, and Rosales and collectively referred to as the nitrogen-fixing clade (Soltis *et al.*, 1995). Species belonging to the orders Rosales, Fagales and Cucurbitales can, almost exclusively, engage an endosymbiosis with nitrogen-fixing gram-positive bacteria of the *Frankia* genus and are classified as actinorhizal plants (Dawson, 2008; Schwintzer, 2012). Differently, plant species belonging to the legume family (Fabaceae) in the Fabales order have evolved an endosymbiosis with a group of gram-negative nitrogen-fixing bacteria collectively called rhizobia. Currently only a single exception to this rule is known as a small group of tropical tree species of the Cannabaceae family in the Rosales order can also establish an endosymbiosis with rhizobia (Becking, 1983, 1992; Lancelle & Torrey, 1984; Geurts *et al.*, 2012). These tropical trees belong to a small genus, *Parasponia*, diverged ~100 MYA from legumes, is comprised of only five species, and represent the only non-legume known to establish an endosymbiosis relation with rhizobia (Geurts *et al.*, 2012).

Questions on why only a limited number of plant lineages evolved nitrogen-fixing endosymbiosis have been a major driving force for many genetic studies (Santi *et al.*, 2013; van Velzen *et al.*, 2019; Huisman & Geurts, 2020). Recently, these questions have become even more timely as it was revealed in several legumes species that some genes essential for nodulation are also needed for AM Symbiosis (Stracke *et al.*, 2002; Ané *et al.*, 2004; Lévy *et al.*, 2004; Imaizumi-Anraku *et al.*, 2005; Kanamori *et al.*, 2006; Saito *et al.*, 2007; Groth *et al.*, 2010; Horváth *et al.*, 2011). This suggests that the evolutionary nitrogen-fixing symbiosis

recruited pathways similar or identical to those used by the much older AM symbiosis (Parniske, 2008). If so, this raises the question of how the existing AM symbiosis pathways were rewired to support a nitrogen-fixing nodule symbiosis and why this only occurred in such a limited number of plant species. Here, I will summarize current knowledge in symbiotic signalling and describe the strategy used in this thesis to provide novel insight into the conserved mechanism of AM symbiosis and nodulation in *Parasponia*.

Evolutionary trajectory of the Nitrogen-fixing endosymbiosis

Quantitative and comparative phylogenetic and genomics studies have led to a better understanding of the evolutionary trajectory giving rise to the nitrogen-fixing symbiosis as we see it today (Velzen *et al.*, 2018; Griesmann *et al.*, 2018). Earlier studies hypothesized that nitrogen-fixing endosymbiosis had evolved at least ten times (Soltis *et al.*, 1995; Werner *et al.*, 2014; Geurts *et al.*, 2016). Eight times for the symbiosis with actinorhizal bacteria *Frankia*, and two times for the symbiosis with rhizobia (Werner *et al.*, 2015). This postulate support the fact that the nodule ontogeny is different between actinorhizal plants and legumes, which suggests a specific recruitment of molecular mechanisms (Swensen, 1996; Doyle, 1998). To explain why nodulating species are limited to the nitrogen-fixing clade, a predisposition event early in the divergence of this clade was hypothesized. This predisposition serves as a prerequisite for evolving nodulation independently multiple times, but only in this specific sub-clade (Soltis *et al.*, 1995; Werner *et al.*, 2014).

Phylogenomic comparisons of nodulating and non-nodulating species resulted in the revival of a second hypothesis on the evolutionary trajectory on nodulation (Soltis *et al.*, 1995; Velzen *et al.*, 2018; Griesmann *et al.*, 2018). This hypothesis states that, not a predisposition event followed by parallel gain-of nodulation, but a single gain-of nodulation followed by massive loss is a more likely scenario (Velzen *et al.*, 2018; Griesmann *et al.*, 2018; van Velzen *et al.*, 2019). The main finding of both studies was the occurrence of multiple independent loss-of essential nodulating genes in all analyzed non-nodulating species of the nitrogen-fixing clade. These genes include putative orthologs of *NOD FACTOR PERCEPTION* (*NFP*), the receptor for bacterial secreted lipo-chitooligosaccharides (LCO) signal molecules, the transcription factor *NODULE INCEPTION* (*NIN*), essential for nodule organogenesis, and the coil-coiled protein

RHIZOBIUM POLAR GROWTH (*RPG*), crucial for intracellular bacterial infection in model legumes like *Medicago truncatula* and *Lotus japonicus*. The comparison of *P. andersonii* and *M. truncatula* transcriptomes revealed ~290 orthologous gene with a nodule enhanced expression profile shared between both species (Velzen *et al.*, 2018), demonstrating that nodulating species utilize a shared subset of genes. Such a conserved genetic network is in agreement with a single gain-massive loss hypothesis.

Symbiotic LCO Signalling

The initiation of symbiotic interaction starts with a chemical signal exchange in the rhizosphere between the microsymbiont and the host plant (Schultze & Kondorosi, 1998; Fox *et al.*, 2001). Among the best studied chemical signals released by the plant root to attract AM fungi or rhizobia are strigolactones and flavonoids, respectively. Strigolactones are phytohormones regulating root and shoot branching (Ruyter-Spira *et al.*, 2013). Furthermore, strigolactones are perceived by AM Fungi and stimulate fungal mitochondrial activity and respiration, resulting in increased hyphal branching (Akiyama *et al.*, 2005; Besserer *et al.*, 2006, 2008). It is believed that this strigolactone triggered branching of AM fungi increases the possibility of fungal contact with the host root (Parniske, 2005). In many host plant species, gene regulating strigolactone biosynthesis, such as *DWARF27*, and transport, such as *PDR*1, are upregulated under phosphate starvation stress (Liu *et al.*, 2011; Kretzschmar *et al.*, 2012). Likely to enhance strigolactone secretion, linking strigolactones directly to the phosphate status of the host plant (Yoneyama et al., 2007, lópez-ráez et al., 2008). Similar to AMF, the nitrogen-fixing symbiosis under N-limiting conditions is initiated by flavonoids secreted into the rhizosphere to attract compatible bacteria (Liu & Murray, 2016; Coskun *et al.*, 2017).

In response to these plant signals (i.e. stigolactones or flavonoids), AM fungi and rhizobia release fungal and bacterial signals that perceive by the host plant to facilitate fungal colonization or nodule formation. Both processes demonstrate that plants have developed a very efficient strategy to overcome nutrient stress (phosphate starvation or nitrogen deficiency) by recruiting microbial symbionts such as AM fungi or rhizobia. The best studied fungal signals are chitin-based compounds such as lipo-chitooligosaccharides (LCOs) or short-

chain chitooligosaccharides (COs), collectively referred to as Myc factors (Maillet *et al.*, 2011; Genre *et al.*, 2013). Secretion of fungal COs (mainly CO4 and CO5) was shown to be stimulated by strigolactone treatments and the application of COs to plant roots induced symbiotic responses, such as calcium spiking, in multiple hosts (Genre *et al.*, 2013). Mycorrhizal LCOs are structurally similar to rhizobial LCOs, which are also known as Nod Factors (Maillet *et al.*, 2011; Limpens *et al.*, 2015). Nod factors were first characterized in the early 1990s. They were shown to be lipo-chitooligosaccharides (LCOs) consisting of four to five β-1,4-linked N-acetyl-D-glucosamine with an N-acyl group at the non-reducing terminal residue (Lerouge *et al.*, 1990; Dénarié *et al.*, 1996). The complexity of LCOs synthesized by certain rhizobia determines host specificity, and is the result of substitutions on the terminal or non-terminal residues of rhizobial LCOs (Spaink *et al.*, 1991; Masson-Boivin *et al.*, 2009; Masson-Boivin & Sachs, 2018). Host specificity can vary between different rhizobial species as some can nodulate only a few and others a broad range of host plants. For example, *Sinorhizobium fredii* NGR234 and *Rhizobium tropici* CIAT899 can interact with a broad range of hosts, likely due to the large number of structurally different LCO molecules produced by these species (Price *et al.*, 1992, 1996; Folch-Mallol *et al.*, 1996; Pueppke & Broughton, 1999; Morón *et al.*, 2005; Estévez *et al.*, 2009). The non-legume *Parasponia andersonii* also uses the LCO-induced signalling pathway for induction of its nodulation process (Marvel, 1987; Camp *et al.*, 2011). *P. andersonii* also has a relatively broad host range (Op den Camp *et al.*, 2012). In contrast, AM fungi have a far broader host range compared to rhizobia. This suggests either a less stringent host selection on Mycorrhizal LCOs and COs, or Nod-factor signalling was also equally broad when it first evolved. It is also possible that AM fungi have the ability to produce an even broader range of different LCOs and COs which might allow them to associate with a wide variety of plant species (Oldroyd, 2013).

The filamentous gram positive *Frankia* spp. can induce symbiotic association with actinorhizal plants (Wall, 2000; Schwintzer, 2012; Van Nguyen & Pawlowski, 2017). Phylogenetically, *Frankia* species can be divided into three main clusters, named cluster I, II and III (Nguyen *et al.*, 2016). Cluster II is thought to represent the most basal lineage, and species in this cluster have a broad range of host plants belonging to four families within the orders Rosales and Cucurbitales. Like rhizobia, *Frankia* can make LCO signal molecules similar to Nod-factors which activates symbiotic processes in the host plant. Homologs of rhizobium LCO

biosynthesis genes, *nodABC*-like genes have been identified in the genome of *Frankia* strains belonging to cluster II. Moreover, it was demonstrated that the *Frankia* Dg1 *nodC* can complement the *Rhizobium leguminosarum nodC* mutant, demonstrating *Frankia* Dg1 *nodC* can function as an N-acetyl glucosamine transferase (Persson *et al.*, 2015). In addition, some *Frankia* genomes contain homologs of rhizobium sulfotransferase gene *nodH* (Normand *et al.*, 1996; Sen *et al.,*2014; Persson *et al.*, 2015; Nguyen *et al.*, 2016). However, due to the technical difficulties of culturing *Frankia* cluster II strains, no LCO-like molecules from such strains have ever been structurally identified (Persson *et al.*, 2015).

On the other hand, genes known to be required for Nod factor biosynthesis have not been identified in strains belonging to clusters I or III (Normand *et al.*, 2007; Van Nguyen & Pawlowski, 2017), suggesting nodulation with *Frankia* species belonging to these clusters is independent of LCO signalling. Nevertheless, signalling molecules able to induce symbiotic responses were identified, but not characterised, in cluster I *Frankia* strains (ACN14a and CcI3) (Chabaud *et al.*, 2016). Different from rhizobia Nod-factors that are amphiphilic and chitinase-sensitive, these non-characterized signal molecules are hydrophilic and resistant to chitinase degradation (Chabaud *et al.*, 2016). This demonstrates that chemically, signal molecules from cluster I are distinct from rhizobium LCOs. Nevertheless, if cluster II is the more ancient cluster of *Frankia* spp. it is likely that LCO signalling is the ancestral mechanism of nodule initiation in the symbiosis between actinorhizal plants and *Frankia*, and the uncharacterised molecules later adaptations. Overall, despite some differences, there are commonalities in the symbiosis signalling mechanisms that are needed in establishing nitrogen-fixing symbiosis on legumes and actinorhizal species (Markmann *et al.*, 2008; Gherbi *et al.*, 2008; Hocher *et al.*, 2011).

Common Symbiosis Signalling Pathway

In model legumes such as *M. truncatula* and *L. japonicus*, the analysis of mutants impaired in nodule formation led to the discovery of genes encoding for Nod factor perception (Catoira *et al.*, 2000; Madsen *et al.*, 2010). These include, plant Lysin-motif (LysM) receptor(s)-like kinases *MtLYK3/LjNFR1* and *MtNFP/LjNFR5*, which combined as a heterodimer form the Nod-factor receptor (Madsen *et al.*, 2003; Radutoiu *et al.*, 2003; Limpens *et al.*, 2003; Arrighi *et al.*,

2006; Broghammer *et al.*, 2012; Moling *et al.*, 2014). These Nod factor receptors are located at the plasma membrane of epidermal cells, where their LysM domains, likely positioned outwards, are essential for LCO binding. In *P. andersonii*, the orthologous of these receptors, *PanNFP2* and *PanLYK3* are also required for nodulation **(Chapter 3)**. Interestingly, in *P. andersonii PanLYK3* functions also in AM symbiosis as well as in chitin-triggered immune signalling **(Chapter 3)**.

It is hypothesized that in Legumes LysM receptors are also required to perceive Myc factors. This is supported by the observation that non-sulfated Myc factors trigger lateral root formation in wild-type *M. truncatula*, but fail to do so in the *Mtnfp* mutant (Maillet *et al.*, 2011). Still, mycorrhization occurs in this mutant, suggesting that functionally redundant receptors could be involved in Myc factors perception (Maillet *et al.*, 2011). In rice, outside the nitrogen-fixing clade of plant species, Myc factors were shown to be perceived by the LysM receptor kinase *OsCERK1* (Miyata *et al.*, 2014; Zhang *et al.*, 2015). *OsCERK1* is the ortholog of the *Arabidopsis AtCERK1* gene, and functions as a co-receptor for chitin-triggered immune responses by interacting with co-receptors *OsCEBiP* and *AtLYK4/5* in Rice and Arabidopsis, respectively (Miya *et al.*, 2007; Shimizu *et al.*, 2010; Cao *et al.*, 2014). In the rice *Oscerk1*, but not in the *Oscebip* mutant, reduced mycorrhization was observed, indicating that *OsCERK1* is likely a co-receptor involved in both symbiosis and plant immunity responses (Miyata *et al.*, 2014; Zhang *et al.*, 2015), similar with *PanLYK3*.

In the case of COs, a recent study identified *M. truncatula MtLYK9*, *MtLYR4* and *L. japonicus LjLYS6* LysM domain containing receptor-like kinases as a receptor for longer chain COs (CO7/8) to trigger defence responses (Bozsoki *et al.*, 2017). Mutations in any of these receptors resulted in impaired chitin-triggered immune responses, while still allowing a successful, although slightly reduced, AM symbiosis (Bozsoki *et al.*, 2017; Gibelin-Viala *et al.*, 2019). Therefore, it is proposed that complexes containing different LysM receptor kinases are required in *M. truncatula* and *L. japonicus* to distinguish between LCOs, short and long COs (Bozsoki *et al.*, 2017).

Downstream of LCO perception, a signalling cascade leading to nodule formation and bacterial infection or AM fungi penetration and intraradical colonization is activated. A large

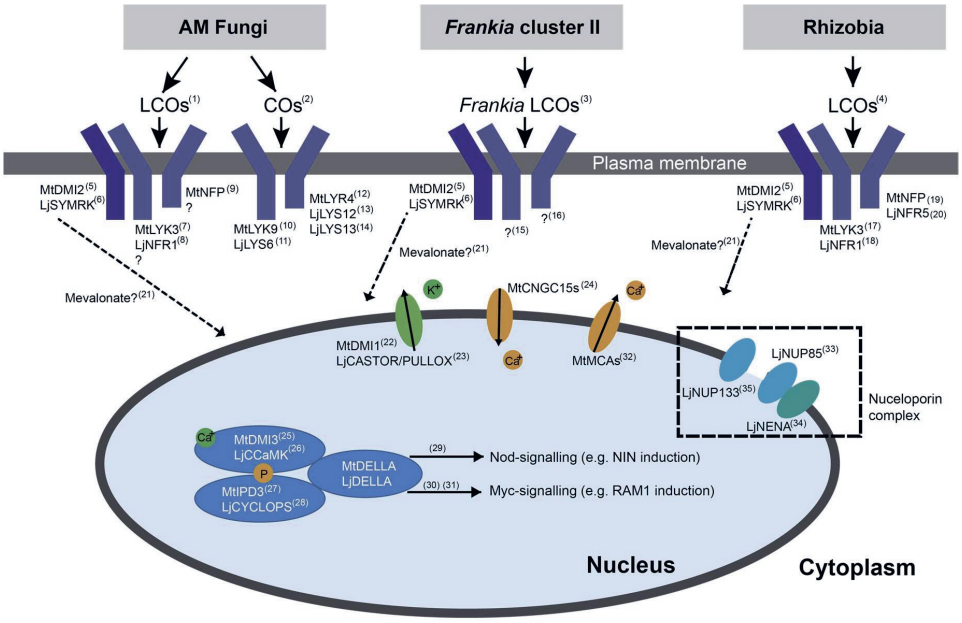

Figure 1. Common Symbiotic Signalling Pathway. This signalling pathway is essential for a successful AM symbiosis as well as for the rhizobium-legume and Frankia-actinorhizal plants symbiosis. To initiate the signalling, Myc factors (Myc-LCOs and Myc-COs) (1,2), Frankia LCOs (3), and Nod-factors (Nod-LCOs) (4) are perceived by plant LysM receptors (7 – 20). These are though to form a complex with MtDMI2/LjSYMRK (5, 6), which interact with a 3-hydroxy-3-methylglutaryl coenzyme A reductase 1 (MtHMGR, 21). This interaction generate mevalonate as a potential secondary messenger to the nucleus (21). Activation of the CSSP eventually leads to nuclear calcium spiking. This is mediated by nuclear envelope located potassium (22, 23), calcium channel (24) and a calcium-transporting ATPase (32). In addition, components of the nucleoporin complex are required for calcium spiking (33, 34, 35). Calcium spiking is decoded by the calmodulin/calcium-dependent protein kinase MtDMI3/LjCCaMK (25, 26), leading to phosphorylation and activation of transcription factor MtDMI3/MtDMI3like/LjCYCLOPS (27, 28). MtIPD3s or MtCYCLOPS interacts with *Medicago* or *Lotus* DELLA protein to initiate downstream signalling of Rhizobium or Frankia symbiosis (29) or AM symbiosis (30,31).

portion of the signalling network induced by rhizobia overlaps with that controlling endomycorrhizal symbiosis, revealing a single pathway used by host plants to establish endosymbiosis with either AM fungi or N_2-fixing bacteria. This genetic network is therefore referred to as the common symbiosis signalling pathway (CSSP, see **Figure 1)** (Oldroyd & Downie, 2006; Oldroyd, 2013). Genetic studies revealed that the CSSP consist, among others, of the LRR-type transmembrane receptor kinase MtDMI2/LjSYMRK, the nuclear-localized potassium channels MtDMI1/LjCASTOR and LjPOLLUX, nuclear pore complex proteins such as LjNUP133, LjNUP85, and LjNENA, the calcium calmodulin dependent kinase MtDMI3, and the CCAMK interacting transcription factor MtIPD3/LjCYCLOPS. Knockout mutations in any of

these genes led to major defects in, or even complete loss-of, nodule initiation and AM fungi colonization (Kistner *et al.*, 2005; Parniske, 2008; Oldroyd, 2013).

The CSSP starts from the LRR-transmembrane receptor kinase LjSYMRK/MtDMI2, which interacts with the kinase domain of LjNFR5/MtNFP to form a complex. SYMRK is essential for both rhizobia and AM symbiosis in legumes (Endre *et al.*, 2002; Stracke *et al.*, 2002; Limpens *et al.*, 2005). *P. andersonii symrk* mutant lines revealed a conserved function as also in this line, nodule and arbuscule formation were abolished **(Chapter 4)**. A downstream signalling cascade of LjSYMRK/MtDMI2, likely involves an enzyme in the mevalonate biosynthesis MtHMGR1 (Kevei *et al.*, 2007; Venkateshwaran *et al.*, 2015), and induces oscillations of the calcium concentration in the perinuclear region. This so called calcium spiking is dependent on nuclear based proteins such as the calcium-dependent adenosine triphosphate (MtMCA8), the potassium (LjCASTOR/MtDMI1, LjPOLLUX) and calcium channels (MtCNGC15a-c) (Stracke *et al.*, 2002; Ané *et al.*, 2004; Imaizumi-Anraku *et al.*, 2005; Chen *et al.*, 2009; Capoen *et al.*, 2011; Charpentier *et al.*, 2016; Kim *et al.*, 2019) and is a reoccurring theme in both types of endosymbiosis (Ehrhardt *et al.*, 1996; Navazio *et al.*, 2007; Granqvist *et al.*, 2015; Charpentier *et al.*, 2016). Additionally, components of the nuclear pore, such as NUCLEOPORIN 85 (LjNUP85), LjNUP133, and LjNENA, have been shown to be essential for calcium spiking in *L. japonicus* (Kanamori *et al.*, 2006; Saito *et al.*, 2007; Groth *et al.*, 2010). The LCO induced calcium oscillation signal is likely decoded by a nuclear localized calcium and calmodulin dependent kinase CCaMK (MtDMI3 in *M. truncatula*) (Lévy *et al.*, 2004; Sieberer *et al.*, 2009).

Activation of CCaMK triggers a transcriptional cascade, starting with the interacting protein LjCYCLOPS/MtIPD3 (Yano *et al.*, 2008; Limpens & Bisseling, 2014; Singh *et al.*, 2014). This transcription factor is activated by phosphorylation (Ovchinnikova *et al.*, 2011). LjCYCLOPS/MtIPD3 is currently considered to be the last shared component between rhizobium and AM fungi in the CSSP. Downstream of the CSSP, the signalling subsequently diverges and leads to very different transcriptional reprogramming and outcomes for both symbiosis.

In legumes, LCO perception activates the transcription factor CYCLOPS, which binds to the promoters of *NODULE INCEPTION* (*NIN*) and *ETHYLENE RESPONSIVE FACTOR REQUIRED FOR*

NODULATION 1 (*ERN1*) and *ERN2* to activate their transcription (Schauser *et al.*, 1999; Kistner & Parniske, 2002; Yano *et al.*, 2008; Laloum *et al.*, 2013; Singh *et al.*, 2014; Cerri *et al.*, 2016, 2017). These genes are not transcriptionally activated during AM fungi symbiosis, and are responsible for a large part of transcriptional reprograming required for rhizobium infection and nodule formation (Cerri *et al.*, 2016, 2017). Arbuscular mycorrhization triggers the expression of a different transcription factor, the GRAS-type transcription factor *REQUIRED FOR ARBUSCULAR MYCORRHYZA 1* (*RAM1*) (Gobbato *et al.*, 2013; Pimprikar *et al.*, 2016). RAM1 interacts with two other GRAS-type transcription factors; *NODULATING SIGNALLING PATHWAY 1* (*NSP1*) and *NSP2* (Xue *et al.*, 2015; Hohnjec *et al.*, 2015). During rhizobia symbiosis, NSP1 and NSP2 form either homo- or heterodimers that are important activating Nod-factor-responsive genes, such as *EARLY NODULIN 11* (*ENOD11*), *NIN* and *ERN1* (Hirsch *et al.*, 2009; Cerri *et al.*, 2012; Kawaharada *et al.*, 2017) and mutants of these genes cannot form root nodules (Wais *et al.*, 2000; Lin *et al.*, 2018), indicating that there might still be some debate to what extent NSPs can be considered part of the CSSP. It was recently demonstrated that the nodulation specific function of NSP1 and NSP2 is conserved in *P. andersonii* (van Zeijl *et al.*, 2018), suggesting the recruitment of the AM fungi related signalling pathways for nitrogen-fixing nodule symbiosis could have also occurred outside the legume family. This is in part also supported by the fact that in the actinorhizal plant species *Casuarina glauca* and *Datisca glomerata*, a symbiotic function for CgSYMRK/DgSYMRK, CgCCaMK and CgNIN has been demonstrated (Markmann *et al.*, 2008; Gherbi *et al.*, 2008; Clavijo *et al.*, 2015). Furthermore, *Frankia* induced signalling triggers a Ca^{2+} oscillation response in *C. glauca* and *Alnus glutinosa* (Granqvist *et al.*, 2015; Chabaud *et al.*, 2016). Combined, this suggest that evolution of a nitrogen-fixing symbiosis with rhizobium or *Frankia* is either shared, or strongly guided by genetic constrains.

Interestingly, mutants in *ccmk*, *cyclops*, *symrk* or *nsp1* can be complemented by homologs of non-nodulating but mycorrhized plant species (Markmann *et al.*, 2008; Banba *et al.*, 2008; Yokota *et al.*, 2010; Saha *et al.*, 2016). This indicates a conserved function of the CSSP between mycorrhized and nodulating plants, which again raises the question: Why can nodules not be triggered in all plant species able to establish a symbiosis with AM fungi?

Efforts to engineer nitrogen fixing symbiosis

Legumes represent the largest family of nodulating species and it includes important crops such as soyebean (*Glycine max*), common bean (*Phaseolus vulgaris*), chickpea (*Cicer arietinum*), lens (*Lens culinaris*) and Pea (*Pisum sativum*). Due to the high nitrogen-fixing efficiency of these legume crops (e.g. up to 100-300 kg/hectare of fixed nitrogen annually) (Wani *et al.*, 1995), they do not require artificial nitrogen fertilizers.

This leads to the question whether it is possible to engineer nitrogen-fixing symbiosis and extend its host range to non-nodulating crops (e.g. wheat, maize, and rice). Extending symbiotic nitrogen fixation to non-legumes has been one of the holy grails of plant breeding ever since the nitrogen-fixing symbiosis was first discovered. Cultivation of rhizobia from legumes to inoculate other plant species has previously been attempted (Pankievicz *et al.*, 2019). However, none of these attempts were successful.

The most achievable way to engineer a nitrogen-fixing root nodule symbiosis is to mimic an existing symbiosis by transferring genes from nodulating to non-nodulating plant species (Huisman & Geurts, 2020). However, most research on nodulation has been done on papilionoid legumes, initially focusing on crops but then mainly on models like *M. truncatula* and *L. japonicus*, resulting in a high papilionoid-bias in our understanding of nodulation. One strategy to work around this bias is to identify a core set of nodulation genes by expanding the genetic studies to non-model species of the Rosales, Cucurbitales and Fagales orders. Since not only legumes can be nodulated by nitrogen-fixing bacteria, it suggests that having knowledge only of legumes is limiting engineering opportunities. Exploiting a core set of nodulation genes on actinorhizal plants and *Parasponia*, will lead to a better understanding of the conserved genes regarding this process in relation to *Frankia* and Rhizobia, respectively, outside the legume family. Having actinorhizal plants and *Parasponia* as a study object can provide alternative, and possibly simpler, blueprints for the nitrogen-fixing nodulation trait. Furthermore, functional studies in other nodulating clades may also offer information on lineage-specific adaptations in legumes which are therefore less interesting to focus on.

To transfer the nitrogen-fixing nodulation trait to other plant species, it is important to know

which genes are responsible and how they translate the perception of bacterial signal molecules into transcriptional and cellular responses. There are two different approaches that have been proposed to identify the core set of nodulation genes. First, comparative analyses of nodule transcriptomes (Hocher *et al.*, 2011; Battenberg *et al.*, 2018; Salgado *et al.*, 2018; Velzen *et al.*, 2018). This strategy identified sets of symbiosis genes with a nodule-enhanced expression profile. The second strategy is comparative phylogenetic analysis, where genomes of nodulating and non-nodulating species in the nitrogen-fixing clade were compared (Velzen *et al.*, 2018; Griesmann *et al.*, 2018). This latter approach revealed that NFP, NIN and RPG, essential for nodulation, are present in plant species outside the nitrogen-fixing clade, but lost in non-nodulating species within this clade (van Velzen *et al.*, 2019; Shen & Bisseling, 2020). This suggest these genes might have undergone neo-functionalisation during the evolution of nodulation, and therefore should be considered primary engineering targets. In addition to these three, four other genes (i.e. *LEK1*, *CRK11*, *DEF1* and *GAT1*) were identified with, although less well known functions in nodulation, similar profiles of presence and absence compared to *NFP*, *NIN* and *RPG* (Velzen *et al.*, 2018). One of the genes, a putative GAMMA (γ) AMINOBUTIRIC ACID TRANSPORTER (*GAT1*) is highly, and likely exclusively, expressed in *P. andersonii* nodules **(Chapter 5)**.

Last, but not least, plant species targeted for engineering nitrogen-fixing symbiosis need to meet a number of requirements. Ideally, such species should be amendable to laboratory experimentation, having established protocols for synchronized seed germination and quantitative nodulation assays. From a genetic point-of-view, a relatively small diploid genome that is fairly homozygotic would be preferred, as well as the ability to be (stable) genetically altered (Huisman & Geurts, 2020). Engineering plants is generally more challenging than manipulating bacteria, primarily due to generation time and the bottleneck of plant transformation (Pankievicz *et al.*, 2019).

The *Parasponia-Trema* experimental model system

Parasponia represents a phylogenetic clade consisting of five tropical tree species indigenous to the Malay Archipelago (Becking, 1992). Phylogenetic reconstruction indicates that the *Parasponia* lineage is embedded in the non-nodulating genus *Trema* (Yang *et al.*, 2013; Velzen

et al., 2018). These species are so closely related that in rare cases inter-specific crosses are possible (Velzen *et al.*, 2018). Both genera grow in areas where vegetation has been cleared due to natural or man-made disturbance, such as landslides, volcanic ash deposits and forest gaps (Elias, 1970; Soepadmo, 1974; Vázquez-Yanes, 1998). *Parasponia* seems to prefer nitrogen-poor eroded soils and is capable of forming dense stands on fresh deposits of volcanic ash, whereas *Trema* has a more widespread distribution (Soepadmo, 1974; Becking, 1992). Besides scare in nitrogen, *Parasponia* habitats are often low in phosphorous as well as other essential nutrients (Akkermans *et al.*, 1978; Trinick, 1980).

Working with non-model species is often difficult as growth and transformation are mostly lacking. For *P. andersonii*, such protocols have been established (van Zeijl et al., 2018; Wardhani et al., 2019), and similar protocols were recently established for *T. orientalis* (unpublished). The growing conditions for *P. andersonii* in both the laboratory and in glass houses has been established (Wardhani et al., 2019), and these conditions are also suitable for growing *T. orientalis*. Additionally, *P. andersonii* and *T. orientalis* also provide a powerful tool for a comparative study regarding nitrogen-fixing nodule symbiosis. It is currently believed that during evolution *T. orientalis* lost its nodulation trait but kept its ability to be colonized by AM Fungi. Combined, *P. andersonii* and *T. orientalis* are thus perfect models to study genes specifically needed for nodulation and/or mycorrhization. For this purpose, a quantitative mycorrhization assays for these plant species have to be developed (**Chapter 2**).

Thesis Outline

The aim of this thesis is to get insight into the conserved mechanisms of nodulation and mycorrhization in a non-legume plant, and to identify components of the core genetic network underlying the nitrogen-fixing nodulation and AM fungi mycorrhization trait. To do so, I adopted *Parasponia andersonii* and *Trema orientalis* as a comparative system and applied different strategies, including phylogeny reconstruction and reverse genetic approaches.

In **Chapter 2**, I describe the development of a quantitative mycorrhization assay for *P. andersonii*. For this, we used established methods for other model plant species, which were optimized by determining the ideal conditions for *P. andersonii* to interact with the AM fungus

Rhizophagus irregularis. The development of such an assay is justified by the fact that in some mutants, mycorrhization phenotypes are mild and require a robust, yet sensitive, quantitative assay. We demonstrate that *P. andersonii* is well-mycorrhized at low exogeneous phosphate levels, and that a close pot system can be used to avoid cross-contamination with rhizobia. Furthermore, we also show that the number of spores needed for *P. andersonii* mycorrhization is dependent on the plant starting material, as seedlings require less fungal spores to be colonized compared to tissue culture explants.

In **Chapter 3**, I focus on the role of the LysM-type receptor gene family in *P. andersonii*. We identified PanLYK3 a LYK-type receptor, the homolog of *M. truncatula* LYK3. We showed that PanLYK3 has a dual function in symbiosis signalling and plant immunity. Further, we investigate the role of PanNFP1 and the newly identified PanNFP2 in LCO signalling. We show that PanNFP1 and PanNFP2 are both, to different extents, needed for nodulation but have no clear function in mycorrhization of *P. andersonii*.

In **chapter 4**, I explore the SYMRK gene function during *P. andersonii* symbiosis, either with rhizobia or AM fungi. By reverse genetic, we showed that in *P. andersonii* SYMRK is essential for both nitrogen-fixing nodule and AM fungi symbiosis. These findings suggest that CSSP defines a conserved genetic basis for nodulation and mycorrhization in *Parasponia*, similar as found in legumes and actinorhizal plants. In this chapter, I also investigate a mutation located in the splice donor site of intron 12 in the *TorSYMRK* gene found in *T. orientalis* accession RG33. This SNP potentially leads to a premature stop codon in the mRNA, encoding a SYMRK receptor lacking a functional kinase domain. We showed that, despite this mutation, *T. orientalis* RG33 can still be mycorrhized.

In **chapter 5**, I investigate the role of GAMMA (γ) AMINOBUTIRIC ACID TRANSPORTER1 (GAT1) during nodulation in *P. andersonii*. *PanGAT1* is exclusively expressed in *P. andersonii* nodules, and one of the seven genes lost in *Trema* species. By reverse genetics, we showed that PanGAT1 is not essential for *P. andersonii* nodulation. No nodule related phenotypes could be observed in any of the *Pangat1* mutant lines created during this research. Therefore we conclude that GAT1 should not be a primary target in the ongoing effort to engineer the nodulation trait in *Trema* species.

In **chapter 6**, I summarize the results obtained from all experimental chapters. I will reflect on their significance and integrate them with published data. I will also provide a future perspective on research aimed at unraveling the conserved mechanisms involved in nodulation and mycorrhization in *Parasponia*, and reflect on the steps I feel need to be taken to transfer the nodulation trait to non-nodulating crops.

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CHAPTER 2

2

A quantitative endomycorrhization assay for *Parasponia andersonii*

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Abstract

Parasponia species (Cannabaceae) are the only known non-legumes able to establish a nitrogen-fixing nodule symbiosis with rhizobium. Also, *Parasponia* species can interact with arbuscular mycorrhizal fungi (AMF), which enhances uptake of nutrients, especially phosphates. The interaction between plants and AMF is generally hard to quantify because the success of this endosymbiosis depends on the condition of both partners as well as environmental factors. Endomycorrhization assays in *Parasponia* still rely on methods as established for model plant species. However, those methods could mask specific phenotypes because each plant species may require specific conditions to develop an efficient AMF symbiosis. Here, we did serial methodological optimizations to determine optimal conditions for the interaction between *Parasponia andersonii* and the AMF *Rhizophagus irregularis*. By quantifying four parameters of mycorrhization efficiency, we show that in two concentrations of exogenous phosphate (20 µM and 40 µM), the interaction between *P. andersonii* and *R. irregularis* occurred very efficiently. In addition, the use of a close pot system to avoid any contamination during the assay did not affect mycorrhization efficiency nor plant growth parameters. Furthermore, quantification of mycorrhization efficiency from two different plant materials -seedling and tissue culture plantlets- revealed that vegetative propagated *P. andersonii* requires a double amount of spores compared to generatively propagated plantlets to obtain a similar level of AMF colonization. Taken together, the newly developed assay will allow quantitative phenotyping of the AMF symbiosis in *P. andersonii* mutants.

Introduction

The vast majority of all land plants establish an endosymbiosis with arbuscular mycorrhiza fungi (AMF) belonging to a single phylum, the Glomeromycota (SCHÜßLER *et al.*, 2001; Hibbett *et al.*, 2007). AMF provides plants access to soil nutrient resources by its extraradical mycelium. In return, the fungus receives carbon in the form of sugar and lipids from the plant (Luginbuehl *et al.*, 2017). The exchange of those components occurs in the fungal haustoria, so-called arbuscules, that are formed inside the cortical root cells. Arbuscules dramatically enlarge the surface area of contact between both organisms and the exchange of nutrients across the peri-arbuscular interface (Yang & Paszkowski, 2011). AMF forms an extensive syncytial hyphal network that directly connects the arbuscules in the root cortex with the nutrient absorbing extraradical mycelium extended in the soil (Smith *et al.*, 2011; Marschner, 2011). It enables the host plant to reach water and nutrient deposits it would otherwise not be able to, leading to increased fitness and growth (Barea *et al.*, 2002).

The primary nutrient of which acquisition from the soil is improved through AMF is inorganic phosphate (Pi) (Javot *et al.*, 2011; Smith & Smith, 2011; Walder *et al.*, 2015). In plants, Pi is the second most crucial nutrient for growth after nitrogen. While Pi is generally abundant in the soil, due to its low mobility, it is one of the nutrients most easily depleted in the area directly surrounding the plant root (Bieleski, 1973; Schachtman *et al.*, 1998; Vance, 2001). Such depletion may lead to Pi deprivation in the plant resulting in to a plethora of phenotypic responses caused by phosphate deficiency (Tinker & Nye, 2000; Balzergue *et al.*, 2013). AMF can obtain Pi from the soil using high-affinity Pi transporters expressed in the extraradical mycelium (Harrison & van Buuren, 1995). Once taken up by the extraradical mycelium, Pi is converted to polyphosphates and translocated along the hyphae towards the arbuscule. Here, polyphosphates are depolymerized, and Pi is transferred to the cortical root cells in exchange for sugars and/or lipids (Ohtomo & Saito, 2005; Jiang *et al.*, 2017; Keymer *et al.*, 2017). Calculations regarding the contribution of AMF to the total Pi uptake suggest that AMF are the dominant source for Pi uptake under low Pi conditions (Smith *et al.*, 2004; Yang *et al.*, 2012).

The establishment of the AM symbiosis is dependent on environmental conditions. These conditions include the availability of exogenous Pi, which in high abundancy can completely

inhibit the symbiotic interaction with AMF (Thomson *et al.*, 1986; Breuillin *et al.*, 2010; Bonneau *et al.*, 2013). This inhibition occurs when sufficient Pi can be acquired through direct uptake by the plant root and is considered as a strategy from the plant to avoid the carbon cost of symbiosis (Bago *et al.*, 2000; Nagy *et al.*, 2009). In addition, the biosynthesis and exudation of strigolactones are negatively affected by high Pi availability (Yoneyama *et al.*, 2007; López-Ráez *et al.*, 2008). Strigolactones are secreted by the plant root into the rhizosphere, where they act as signal molecules to induce morphological and developmental responses in the fungus such as hyphal branching (Akiyama *et al.*, 2005; Besserer *et al.*, 2006).

It was previously reported that AMF can colonize *Parasponia andersonii* (Camp *et al.*, 2011; Velzen *et al.*, 2018; Bu *et al.*, 2020). *Parasponia* species are tropical trees belonging to a genus in the Cannabaceae. This genus comprises five species, all native to the Malay archipelago (Soepadmo, E., 1974; Doyle, 1998). All five *Parasponia* species grow predominantly on the slopes of volcanic hills, which are poor in organic nitrogen content, making *Parasponia* a pioneer plant. *Parasponia* trees can reach lengths of up to 10 meters and can grow roughly 45 cm per month (Becking, 1979).

Parasponia species are the only non-legumes that can establish a nitrogen-fixing root nodule symbiosis with rhizobia, making them interesting models for comparative studies on rhizobia nodulation. Phylogenomic studies indicated that the nodulation trait evolved only once, which implies *Parasponia,* legumes and other nodulating plants share a core genetic network essential for nodulation. Studies in legumes showed that nodulation is founded on the AMF symbiosis. It was found that rhizobium and AMF secrete a structurally highly similar lipo-chitooligosaccharide signal molecule, known Nod factor in the case of rhizobium and Myc factor in the case of AMF (Streng *et al.*, 2011). Subsequently, several plant genes have been identified that act in the establishment of both symbioses. These genes encode proteins of a signaling cascade and are referred to as the common symbiotic signaling pathway (Ivanov *et al.*, 2012; Sun *et al.*, 2015). In addition, several genes were identified to function only in nodulation or mycorrhization, demonstrating that there is some degree of divergence between the genetic networks controlling both symbioses (Oldroyd, 2013). Until now, research on the common signaling pathway in *Parasponia* primarily focused on nodulation (Geurts *et al.*, 2012, 2016; van Zeijl *et al.*, 2018; Velzen *et al.*, 2018). As a result, our

understanding of the duality of the common signaling pathway in non-legume species that nodulate with rhizobia is limited.

Here, we aim to establish a quantitative AM symbiosis assay for *P. andersonii.* So far, all attempts to study the association between AMF and *Parasponia* have used methods optimized for plant species like rice (*Oryza sativa*) or *Medicago truncatula* (Sharma, personal communication). We provide a method optimized to perform a quantitative analysis of the *P. andersonii* AM symbiosis using a commercial *Rhizophagus irregularis* spore suspension of the reference strain DAOM197198. We demonstrate that the optimal exogenous phosphate concentration for *P. andersonii* mycorrhization lies between 20 and 40 µM Pi. Moreover, we show that vegetative propagated *P. andersonii* plantlets require a higher amount of spores compared to seedlings. Additionally, we show that a closed pot system has no negative effect on either plant growth or mycorrhization efficiency compared to an open pot system, demonstrating that such closed pots can be used during mycorrhization to avoid cross-contamination with rhizobia. Combined, our results demonstrate that a more robust and reproducible mycorrhization assay for *P. andersonii* can be achieved by making a few specific adjustments to available mycorrhization protocols.

Results

Optimization of the AMF - *Parasponia andersonii* interaction

We set out to develop a *P. andersonii* quantitative mycorrhization assay to find the most optimal conditions for it to interact with AMF. Elevated levels of organic phosphate (Pi) limit the establishment of AMF symbiosis. On the other hand, limited Pi availability could severely hinder plant growth prior the interaction with AMF. To maneuver this fine line, we tested two relatively low concentrations of exogenous Pi (20 µM and 40 µM) for their effect on AM symbiosis and plant development. Previous studied on the *P. andersonii*-AMF interaction relied on dried inoculum of *R. irregularis*. However, as this has proven to be a highly variable source of AMF, we chose to use the commercially available *R. irregularis* spores suspension of the DAOM 197198 reference strain (Agronutrition, Carbonne, France). The use of this inoculum allowed application of a standardized amount of spores for plant inoculation.

Determining the optimal exogenous phosphate concentration was done in parallel to testing the effect of a closed pot system on AM symbiosis. In previous experiments, plants were grown in a open pot system. However, since *P. andersonii* can also engage in an endosymbiotic relation with nitrogen-fixing rhizobia bacteria, such open pot system is prone for cross contamination. As AMF and rhizobia activate the same signaling cascade (Oldroyd, 2013), avoiding rhizobium contamination is especially relevant when analyzing the effect of

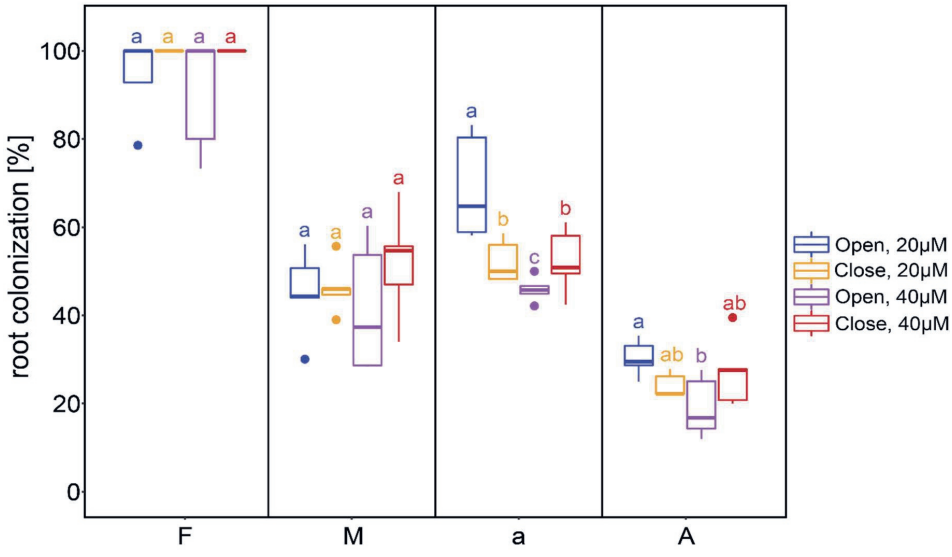

Figure 1: Mycorrhization efficiency of *P. andersonii* inoculated with 1000 spores of *R. irregularis* DAOM 197198 in an open or close pot system using either 20 or 40 µM of phosphate. Mycorrhization efficiency represented by; frequency of mycorrhiza in the root system (F%), the intensity of colonization in the root system (M%), the arbuscule abundance in the observed root segments (a%), and the arbuscule abundance in the root system (A%). Data shown represent five biological replicates. Different letters above the boxes indicate statistical significance ($p < 0.01$) as determined by the Kruskal-Wallis test in combination with Fisher's post-hoc test.

mycorrhization on transcriptional and metabolomic changes. To avoid contamination with rhizobia, we tested a closed pot system.

Mycorrhization efficiency was determined as previously described by Trouvelot and colleagues (Trouvelot *et al.*, 1986). In this method four parameters are quantified; (i) the frequency of mycorrhiza in the root system (F%), (ii) the intensity of mycorrhizal colonization in the root system (M%), (iii) arbuscule abundance in the observed root segments (a%), and

(iv) arbuscule abundance in the whole root system (A%). Two weeks old *P. andersonii* seedlings were transferred to either open or closed sterile 1 L pots. Each pot was filled with 800 gram of sterilized river sand supplemented with 70 mL ½ strength modified Hoagland solution containing either 20 µM or 40 µM K_2PO_4. Plants were inoculated with 1 mL of *R. irregularis* spores solution (1,000 spores·mL^{-1}). All roots were analyzed six weeks post-inoculation (WPI) **(Figure 1)**.

In this experiment, we found that *P. andersonii* plants were well mycorrhized under all four conditions. The frequency of mycorrhiza (F%) reached almost 100%, indicating that all 50 root segments were colonized by AMF. In addition, we observed that the intensity of mycorrhizal colonization (M%) varied between 40 and 50% without any statistical differences between the tested conditions. When analyzing the arbuscule abundance (a%), plants grown in the open system under 20 µM of phosphate had slightly more arbuscules compared to other conditions tested. Though, this effect was not observed when corrected for the total root system (A%) **(Figure 1)**. Combined, these result suggests (i) that closed pots can be used for *P. andersonii* mycorrhization experiments on and, (ii) that the use of 1,000 spores per plant leads to saturation in the mycorrhization efficiency. Since no clear difference in mycorrhization efficiency was observed between plants grown with either 20 or 40 µM exogenous Pi, we aimed to analyze plant performance to select a most optimal Pi condition for our future experiments.

Mycorrhization of *P. andersonii* effects plant growth

To determine the optimal exogenous phosphate concentration to conduct *P. andersonii* mycorrhization experiments, we analyzed the effect of phosphate levels -with and without AMF- on plant performance. Plants were inoculated with 1 mL of *R. irregularis* spores solution (1000 spores·mL-1), or 1 mL of sterilized water (mock) and grown for six weeks. Plant growth was analyzed by determining the number of true leaves, fresh shoot weight, and fresh root weight **(Figure 2)**. Neither the available phosphate nor the open or closed system had any effect on plant growth under mock treatment. However, as expected, mycorrhization had a clear positive effect on plant growth and development. Except for true leaves under 40 µM of phosphate in both systems, all measured parameters increased upon mycorrhization **(Figure 2I-K)**. When analyzing the plants grown under 40 µM phosphate in more detail, we observed that the total fresh shoot mass increased, without effecting the number of leaves

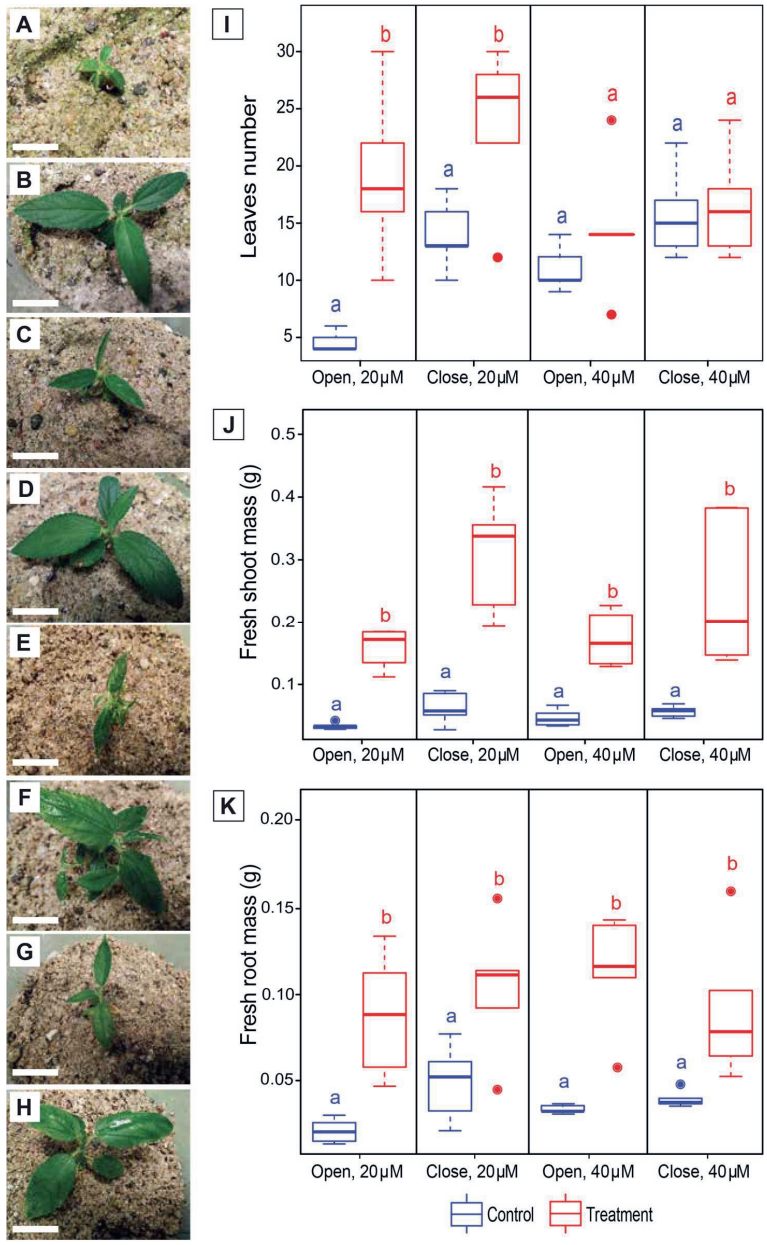

Figure 2: The effect of *R. irregularis* colonization on *P. andersonii* plant growth and development. **(A-H)** *P. andersonii* plants that were grown **(A)** uninoculated in an open pot system with 20 µM phosphate, **(B)** inoculated in an open pot system with 20 µM phosphate, **(C)** uninoculated in an open pot system with 40 µM phosphate, **(D)** inoculated in an open pot system with 40 µM phosphate, **(E)** uninoculated in a close pot system with 20 µM phosphate, **(F)** inoculated in a close pot system with 20 µM phosphate, **(G)** uninoculated in a close pot system with 40 µM phosphate, **(H)** inoculated in a close pot system with 40 µM phosphate. **(I)** Number of true leaves, **(J)** fresh shoot mass, and **(K)** fresh root mass 6 wpi. Data shown represent five biological replicates (n=5). Different letters above the boxes indicate statistical significance ($p < 0.05$) as determined by student t-test. Scale bar equal to 1 cm.

formed. This indicated that the overall size of these leaves must have increased substantially **(Figure 2I, K)**.

