Duplication of Symbiotic Lysin Motif Receptors Predates the Evolution of Nitrogen-Fixing Nodule Symbiosis

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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 (N₂) 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 nonleguminous crop species (Burrill and Hansen, 1917; Rogers and Oldroyd, 2014; Huisman and Geurts, 2019).

The Frankia spp. and rhizobium nitrogen-fixing nodulation trait occurs in 10 paraphyletic lineages within the orders Fabales, Fagales, Cucurbitales, and Rosales, collectively known as the nitrogen-fixing clade.
Duplication in LysM Receptors Predates Nodulation

RESULTS

Phylogeny Reconstruction of Orthogroups Representing LysM-Type LCO Receptors

To obtain insight into the LysM-type receptor family of *P. andersonii*, we analyzed it phylogenetically. We identified 16 *P. andersonii* genes encoding putative LysM-type receptors that grouped in all except one known orthogroups (Supplemental Fig. S1; Supplemental Table S1). Genetic studies in legumes uncovered only two orthogroups that contain proteins with a known function in rhizobium LCO signaling; these are named LYK-I and LYR-IA (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 MiLYK3/LjNFR1 and LjNFR5 (Limpens et al., 2003; Radutoiu et al., 2003; Murakami et al., 2018). Besides these, the LYK-I orthogroup also includes chitin innate immune receptors of *M. truncatula* MiLYK9/MtCERK1, L. japonicus LjCERK6, Arabidopsis (*Arabidopsis thaliana*) AtCERK1, tomato (*Solanum lycopersicum*) SlCERK1, and rice OsCERK1 (Limpens et al., 2003; Miya et al., 2007; Wan et al., 2008; Shimizu et al., 2010; Miya 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 in AM symbiosis (Miya et al., 2014; Zhang et al., 2015; Feng et al., 2019; Gibelin-Viala et al., 2019). Two *P. andersonii* 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 into the evolutionary relationships of these genes when compared with LCO and CO receptors. Notably, LysM-type receptors of the recently sequenced nodulating actinorhizal plants and nonnodulating relatives were included (Griesmann et al., 2018). The resulting phylogeny largely resembles rosid species trees as 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 into 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 occurred in an ancestral eudicot (Fig. 1; Supplemental Data Set S1). The only studied member in the LYK-Ia orthogroup is tomato *SILYK12*, and knockdown of this gene by virus-induced gene silencing substantially 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 the genus *Parasponia*, no gene duplications occurred in the LYK-Ib clade (represented by *PanLYK3*) or in the LYK-Ia clade (represented by *PanLYK1*). In contrast, *P. andersonii* *PanLYK3* experienced a duplication of exclusively the first exon. To determine whether this duplication is specific for the *Parasponia* genus, we analyzed the LYK3 genomic region of two additional *Parasponia* spp. and three nonnodulating species of the closely related genus *Tremia*. This revealed that the duplication of LYK3 exon 1 is present in all species investigated and occurred twice, where the most distal exon 1 copy was lost in *P. andersonii* (Fig. 2A; Supplemental Fig. S2A). The encoded pre-mRNAs both splice into a shared second exon (Fig. 2). Each exon 1 copy contains a putative transcription and translation start site, which allows for differential expression of the variants (Fig. 2, B and C). Genes of the LYK-I clade 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 domains. Therefore, the *P. andersonii* *PanLYK3* gene encodes two protein variants, named PanLYK3.1 and PanLYK3.2, that differ in their extracellular domain (Supplemental Fig. S2B).

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* spp. harbor 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., 2018). *PanNFP1* and *PanNFP2* originated from an ancient duplication. Phylogenetic reconstruction, including additional nodulating and nonnodulating 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 (Fig. 3; Supplemental 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* spp. 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* spp. of cluster I or cluster III that lack LCO biosynthesis genes, such as *Alnus glutinosa* and *Casuarina glauca* (Fig. 3; Pawlowski and Demchenko, 2012; Griesmann et al., 2018; Salgado et al., 2018; Nguyen et al., 2019). In line with what was reported for the nonnodulating Rosales species (van Velzen et al., 2018), NFP-II-type pseudogenes can be found in the genomes of the nonnodulating Fagales spp. Chinese chestnut (*Castanea mollissima*) and European beech (*Fagus sylvatica*). 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.

**P. andersonii** *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 *P. andersonii*. We noted that, in contrast to *PanLYK3*, *PanLYK1* is exclusively expressed in roots and nodule tissue (Fig. 2B), suggesting that this gene may also function in a symbiotic context. Therefore, we decided to include this gene in further studies. Finally, we also included *PanNFP1*, since an earlier study based on RNA interference (RNAi) in transformed *P. andersonii* roots showed that
Figure 1. Phylogeny reconstruction of 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 coerulea*, a basal eudicot in the Ranunculales. A subset of proteins is unresolved. *P. andersonii* proteins are in boldface. Genuses *Parasponia* and *Trema* LYK3.1 and LYK3.2 represent protein variants of LYK3. Deduced pseudoproteins are depicted in gray/strikethrough. Proteins with known functions in nodulation, mycorrhization, and/or chitin innate immune response.
this gene functions in nodulation (Op den Camp et al., 2011). To test whether these four *P. andersonii* genes can function as rhizobium LCO receptors, we conducted two complementary experiments. First, we introduced *P. andersonii* receptor pairs into a *L. japonicus Ljnfr1;Ljnfr5* double mutant, aiming to determine whether these *P. andersonii* receptors can transcomplement for LCO-induced Ca$^{2+}$ oscillation. Second, we generated CRISPR-Cas9 knockout mutants in *P. andersonii* to study their role in nodulation.

We selected *L. japonicus* for transcomplementation studies because its microbial host *Mesorhizobium loti* strain R7A can also nodulate *P. andersonii* (Supplemental Fig. S3, A–C). By using *Agrobacterium rhizogenes*-mediated root transformation, we tested six combinations of *P. andersonii* heterodimeric receptor pairs under the control of the promoter and terminators of *Ljnfr1* and *Ljnfr5* (Fig. 4). These promoters were shown to be functional in complementation of the *L. japonicus Ljnfr1-1;Ljnfr5-2* double mutant (Supplemental Fig. S3, D–H). For the transcomplementation constructs, we included the nucleus-localized calcium sensor R-GECO1.2, allowing visualization of nuclear Ca$^{2+}$ oscillations (Zhao et al., 2011). In wild-type *L. japonicus* 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 (Supplemental Fig. S3, I and J; Supplemental Movie S1; Miwa et al., 2006). Analyzing the transgenic roots expressing *P. andersonii* receptor combinations revealed that nine out of 11 tested combinations elicit Ca$^{2+}$ oscillation, although less regular in shape and frequency when compared with the positive control (Fig. 4B; Supplemental Movie S2). Interestingly, the receptor combinations *PanLYK1;Ljnfr5* and *Ljnfr1;PanNFP2* did not elicit any Ca$^{2+}$ oscillation response, whereas both *P. andersonii* receptors are, at least partially, functional as an *M. loti* LCO receptor when combined with a *P. andersonii* counterpart (Fig. 4B). Upon inoculation with *M. loti* R7A, only nodule-like structures were observed on roots transcomplemented with different *P. andersonii* receptor combinations (4 weeks postinoculation [wpi]) but not with heterologous receptor pairs (Supplemental 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 (Supplemental Fig. S3, K–P). Taken together, the transcomplementation studies of a *L. japonicus Ljnfr1;Ljnfr5* mutant indicated that all four *P. andersonii* receptors, *PanLYK1*, *PanLYK3*, *PanNFP1*, and *PanNFP2*, have