Similar to the observed mycorrhization efficiency, the closed pot system did not affect plant growth. As no clear differences could be observed in plant development between 20 µM and 40 µM phosphate application, we decided to use the 20 µM phosphate concentration in future experiments. Taken together, this means that the protocol for *P. andersonii* mycorrhization assays will be based on closed pot, holding 800 mL sterilized sand supplemented with 70 mL ½ strength Hoagland containing 20 µM of phosphate. However, the optimum number of *R. irregularis* spores still need to be determined.

Determining the *R. irregularis* spore number for *P. andersonii* mycorrhization

To determine the optimal spore concentration to use in the *P. andersonii* mycorrhization assay, we performed an experiment inoculating plants with 1 mL of 4 different concentrations

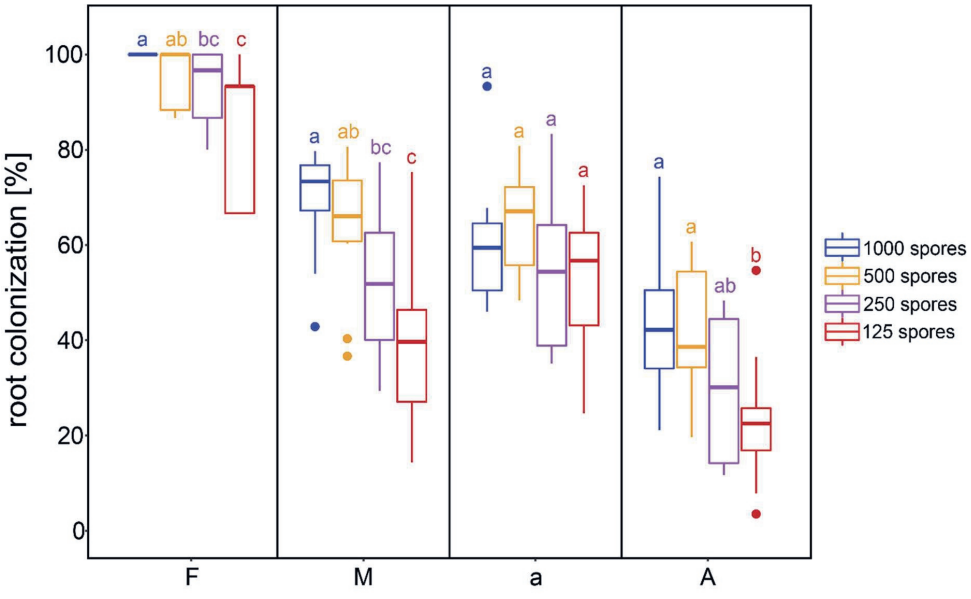

Figure 3: The effect of *R. irregularis* spore number on mycorrhization efficiency *P. andersonii* seedlings. Mycorrhization efficiency represented by; frequency of mycorrhiza in the root system (F%), the intensity of colonization in the root system (M%), the arbuscule abundance in the observed root segments (a%), and the arbuscule abundance in the root system (A%). Different letters above the boxes indicate statistical significance ($p < 0.01$) as determined by the Kruskal-Wallis test in combination with Fisher's post-hoc test. (Data shown represents ten biological replicates from two independent experiments (n=2x5), data from the individual experiment can be found in supplemental **Figures S1A and S1B**).

of spores (*i.e.* 1000, 500, 250, 125 spores·mL^{-1}). Inoculated seedling were grown for 6 weeks in a closed pot system. subsequently, the mycorrhization efficiency was determined as previously described **(Figure 3)**.

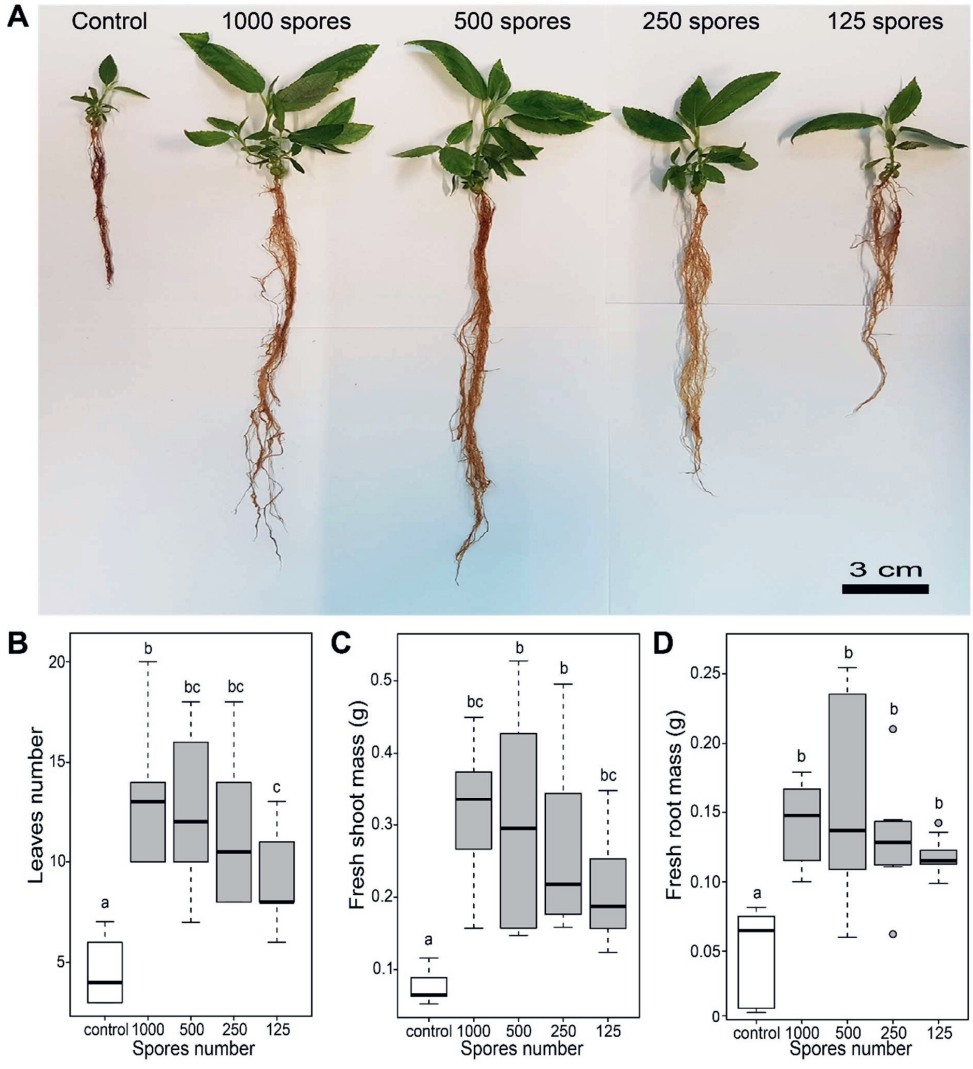

Figure 4: The effect of *R. irregularis* spore number on *P. andersonii* plant growth and development. Plants inoculated with mock, 1000, 500, 250 or 125 *R. irregularis* spores. Shown are; **(A)** Photograph of 6-weeks-old *P. andersonii* plants **(B)** number of true leaves, **(C)** fresh shoot mass, **(D)** fresh root mass. Different letters above the boxes indicate statistical significance ($p < 0.05$) as determined by ANOVA in combination with Tukey post-hoc test. (Data shown represents ten biological replicates from two independent experiments (n=2x5), data from the individual experiment can be found in supplemental **Figures S2A and S2B**).

Similar to our previous results, the frequency of mycorrhiza (F%) with 1000 spores per plant was saturated. In addition, F% on plants inoculated with 500 spores was also saturated, however, with this number of spores we could observe variation between replicates. When spore numbers was lowered even further (*i.e.,* 250 and 125 spores) F% was reduced by 4% and 10%, respectively. A similar effect could be observed for the intensity of mycorrhizal colonization in the roots system (M%), where M% did not significantly differ between 1000 and 500 spores. This parameter was clearly lower when 250 and 125 spores were used. Interestingly, we did not observe any significant differences in the arbuscule abundance in observed root segments (a%) when using either 250 or 125 spores. However, when normalized for the root system (A%), the spore dilution effect in arbuscule abundance in the root system (A%) was observed.

Next, we investigated whether reduced spore numbers effects AMF stimulated plant growth promotion, as described for 1000 spores **(Figure 2)**. Again we quantified the number of true leaves, the fresh shoot weight, and the fresh root weight **(Figure 4)**. As seen previously when used 1000 spores, all plants inoculated with AMF performed significantly better compared to mock treatment **(Figure 4)**. With the exception of the number of true leaves **(Figure 4B)**, no differences between plant performance under different spore numbers were observed **(Figure 4B-D)**. This demonstrates that *P. andersonii* benefits strongly from AMF even when spore numbers are relatively low.

Taking together, our results indicate that to test mycorrhization efficiency in *P. andersonii* seedlings, an inoculum containing between 250 or 125 spores per mL solution will be most suitable. As the risk of saturating the mycorrhization efficiency remains at higher spore numbers, we selected to use 125 spores for our future assays.

Mycorrhization assay using *P. andersonii* from tissue culture propagation

P. andersonii are fast growing tropical trees, wind pollinated, and can be monoecious or diecious (Soepadmo, E., 1974). This complicates the production of homozygote seeds. For this reason, we set up a vegetative *in vitro* maintenance system of transgenic lines (van Zeijl *et al.*, 2018). As a result, no transgenic seeds will be available, and mycorrhization assays on mutants and RNAi lines will have to be conducted on tissue culture material.

In general, seedlings display some different characteristics compared to explants propagated through tissue culture. This holds especially true for its root system architecture. In seedling plants, the root consists of one main root that gives rise to several lateral roots. In contrast,

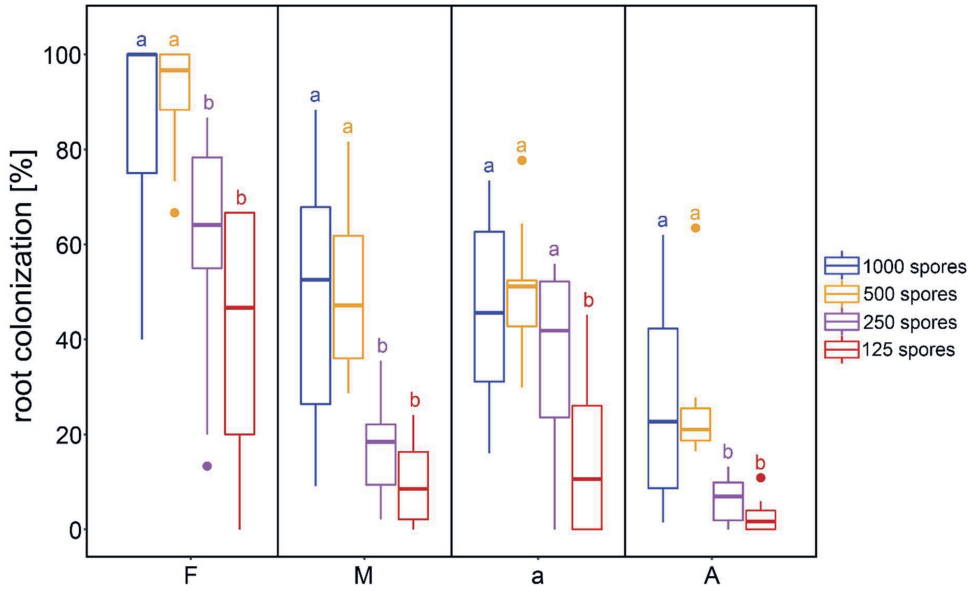

Figure 5: The effect of different number of *R. irregularis* spores on mycorrhization efficiency of *P. andersonii* explants. Mycorrhization efficiency represented by; frequency of mycorrhiza in the root system (F%), the intensity of colonization in the root system (M%), the arbuscule abundance in the observed root segments (a%), and the arbuscule abundance in the root system (A%). Different letters above the boxes indicate statistical significance ($p < 0.01$) as determined by the Kruskal-Wallis test in combination with Fisher's post-hoc test. (Data shown represents ten biological replicates from two independent experiments (n=2x5), data from the individual experiment can be found in supplemental **Figures S3A and S3B**).

the root system of tissue culture explants consists of several roots that originate from the cutting edge were the plantlet was separated from the *in vitro* culture. Based on these differences, we cannot rule out the mycorrhization assay optimized for seedlings, it needs additional optimization when vegetative propagated plantlets are used. To test this, vegetative propagated plantlets were selected that were similar in size. However, we observed that after rooting, the number of leaves and roots of these plantlets were variable prior AMF inoculation. Therefore, we focused only on quantifying the mycorrhization efficiency and did not investigate any growth parameters during this experiment. We postulate that the increased root mass of vegetative propagated plantlets when compared to seedlings at the start of the experiment might require a different number of *R. irregularis*

spores. To test this, we grew explants in the closed pot system with 20 µM of phosphate, inoculated with either mock, 1000, 500, 250, or 125 spores. Plants were harvested six weeks post-inoculation, and mycorrhization efficiency was characterized **(Figure 5)**. The frequency of mycorrhiza in the root system (F%) in plants inoculated with 1000 spores was again saturated. However, we observed high variability within replicates even at this number of spores. This variability was caused by the fact that in one of the two trials, some root segments were poorly colonized. This affected all observed parameters. As expected, the level of F% reduced along with the reduction in the number of spores added to the plantlets. When inoculated with 125 spores, we observed that in one of the trials, the arbuscule abundance in the root system was almost zero **(Figure S3)**. In addition, plantlets inoculated with either 250 or 125 spores did not significantly differ in any of the parameters except for the arbuscule abundance in the observed root segments (a%). We concluded that if we were to use 125 spores during mycorrhization assays on vegetative propagated plantlets, we run the risk that the experiment may fail because of low number of arbuscules in control plantlets. Therefore, we decided to use 250 spores during our mycorrhization assays on *P. andersonii* propagated plantlets.

Discussion

The interaction between AMF and host plants is difficult to quantify. This is because not only does a successful interaction involve many factors from both plant and fungal partners, it also depends heavily on environmental conditions. To study mycorrhization on *P. andersonii*, we developed a standardized quantitative mycorrhization assay with an optimized level of phosphate and number of spores.

P. andersonii mycorrhization requires low phosphate

Until now, mycorrhization assays on *P. andersonii* relied on methods optimized for other plant models, such as rice and *M. truncatula*. Phosphate availability is a crucial factor in mycorrhiza symbiosis (Balzergue *et al.*, 2011). The inhibitory effect of high phosphate on AM symbiosis is considered a general phenomenon. As a result, most plant species only will interact with symbiotic AMF at relatively low exogenous phosphate levels. But the precise concentrations that is inhibiting may vary between host plant species (Carbonnel & Gutjahr, 2014; Kobae *et*

al., 2016). For example, in rice, the optimal level of phosphate for mycorrhization has been reported to lay between 5 to 25 µM Pi (Paszkowski *et al.*, 2002), whereas 20 µM has been determined to be the optimal concentration for *M. truncatula* mycorrhization (Branscheid *et al.*, 2010; Zeng *et al.*, 2018).

Here, we demonstrate that *P. andersonii* can be well mycorrhized in a range of 20 to 40 µM Pi. This suggests that, although *P. andersonii* has different physiological characteristics compared to rice and *M. truncatula*, low phosphate availability in this species also plays a pivotal role in the establishment of the AM symbiosis. In *M. truncatula*, relatively high levels of Pi are needed to suppress the AM symbiosis (Balzergue *et al.*, 2013), as 1,000 and 1,300 µM Pi had only moderate effects on root colonization (Branscheid *et al.*, 2010; Bonneau *et al.*, 2013). Moreover, 750 µM Pi is sufficient to abolish the AM symbiosis almost completely in pea (Balzergue *et al.*, 2011), and a similar effect is obtained when Petunia is grown using 10,000 µM Pi (Breuillin *et al.*, 2010). AM symbiosis in rice seems to be more sensitive to elevated Pi concentrations, as mycorrhization is already strongly reduced at 500 µM Pi (Paszkowski *et al.*, 2002). In the case of *P. andersonii*, currently no data at which phosphate concentration, AM symbiosis will be totally abolished.

A closed growth system is suitable for mycorrhization assays

As *P. andersonii* can also be nodulated, we need a growth system where plants can be mycorrhized efficiently without possible rhizobia contamination. On the other hand, the growth system should also not have a negative effect on plant growth. So far, there are no reports on the effect of nodulation on the efficiency of mycorrhization in *P. andersonii*, or vice versa. However, as the signalling cascade leading to the establishment of both symbioses are interwoven, cross contamination is not desirable. In the closed pot system, nodules on mycorrhized or non-mycorrhized *P. andersonii* control plants were not observed, demonstrating that this system prevents rhizobium contamination. Also, we observed no differences in the mycorrhization efficiency between the open or closed pot system. Our data also showed that the use of a closed pot system has no negative effect on *P. andersonii* growth. Therefore, we conclude that we best use the closed system for *P. andersonii* mycorrhization assays.

***P. andersonii* benefits from AM symbiosis**

Plant growth responses as a result of mycorrhizal association can range from highly positive to extremely negative. This is because the interaction between plant and fungus can vary from what could be considered parasitism to neutral, or a full symbiotic mutualism. The environmental conditions, especially the abundance of soil nutrients, determine the position of outcome of the AM association (Johnson *et al.*, 1997). Based on the analysis of inter-/intracellular AM structures and plant growth parameters, we demonstrated that *P. andersonii* and *R. irregularis* are compatible resulting in a mutualistic endosymbiosis.

***R. irregularis* spores are a good AMF source to use in *P. andersonii* mycorrhization**

It has been reported that inoculation with spores, often regarded as less virulent, results in a relatively slow progression of the AMF infection (David-Schwartz *et al.*, 2001; Kumar *et al.*, 2020). In this study, we tested a *R. irregularis* spores suspension of the reference strain DAOM 197198 and demonstrated that inoculation with 1000 spores results in saturated mycorrhization levels 6 wpi. This indicates that the DAOM 197198 spores suspension is a good inoculum source. The differences between our results and the mycorrhiza colonization efficiency reported before, could be explained by either the plant species used, method of spore application and/or the experimental condition. For example, it is common practice to mix two-thirds of spores with the substrate and apply the additional one-third close to the seedling. In our set-up, all spores are added directly to the plant root. Thereby, possibly creating a condition where all spores can interact directly with the root after germination and start the infection process without delay.

The saturated mycorrhization levels demonstrate that the system is working well. However, a saturated assay could potentially mask any quantitative differences in mycorrhization efficiency between mutants and/or treatments. The precise phenotyping of AMF interactions remains difficult when it comes to limited differences between samples. It was previously reported that high spore density can mask minor differences in mycorrhizal efficiency (Delaux *et al.*, 2013). One advantage of using spore inoculum is the ability to precisely quantify the density of spores added in the assay, and we were able to avoid saturation by reducing the number of spores by controlled dilution steps.

As expected, reducing the number of spores positively correlated with a reduction in the level of mycorrhiza efficiency parameters (M%, a%, and A%). Moreover, a reduced number of spores also highlighted phenotypical differences more clearly between treatments. For example, spores number optimization revealed a quantitative difference between the spores needed for optimal mycorrhization of tissue culture explants and seedlings. The tissue culture explants require a double amount of spores compared to the seedlings. We noticed that at six weeks post-inoculation, the root system on tissue culture plantlets were also larger compared to the seedling plants. In tissue culture plantlets, lateral roots are formed from many crown roots. It is therefore, likely these explants create a denser root system, compared to the seedling plants in which lateral roots only emerged from one primary root. It cannot be excluded that a difference in root architecture and root volume already exists at the start of the experiment and that such denser root system on tissue culture plantlets requires a higher starting spore number to be mycorrhized effectively. It is reported that AMF prefer to colonize lateral roots over primary root or crown roots. It was suggested this is due to a higher sturdiness and lignin content in roots with anchoring function (Hepper, 1985; Amijee *et al.*, 1993; Paszkowski & Gutjahr, 2013). This would suggest that more lateral roots could lead to higher mycorrhization potential, possibly further explaining why in our assays tissue culture explants systematically require higher spore numbers.

Taken together, through a series of optimizations, we provide a robust quantitative mycorrhization assay for *P. andersonii*. This protocol can be used on seedlings as well as tissue culture plantlets, and it will allow us to study the role of specific genes during *P. andersonii* mycorrhiza symbiosis, both qualitative and quantitative. In addition, these protocols can also be used to study related species (e.g., *Parasponia rigida* or *Trema orientalis*). Moreover, the assay presented here, could serve as a reference to develop similar assays for other woody species.

Materials and Methods

Plant materials and growth conditions

All experiments were performed using *P.andersonii* WU1. *Parasponia* trees are grown in a greenhouse at 28ºC, 85% humidity, and a 16/8 h day/night (Velzen *et al.*, 2018; Wardhani *et al.*, 2019). *Parasponia* seedlings and *Parasponia* in vitro tissue culture were obtained and maintained according to Op den Camp et al. 2011 (Camp *et al.*, 2011; van Zeijl *et al.*, 2018; Wardhani *et al.*, 2019). *Parasponia* seedlings and rooted tissue culture plants for mycorrhization assay were transferred to sterile polypropylene containers 1L, fitted with a gas exchange lid (OS140BOX, Duchefa Biochemie, Netherlands). Pots were filled with 800 g of sterilized steamed river sand and supplemented with 70 mL of ½ strength modified Hoagland medium (Wardhani *et al.*, 2019) containing either 20 µM or 40 µM potassium phosphate. Sand and medium were mixed directly by vigorously shake. In the Open pot system, plants were watered with sterilized demineralize water every two weeks.

Spores inoculum preparation

Mycorrhization assays were performed using a commercial spore of *Rhizopagus irregularis* (Agronutrion-DAOM197198). Spore inoculum was prepared by pipetting the required amount of spore suspension onto the stack of polyester filters (top to bottom: 210 µm, 120 µm, and 36 µm mesh size). Filters were rinsed 3x with 100 mL of autoclaved demineralized water. Spores retained in the 36 µm filter were rinsed with autoclaved demineralized water for at least 6x. Spores were resuspended with autoclaved demineralized water at the same volume of initial spore suspension that being used then collected in a sterile tube. Spores then were counted under a bright-field microscope and converted into a ratio of spores/mL. The spore suspension was diluted until they reached the required number of spores. The spore suspension was stored at 4ºC before used.

Spores inoculation

2 weeks old of *Parasponia* seedlings growing on Schenk and Hildebrandt medium (Duchefa) without sucrose or 3-4 weeks old of rooted *Parasponia* planlets growing in rooting medium

were taken out. One *Parasponia* seedling or planlet was placed in each pot. 1 mL spore suspension were pipetted directly onto the plant root. After 6 weeks, plants were harvested.

Plants Harvest

6 weeks *Parasponia* plants were taken out from the pots, and roots were washed with running water to remove as much sand as possible. Shoots and roots were separated then dried with tissue paper. After shoots and roots were relatively dry, those organs were weighed to quantify the fresh biomass.

Trypan Blue staining

Roots were cut into 1 cm long pieces and boiled in 10% KOH (w/v) for 20 min at 90^{o}C. Boiled roots were then placed on a cell strainer with 100 µm mesh size and rinsed 3x with 50 mL of water. Roots were stained with 0.05% (w/v) trypan blue in lactoglycerol (300 mL of lactic acid; 300 mL of glycerol; and 400 mL of demineralized water) for 5 min at 90^{o}C in a water bath or heating block. Stained roots were transferred to 30% glycerol, and root samples can be stored at room temperature.

Quantification of mycorrhization

25-50 root fragments were placed on a single microscope slide. 30% glycerol was added, and roots were covered with a cover glass and pressed until root fragments became flat. Mycorrhization efficiency was examined according to Trouvelot et al. (Trouvelot *et al.*, 1986) using a bright-field microscope. The mycorrhization efficiency was quantified based on four classes (%F, %M, a% and %A), which allows rapid estimation of the frequency of mycorrhiza, the intensity of mycorrhizal colonization, and arbuscules abundance in the root system.

Statistical analysis

Graphs and statistical analysis were performed using R studio 1.1.456. Ramf R package (Chiapello *et al.*, 2019) were used to analyze and display of quantitative AM fungal root colonization data. Statistical tests on four classes of mycorrhization efficiency was done using Kruskal-Wallis test in combination with the post-hoc test using the criterion of Fisher's least

significant difference. Statistical significance was defined as a $p<0.01$. Statistical test on plant growth parameters was done using One Way Analysis of variance (ANOVA) and a Tukey post-hoc test for multiple comparisons. Statistical significance for these parameters was defined as a $p<0.05$.

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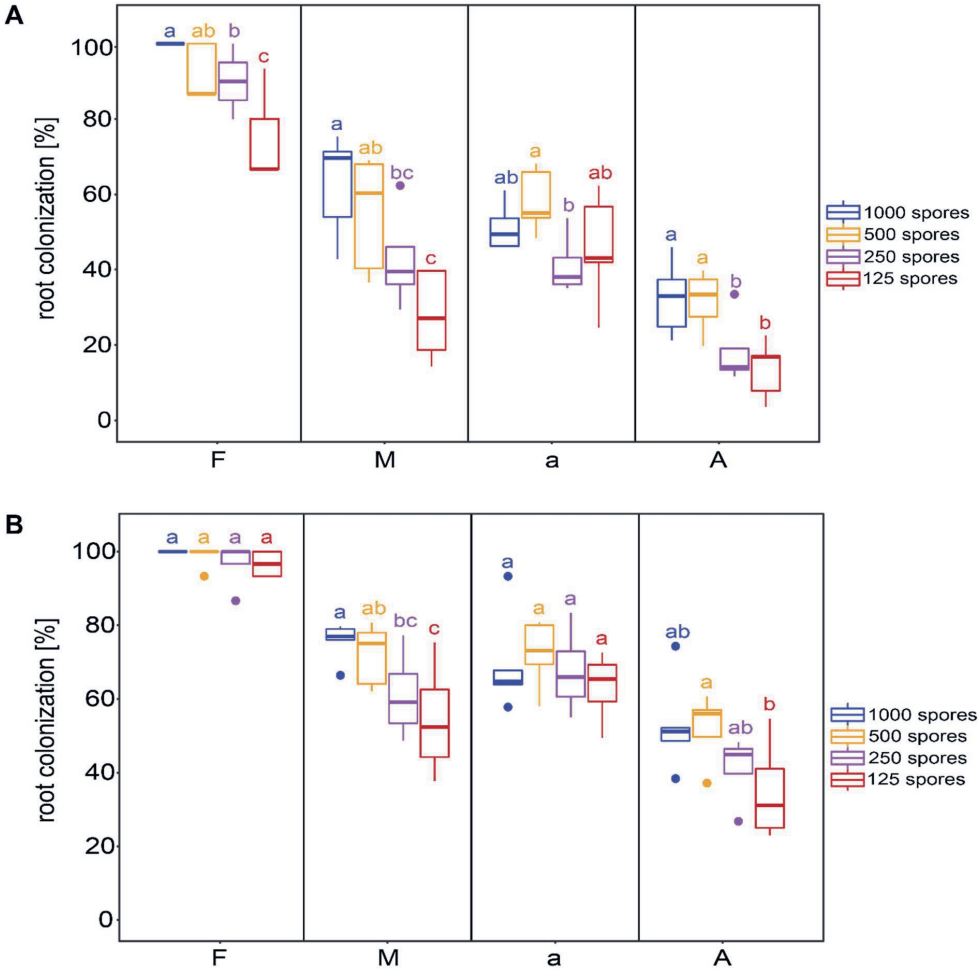
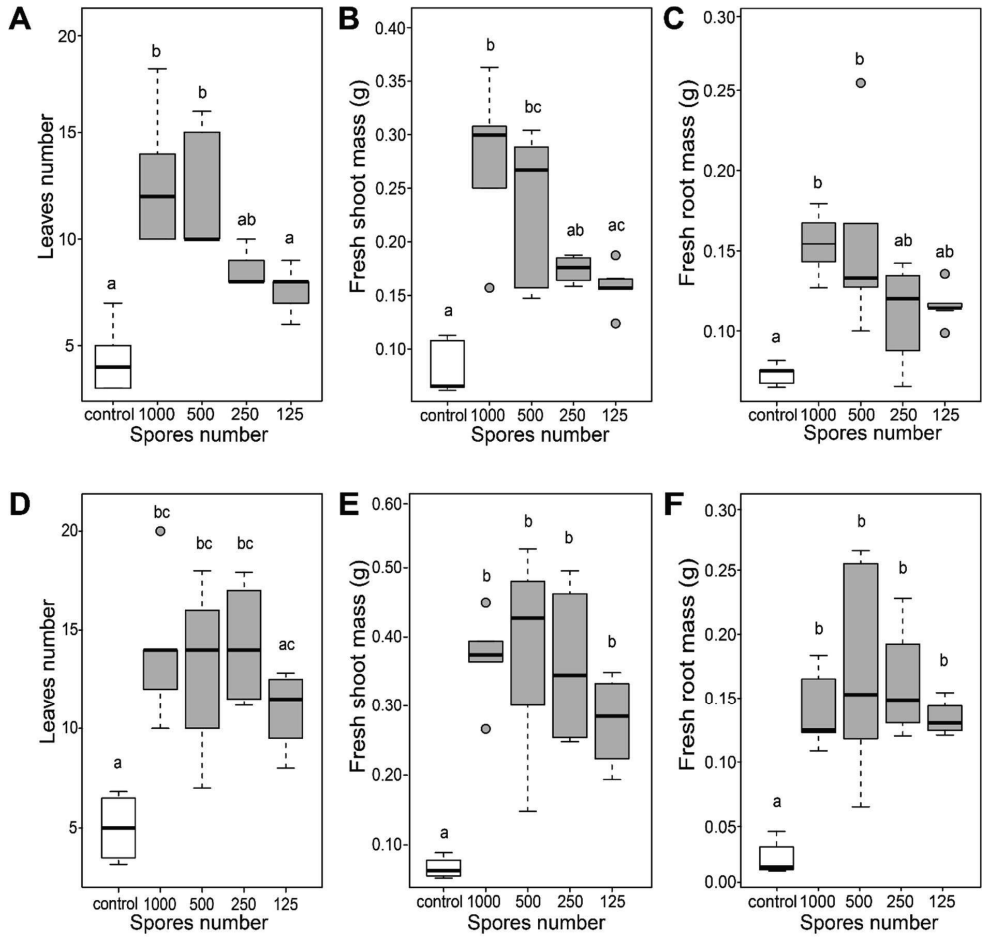
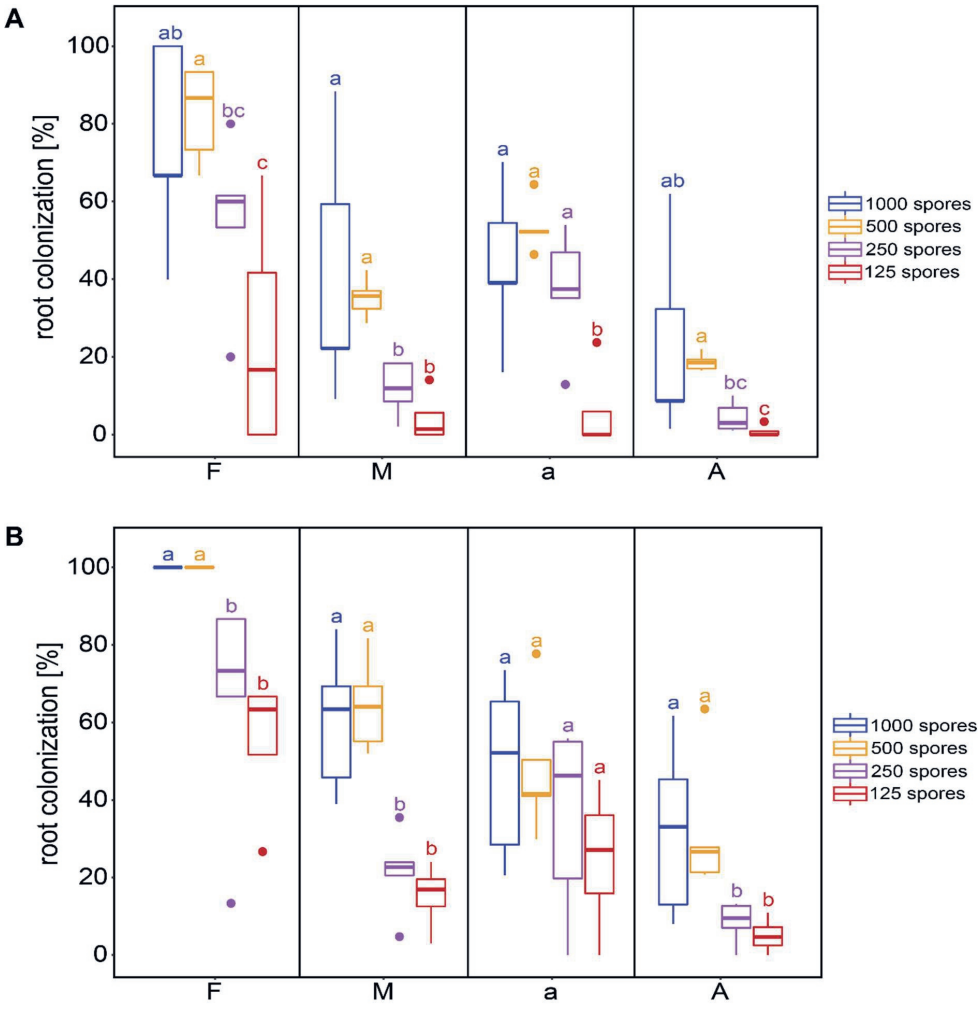
Supplemental data



Supplemental Figure S1: The effect of different number of *R. irregularis* spores on mycorrhization efficiency of *P. andersonii* plants from seeds. Mycorrhization efficiency represented by; frequency of mycorrhiza in the root system (F%), the intensity of colonization in the root system (M%), the arbuscule abundance in the observed root segments (a%), and the arbuscule abundance in the root system (A%). Data shown represent five biological replicates per experiment, from two independent experiments. **(A)** from the first experiment (n=5) and **(B)** from the second experiment (n=5). Different letters above the boxes indicate statistical significance ($p < 0.01$) as determined by the Kruskal-Wallis test in combination with Fisher's post-hoc test.



Supplemental Figure S2: The effect of different number of *R. irregularis* spores on *P. andersonii* plant growth and development. Data shown represent five biological replicates per experiment from two independent experiments. The effect of different number of spores on true leaves number, fresh shoot mass, and fresh root mass **(A-C)** from the first experiment (n=5), and **(D-F)** from the second experiment (n=5). Different letters above the boxes indicate statistical significance ($p < 0.05$) as determined by ANOVA in combination with Tukey post-hoc test.



Supplemental Figure S3: The effect of different number of *R. irregularis* spores on mycorrhization efficiency of *P. andersonii* explants. Mycorrhization efficiency represented by; frequency of mycorrhiza in the root system (F%), the intensity of colonization in the root system (M%), the arbuscule abundance in the observed root segments (a%), and the arbuscule abundance in the root system (A%). Data shown represent five biological replicates per experiment. **(A)** from the first experiment (n=5) and **(B)** from the second experiment (n=5). Different letters above the boxes indicate statistical significance ($p < 0.01$) as determined by the Kruskal-Wallis test in combination with Fisher's post-hoc test.

CHAPTER 3

3

Duplication of symbiotic Lysin Motif-receptors predates the evolution of nitrogen-fixing nodule symbiosis

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Abstract

Rhizobium nitrogen-fixing root nodule symbiosis occurs in two taxonomic plant lineages: legumes (Fabaceae) and *Parasponia* (Cannabaceae). Both symbioses are initiated upon the perception of rhizobium-secreted lipo-chitooligosaccharide (LCOs), called Nod factors. Studies in the model legumes *Lotus japonicus* (lotus) and *Medicago truncatula* (medicago) showed that rhizobium LCOs are perceived by a heteromeric receptor complex of distinct Lysin Mptif (LysM)- type transmembrane receptors named NOD FACTOR RECEPTOR1 (LjNFR1)-LjNFR5 (lotus) and LYSM DOMAIN CONTAINING RECEPTOR KINASE3 (MtLYK3)-NOD FACTOR PERCEPTION (MtNFP) (medicago). Recent phylogenomic comparative analyses indicated that the nodulation trait of legumes, *Parasponia* as well as of so-called actinorhizal plants that establish a symbiosis with diazotrophic *Frankia* bacteria, share an evolutionary origin about 110 million years ago. However, the evolutionary trajectory of LysM-type LCO receptors remains elusive. By conducting phylogenetic analysis, trans-complementation studies, and CRISPR-Cas9 mutagenesis in *Parasponia andersonii* (*Parasponia*), we obtained insight of LCO receptors essential for nodulation. We identified four LysM-type receptors controlling nodulation in *P. andersonii*: PanLYK1, PanLYK3, PanNFP1 and PanNFP2. These genes evolved upon ancient duplication events predating and coinciding with the origin of nodulation. Phylogenetic and functional analysis associated the occurrence of a functional NFP2-orthologous receptor to LCO-driven nodulation. Legumes and *Parasponia* use orthologous LysM-type receptors to perceive rhizobium LCOs, suggesting a shared evolutionary origin of LCO-driven nodulation. Furthermore, we found that both PanLYK1 and PanLYK3 are essential for intracellular arbuscule formation of mutualistic endomycorrhizal fungi. PanLYK3 also acts as a chitin oligomer receptor essential for innate immune signalling, demonstrating functional analogy to CHITIN ELECITOR RECEPTOR KINASE (CERK)-type receptors.

Introduction

Nitrogen availability is a critical factor for plant growth, but fixed nitrogen in the form of nitrate or ammonia in soils is limited. Plants have acquired different strategies to overcome this limitation. One such strategy is establishing a nodule endosymbiosis with nitrogen-fixing *Frankia* or rhizobium bacteria. Inside nodules, physiological conditions are created that allow the bacteria to convert atmospheric dinitrogen (N_2) into ammonia that can be used by the plant. Carbohydrates of plant origin fuel this energy demanding process. The unique character of nitrogen-fixing nodule symbiosis has raised the interest of plant researchers for more than a century, ultimately aiming to transfer this trait to non-leguminous crop species (Burrill and Hansen, 1917; Rogers and Oldroyd, 2014; Huisman and Geurts, 2019).

The *Frankia* and rhizobium nitrogen-fixing nodulation trait occurs in ten paraphyletic lineages within the orders Fabales, Fagales, Cucurbitales and Rosales, collectively known as the nitrogen-fixing clade (Soltis et al., 1995). Based on phylogenomic comparisons of nodulating and non-nodulating plant species it is hypothesized that the nitrogen-fixing nodule symbiosis with rhizobium or *Frankia* bacteria has a shared evolutionary origin, dating to about 110 million years ago (Griesmann et al., 2018; van Velzen et al., 2018a; van Velzen et al., 2018b). Subsequently, the nodulation trait most probably was lost multiple times, which is associated with pseudogenization of two key genes essential for nodule organogenesis and bacterial infection; the transcription factor *NODULE INCEPTION* (*NIN*) and the coiled-coil protein-encoding gene *RHIZOBIUM POLAR GROWTH* (*RPG*) (Griesmann et al., 2018; van Velzen et al., 2018b). These two genes likely experienced genetic adaptations, allowing them to function exclusively in nodulation. However, insight in the evolutionary trajectory of signalling receptors involved in recognition of bacterial signals and subsequent activation of the pathways leading to nodule organogenesis and bacterial infection remains elusive.

The nitrogen-fixing nodulation trait is best studied in the legume models *Lotus japonicus* (lotus) and *Medicago truncatula* (medicago) (Fabaceae, Fabales). Both these legumes recognize their rhizobium microsymbionts by the structural characteristics of secreted lipo-chitooligosaccharides (LCOs, also known as Nod factors). Perception of these molecules triggers nodule development (Wang et al., 2012). LCO signalling is also the basis of rhizobium-induced nodulation in the non-legume *Parasponia* (Cannabaceae, Rosales) (Marvel et al.,

1987; Op den Camp et al., 2011; van Velzen et al., 2018b). Additionally, it was found that diazotrophic *Frankia* strains of a basal taxonomic lineage (so-called cluster-II strains) possess LCO biosynthesis genes, but the nodulating strains of two other taxonomic clusters do not (Pawlowski and Demchenko, 2012; Persson et al., 2015; Nguyen et al., 2016; Van Nguyen et al., 2019). LCOs, as well as chitin oligomers (COs), are also used by arbuscular mycorrhiza (AM) fungi to signal their hosts (Maillet et al., 2011; Genre et al., 2013). Perception of these AM signals requires a plant LysM-type receptor that also is essential for chitin innate immune signalling; e.g. OsCERK1 in rice (*Oryza sativa*) (Miyata et al., 2014; Zhang et al., 2015, He et al., 2019). This suggests that nodulating bacteria co-opted LCO signalling from the widespread AM symbiosis and/or innate immune signalling (Parniske, 2008; Gough and Cullimore, 2011; Geurts et al., 2012).

Genetic and biochemical studies in lotus and medicago demonstrated that rhizobium LCOs are perceived specifically by a heteromeric complex containing two distinct LysM-type receptors, named NOD FACTOR RECEPTOR1 (LjNFR1) and LjNFR5 in lotus, and LYSM DOMAIN CONTAINING RECEPTOR KINASE3 (MtLYK3) and NOD FACTOR PERCEPTION (MtNFP) in medicago (Limpens et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003; Arrighi et al., 2006; Radutoiu et al., 2007; Broghammer et al., 2012). Other receptors may modulate the LCO response, such as LjNFRe, a homolog of LjNFR1 in lotus (Murakami et al., 2018). The LysM-type receptor family can be divided into two subclasses; named LYK and LYR, characterized by having a functional or dead kinase domain (Arrighi et al., 2006). Together these make up 11 orthogroups, two of which include legume LCO receptors (Buendia et al., 2018). Within legumes, the orthogroup that includes LjNFR1/MtLYK3 (named LYK-I clade) expanded upon gene duplications, allowing functional separation of rhizobium-induced signalling, AM symbiosis and chitin-triggered innate immune responses (De Mita et al., 2014; Bozsoki et al., 2017; Buendia et al., 2018; Gibelin-Viala et al., 2019). Likewise, the *LjNFR5*/*MtNFP* (orthogroup LYR-IA) experienced a gene duplication early in the legume clade (Young et al., 2011; Buendia et al., 2018).

Data on symbiotic LysM-type receptors in nodulating non-legumes are scarce. Only in *Parasponia andersonii* (*Parasponia*) has a receptor functioning in nodulation been identified; named *PanNFP1*, which is a close homolog of *LjNFR5/MtNFP* (Op den Camp et al., 2011). Besides *PanNFP1*, *Parasponia* species possess a homologous receptor, named *NFP2*, which is

more closely related to *LjNFR5/MtNFP* and transcriptionally activated in root nodules. Interestingly, this receptor is pseudogenized in non-nodulating Rosales species (van Velzen et al., 2018b). To obtain insight into the evolution of LysM-type LCO receptors that are essential for nodulation, we used *Parasponia* as a comparative system to legumes. The genus *Parasponia* represents five tropical tree species, which form nitrogen-fixing nodules with LCO producing rhizobium species that also nodulate legumes (van Velzen et al., 2018b). *Parasponia* and legumes diverged at the root of the nitrogen-fixing clade >100 million years ago (Li et al., 2015; van Velzen et al., 2018a). The microbial symbionts of the ancestral nodulating plants remain elusive, and it is probable that *Parasponia* and legumes accepted rhizobium as a microbial partner in parallel (van Velzen et al., 2018a). In line with this, *Parasponia* provides a unique comparative system to obtain insight in evolutionary trajectories of different LCO receptors that are essential for nodulation.

Results

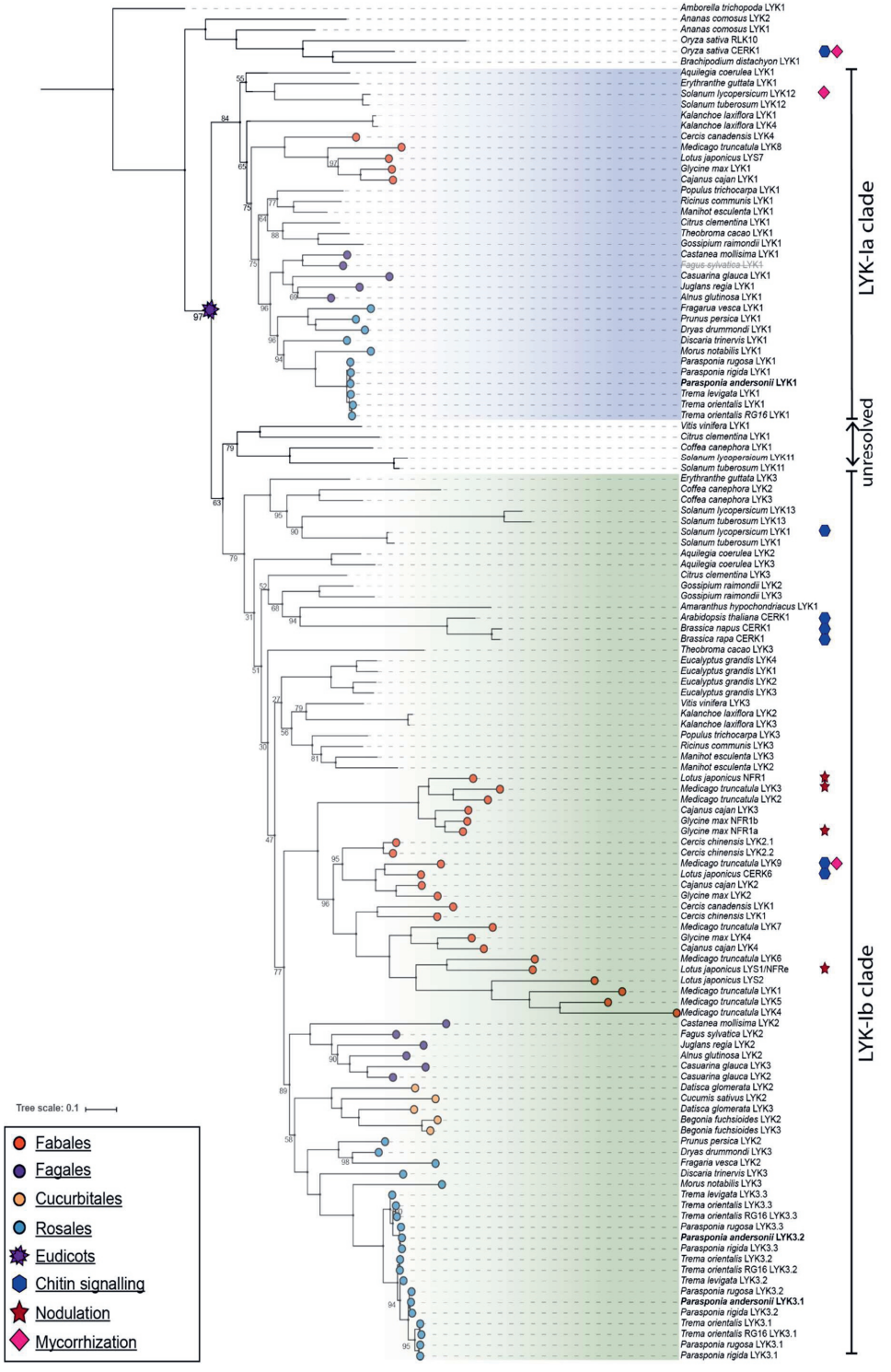
Phylogeny reconstruction of orthogroups representing LysM-type LCO receptors

To obtain insight into the LysM-type receptor family of *Parasponia*, we analysed it phylogenetically. We identified 16 *Parasponia* genes encoding putative LysM-type receptors that grouped in all known orthogroups except one **(Figure S1; Table S1)**. Genetic studies in legumes uncovered only two orthogroups that contain proteins with a known function in rhizobium LCO signalling; which are named LYK-I and LYR-IA, respectively (Buendia et al., 2018). *P. andersonii* has two gene copies in both these orthogroups.

LYK-I is the largest orthogroup, containing the functional legume LCO receptors *MtLYK3/LjNFR1* and *LjNFR*e (Limpens et al., 2003; Radutoiu et al., 2003; Murakami et al., 2018). Besides these, the LYK-I orthogroup includes also chitin innate immune receptors of medicago *MtLYK9/MtCERK1,* lotus *LjCERK6*, arabidopsis (*Arabidopsis thaliana*) *AtCERK1*, tomato (*Solanum lycopersicum*) *SlLYK1* and rice *OsCERK1* (Limpens et al., 2003; Miya et al., 2007; Wan et al., 2008; Shimizu et al., 2010; Miyata et al., 2014; Zhang et al., 2015; Bozsoki et al., 2017; Carotenuto et al., 2017; Liao et al., 2018; Gibelin-Viala et al., 2019; He et al., 2019). OsCERK1 and MtLYK9/MtCERK1 have also been found to function also in AM symbiosis (Miyata et al., 2014; Zhang et al., 2015; Feng et al., 2019; Gibelin-Viala et al., 2019). Two *Parasponia* genes are part of this orthogroup; named *PanLYK1* and *PanLYK3*.

A more exhaustive phylogenetic reconstruction was conducted using gene orthologs of additional species, to obtain insight in the evolutionary relationships of these genes when compared to LCO and CO receptors, Notably, LysM-type receptors of the recently sequenced nodulating actinorhizal plants and non-nodulating relatives were included (Griesmann et al., 2018). The resulting phylogeny largely resembled the Rosid species trees as was reconstructed on the basis of plastid-coding genes (Wang et al., 2009; Gonçalves et al., 2019). Our analysis revealed that *PanLYK1* and *PanLYK3* originated from an ancient duplication, dividing this orthogroup in two subgroups that we named LYK-Ia and LYK-Ib. This duplication does not coincide with the birth of the nitrogen-fixing clade, but rather has occurred in an ancestral eudicot **(Figure 1; Data set S1)**. The only studied member in the LYK-Ia orthogroup is tomato *SlLYK12*, and knockdown of this gene by virus-induced gene silencing (VIGS) significantly reduces mycorrhizal colonization (Liao et al., 2018). The LYK-Ib clade represents several functionally characterized genes, including the chitin innate immune receptors and legume rhizobium LCO receptors. Legumes exhibit an increased number of genes in the LYK-Ib subclade, which are the result of tandem duplications (Limpens et al., 2003; Radutoiu et al., 2003; Zhu et al., 2006). These duplications may have driven neofunctionalization of LCO receptors in legumes (De Mita et al., 2014). In *Parasponia*, no gene duplications have occurred in the LYK-Ib clade (represented by *PanLYK3*) nor the LYK-Ia clade (represented by *PanLYK1*). In contrast, *P. andersonii PanLYK3* experienced a duplication exclusively of the first exon. To determine whether this duplication is specific for the *Parasponia* genus we analysed the *LYK3* genomic region of two additional *Parasponia* and three non-nodulating species of the closely related genus *Trema*. This revealed that the duplication of *LYK3* exon1 is present in all species investigated and occurred twice, where the most distal exon 1 copy was lost in *P. andersonii* **(Figure 2A, Figure S2A)**. The encoded pre-mRNAs both splice into a shared second exon **(Figure 2)**. Each exon1 copy contains a putative transcriptional and translation start site, which allows for differential expression of the variants **(Figure 2B-C)**. Genes of the LYK-I clade

Figure 1. Phylogeny reconstruction the LYK-I orthogroup, containing known CO and LCO receptors, based on 127 sequences from 47 species. Two main subgroups are recognized in Eudicots, LYK-Ia (blue) and LYK-Ib (green). Note the presence of both variants in *Aquilegia coeralia*, a basal Eudicot in the Ranunculales. A subset of proteins is unresolved. *Parasponia* proteins are in bold. *Parasponia* and *Trema* LYK3.1 and LYK3.2 represent protein variants of LYK3. Deduced pseudo-proteins are depicted in grey/strikethrough. Proteins with known functions in nodulation, mycorrhization, and/or chitin-innate immune signalling are indicated. Bootstrap values indicate IQ-tree UF-bootstrap support%, values >98 are not shown. Tree scale bar represents substitutions per site. A complete list of species and accession numbers can be found in **Data set S1**.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Ananas comosus LYK2
Ananas comosus LYK1
Oryza sativa RLK10
Oryza sativa CERK1
Brachipodium distachyon LYK1
Aquilegia coerulea LYK1
Erythranthe guttata LYK1
Solanum lycopersicum LYK12
Solanum tuberosum LYK12
Kalanchoe laxiflora LYK1
Kalanchoe laxiflora LYK4
Cercis canadensis LYK4
Medicago truncatula LYK8
Lotus japonicus LYS7
Glycine max LYK1
Cajanus cajan LYK1
Populus trichocarpa LYK1
Ricinus communis LYK1
Manihot esculenta LYK1
Citrus clementina LYK1
Theobroma cacao LYK1
Gossipium raimondii LYK1
Castanea mollisima LYK1
Fagus sylvatica LYK1
Casuarina glauca LYK1
Juglans regia LYK1
Alnus glutinosa LYK1
Fragaria vesca LYK1
Prunus persica LYK1
Dryas drummondi LYK1
Discaria trinervis LYK1
Morus notabilis LYK1
Parasponia rugosa LYK1
Parasponia rigida LYK1
Parasponia andersonii LYK1
Trema levigata LYK1
Trema orientalis LYK1
Trema orientalis RG16 LYK1
Vitis vinifera LYK1
Citrus clementina LYK1
Coffea canephora LYK1
Solanum lycopersicum LYK11
Solanum tuberosum LYK11
Erythranthe guttata LYK3
Coffea canephora LYK2
Coffea canephora LYK3
Solanum lycopersicum LYK13
Solanum tuberosum LYK13
Solanum lycopersicum LYK1
Solanum tuberosum LYK1
Aquilegia coerulea LYK2
Aquilegia coerulea LYK3
Citrus clementina LYK3
Gossipium raimondii LYK2
Gossipium raimondii LYK3
Amaranthus hypochondriacus LYK1
Arabidopsis thaliana CERK1
Brassica napus CERK1
Brassica rapa CERK1
Theobroma cacao LYK3
Eucalyptus grandis LYK4
Eucalyptus grandis LYK1
Eucalyptus grandis LYK2
Eucalyptus grandis LYK3
Vitis vinifera LYK3
Kalanchoe laxiflora LYK2
Kalanchoe laxiflora LYK3
Populus trichocarpa LYK3
Ricinus communis LYK3
Manihot esculenta LYK3
Manihot esculenta LYK2
Lotus japonicus NFR1
Medicago truncatula LYK3
Medicago truncatula LYK2
Cajanus cajan LYK3
Glycine max NFR1b
Glycine max NFR1a
Cercis chinensis LYK2.1
Cercis chinensis LYK2.2
Medicago truncatula LYK9
Lotus japonicus CERK6
Cajanus cajan LYK2
Glycine max LYK2
Cercis canadensis LYK1
Cercis chinensis LYK1
Medicago truncatula LYK7
Glycine max LYK4
Cajanus cajan LYK4
Medicago truncatula LYK6
Lotus japonicus LYS1/NFRe
Lotus japonicus LYS2
Medicago truncatula LYK1
Medicago truncatula LYK5
Medicago truncatula LYK4
Castanea mollisima LYK2
Fagus sylvatica LYK2
Juglans regia LYK2
Alnus glutinosa LYK2
Casuarina glauca LYK3
Casuarina glauca LYK2
Datisca glomerata LYK2
Cucumis sativus LYK2
Datisca glomerata LYK3
Begonia fuchsioides LYK2
Begonia fuchsioides LYK3
Prunus persica LYK2
Dryas drummondi LYK3
Fragaria vesca LYK2
Discaria trinervis LYK3
Morus notabilis LYK3
Trema levigata LYK3.3
Trema orientalis LYK3.3
Trema orientalis RG16 LYK3.3
Parasponia rugosa LYK3.3
Parasponia andersonii LYK3.2
Parasponia rigida LYK3.3
Trema orientalis LYK3.2
Trema orientalis RG16 LYK3.2
Trema levigata LYK3.2
Parasponia rugosa LYK3.2
Parasponia andersonii LYK3.1
Parasponia rigida LYK3.2
Trema orientalis LYK3.1
Trema orientalis RG16 LYK3.1
Parasponia rugosa LYK3.1
Parasponia rigida LYK3.1
LYK-Ia clade
unresolved
LYK-Ib clade
Tree scale: 0.1
Fabales
Fagales
Cucurbitales
Rosales
Eudicots
Chitin signalling
Nodulation
Mycorrhization

have a highly conserved intron-exon structure (Zhang et al., 2009). In most cases, the first exon encodes the extracellular domain comprising the signal peptide and three LysM motifs. So, the *P. andersonii PanLYK3* gene encodes two protein variants named PanLYK3.1 and PanLYK3.2 that differ in their extracellular domain **(Figure S2B)**.

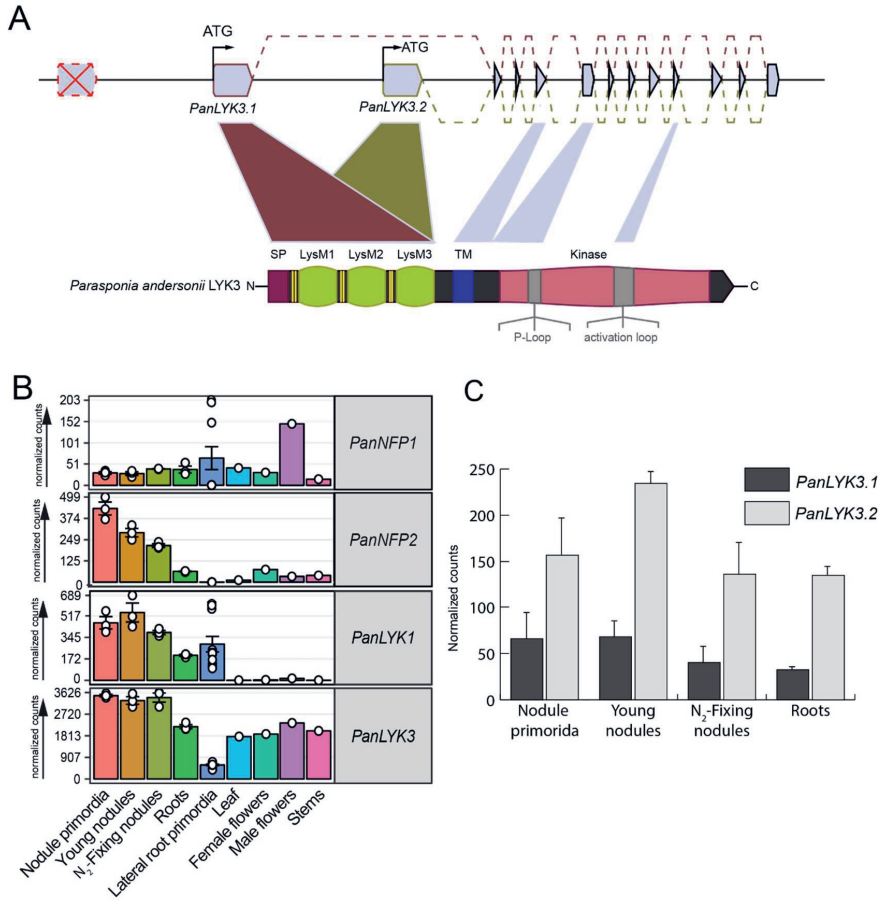


Figure 2. Gene structure and expression of *Parasponia PanLYK3* (**A**) Structure of the *PanLYK3* gene model and encoded proteins. *PanLYK3* possesses two protein variants of exon 1, which differ in the extracellular region containing the LysM-domains. A third copy of exon1 is lost in *P. andersonii.* (**B**) Expression profile of *PanNFP1, PanNFP2*, *PanLYK1* and *PanLYK3* in different plant tissues. Expression is given in DESeq2-normalized read counts; error bars represent SE of biological replicates. Dots represent individual expression levels. The analysis is based on data presented in Van Velzen *et. al.* 2018 (van Velzen et al., 2018b). (**C**) Relative expression of the *PanLYK3.1* and *PanLYK3.2* transcriptional variants based on RNA-seq reads splicing into the second exon. Data are represented as mean ± SE (n=3). The analysis is based on data presented in Van Velzen *et. al.* 2018 (van Velzen et al., 2018b).

The LYR-IA orthogroup represents the legume LCO receptors MtNFP, LjNFR5 and pea (*Pisum sativum*) PsSYM10 (Madsen et al., 2003; Arrighi et al., 2006; Buendia et al., 2016; Miyata et

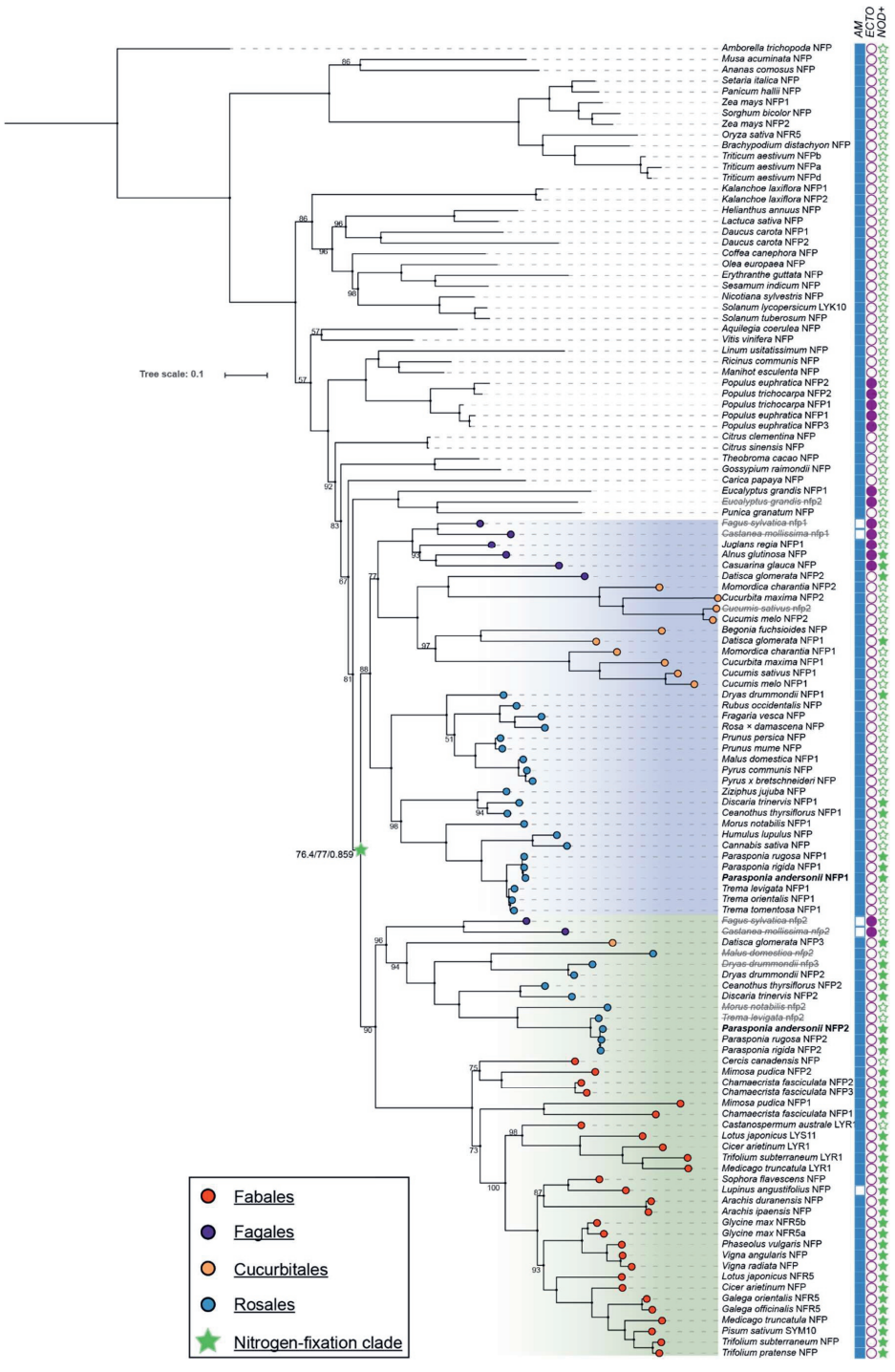
al., 2016). Previously, we have shown that *Parasponia* species harbour two genes in this orthogroup, *PanNFP1* and *PanNFP2* in *P. andersonii,* of which the latter is more closely related to *MtNFP*/*LjNFR5* (van Velzen et al., 2018b). *PanNFP1* and *PanNFP2* originated from an ancient duplication. Phylogenetic reconstruction including additional nodulating and non-nodulating species supported the occurrence of NFP-I and NFP-II subclades in the LYR-IA orthogroup and showed that this duplication associates with the origin of the nitrogen-fixing clade **(Figure 3; Data set S2)**. Several Actinorhizal species possess gene copies in both NFP subclades; including *Datisca glomerata*, *Dryas drummondii*, and *Ceanothus thyrsiflorius.* All these species nodulate with diazotrophic *Frankia* species of taxonomic cluster-II, which possess LCO biosynthesis genes. An NFP-II-type orthologous gene is notably absent in Actinorhizal species that are exclusively nodulated by *Frankia* species of cluster-I or cluster-III that lack LCO biosynthesis genes; e.g. *Alnus glutinosa* and *Casuarina glauca* **(Figure 3)** (Pawlowski and Demchenko, 2012; Griesmann et al., 2018; Salgado et al., 2018; Van Nguyen et al., 2019). In line for what was reported for the non-nodulating Rosales species (van Velzen et al., 2018b), NFP-II-type pseudogenes can be found in the genomes of the non-nodulating Fagales species *Castanea mollissima* and *Quercus fagus*. This shows a strict association of the presence of a functional NFP-II-type gene and LCO-driven nodulation, suggesting that the NFP-II subclade represents LCO receptors that function exclusively in nodulation.

***Parasponia* PanNFP1, PanNFP2, PanLYK1 and PanLYK3 can perceive rhizobium LCOs**

Based on the orthologous relation to legume LCO receptors, we considered *PanLYK3* (both variants) and *PanNFP2* as the most likely candidates to encode rhizobium LCO receptors in *Parasponia*. We noted that, in contrast to *PanLYK3*, *PanLYK1* is exclusively expressed in roots and nodule tissue **(Figure 2B)**, suggesting this gene may also function in a symbiotic context. Therefore, we decided to include this gene in further studies. Finally, we included also *PanNFP1*, since an earlier study based on RNA interference (RNAi) in transformed *Parasponia* roots showed that this gene functions in nodulation (Op den Camp et al., 2011). To test whether these four *Parasponia* genes can function as rhizobium LCO receptors, we conducted two complementary experiments. First, we introduced *Parasponia* receptor pairs into a lotus *Ljnfr1;Ljnfr5* double mutant aiming to determine whether these *Parasponia P. andersonii* receptors can trans-complement for LCO-induced Ca^{2+} oscillation. Second, we generated CRISPR-Cas9 knockout mutants in *Parasponia* to study their role in nodulation.

We selected lotus for trans-complementation studies as its microbial host *Mesorhizobium loti* strain R7A can also nodulate *Parasponia* **(Figure S3A-C)**. By using *A. rhizogenes*-mediated root transformation, we tested six combinations of *Parasponia* heterodimeric receptor pairs under control of the promoter and terminator of lotus LjNFR1 and LjNFR5 **(Figure 4A)**. These promoters showed to be functional in complementation of the *Ljnfr1-1;Ljnfr5-2* double mutant **(Figure S3D-H)**. For the trans-complementation constructs, we included the nuclear localized calcium sensor R-GECO1.2, allowing visualization of nuclear Ca^{2+} oscillations (Zhao et al., 2011). In wild-type lotus roots, Ca^{2+} oscillation was most strong in young root hair cells, whereas this response is not recorded in the *Ljnfr1-1;Ljnfr5-2* double mutant **(Figure S3I,J; movie S1)** (Miwa et al., 2006). Analysing the transgenic roots expressing *Parasponia* receptor combinations revealed that nine out of eleven tested combinations elicit Ca^{2+} oscillation, although less regular in shape and frequency when compared to the positive control **(Figure 4B; movie S2)**. Interestingly, the receptor combinations *PanLYK1;LjNFR5* and *LjNFR1;PanNFP2* did not elicit any Ca^{2+} oscillation response, whereas both *Parasponia* receptors are -at least partially- functional as an *M. loti* LCO receptor when combined with a *Parasponia* counterpart **(Figure 4B)**. Upon inoculation with *M. loti* R7A, only nodule-like structures were observed on roots trans-complemented with different *Parasponia* receptor combinations (4 weeks post-inoculation), but not with heterologous receptor pairs **(Table S2)**. We sectioned the largest nodule-like structures, which were present on *PanLYK3.2;PanNFP2* and *PanLYK1;PanNFP1* transformed plants. This showed the absence of intracellular rhizobium infections **(Figure S3K-P)**. Taken-together, the trans-complementation studies of a lotus *Ljnfr1;Ljnfr5* mutant indicated that all four *Parasponia* receptors -*PanLYK1, PanLYK3, PanNFP1* and *PanNFP2*- have the potential to function as a receptor for *M. loti* LCOs, but none could fully trans-complement a lotus *Ljnfr1-1;Ljnfr5-2* double mutant for nodulation.

Figure 3. Phylogeny reconstruction of LYRI-A orthogroup, containing known legume LCO receptors, based on 122 sequences from 87 species. A gene duplication in the root of the nitrogen-fixing clade is recognized; resulting in two subclades named NFP-I (blue) and NFP-II (green). The symbiotic capacities of the species are marked by filled (positive) and unfilled (negative) symbols: AM symbiosis (blue squares), ectomycorrhizal symbiosis (purple circles) and nodulation (green stars). *Parasponia* PanNFP1 and PanNFP2 are in bold. Deduced psuedo-proteins are depicted in grey/strikethrough. Values indicate IQ-tree UF-bootstrap support%. values >98 are not shown. Branch support for the nitrogen-fixing clade indicates aSH-aLRT / UF-Bootstrap / approximate Mr.Bayes support, respectively. Tree sScale bar represents substitutions per site. A list of species and accession numbers can be found in **Data set S2**.


AM
ECTO
NOD+
Amborella trichopoda NFP
Musa acuminata NFP
Ananas comosus NFP
Setaria italica NFP
Panicum hallii NFP
Zea mays NFP1
Sorghum bicolor NFP
Zea mays NFP2
Oryza sativa NFR5
Brachypodium distachyon NFP
Triticum aestivum NFPb
Triticum aestivum NFPa
Triticum aestivum NFPd
Kalanchoe laxiflora NFP1
Kalanchoe laxiflora NFP2
Helianthus annuus NFP
Lactuca sativa NFP
Daucus carota NFP1
Daucus carota NFP2
Coffea canephora NFP
Olea europaea NFP
Erythranthe guttata NFP
Sesamum indicum NFP
Nicotiana sylvestris NFP
Solanum lycopersicum LYK10
Solanum tuberosum NFP
Aquilegia coerulea NFP
Vitis vinifera NFP
Linum usitatissimum NFP
Ricinus communis NFP
Manihot esculenta NFP
Populus euphratica NFP2
Populus trichocarpa NFP2
Populus trichocarpa NFP1
Populus euphratica NFP1
Populus euphratica NFP3
Citrus clementina NFP
Citrus sinensis NFP
Theobroma cacao NFP
Gossypium raimondii NFP
Carica papaya NFP
Eucalyptus grandis NFP1
Eucalyptus grandis nfp2
Punica granatum NFP
Fagus sylvatica nfp1
Castanea mollissima nfp1
Juglans regia NFP1
Alnus glutinosa NFP
Casuarina glauca NFP
Datisca glomerata NFP2
Momordica charantia NFP2
Cucurbita maxima NFP2
Cucumis sativus nfp2
Cucumis melo NFP2
Begonia fuchsioides NFP
Datisca glomerata NFP1
Momordica charantia NFP1
Cucurbita maxima NFP1
Cucumis sativus NFP1
Cucumis melo NFP1
Dryas drummondii NFP1
Rubus occidentalis NFP
Fragaria vesca NFP
Rosa × damascena NFP
Prunus persica NFP
Prunus mume NFP
Malus domestica NFP1
Pyrus communis NFP
Pyrus x bretschneideri NFP
Ziziphus jujuba NFP
Discaria trinervis NFP1
Ceanothus thyrsiflorus NFP1
Morus notabilis NFP1
Humulus lupulus NFP
Cannabis sativa NFP
Parasponia rugosa NFP1
Parasponia rigida NFP1
Parasponia andersonii NFP1
Trema levigata NFP1
Trema orientalis NFP1
Trema tomentosa NFP1
Fagus sylvatica nfp2
Castanea mollissima nfp2
Datisca glomerata NFP3
Malus domestica nfp2
Dryas drummondii nfp3
Dryas drummondii NFP2
Ceanothus thyrsiflorus NFP2
Discaria trinervis NFP2
Morus notabilis nfp2
Trema levigata nfp2
Parasponia andersonii NFP2
Parasponia rugosa NFP2
Parasponia rigida NFP2
Cercis canadensis NFP
Mimosa pudica NFP2
Chamaecrista fasciculata NFP2
Chamaecrista fasciculata NFP3
Mimosa pudica NFP1
Chamaecrista fasciculata NFP1
Castanospermum australe LYR1
Lotus japonicus LYS11
Cicer arietinum LYR1
Trifolium subterraneum LYR1
Medicago truncatula LYR1
Sophora flavescens NFP
Lupinus angustifolius NFP
Arachis duranensis NFP
Arachis ipaensis NFP
Glycine max NFR5b
Glycine max NFR5a
Phaseolus vulgaris NFP
Vigna angularis NFP
Vigna radiata NFP
Lotus japonicus NFR5
Cicer arietinum NFP
Galega orientalis NFR5
Galega officinalis NFR5
Medicago truncatula NFP
Pisum sativum SYM10
Trifolium subterraneum NFP
Trifolium pratense NFP
Tree scale: 0.1
76.4/77/0.859
NFP-I clade
NFP-II clade
Fabales
Fagales
Cucurbitales
Rosales
Nitrogen-fixation clade

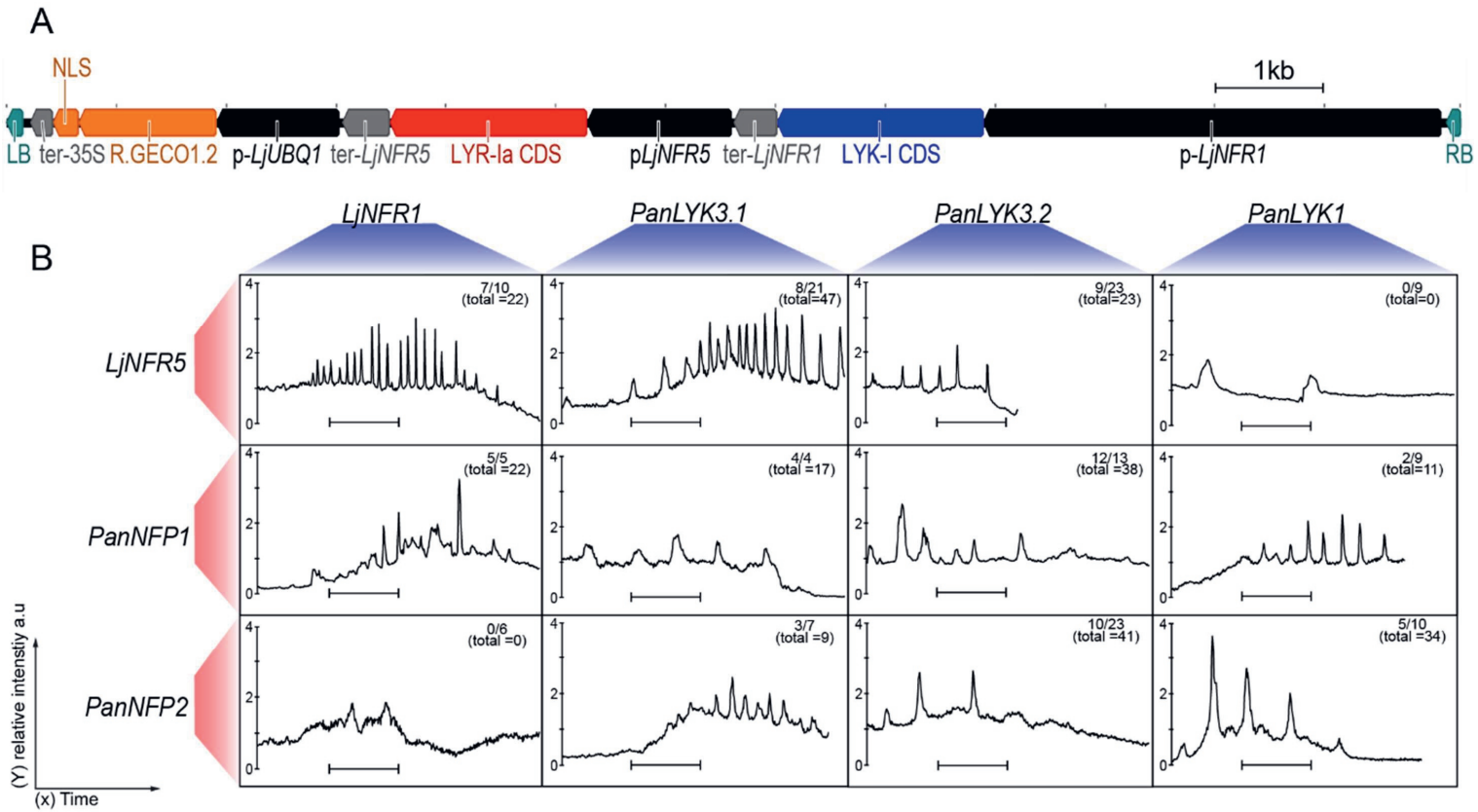

A
NLS
1kb
LB
ter-35S
R.GECO1.2
p-LjUBQ1
ter-LjNFR5
LYR-Ia CDS
pLjNFR5
ter-LjNFR1
LYK-I CDS
p-LjNFR1
RB
B
LjNFR1
PanLYK3.1
PanLYK3.2
PanLYK1
LjNFR5
PanNFP1
PanNFP2
7/10 (total =22)
8/21 (total=47)
9/23 (total=23)
0/9 (total=0)
5/5 (total =22)
4/4 (total =17)
12/13 (total =38)
2/9 (total=11)
0/6 (total =0)
3/7 (total =9)
10/23 (total =41)
5/10 (total =34)
(Y) relative intenstiy a.u
(x) Time

Figure 4. *Parasponia PanNFP1*, *PanNFP2*, *PanLYK1* and *PanLYK3* complement a lotus *Ljnfr1;Ljnfr5* mutant for rhizobium-induced Ca^{2+} oscillation. **(A)** Schematic representation of the T-DNA region of the binary construct used for A. rhizogenes-based root transformation of a lotus *Ljnfr1;Ljnfr5* double mutant. cDNA clones of LYK-I (marked blue) or LYR-Ia type genes (marked red) were cloned in identical fashion. cDNA clones were inserted between native promoter (marked black) pLjNFR1 (4,171bp) or pLjNFR5 (1,314bp), and native terminator (marked gray) sequences ter-LjNFR1 (394 bp) or ter-NFR5 (432 bp). pLjUBQ1::R.GECO1.2-nls:CaMV35S-ter (marked orange) was used to visualize nuclear calcium oscillation. The left border (LB) and right border (RB) (marked green) flank the T-DNA region. **(B)** Representative traces of nuclear Ca^{2+} oscillation, as observed in different combinations of LYK-I (red) and LYR-Ia (blue) type receptors introduced in a lotus *Ljnfr1;Ljnfr5* double mutant. Note that the receptor combinations *PanLYK1;LjNFR5* and *LjNFR1;PanNFP2* didn't complement for Ca^{2+} oscillation. Traces were recorded ~10 min post-application of LCOs extracted from *M. loti* R7A (~10^{-9} M). Numbers denote spiking roots vs the number of roots analysed. The number in brackets denotes the total number of spiking nuclei observed. Scale bar = 10 minutes. Y-axis is the relative fluorescence intensity compared to defined baseline in arbitrary units.

Parasponia PanNFP1, PanNFP2, PanLYK1 and *PanLYK3* function in nodulation

We recently established an efficient *Agrobacterium tumefaciens*-mediated transformation protocol for *Parasponia*, which allows the generation of CRISPR-Cas9 mutant plantlets in a ~3 month timeframe (van Zeijl et al., 2018; Wardhani et al., 2019). This enabled us to test by mutagenesis whether *PanLYK1, PanLYK3, PanNFP1* and *PanNFP2* are essential for rhizobium-induced nodule formation. We aimed to generate small deletions of 100-300 bp in the area covering the LysM domains by using two or three single guide RNAs (sgRNAs) that have no potential high identity off-targets. In the case of *PanLYK3* the transmembrane domain was targeted in order to mutate both alternative start variants. Additionally, we targeted specifically *PanLYK3.1* and *PanLYK3.2* by designing specific guides on the first exon. Selected single guides only had off-targets with at least three mismatches or two indels, based on alignments to the *Parasponia* reference genome. Shoots regenerated after *A. tumefaciens*-mediated co-cultivation were genotyped using PCR and subsequent sequence analysis to detect potential mutations at the CRISPR target sites. Only T_0 shoots with a >75 bp deletion between the two target sites or edits generating a frameshift were considered for propagation and subsequent further evaluation. At least two independent mutant alleles were generated per gene, with the exception of *Panlyk3.1* for which only a single suitable allele could be identified **(Data set S3)**. Putative off-target sites that occur in coding sequence regions were amplified by PCR and subsequently sequenced by sanger sequencing. Subsequently, *PanNFP1* was sequenced in *PanNFP2* lines and *PanNFP2* in *PanNFP1* lines **(Data set S3)**. No off-target mutations at these locations were identified. The selected tissue culture lines were *in vitro* propagated and rooted, so they could be used for experimentation.

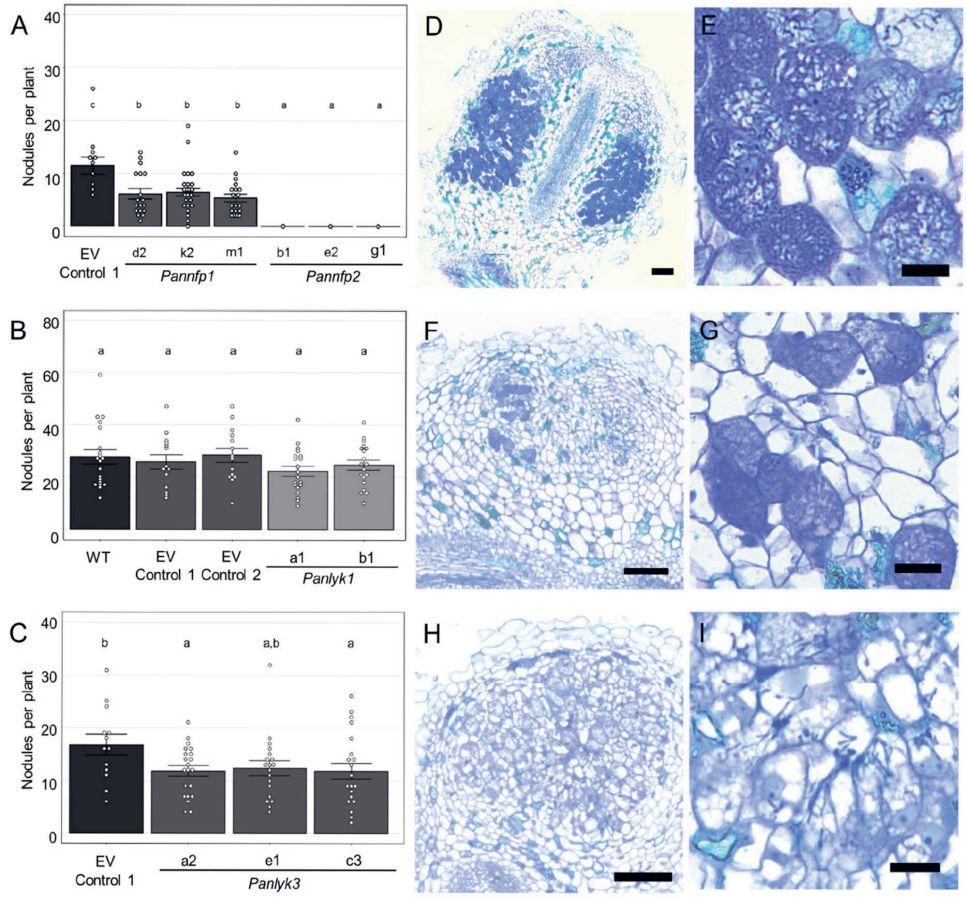


Figure 5. *Parasponia Pannfp1*, *Pannfp2* and *Panlyk3* mutants are affected in nodulation. Data are represented as mean ± SE, dots represent individual data points. Letters denote statistical significance based on one-way ANOVA and Tukey post-hoc contrasts P>0.05.

(**A**) Nodule numbers in *Parasponia* CRISPR-Cas9 mutant lines *Pannfp1* d2 (n=18), k2 (n=31) and m1 (n=19) and *Pannfp2* b1 (n=19), e2 (n=10) and g1 (n=9), 5 wpi with *M. plurifarium* BOR2. EV Control 1 (n=12) represents a positive control line transformed with a binary vector not containing sgRNAs. (**B**) Nodule numbers in *Parasponia* CRISPR-Cas9 mutant lines *Panlyk1* a1 (n=19) and b1 (n=20), 5 wpi with *M. plurifarium* BOR2. EV control 1 (n=14) and EV control 2 (n=14) represent two independent positive control lines transformed with a binary vector not containing sgRNAs. WT (n=20) represent untransformed plantlets. (**C**) Nodule numbers in *Parasponia* CRISPR-Cas9 mutant lines *Panlyk3* a2 (n=21), c3 (n=21) and e1 (n=19), 5 wpi with *M. plurifarium* BOR2. EV Control 1 (n=14). (**D,E,F,G,H,I**) Toluidine blue-stained section of representative nodules grown with *M. plurifarium* BOR2 (**D**) Wild type *Parasponia* transformed with an EV-1 construct expressing Cas9. Scale bar: 100 µm. (**E**) Infected nodule cells containing fixation threads formed on EV-1 plants. Scale bar: 20 µm. **(F)** Infected nodule of *Panlyk3* line e2. Note patches of infected cells. Scale bar: 100 µm. (**G**) infected nodule cells of the *Panlyk3* line e2 containing fixation threads. Scale bar: 20 µm. (**H**) Empty nodule of *Panlyk3* line e2. Note the absence of fully infected cells. Scale bar: 100 µm. (**I**) Nodule cells of the *Panlyk3* line e2 containing infection threads but no fixation threads. Scale bar: 20 µm.

We compared the nodulation phenotype of *Panlyk1, Panlyk3, Pannfp1* and *Pannfp2* knockout mutants in independent experiments, using empty vector (EV) transformed lines as control **(Figure 5; Figure S4)**. All three independent *Pannfp2* mutant lines showed to be unable to form nodules or nodule-like structures (5 weeks post inoculation, wpi) with strain *Mesorhizobium plurifarium* BOR2, demonstrating the requirement for this gene in the nodulation trait **(Figure 5A)**. Additionally, we noted a reduced nodulation efficiency of all three independent *Pannfp1* mutant lines. This is in line with earlier findings using RNAi to target *PanNFP1* in *A. rhizogenes*-transformed *Parasponia* roots (Op den Camp et al., 2011), demonstrating that *Pannfp1* controls nodulation efficiency, but is not essential for rhizobium intracellular infection. Previously, we reported that *PanNFP1* RNAi-nodules have a strong infection phenotype when inoculated with the *Sinorhizobium fredii* strain NGR234 (Op den Camp et al., 2011). We did not observe such an infection phenotype in nodules induced by *M. plurifarium* BOR2 on *Pannfp1* knockout mutant plants **(Figure S4)**. In order to determine whether the *Pannfp1* infection phenotype is strain dependent, we nodulated plants also with *S. fredii* NGR234. This strain showed to be less optimal under the chosen conditions (agroperlite supplemented with EKM medium and *S. fredii* NGR234.pHC60 at OD 0.05). In an effort to optimize nodulation efficiency with this strain we used river sand and scored nodulation 8 weeks post-inoculation. Under these conditions, no difference between *Pannfp1* and EV-control was observed. Nodules formed on *Pannfp1* mutant plants were infected normally **(Figure S4)**.

Similarly to *Pannfp1* mutant plants inoculated with *M. plurifarium* BOR2, we found a reduced nodulation efficiency in *Parasponia Panlyk3* knockout mutants, but not in *Panlyk3.1* and *Panlyk3.2* variant specific mutant alleles, nor in *Panlyk1* mutants **(Figure 5; Figure S4)**. To determine whether nodules formed on *Panlyk1* and *Panlyk3* mutants have an infection phenotype, we analysed thin sections. In contrast to legumes, *Parasponia* doesn't guide rhizobia in infection threads towards the nodule primordia. Instead, rhizobia enter via apoplastic cracks in epidermis and cortex, and only form infection threads to penetrate nodule cells. Once inside, infection threads develop into fixation threads, which are wider - having two phyla of bacteria aligned compared to one in infection threads- and possess a thinner cell wall (Lancelle and Torrey, 1984; Lancelle and Torrey, 1985). *Panlyk1* mutant nodules showed no defects in infection thread structure or the transition from infection

threads to fixation threads. In the case of *Panlyk3*, nodules were relatively small and had diverse phenotypes. Out of 45 sectioned nodules of the line *Panlyk3*-e2, 22 were infected like wild type, 15 contained only infection threads, but no fixation threads, and 8 showed an intermediate phenotype with few infected cells **(Figure 5 F-I. Figure S4)**. To confirm that the infection phenotype is a result of a full *Panlyk3* knockout mutation, we sectioned 28 nodules of the independent knockout line *Panlyk3*-c3. This revealed similar results; 11 nodules normally infected, 11 contained only infection threads and 6 nodules with an intermediate phenotype. Next, we determined whether this infection phenotype is controlled specifically by or either *PanLYK3.1* or *PanLYK3.2* which showed not to be the case **(Figure S4)**. As ~50% of the nodules formed on the *Parasponia Panlyk3* mutant plants displayed a wild-type phenotype, it suggests redundancy in gene functioning. Interestingly, *S. fredii* NGR234 could not nodulate *Panlyk3* mutants, which suggest this strain is fully dependent on PanLYK3 controlled signal transduction (**Figure S4**).

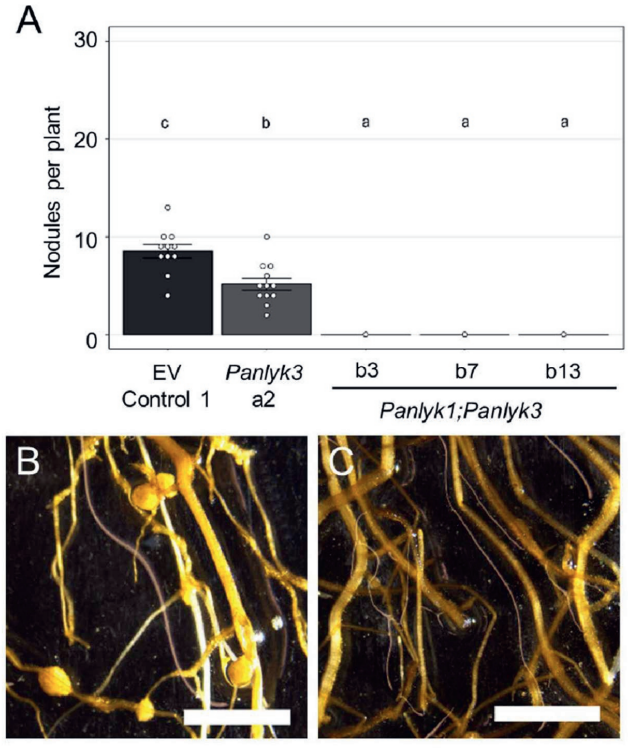


Figure 6. *Parasponia PanLYK1* and *PanLYK3* act redundantly in nodulation.
(**A**) Average nodule numbers per plant in EV control 1 (n=11) and re-transformed *Panlyk3* a2 line (n=12) and *Panlyk1;Panlyk3* double mutant lines b3 (n=10), b7 (n=5) and b13 (n=10), 5 wpi with *M. plurifarium* BOR2. Data are represented as mean ± SE, dots represent individual data points. Letters denote statistical significance based on one-way ANOVA and Tukey post-hoc contrasts P>0.05. (**B**) Roots with nodules of EV control 1, 5 wpi with *M. plurifarium* BOR2. Scale bar: 5 mm. (**C**) Roots without nodules of the *Panlyk1;Panlyk3* double mutant (line b3) 5 wpi with *M. plurifarium* BOR2 Scale bar: 5 mm

As *Parasponia* did not experience any gene duplication events in the LYK-Ib clade, *PanLYK1* in the LYK-Ia clade is the closest homolog of *PanLYK3*. In order to investigate whether the *PanLYK1* gene is functionally redundant with *PanLYK3* in case of *M. plurifarium* BOR2 inoculation, we generated a *Panlyk1;Panlyk3* double mutant. To do so, a binary construct with the two sgRNAs targeting *PanLYK1* was used for re-transformation of the *Panlyk3* mutant (line a2). We obtained three independent *Panlyk1;Panlyk3* mutants **(Data set S3)**. *M. plurifarium* BOR2 inoculation experiments revealed that all *Panlyk1;Panlyk3* double mutant lines were

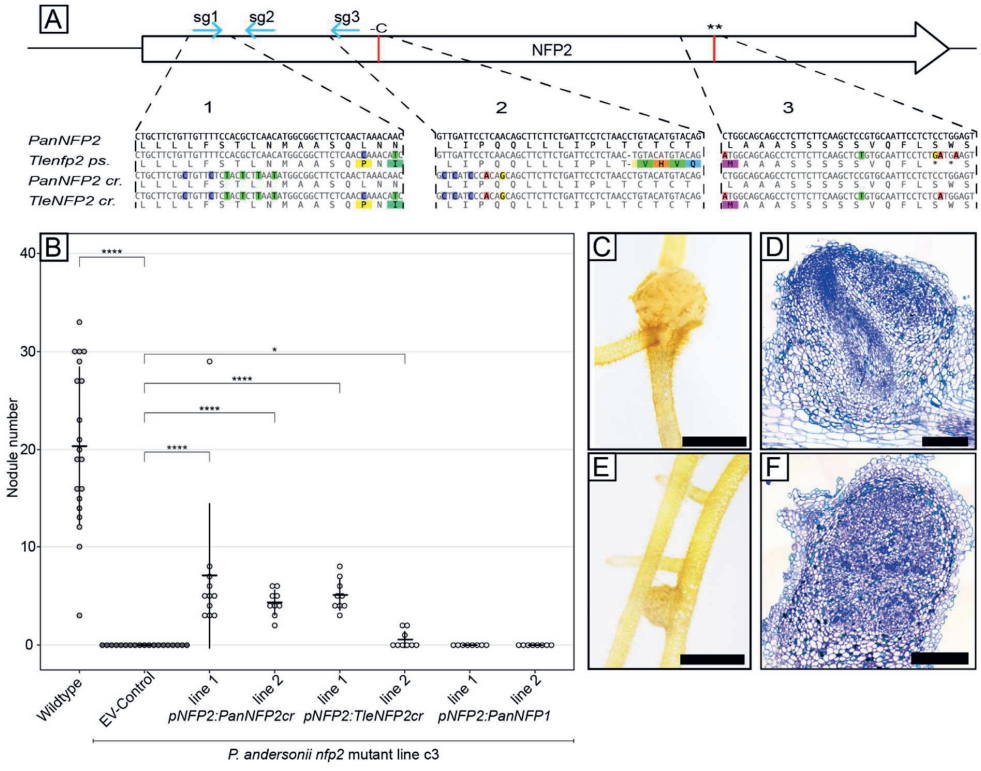


Figure 7: A repaired *Trema levigata nfp2* pseudogene can replace *PanNFP2* for nodule formation. (A) Schematic representation of NFP2 coding region with indicated replacements to avoid CRISPR targeting of inserted *NFP2* genes of *P. andersonnii* (*PanNFP2cr*) and a repaired *T. levigata* (*TleNFP2cr*). Blue arrows: Guide RNA target sites. Red lines: *Trema levigata* mutations. Region 1. Replacement of six codons at the sg1 site. Region 2. Replacement of five codons at the sg3 site plus repair of the *T. levigata* indel (red line). Region 3. Repair of the double stop codon in *Trema levigate* (red line, black asterisks). The replacement of five codons at the sg2 site is not shown. **(B)** *PanNFP2cr* and repaired *TleNFP2cr* can restore nodulation in the *Pannfp2* mutant line C3 when driven by the *PanNFP2* promoter, whereas *PanNFP1* cannot. Nodulation scored 5 wpi with *M. plurifarium* BOR2. Error bars represent the SD of the mean, statistical significance by Mann–Whitney–Wilcoxon (MWW). P>0.05 not significant (ns), P<0.05 *, P<0.01 **, P<0.001 ***, P<0.0001 ****. **(C,D)** Nodule and section of a *pNFP2:PanNFP2cr* line 1. **(E,F)** Nodule and section of *pNFP2:TleNFP2cr* line 1. **(C,E)** scale bar 2mm **(D,F)** scale bar 100µm.

unable to form any nodule or nodule-like structure **(Figure 6)**. To confirm that the nodulation minus phenotype in the *Panlyk1;Panlyk3* lines is not due to any off-target mutation, we conducted complementation studies using *A. rhizogenes*-mediated root transformation. As the putative promoter of *PanLYK3* is rather complex due to the occurrence of alternative transcriptional start sites **(Figure 2)**, we used the *LjNFR1* promoter, as well as the constitutive *AtUBQ10* and *CaMV35S* promoters, to drive a CRISPR-resistant allele of *PanLYK3.1* (*PanLYK3cr*). Compound plants carrying transgenic roots expressing *PanLYK3cr* could be nodulated by *M. plurifarium* BOR2 **(Figure S5)**. Together, this showed that in *Parasponia*, *PanLYK1* and *PanLYK3* act redundantly in root nodule formation. (For complementation studies of *Pannf2*, see below).

The results demonstrate that *Parasponia PanLYK1, PanLYK3, PanNFP1* and *PanNFP2* function in rhizobium LCO-driven nodulation. *PanLYK3* and *PanNFP2* are orthologous to legume *LjNFR1/MtLYK3* and *LjNFR5/MtNFP*, indicating a shared evolutionary origin of LCO-driven nodulation in both taxonomic lineages. As *PanLYK1* and *PanLYK3* evolved from a duplication predating the emergence of the nitrogen-fixing clade, it suggests that LCO signalling is an ancestral function of these LYK-I receptors.

A repaired *Trema levigata NFP2* pseudogene, but not *PanNFP1,* can functionally complement a *Parasponia andersonii npf2* mutant

PanNFP1 and *PanNFP2* differ in expression pattern. Whereas both genes are expressed in root tissue, only *PanNFP2* is upregulated in nodules **(Figure 2)** (Van Velzen et al., 2018b). We questioned whether the difference in symbiotic functioning between both genes is the result of regulatory evolution. To test this, we first identified a functional promoter region of *PanNFP2*. *A. tumefaciens* mediated transformation showed that a 2.75 kbps *PanNFP2* upstream region can be used to functionally complement the *Parasponia Pannfp2* mutant when using a *PanNFP2* CRISPR-resistant allele (*PanNFP2cr*). Two independent lines formed 7±7 and 4±1 nodules 5 weeks post inoculation with *M. plurifarium* BOR2 **(Figure 7)**. However, when we used *PanNFP1* driven by the *PanNFP2* promoter, no trans-complementation of the *Parasponia nfp2* mutant phenotype was observed. This suggests that there is a functional difference in the encoded *PanNFP1* and *PanNFP2* receptors.

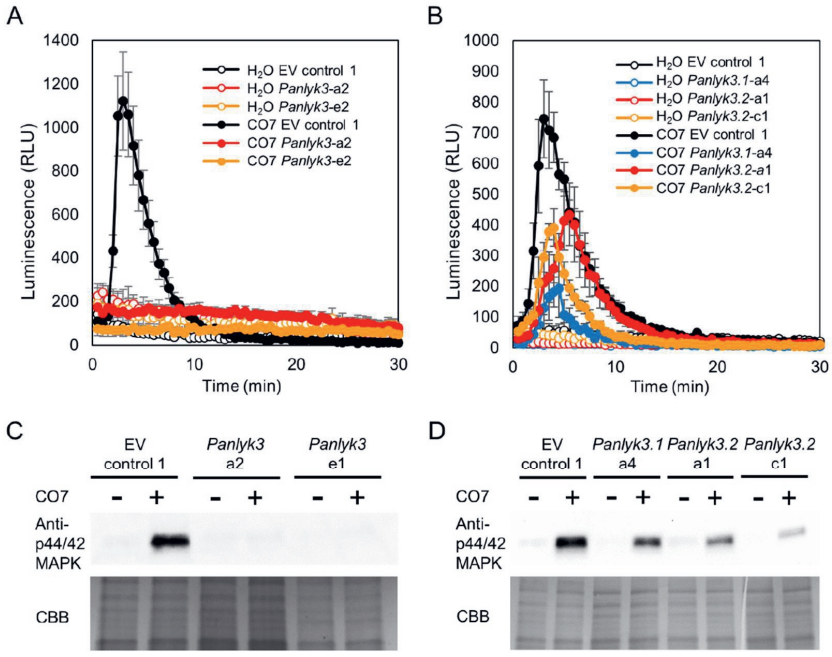


Figure 8. *Parasponia PanLYK3* is essential for chitin triggered immunity responses in roots. **(A,B)** Production of ROS measured upon treatment with 100 µM CO7 (filled circle) or H_2O (open circle) with (**A**) EV control 1 plants (black), *Panlyk3* line a2 (red) and *Panlyk3* line e1 (orange) (**B**) EV control 1 plants (black), *Panlyk3.1* line a4 (blue), *Panlyk3.2* line a1 (red) and *Panlyk3.2* line c1 (orange). For A and B, data are the average of at least three independent biological replicates ±SE. Luminescence is measured in relative light units RLU. **(C,D)** Phosphorylation of mitogen activated protein kinase (MAPK) analysed by immunoblot using an anti-p44/42 MAPK antibody upon treatment with 100 µM CO7 (upper panel). Equal loading was confirmed by CBB staining (bottom panel). Results shown are a representative out of three independent experiments. (**C**) MAPK phosphorylation in root pieces of EV control 1, *Panlyk3* line a2 and *Panlyk3* line e1. (**D**) EV control 1, *Panlyk3.1* line a4, *Panlyk3.2* line a1 and *Panlyk3.2* line c1.

Next, we questioned whether the *nfp2* pseudogene as present in several non-nodulating Rosales species may have encoded a functional symbiosis receptor. To test this, we focussed on the the *nfp2* pseudogene of *Trema levigata*, as it has only three mutations that cause a disturbance of the open reading frame **(Figure 7)**. We repaired these three mutations, using *PanNFP2* as a template, resulting in an engineered CRISPR resistant *TleNFP2cr* that encodes for a LysM-type receptor protein of 582 amino acids, similar as *PanNFP2* of *Parasponia*. We tested whether *TleNFP2cr* driven by the *PanNFP2* promoter can trans-complement the *Parasponia Pannfp2* mutant. *A. tumfaciens* transformation resulted in two lines that can form functional root nodules 5 weeks post-inoculation with *M. plurifarium* BOR2. This supports the

hypothesis that *T. levigata nfp2* encoded a functional symbiosis receptor prior to the pseudogenization of this gene.

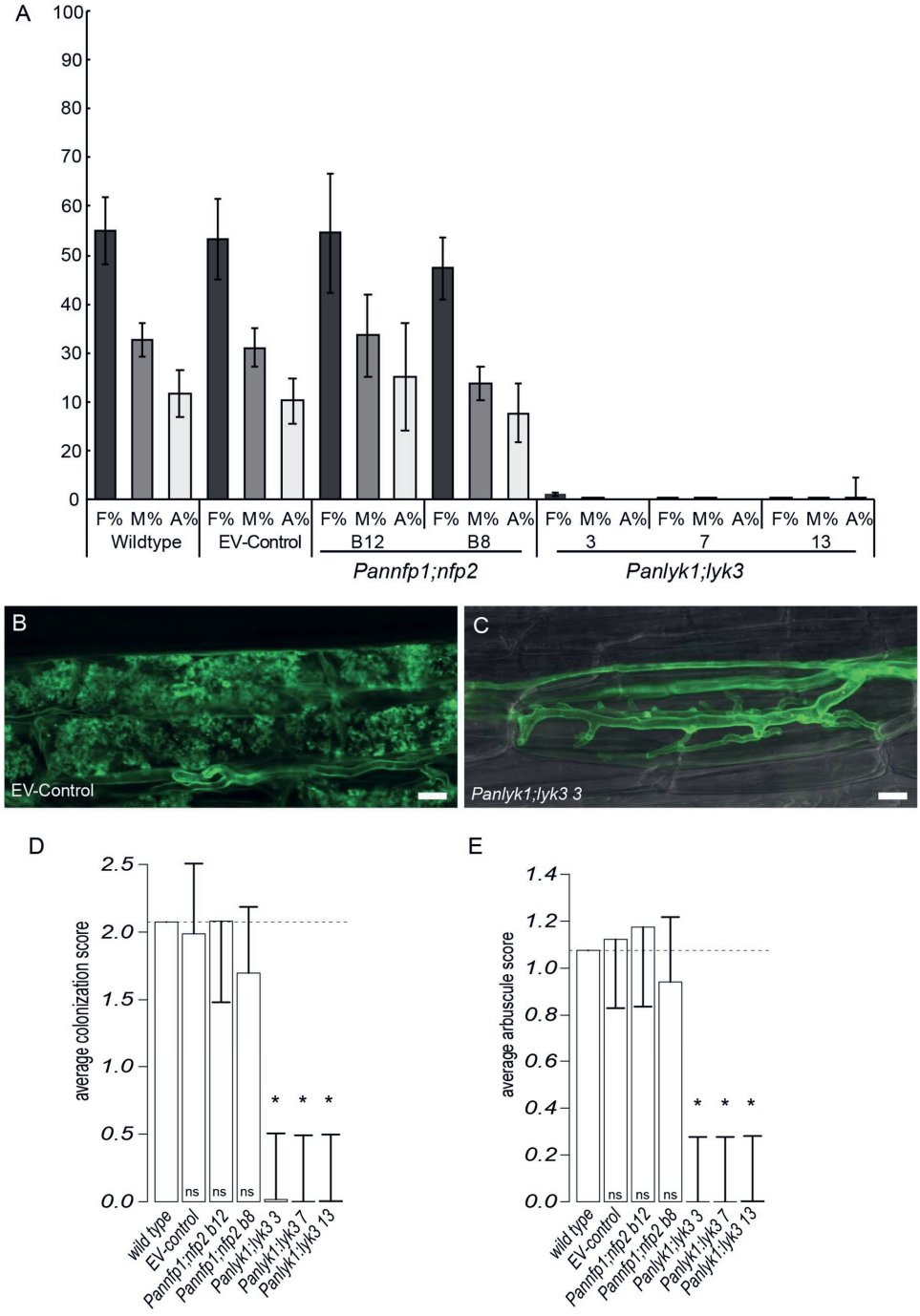


Figure 9. *Parasponia PanLYK1* and *PanLYK3* act redundantly in arbuscular mycorrhization. **(A)** The *Parasponia Panlyk1;Panlyk3* double mutant shows a strongly reduced colonization compared to wildtype and control *Parasponia* roots. *Parasponia Pannfp1;Pannfp2* mutants are not significantly affected. Frequency and Arbuscule abundance classes according to Trouvelot et. al. 1986 (Trouvelot, A, Kough J L, Gianinazzi-Pearson V, 1986). F%: colonization frequency in the root system. M%: intensity of mycorrhizal colonization. A%: Arbuscule abundance in the root system. Error bars represent the SE of 10 biological replicates scored at 6 weeks post inoculation using 250 spores of *Rhizophagus irregularis* strain DOAM197198 (Trouvelot, A, Kough J L, Gianinazzi-Pearson V, 1986). **(B)** Highly branched arbuscules formed in EV-Control plants 6 weeks PI stained with WGA-alexa488. Scale bar 10 µm. **(C)** Phenotype of stunted arbuscules formed in the *Panlyk1;Panlyk3* double mutant stained with WGA-alexa488. Scale bar 10 µm **(D,E)** Statistical analysis of raw (observed) data. **(D)** Mean colonization frequency score (classes 0 to 5) and **(E)** Mean arbuscule score (class 0 to 3). Class presented in Trouvelot et. al. 1986 (Trouvelot, A, Kough J L, Gianinazzi-Pearson V, 1986). Reduced mycorrhizal colonization and arbuscule formation in *Panlyk1;Panlyk3* mutants is considered significant compared to wild type. Error bars represent the Bonferroni corrected Least Significant Difference. Error bars non overlapping with mean wild type value are considered significant. Dashed line indicates mean wild type score.

Parasponia PanLYK3 is essential for chitin triggered immune responses and controls AM symbiosis in coherence with *PanLYK1*

Next, we aimed to determine whether the *Parasponia* LysM-type receptors that control nodulation are also involved in other processes, as this may provide insights into ancestral functions of these genes. Some LysM-type receptors of the LYK-I clade are known to function in chitin-triggered immunity and/or the arbuscular mycorrhizal symbiosis; e.g. lotus LjCERK6, medicago MtLYK9/MtCERK1, arabidopsis AtCERK1, tomato SlLYK1 and rice OsCERK1 **(Figure 1)** (Miya et al., 2007; Wan et al., 2008; Shimizu et al., 2010; Bozsoki et al., 2017; Liao et al., 2018; Feng et al., 2019; Gibelin-Viala et al., 2019a; He et al., 2019). Similarly, some experimental evidence using transient silencing assays indicated that LysM-type receptors of the LYR-IA clade function in mycorrhization, including *Parasponia* PanNFP1 (Op den Camp et al., 2011). In line with this, we aimed to confirm this phenotype in stable *Pannfp1* knockout mutants, and determine whether other *Parasponia* symbiotic LysM-type receptors may function also in AM symbiosis and/or chitin-induced innate immunity signalling.

First, we investigated whether the *Parasponia* LysM-type receptors mutants are affected in chitin-triggered immunity responses. To do so, two complementary assays were used; a chitin-induced ROS-burst production and MITOGEN-ACTIVATED PROTEIN KINASE3 (MAPK3) / MAPK6 phosphorylation assay. Chitin heptamers (CO7) effectively induced a ROS burst in *Parasponia* root segments at concentrations of <1 µM when incubated at 28°C, the regular growth temperature of *Parasponia* species. **(Figure 8A, Figure S6B)**. To test whether ROS bursts can also be triggered by rhizobium LCOs, we used the extracts of *M. loti* R7A and *Rhizobium tropici* CIAT899. These two strains can nodulate *Parasponia* but produce

structurally different LCOs (López-Lara et al., 1995; Folch-Mallol et al., 1996). However, neither triggered a ROS burst in *Parasponia* roots **(Figure S6A)**. To determine whether CO7-induced ROS bursts were associated with phosphorylation of *Parasponia* MAPK3 and MAPK6 homologs, we used an anti–phospho-p44/42 HsMAPK antibody, which detects phosphorylated MAPK3 and MAPK6 of different plant species (Yamaguchi et al., 2013; Bozsoki et al., 2017). *Parasponia* possesses a single *PanMAPK3* and a single *PanMAPK6* gene, which each encodes a protein with a conserved Thr202/Tyr204 phosphorylation site **(Figure S6C)**. Upon CO7 application (100 µM, 10 min.), a MAPK3/6 phosphorylation pattern can be detected, which is not observed upon application of *M. loti* or *R. tropici* LCO extracts **(Figure 8C; Figure S6D)**. Next, we determined whether *Parasponia* LysM-type receptor mutants are affected in responses to chitin CO7 oligomers. *Pannfp1*, *Pannfp2* and also a newly created *Pannfp1;Pannfp2* double mutant showed a wild-type ROS-burst and MAPK3/6 phosphorylation profile **(Figure S6; Data set S3)**. Similarly, the *Panlyk1* mutant showed a ROS burst and MAPK3/6 phosphorylation profile, as did wild-type root segments **(Figure S6E,F)**. In contrast, *Parasponia Panlyk3* mutant lines lacked a chitin triggered ROS-burst and showed no p44/42 MAPK phosphorylation **(Figure 8)**. Individual exon knockout *Panlyk3.1* or *Panlyk3.2* mutants both showed ROS production and MAPK3/6 phosphorylation upon application of 100 µM CO7, however at reduced levels **(Figure 8B, D)**. Taken together, these data show that *PanLYK3* - which is the only *Parasponia* gene in the LYK-Ib clade is essential for chitin innate immune signalling in roots.

Studies in *Parasponia*, tomato, medicago and rice revealed that LYR-IA and LYK-I putative orthologous genes have functions in AM symbiosis (Miyata et al., 2014; Zhang et al., 2015; Buendia et al., 2016; Miyata et al., 2016; Carotenuto et al., 2017; Liao et al., 2018; Feng et al., 2019; Gibelin-Viala et al., 2019; He et al., 2019). Interestingly, we noted that the NFP-I-type gene is pseudogenized in European beech (*Fagus sylvatica*) and Chinese chestnut (*Castanea mollissima*). Both species have lost AM symbiosis in favour of an ectomycorrhizal symbiosis **(Figure 3)** (Werner et al., 2018). We conducted an RNA-sequencing experiment on *Parasponia* roots mycorrhized by *Rhizophagus irregularis* strain DOAM197198. Several marker genes for mycorrhization showed to be enhanced in expression in mycorrhized *Parasponia* root samples; including *PanSTR1, PanSTR2, PanPT4, PanVPY, PanD27, PanRAD1* and *PanRAM1* **(Figure S7)**. Also, this suggested that *PanNFP1* is expressed higher than

PanNFP2 under these conditions **(Figure S7)**. However, no significant differential regulation of any of the studied LysM-type receptor encoding genes was detected between phosphate starved control roots and mycorrhized root samples **(Figure S7)**. To determine whether *Parasponia* symbiotic LysM-type receptors also function in AM symbiosis, we conducted three independent experiments using *in vitro* propagated mutant plantlets inoculated with 250 spores of *R. irregularis* DOAM197198. The average colonization and arbuscule formation frequency were scored 6 weeks post-inoculation. These experiments revealed substantial variation in mycorrhization efficiency between replicates, though no clear impaired AM symbiosis phenotype could be observed in any of the single mutants, including *Pannfp1*. Strikingly, *Panlyk1* showed a significant increase in colonization and arbuscule frequency **(Figure S8ABC)**. Analysing both double mutants -*Pannfp1;Pannfp2* and *Panlyk1;Panlyk3*- revealed a strong AM symbiosis phenotype only in the latter **(Figure 9, Figure S8)**. The fungal colonization of the *Panlyk1;Panlyk3* mutant was severely affected with only a few infections observed. Confocal imaging of WGA-alexa488 stained roots showed that besides the level of colonization, also the morphology of the few arbuscules that were formed was affected in *Panlyk1;Panlyk3* plants. In wild type plants, many cortical cells were filled with arbuscules that were finely branched and occupied most of the cell. In contrast, the few hyphae that enter cortical cells in the *Panlyk1;Panlyk3* mutant were unable to form mature arbuscules, either because the fungus fails to switch to fine branching, or because a limited number of fine branches is made **(Figure 9)**. As both *Panlyk1* and *Panlyk3* single mutant plants do not show such impaired mycorrhizal phenotype, we conclude that both genes function in conjunction to control mycorrhizal infection.

Taken together, these experiments revealed that PanLYK1 and PanLYK3 can function in multiple processes, including rhizobium nodulation (PanLYK1 and PanLYK3) arbuscular mycorrhizal symbiosis (PanLYK1 and PanLYK3) and chitin innate immune signalling (PanLYK3). This suggests that no subfunctionalization of these receptors is required to allow functioning in the rhizobium nitrogen-fixing nodulation trait.

Discussion

We used *Parasponia* as a comparative system to legumes to obtain insight into the evolutionary trajectory of LysM-type rhizobium LCO receptors. By conducting phylogenetic

analysis, trans-complementation studies in a lotus LCO receptor double mutant, and CRISPR-Cas9 mutagenesis in *Parasponia*, we identified four LysM-type receptors that function in LCO-driven nodulation in a non-legume. Two of these, *PanLYK3* and *PanNFP2*, are putative orthologs to known legume rhizobium LCO receptors *LjNFR1/MtLYK3* and *LjNFR5/MtNFP,* respectively. As the *Parasponia* and legume lineages diverged early in the nitrogen-fixing clade (>100 MYA), the use of orthologous genes for rhizobium LCO perception supports the hypothesis of a shared evolutionary origin of LCO-driven nodulation. In contrast to legumes, symbiotic LysM-type receptors in *Parasponia* did not experience recent duplication events. Instead, the *Parasponia* symbiotic LysM-type LCO receptors evolved following two ancient duplications. We hypothesize that the *PanNFP1* - *PanNFP2* duplication associates with the origin of the nitrogen-fixation clade, whereas in case of *PanLYK1* and *PanLYK3* the duplication occurred prior the birth of the nitrogen-fixing clade. This makes it most probable that the capability of these receptors to perceive LCOs predates the evolution of the nitrogen-fixing nodulation trait.

Currently, the *NFP1-NFP2* duplication can not yet precisely dated because legumes do not possess an NFP-I-type gene. This can be explained in two scenarios. (i) The *NFP1-NFP2* duplication occurred in the root of the nitrogen-fixing clade, and subsequently, the NFP-I-type gene got lost in the Fabales lineage. (ii) The *NFP1-NFP2* duplication occurred in an ancestor of the Fagales-Cucurbitales-Rosales lineages after the divergence of the Fabales order. The recent finding that ectopic expression of the *NFP*-type gene of two species outside of the nitrogen fixing clade (*Petunia hybrida PhLYK10* and tomato *SlLYK10*) can -at least partially- *trans* complement the medicago and lotus *Mtnfp* and *Ljnfr5* mutant phenotypes demonstrates that LCO receptor functionality is ancestral to the *NFP1-NFP2* duplication (Girardin et al., 2019). The putative promoters of *PhLYK10* and *SlLYK10* show a nodule-enhanced expression profile similar to that reported for *PanNFP2 (Girardin et al., 2019)*, which may support the second scenario, where the duplication of *NFP1-NFP2* has occurred only after the divergence of the Fabales clade. However, for such a scenario, it is essential that Fabales represents the most basal lineage in the nitrogen-fixing clade. To date, this remains unknown. For example, a recent phylogenetic study suggests, although with limited statistical support, that Fabales in sister to Fagales (Koenen et al 2019). The phylogenetic analysis presented here **(Figure 3)** suggests that the first scenario is most probable (aSH-

aLRT/UF-Bootstrap/approximate with Mr.Bayes support 76.4/77/0.859). Additionally, we searched for amino acid motifs in NFP-I and NFP-II type proteins and found an indel region in legume and non-legume NFP-II type proteins that is distinct from NFP-I **(Figure S9)**. This also supports the hypothesis that NFP1-NFP2 duplicated at the root of the nitrogen-fixing clade. However, additional experiments are needed to definitively reject either scenario.

Trans complementation studies in a lotus *Ljnfr1;Ljnfr5* double mutant showed that *Parasponia* LCO receptors can only partially restore LCO signalling. This only partial complementation we did not anticipate, because of the shared microsymbiont *M. loti* that can nodulate *Parasponia* as well as lotus. One explanation for this limited functionality may be that such receptors function in larger multiprotein membrane domain complexes. In such a case, the *Parasponia* LCO receptors are not adapted to interact with associated lotus proteins. Additionally, legumes and *Parasponia* have diverged in the mode of rhizobium infection. Whereas rhizobium penetrates *Parasponia* roots apoplastically by crack entry, legumes are generally infected intracellularly via curled root hair cells. Phenotypic analysis of rhizobium infection in legumes suggests that a specific LCO receptor is involved in this process, the so-called entry receptors (Ardourel et al., 1994). Such entry receptors have not yet been fully characterized, but *MtLYK3* carry out such functions, as they control rhizobium infection (Limpens et al., 2003; Smit et al., 2007). It remains elusive whether such entry receptor functioning requires specific adaptations that did not occur in the *Parasponia LYK3* ortholog.

We showed that an engineered *T. levigata TleNFP2* receptor can functionally complement the *Parasponia Pannfp2* mutant, whereas *PanNFP1* cannot. This suggests that the NFP1 and NFP2 receptor proteins have functionally diverged. Based on the finding that NFP orthologous protein of *P. hybrida* (PhLYK10) and tomato (SlLYK10) can complement lotus *Ljnfr5* and medicago *Mtnfp* mutants, it can be hypothesized that in *Parasponia* especially *PanNFP1* has experienced protein adaptations. However, it should be noted that the *trans* complementation studies presented here were conducted using the native *PanNFP2* promoter, whereas studies conducted with *PhLYK10* and *SlLYK10* were conducted with *CAMV35S* (Girardin et al., 2019). Such overexpression may mask differences in substrate affinity and/or specificity, under which native transcriptional regulation are biologically relevant. Our data demonstrate that the ancestor of *T. levigata* possessed a NPF2 receptor

that can function in nodulation.

Mutant analysis in legumes demonstrated that rhizobium nodulation co-opted elements of an AM signalling pathway, including the LRR-type transmembrane receptor kinase lotus SYMBIOTIC RECEPTOR KINASE (LjSYMRK)/Medicago DOES NOT MAKE INFECTIONS2 (MtDMI2), the nuclear envelope located cation ion channels LjCASTOR, LjPOLLUX/MtDMI1, the nuclear localized CALCIUM CALMODULIN KINASE LjCCaMK/MtDMI3, and the transcription factor LjCYCLOPS/Medicago INTERACTING PROTEIN OF DMI3 (MtIPD3) (Geurts et al., 2012). However, in legumes, rhizobium and AM fungi were shown to have independent perception mechanisms to active this common symbiosis signalling pathway. In lotus and medicago, these consist of LjNFR1-LjNFR5 / MtLYK3-MtNFP for rhizobium LCOs and MtLYK9/MtCERK1 for AM signals (Geurts et al., 2012; Feng et al., 2019; Gibelin-Viala et al., 2019b). MtLYK3 and MtLYK9/MtCERK1 both belong to the LYK-Ib clade and evolved upon legume specific duplication events **(Figure 1)** (De Mita et al., 2014). The strong phenotype in AM and nodule symbiosis of the *Parasponia Panlyk1;Panlyk3* knockout mutant demonstrates that such subfunctionalization is not causal for the evolution of rhizobium LCO receptors. In *Parasponia*, both receptors function in conjuction to control AM and rhizobium nodulation. Additionally, PanLYK3 acts as a chitin innate immune receptor. Such multifunctionality has also been reported for MtLYK9/MtCERK1 in medicago and OsCERK1 in rice, which function both in AM symbiosis and chitin innate immune signalling (Miyata et al., 2014; Carotenuto et al., 2017; Feng et al., 2019; Gibelin-Viala et al., 2019). As monocots did not experience the LYK-Ia / LYK-Ib duplication, it demonstrates that committing multiple functions in symbiosis and innate-immunity was ancestral to species of the nitrogen fixing clade, but functionally diverted in the legume lineage.

The presence of NFP-type genes (LYR-IA orthogroup) in species outside of the nitrogen-fixing clade associates with the ability to establish an AM symbiosis **(Figure 3)** (Delaux et al., 2014; Gough et al., 2018). However, corresponding mutants have only a relatively weak phenotype in AM symbiosis (Buendia et al., 2016; Miyata et al., 2016; Girardin et al., 2019). Upon duplication of this gene, the NFP-I and NFP-II subclades may have inherited the ancestral function. As both the *Parasponia* PanNFP1 and PanNFP2 receptors can partially complement LCO-induced Ca^{2+} oscillation in the lotus *Ljnfr1;Ljnfr5* double mutant, it supports that receptors of the NFP-I and NFP-II clades can act as an LCO receptor, which may reflect the

ancestral function. Our observation that the presence of a functional gene in the NFP-II clade strictly associates with LCO-based nodulation suggests that this gene was co-opted to function in this trait. The importance of this LysM-type LCO receptor in the nitrogen-fixing nodulation trait is underlined by the complete block of nodulation in knockout mutants in legumes (e.g. lotus *Ljnfr5*, medicago *Mtnfp* and pea *Pssym10*) and *Parasponia* (*Parasponia Pannfp2*) (Madsen et al., 2003; Arrighi et al., 2006). As *Parasponia* and legumes diverged at the root of the nitrogen-fixing clade, it suggests that the adaptations in the NFP-II clade are ancient and may have coincided with the birth of the nodulation trait.

The NFP-I type gene retained -at least in part- its ancestral function, indicated by its presence in non-nodulating species in the nitrogen-fixing clade that can establish an AM symbiosis. In cases where AM symbiosis is replaced by an ectomycorrhizal symbiosis such as in *Fagus sylvatica* or *Castanea mollissima,* the NFP-I type gene pseudogenized. However, phenotypic studies in stable *Parasponia* mutants could not support the functioning of *PanNFP1* in AM symbiosis. These findings contradict our earlier observation that this gene functions in arbuscule formation (Op den Camp et al., 2011). The reason for this discrepancy may be due to the used RNAi construct used, which may have off-target effects (van Velzen et al., 2018b). To determine whether this is the case, we have studied the expression of LysM-type RLK genes in two independent *PanNFP1* RNAi experiments. This revealed significant knockdown not only of *PanNFP1*, but also *PanNFP2*, which can explain the strong rhizobium nodulation and infection phenotype as reported by Op den Camp et al. (2011). We also found variable expression levels of other LysM-RLKs, including *PanLYK1* and *PanLYK3*, which may explain the reported mycorrhization phenotype on *PanNFP1* RNAi roots **(Figure S10)**. Studies presented here, using CRISPR-Cas9 knockout mutant lines revealed substantial biological variation in mycorrhization efficiency of *Parasponia* roots, which may have hindered the observation of minor quantitative AM symbiosis phenotypes. To rule out that PanNFP1 and PanNFP2 may function redundantly to control AM symbiosis, we analysed also a *Pannfp1;Pannfp2* double mutant. Also, these lines showed to be effectively mycorrhized. Therefore, we conclude that our current mutant phenotype analysis does not find support for essential functioning of *Parasponia* PanNFP1 and PanNFP2 in AM symbiosis.

The study presented here provided insight into the evolutionary trajectory of symbiotic LCO LysM-type receptors. By using *Parasponia* as a comparative system to legumes, we revealed

two ancestral duplications of LysM-type LCO receptors that predate, and coincide with, the evolution of nitrogen-fixing nodules. The strict association of genes in the NFP-II clade with LCO-driven nodulation strongly suggests that this gene was co-opted to function specifically in this symbiosis, making NFP2s a target in approaches to engineer LCO-driven nodulation in non-leguminous plants.

Materials & Methods

LysM-type receptor phylogeny reconstructions

Orthogroups containing LysM-type receptor kinases of *Parasponia*, generated in a previous study (van Velzen et al., 2018b), were combined and re-aligned into a single alignment using MafftV7.017. MrBayes3.2.6 was used to calculate phylogenetic relations under default parameters in Geneious R8.1.9 (Biomatters Ltd, UK) (Huelsenbeck and Bollback, 2001). Clades were named as published previously (Huelsenbeck and Bollback, 2001; Buendia et al., 2018). For clade LYK-I and LYR-IA additional putative orthologs were collected from Phytozome and NCBI databases using BLAST with AtCERK and MtNFP protein sequences as query **(Table S1)**. Available Genomes from Fabales, Fagales, Cucurbitales and Rosales species were downloaded and local BLAST analysis was conducted using Geneious R8.1.9 (Biomatters Ltd, UK) to search for additional unannotated LYK-I and LYR-IA protein sequences. Pseudogenes were annotated manually based on the closest functional ortholog so that a protein sequence could be deduced. Correct protein sequences were aligned using MAFFTV7.017 and subsequently manually curated. The deduced amino acid sequence was subsequently added to the alignment if the alignment length was at least 70% of the *Parasponia* protein. Phylogenetic analysis was performed using IQ-tree (Nguyen et al., 2015; Trifinopoulos et al., 2016), running the modelfinder extension to find the best substitution models (Kalyaanamoorthy et al., 2017). Branch support analysis was done using Sh.aLRT 1000 replicates, UF-BOOTSTRAP support 1000 iterations (Kalyaanamoorthy et al., 2017; Hoang et al., 2018) and approximate Bayes support. Branch supports shown are UF-Bootstrap support%. Best fit model for the LYK-I clade: JTT+I+G4. Best fit model for LYR-IA clade: JTT+I+G4. Resulting tree files were loaded into Interactive Tree Of Life (iTOL) v3 for editing (Letunic and Bork, 2016). The analysis was run at least three times. Trees were rooted to outgroup angiosperm species *Amborella trichopoda*. UF Bootstrap Branch supports >98 were omitted for visual clarity. Gene names,

accession numbers and alignment file of identified homologs can be found in **Data set S1** for LYK-I and **Data set S2** for LYR-IA, and **Table S1** for *Parasponia*.

LYK3 alignment and variant detection

Genomic LYK3 regions of *Parasponia, P. rigida, P. rugosa, Trema orientalis* RG16*, T. orientalis* RG33, and *Trema levigata* were extracted from the respective assemblies (van Velzen et al., 2018b) and Aligned using MAFFTV7.0.17 implemented in Geneious R8.1. Coding sequences of *Parasponia, P.rigida, P.rugosa LYK3* protein variants were translated and aligned using MAFFTV7.0.17 implemented in Geneious R8.1 **(Data set S1)**.

Vector constructs

All vectors generated for this study were created using golden gate cloning (Engler et al., 2009). Backbones and binary vectors were derived from the golden gate molecular toolbox (Engler et al., 2014). *Parasponia* LysM-type receptor cDNA clones were sequence synthesized as level 0 modules, including silent mutations in golden gate BsaI or BpiI restriction sites. Golden gate compatible clones of LjNFR1 and LjNFR5 promoters, CDS and terminators were obtained from Arhus University, Denmark. The calcium signalling reporter pLjUBQ1:R-GECO1.2 was published previously (Kelner et al., 2018). The generation and assembly of *Parasponia* CRISPR constructs were done as published previously (van Zeijl et al., 2018). For hairy root transformation, a modified level 2 standard vector carrying spectinomycin instead of kanamycin resistance was created. All sgRNAs were expressed using the AtU6 promoter. All Golden Gate binary vectors were verified by restriction digestion and DNA sequencing before transformation. A list of primers and constructs can be found in **Table S3 and S4**.

Genotyping and off-target analysis

All sgRNA targets were designed using the Geneious R10 CRISPR design tool, which picks targets on the principles described in Doench et al. (2014). To be selected Guide RNAs must have no potential target sites in the genome with (i) Less than three mismatches or (ii) less than two indels. Known off-target locations in CDS regions were PCR amplified and sequenced. No off-target mutations at these sites were detected. Genotypes and known off-target locations of CRISPR mutants used in this study can be found in **Data set S3**. Primers

used for the creation of sgRNAs and subsequent sequencing of mutants and off-targets are listed in **Table S4**.

Bacterial strains

We used *Mesorhizobium plurifarium* BOR2 (van Velzen et al., 2018b) and *Sinorhizobium fredii* NGR234.pHC60 expressing GFP (Trinick and Galbraith, 1980; Cheng and Walker, 1998; Op den Camp et al., 2011) for *Parasponia* inoculation experiments. *M.loti* R7A.pHC60 (Cheng and Walker, 1998; Sullivan et al., 2002) was used for lotus inoculations. *M. loti* R7A and *Rhizobium tropici* CIAT899 (Martínez-Romero et al., 1991) containing plasmid pMP604 (Spaink et al., 1989) were used for LCO extraction. *A. rhizogenes* strain AR10 (Hansen et al., 1989b; Martínez-Romero et al., 1991) was used for lotus root transformation. *Agrobacterium tumefaciens* strain AGL-1 (Lazo et al., 1991) was used in *Parasponia* transformation. *Agrobacterium* sp. MSU440 was used for *Parasponia* hairy root transformations (Cao et al., 2012). The *Escherichia coli* strain DH5α was used to propagate plasmids and in all subsequent cloning steps.

Rhizobium LCO isolation

To isolate rhizobium LCOs the plasmid pMP604 containing an auto-active NodD protein was introduced in *M. loti* R7A and *R. tropici* CIAT899 (Spaink et al., 1989; López-Lara et al., 1995). LCOs were extracted from a 750 ml liquid culture, OD600=0.5, grown at 28°C in minimal medium (5.75 mM K_2HPO_4, 7.35 mM KH_2PO_4, 5.9 mM KNO_3, 460 nM $CaCl_2$, 37.5 µM $FeCl_3$, 20.5 nM biotin, 2.07 mM $MgSO_4$, 2.9 nM Thiamine HCl, 8.1 nM Nicotinic acid, 4.8 nM Pyridoxine HCl, 2.8 nM Myo-inositol, 4.6 nM Panthotenate and 1% w/v sucrose) by the addition of 150 mL 1-butanol and 1h shaking. The butanol phase was transferred and subsequently evaporated (water bath 40˚C). Pellet was dissolved in 75 mL methanol, tested for Nod-factor activity and stored at -20˚C for later use. The concentration of active LCOs was estimated by using *LjNIN* induction in lotus wild type Gifu roots, 3h post-application. The lowest active dilution was estimated to be ~10^{-10} M.

Lotus japonicus Agrobacterium rhizogenes root transformation

Lotus *Ljnfr1-1;Ljnfr5-2* double mutants (Madsen et al., 2003; Radutoiu et al., 2003) were used for LysM complementation assays and 'Gifu' wild-type as control. Seedlings for *A. rhizogenes* root transformation were moved to fresh half-strength B5 medium and co-cultivated for 1 week as described previously using *A. rhizogenes* strain AR10 (Stougaard et al., 1987; Hansen et al., 1989a; Stougaard, 1995). During root emergence plants were grown on 1% agar plates half-strength B5 media containing 0.03% w/v cefotaxime and 1% w/v sucrose. Plants were screened for transformed roots using nuclear-localized R.GECO1.2 fluorescence. Shoots with transformed roots were grown in Agroperlite (Maasmond-Westland, Netherlands) supplemented with modified ½ Hoagland's medium (Hoagland et al., 1950) containing 0.56 mM NH_4NO_3 and inoculated with *M. loti* R7A.pHC60 (expressing GFP) at OD600= 0.05. Plants were grown at 21°C under a 16h light/8h dark regime. For calcium oscillation analysis transformed plants were grown on ½ Hoagland's plates with 1% agar containing 0.56 mM NH_4NO_3 for 1 week. Plants were moved to N-free ½ hoaglands medium 1 week prior to imaging.

Calcium oscillation quantification

Calcium spiking experiments were performed on a Leica TCS SP8 HyD confocal microscope equipped with a water lens HC plan-Apochromat CS2 40x/1.0. Transformed root segments expressing R-GECO1.2 were selected and incubated with 500x diluted LCO extract (estimated to represent ~10^{-9} M) in nitrate-free ½ Hoagland's medium (Hoagland et al., 1950) on a glass slide with coverslip. Images were are taken at 5s intervals for a minimum of 20 minutes per sample using an excitation wavelength of 552 nm and emission spectrum 585-620 nm. It is possible to monitor a large number of nuclei per root sample. However, only epidermal and especially root hairs showed to be responsive. Therefore, total nuclei numbers vary largely between samples. Video recordings of Imaged root samples were exported to ImageJ1.50i (Collins, 2007). The Geciquant ImageJ plugin was used for background subtraction and region of interest (ROI) selection (Srinivasan et al., 2015). Average pixel intensity of ROIs (individual nuclei) were measured. Average pixel values (0-255) per nucleus were plotted and a background R-GECO1.2 fluorescence baseline of 2x 1 minute (2 regions of 12 frames) was selected manually in a region of the trace where no spikes were occurring. Only nuclei with a

minimum of three spikes with an amplitude of over 1.5 times background were considered as positive.

Parasponia growth conditions for propagation, transformation, mycorrhization and nodulation

Sequenced *Parasponia* WU1 trees or its direct descendants, were used in all experiments (Op den Camp et al., 2011; van Velzen et al., 2018b). Prior to transformation or transfer to tissue culture *Parasponia* trees are grown in a conditioned greenhouse at 28°C, 85% humidity and a 16/8 h day/night regime. *Parasponia in vitro* propagation, transformation, CRISPR-Cas9 mutagenesis and nodulation assays were done according to Van Zeijl et al 2018 (van Zeijl et al., 2018). *P.andersonii* hairy root transformations were performed according to Cao et al 2012 (Cao et al., 2012).

Parasponia Nodulation assay and analysis

Rooted tissue culture plantlets for phenotyping assays were grown in crystal-clear polypropelene containers (1 L), with a gas exchange filter (OS140BOX, Duchefa Biochemie, Netherlands). Pots were half-filled with agraperlite (Maasmond-Westland, 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] (Becking, 1983). For nodulation assays, modified EKM medium (Becking, 1983) was inoculated with rhizobia (OD600 = 0.025) prior to planting the shoots. For inoculation with strain *S. fredii* NGR234.pHC60, containers were half-filled with sterilized river sand and watered with modified EKM-medium containing the bacteria at an OD600 = 0.05. All Nodules were fixed in buffer containing 4% paraformaldehyde mixed with 3% v/v glutaraldehyde in 50 mM phosphate (pH = 7.4). A vacuum was applied for 2 hours during a total 48h incubation. Fixed nodules were embedded in Plastic, Technovit 7100 (Heraeus-Kulzer, Germany), according to manufacturer's recommendations. Sections (5 µm) were made using a RJ2035 microtome (Leica Microsystems). Sections were stained using 0.05% Toluidine Blue O. Images were taken with a DM5500B microscope equipped with a DFC425c camera (Leica microsystems).

***Parasponia* mycorrhization assay**

For mycorrhization experiments, pots were half-filled with sterilized river sand, watered with modified ½ strength Hoagland's medium containing 20 µM potassium phosphate. Pots were inoculated with 250 spores of *Rhizopagus irregularis* (Agronutrion-DAOM197198). In all experiments, plantlets in pots with closed lids were placed in a climate room at 28°C, 16/8 h day/night. Plants were watered with sterilized demineralized water. Plants harvested 6 weeks post inoculation with *Rhizopagus irregularis* (Agronutrion-DAOM197198). Root segments were treated with 10% (w/v) KOH and incubated at 90°C for 20 minutes. The root samples were then rinsed 6 times with water and stained with trypan blue at 90°C for 5 minutes. For each mutant, ten plants were assessed and from each plant 30 root segments (each segment of approx. 1 cm long) were examined and mycorrhizal structures (hyphae, vesicles and arbuscules) were determined using magnified line intersect method (Trouvelot, A, Kough J L, Gianinazzi-Pearson V, 1986) using a Leica CTR6000 microscope. For staining with WGA-Alexafluor 488 (Molecular Probes, Thermo Fisher Scientific, Waltham, MA, USA), roots were incubated in 10% (w/v) KOH at 60°C for 3 h. Then, roots were washed three times in phosphate-buffered saline (PBS) (150 mM NaCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4, pH 7.4), and incubated in 0.2 $\mu g.ml^{-1}$ WGA-Alexafluor 488 in PBS at room temperature for 16 h. For RNA-isolation *Parasponia* WT plants were grown according to conditions above. RNA was isolated according to protocols published in (Op den Camp et al., 2011; van Velzen et al., 2018b). Mock inoculated plants were harvested as control. Three independent biological replicates were taken per sample. Expression was determined using RNA-seq. Reads were mapped using kallisto (Bray et al., 2016). Expression values and differential expression were determined using sleuth (Pimentel et al., 2017). Differentially expressed genes (Benjamini-Hochberg multiple testing corrected q-value <= 0.05).

qPCR analysis of panNFPi cDNA samples

PanNFPi cDNA samples were generated previously (Op den Camp et al., 201). qPCR was performed in 10 µl reactions using 2x iQ SYBR Green Super-mix (Bio-Rad, United States). PCR reaction was executed on a CFX Connect optical cycler, according to the manufacturer's protocol (Bio-Rad, United States). Three technical replicates per cDNA sample were used. Data analysis and statistical analysis of biological replicates was performed using CFX

Manager 3.0 software (Bio-Rad, United States). Gene expression was normalized against Reference genes *PanACTIN* and *PanEF1alpha.* Primers can be found in **Table S4**.

ROS assay

Parasponia plantlets were grown on rooting medium (van Zeijl et al., 2018) for 4 weeks at 28°C before the treatment. Roots, submerged in water, were cut into approximately 1cm pieces. Each well of a black 96 well flat bottom polystyrene plate (Nunc) was filled with 10 root pieces. 10 replicates per line were analysed. After filling the wells, it was kept 5 hours in 28 °C. After incubation, the water was replaced with 100 µl of assay solution containing 0.5 µM L-012 (FUJIFILM Wako Chemicals), 10 µg/ml Horseradish peroxidase (Sigma) and respective elicitors (CO7 (ELICITYL) or LCOs extracted from *M. loti* or *R. tropici*) at described concentrations. As a mock treatment, 100 µl H_2O was added. The light emission was immediately measured at 30 second intervals for 30 minutes, using a Clariostar multi well-plate reader. All data are the average of at least three independent biological replicates.

Protein extraction from *Parasponia* and western blotting

Parasponia plantlets were grown on rooting medium (van Zeijl et al., 2018) for 4 weeks at 28°C before the treatment. About 200mg of roots were cut while submerged in water and collected in a PCR-tube. Root segments were incubated for 5 hours at 28 °C before treatment. Root pieces were treated with water containing 100 µM CO7 (ELICITYL) for 10 min. After incubation, roots were immediately frozen in liquid nitrogen. Samples were homogenized using metal beads. Total root protein was extracted in a buffer containing 50 mM Tris·HCl (pH 7.5), 150mM KCl, 1mM EDTA (pH 7.5), 0.1% Triton X-100, 1 mM DTT, complete protease inhibitors (Roche), and phosstop (Roche). Amounts of extracted protein were measured with Qubit (Thermo fisher Scientific) and equal amounts of protein ~20 µg were electrophoresed by Mini-PROTEAN TGX stain free gels (BIORAD). A Trans-Blot Turbo Transfer system was used for blotting. To visualize phosphorylated MPK3/MPK6, the antibody for anti-phospho-p44/42 MAPK was used (no. 4370; Cell Signalling Technology). Anti-rabbit antibody (no. 7054; Cell Signalling Technology) were used as secondary antibody. Equal loading was confirmed by CBB staining.

Quantification and statistical analysis

Nodule number was quantified as Mean nodule number ± SE for all experiments. Replicate number is denoted in figure or figure legend. Additionally, all individual data points were plotted for graphical visualization of variation. Graphs and statistical analysis were performed using R studio 1.1.456 for nodulation experiments. Statistical tests on nodule numbers was done using One Way Analysis of variance (ANOVA) and a Tukey post-hoc test for multiple comparisons. Statistical significance was defined as a $p<0.05$. Levenes test for homogeneity of variance was used prior to running a one-way ANOVA. In cases where normality assumption was violated, alternative tests such as Mann–Whitney–Wilcoxon (MWW) were used as denoted in the figure legends. For the mycorrhization experiment a standard linear model was used to estimate the difference, and the corresponding least significant differences (LSD), of the knockout mutants with the wild type control. The LSD with respect to the control was Bonferroni adjusted to correct for multiple testing.

Accession numbers

Sequence data from this article can be found in the GeneBank/EMBL data libraries under accession numbers as mentioned in **Table S1**.

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Supplemental Data

The supplemental data belonging to this chapter are available online at plant Physiology: https://doi.org/10.1104/pp.19.1042

Supplemental Figure S1. Phylogeny reconstruction of orthogroups representing LysM-type receptors.

Supplemental Figure S2. Duplication of the *LYK3* first exon is conserved among *Parasponia* and *Trema* subspecies.

Supplemental Figure S3. Complementation of a lotus *Ljnfr1;Ljnfr5* mutant for LCO-induced calcium oscillation.

Supplemental Figure S4. Nodulation is affected in *Parasponia Pannfp1*, *Pannfp2* and *Panlyk3* CRISPR-Cas9 mutants.

Supplemental Figure S5. Complementation of *Parasponia Panlyk1*;*Panlyk3* double mutant.

Supplemental Figure S6. CO7 triggered ROS production and MPK phosphorylation in *Parasponia* mutant lines.

Supplemental Figure S7. Expression of *Parasponia* LysM-type receptors during mycorrhization.

Supplemental Figure S8. *Parasponia* LysM-type receptor can establish arbuscular mycorrhizal symbiosis.

Supplemental Figure S9. Conserved indel in NFP-II type receptor proteins

Supplemental Figure S10. The *PanNFP1* RNAi construct has off target activity on *PanNFP2* and other LysM-type receptor kinases.

Supplemental Table S1. *Parasponia andersonii* LysM-type receptors.

Supplemental Table S2. Trans-complementation of lotus *Ljnfr1;Ljnfr5* for nodulation.

Supplemental Table S3. Construct generated in this study.

Supplemental Table S4. Primers used in this study.

Supplemental Data Set S1. Sequence alignment of LYK-I type receptors in fasta format.

Supplemental Data Set S2. Sequence alignment of LYR-Ia type receptors in fasta format.

Supplemental Data Set S3. Genotyping and off target analysis of *Parasponia* CRISPR-Cas9 mutants generated in this study.

Supplemental Movie S1. Calcium spiking in root hairs of lotus *Ljnfr1-1;Ljnfr5-2* double mutant complemented with *LjNFR1;LjNFR5.*

Supplemental Movie S2. Calcium spiking in roor hairs of lotus *Ljnfr1-1;Ljnfr5-2* double mutant trans-complented with *PanLYK3.1;LjNFR5*.

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CHAPTER 4

An extremely rare non-canonical splice site found in *Trema orientalis* SYMRK does not affect its dual symbiotic functioning

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Abstract

Plants with nitrogen-fixing nodules occur in ten related taxonomic lineages interspersed with lineages of non-nodulating species. Nodules result from an endosymbiosis between the plant and diazotrophic bacteria, rhizobia in the case of legumes and *Parasponia,* or *Frankia* in the case of actinorhizal plants. Phylogenomic studies indicate that nodulation has a single evolutionary origin and that loss of this trait is associated with pseudogenization of nodulation-specific genes. The molecular signalling and cellular mechanisms critical for nodulation have been co-opted from the much older plant-fungal arbuscular endomycorrhizal symbiosis. Studies in legumes and the actinorhizal plants uncovered a key component in the symbiotic signalling complex, the LRR-type SYMBIOSIS RECEPTOR KINASE (SYMRK). SYMRK is essential for nodulation and arbuscular endomycorrhizal symbiosis. To our surprise, however, despite its arbuscular endomycorrhizal symbiosis capacities, we observed a seemingly critical mutation in a 5'-intron donor splice site in the *SYMRK* gene of *Trema orientalis,* the non-nodulating sister species of *Parasponia*. This led us to investigate the symbiotic functioning of *SYMRK* in the *Trema-Parasponia* lineage and we address the question of to what extent a single nucleotide polymorphism in an 5'-intron donor splice site affects the symbiotic functioning of *SYMRK*. We show that *SYMRK* is essential for nodulation and endomycorrhization in *Parasponia*. Subsequently, it is revealed that the 5'-intron donor splice site of *SYMRK* intron 12 is variable and in most dicotyledon species doesn't contain the canonical dinucleotide 'GT' signature but the much less common motif 'GC'. Strikingly, in *T. orientalis,* these dinucleotides converted into an extremely rare non-canonical 5'-intron donor splice site 'GA'. This *SYMRK* allele, however, is fully functional and spreads in the *T. orientalis* population in Malaysian Borneo. We conclude that *SYMRK* functioning is highly conserved in legumes, actinorhizal plants, and *Parasponia*. The gene possesses a non-common 5'-intron donor GC donor splice site in intron 12, which is converted into a GA in *T. orientalis* accessions of Malaysian Borneo. The *Parasponia-Trema* comparative system provides an experimental system to study splicing efficiency and the evolution of non-canonical splice sites.

Introduction

Plants have evolved a range of mutualistic endosymbiotic partnerships with microbes to enhance nutrient uptake. The most ancient mutualistic endosymbiosis is the interaction between plant roots and Glomeromycota fungi, also known as arbuscular mycorrhizal (AM) fungi, which evolved more than 450 million years ago (Remy *et al.*, 1994). Even today, AM endosymbiosis still occurs in ~80% of all higher plants (Brundrett & Tedersoo, 2018). Besides AM symbiosis, several plant lineages evolved additional or even alternative mutualistic endosymbiotic interactions, like orchid mycorrhiza, ericoid mycorrhiza, and diazotrophic rhizobia or *Frankia* bacteria hosted in root nodules. Interestingly, the evolution of these mutualistic endosymbiotic partnerships co-opted a signalling pathway critical for AM symbiosis. This pathway, known as the common symbiosis signalling pathway, is highly conserved and can be found in angiosperms, gymnosperms, monilophytes, and bryophytes species (Radhakrishnan *et al.*, 2020).

The common symbiosis signalling pathway was first discovered in pea (*Pisum sativum*), showing to be critical for AM symbiosis and rhizobium-induced nodulation (Duc *et al.*, 1989). Subsequent molecular genetic characterization in the legume models *Lotus japonicus* and *Medicago truncatula* revealed the pathway consists of four conserved components stretching from an LRR-type transmembrane receptor kinase down to the transcription factor *LjCYCLOPS/MtIPD3* (Parniske, 2008; Oldroyd, 2013). The LRR-type receptor kinase is generally called SYMRK (SYMBIOSIS SIGNALLING RECEPTOR KINASE). Its extracellular structure varies between different species, but in incase of eudicots possess a malectin domain, a conserved GDPC motif, and 2-3 LRR domains linked to a canonical intracellular serine-threonine kinase domain (Endre *et al.*, 2002; Stracke *et al.*, 2002; Markmann *et al.*, 2008; Kosuta *et al.*, 2011). The malectin domain is cleaved in the absence of symbiotic signalling (Antolín-Llovera *et al.*, 2014; Pan *et al.*, 2018). Studies in *L. japonicus* showed that the remaining part of the SYMRK protein interacts with the LysM-type transmembrane receptor LjNFR5 (Antolín-Llovera *et al.*, 2014; Pan *et al.*, 2018). LjNFR5 is part of the receptor complex essential for recognizing rhizobium secreted lipo-chitooligosaccharide (LCO) signal molecules (Madsen *et al.*, 2003; Gysel *et al.*, 2021). Legume *symrk* knockout mutants are blocked in rhizobium LCO-induced signalling through the common symbiosis signalling pathway. Subsequently, nodule

formation is not initiated, nor is infection initiated in *symrk* mutants (Catoira *et al.*, 2000; Endre *et al.*, 2002; Stracke *et al.*, 2002; Indrasumunar *et al.*, 2015). LjSYMRK is also found to interact with the innate immune receptor LjBAK1 (BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED RECEPTOR KINASE 1), which may allow repression of immune responses upon infection upon symbiotic infection (Feng *et al.*, 2021). Such a role is supported in *symrk* mutant analysis, revealing fortification of the plant cell wall upon *Glomus mosseae* AM fungus interaction in a specific *M. truncatula* mutant and in RNA interference (RNAi) roots upon rhizobium infection (Calantzis *et al.*, 2001; Limpens *et al.*, 2005).

Studies on *SYMRK* in non-legumes are limited. RNAi Knockdown studies in the actinorhizal plant *Casuarina glauca* and *Datisca glomerota* showed that, like in legumes, *SYMRK* is essential for nodulation (Markmann *et al.*, 2008; Gherbi *et al.*, 2008). This finding demonstrates that the common symbiosis signalling pathway defines a conserved genetic basis for nodulation with rhizobia or *Frankia*. More recent phylogenomic studies support the hypothesis that the nodulation trait has a single evolutionary origin in the last common ancestor of the orders Fabales, Fagales, Cucurbitales and Rosales, representing all ten nodulating plant lineages (Gherbi *et al.*, 2008; Griesmann *et al.*, 2018; van Velzen *et al.*, 2019). The occurrence of non-nodulating lineages in these four taxonomic orders allowed the identification of nodulation-specific genes, as such genes are prone to pseudogenization from the moment a plant lineage loses the nodulation trait.

We identified seven of such nodulation-specific genes by comparing nodulating *Parasponia* species to their non-nodulating sister species of the genus *Trema* (Velzen *et al.*, 2018). Among these is an *NFP*/*NFP5* orthologous LysM-type receptor named *NFP2* that is essential for nodulation in *Parasponia* (Rutten *et al.*, 2020). To our surprise, however, we identified also a seemingly critical mutation in *SYMRK* of *Trema orientalis* (accession RG33; *TorSYMRK*^{RG33}), which originates from the Sabah Province in Malaysian Borneo. It suggests that the *TorSYMRK*^{RG33} allele experiences pseudogenization, despite the fact *T. orientalis* accession RG33 still can establish an AM symbiosis (Velzen *et al.*, 2018). *TorSYMRK*^{RG33} has a conserved gene structure, though has a mutation in the conserved dinucleotide motif in the 5'-donor splice site of intron 12, converting this generally highly conserved dinucleotide motif into 'GA'. This led us to investigate the symbiotic functioning of *SYMRK* in the *Trema-Parasponia*

lineage. We addressed the question of to what extent a seemingly critical SNP in an intron donor splice site affects the symbiotic functioning of *TorSYMRK*.

Results

Trema orientalis and *Parasponia andersonii* differ in colonization by arbuscular mycorrhiza fungi

Since *SYMRK* is known to be important for arbuscular mycorrhization in a range of species (Endre *et al.*, 2002; Stracke *et al.*, 2002; Capoen *et al.*, 2005; Gherbi *et al.*, 2008), we first questioned whether *T. orientalis* accession RG33 can be effectively mycorrhized. To investigate this, we compared the mycorrhization dynamics of *T. orientalis* RG33 to *P. andersonii* (accession WU1). Both species are close relatives that diverged less than 20 million years ago (van Velzen *et al.*, 2019), though have a somewhat different root architecture. *T. orientalis* plantlets have a shorter main root, whereas its lateral roots are longer when compared to *P. andersonii* **(Figure S1)**.

To compare the mycorrhization efficiency, seedlings of both species were inoculated with 125 spores of *Rhizophagus irregularis* DOAM197198. Mycorrhization was quantified for 6 weeks, focussing on the frequency of mycorrhizal in the root system (F%), the intensity of mycorrhizal colonization in the root system (M%), the arbuscule abundance in the root system (A%), and arbuscule abundance in infected root segments (a%) (Trouvelot *et al.*, 1986). This revealed a clear difference in mycorrhization colonization dynamics between both species. The root system of *P. andersonii* is broadly colonized, showing an abundant presence of hyphae already 4 weeks post-inoculation (F% >80%, M% >50%, **Figure 1**). In contrast, *T. orientalis* RG33 showed a reduced mycorrhizal infection and a low abundance of mycorrhizal hyphae in the root (F% <20%, M% <10%, 4 weeks post-inoculation) **(Figure 1A)**. These reduced mycorrhizal infection rates of *T. orientalis* RG33 were also reflected in a reduced number of arbuscules found in the root system (A%). However, when evaluating root segments that were infected, the arbuscule abundance (a%) showed to be comparable to *P. andersonii* **(Figure 1B)**. Taken together, this indicates that *T. orientalis* RG33 is infected less frequently by *R. irregularis* DOAM197198 when compared to *P. andersonii*. But once infected, the number of arbuscules formed in the infected segment of the root is similar between both species.

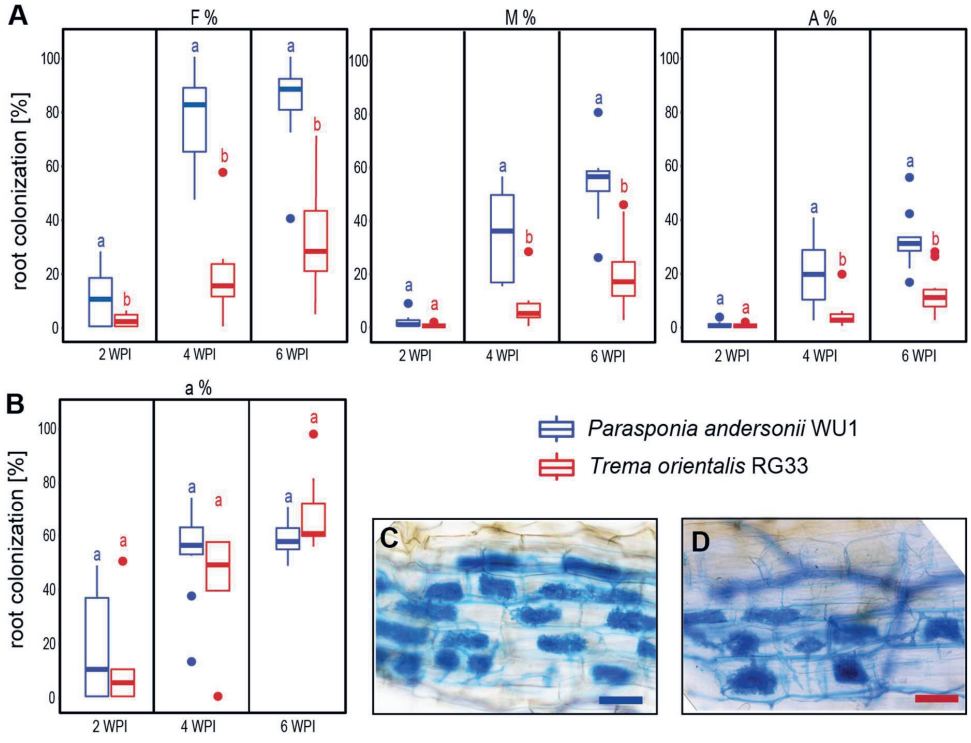

Figure 1. *Trema orientalis* accession RG33 and *Parasponia andersonii* accession WU1 differ in mycorrhiza colonization. (**A**) Comparison of mycorrhization efficiency in the root system of *P. andersonii* WU1 (blue) and *T. orientalis* RG33 (red) at 2, 4 and 6 weeks post-inoculation with *Rhizophagus irregularis* DOAM197198. F%: The frequency of mycorrhiza in the root system. M%: the intensity of mycorrhizal colonization in the root system. A%: Arbuscule abundance in the root system. (**B**) a%: Arbuscule abundance in 50 randomly selected 1 cm of root segments. Data shown represents 10 biological replicates, for each 50 x 1cm segment that has been analyzed. Analysis done according to Trouvelot *et al.* (1986). Different letters above the boxes indicate statistical significance ($p < 0.01$) as determined by the Kruskal-Wallis test in combination with Fisher's post-hoc test. (**C**) Trypan blue-stained *P. andersonii* and (**D**) *T. orientalis* root segment visualising *R. irregulates* arbuscules 6 weeks post-inoculation. Size bar = 50 µm.

Parasponia andersonii SYMRK is essential for arbuscular mycorrhization and nodulation

As *T. orientalis* RG33 can establish an arbuscular mycorrhizal symbiosis, we questioned whether *SYMRK* represents a single copy gene in the *Trema-Parasponi*a taxonomic lineage. We analysed genome sequences of species in a broad phylogenetic context. As an outgroup, the closest *SYMRK* paralogs of *P. andersonii* and *T. orientalis* were included. This revealed that *SYMRK* is a single copy gene in the *Parasponia - Trema* lineage **(Figure S2)**.

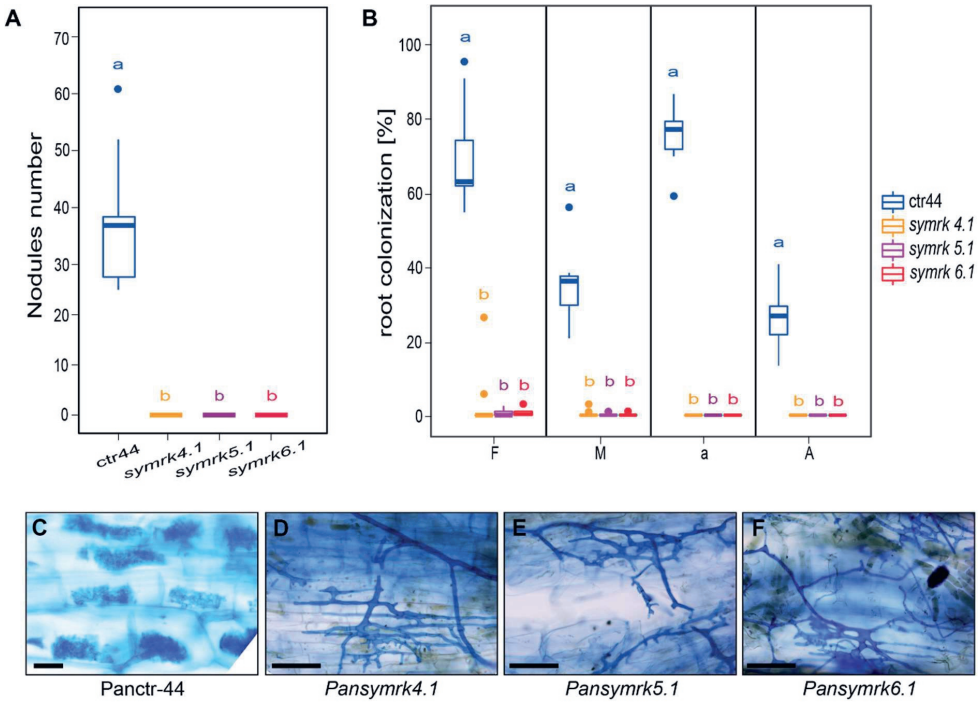

Figure 2: *Parasponia andersonii PanSYMRK* is essential for mycorrhization and nodulation. (**A**) Nodule numbers formed in *P. andersonii* empty vector (ev) control line (ctr44) and three *Pansymrk* mutant lines, 6 weeks post-inoculation with *Mesorhizobium plurifarium* BOR2. (**B**) Mycorrhization efficiency in the root system of *P. andersonii* ev-control and three independent *Pansymrk* mutant lines 6 weeks post-inoculation with *Rhizophagus irregularis* DOAM197198. F%: The frequency of mycorrhiza in the root system. M%: the intensity of mycorrhizal colonization in the root system. A%: Arbuscule abundance in the root system. a%: Arbuscule abundance in mycorrhizal parts of root segments. Data shown represents 10 biological replicates. For ctr44, *Pansymrk4.1*, *Pansymrk5.1*, and *Pansymrk6.1*, 50, 417, 760, and 1085 x 1 cm segment, respectively, has been analyzed. Analysis was done according to Trouvelot *et al*. (Trouvelot *et al.*, 1986)(**C-F**): Trypan blue-stained *P. andersonii* ev-control (**C**), *Pansymrk*-4.1 (**D**), *Pansymrk*-5.1 (**E**), and *Pansymrk*-6.1 (**F**) root segment visualizing *R. irregulates* infections 6 weeks post-inoculation. Size bar = 25 µm.

Mutant analysis In legumes and the actinorhizal species *C. glauca* showed that *SYMRK* commits a dual role in the establishment of arbuscular mycorrhizal symbiosis and nodulation (Catoira *et al.*, 2000; Endre *et al.*, 2002; Stracke *et al.*, 2002; Demchenko *et al.*, 2004; Gherbi *et al.*, 2008; Indrasumunar *et al.*, 2015). To determine whether *SYMRK* in the *Parasponia-Trema* lineage fulfils a similar dual role, we generated CRISPR/Cas9 *symrk* knockout mutants in *P. andersonii.* In total, three knockout mutant lines (homozygous line *Pansymrk*4.1, and bi-allelic lines *Pansymrk*5.1 and *Pansymrk*6.1) were obtained by targeting the fourth and fifth coding exon using two single guide RNAs (sgRNAs) **(Figure S3A)**. All mutant alleles contain large deletions, and encode only a fragment of the extracellular domain **(Figure S3B)**.

To determine whether SYMRK commits a key symbiotic function in *Parasponia,* we first studied the nodulation phenotype of the Pan*symrk* mutants. Pan*symrk*4.1, Pan*symrk*5.1 and Pan*symrk*6.1 plantlets were inoculated with *Mesohizobium plurifarium* BOR2 and the nodulation phenotypes were examined six weeks post-inoculation. The transgenic empty vector control plants (ctr-44) were effectively nodulated, having nodule numbers ranging from 25 to 61 per plant. In contrast, the three Pan*symrk* mutant lines were unable to nodulate **(Figure 2A)**. Next, we investigated the role of *PanSYMRK* in arbuscular mycorrhizal symbiosis. Pan*symrk*4.1, Pan*symrk*5.1, Pan*symrk*6.1, and crt-44 control plantlets were inoculated with an *R. irregularis* DAOM 197198 spore suspension. Mycorrhization phenotypes were examined six weeks post-inoculation by quantifying four parameters; F%, M%, a%, and A%, as described above. The empty vector control plants (ctr-44) interacted normally with the applied symbiont, with F%, M%, a% and A% of 65,4%, 36,8%, 77,1%, and 26,1%, respectively **(Figure 2B,C)**. Although some intraradical hyphae were found in a minority of the Pan*symrk* root segments (7 out of 417, 6 out of 760, and 9 out of 1085 segments, respectively) **(Figure 2B, D-F)**, no arbuscules were observed in any of the tested *Pansymrk* mutant plantlet's roots. Taken together, this demonstrates that *SYMRK* is essential for nodulation and arbuscular mycorrhization of *P. andersonii* roots.

The GA mutation of the 5'-donor splice in intron 12 does not affect *SYMRK* functionally

As *T. orientalis* RG33 -possessing a single *SYMRK* copy- can be mycorrhized effectively, it suggests that the *TorSYMRK^{RG33}* allele encodes a functional protein to support this plant-fungus symbiosis. The *M. truncatula* R38 *dmi2/symrk* mutant revealed that the SYMRK requirements differ between mycorrhizal colonization and rhizobium nodulation (Endre *et al.*, 2002). *M. truncatula* R38 possesses a missense mutation converting a glycine to glutamic acid mutation at position 794 of the protein (Endre *et al.*, 2002). This mutation affects the kinase phosphorylation activity and the capacity of the protein to interact with potential downstream target 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase1 (HMGR) (Kevei *et al.*, 2007; Jayaraman *et al.*, 2017). This *SYMRK* mutation affects nodulation but not mycorrhization, suggesting a functional kinase domain is less important for the latter interaction (Endre *et al.*, 2002). As the *T. orientalis SYMRK^{RG33}* may encode -at least in part- a truncated SYMRK protein lacking essential domains of the kinase motif **(Figure 3A)**, we question to what extent this allele could function in nodulation. To investigate this, we first

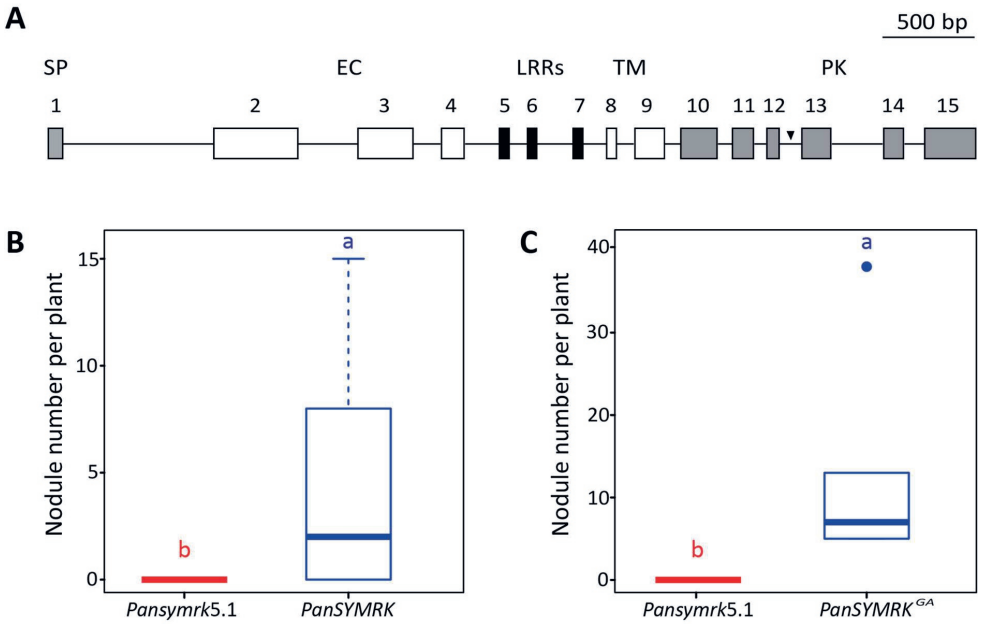

Figure 3: A full length *TorSYMRK*^{RG33} can trans-complement a *Parasponia andersonii symrk* mutant. (**A**) Structure of *TorSYMRK*^{RG33} gene and encoded protein**.** Exon are depicted as boxes, introns as a black line. SP: predicted signal peptide; EC: extracellular domain; LRR: leucin-rich repeat motifs; TM: transmembrane domain; PK: protein kinase domain. Indicated by a black arrow head is the position of the mutation in the intron 12 (**B**) *P. andersonii Pansymrk*5.1 functional complementation with pPanSYMRK:*PanSYMRK*gene using *A. rhizogenes*-mediated root transformation. (**C**) *P. andersonii Pansymrk*5.1 complementation with pPanSYMRK:*PanSYMRK*^{GA} gene using *A. rhizogenes*-mediated root transformation. Different letters above the boxes indicate statistical significance ($p < 0.05$) as determined by student t-test.

identified the native promoter region of *P. andersonii*. Using *A. rhizogenes* root transformation, we showed that a ~3 kb upstream region -including the 5'UTR of *PanSYMRK* driving the *PanSYMRK* gene 6.05 kb could functionally complement *Pansymrk* 5.1 mutant. On average 4.9 nodules/plant at 8 wpi **(Figure 3B)**. Next, we used this promoter to drive *PanSYMRK* gene harbouring a GA mutation at the donor site of intron 12, mimicking *TorSYMRK*^{RG33} allele to determine its functionality in the *P. andersonii Pansymrk* 5.1 mutant background. Using *A. rhizogenes* root transformation, we found full complementation of *Pansymrk* 5.1 mutant. On average, 13.6 nodules/plant were formed at 8 wpi **(Figure 3C)**. Sections of these nodules revealed a wild-type cytoarchitecture, including a large zone of cells possessing fixation threads. This shows that the GA point mutation at the donor site of intron 12 is not affecting *SYMRK* gene functionality.

Table 1. Splicing efficiency of *Trema orientalis* and *Parasponia andersonii SYMRK* in the root zone susceptible for symbiotic engagement. Quantification based on RNA-seq samples of three biological replicates.

SYMRK	*T. orientalis*	root susceptible zone		*P. andersonii*	root susceptible zone	
	length (bp)	Coverage	s.d.	length (bp)	Coverage	s.d.
exon1	99	28,1	8,9	99	94,6	39,6
intron1	765	0,0	0,1	912	0,1	0,1
exon2	525	35,2	13,2	525	96,5	17,6
intron2	339	0,0	0,0	341	0,1	0,2
exon3	475	51,2	9,1	475	114,6	17,1
intron3	351	0,0	0,0	364	0,0	0,0
exon4	150	59,3	23,4	150	127,0	24,9
intron4	188	0,1	0,1	175	0,1	0,2
exon5	71	89,2	27,6	71	166,1	29,4
intron5	103	0,3	0,2	102	1,0	0,7
exon6	68	84,4	20,7	68	133,1	22,2
intron6	202	2,7	0,5	319	0,2	0,2
exon7	71	70,7	18,3	71	125,0	13,2
intron7	133	0,0	0,0	134	0,3	0,4
exon8	71	62,2	16,9	71	138,8	15,5
intron8	97	0,9	1,1	86	0,1	0,1
exon9	193	76,1	16,1	193	142,9	22,8
intron9	98	1,9	0,6	98	0,9	0,9
exon10	235	79,6	13,5	235	155,3	18,6
intron10	83	0,1	0,1	90	0,3	0,6
exon11	126	100,8	13,3	126	158,0	35,0
intron11	65	1,3	1,9	83	10,5	4,3
exon12	86	91,8	19,3	86	158,9	34,5
intron12	118	4,6	1,0	118	0,2	0,3
exon13	189	109,4	13,8	189	159,3	16,9
intron13	276	1,6	1,0	273	0,7	0,6
exon14	132	155,1	7,4	132	255,8	22,9
intron14	112	4,2	4,3	112	0,4	0,7
exon15	326	135,4	9,4	326	207,6	13,0

A GA 5'-donor splice site is extremely rare though effectively spliced in *TorSYMRK^{RG33}*

We questioned how effective an intron that possesses a GA as the first two nucleotides of a donor splice site is actually spliced. To determine this, we aimed to compare the coverage of RNAseq reads of the 15 exons and 14 introns of the *SYMRK* gene of *T. orientalis* and *P. andersonii*. In both species, *SYMRK* is highly similar, though introns show some variation in length **(Table 1)**. *SYMRK* is known to be expressed in the root (Stracke *et al.*, 2002). We grow *T. orientalis* and *P. andersonii* seedlings *in vitro* on a low nitrate medium and subsequently

Table 2. Frequency of predicted canonical and non-canonical donor splice sites in *Trema orientalis*, *Parasponia andersonii*, *Lotus japonicus*, *Medicago truncatula* and *Arabidopsis thaliana* gene models.

donor splice	*T. orientalis*		*P. andersonii*		*M. truncatula*		*L. japonicus*		*A. thaliana*	
site	Total	%	Total	%	Total	%	Total	%	Total	%
AA	27	0,03%	28	2,95%	0	0	7	2,62%	4	3,15%
AC	8	0,01%	2	0,21%	0	0	31	11,61%	0	0,00%
AG	14	0,01%	11	1,16%	0	0	44	16,48%	1	0,79%
AT	59	0,06%	47	4,96%	0	0	60	22,47%	91	71,65%
CA	19	0,02%	3	0,32%	0	0	15	5,62%	0	0,00%
CC	1	0,00%	0	0,00%	0	0	0	0,00%	0	0,00%
CG	2	0,00%	0	0,00%	0	0	5	1,87%	1	0,79%
CT	66	0,06%	44	4,64%	0	0	26	9,74%	0	0,00%
GA	10	0,01%	5	0,53%	0	0	62	23,22%	12	9,45%
GC	551	0,53%	546	57,59%	1044	0,80%	1523	570,41%	1193	0,93%
GG	59	0,06%	46	4,85%	0	0	4	1,50%	0	0,00%
GT	102252	98,18%	100857	98,54%	128794	99,20%	113255	98,39%	126501	98,96%
TA	650	0,62%	425	44,83%	0	0	39	14,61%	0	0,00%
TC	3	0,00%	6	0,63%	0	0	25	9,36%	0	0,00%
TG	383	0,37%	264	27,85%	0	0	4	1,50%	0	0,00%
TT	40	0,04%	72	7,59%	0	0	7	2,62%	30	23,62%
Canonical	102813	98,72%	101408	99,07%	129838	100,00%	114840	99,77%	127706	99,90%
Non-canonical	1331	1,28%	948	0,93%	0	0	267	0,23%	127	0,10%
Grand Total	**104144**	**100,00%**	**102356**	**100,00%**	**129838**	**100,00%**	**115107**	**100,00%**	**127833**	**100,00%**

isolated 1 cm regions of roots just above the root meristemic zone. RNA extracted from these samples was sequenced (in triplicates), mapped, and analysed. To our surprise, this uncovered a splice variant of *P. andersonii PanSYMRK*, based on an alternative splicing acceptor site of intron 11, though which we decided not to study it any further **(Figure S4)**. When focussing on intron 12, we found a coverage of 4.6±1.0 reads for *TorSYMRK*^{RG33}, whereas in *P. andersonii,* the coverage of this intron is only 0.2±0.3 reads **(Table 1)**. This may suggest that *SYMRK* intron 12 is spliced less efficient in *T. orientalis* when compared to *P. andersonii.* However, similar variance is observed also for other introns, which possess canonical splice sites **(Table 1)**. Therefore, we conclude that *SYMRK*^{RG33} intron 12 is effectively spliced, despite a non-canonical GA dinucleotide motif in the donor splice site.

Next, we questioned how unique a GA donor splice site is in plants. For this, we analysed all annotated introns in *T. orientalis*, *P. andersonii,* as well as in the model plant species *L. japonicus, M. truncatula*, and *Arabidopsis thaliana*. This showed that a GA donor splice site is extremely rare, varying from none in the annotated gene models of *M. truncatula* to 62 in *L. japonicus* **(Table 2)**. When analyzing the gene models carrying GA donor splice side introns, we noted three interesting aspects. (i) The GA intron splice sites are not conserved among

orthologous genes of the five species, not even in both legumes, (ii) none of the genes have been functionally characterized, and (iii) some gene models possess multiple GA donor splice site introns. This let us to conclude that a GA dinucleotide motif in the donor splice site is extremely rare in dicot plants and has not been described in any characterized gene of the five species studied.

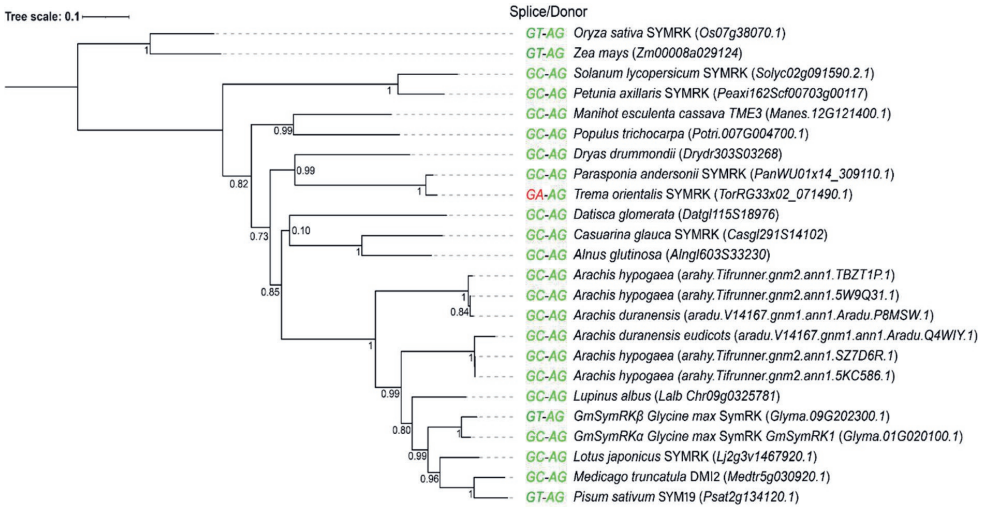

Figure 4. Phylogeny of *SYMRK* including the splice site dinucleotide motifs for intron 12. Phylogeny was reconstructed based on an alignment of SYMRK orthologous proteins from 19 species. Terminals are labelled by their gene name or gene identifier. The non-canonical GC donor splice site is common in *SYMRK* intron 12 of dicotyledon species, except in *Glycine max* SYMRKβ and *Pisum sativum* SYM19, where GC is substitute by GT. In contrast, only *Trema orientalis* RG33 posses a GA motif in this position (highlighted in red).

The *SYMRK*^{RG33} allele is common in *Trema orientalis* of Malaysian Borneo

As *T. orientalis* RG33 possesses an extremely rare GA motif at the donor splice site of intron 12, we question to what extent such polymorphism is unique to *SYMRK*. First, we analysed *SYMRK* orthologs of 19 species representing monocots and major clades of dicots, including species of the Fabales, Fagales, Cucurbitales, and Rosales. This showed that a non-canonical GC donor splice site is common in *SYMRK* intron 12 of dicotyledon species **(Figure 4)**. Though, none of the analysed *SYMRK* genes possesses an extremely rare GA motif at this position. Subsequently, we analysed *SYMRK* of the *Parasponia-Trema* species complex. Among others, *T. orientalis* accession RG33 was collected during an expedition in Sabah Province, Malaysian

Borneo in 2012 (Merckx *et al.*, 2015; Velzen *et al.*, 2018). We analyzed 27 additional *T. orientalis* individuals collected from five distinct locations in Malaysian Borneo **(Figure 5A)**. All possess the rare GA intron 12 donor splice, whereas this mutation is absent in *Trema* and *Parasponia* accessions sampled outside Borneo **(Figure 5B; Table S1)**. This demonstrates that the *SYMRK*^{RG3} allele is not unique, though it associates with the Borneo *T. orientalis* population.

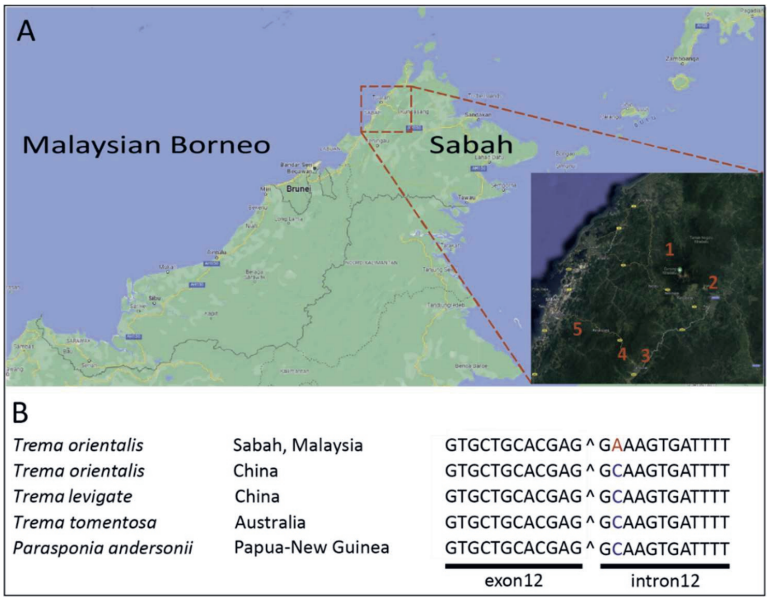

Figure 5. *SYMRK* intron 12 unique non-canonical donor splice site occurs in a *Trema orientalis* population endogenous to Sabah, Malaysia. (**A**) Locations of 28 *Trema orientalis* specimens collected in Malaysian Borneo, province of Sabah. 1: Sayap, 2: Poring, 3: Mahua, 4: Gunung Alab, and 5: Inobong. Plants were collected in 2012 as described in Merckx et al. (Merckx *et al.*, 2015)(see also **Table S1**). (**B**) The 'GA' donor splice site of intron 12 is unique to *Trema orientalis* of Sabah, Malaysia, whereas related accessions and species possess a non-canonical 'GC' at this position in *SYMRK*.

Discussion

The LRR-type receptor kinase SYMRK is a critical component in the common symbiosis signalling pathway controlling endosymbioses. In legumes, SYMRK is essential for rhizobium LCO-induced signalling. We identified a seemingly critical mutation of the conserved dinucleotide motif in the 5'-donor splice site in *T. orientalis SYMRK* accession RG33. *T. orientalis* is a non-nodulating relative of nitrogen-fixing *Parasponia* species and has experienced pseudogenization of several key nodulation genes (Velzen *et al.*, 2018). Here we

show that despite the loss of a conserved splice site motif, *TorSYMRK*^{RG33} remains a functional allele that can be effectively spliced. The dominant occurrence of the *TorSYMRK*^{RG33} allele in the Malaysian Borneo *T. orientalis* population underlines the splice site mutation is not affecting the fitness of the tree.

Splicing is a highly conserved process in eukaryotes, requiring a spliceosome complex consisting of five small nuclear RNAs and several proteins. The vast majority of introns are spliced by the so-called U2-type spliceosome, recognizing two highly conserved di-nucleotide motifs at the start and end of the intron sequence, namely GT-AG. Bioinformatic studies in plant, animal, and fungal species indicate that in less than 2% of cases, alternative dinucleotide motifs are used, among which GC-AG is the most abundant non-canonical splice motif representing 1.5% of all introns annotated in plant gene models (Parada *et al.*, 2014; Frey & Pucker, 2020). The GA-AG splicing motif, as found in *TorSYMRK*^{RG33} intron 12, is reported to occur in >0.03% of the cases (Frey & Pucker, 2020). This frequency is in line with what we found when analysing the predicted gene models of *T. orientalis, P. andersonii, L. japonicus, M. truncatula,* and *A. thaliana* **(Table S2)**. To our knowledge, none of these gene models has been verified experimentally. In this regard, *TorSYMRK*^{RG33} represents the first plant gene in which a GA-AG dinucleotide splicing motif is confirmed to be functional.

The selection mechanisms driving the evolution of rare non-canonical splice sites remain elusive. The GA-AG dinucleotide splicing motif was found in higher frequency in two non-related animal species; the copepod *Eurytemora affinis* and the tunicate *Oikopleura dioica* (Lee, 2016; Robertson, 2017; Frey & Pucker, 2020). However, it remains unknown whether both species have gained these by convergent evolution or, alternatively, it is an ancestral trait that is preserved in only a few species (Frey & Pucker, 2020). In the case of *SYMRK*, we noted that in related species, *SYMRK* intron 12 possesses the more common non-canonical GC-AG dinucleotide splice motif. This may lead to the hypothesis that such GC-AG motif is the ancestral state allowing the evolution of the even more rare GA-AG motif. We inserted the GC to GA mutation in the *P. andersonii* SYMRK gene and showed that this variant is fully functional when expressed under its native promoter. This suggests that a simple single nucleotide polymorphism is sufficient to allow the evolution of the GA-AG dinucleotide splicing motif in *TorSYMRK*^{RG33}.

By making CRISPR-Cas9 *P. andersonii* mutants, we demonstrated for the first time that SYMRK commits a similar dual symbiotic role in nodulation and AM symbiosis in non-legumes as reported for legumes. An earlier RNAi study in the actinorhizal plant *C. glauca* provided evidence that *SYMRK* is also critical for *Frankia*-induced nodulation (Gherbi *et al.*, 2008). Taken together, it supports the hypothesis that *SYMRK* -and other components of the common symbiosis signalling pathway- have been recruited to function in nodulation in a common ancestor that lived before the divergence of the Fabales, Fagales and Rosales orders.

The *Parasponia-Trema* comparative system is established to obtain insight into the evolutionary trajectory of the nodulation trait. It uncovered a number of genes critical for rhizobium-induced nodulation in a non-legume (Velzen *et al.*, 2018; Bu *et al.*, 2020; Rutten *et al.*, 2020). Eventually, *Trema* species can serve as an experimental test system to uncover essential genes to rebuild the nodulation trait. Additionally, however, we demonstrated that the *Parasponia-Trema* comparative system is equally valuable to uncovering the functionality of rare non-canonical splicing motifs.

Material and Methods

Plant materials and growth conditions

P. andersonii WU1 and *T. orientalis* RG33 were grown and maintained as described previously (van Zeijl *et al.*, 2018; Wardhani *et al.*, 2019). Plantlets for nodulation and mycorrhization assay were vegetatively propagated *in vitro* and rooted (van Zeijl *et al.*, 2018; Wardhani *et al.*, 2019).

Mycorrhization assays and trypan blue staining

Mycorrhization assays were performed using a commercial spore of *Rhizopagus irregularis* (Agronutrion-DAOM197198, Carbonne, France). Spores inoculum, inoculation, and trypan blue staining were prepared and performed as described previously (Wardhani *et al.*, 2019; **Chapter 2**).

Quantification of mycorrhization

To quantify mycorrhization, a minimum of ~50 cm roots for each sample were cut into 1 cm fragments. 25-30 root fragments were placed on a single microscope slide, and 30% glycerol was added. Roots were covered with a cover glass and pressed until root fragments became flat. The frequency of mycorrhiza (%F), the intensity of mycorrhizal colonization (%M), and arbuscules abundance (%A) in the root system were scored and calculated according to Trouvelot et al. (Trouvelot *et al.*, 1986).

Nodulation assay

According to the previous reports, plantlets for nodulation assay were inoculated with *Mesorhizobium plurifarium* BOR2 (OD600 = 0.05) (van Zeijl *et al.*, 2018; Velzen *et al.*, 2018; Wardhani *et al.*, 2019). Six weeks *Parasponia* plants were removed from the pots, and roots were washed with running water to remove perlite. Nodules were counted and stored for the following purposes. For the complementation study, after 8 weeks of incubation, plant roots were examined under fluorescent stereo microscopy, where nodule number was quantified for each transgenic root (Wardhani *et al.*, 2019).

Root growth assay

Five seedlings of *P. andersonii* and *T. orientalis* RG33 were grown on ½ strength modified Hoagland medium in 12 cm square plates. Plants were grown vertically at a 60-degree angle for 21 days at 28°C, 16/8h day-night regime. The primary root was determined as the main root that emerged from cotyledon, whereas lateral roots were determined as roots that emerged from the primary root. Per plants, primary root length, the average number of lateral roots, and lateral root density (per cm main root) were determined 21 days post germination. Primary root growth was measured by following its development every day for 21 days post germination. The average lateral root length was determined by measuring its size in five selected lateral roots 21 days post germination.

Vectors and Constructs

Single-guide RNAs (sgRNAs) were designed using the 'Find CRISPR Targets' function implemented in Geneious software v 9.1.5 (Biomatters, New Zealand) and subsequently checked against the *P. andersonii* genome for high identity off-targets. For CRISPR/Cas9-mediated mutagenesis and complementation studies, binary transformation constructs were created using Golden Gate assembly as described previously (van Zeijl *et al.*, 2018; Wardhani *et al.*, 2019), and a list of constructs generated from both studies is listed in **Supplemental Table S3**. For CRISPR/Cas9-mediated mutagenesis, two sgRNAs were used to target the fourth and the fifth coding exon of *PanSYMRK* **(Supplemental Figure 3)**. Selected sgRNAs were amplified using sequence-specific forward primers and a universal reverse primer **(Supplemental Table S4)**, using Addgene plasmid no. 46966 as template (Nekrasov *et al.*, 2013). To allow for Golden Gate cloning, BpiI and BSaI restriction sites in the putative promotor sequence of *PanSYMRK* were mutated by introducing single nucleotide substitution (Engler *et al.*, 2014). For the complementation study, the sequence of *Parasponia SYMRK* promoter, 5' untranslated region (5' UTR), genomic DNA, 3' untranslated region (3' UTR), and terminator were synthesized. Also, a modified version of *Parasponia SYMRK* genomic DNA were synthesized where is harboring a point mutation at the donor splice site of the 12th intron, mimicking *Trema orientalis SYMRK*. (Invitrogen, Thermo Fisher Scientific, United States).

Plant transformation

Agrobacterium tumefaciens-mediated transformation and genotyping were done based on previously published protocols (van Zeijl *et al.*, 2018; Wardhani *et al.*, 2019). Primers used for genotyping are listed in **Supplemental Table S4**. Hairy root transformation was performed according to Cao et al. (Cao *et al.*, 2012), where *Agrobacterium rhizogenes* harboring plasmid DNA of interest was used to infect micropropagated plants wounded on their base. Infected plants were grown on agar plates of Schenk and Hildbrandt medium (SH medium) (Schenk & Hildebrandt, 1972) and incubated at 21°C for one week on a 16/8h light/dark regime. Transformed plants were transferred to agar plates of SH medium supplemented with, 10 g sucrose/L as well as cefotaxime 100 μg/mL, and kanamycin 50 μg/mL antibiotics and then incubated at 21°C for one more week. Plates were incubated at 28°C for two weeks, and plants were checked under fluorescence stereo microscopy for looking transgenic roots.

RNA Sequencing

For RNA isolation, tissue was harvested from ~1 cm region just above the meristematic zone 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 in triplicate as previously described (Velzen *et al.*, 2018). Library preparation and RNA sequencing was conducted by B.G.I (Schenzhen, China). Mapped RNA-sequencing data covering the SYMRK gene in *Parasponia andersonii* and *Trema orientalis* were visualized using Intergrative Genomics Viewer (IGV) (Robinson *et al.*, 2011). Based on the different splice sites, two SYMRK splice variants were manually constructed. Functional protein domains for these variants were annotated using InterProScan 5 (Jones *et al.*, 2014).

Phylogenetic reconstruction

The proteomes of 51 plant species belonging to the Nitrogen fixation clade and two species belonging to the Solanales and Poales were clustered using OrthoFinder v2.5.4. (Emms & Kelly, 2019). The SYMRK orthogroup was found by searching for the *Lotus japonicus* SYMRK (Lj2g3v1467920.1) gene identifier. SYMRK orthologous proteins were extracted and aligned using Clustal Omega 1.2.3. (Sievers *et al.*, 2011). A phylogenetic SYMRKt tree was constructed

using PhyML 3.0 (Guindon *et al.*, 2010) with LG substitution model 1000 bootstrap replicates and rooted on the two Poales outgroup species. The tree was visualized using Interactive Tree Of Life (iTOL) tree viewer (Letunic & Bork, 2007). A sub-selection of 19 species was extracted from the SYMRK orthogroup, and a tree was constructed using the same methods described above. Based on the SYMRK gene models for these 19 species, the splice site for SYMRK intron 12 was added.

Statistical analysis

Graphs and statistical analysis for mycorrhization quantification were performed using R studio 1.1.456. Ramf R package were used to analyze and display of quantitative AM fungal root colonization data (Chiapello *et al.*, 2019). Statistical tests on three classes of mycorrhization efficiency were done using Kruskal-Wallis test in combination with the post-hoc test using Fisher's least significant difference criterion. Statistical significance was defined as a $p<0.01$. A statistical test on root growth assays and for nodules number quantification on complementation study were done using a student t-test. Statistical significance for these parameters was defined as a $p<0.05$.

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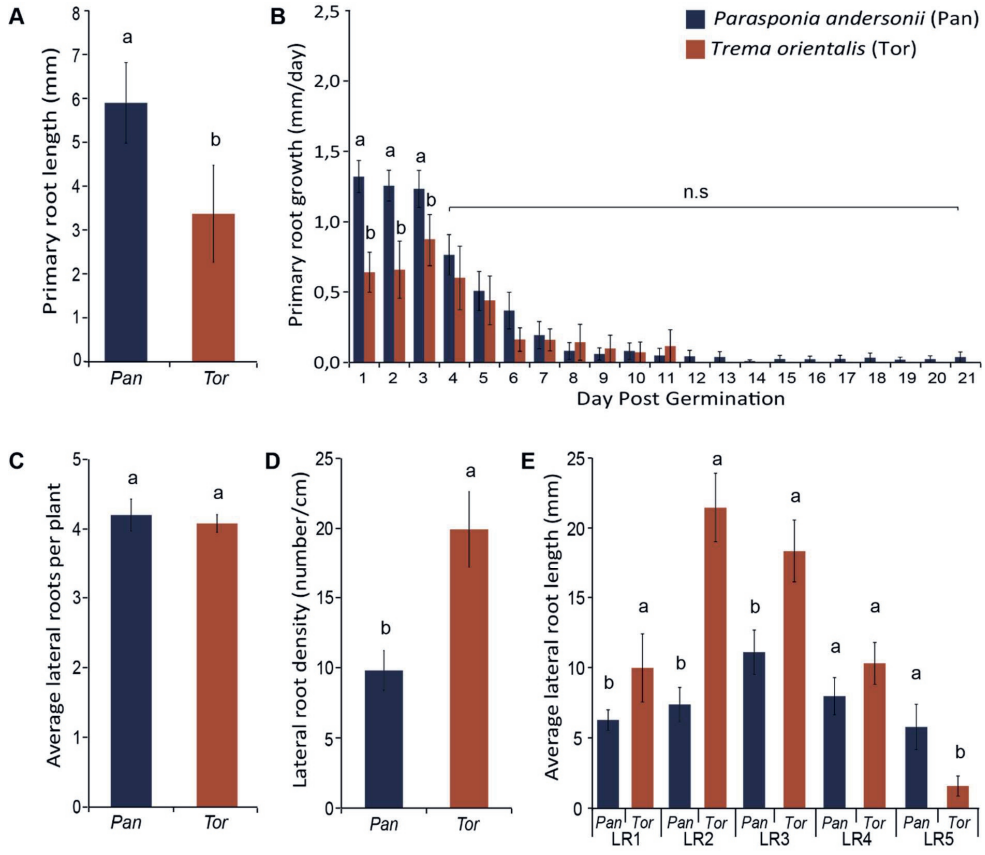
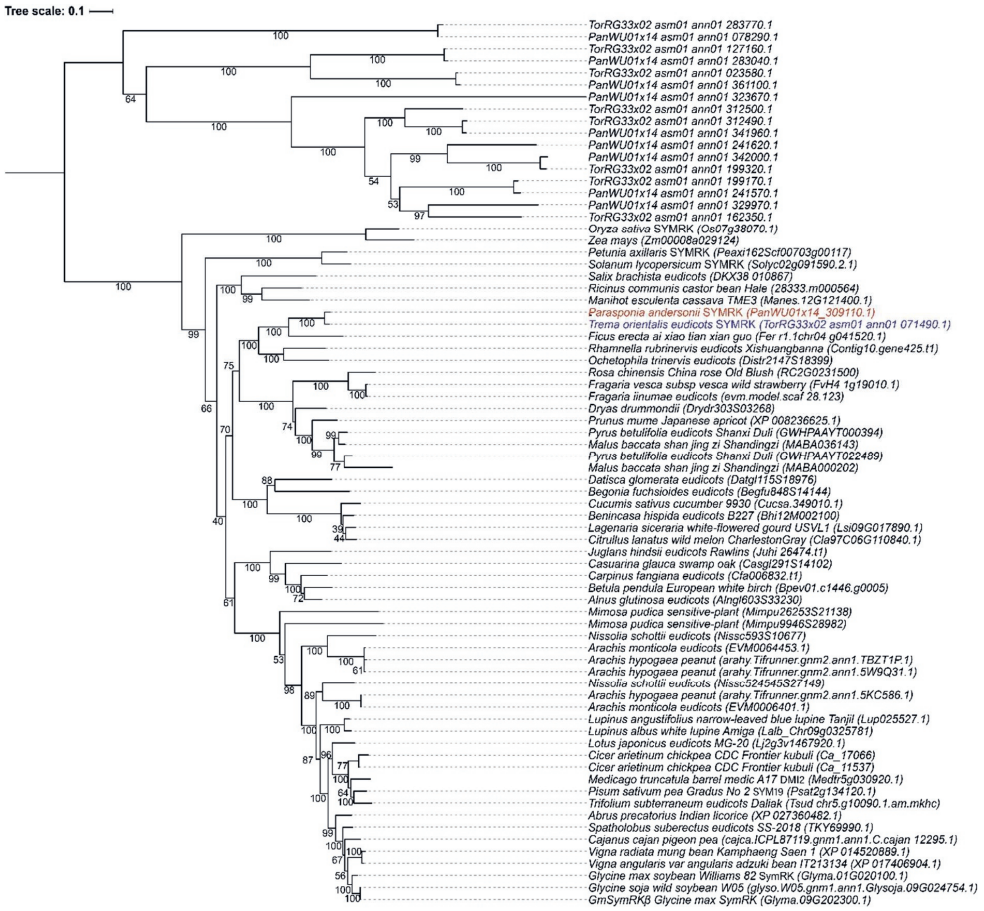
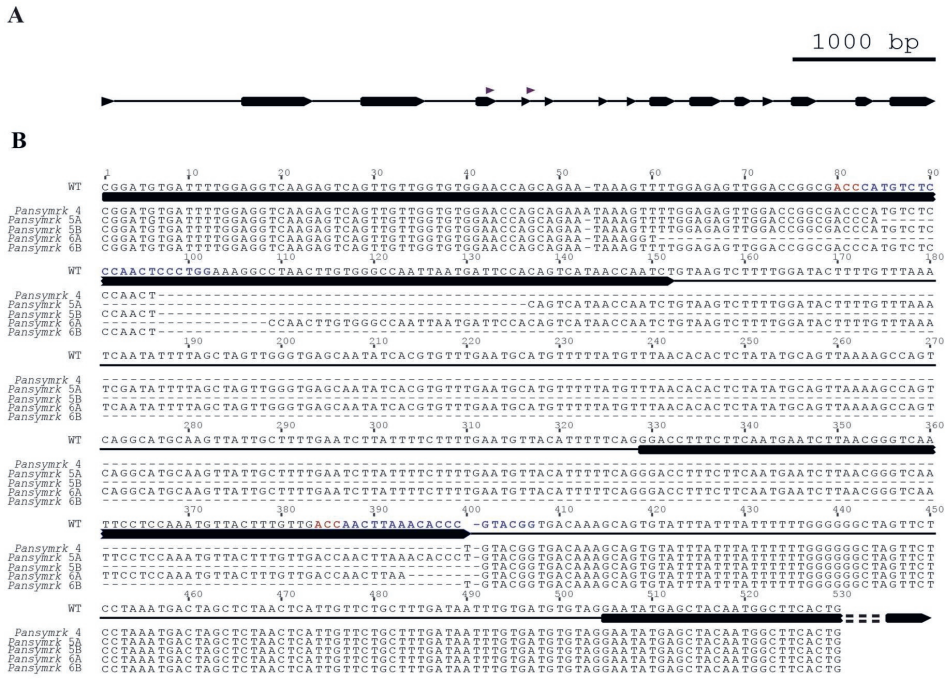
Supplemental Information



Supplemental Figure S1: Root architecture of *Trema orientalis* accession RG33 and *Parasponia andersonii* accession WU1 differs. (**A**) Primary root length 21 days post-germination. (**B**) Growth of primary root 1-21 days post-germination (mm/day) (**C**) The average number of lateral roots per plant, and (**D**) lateral root density (cm^{-1} main root) 21 days post-germination (**E**) Average lateral root length in five selected root 21 days post-germination (mm). n=5 +/- s.e. Different letters above the bars indicate statistical significance ($p < 0.05$) as determined by student t-test. Tor, red: *T. orientalis* RG33, Pan, blue: *P. andersonii* WU1. Plants were grown *in vitro* on ½ strength modified Hoagland medium in 12 cm square plates.



Supplemental Figure S2: Phylogenetic tree of the SYMRK orthogroup. Phylogeny was reconstructed on an alignment of SYMRK protein from 51 plant species belonging to the Nitrogen fixation clade and two species belonging to the Solanales and Poales. In addition, SYMRK-like genes in Trema and Parasponia are added to show that these genes are outside the orthogroup. Therefore, *SYMRK* is a single copy gene in *Trema* and *Parasponia*. Branch support is indicated by posterior probabilities. Terminals are labelled by their name gene or genbank identifier.



Supplemental Figure S3: *Parasponia andersonii symrk* CRISPR-Cas9 mutant alleles. (**A**) Structure of *PanSYMRK* gene spanning 7,280 bp and possessing 15 exons and 14 introns. Indicated by a purple arrowheads are the positions of two sgRNAs in exon 4 and 5. (**B**) Sequence alignment of the fourth and fifth exons of *PanSYMRK* in wild type (WT) and *pansymrk* mutants (line 4, 5, & 6). Line 4 is homozygote whereas line 5 and 6 are bi-allelic. In bi-allelic mutant lines, both alleles (A and B) are shown. Highlighted in blue and red are the sgRNA target sites and PAM sequences, respectively.

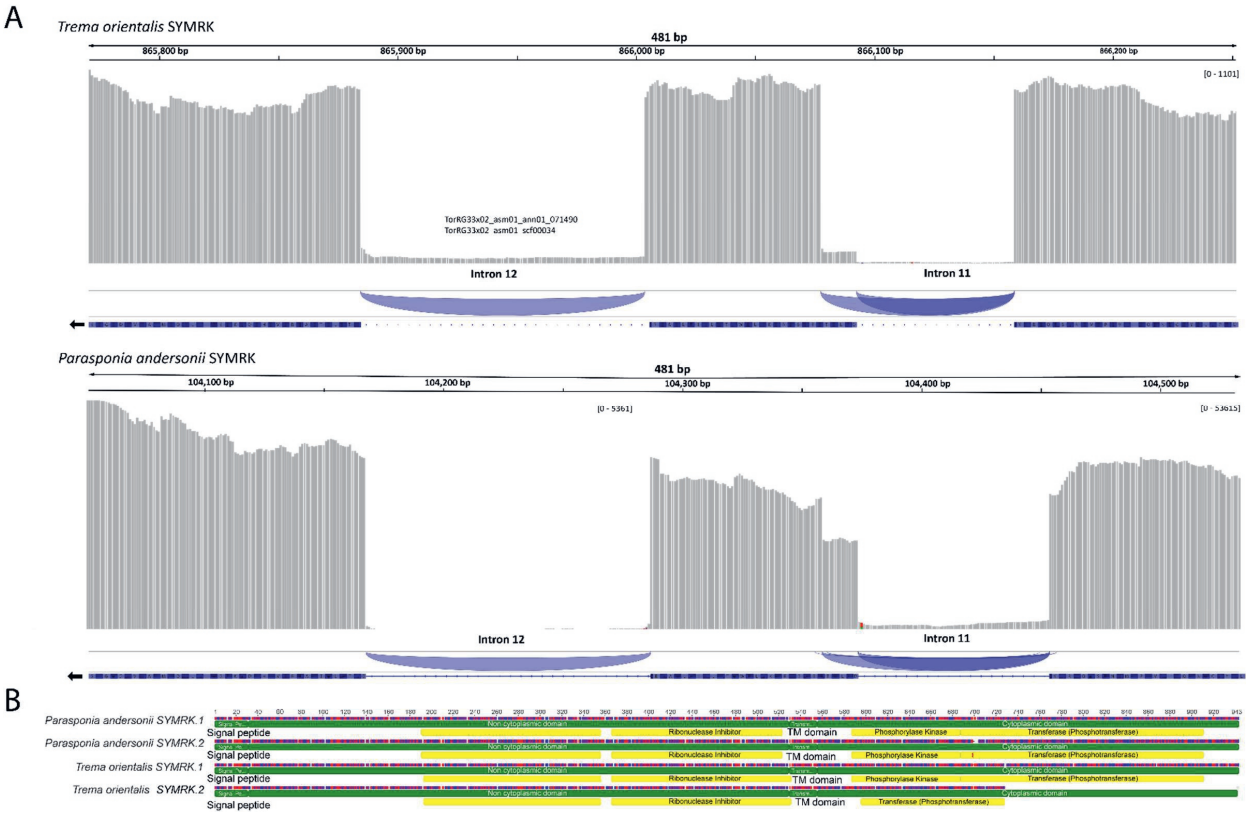


Figure S4: (**A**) Mapping of root RNA sequence reads to *SYMRK* gene models of *T. orientalis* and *P. andersonii*. Note: gene models are presented in reverse orientation. (**B**) Structure of *T. orientalis* and *P. andersonii SYMRK* splice variants.

Supplemental Table S1. Twenty-eight *Trema orientalis* individuals collected at 5 locations in Sabah, Malaysia, all possess a non-canonical donor splice site at intron 12.

species	accession	intron 12 donor splice site	origin	GPS MGRS	Latitude & Longitude	altitude (meter)
Trema orientalis	RG1	CACGAG^GAAAGT	Malaysia, Sabah, Poring	50NMM 56071 58651	5.958664, 116.603094	1018
Trema orientalis	RG2	CACGAG^GAAAGT	Malaysia, Sabah, Poring	50NMM 67379 68352	6.046485, 116.705216	540
Trema orientalis	RG4	CACGAG^GAAAGT	Malaysia, Sabah, Poring	50NMM 67379 68352	6.046485, 116.705216	540
Trema orientalis	RG5	CACGAG^GAAAGT	Malaysia, Sabah, Poring	50NMM 67494 68312	6.046124, 116.706255	496
Trema orientalis	RG8	CACGAG^GAAAGT	Malaysia, Sabah, Poring	50NMM 67137 68377	6.046710, 116.703029	499
Trema orientalis	RG9	CACGAG^GAAAGT	Malaysia, Sabah, Poring	50NMM 67137 68377	6.046710, 116.703029	499
Trema orientalis	RG16	CACGAG^GAAAGT	Malaysia, Sabah, Sayap	50NMM 51863 83196	6.180670, 116.564897	852
Trema orientalis	RG17	CACGAG^GAAAGT	Malaysia, Sabah, Sayap	50NMM 51942 83438	6.182860, 116.565609	716
Trema orientalis	RG18	CACGAG^GAAAGT	Malaysia, Sabah, Sayap	50NMM 51951 83446	6.182932, 116.565690	802
Trema orientalis	RG19	CACGAG^GAAAGT	Malaysia, Sabah, Sayap	50NMM 51893 82920	6.178174, 116.565170	787
Trema orientalis	RG20	CACGAG^GAAAGT	Malaysia, Sabah, Sayap	50NMM 51936 82479	6.174185, 116.565562	835
Trema orientalis	RG23	CACGAG^GAAAGT	Malaysia, Sabah, Poring	50NMM 67802 68279	6.045827, 116.709038	467
Trema orientalis	RG27	CACGAG^GAAAGT	Malaysia, Sabah, Poring	50NMM 68156 68282	6.045856, 116.712237	445
Trema orientalis	RG28	CACGAG^GAAAGT	Malaysia, Sabah, Poring	50NMM 67752 68251	6.045573, 116.708587	461
Trema orientalis	RG29	CACGAG^GAAAGT	Malaysia, Sabah, Mahua	50NMM 34597 40705	5.796153, 116.409250	1065
Trema orientalis	RG31	CACGAG^GAAAGT	Malaysia, Sabah, Mahua	50NMM 34905 40499	5.794292, 116.412034	1046
Trema orientalis	**RG33**	**CACGAG^GAAAGT**	**Malaysia, Sabah, Mahua**	**50NMM 35073 40387**	**5.793281, 116.413552**	**1050**
Trema orientalis	RG34	CACGAG^GAAAGT	Malaysia, Sabah, Mahua	50NMM 36940 38236	5.773840, 116.430435	895
Trema orientalis	RG36	CACGAG^GAAAGT	Malaysia, Sabah, Mahua	50NMM 36887 38255	5.774012, 116.429956	887
Trema orientalis	RG37	CACGAG^GAAAGT	Malaysia, Sabah, Mahua	50NMM 36703 38442	5.775701, 116.428292	895
Trema orientalis	RG38	CACGAG^GAAAGT	Malaysia, Sabah, Gunug Alab	50NMM 27210 43115	5.817880, 116.342505	1827
Trema orientalis	RG39	CACGAG^GAAAGT	Malaysia, Sabah, Gunug Alab	50NMM 27221 43132	5.818034, 116.342604	1830
Trema orientalis	RG40	CACGAG^GAAAGT	Malaysia, Sabah, Gunug Alab	50NMM 26424 40871	5.797573, 116.335429	1560
Trema orientalis	RG41	CACGAG^GAAAGT	Malaysia, Sabah, Gunug Alab	50NMM 27548 38135	5.772835, 116.345609	1340
Trema orientalis	RG45	CACGAG^GAAAGT	Malaysia, Sabah, Gunug Alab	50NMM 29782 35934	5.752948, 116.365808	1004
Trema orientalis	RG50	CACGAG^GAAAGT	Malaysia, Sabah, Crocker Rang, Inobong	50NMM 03620 49526	5.875581, 116.129347	261
Trema orientalis	RG52	CACGAG^GAAAGT	Malaysia, Sabah, Crocker Rang, Inobong	50NMM 03744 50645	5.885704, 116.130451	205
Trema orientalis	RG53	CACGAG^GAAAGT	Malaysia, Sabah, Crocker Rang, Inobong	50NMM 03738 50648	5.885736, 116.130401	205
Trema orientalis	WU41	CACGAG^GCAAGT	China	not determined	not determined	not determined
Trema orientalis	WU42	CACGAG^GCAAGT	China	not determined	not determined	not determined
Trema orientalis	WU43	CACGAG^GCAAGT	China	not determined	not determined	not determined
Trema orientalis	WU44	CACGAG^GCAAGT	China	not determined	not determined	not determined
Trema orientalis	WU45	CACGAG^GCAAGT	China	not determined	not determined	not determined
Trema levigata	WU50	CACGAG^GCAAGT	China	not determined	not determined	not determined
Trema tomentosa	WU10	CACGAG^GCAAGT	Australia	not determined	not determined	not determined
Parasponia andersonii	WU1	CACGAG^GCAAGT	Papua-New Guinea	not determined	not determined	not determined

Supplemental Table S2. Frequency of intron with GA donor sites in several plants genome

Species name	Gene ID	Description	Σ introns with GA	Intron number with GA donor
Trema orientalis	TorRG33x02_asm01_ann01_006210	na	1	1
	TorRG33x02_asm01_ann01_024300	na	3	1,2,3
	TorRG33x02_asm01_ann01_098400	na	2	1,2
	TorRG33x02_asm01_ann01_119330	na	1	1
	TorRG33x02_asm01_ann01_177120	na	1	1
	TorRG33x02_asm01_ann01_178050	na	1	1
	TorRG33x02_asm01_ann01_315370	na	1	1
	TorRG33x02_asm01_ann01_071490	Symbiosis receptor kinase (SYMRK)	1	12
Parasponia andersonii	PanWU01x14_asm01_ann01_022890.1	na	1	1
	PanWU01x14_asm01_ann01_040010.1	na	2	1,2
	PanWU01x14_asm01_ann01_044300.1	na	1	1
	PanWU01x14_asm01_ann01_230920.1	na	1	1
Lotus japonicus GIFU	LotjaGi1g1v0060300	Disease resistance protein	19	4,5,6,7,8,9,10,11,12,13,14,15 ,16,17,18,19,20,21,22,23
	LotjaGi2g1v0134700	na	1	4
	LotjaGi2g1v0299800	F-box protein interaction domain protein	21	1,2,3,4,5,6,7,8,9,10,11,12,13, 14,15,16,17,18,19,20,21
	LotjaGi3g1v0227900	na	11	2,3,4,5,6,7,8,9,10,11,12
	LotjaGi4g1v0079400	na	5	2,3,4,5,6
Medicago truncatula	No intron splice donor sites with GA found			
Arabidopsis thaliana	AT5G40560	Degradation of periplasmic proteins 13 (DEG13)	8	1,2,3,4,5,6,7,8
	AT2G17770	Basic region/leucine zipper motif 27 BZIP27	2	3,4
	AT4G35900	na	2	2,3

Supplemental Table S3. List of Golden Gate constructs used in this study.

Construct	Description	Level	Backbone	contains[1]
1	*nptII* resistence cassette	1	pICH47802	pICSL70004:*nptII*
2	$35S_{pro}$:ΩNLS-Cas9:$35S_{ter}$	1	pICH47742	pICH41388:$35S_{pro}$, pAGM5331:ΩNLS, pICH41308::aCas9, pICH41414:$35S_{ter}$
3	PanSYMRKsgRNA1	1	pICH47852	pICSL01009:AtU6p, corresponding PCR amplicon
4	PanSYMRKsgRNA2	1	pICH47772	pICSL01009:AtU6p, corresponding PCR amplicon
5	CRISPR_ctrl	2	pICSL4723	1R: construct 1, 2F: construct 2, end-link pICH41744
6	CRISPR_PanSYMRK	2	pICSL4723	1R: construct 1, 2F: construct 2, 3F: construct 3, 4F: construct 4; end-link pICH41766
7	PanSYMRK $Part1_{Pro}$:$SYMR_{gene}$:$SYMRK_{ter}$	1	pICH47742	EC75120:$SYMRK_{Pro}$, EC75123: $SYMRK_{gene}$ part1, EC75124: $SYMRK_{gene}$ part2, EC75122: $SYMRK_{ter}$
8	PanSYMRK $Part1_{Pro}$:$SYMRK_{gene-GA}$:$SYMRK_{ter}$	1	pICH47742	EC75120:$SYMRK_{Pro}$, EC75123: $SYMRK_{gene}$ part1, EC75125: $SYMRK_{gene-GA}$ part2, EC75122: $SYMRK_{ter}$
9	PanSYMRK $Part2_{pro}$	1	pICH47732	EC75121:$SYMRK_{Pro}$ part2
10	$35S_{Pro}$:$erGFP_{gene}$:$t35S_{ter}$	1	pICH47831	pICH51277:$35S_{Pro}$, EC74047:$erGFP_{gene}$, pICH41414:$35S_{ter}$
11	$35S_{Pro}$:$erGFP_{gene}$:$t35S_{ter}$	2	MOB215_pICSL4723_ modified to Spec R	Dummy1,Dummy2,Dummy3,1R: Construct 10
12	$PanSYMRK_{Pro}$ Part2, $SYMRK_{Pro}$ Part1, $SYMRK_{gene}$:$SYMRK_{ter}$, $35S_{Pro}$:$erGFP_{gene}$:$35S_{ter}$	2	MOB215_pICSL4723_ modified to Spec R	1F: Construct 9, 2F: Construct 7, Dummy 3, 4R: Construct 10

[1]Position and orientation in level 2 Golden gate modules is depicted by a number followed by either F or R for forward or reverse orientation

Supplemental Table S4. Primers used in this study.

Name	Purpose	Sequence
sgRNA-Rv	CRISPR assembly	tgtggtctccaAGCGTAATGCCAACTTTGTAC
PanSYMRK_sgRNA1	CRISPR assembly	tgtggtctcaattGACCCATGTCTCCCAACTCCCgttttagag ctagaaatagcaag
PanSYMRK_sgRNA2	CRISPR assembly	tgtggtctcaattGACCAACTTAAACACCCTGTAgttttagag ctagaaatagcaag
geno_PanSYMRK-KO-Fw	Genotyping CRISPR mutants	TTCCAGTTTGGGGCCCATTT
geno_PanSYMRK-KO-Rv	Genotyping CRISPR mutants	GGAGGAAGAAGGAAAGTCCGG

CHAPTER 5

5

GAMMA (γ)-AMINOBUTYRIC ACID TRANSPORTER1 (*PanGAT1*) is non-essential for nodulation in *Parasponia andersonii*

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Abstract

γ-AMINOBUTYRIC ACID (GABA) TRANSPORTER 1 (*GAT1*) is one of the seven genes consistently lost in the non-nodulating *Trema* species. In legumes, GAT1 functions in the transport of GABA, a non-protein incorporated amino acid, from the extracellular compartment to the cytosol of infected cells. It is hypothesized that this GABA provides an energy source for symbiotic bacteroids. *Parasponia andersonii* has three genes encoding putative GABA transporters (i.e. *PanGAT1*, *PanGAT2*, and *PanGAT3*). However, only *PanGAT1* is exclusively expressed in *P. andersonii* root nodules and lost in all analyzed *Trema* species. This raises the question of whether PanGAT1 has a function during nodulation. To answer this question, four bi-allelic *Pangat1* knockout mutants were created using CRISPR-Cas9. Mutant plants were grown and inoculated with *Mesorhizobium plurifarium* BOR2. Several nodule related phenotypes (e.g. nodule number, size, cytoarchitecture, bacterial and GABA content) were analyzed. We show that, *Pangat1* knockout leads to no obvious phenotypes in any of our analyses of *P. andersonii* nodulation. From this, we conclude that PanGAT1 is not essential for nodulation, and therefor GAT1 is not a primary target gene in the ongoing effort to re-engineering *Trema* species.

Introduction

A phylogenetic study comparing nodulating *Parasponia* to non-nodulating *Trema* species (Cannabaceae) revealed seven nodule-specific expressed genes present in *Parasponia andersonii* to be lost in *Trema* (Velzen *et al.*, 2018). Among these seven is a gene we named *Parasponia andersonii GAT1* (Figure 1). *GAT1* encodes for a putative Gamma (γ)-aminobutyric acid (GABA) transporter, homologous to *AtGAT1*. *AtGAT1* belongs to a small family of GABA specific transporters that in *A. thaliana* that consists of 2 members (Wipf *et al.*, 2002; Meyer *et al.*, 2006). *AtGAT1* was the first GABA transporter to be identified in plants using the model species *Arabidopsis thaliana* (Meyer *et al.*, 2006) and its functional characterization in a heterologous system demonstrated that *AtGAT1* is an H^+-driven transporter specific for GABA. The *AtGAT1* protein is located on the plasma membrane of *A. thaliana* cells (Meyer *et al.*, 2006), and it has been suggested that here *AtGAT1* facilitates GABA uptake into the plant cell (Meyer *et al.*, 2006; Batushansky *et al.*, 2015).

GABA is a four-carbon (C) non-proteinogenic amino acid, which can be found ubiquitously in the cytosol of all prokaryotic and eukaryotic organisms (Shelp *et al.*, 2009; Ramos-Ruiz *et al.*, 2018). GABA was first discovered in 1949 in potato tubers (Fait *et al.*, 2008). However, later it was demonstrated that GABA is the most abundant inhibitory neurotransmitter in mammalian brain (Bowery & Smart, 2006). As a consequence, GABA related research in animal or human related fields is currently much more advanced compared to plants (Žárský, 2015; Ramesh *et al.*, 2017). Research that led to fundamental insights and discoveries related to GABA in the animal kingdom that proved to be appliable for plants as well. As such we know that in both animal and plant cells, GABA can be synthesized via a short pathway that bypasses two steps of the tricarboxylic acid cycle (TCA cycle). A short pathway commonly referred to as the GABA shunt (Wang *et al.*, 2006; Fait *et al.*, 2008; Michaeli & Fromm, 2015). Apart from these similarities, there are also differences. Although in animal cells the GABA shunt seems to be the dominant pathway, in plant cells, GABA is mainly synthesized by three additional routes. In plants, GABA can either be formed by; (1) an irreversible metabolic reaction where glutamate is catalyzed to GABA by the cytosolic GLUTAMATE DECARBOXYLASE (GAD) (Ramos-Ruiz *et al.*, 2019), (2) through polyamine degradation by an enzyme called DIAMINE OXIDASE (Yang *et al.*, 2018; Li *et al.*, 2021), or (3) a non-enzymatically conversion of proline to GABA under oxidative stress (Podlešáková *et al.*, 2019).

It has been suggested that in plants GABA has at least two main functions (Ramos-Ruiz *et al.*, 2019). Firstly, it is believed that GABA can act as a substrate for a variety of metabolomic pathways. Among such metabolic roles are; the provision of carbon skeletons and energy, the regulation of cytosolic pH, and the involvement in nitrogen metabolism (Fait *et al.*, 2008; Michaeli & Fromm, 2015; Ramos-Ruiz *et al.*, 2019). As such, GABA could play a central role interfacing several primary and secondary metabolomic pathways in plants (Bouché & Fromm, 2004). Additionally, GABA has been reported to accumulate in various plant tissues (i.e. shoots, tubers, flowers, fruits, roots, and nodules) under (a)biotic stress conditions (Serraj *et al.*, 1998; Shelp *et al.*, 2009; Sulieman, 2011; Podlešáková *et al.*, 2019; Li *et al.*, 2021). This suggests that GABA might also be involved in signaling pathways in response to these stresses (Ramos-Ruiz *et al.*, 2019; Li *et al.*, 2021).

In legumes, GABA is found in relatively high concentrations in nodules (Sulieman & Schulze, 2010). This GABA is mostly located in the cells of the fixation zone (Booth *et al.*, 2021). The precise functioning of GABA in root nodules remains elusive. Again, a dual role has been proposed where GABA either acts as a signaling molecule and/or a carbon input into the bacteroid metabolism (Bouché & Fromm, 2004; Ramos-Ruiz *et al.*, 2019). Support for this a role in bacteroid metabolism can be found in the fact that GABA is also present in these bacteroids. Bacteroids are rhizobium bacteria terminally differentiated in their symbiotic state (Streeter, 1987; Fitzmaurice & O'Gara, 1993; White *et al.*, 2009b). It has been proposed that rhizobia lost the ability produce GABA, and therefore any GABA detected in these bacteroids is likely of host plant origin (White *et al.*, 2009b; Sulieman & Schulze, 2010). The basis for this hypothesis is the fact that the before mentioned GLUTAMATE DECARBOXYLASE (GAD) enzyme, essential for the conversion of glutamate to GABA, is absent from the rhizobia genome (Jin *et al.*, 1990; Fitzmaurice & O'Gara, 1993; Labidi *et al.*, 2000) although present in various other bacterial species (Feehily & Karatzas, 2013). Combined with the fact that external GABA feeding into the phloem sap of the host plant results in a proportionally short term increase in nodule activity by increased nitrogen fixation (Sulieman & Schulze, 2010; Booth *et al.*, 2021) this suggests a more direct role of GABA during nodulation. In such a scenario, GABA transport from phloem or extracellular compartments into the cytosol of rhizobium infected nodule cells and subsequently bacteroids is likely mediated by plant encoded GABA transporters. However, the nature of such transporters remains elusive.

Here, we investigated the putative role of GAT1 during nodulation using the model *Parasponia andersonii*. Based on its conservation in *Parasponia* and absence from *Trema*

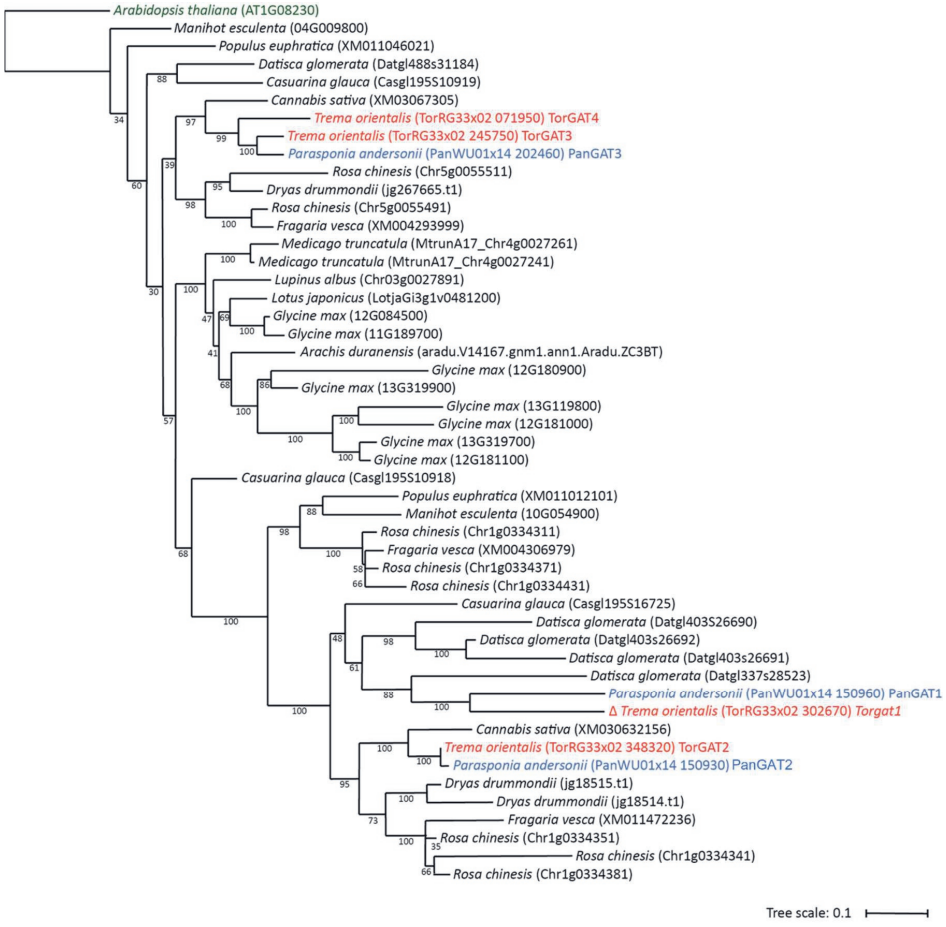

Figure 1. **Phylogenetic tree of the GABA Transporter (GAT) family orthology group rooted to *Arabidopsis thaliana* GAT1 (in green)**. Putative *Parasponia andersonii* GAT genes (in blue) and *Trema orientalis* RG33 (in red), Δ indicates locus is a pseudogene. Branch numbers indicate bootstrap values. Gene identifier number between brackets (Klein *et al*., 2022).

species, we hypothesize that PanGAT1 has a symbiotic function which might be required for nodulation. We further investigate the phylogeny and expression profile of *PanGAT1*. Additionally, to test whether PanGAT1 has symbiotic functions we created four *Pangat1* mutant alleles lines using CRISPR/CAS9 mutagenesis. These mutants were characterized for several nodules relative phenotypes (e.g. nodule numbers, nodule size and bacteria content) to determine the possible function of PanGAT1 during nodulation in *Parasponia andersonii*.

Results

Phylogenetic analysis of GABA Transporters in *P. andersonii*

It was previous reported that the genomes of three *Parasponia* and two *Trema* species encode three putative GABA transporters (Velzen *et al.*, 2018). Phylogenetic analysis suggests that only two of these three copies can be considered orthologous to each other **(Figure 1)**.

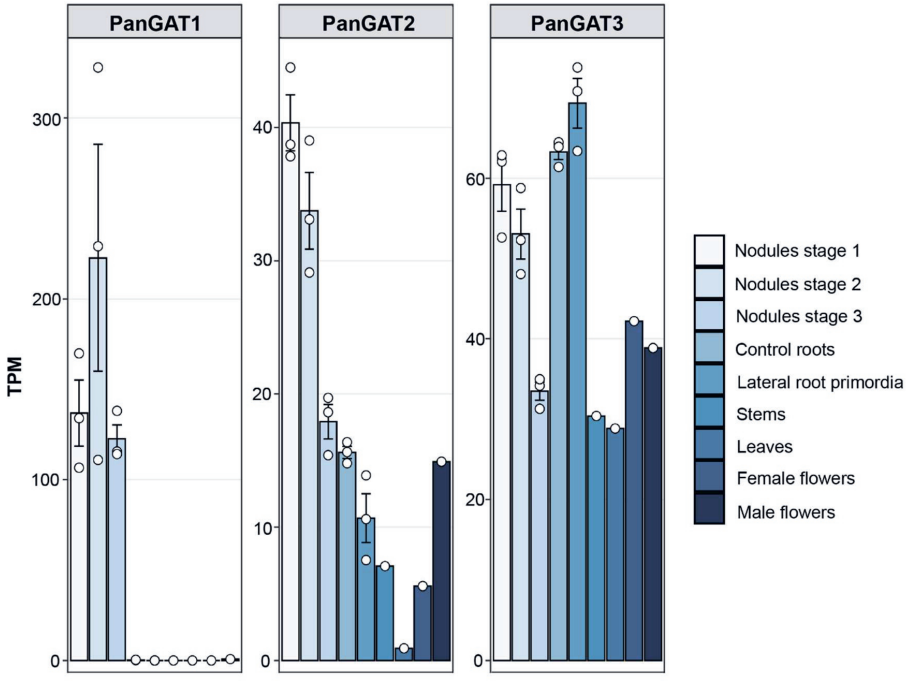

Figure 2. Expression profile of *PanGAT1*, *PanGAT2* , *PanGAT3* in 9 different *Parasponia andersonii* tissues in transcripts per million (TPM) (Velzen *et al.*, 2018). Expression is given in DESeq2 normalized read counts, error bars represent standard error of three biological replicates. Dots represent individual expression levels.

This indicates that putative GABA transporter 1 (GAT1) was loss in *T. orientalis* and *T. tomatosa*, but not in any of the sequenced *Parasponia* spp., suggesting a function for GAT1 during *Parasponia* nodulation (Velzen *et al.*, 2018). Here we focus on *P. andersonii* GAT1 (PanGAT1) for further investigating this putative role.

Expression analysis of *GAT* genes in *Parasponia* tissues

To investigate a possible nodule specific function for PanGAT1, we first turned our attention to the expression domain of all three *P. andersonii* GAT genes. For this we queried our previously constructed *P. andersonii* expression atlas (Parasponia.org, (Velzen *et al.*, 2018; Holmer *et al.*, 2019)). In this atlas we have 9 tissue/organ samples including; 3 stages of nodules (young, intermediate and mature), control roots, lateral root primordia, stems, leaves, female flowers, and male flowers. *PanGAT1* is highly expressed in all three nodule stages, with an apparent optimal at stage 2. Interestingly, no expression of *PanGAT1* could be detected in any of the other tissues present in our database **(Figure 2)**. In contrast, although

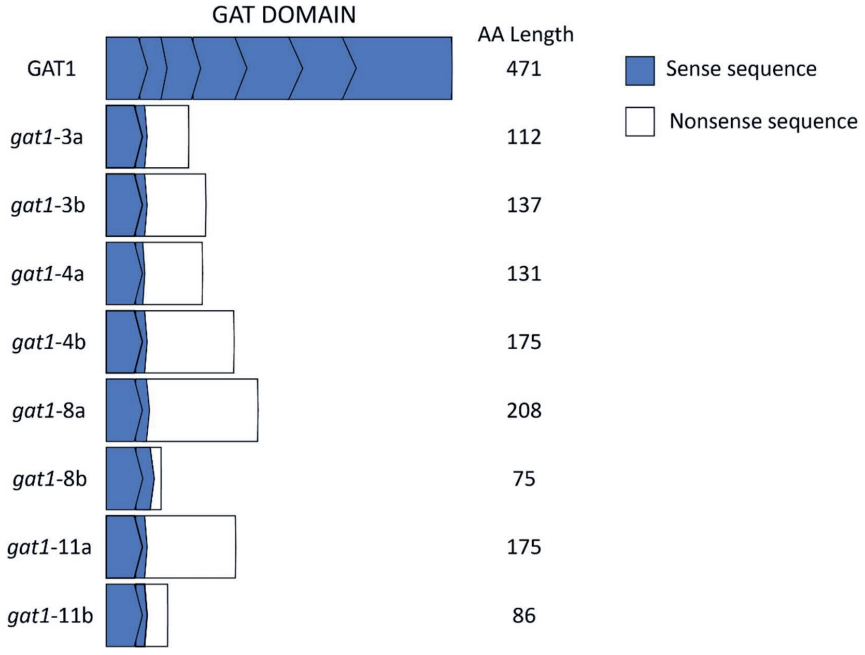

Figure 3. Schematic representation of the PanGAT1 amino acid (AA) sequence of wild type and our four bi-allelic *Pangat1* mutants. Closed blue bar represents the wild-type sense sequence, internal lines exon boundaries, open white bars indicates a nonsense sequence due to a frameshift. Bar length and AA number indicative of the protein length until the (premature) stop codon.

also relatively high expressed in nodules, both PanGAT2 and PanGAT3 expression can be detected in most, if not all, tissues in our database **(Figure 2)**. Combined this indicates that all three PanGAT genes are expressed in *Parasponia* nodules, although only *PanGAT1* expression might be limited to nodule tissue.

Knockout of *PanGAT1* does not lead to obvious nodulation phenotypes

To functionally test PanGAT1 we generated mutants using CRISPR/CAS9 as previously described for *P. andersonii* (van Zeijl *et al.*, 2018a; Wardhani *et al.*, 2019). This resulted in the creation of four independent *Pangat1* mutant lines (e.g. *Pangat1-3*, *Pangat1-4*, *Pangat1-8*, and *Pangat1-11*, **supplemental Figure S1)**. These four lines containing independent mutations early in the PanGAT1 coding sequence, resulting in frameshifts and premature stop codons deleting most of the so called GAT domain and likely abolishing any functional protein **(Figure 3 and supplemental Figure S2)**. All four lines were selected for further experimentation.

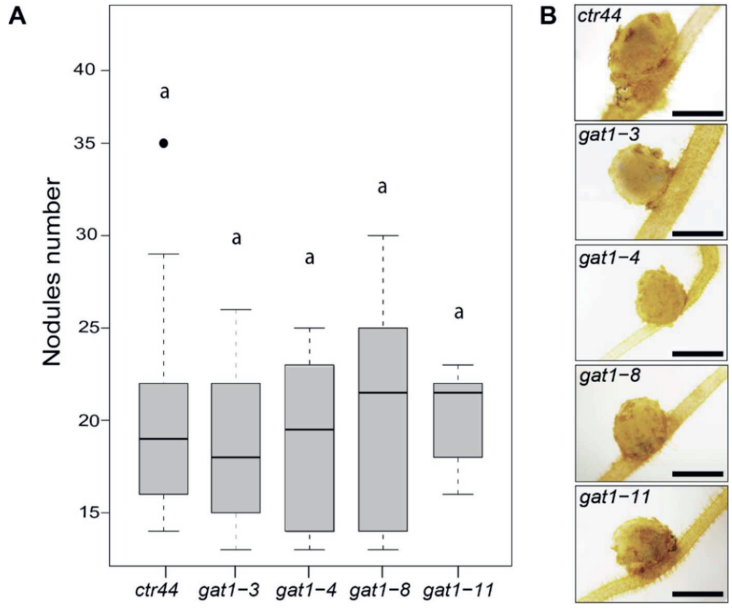

Figure 4. **Phenotype of the four independent *Pangat1* biallelic mutant lines at 6 weeks post inoculation with *Mesorhizobium plurifarium* BOR2. (A)** Total nodules number per plant, EV-control *(ctr44)*, *(pan)gat1-3*, *4*, *8*, *11* mutant lines, n=10, similar letters show no significant differences between these lines as determined by ANOVA in combination with Tukey post-hoc test. **(B)** Representative images of the macroscopic morphology of the nodules found on EV-control *(cntr44)* and the *(pan)gat1-3*, *4*, *8*, *11* mutant lines, scale bars 1mm.

As our *P. andersonii* tissue culture does not enable us to generate prodigy from our primary transformed (T_0) lines we used and a constitutively expressing CRISPR/CAS9 and antibiotic resistance line without any CAS9 guide RNAs (ctr44) as an empty vector control. All primary *Pangat1* mutant and ctr44 lines were maintained in tissue culture, and rooted explants used

for the experimentation as previously described (van Zeijl *et al.*, 2018a; Wardhani *et al.*, 2019). Rooted plantlets were inoculated with the *P. andersonii* compatible rhizobium strain *Mesorhizobium plurifarium* BOR2 (van Zeijl *et al.*, 2018a; Velzen *et al.*, 2018; Wardhani *et al.*, 2019) and grown for an additional 6 weeks before scoring any nodulation phenotypes. To our surprise, we found no effect on nodule numbers in any of the four *Pangat1* knockout lines compared to our empty vector control **(Figure 4A)**. Also, when examining the morphology of these nodules macroscopically, no obvious defects were observed **(Figure 4B)**.

PanGAT1 knockout does not affect nodule cytology

To investigated whether a PanGAT1 knockout effected nodule development, we investigated the cytoarchitecture of the four knockout mutant lines nodule. Also here, semi-thin section of nodules taken from any of the four *Pangat1* mutant lines revealed no phenotype in their cytology. *Pangat1* nodules have a normal ontogeny consisting of an apical meristem, followed by several layers that contain infection threads **(Figure 5A)**. Below this infection zone, 2-3 cell layers of infected cells can be found that are larger compared to non-infected cells **(Figure 5B)**. These cells are immediately followed by cells that are packed with fixation threads **(Figure 5B)**. Also here, no difference were observed between any of the lines. As such, *Pangat1* mutant nodules are not anatomically different from EV-control nodules.

As there are no obvious phenotypes in nodule morphology or cytoarchitecture in any of the *Pangat1* mutants analyzed, we questioned whether PanGAT1 could have a more specific role in nodule functioning downstream of organogenesis. For this, we zoomed in a bit more to investigate the effect of PanGAT1 knockout on the volume, bacterial density, and GABA content of these *Pangat1* nodules.

It has been suggested that GABA can act as a rhizobial energy source (White *et al.*, 2009b; Prell *et al.*, 2009). If so, GABA needs to be transported from the plant to the rhizobia inside the nodule cells. It is tempting to assume that such a transport could be mediated by PanGAT1. If this is the case, blocking GABA transport in *P. andersonii* facilitated by PanGAT1 could negatively affected the size of nodules formed on *Pangat1*. We did observe some differences in nodule volume in some of our mutants. Although, there was a lot of variation in and between the mutant lines. In contrast to our hypothesis, *Pangat1-3* and *Pangat1-11* nodules were slightly, though significant, larger compared to those on ctr44. On the other

hand, *Pangat1-8* was not significant different compared to either ctr44 or *Pangat1-11* and the nodule volume of *Pangat1-4* was identical to pangat1-8 and ctr44 **(Figure 6A)**. As all four lines have mutations more or less effecting Pangat1 similarly, it is not likely that these differences in nodule volume can be contributed to a knockout of PanGAT1.

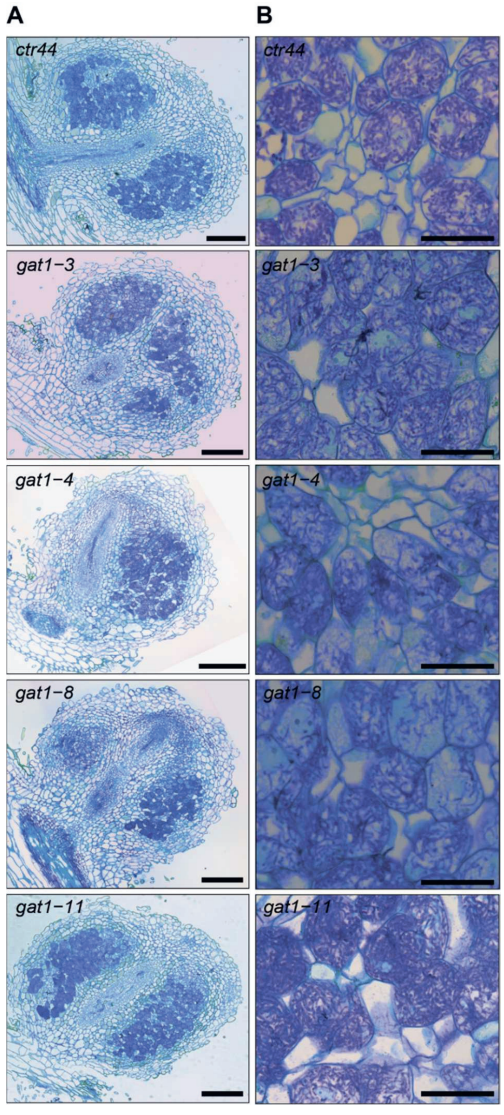

Figure 5. Representative cytoarchitecture images of nodules inoculated with *Mesorhizobium plurifarium* BOR2 taken from ctr44 (control) and the four independent *Pangat1* knockout lines. (A) overview section, scale bars 200µm. **(B)** fixation zone closeup, scale bars 50 µm.

Next, we wondered if the effect of PanGAT1 knockout might be more subtle. GABA is likely not be the only energy source available to the bacteroids inside the nodule cells. If so, reduced GABA transport capacity in a *Pangat1* mutant might have a limited effect bacterial abundancy inside the nodule only without effecting its average volume. To investigate this we measured colony forming units (CFU) present per volume nodule (mm^3). We observed that knockout of *Pangat1* did not affect bacterial densities. In general, bacteria numbers per nodule volume from the mutant lines were slightly, but not statistically lower compared to the control line although there was some statistical differences among the mutant lines themselves **(Figure 6B)**. Again likely to be due to other factors than *Pangat1* knockout.

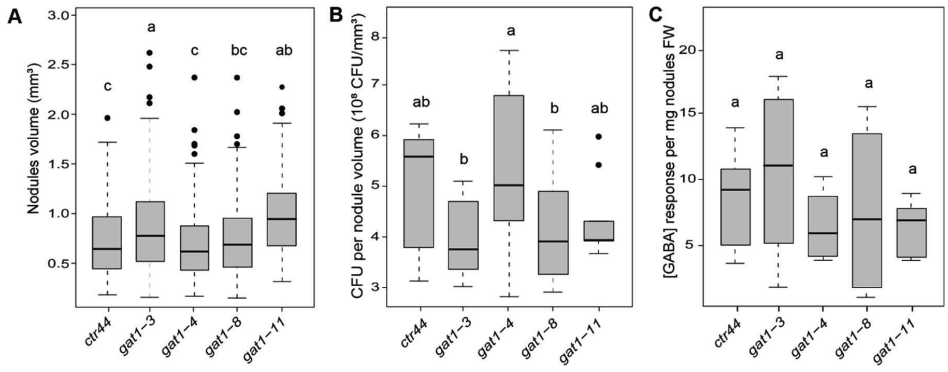

Figure 6. Nodulation related phenotype of ctr44 (EV-control) and the four independent *Pangat1* knockout lines 4 weeks post inoculation with Mesorhizobium plurifarium BOR2. (A) Nodules volume (mm^3) **(B)** Colony Forming Units (CFU) per nodule volume mm^3, and **(C)** GABA concentration (response per mg nodules fresh weight (FW)), ctr44 (control) and *Pangat1*-3, 4, 8, 11 mutant lines, n=10, different letters above the boxes indicate statistical significance ($p < 0.05$) as determined by ANOVA in combination with Tukey post-hoc test.

The lack of clear phenotype made us question if PanGAT1 knockout leads to reduced levels of GABA in *P. andersonii* nodules. For this we set out to measure GABA content inside mature nodules using MRM-UPLC-MS/MS. To our surprise, no significant difference in GABA levels were found when comparing ctr44 to any of the *Pangat1* knockout lines **(Figure 6c)**.

Discussion

GAT1 was previously identified as one of the seven genes lost in *Trema* but retained in *Parasponia* species (Velzen *et al.*, 2018). It is hypothesized that the loss of GAT1 in *Trema* is linked to the loss of the nodulation trait in this plant family. This would thus indicate that GAT1 is required for nodulation in *Parasponia*. However when mutated in *Parasponia andersonii*, no obvious nodulation phenotypes were observed in any of the independent *Pangat1* knock out mutants. At this point we cannot rule out an effect on the nitrogen fixation rate in *Pangat1* nodules due to the fact that our attempts to perform an acetylene reduction assay (ARA) failed. Nevertheless, the observation that traits related to nodule functioning (e.g. nodule ontogeny and infection, bacterial content, nodule size and nodule numbers) are also not affected in the *Pangat1* mutants suggests that no phenotypes are to be expected here as well. Combined, this suggests, if any, only a minor role for GAT1 during nodulation in *P. andersonii*.

The fact that none of the analysed nodule traits showed any significant deviation from the empty vector control could lead to two hypotheses; either PanGAT1, and possibly GABA, is not needed for nodulation in *P. andersonii*, or other genes can act redundantly and complement any effect *Pangat1* knock out might have on nodulation.

When looking at this first option, there are several reports on how GABA is thought to be involved during nodulation in model legumes. Here nodule activity is regulated by the N-requirement of the shoot (Hartwig, 1998; Fischinger *et al.*, 2010; Schwember *et al.*, 2019). A nitrogen flux in the phloem coordinates a whole-plant N-based feedback mechanism, balancing shoot demand with nitrogen fixation activity in the nodule (Parsons *et al.*, 1993; Ruffel *et al.*, 2008). It has been reported that in legumes, a similar correlation exists between the phloem flux of specific amino acids (e.g. GABA) and the nitrogen fixation activity in the nodule (Neo & Layzell, 1997; Sulieman & Schulze, 2010; Yamashita *et al.*, 2019). However, the nature of such signal and the mechanism by which it could be regulating nodule activity remain to be determined. It has been suggested that GABA acts directly as an energy supply for the bacteroids, bypassing the so called decarboxylating arm of the TCA cycle (White *et al.*, 2009a; Prell *et al.*, 2009; Sulieman, 2011; Seifikalhor *et al.*, 2019). It was demonstrated by Prell and colleagues (Prell *et al.*, 2009) that rhizobium strains that have no access to GABA are still able to symbiotically fix nitrogen. This suggests that, while GABA catabolism might still provide energy, it is not essential for nitrogen fixation. In addition, GABA might have a more indirect effect on nodulation, acting as a communication signal in the plant. GABA concentrations are known to be affected by various stresses, and thus could act as a signal to the root on the stress status of the shoot. In such case, GABA fluctuations might be observed when plants are grown and nodulated under various conditions. However, any findings on GABA functioning during nodulation in legumes cannot be simply projected on *P. andersonii*, as they need to be experimentally validated before being used to address GABA related questions in this non legumes species. It might still be possible that *PanGAT1* and GABA have no important function during nodulation in *P. andersonii*. Nevertheless, The specific expression domain of *PanGAT1*, exclusively expressed in root nodules, seems to be in conflict with no involvement of this gene during nodulation.

The second option, gene redundancy, is an attractive explanation for the observed lack of *Pangat1* related phenotypes. As mentioned before, we observed that *P. andersonii* contains

not one, but three paralogues copies of GAT (i.e. *PanGAT1*, *PanGAT2* and *PanGAT3*). Although, only *PanGAT1* is highly upregulated in *P. andersonii* nodules compared to control roots, we observed the expression of *PanGAT2* and *PanGAT3* to be relatively high as well. It is further possible that expression levels of *PanGAT2* and *PanGAT3* are further increased in a *Pangat1* mutant background. Unfortunately, we did not test this, but the expression levels of *PanGAT2* and *PanGAT3* could be already high enough in wild-type root nodules for these genes to act redundantly to *PanGAT1* and thus could both compensate for the loss of *PanGAT1* function when this gene is knocked out. This would also explain why we were unable to detect any effect on GABA levels in whole nodules harvested from the *Pangat1* mutants. Although if *PanGAT1* functions as a directional GABA transporter, miss localisation of GABA in *Pangat1* mutant nodules cannot be detected by analysing whole nodules and can therefore not be ruled out at this point. Nevertheless, if directional transport by *PanGAT1* would be needed during *P. andersonii* nodulation, this would likely lead to a nodulation phenotype.

In addition to the GAT transporter family, two low-affinity transporters are described in plants; AMINO ACID PERMEASE 3 (AAP3) and PROLINE TRANSPORTER *2* (ProT2) (Breitkreuz *et al.*, 1999; Meyer *et al.*, 2006; Yang *et al.*, 2020; Li *et al.*, 2021). Both transporters mediate the influx of GABA, however, as their names suggest, AAP3 and ProT2 are non-specific in which amino acids transport or have a preference for transporting proline, respectively (Breitkreuz *et al.*, 1999). Unlike AtGAT1, they do not have a high affinity for transporting GABA (Meyer *et al.*, 2006). Recently, an additional family of GABA transporters, the ALUMINIUM-ACTIVATED MALATE TRANSPORTERS (ALMTs), was identified in wheat (*Triticum aestivum*) (Ramesh *et al.*, 2015). Different from GAT1, ProT2, or AAP3, ALMTs can facilitate bidirectional GABA transport between the cytosol and apoplast (Ramesh *et al.*, 2015, 2018; Žárský, 2015). ALMTs have been found in a wide range of tissues from a number of different plant species. In addition, ALMTs have been characterized in model legumes such as *L. japonicus*, *M. truncatula*, and *G. max* where, unlike for GAT1, there is evidence that ALMTs are expressed in root nodules of these species (Serraj *et al.*, 1998; Takanashi *et al.*, 2016; Booth *et al.*, 2021). It is therefore possible that members from this family in *P. andersonii* fulfill a similar function. Adding to a growing list of genes that can act redundantly and further masking any putative phenotype of PanGAT1 knock out. If focusing on the role of GABA during *P. andersonii* nodulation, it would be prudent to annotate and analyse any and all genes that could be involved in GABA

translocation. In such a study, double, triple or even higher combinations of mutants might be needed to tackle these types of gene redundancies. Nevertheless, our aim was to study PanGAT1 because its phylogenetic distribution within the *Parasponia* and Trema species, we therefore did not include any additional GABA transporters in our research.

The aim of our study was to investigate if an engineering strategy aimed at restoring nodulation in *Trema* needs to consider GAT1. Our results indicate that this is not the case. In light of any engineering in *Trema*, a lack of a nodulation phenotype is more important than understanding why no nodulation phenotype in the *Pangat1* mutants can be observed. It is likely that PanGAT1 functions in nodulation, but that it does so in concert with, and redundant to, other GABA transporters. In that sense, GABA transport might still be an important component in successfully engineering a functional nodule on *Trema*, but this might be achieved by modulating genes from the GAT family present still in *Trema*.

Our hypothesis was that PanGAT could be causally linked to the loss of nodulation in *Trema*. However, our results seem to suggest differently. The emerging scenario is that, from the GAT GABA transporter family PanGAT1 became exclusively used during nodulation, whereas PanGAT2 and PanGAT3 kept a broader function in GABA translocation in other parts of the plant. This is reflected in our RNA sequencing data where PanGAT1 expression seems to be restricted to nodules. When *Trema* lost its ability to nodulate, genes that were exclusively used during this process lost their evolutionary pressure to be maintained. Theoretically this is could have led to neo-functionalisation or gene loss. In case of GAT1 the latter seems to be the case. Thus, PanGAT1 is not needed to retain nodulation, nodulation is needed to keep PanGAT1.

Material and Methods

Plant materials and growth conditions

All experiments were performed using *P. andersonii* WU1 or offspring thereof (Velzen *et al.*, 2018; Wardhani *et al.*, 2019). Plants were grown and maintained as described previously (van Zeijl *et al.*, 2018b; Wardhani *et al.*, 2019). Planlets for nodulation assays were multiplied via in vitro propagation and rooted when needed.

Bacteria inoculation

Rooted planlets were inoculated with *Mesorhizobium plurifarium* BOR2 (OD_{600} = 0.05) (van Zeijl *et al.*, 2018b; Velzen *et al.*, 2018; Wardhani *et al.*, 2019). Nodules were harvested 6 weeks post-inoculation. Binoculars equipped with a Nikon camera (DS-Fi2) was used to image each nodule individually. To measure nodule volume, the area and perimeter of each nodule were extracted from these images using FIJI (Schindelin *et al.*, 2012). The corresponding prolate spheroid volume of each nodule was measured by using the best-fitted ellipse to calculate this volume based on a previously developed formula (Bubeníčková *et al.*, 2014).

Bacterial counting inside nodules

To determine CFU per nodule volume, all the nodules were surface sterilized with 96% ethanol for 20 s, then 4% sodium hypochlorite for 1 min, and washed seven times with sterile water. A nodule was crushed in 150 µL of 0.9% sterile saline solution. Fifty microliters of the crushed nodule were diluted in series and both 10,000 and 100,000 fold dilutions were streaked on PSY plates with sterile glass beads and incubated at 28°C for 7 days. Colonies were counted to determine total CFU Rhizobia per nodule.

Expression analysis of GAT genes on *Parasponia andersonii* tissues

Expression analysis of PanGAT genes was carried out by mining the *Parasponia* transcription atlas (Velzen *et al.*, 2018; Holmer *et al.*, 2019).

Vectors and constructs

Binary constructs generated for this study were created using Golden Gate cloning. Backbones and several inserts were derived from the golden gate molecular toolbox (Engler *et al.*, 2009, 2014). Modifying the sequence of interests by removal BsaI or BpiI sites was carried out as described in Engler et al 2014 (Engler *et al.*, 2014). The generation and assembly of CRISPR-Cas9 construct for *Parasponia* were done as published previously (van Zeijl *et al.*, 2018b; Wardhani *et al.*, 2019). Vectors were verified using restriction digestion and sequencing of the sg target-containing region. Guide RNAs were designed using the CRISPR design tool implemented in Geneious Software R10 (Biomatters, Auckland, New Zealand), which is based on variables described in Doench et al 2014 (Doench *et al.*, 2014). Selected guide target sites have no potential off-targets in the *Parasponia* genome with less than two indels or three mismatches. A list of constructs generated for this study is listed in **(Table S1)**.

Genotyping

CRISPR deletions were detected using Phire plant direct PCRs (Thermo fisher, F130WH) using the manufacturer's recommendations. Target sites were amplified with primers flanking the deletion site **(Table S2)**, and potential deletion candidate shoots were re-genotyped during shoot propagation to ensure line homogeneity.

GABA Extraction and quantification by liquid chromatography-tandem mass spectrometry

y-Aminobutyric acid (GABA) was extracted and analysed as previously described for ACC (Bours *et al.*, 2013) with modifications. For the extraction of GABA from *P. andersonii* nodules, ~20 mg of snap-frozen plant material were used per sample. Tissue was ground to a fine powder at -80˚C using 3-mm stainless steel beads at 50 Hz for 2*30 seconds in a TissueLyser LT (Qiagen, Germantown, USA). Ground samples were extracted with 1 mL of cold extraction solvent (water:ethanol, 20:80 [v/v]) and sonicated for 15 seconds at RT in a Branson 3510 ultrasonic bath (Branson Ultrasonics). [2H6]-GABA (0.1 nmol/mL) as an internal standard.

Analysis of GABA in Arabidopsis leaf extracts was performed by comparing retention times and mass transitions with those of standard GABA (Sigma) using a Waters Xevo TQs tandem

quadruple mass spectrometer as previously described (Gühl *et al.*, 2021) with the following modifications. Chromatographic separation was achieved on an Acquity UPLC HSS T3 column (100 × 2.1 mm, 1.8 µm; Waters) by applying a methanol-water gradient to the column, starting from 5% (v/v) methanol for 0.1 min and rising to 70% (v/v) methanol at 17.00 min, followed by a 1 min gradient to 100% (v/v) methanol, before going back to 5% (v/v) methanol using a 1 min gradient. The column was equilibrated for 2 min, using this solvent composition prior to the next run. The run time was 20 min. The column was operated at 40°C with a flow rate of 0.25 mL min−1. Sample injection volume was 5 µL. For identification, the following MRM transitions were selected: mass-to-charge ratio (m/z) 274.20 > 170.98 at a collision energy of 15 eV and m/z 274.20 > 116.00 at 35 eV for GABA, and (m/z) 280.20 > 170.98 at a collision energy of 15 eV and m/z 280.20 > 116.00 at 35 eV for [2H6]GABA. Cone voltage was set to 50 eV. GABA was quantified using a calibration curve with known amounts of standards and based on the ratio of the area of the MRM transition m/z 274.20 > 17.96 for GABA to the MRM transition m/z 280.20 > 170.96 for [2H6]GABA. Data acquisition and analysis were performed using MassLynx 4.1 software (Waters). The summed area of all the corresponding MRM transitions was used for statistical analysis.

Histochemical analysis, microtome sectioning and microscopy

Nodules were harvested and fixed in 4% paraformaldehyde (w/v), 5% glutaraldehyde (v/v) in 50 mM sodium phosphate buffer (pH 7.2) at 4^{o}C for 24h. Subsequently, the samples were dehydrated using an ethanol series and embedded in Technovit 7100 (Heraeus Kulzer, Hanau, Germany) according to the manufacturer's instructions. Semithin sections were cut using a Leica Ultra-cut microtome (Leica Microsystem, Wetzlar, Germany) to 5 µm thickness. Sections were stained with 0.05% Toluidine Blue. Images were photographed using a Leica DM5500B microscope equipped with a DFC425C camera (Leica Microsystem, Germany).

Statistical analysis

Graph visualization and statistical analysis were generated using R studio version 1.1.463. Statistical analysis was performed using One-Way Anova, followed by post-hoc Tukey HSD ($p<0.05$). Bars represent mean ± SE for all experiments. Elements in the boxplot illustrate the lowest and highest data points, the sample median, and the first and third quartiles. Bars

labelled with identical letters are indicated to be not significantly different.

Phylogenetic reconstruction

Phylogvenetic tree reconstruction was performed using FasTree implemented in Geneious Software v 8.1 (Price *et al.*, 2009). As input for phylogenetic reconstruction a protein alignment generated using MAFFT alignment (Katoh *et al.*, 2002) was used. Pretein sequences of *P. andersonii* and *T. orientalis* were retrieved from www.parasponia.org, and for *Arabidopsis thaliana*, *Manihot esculenta*, *Populus euphratica*, *Datisca glomerota*, *Casuarina gluca*, *Cannabis sativa*, *Rosa chinesis*, *Dryas drummondii*, *Medicago truncatula, Lupinus albus*, *Lotus japonicus*, *Glycine max*, *Arachis duranensis*, and *Fragraria vesca* were retrieved from https://phytozome.jgi.doe.gov/pz/portal.html.

Author Contributions

Conseptualization, Y.P.R., W.K. and R.G.; Methodology, Y.P.R and W.K.; Investigation. Y.P.R., J.K and W.K.; Writing – Original Draft, Y.P.R.; Writing – Review & Editing, Y.P.R., W.K., and R.G.; Supervision, R.G and W.K.

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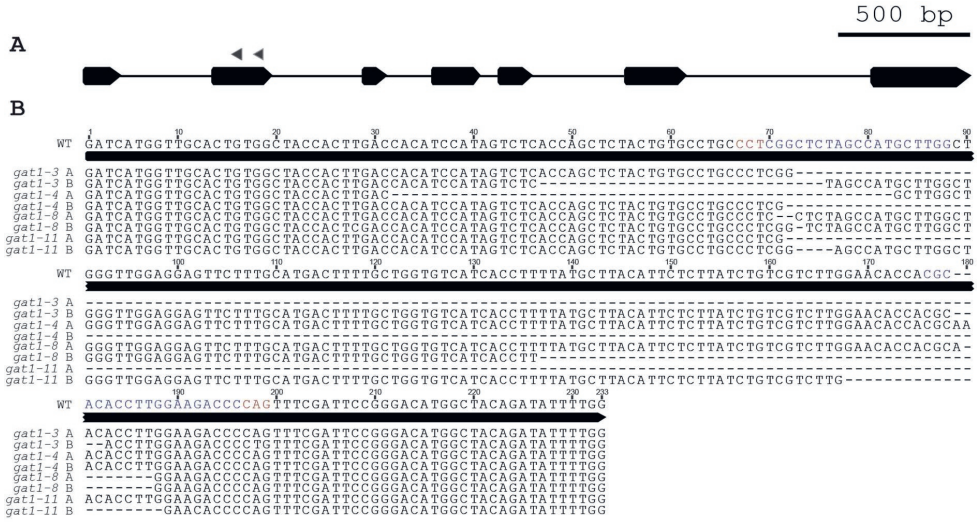
Supplemental data



Supplemental Figure S1. ***Pangat1* CRISPR mutant alleles. (A)** Schematic representation of *PanGAT1* gene model. Indicated by a grey arrowheads are the location of two sgRNA target sites used for DNA cleavage. **(B)** Sequence alignment of the second exon of *PanGAT1* in wild type (WT) and *pangat1* mutants (line 3, 4, 8 & 11). In bi-allelic mutant lines, both alleles (A and B) are shown. Highlighted in blue and red are the sgRNA target sites and PAM sequences, respectively.

PanGAT1		MGSEHDEVTPNSMISNSGFLVCELENGVLKGAPPPKEYGAGALSVLEPRGSWLHCGYHLTTSIVSPALLCLPSALAMLG WVGGVLCMTFAGVITFYAYILLSVVLEHHAHLGRPQFRFRDMATDILGPGWGKYFVGPLQLGLCYSVVVAFTLLGGQSL KFIYLLSNPTGTMKLYNFTIIFGALMLFLVQIPSLHSLRHINLVSLNLCLAFSVCVTIGSIYIGNCELGFKKNNTIMEG QGSQSILAAFFAISIVATTFSSGVFPQIQATIAPPVKTNMFKGICISYSVIVSTYYSVAISGYWAFGNRSKGSVLANFI GDDHKPLLPTWFLLITNLFILLQVSAATLVYLQPTNQLLEEKFIDPKSDQFSGPNIVRRLFLRLISVIIATTLAAMLPF FGDLMALFGAFGCIPLDFILPFIFYNLTFKPSNRSFIFWVNTLIAVICLFMAGIGAIASLYKILSDAKMFRLFPNM
pangat1_3	a	MGSEHDEVTPNSMISNSGFLVCELENGVLKGAPPPKEYGAGALSVLEPRGSWLHCGYHLTTSIVSPALLCLPSDTLED PSFDSGTWLQIFWDPDGENTLWAHFNLGYATVL*
	b	MGSEHDEVTPNSMISNSGFLVCELENGVLKGAPPPKEYGAGALSVLEPRGSWLHCGYHLTTSIVSSHAWLGWRSSLHD FCWCHHLLCLHSLICRLGTPRTLEDPSFDSGTWLQIFWDPDGENTLWAHFNLGYATVL*
pangat1_4	a	MGSEHDEVTPNSMISNSGFLVCELENGVLKGAPPPKEYGAGALSVLEPRGSWLHCGYHLTLGWVGGVLCMTFAGVITFY AYILLSVVLEHHANTLEDPSFDSGTWLQIFWDPDGENTLWAHFNLGYATVL*
	b	MGSEHDEVTPNSMISNSGFLVCELENGVLKGAPPPKEYGAGALSVLEPRGSWLHCGYHLTTSIVSPALLCLPSTPWKTP VSIPGHGYRYFGTRMGKILCGPTSTWAMLQCCSCLHSSRRAKSQVHLLALQSNWNNEAVQFYYNLRSLNAVFGTNPIAP LPQAHQSCLSKPLPCF*
pangat1_8	a	MGSEHDEVTPNSMISNSGFLVCELENGVLKGAPPPKEYGAGALSVLEPRGSWLHCGYHLTTSIVSPALLCLPSSSHAWL GWRSSLHDFCWCHHLLCLHSLICRLGTPRRKTPVSIPGHGYRYFGTRMGKILCGPTSTWAMLQCCSCLHSSRRAKSQVH LLALQSNWNNEAVQFYYNLRSLNAVFGTNPIAPLPQAHQSCLSKPLPCF*
	b	MGSEHDEVTPNSMISNSGFLVCELENGVLKGAPPPKEYGAGALSVLEPRGSWLHCGYHLTTSIVSPALLCLPSV*
pangat1_11	a	MGSEHDEVTPNSMISNSGFLVCELENGVLKGAPPPKEYGAGALSVLEPRGSWLHCGYHLTTSIVSPALLCLPSTPWKTP VSIPGHGYRYFGTRMGKILCGPTSTWAMLQCCSCLHSSRRAKSQVHLLALQSNWNNEAVQFYYNLRSLNAVFGTNPIAP LPQAHQSCLSKPLPCF*
	b	MGSEHDEVTPNSMISNSGFLVCELENGVLKGAPPPKEYGAGALSVLEPRGSWLHCGYHLTTSIVSPALLCLPSEPCLAG LEEFFA*

Supplemental Figure S2. Amino acid (AA) sequences of wild type and four bi-allelic *Pangat1* mutants. Black letters represents the wild-type sense sequence, and red letters indicates a nonsense sequence due to a frameshift, asterisk indicates a premature stop codon.

Supplemetal Table S1. List of Golden Gate constructs used in this study.

Construct	Description	Level	Backbone	contains[1]
1	*nptII* resistence cassette	1	pICH47802	pICSL70004:*nptII*
2	$35S_{pro}$:ΩNLS-Cas9:$35S_{ter}$	1	pICH47742	pICH41388:$35S_{pro}$, pAGM5331:ΩNLS, pICH41308::aCas9, pICH41414:$35S_{ter}$
3	PanGAT1sgRNA1	1	pICH47831	pICSL01009:AtU6p, corresponding PCR amplicon
4	PanGAT1sgRNA2	1	pICH47822	pICSL01009:AtU6p, corresponding PCR amplicon
5	CRISPR_ctrl	2	pICSL4723	1R: construct 1, 2F: construct 2, end-link pICH41744
6	CRISPR_PanGAT1	2	pICSL4723	1R: construct 1, 2F: construct 2, 3F: construct 3, 4F: construct 4; end-link pICH41766

[1]Position and orientation in level 2 Golden gate modules is depicted by a number followed by either F or R for forward or reverse orientation

Supplemental Table S2. Primers used in this study

Name	Purpose	Sequence
sgRNA-Rv	CRISPR assembly	tgtggtctccaAGCGTAATGCCAACTTTGTAC
PanSYMRK_sgRNA1	CRISPR assembly	tgtggtctcaattGCCAAGCATGGCTAGAGCCGgttttagag ctagaaatagcaag
PanSYMRK_sgRNA2	CRISPR assembly	tgtggtctcaattGGGGTCTTCCAAGGTGTGCGgttttagag ctagaaatagcaag
geno_PanGAT1_F	Genotyping CRISPR mutants	TGCAACTGGTTTTGGCGATG
geno_PanGAT1_R1	Genotyping CRISPR mutants	ACGCGGGTATTGAAAGAGAGG
geno_PanGAT1_R2	Genotyping CRISPR mutants	TCAGGAGAAAAACCCAGGGA

CHAPTER 6

6

General Discussion

Yuda Purwana Roswanjaya

Introduction

Plants rely on symbiosis with microorganisms in the soil to increase their access to scarce nutrients. Nitrogen and phosphorous are often the limiting factor for plant growth and development. To cope with this nutrient shortage, most plants establish symbiotic relationships with microorganisms (Parniske, 2000). Symbiosis is an integral aspect of plant nutrition and classically refers to any relationship of organisms living together, regardless of the resulting benefits or cost this brings to both partners. Endosymbiosis is the most intimate form of symbiosis (Martin & Schwab, 2013). During endosymbiosis, all or part of the microbe is hosted within a plant cell, which allows a targeted exchange of nutrients between the two partners. The most widespread and ancient form of endosymbiosis is the interaction of plants with arbuscular mycorrhizal (AM) fungi, which form an extension to the plant root system and help the plant to take up nutrients. Several other endosymbioses that evolved later use mechanisms and core gene sets involved in AM symbiosis. One well studied example of such co-option of genetic network of AM symbiosis is the interaction between nitrogen-fixing rhizobia and nodulating plants.

Phosphate is a crucial nutrient for plant growth that is limited by its low solubility and low diffusion in the soil (Smith & Smith, 2011; Walder *et al.*, 2015). This nutrient is quickly depleted in the soil directly surrounding the plant root (Lewis & Quirk, 1967). To increase the uptake of this limited phosphate, most of the plant species utilize a symbiosis with AM fungi to increase the total volume of soil from which nutrients can be taken up. AM fungi extend beyond this depleted zone and are less prone to create depletion zones themselves due to their small diameter. AM fungi also transfer more mobile nutrients like nitrogen to the plant (Leigh *et al.*, 2009), but the importance of mycorrhiza for nitrogen nutrition seems to be limited (Smith & Read, 2010). Besides increasing access to nutrients, mycorrhiza can enhance water uptake (Augé, 2001) and induce plant resistance to biotic stresses (Pozo *et al.*, 2010).

Besides a symbiosis with AM fungi, some plant species can establish also a nodule symbiosis with nitrogen-fixing bacteria to overcome nitrogen deficiency. Nodulating plants are only found in four related taxonomic orders that form a monophyletic lineage: the so-called nitrogen-fixing clade representing Fagales, Fabales, Cucurbitales, and Rosales (Soltis *et al.*,

1995). Within the nitrogen fixing clade, 10 out of 28 plant families comprise species that can establish nitrogen-fixing nodule symbiosis (Soltis *et al.*, 1995). Most families contain only a few nodulating plant species. For example, in the cannabis family (Cannabaceae, order Rosales) *Parasponia* is the only nodulating genus representing five species, whereas the other nine genera in this family represent species that are unable to do so. In this respect, the legume family (Fabaceae, order Fabales) is an exception. This family comprises over 20,000 species divided over 750 genera, of which most possess the nitrogen-fixing nodule symbiosis trait. Taken together, in the nitrogen fixing clade, lineages of nodulating plants are dispersed as this clade also represents many lineages of non-nodulating species.

To establish a nitrogen-fixing nodule symbiosis, plants associate with one of two different types of diazotrophic microsymbionts. Legumes interact with a group of gram-negative bacteria collectively known as rhizobia. Also, *Parasponia* species establish a nitrogen-fixing symbiosis with rhizobia. The remaining nodulating plants associate with gram-positive filamentous *Frankia* species and therefore are collectively called actinorhizal plants. Intriguingly, *Parasponia* and legumes that both interact with rhizobia do not represent a monophyletic group, but diverge ~100 million years ago and are interspersed with lineages that nodulate with *Frankia*. Also, there is a significant phenotypic variation in legume, *Parasponia* and actinorhizal nodules, especially in nodule ontogeny, infection mode, and the way micro-symbionts are hosted. Originally, this led to speculations whether nitrogen-fixing nodule symbiosis evolved multiple times independently in a divergent manner, preceded by a predisposition in the last common ancestor of the nitrogen fixing clade (Swensen, 1996; Doyle, 1998, 2011, 2016; Werner *et al.*, 2014; Li *et al.*, 2015; Martin *et al.*, 2017). However, phylogenomic studies found strong evidence that nodulation evolved only once in the root of the nitrogen fixing clade followed by massive parallel loss (Velzen *et al.*, 2018; Griesmann *et al.*, 2018; van Velzen *et al.*, 2019). In this chapter, I will discuss the results described in this thesis in which I use *Parasponia* as a comparative system, and what these findings imply concerning conserved mechanisms in nodulation and mycorrhization.

Comparative studies to identify components of the core genetic network on nodulation and mycorrhization

In the last two decades, it was observed from mutant analysis in legumes and transcriptomic studies on legumes and non-legumes that there is a conserved common symbiosis signalling pathway (CSSP), which is used for nodulation and mycorrhization. (Markmann *et al.*, 2008; Gherbi *et al.*, 2008; Camp *et al.*, 2011; Hocher *et al.*, 2011; Tromas *et al.*, 2012; Svistoonoff *et al.*, 2013, 2014; Granqvist *et al.*, 2015; Fabre *et al.*, 2015; Chabaud *et al.*, 2016). Phylogenomic studies revealed a correlation between presence of key CSSP genes -like *SYMRK*, *CCaMK,* and *CYCLOPS*- in plant species that can establish intracellular endosymbiosis such as AM and nitrogen-fixing nodule symbiosis, and the loss of these genes in plants which do not engage in any type of those intracellular infection symbiosis. This defines the CSSP as a universal signalling pathway for intracellular mutualistic symbiosis in plants (Radhakrishnan *et al.*, 2020).

There are 126 symbiotic nodulation genes (including genes those of the CSSP) that have been identified through mutant analysis in legumes (Velzen *et al.*, 2018; Roy *et al.*, 2020). These genes have diverse functions, ranging from symbiotic signalling, transcriptional regulation leading to nodule organogenesis, systemic signalling to control nodule numbers, guiding rhizobium infection, formation of symbiosomes, maturation and senescence of the nodule. Orthology assessment can be used to assess the commonalities of the genetic basis of nodulation in the model legume *Medicago truncatula* and the non-legume *Parasponia andersonii* (Velzen *et al.*, 2018). This approach can also determine whether *Parasponia*-rhizobium symbiosis requires the same genetic signalling pathway as has been uncovered legumes. Molecular insights in the nodulation trait of non-legumes can also be retrieved from comparative studies. These studies, for example can be used to identify orthologous genes that show a conserved expression profile in nodules. In *P. andersonii*, 1,719 genes were identified to have a nodule enhanced expression. By comparing the transcriptome of *M. truncatula* and *P. andersonii*, ~290 shared nodule-enhanced genes could be identified (Velzen *et al.*, 2018). This suggests that the root nodulation in both species is orchestrated by similar transcriptional networks. Some of these genes have been identified also in forward genetic approaches in model legumes; such as the transcription factors *CYCLOPS* and *NODULE*

INCEPTION (*NIN*). Interestingly, within this 290 commonly-recruited gene set, only 26 have previously been identified in legumes as symbiotic genes, indicating that a large part of this core genetic basis of nodulation remains to be uncovered. Therefore, a combination of omics studies together with genetics in models and non-models is essential to obtain mechanistic insights and to identify the symbiosis genes that belong to the core genetic network controlling nodulation as well as mycorrhization. Further, comparative genetic analysis can highlight the information on the specific adaptations that occurred in a specific clade. These lineage-specific adaptations provide insights on the degree of flexibility of how symbiotic signalling is integrated (Huisman & Geurts, 2020).

The nodulating non-legume *Parasponia* comprise five species that are phylogenetically embedded within the non-nodulating *Trema* genus (Yang *et al.*, 2013; Velzen *et al.*, 2018), indicating a close relationship. Therefore, these two groups of species are ideal for a comparative study to identify genetic adaptations associated with the nodulation trait. Comparative genome analysis revealed that *Parasponia* and *Trema* species are highly similar in genetic make up (Velzen *et al.*, 2018). To get insight into the molecular-genetic changes underlying the evolution of nitrogen-fixing nodule symbiosis, comparative studies on *Parasponia* and non-nodulating species in Rosales order -including *Trema*- were done (Velzen *et al.*, 2018). This revealed the pseudogenization or even loss of three nodulation genes in *Trema* and other non-nodulating Rosales species. Among the lost genes are *NOD FACTOR PERCEPTION2* (*NFP2*) encoding a LysM-type receptor involved in recognizing rhizobial lipochitooligossacharide (LCO) signal molecules, *NIN* encoding a LCO responsive transcription factor that is essential for nodule organogenesis and bacterial infection, and *RHIZOBIUM DIRECTED POLAR GROWTH* (*RPG*) encoding a coil-coiled protein that functions in rhizobium intracellular infection threads elongation. These findings were supported by a complementary study comparing in total 37 nodulating and non-nodulating species covering the nitrogen fixing clade (Griesmann *et al.*, 2018). As *NFP2*, *NIN* and *RPG* are only expressed in a symbiotic context and commit specific function in nodulation, loss of these gene in non-nodulating species of the nitrogen fixing clade suggests that these species have lost the capacity to form nitrogen-fixing nodule. These findings are in line with the hypothesis that the nitrogen-fixing nodulation trait evolved only once in the root of the nitrogen fixing clade, followed by massive parallel loss of the trait (van Velzen *et al.*, 2019). Outside of the nitrogen

fixing clade, putative orthologs of *NIN*, *RPG,* and *NFP* can be found. In these species, they likely perform a function independent of symbiotic interaction with rhizobia or *Frankia*, and this function possibly lies in the interaction with arbuscular mycorrhizal fungi. In addition to *NIN*, *RPG* and *NFP2*, in *P. andersonii*, four other genes were identified with similar profiles of presence and absence in the nodulating and non-nodulating sister species (Velzen *et al.*, 2018). One of the genes, a putative *GAMMA (γ) AMINOBUTIRIC ACID TRANSPORTER* (*GAT1*) is exclusively expressed in *P. andersonii* nodules **(Chapter 5)**.

Evolution of LysM-type receptor kinase in the nitrogen fixing clade

The putative LCO-receptor NFP2 is one among the symbiosis genes that is lost in *Trema* (Velzen *et al.*, 2018). *Parasponia* NFP2 belongs to the so-called LYR-Ia clade, which have representatives in most plant species in or outside the nitrogen fixing clade. Phylogenetic comparisons uncovered a duplication in the LYR-Ia orthogroup at the root of the nitrogen fixing clade, resulting two subclades named NFP-I and NFP-II **(Figure 1)**. The NFP-II subclade comprises exclusively genes of plant species that are nodulated by Nod factor producing rhizobia (legumes and *Parasponia*), and Actinorhizal plants that are nodulated by *Frankia* cluster-II strains. The latter have found to possess Nod factor bio-synthesis genes, suggesting these *Frankia* species produce LCOs as well (Normand *et al.*, 1996; Van Nguyen & Pawlowski, 2017). In contrast, NFP-I orthologous genes are present in most species, except Fabales, suggesting the Fabales ancestral species lost the NFP-I gene copy. In legumes this loss coincided with a duplication of the NFP-II gene clade, giving rise to LCO receptors that function in nodulation (e.g. *LjNFR5* in *Lotus japonicus* and *MtNFP* in *Medicago truncatula*), and a gene copy that is responsive arbuscular mycorrhization (*LjLYS11* and *MtLYR1*) (Gomez *et al.*, 2009; Young *et al.*, 2011; Rasmussen *et al.*, 2016). Interestingly, we noted that the occurrence of a functional NFP-I type receptor associates with plant species that can establish an AM symbiosis. For example, both genes encoding the NFP-I and NFP-II type receptors pseudogenized in *Castanea mollisima* and *Fagus sylvatica* (Fagales), two species that establish ectomyorrhizal symbiosis instead of endomycorrhiza, nor can from nodules **(Chapter 3)**. This let us to investigate whether the NFP-I type receptor is essential for AM symbiosis in *P. andersonii.* We created CRISPR-Cas9 mutants in *PanNFP1*, though these knock out plants were not affected in AM efficiency. Instead, we observed a 50% reduction in nodulation

efficiency, indicating that this LysM-type receptor is functioning in nodulation. Even a CRISPR-Cas9 *Pannfp1;Pannfp2* double mutant didn't reveal a phenotype in AM symbiosis, demonstrating that in *Parasponia* LYR-Ia clade LysM-type receptors are not essential for this interaction.

A second clade of LysM-type receptors, LYK-I, is also of outstanding importance for LCO perception. Legumes possess a highly specific LysM-type receptor for rhizobium Nod factor signalling; named MtLYK3/LjNFR1 in *M. truncatula* and *L. japonicus* **(Figure 1)**. This receptor evolved on a series of gene duplications in the Fabales order (De Mita *et al.*, 2014). These duplications also create several other LysM-type receptors, such as MtLYK9/LjCERK6, which are important for chitin-induced immunity and AM symbiotic signalling (Bozsoki *et al.*, 2017; Leppyanen *et al.*, 2018; Gibelin-Viala *et al.*, 2019; Feng *et al.*, 2019). This suggests a functional overlap of LCO and chitin signalling in AM symbiosis, similar as is reported for rice were a single receptor -named OsCERK1- functions in chitin-induced immunity and AM symbiosis (Miyata *et al.*, 2014; Zhang *et al.*, 2015). In *M. truncatula*, a *Mtlyk9;Mtnfp* double mutant is significantly affected in AM colonization, whereas both single mutants do not show such phenotype (Feng *et al.*, 2019). It underlines the dual function of MtLYK9, and it indicates a role of MtNFP in AM symbiosis.

The duplication and subsequent divergence of into a genuine rhizobium LCO receptor and a CERK-type receptor has not occurred in nodulating non-legumes. Compelling, *Parasponia PanLYK3* encodes a trifunctional receptor that is also essential for chitin innate immune signalling. Although *Panlyk3* knockout mutants are still able to nodulate, they severely affected in rhizobium infection. Interestingly *PanLYK3*, is partially functionally redundant with *PanLYK1*; another conserved member of the LYK-I clade. Only a double *Panlyk1;Panlyk3* mutant showed to be fully blocked in nodule formation and AM interactions **(Chapter 3)**. The *PanLYK1 – PanLYK3* duplication showed to be ancient and associates with the birth of eudicots. The duplication gave rise to the LYK-Ia and LYK-Ib subclades **(Figure 1)**. The partially redundant functioning of *PanLYK1* and *PanLYK3* in AM symbiosis suggests that this may also be the case other dicot species. Outside the nitrogen fixing clade, chitin signalling pathway appears to be the major function of LYK-Ib clade members, such as *Arabidopsis thaliana* AtCERK1 or tomato SlLYK1 (Miya *et al.*, 2007; Liao *et al.*, 2018). Tomato SlLYK12, a

representative member of the LYK-Ia clade showed to be involved in AM infections (Liao *et al.*, 2018). This supports the ancient functioning of LYK-Ia and LYK-Ib subclades in arbuscular mycorrhization **(Chapter 3)**.

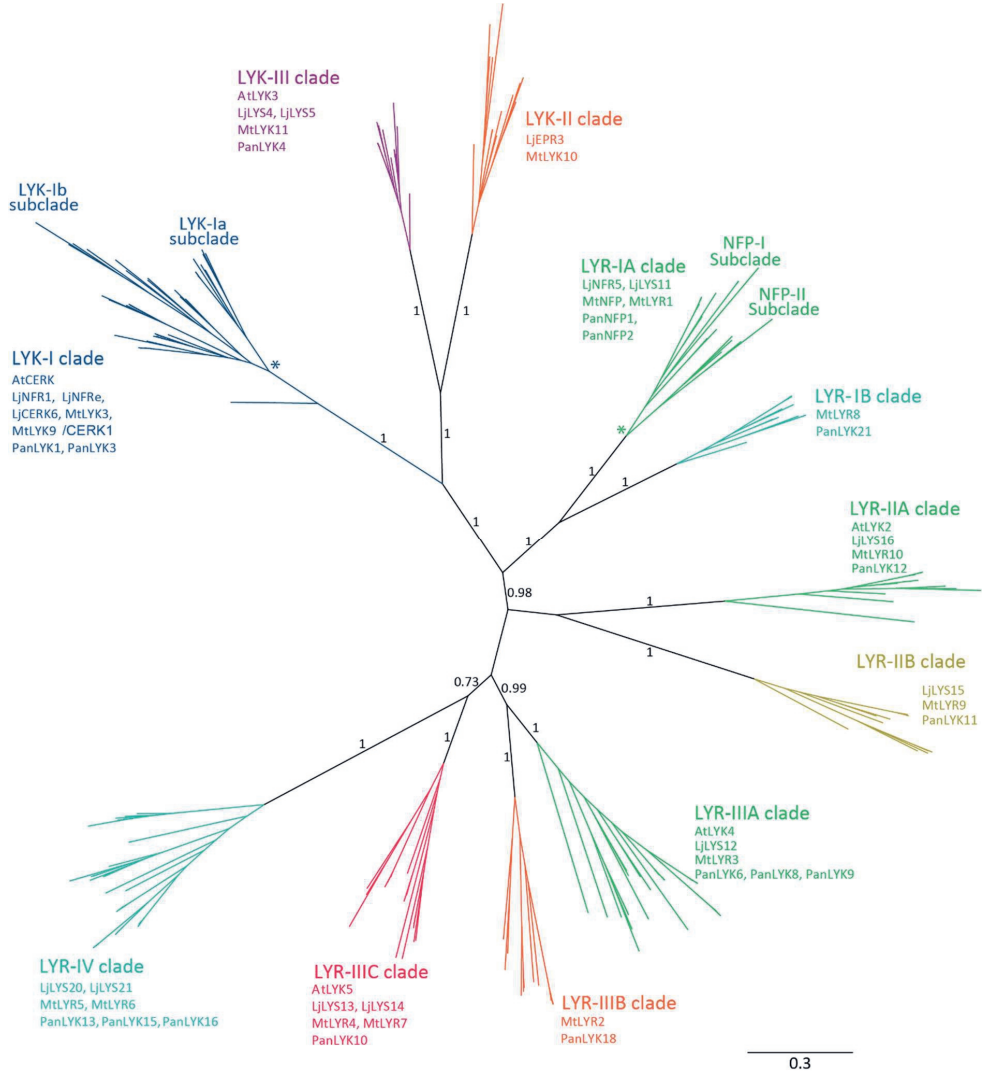


Figure 1. Phylogeny reconstruction of orthogroups representing LysM-type receptors. Identified orthogroups are marked in different colours and named according to previously described phylogenetic grouping (Buendia *et al.*, 2018). Indicated are the known receptors from *Arabidopsis thaliana* (At), *Lotus japonicus* (Lj), *Medicago truncatula* (Mt), and the identified *Parasponia andersonii* (Pan) proteins. Protein sequences of all species were aligned using MrBAYES3.2.6 Bayesian inference implemented in Geneious R8.1.2. MrBayes. Posterior Branch Supports shown only for branches leading to clades. Indicated by asterix is the location of duplications. Tree scale and scale bar indicate substitutions per site.

Is PanGAT1 a core gene in a genetic network of nitrogen-fixing symbiosis?

Like the key symbiosis genes, *NFP2*, *NIN* and *RPG*, we found that the putative Gamma (γ)-aminobutyric acid (GABA) transporter GAT1 associates with the nodulation trait. GABA can be found in almost all plant tissues, including nodules (Sulieman, 2011), indicating that GABA transporters are required in this symbiotic organ. GABA transporters can transport GABA across the plasma membrane. *A. thaliana* grows efficiently on GABA as a sole nitrogen source, thereby providing evidence for the existence of GABA transporters in plant roots (Breitkreuz *et al.*, 1999). Two low-affinity GABA transporters were identified, amino acid permease 3 (AAP3) and proline transporters 2 (ProT2). These two GABA transporters can transport also proline (Breitkreuz *et al.*, 1999; Yang *et al.*, 2020; Li *et al.*, 2021). Later, it was shown that AtGAT1 is a transporter with a high affinity to GABA (Meyer *et al.*, 2006). We found three copies of putative high-affinity GABA transporters in *P. andersonii,* named PanGAT1, PanGAT2, and PanGAT3. In a comparative study between *Parasponia* and *Trema* species, only *GAT1* pseudogenized in *Trema* (Velzen *et al.*, 2018). Interestingly, among these three genes, only PanGAT1 is nodule-specific and highly upregulated in this organ **(Chapter 5)**. This suggests a possible role for PanGAT1 in nitrogen-fixing nodule symbiosis in *Parasponia*. However, forward genetic studies on this gene suggest that PanGAT1 is not essential for nodulation **(Chapter 5)**. No nodule-related phenotype could be observed in *Pangat1* mutant lines, implying that PanGAT1 is not a core gene in a genetic network of nitrogen-fixing symbiosis. The fact the *Pangat1* mutants show no phenotype in nodulation could suggest that GABA transport is not essential for nodulation in *P. andersonii*. Alternatively, the lack of phenotype can be the result of gene redundancy; other GAT genes can compensate for the loss of PanGAT1. Since PanGAT1 is the first putative GABA transporter reported to be specifically expressed in nodules, a comparison to legumes species regarding the role of GABA in nodulation is relevant.

The conserved symbiotic function of SYMRK in nodulation and mycorrhization

In legumes, the LRR-type receptor kinase SYMRK is essential for root endosymbiosis with rhizobia and the formation of an AM symbiosis (Endre *et al.*, 2002; Stracke *et al.*, 2002). Studies on *Casuarina glauca* revealed that this gene is also required for nodulation in actinorhizal plants (Gherbi *et al.*, 2008). Trans-complementation studies in legumes and RNAi

studies in *Datisca glomerata* showed that SYMRK in this actinorhizal plant can function also in AM symbiosis (Markmann et al 2008). This supports the finding of a shared genetic basis of the different type of endosymbioses. To study role of the *SYMRK* gene in *P. andersonii*, we used a reverse genetic approach by creating a CRISPR-Cas9 knockout mutants of PanSYMRK. Phenotyping these mutants showed that PanSYMRK is essential for nitrogen-fixing symbiosis as well as AM symbiosis **(Chapter 4**). Root nodules and mycorrhizal infections are totally abolished in these mutants. In some *Pansymrk* mutant plants extensive development of extraradical hyphae was observed upon inoculation with the AM fungus *Rhizophagus irregularis*. These hyphae grow along the epidermis cells and formed appressoria that were frequently associated with an abnormal, swollen hyphal structure. However, most fungal infection attempts were arrested in the epidermal cells and aborted, indicating that the mutant epidermal cells cannot support fungal infection. The failure of fungi to penetrate the epidermis resulting in a very low level of intraradical colonization **(Chapter 4)**. A similar phenotype is also observed in legume *symrk* mutants and actinorhizal *Dgsymrk* RNAi lines (Markmann *et al.*, 2008). Taken together, these findings support a conserved symbiotic functioning of SYMRK.

Interestingly, in *Trema orientalis* we observed a seemingly critical mutation in the 5'-donor splice site of intron 12 of TorSYMRK. We showed that *T. orientalis* can be mycorrhized, though less effective when compared *P. andersonii* **(Chapter 4)**. In-depth analysis showed that TorSYMRK represents a functional gene, carrying a very rare non-canonical 'GA' 5'-donor splice site in intron 12. This TorSYMRK allele can functionally complement the *P. andersonii Pansymrk* knockout mutant. Therefore, it remains elusive what causes the differences mycorrhization efficiency between *P. andersonii* and *T. orientalis*.

Future efforts for engineering of nodulation

Nitrogen-fixing crop plants are crucial to sustainable agriculture because they can reduce chemical fertilizer application. This sparked research on engineering the nodulation capacity in non-nodulating crops. To achieve such an ambitious objective, it is essential to identify genetic adaptations critical for the nodulation trait. To this end, we aimed to identify the core set of nodulation genes conserved in nodulating species of different taxonomic clades. One

approach to identifying such a core set of nodulation genes is to expand the genetic studies to non-model species of the Rosales, Cucurbitales, and/or Fagales orders. Such a strategy is also relevant and complementary to studies in non-legume model systems and will minimize a bias towards a focus on legume-specific adaptations. We developed *Parasponia* into such a complementary system. The species has a relatively short seed-to-seed generation time of 6 months, though even more importantly, it can be efficiently transformed and subsequently propagated *in vitro*. I developed a quantitative arbuscular mycorrhization assay for *P. andersonii* **(Chapter 2)**, which allowed us to identify *PanLYK1* and *PanLYK3* as critical LysM-type receptors to establish endosymbiosis with AM fungi and rhizobia. Also, we showed that *PanNFP2* functions exclusively in LCO-induced nodulation. These findings pinpoint these receptors as important targets for engineering the nodulation trait.

Another important aspect to be considered in an engineering approach is the selection of a plant species that can serve as a model for engineering the nitrogen-fixing nodulation trait. Such plant species should be easy to work with in laboratory conditions, have established protocols for seed germination and plant growth, have a relatively small diploid genome that is fairly homozygotic, and can be transformed (Huisman & Geurts, 2020). We propose *Trema* species as such an engineering model. *Trema* is a non-nodulating sister plant of *Parasponia* and most probably lost the nodulation trait ~20 million years ago. In *Trema* species, only a limited number of nodulation genes have been pseudogenized and studies presented in this thesis showed that not even all are critical for nodulation (e.g. the GABA transporter GAT1). Therefore, *Trema* sp. represents an excellent models aiming to repair the nodulation trait.

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Summary

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Summary

Arbuscular mycorrhiza (AM) fungi, which belong to the Glomeromycota subphylum, can form a symbiotic interaction with the majority (>80%) of land plants. AM fungi can benefit their hosts in multiple ways. The primary advantage is that AM fungi facilitate uptake of mineral nutrients such as phosphate, nitrogen, and microelements from the soil by developing extensive extraradical hyphal mycelium, which expands the absorption surface of the host root system. Furthermore, AM fungi provide protection against plant pathogens by improving host fitness and competing for colonization sites with pathogens or activating systemic acquired resistance. Additionally, they affect the microbiome surrounding the roots and improve soil structure. In return for that benefits, AM fungi receive considerable amounts (up to 20%) of photosynthetic carbohydrates and/or lipids from the plant. In fact, AM fungi have become obligate biotrophs that fully depend on their host plants to complete their life cycle.

Besides a symbiosis with AM fungi, a more recent symbiosis association evolved between a much smaller group of plant species with nitrogen-fixing rhizobia or *Frankia* bacteria. The difference between both symbioses is the formation of a new organ on the host plant root in case the interaction with nitrogen-fixing bacteria. This organ is the so-called root nodule in which the nitrogen-fixing bacteria are hosted. Inside nodules, plants provide the optimal conditions for the bacteria to convert atmospheric nitrogen (N_2) into ammonia, which is provided to the plant host in exchange for photosynthates. The nitrogen-fixing nodule symbiosis occurs exclusively in a subset of genera of 10 taxonomic families. These nodulating plant genera form a paraphyletic clade in the orders Fabales, Fagales, Cucurbitales, and Rosales, collectively known as 'Nitrogen Fixing Clade' (NFC). However, phylogenomic studies indicate that this phylogenetic pattern of nodulating and non-nodulating plants is the result of a single evolutionary gain of the nodulation trait at the root of the NFC, followed by parallel loss of nodulation in many lineages.

Studies on the model legumes *Medicago truncatula* and *Lotus japonicus* showed the nodule formation is initiated upon the perception of rhizobial secreted lipochitooligosaccharides (LCOs) signal, which is known as Nodulation (Nod) factor. This signal is structurally related to the symbiotic signals produced by AM fungi, which are also perceived by the plant root to initiate fungal colonization and arbuscules formation. Rhizobial and mycorrhizal LCOs are

recognized by specific LysM-type receptor complexes and activate a signalling pathway that is largely shared between both symbioses. Therefore the signalling pathway is called the Common Symbiosis Signalling Pathway (CSSP).

A main aim in nitrogen-fixing nodulation research is to engineer the nodulation capacity into non-nodulating crops. To this end, it is important to identify critical genetic adaptations underlying the nodulation trait. Research on nodulation has been done on legumes, mainly on models like *M. truncatula* and *L. japonicus*, resulting in legume-biased knowledge. One strategy for defining a core set of nodulation genes is to expand the genetic studies to non-model species of the Rosales, Cucurbitales, and/or Fagales orders. For this purpose, we selected *Parasponia andersonii* as a complementary research system. The single evolutionary gain hypothesis implies that the core genetic adaptations underlying the nodulation trait are conserved in all nodulating plant species and activated via the CSSP network. In this thesis, I investigated conserved mechanisms in nodulation and mycorrhization in *Parasponia*.

One essential aspect of a plant research system is the availability of reliable protocols to study rhizobium and AM symbioses. In **Chapter 2**, I describe the development of a quantitative mycorrhization assay for *P. andersonii*. For this, I determined the conditions for *P. andersonii* to interact with the AM fungus *Rhizophagus irregularis*. The development of such an assay is justified by the fact that in some mutants, mycorrhization phenotypes are weak and require a robust yet sensitive quantitative assay. We demonstrate that *P. andersonii* is well-mycorrhized at low exogenous phosphate levels and that a close pot system can be used to avoid cross-contamination with rhizobia. Furthermore, We also show that the number of spores needed for *P. andersonii* mycorrhization depends on the plant starting material, as seedlings require less fungal spores to be colonized than tissue culture explants.

In **Chapter 3**, we discovered that *Parasponia* uses at least four LysM-type receptors for rhizobial Nod factor recognition. Of these four receptors, two are of LYK-I type, named PanLYK1 and PanLYK3. We showed that PanLYK3 has a triple function; controling nodulation, AM symbiosis, and chitin innate immune signalling. The second receptor, PanLYK1 has no phenotype when mutated. However, a double *Panlyk1;Panlyk3* mutant is completely blocked in nodule formation and arbuscular mycorrhizal infection. This indicates that there is -in part- functional overlap in symbioses signalling between these two LYK-I type receptors. The other two receptors, PanNFP1, and PanNFP2 belong to the LYR-Ia clade of LysM receptors, which

possess an inactive kinase domain. The pseudogenization of *NFP2* in non-nodulating plant species of the NFC indicates that this receptor is committed to function exclusively in nodulation. While *Parasponia Pannfp2* mutants cannot be infected by rhizobia or form nodules, they have no apparent phenotype in AM symbiosis. This confirms that this receptor is nodulation specific.

In legumes, the LRR-type receptor kinase SYMRK is essential for nodulation and mycorrhization. In **Chapter 4**, I show that this also the case in a non-legume. *P. andersonii SYMRK (PanSYMRK)* is essential for both nitrogen-fixing nodule and AM fungi symbiosis. In *Pansymrk* knockout mutants, root nodules and arbuscular mycorrhizal infections are totally abolished. These findings suggest that CSSP defines a conserved genetic basis for nodulation and mycorrhization in *Parasponia*, similar as found in legumes and actinorhizal plants. In this chapter, I also show that a very rare non-canonical 'GA' mutation located in the 5'-splice donor site of intron 12 in the *TorSYMRK* gene of *Trema orientalis* does not affect mycorrhization.

Like the LCO receptor NFP2, the Gamma (γ) amonibutyric acid transporter1 (GAT1) was consistently lost in non-nodulating *Trema* species. In *P. andersonii* this transporter is exclusively expressed in nodules, which implies a specific function during nodulation. In **chapter 5**, I characterized nodule phenotypes (e.g., nodule number, size, cytoarchitecture, bacterial and GABA content) in *P. andersonii Pangat1* mutants and revealed that PanGAT1 is not essential for nodulation. Therefore, it suggests that GAT1 is not a primary target gene in the ongoing effort to restore the nodulation trait in *Trema* species.

In **Chapter 6**, I discuss the data generated during my thesis research concerning a conserved mechanism in nodulation and mycorrhization on non-legume *Parasponia*. Following our conclusion that *PanNFP2* and *PanSYMRK* function is conserved in *Parasponia* similar as found in legumes. Further, we suggest that *PanNFP2* is obviously an essential engineering candidate for nodule organogenesis in *Trema* lineages.

Samenvatting

Arbusculaire mycorrhiza (AM) schimmels behoren tot het sub-fylum Glomeromycota en gaan een symbiotische interactie aan met ongeveer 80% van alle landplanten. AM-schimmels kunnen hun waardplant op meerdere manieren ten goede komen. Het meest voor de hand liggende voordeel is dat AM-schimmels de opname van minerale voedingsstoffen zoals fosfaat, stikstof en micro-elementen uit de bodem vergemakkelijken. Dit gebeurd door een uitgebreid extraradicaal mycelium van schimmeldraden te ontwikkelen, welke het absorptieoppervlak van het wortelsysteem van de waardplant velen malen vergroot. Verder zijn er aanwijzingen om aan te nemen dat interacties met AM schimmels ook bescherming bieden tegen plantpathogenen. Er worden vaak drie mogelijke oorzaken voor dit effect genoemd. Zo kan het zijn dat AM schimmels; zorgen voor een verbetering van de groeicondities van een waardplant, waardoor zij minder vatbaar wordt voor infecties of direct concurreren met pathogenen om een gelimiteerd aantal infectieplekken, of door een systemische resistentie in de waardplant te activeren. Ten slotte beïnvloeden AM schimmels het microbioom rond de plantwortels en verbeteren ze de bodemstructuur, waarin de waardplant groeit. In ruil voor al dit krijgt de AM schimmel een aanzienlijke hoeveelheid (tot 20%) koolhydraten en/of lipiden van de waardplant. AM schimmels zijn obligaat biotroop en dus volledig afhankelijk van een waardplant voor het voltooien van hun levenscyclus.

Naast deze symbiose tussen landplanten en AM schimmels, is relatief recent nog een tweede plant-microbe symbiose ontstaan. Deze symbiose vindt plaats tussen een specifieke groep planten en stikstofbindende bacteriën behorende tot de rhizobia of Frankia genera. De stikstofbindende symbiose is gelimiteerd tot een veel kleinere groep plantensoorten en komt uitsluitend voor in een sub-deel van de geslachten van 10 taxonomische families, slechts een kleine 3% van alle landplanten. Deze plantengeslachten vormen een parafyletische clade in de ordes Fabales, Fagales, Cucurbitales en Rosales, welke gezamenlijk bekend staan als de 'Nitrogen Fixing Clade' (NFC). Fylogenetische studies leiden tot de hypothese dat dit uitzonderlijke patroon van knolvormende en niet-knolvormende planten het resultaat kan zijn van één evolutionaire verandering die geleid heeft tot het ontstaan van knolsymbiose vroeg in de NFC, gevolgd door veelvuldig parallel verlies van deze eigenschap in veel van de niet knolvormende geslachten in deze clade. Een groot verschil tussen beide vormen van symbiose is dat bij de laatstgenoemde, een nieuw orgaan op de waardplantwortel wordt

gevormd tijdens de interactie met de bacterie. Dit orgaan is de zogenaamde wortelknol, waarin de stikstofbindende bacteriën worden gehuisvest. In deze wortelknollen zorgt de waardplant voor optimale condities voor de bacteriën om atmosferische stikstof (N2) om te zetten in ammoniak. Deze ammoniak wordt in ruil voor fotosynthaten aan de waardplant geleverd.

Onderzoek uitgevoerd op de modelplanten *Medicago truncatula* en *Lotus japonicus* (beiden vlinderbloemigen), tonen aan dat de vorming van wortelknollen wordt geïnitieerd na de waarneming van door de bacterie uitgescheiden signaalstoffen. Dit signaal zijn organische verbindingen behorende tot de lipochitooligosacchariden (LCO's) en wordt over het algemeen aangeduid als Nodulation (Nod) factoren. Nod factoren zijn chemisch gerelateerd aan de signaalstoffen, geproduceerd door AM schimmels Myc factoren. Ook deze Myc factoren worden door de waardplantwortel waargenomen. Ze induceren echter geen wortelknollen, maar leiden tot schimmelkolonisatie en de initiatie van arbuscules-vorming. Rhizobiale en mycorrhiza-LCO's worden herkend door specifieke LysM-type receptorcomplexen en activeren een signaalroute die grotendeels wordt gedeeld door beide symbiosen. Deze signaalroute wordt daarom ook wel de *Common Symbiosis Signaling Pathway* (CSSP) genoemd.

Het ultieme doel van onderzoek naar stikstofbindende wortelknollen is het overzetten van deze symbiose naar een niet-knolvormend gewas buiten de NFC. Om dit te bereiken is het belangrijk om de genetische aanpassingen, welke kritisch waren tijdens het evolutionaire proces om knolvorming te verwerven, in kaart te brengen. Er is veel hoogwaardig onderzoek gedaan naar knolvorming bij vlinderbloemige, voornamelijk aan plantmodellen zoals *M. truncatula* en *L. japonicus*. Dit heeft helaas als bijvangst dat onze kennis met betrekking tot knolvorming eenzijdig en sterk op vlinderbloemige gefocust is. Één nieuwe strategie voor het definiëren van een kernset aan knolvormingsgenen is om dit soort studies uit te breiden naar niet-modelsoorten behorende tot de Rosales, Cucurbitales en Fagales. Met dit doel in gedachten hebben wij *Parasponia andersonii* geselecteerd als een complementair model. De hypothese dat alle wortelknolsymbiose naar een enkel evolutionair event te herleiden is, zou kunnen betekenen dat; 1) de genetische basisaanpassingen welke hieraan ten grondslag ligt in alle knolvormende plantensoorten geconserveerd is, en 2) deze symbiose geactiveerd kan worden via één gemeenschappelijke symbiose-signaleringsnetwerk. In dit proefschrift heb ik

de mogelijkheid van geconserveerde mechanismen in nodulatie en mycorrhisatie in Parasponia onderzocht.

Een essentieel aspect van een geschikt plantmodel om plant-microbe symbiose te onderzoeken is de beschikbaarheid van betrouwbare protocollen om zowel wortelknol als AM symbiose te bestuderen. In **Hoofdstuk 2** beschrijf ik de ontwikkeling van een kwantitatieve mycorrhisatie test voor *P. andersonii*. Hierbij heb ik de optimale condities bepaald voor *P. andersonii* om te interacteren met de AM schimmel *Rhizophagus irregularis*. De ontwikkeling van zo'n gevoelig test is nodig, omdat in sommige mutanten de mycorrhisatiefenotypes mild zijn en dus een robuuste maar gevoelige kwantitatieve test vereisen. Ik toon aan dat *P. andersonii* goed gemycorrhizeerd kan worden bij een laag exogeen fosfaatniveau, en dat een gesloten potsysteem uiterst geschikt is om kruisbesmetting met rhizobia te voorkomen. Verder demonstreer ik dat het aantal sporen nodig om *P. andersonii* te mycorrhizeren afhangt van het startmateriaal, aangezien zaailingen minder schimmelsporen nodig hebben om gekoloniseerd te worden dan planten verkregen uit weefselkweek.

In **Hoofdstuk 3**, zoemen we in op de LysM-receptor family van P. andersonii. We laten zien dat *P. andersonii* tenminste vier LysM-type receptoren gebruikt voor herkenning van rhizobiale Nod factoren. Van deze vier receptoren behoren er twee tot het LYK-I-type (PanLYK1 en PanLYK3) en twee tot de LYR-Ia-type (PanNFP1 en PanNFP2) LysM-receptoren. Dit laatste type wordt gekenmerkt door een inactief kinasedomein. We demonstreren dat PanLYK3 bij minimaal drie processen betrokken is; de wortelknol symbiose, de AM symbiose en de immuun signalering van chitine. De tweede receptor, PanLYK1 heeft geen waarneembaar fenotype wanneer gemuteerd. Echter, een dubbele mutant Panlyk1Panlyk3 is volledig geblokkeerd in de vorming van wortelknollen en infectie met de AM schimmel. Dit geeft aan dat er een -deels- functionele overlap is in symbiosesignalering tussen deze twee LYK-I-type receptoren en beide vormen van symbiose. De pseudogenisering van NFP2 in niet-knolvormende plantsoorten binnen de NFC geeft aan dat deze receptor uitsluitend in knolvorming functioneert. In tegenstelling tot eerdere berichten kan de *Pannfp2* mutant inderdaad niet worden geïnfecteerd door rhizobia en geen wortelknollen vormen, maar heeft zij geen fenotype in AM symbiose. Dit bevestigt dat NFP2 specifiek is voor knolvorming.

Bij vlinderbloemige is het LRR-type receptorkinase SYMRK essentieel tijdens zowel knolvorming als mycorrhisatie. In **Hoofdstuk 4** laat ik zien dat dit ook het geval is bij de niet-vlinderbloemige *P. andersonii*. In *P. andersonii* is SYMRK (PanSYMRK) essentieel voor zowel het induceren van stikstofbindende wortelknollen als de initiatie van AM symbiose. In *Pansymrk* knock-out mutanten zijn wortelknollen en AM infecties volledig verdwenen. Deze bevindingen suggereren dat CSSP ook in *P. andersonii* een geconserveerde genetische basis definieert voor knolvorming en mycorrhisatie, vergelijkbaar met die van planten in de orde van de Fabales. In dit hoofdstuk laat ik ook zien dat een mutatie welke leidt tot een zeer zeldzame 5'-splice donorplaats (GA) bij intron 12 in *TorSYMRK* geen invloed heeft op mycorrhisatie.

Net als de LCO-receptor NFP2 is ook de Gamma (γ) amonibutyric acid transporter (GAT1) consequent verloren gegaan in alle niet-knolvormende Trema-soorten. In *P. andersonii* wordt deze GABA transporter uitsluitend tot expressie gebracht in wortelknollen, wat een specifieke functie impliceert in de wortelknolsymbiose. In **Hoofdstuk 5** karakteriseer ik wortelknol gerelateerde fenotypes (bijv. aantal wortelknollen, grootte, cyto-architectuur, bacteriële- en GABA inhoud) van de *P. andersonii gat1* mutanten (*Pangat1*) en onthul dat PanGAT1 niet essentieel is voor knolvorming. Dit leidt tot de conclusie dat GAT1 geen primair doelwit hoeft te zijn in de voortdurende pogingen om de wortelknolsymbiose in Trema-soorten te herstellen.

In **Hoofdstuk 6** bespreek ik de resultaten die tijdens mijn thesisonderzoek zijn gegenereerd met betrekking tot een geconserveerd mechanisme tussen de wortelknol symbiose en mycorrhisatie in de niet-vlinderbloemige soort *Parasponia andersonii*. Ik reflecteer op het feit dat de functies van NFP2 en SYMRK geconserveerd zijn binnen *Parasponia andersonii*, vergelijkbaar met hun respectievelijke functies in vlinderbloemige. Verder concludeer ik dat *PanNFP2* een essentieel kandidaat gen is voor genetische modificatie in Trema soorten met als doel het herstel van de wortelknolsymbiose.

Ringkasan

Jamur arbuskular mikoriza, termasuk ke dalam sub-filum Glomeromycota, dan dapat membentuk interaksi simbiotik dengan sebagian besar (>80%) tanaman. Jamur arbuskular mikoriza dapat memberikan manfaat pada tanaman inangnya melalui berbagai cara. Manfaat utamanya adalah memfasilitasi penyerapan mineral seperti fosfat, nitrogen, dan mikroelemen dari dalam tanah melalui pembentukan hifa ekstraradikal yang ekstensif, yang meningkatkan luas permukaan penyerapan dari sistem perakaran tanaman inang. Lebih jauh, jamur arbuskular mikoriza menyediakan perlindungan terhadap patogen tanaman melalui peningkatan kebugaran tanaman inang dan berkompetisi untuk mendapatkan titik kolonisasi dengan patogen atau dengan mengaktifkan resistensi sistemik. Jamur arbuskular mikoriza juga mempengaruhi mikrobiom di sekitar perakaran dan meningkatkan struktur tanah. Sebagai timbal baliknya, jamur arbuskular mikoriza menerima sejumlah (lebih dari 20%) karbohidrat hasil fotosintesis dan atau lemak dari tanaman. Jamur arbuskular mikoriza merupakan biotrof obligat yang sepenuhnya bergantung pada tanaman inangnya untuk memenuhi siklus hidupnya yang lengkap.

Selain simbiosis dengan jamur arbuskular mikoriza, asosiasi simbiosis yang relatif lebih baru melibatkan antara sekelompok kecil spesies tanaman dengan bakteri pemfiksasi nitrogen, dari kelompok rhizobia atau Frankia. Perbedaan diantara kedua jenis simbiosis adalah pembentukan organ baru pada akar tanaman inang pada interaksi dengan bakteri pemfiksasi nitrogen. Organ ini disebut dengan nodul dimana bakteri pemfiksasi nitrogen hidup dan berkembang biak. Di dalam nodul, tanaman menyediakan kondisi yang optimal untuk bakteri untuk merubah nitrogen atmosferik menjadi amonia, yang diberikan ke tanaman dan ditukar dengan hasil fotosintesis. Simbiosis dengan bakteri pemfiksasi nitrogen terjadi secara eksklusif pada subset genus dari 10 tingkatan takson famili. Genus dari tanaman yang mampu membentuk nodul ini membentuk kelompok parafiletik dalam ordo Fabales, Fagales, Cucurbitales, dan Rosales, yang secara bersama-sama dikenal dengan “Kelompok Pemfiksasi Nitrogen”. Meskipun begitu, studi filogenomik mengindikasikan bahwa pola filogentik dari tanaman pembentuk nodul dan bukan pembentuk nodul berasal dari nenek moyang yang semuanya dapat membentuk nodul, yang kemudian diikuti dengan kehilangan paralel dari sifat ini di banyak garis keturunan.

Studi pada tanaman model *Medicago truncatula* dan *Lotus japonicus* menunjukan bahwa proses pembentukan nodul diinisiasi dengan penangkapan sinyal yang disekresikan oleh rhizobia berupa lipochitooligosakarida (LCO), yang dikenal dengan faktor nodulasi. Sinyal ini secara struktur mirip dengan sinyal simbiotik yang dihasilkan oleh jamur arbuskular mikoriza, yang juga diterima oleh akar tanaman untuk memulai kolonisasi jamur dan pembentukan arbuskul. LCO yang dihasilkan oleh rhizobia maupun mikoriza dikenali oleh reseptor spesifik Lys-M kompleks dan mengaktifasi jalur sinyaling yang secara luas bersinggungan diantara kedua simbiosis. Oleh karenanya, jalur sinyaling ini disebut *Common Symbiosis Signaling Pathway* (CSSP).

Tujuan utama dari penelitian mengenai nodulasi-fiksasi nitrogen adalah untuk memindahkan kapasitas nodulasi pada tanaman hortikultura yang tidak bisa membentuk nodul. Untuk itu, sangat penting untuk mengidentifikasi adaptasi gentik penting yang mendasari sifat dari nodulasi itu sendiri. Penelitian mengenai nodulasi sudah banyak dilakukan pada legum, khususnya pada tanaman model seperti *M. truncatula* dan *L. japonicus*, menghasilkan pengetahuan yang bias karena hanya terbatas pada nodulasi di tanaman legum. Salah satu strategi untuk mencari kelompok gen inti pada proses nodulasi adalah dengan memperluas studi genetik pada spesies bukan model di ordo Rosales, Cucurbitales, dan Fagales. Untuk kepentingan ini, kami memilih *Parasponia andersonii* sebagai sebuah sistem yang bisa melengkapi. Hipotesis bahwa pada awalnya semua tanaman dapat membentuk nodul mengindikasikan bahwa adaptasi inti genetik yang mendasari sifat nodulasi adalah lestari pada semua spesies tanaman yang mampu membentuk nodul dan diaktifasi melalui *common simbiosis signalling pathway*. Di tesis ini, saya menginvestigasi mekanisme yang lestari pada nodulasi dan mikorisasi di tanaman *Parasponia*.

Salah satu aspek penting dalam penelitian mengenai nodulasi dan mikorisasi pada tanaman adalah ketersediaan protokol untuk mempelajari simbiosis dengan rhizobium dan jamur arbuskular mikoriza. **Di BAB 2**, saya menjelaskan pengembangan uji kuantitatif mikoriza untuk *P. andersonii*. Untuk itu, saya mencari kondisi yang optimal untuk *P. andersonii* agar dapat berinteraksi dengan jamur mikoriza *Rhizophagus irregularis*. Pengembangan uji ini didasari oleh kenyataan bahwa di beberapa mutan, fenotif mikorisasi sangat lemah dan membutuhkan uji yang ajeg tetapi sensitif. Hasil kami menunjukan bahwa *P. andersonii* dapat termikorisasi dengan baik pada level fosfat yang rendah dan sistem pot tertutup dapat

digunakan untuk menghindari kontaminasi silang dengan rhizobia. Lebih jauh, kami juga menunjukan bahwa jumlah spora yang dibutuhkan untuk memikorisasi *P. andersonii* tergantung pada material awal tanaman, bibit dari biji membutuhkan spora jamur yang lebih sedikit untuk terkolonisasi dibandingkan dengan eksplan yang diperoleh dari kultur jaringan.

Di BAB 3, kami menemukan bahwa *Parasponia* menggunakan setidaknya 4 tipe LysM reseptor untuk mengenali faktor nodulasi yang dihasilkan rhizobia. Dari empat reseptor ini, dua adalah tipe LYK-I, yang dinamai PanLYK1 dan PanLYK3. Kami menunjukan bahwa PanLYK3 memiliki tiga jenis fungsi; mengontrol nodulasi, simbiosis dengan mikoriza, dan sinyaling imun bawaan dari kitin. Reseptor kedua, PanLYK1 tidak memiliki fenotif ketika dimutasi. Namun, mutan *panlyk1;panlyk3* secara nyata memblokir pembentukan nodul dan infeksi mikoriza. Ini mengindikasikan bahwa terdapat sebagian fungsi yang tumpang tindih dalam sinyaling simbiosis diantara kedua tipe LYK1 reseptor. Dua reseptor lainnya, PanNFP1, dan PanNFP2 merupakan bagian dari LYR-Ia pada reseptor LysM, yang memiliki domain kinase yang tidak aktif. Pseudogenisasi dari NFP2 pada tanaman yang tidak dapat membentuk nodul mengindikasikan bahwa reseptor ini memiliki fungsi yang eksklusif dalam nodulasi. Di saat mutan Parasponia *Pannfp2* tidak dapat diinfeksi oleh rhizobia atau membentuk nodul, mutan reseptor tersebut tidak memiliki fenotif yang jelas dalam mikoriza simbiosis. Hal ini mengkonfirmasi bahwa receptor ini merupakan nodul spesifik.

Pada legum, tipe LRR receptor kinase SYMRK sangat penting untuk nodulasi dan mikorisasi. **Di BAB 4**, saya menunjukan bahwa pada kasus non legum *P. andersonii* SYMRK (PanSYMRK), berperan penting baik untuk simbiosis dengan rhizobia pemfiksasi nitrogen dan juga dengan jamur mikoriza. Pada mutan *Pansymrk*, pembentukan nodul dan infeksi mikoriza secara nyata tidak dapat terjadi. Penemuan ini menunjukan bahwa *Common Symbiosis Signaling Pathway* merupakan dasar genetik yang lestari untuk nodulasi dan mikorisasi pada *Parasponia*, sama halnya seperti yang ditemukan pada legum dan tanaman aktinoriza. Di bab ini, saya juga menunjukan bahwa mutasi non-canonical 'GA' yang sangat langka terletak pada 5'-splice site donor dari intron 12 di gen TorSYMRK tanaman *Trema orientalis* tidak memiliki pengaruh pada proses mikorisasi.

Seperti halnya reseptor LCO NFP2, Gamma (γ) amonibutyric acid transporter1 (GAT1) secara konsisten hilang pada spesies non-nodulasi *Trema*. Pada *P. andersonii*, transporter ini secara eksklusif terekspresi hanya pada nodul, yang mengimplikasikan fungsi yang spesifik pada

proses nodulasi. **Di BAB 5**, saya mengkarakterisasi fenotif dari nodul (jumlah nodul, ukuran nodul, citoarsitektur, jumlah bakteri dan kandungan GABA) pada mutan *P. andersonii* Pangat1 dan menghasilkan kesimpulan bahwa PanGAT1 tidak esensial untuk proses nodulasi. Berdasarkan hal tersebut, ini menunjukan bahwa GAT1 bukan merupakan target utama dari gen yang akan ditransfer ke spesies *Trema* untuk mengembalikan sifat nodulasi.

Di BAB 6, saya mendiskusikan data yang dihasilkan selama penelitian thesis ini terutama mengenai mekanisme lestari dari nodulasi dan mikorisasi pada non-legum *Parasponia*. Kesimpulan dari penelitian kami adalah PanNFP2 dan PanSYMR fungsinya lestari pada *Parasponia*, seperti yang ditemukan pada legum. Lebih lanjut, kami menyarankan bahwa PanNFP2 secara jelas merupakan kandidat yang menjanjikan untuk merekayasa nodul organogenesi pada tanaman *Trema*.

Appendices

Acknowledgements

I am grateful that my long, up-and-down, not-easy PhD journey has finally reached the finish line. Without support from so many amazing people, I think I would not make it. Here, I would like to acknowledge those who support me not only in my academic life but also in my personal life. First, I would like to thank the **Ministry of Research, Technology and Higher Education of the Republic of Indonesia** for providing me with a RISET-Pro scholarship to pursue a Doctoral degree in Molecular Biology Group, Wageningen University & Research.

René, it was in 2014, in Puncak, Bogor, Indonesia, when we first met. I remembered your question about whether I had experience doing research on molecular level. Even though, at that time, I only had a little background in the field that I know better now, you still gave me an opportunity to work on the *Parasponia* project under your supervision as a PhD student. Thank you for your trust, patience, understanding, and support in guiding me to do this project until the end. Your critical comments, advice, our countless discussions, your demands for better results (I had to repeat the mycorrhization assay in *lyk* and *nsp* mutants seven times) have shaped me to become a good independent researcher. As a student, I am sometimes too stubborn to be directed, but you always act in your best capacity as my co-promotor as well as my friend. Thank you for encouraging me to keep going to write this thesis and pushing me to the limit, even when I doubted myself that I could do this. Your optimism in science has inspired me. I can not wait to collaborate with you in the future, and I look forward to having you as a visiting researcher at my institute in Indonesia.

Ton, as a well-known senior scientist, I admire your consistency in doing research. Thank you for being my promotor, a person who always asks unpredictable questions during MOLBI Friday work discussions or rhizosphere group weekly meetings. Sometimes I did not expect those questions, even for a simple question like “why,” and found difficulties getting the answer. Now, I understand that all difficult questions are a way to stimulate my holistic critical thinking about my research.

Wouter, we were just a colleague at first, but when I jumped to mycorrhizal research in my second year, you were always there. I knew mycorrhiza was also new for you, but you were

willing to learn with me. Thank you for your persistent trust that I can do beyond my thought. With you, I could also publish two papers outside *Parasponia* in the prestigious journal: Science and Nature Communication as a co-author. I am very grateful for your help during the writing process. You initiated a weekly meeting, so I had to continue writing in Indonesia. Our countless discussions, quick revision, and suggestions were a spirit for me. You are a person who knows better how struggling I am with this thesis, and every time I felt I wanted to give up, you always came up with solutions or motivation that put me back on track. Since the beginning, you have always said that even though you were not my co-promotor, you would always help me. But, in the end, we managed to make you as my co-promotor officially. I am proud to be your first PhD student.

Luuk, at first, I had difficulties starting a conversation with you because you seemed unreachable. But over time, I realized that you are a good discussion partner and friend, and you were willing to help whenever I got problems in my experiments. Thank you for involving me in the project that became chapter 3 of this thesis. Further, I will never forget your kindness for being the only person who talked to me in English during someone's farewell dinner when everybody insisted on speaking in Dutch. I wish you good luck with your future, whatever path you will take.

Titis, I have known you since 2009 as a colleague at BPPT. As a friend, I felt honored to become a witness to your transformation from a naive girl to an independent woman. You are stubborn and sometimes *menyebalkan*, but on the other side, you know what you want and fight for it. Our interaction showed me a different meaning of friendship. Thank you for being a nice officemate both in Indonesia and the Netherlands, being a great gossip partner in the lab, and being a person to talk with in Bahasa Indonesia when I am tired of speaking in English during working hours. I am also grateful for having you as my paranymph and sharing the stage during my defense. Good luck with any plans you have made for your future, including your love life.

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also thankful that you accepted my request to be my paranymph and became an essential part of the end of my PhD journey. I wish you great success in your career.

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The current and former *Parasponia* team members; **Arjan**, **Robin**, **Rens**, **Trupti**, **Fengjiao**, **Simon**, **Kana**, **Sultan**, and **Rik**. **Arjan**, I would like to thank you for your patience taught me golden gate cloning at the beginning. Also, I enjoyed listening to the music in the Nederland.fm with you at the PT lab, revealing that you remembered all the lyrics from the up-to-date songs. I am sorry I did not make it to meet you when you visited Indonesia. **Robin**, thank you for your kindness and all the help in analyzing data during my first year when my project was still about the *Parasponia* microbiome. **Rens**, thank you for always being available when I need your help with bioinformatic things. **Trupti**, I wish I could do more to help you. Thank you for contributing to Chapter 4. **Fengjiao**, thank you for the friendship and picked me as your paranymph, although in the end, your defense was online, and you could not share the stage with me. I am also grateful to be one of the authors of your published manuscript. **Simon**, from all the friends in the lab, every time you need someone to water your plants because you were not around, you always ask me to do so. Thank you for your trust. **Kana**, I admire your hard work and your persistence. Thank you for your contribution to Chapter 3. **Sultan**, Thank you for your scientific contribution in Chapter 4 and all the help you offered when I was in Indonesia. I wish you great success for the rest of your PhD and your career as a researcher in Saudi Arabia. **Rik**, you came at the right time. You Helped me with the mycorrhization assay on *nsp* and *lyk* mutants, and finally, after seven repeated experiments, we found a phenotype. Thank you for all the help and your input in Chapter 4.

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Maria and **Marie-José**, what would the lab be without you? Thank you for all the administrative work, organization, and ordering stuff. **Marie-José**, thank you for being there when I lost my wallet on the first day I arrived at Wageningen. On Saturday, on holiday, you picked me up in my apartment and brought me to the police station to make a report. It was the sweetest thing from you.

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Furthermore, I would like to thank my other Indonesian friends outside Wageningen. **Kang Kus** and **Mbak Etty**, we came to the Netherlands with the same dream. Thank you for the spirit and encouragement that we shared. **Om Angga** and **Miss Laurenz**, Indonesia we did it! Thank you for all the video calls, jokes, and laughs. I enjoyed my stay in Switzerland during the winter holiday back in 2018. **Rizki**, although we rarely meet in person, thank you for always keeping our friendship valuable. I hope I can visit you again in Muri anytime soon. **The Lanser family: William, Indry, Alexa, Jericko, and Ben**. Thank you for the warm welcome every time I visited you in Amsterdam.

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List of Publications

Yuda Purwana Roswanjaya, Sultan Alhusyani, Joël Klein, Luuk Rutten, Rik Huisman, Trupti Sharma, Wouter Kohlen, Rene Geurts (2022). An extremely rare non-canonical splice site found in Trema orientalis SYMRK does not affect its dual symbiotic functioning. *To be submitted*.

Jienshun Lin, **Yuda Purwana Roswanjaya**, Wouter Kohlen, Jens Stougaard, Dugald Reid (2021). Nitrate restricts nodule organogenesis through inhibition of cytokinin biosynthesis in *Lotus japonicus*. *Nature Communications* 12:6544, doi:10.1038/s41467-021-26820-9.

Luuk Rutten, Kana Miyata, **Yuda Purwana Roswanjaya**, Rik Huisman, Fengjiao Bu, Marijke Hartog, Sidney Linders, Robin van Velzen, Ton Bisseling, Wouter Kohlen, Rene Geurts (2020). The duplication of two symbiotic LysM-receptors predates the evolution of nitrogen fixing symbiosis. *Plant Physiology* pp.01420.2019, doi:10.1104/pp.19.01420

Titis A. K Wardhani, **Yuda Purwana Roswanjaya**, Simon Dupin, Huchen Li, Sidney Linders, Marijke Hartog, Rene Geurts, Arjan van Zeijl (2019). Transforming, genome editing and phenotyping the nitrogen-fixing tropical cannabaceae tree *Parasponia andersonii*. *Journal of Visualized Experiments* (150), e59971, doi:10.3791/59971.

Fengjiao Bu, Luuk Rutten, **Yuda Purwana Roswanjaya**, Olga Kulikova, Marta Rodriguez-Franco, Thomas Ott, Ton Bisseling, Arjan van Zeijl, Rene Geurts (2019). Mutant analysis in the non-legume Parasponia andersonii identifies NIN and NF_YA1 transcription factors as a core genetic network in nitrogen-fixing nodule symbioses. *New Phytologist*, doi:10.1111/nph.16386.

Robin van Velzen, Rens Holmer, Fengjiao Bu, Luuk Rutten, Arjan van Zeijl, Wei Liu, Luca Santuari, Qingqin Cao, Trupti Sharma, Defeng Shen, **Yuda Purwana Roswanjaya**, Titis A K Wardhani, Maryam Selfi Kalhor, Jolle Jensen, Johan van den Hoogen, Berivan Güngör, Marijke Hartog, Jan Hontelez, Jan Verver, Elio Schijlen, Rimi Repin, Menno Schilthuizen, M Eric Schranz, Renze Heidstra, Kana Miyata, Elena Federova, Wouter Kohlen, Ton Bisseling, Sandra Smit, Rene Geurts (2018). Comparative genomics of the non legume Parasponia reveals insights into evolution of nitrogen-fixing rhizobium symbioses. *Proc. Natl. Acad. Sci. USA*. 115:E4700-E4709.

Maximilian Griesmann, Yue Chang, Xin Liu, Yue Song, Georg Haberer, Matthew B. Crook, Benjamin Billault-Penneteau, Dominique Lauressergues, Jean Keller, Leandro Imanishi, **Yuda Purwana Roswanjaya**, Wouter Kohlen, Petar Pujic, Kai Battenberg, Nicole Alloisio, Yuhu Liang, Henk Hilhorst, Marco G. Salgado, Valerie Hocker, Hassen Gherbi, Sergio Svistoonoff, Jeff J. Doyle, Shixu He, Yan Xu, Shanyun Xu, Jing Qu, Qjang Gao, Xiandong Fang, Yuan Fu, Philippe Normand, Alisson M. Berry, Luis G. Wall, Jean-Michel Ane, Katharina Pawlowski, Xun Xu, Huanming Yang, Manuel Spannagl, Klaus F. X. Mayer, Gane Ka-Shu Wong, Martin Parniske, Pierre-Marc Delaux, Shifeng Cheng (2018). Phylogenomic reveals multiple losses of nitrogen-fixing root nodule symbiosis. *Science* 361, eaat1743.

About the Author

Yuda Purwana Roswanjaya, or Yuda, as he fondly known by family, friends, and colleagues, was born in Bandung on December 27th, 1981. He was the first-born of a Sundanese family.

Yuda obtained his Bachelor degree in Biology in 2003 and Master of Biotechnology in 2006 from Bandung Institute of Technology. He graduated cum laude for both titles. His research was focused on food fermented products. During his early career, from 2006 – 2009, he gained his experience in private company sectors, which were related to agriculture. Later, he joined the Agency for the Assessment and Application of Technology (BPPT), which is now known as National Research and Innovation Agency (BRIN), Republic of Indonesia. In 2015, he was granted a scholarship from his agency to pursue a PhD at Wageningen University & Research. He joined a research project related to the non-legume plant *Parasponia andersonii* in Laboratory of Molecular Biology, Department of Plant Sciences, under the supervision of Dr.ir. Rene Geurts.

Yuda is often described as talkative, sociable, enthusiastic, friendly, and outgoing by people around him. He loves being the center of attention and always volunteered to be the master of ceremonies or moderators in various events, from weddings to scientific discussions. Once, he joined “Mojang-Jajaka Bandung,” a competition of the young representatives of Bandung citizens. Although he failed to achieve the title, he was granted “Mr. Congeniality” among the competitors, as he was well-loved by many people.

Behind his extrovert personality, Yuda is also known as a hard-working person. He devoted a lot of time in the laboratory and greenhouse to work on his PhD project. During the most stressful period, he often cooks sophisticated Indonesian cuisine and invites some friends to

have dinner together at his place. During his break, he also enjoyed traveling and shopping to relieve his stress. Finally, in 2022 he managed to complete his PhD, and he is expected to resume his position as a researcher at Research Center for Applied Microbiology at BRIN as soon as possible. He will continue to contribute to Indonesia's agriculture sector through his work.

Written by:

Dr. Nuning Winaris, MSc.

WUR Alumna 2020

Education Statement of the Graduate School
Experimental Plant Sciences

Issued to: Yuda Purwana Roswanjaya
Date: 13 December 2022
Group: Laboratory of Molecular Biology
University: Wageningen University & Research

1) Start-Up Phase	*date*
► **First presentation of your project**	
Title: Trade-offs for gaining nitrogen fixing endosymbiosis	23 Oct 2015
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
► **MSc courses**	
Subtotal Start-Up Phase	1,5

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD Get2Gether, Soest, NL	28-29 Jan 2016
EPS PhD Get2Gether, Soest, NL	09-10 Feb 2017
EPS PhD Get2Gether, Soest, NL	15-16 Feb 2018
► **EPS theme symposia**	
EPS Theme 1 Symposium "Developmental Biology of Plants", Wageningen, NL	21 Jan 2016
EPS Theme 1 Symposium "Developmental Biology of Plants", Wageningen, NL	30 Jan 2018
EPS Theme 1 Symposium "Developmental Biology of Plants", Wageningen, NL	05 Feb 2020
EPS Theme 2 Symposium & Willie Commelin Scholten Day "Interactions Between Plants and Biotic Agents", Leiden, NL	22 Jan 2016
EPS Theme 2 Symposium & Willie Commelin Scholten Day "Interactions Between Plants and Biotic Agents", Wageningen,	23 Jan 2017
EPS Theme 2 Symposium & Willie Commelin Scholten Day "Interactions Between Plants and Biotic Agents", Wageningen,	01 Feb 2019
EPS Theme 2 Symposium & Willie Commelin Scholten Day "Interactions Between Plants and Biotic Agents", Utrecht, NL	04 Feb 2020
EPS Theme 2 Symposium & Willie Commelin Scholten Day "Interactions Between Plants and Biotic Agents', online	09 Feb 2021
EPS Theme 4 Symposium "Genome Biology", Wageningen, NL	13 Dec 2019
► **Lunteren Days and other national platforms**	
Annual meeting Experimental Plant Sciences", Lunteren, NL	10-11 Apr 2017
Annual meeting Experimental Plant Sciences", Lunteren, NL	09-10 Apr 2018
Annual meeting Experimental Plant Sciences", Lunteren, NL	08-09 Apr 2019
► **Seminars (series), workshops and symposia**	
Seminar: Dr. Siobhan Brady, Regulation of root morphogenesis in tomato species in the face of a changing environment	09 Sep 2015
Seminar: Lionel Dupuy, New methods to anylize root-microbe interaction	07 Oct 2015
Seminar: Dr. Jean-Francois Arrighi, Evolution of Nod Factor-independent rhizobium symbiosis	18 Oct 2017
Seminar: Prof. Dr. Giles Oldroyd, Recognition of symbiotic microorganisms by plants	19 Oct 2017
Seminar: Dr. Asaf Levy, Bacteria and the future of agriculture: from sequence to function	22 Feb 2018
Seminar: Prof Owen Atkin, Plant metabolism in a warming world-welcome to the dark side	20 Nov 2019
Seminar: Prof. Dr. Jian Xu, The root of single cell CAPability	02 Dec 2019
Seminar: Dr. Pascal Ratet, Suppression of defense during Medicago root nodule formation	03 Dec 2019
Seminar: Dr Ivan Baxter, Mind the GxE=P; elemental content in plants	09 Dec 2019
Seminar: Dr. Uta Paszkowski and Dr. Martin Parniske, Molecular genetic of arbascular mycorrhiza symbiosis in rice; and Where do you go, symbiosis?	27 Apr 2020
Seminar: Dr. Dugald Reid and Dr. Chao Su, Regulation of cytokinin biosynthesis and signaling during nodulation; and Membrane morphodynamics during Rhizobia infection	25 May 2020
Seminar: Dr. Lena Müller and Dr. Benoit Lefebvre, CLE peptide signaling regulates fungal colonisation in arbuscular mycorrhiza; and Roles and evolution of plant symbiotic signal receptors involved in arbuscular mycorrhiza	29 Jun 2020
Seminar: Prof. Takashi Soyano and Dr. Melanie Rich, Transcriptional regulatory pathways of nodulation processes in Lotus japonicus; and Evolution of lipid exchanges in Arbuscular Mycorrhiza Symbiosis	27 Jul 2020
Seminar: Dr. Florian Frugier and Dr. Chloe Cathebras, Integration of systemic signalling pathways regulating symbiotic nodulation; and Evolution of the nitrogen-fixing root nodule symbiosis	28 Sep 2020
Seminar: Dr. Caroline Gutjahr and Dr.Simona Radutoiu, Arbuscular mycorrhiza development and function; and Understanding how specificity in Nod factor signaling is ensured by legume LysM receptors	30 Nov 2020
Meeting: Wageningen Plant Microbiome Network kick-off programme, Wageningen, NL	29 Jun 2016
Meeting: Wageningen Plant Microbiome Network, Wageningen, NL	13 Dec 2016
Meeting: Wageningen Plant Microbiome Network, Wageningen, NL	21 Feb 2017
Meeting: Wageningen Plant Microbiome Network, Wageningen, NL	18 April 2017
Public lecture: Prof. Alga Zuccaro and Dr. Ikram Blilou, Wageningen, NL	10 Sep 2019
Public lecture: Dr. Jurgen Kleine-Vehn, Wageningen, NL	28 Oct 2019
Public lecture: Prof. Eva Stukenbrock and Dr. Silke Robatzek, Wageningen, NL	28 May 2020
Symposium: Evolution and maintenance of (belowground) cooperation, Amsterdam, NL	04 Feb 2016
Symposium: 1st Wageningen Indonesia Scientific Exposure (WISE), Wageningen, NL	28 Oct 2016
Symposium: INTERWOVEN : How science and art meet belowground, Wageningen, NL	14 Dec 2016
Symposium: Publish for Impact, Wageningen, NL	07 Feb 2017
Symposium: Farewell EPS director Ton Bisseling "The underground Labyrinth: Roots, Friends and Foes", Wageningen, NL	08 Feb 2017
Symposium: 2nd Wageningen Indonesia Scientific Exposure (WISE), Wageningen, NL	08-09 Mar 2017
Symposium: 3rd Wageningen Indonesia Scientific Exposure (WISE), Cibinong, Bogor, ID	05-06 Jul 2018
Symposium: The ecology and management of sustainable and climate-smart rice system, Wageningen, NL	05 Mar 2019
Symposium: 4th Wageningen Indonesia Scientific Exposure (WISE), Wageningen, NL	12 Mar 2019
Symposium: 6th Wageningen PhD Symposium "Science with Impact", Wageningen, NL	25 Oct 2019
Symposium: 3rd WURomics symposium: Advances in plant and food metabolomics, Wageningen, NL	12 Dec 2019
Symposium: Artificial intellegence in plant science and breeding, online	24 Feb 2021
► **Seminar plus**	
► **International symposia and congresses**	
20th International Conference on Molecular Plant-Microbe Interactions, Osaka, JP	11-12 Oct 2018
4th International Molecular Mycorrhiza Meeting, Turin, IT	06-08 Feb 2019
Engineering Nitrogen Symbiosis for Africa (ENSA) Annual Meeting, Montauban, FR	23-26 Sep 2019
4th Adam Kondorosi "Beneficial Plant-Microbe Interactions" Symposium, Gif sur Yvette, FR	26-27 Nov 2019
5th International Molecular Mycorrhiza Meeting, online	05 Aug 2022

CONTINUED ON NEXT PAGE

► **Presentations**	
Poster: Trade-offs for gaining nitrogen fixing endosymbiosis, 1st Wageningen Indonesia Scientific Exposure (WISE) 2016, Wageningen, NL	28 Oct 2016
Poster: The mycorrhization of Parasponia and Trema : A comparative study, 2nd Wageningen Indonesia Scientific Exposure (WISE) 2017, Wageningen, NL	08-09 Mar 2017
Poster: A functional kinase domain of the symbiosis receptor kinase (SYMRK) is not essential for endomycorrhization of tropical Trema trees, Annual meeting Experimental Plant Sciences", Lunteren, NL	09-10 Apr 2018
Poster: The effect on arbuscular mycorrhizal symbiosis of a natural occuring symbiosis receptor kinase (SYMRK) mutant allele in a Borneo Trema Orientalis population, 4th International Molecular Mycorrhiza Meeting, Turin, IT	06-08 Feb 2019
Poster: The effect on arbuscular mycorrhizal symbiosis of a natural occuring symbiosis receptor kinase (SYMRK) mutant allele in a Borneo Trema Orientalis population, 4th Wageningen Indonesia Scientific Exposure 2019 (WISE), Wageningen,	12 Mar 2019
Poster : Analysis of the Parasponia andersonii γ-Aminobutyric Acid Transporter1 (GAT1) mutant reveals no function during nodulation, 6th Wageningen PhD Symposium, Wageningen, NL	25 Oct 2019
Poster : Analysis of a natural occuring symbiosis receptor kinase (SYMRK) mutant allele in Trema orientalis, 4th Adam Kondorosi Symposium, Gif sur Yvette, FR	26-27 Nov 2019
Talk: Occurance of Symbiosis Receptor Kinase (SYMRK) mutant allele in a natural Trema population does not affect arbuscular endomycorrhizal symbiosis, 3rd Wageningen Indonesia Scientific Exposure (WISE) 2018, Cibinong, Bogor, ID	05-06 Jul 2018
Talk: Arbuscular mycorrhizal symbiosis in Trema Orientalis : Effect of a natural-occuring symbiosis receptor kinase (SYMRK) mutant allele, 20th International Conference on Molecular Plant-Microbe Interactions, Osaka, JP	11-12 Oct 2018
► **IAB interview**	
► **Excursions**	
Subtotal Scientific Exposure	23,4

3) In-Depth Studies	*date*
► **Advanced scientific courses & workshops**	
VLAG course "Introduction to R", Wageningen, NL	22-23 May 2018
VLAG course "Applied statistic", Wageningen, NL	30 May - 01 Jun 2018
EPS course "Transcription Factor and Transcriptional Regulation", Wageningen, NL	10-12 Dec 2018
► **Journal club**	
Member of literature discussion group at laboratory of Molecular Biology	2015-2020
► **Individual research training**	
Subtotal In-Depth Studies	5,6

4) Personal Development	*date*
► **General skill training courses**	
EPS Introduction Course, Wageningen, NL	11 Feb 2016
WGS workshop "Reviewing a scientific paper", Wageningen, NL	19 May 2016
WGS PhD Competence Assessment, Wageningen, NL	09 Jun 2016
WGS course "Brain Training", Wageningen, NL	02 Nov 2016
WGS course "Project & Time Management", Wageningen, NL	18 Jan - 01 Mar 2017
WGS PhD Workshop Carousel, Wageningen, NL	07 Apr 2017
WGS workshop "Scientific Publishing", Wageningen, NL	05 Apr 2018
WGS course "Last stretch of the PhD Programme, Wageningen, NL	17 Dec 2019
WGS course "Writing Propositions for your PhD", Wageningen, NL	17 Dec 2019
EPS Postdoc Career Day, Wageningen, NL	07 Feb 2020
Workshop: Scientific integrity, Wageningen, NL	20 Feb 2020
► **Organisation of meetings, PhD courses or outreach activities**	
► **Membership of EPS PhD Council**	
Subtotal Personal Development	3,5

TOTAL NUMBER OF CREDIT POINTS*	34

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS with a minimum total of 30 ECTS credits.

** A credit represents a normative study load of 28 hours of study.*

Colophon

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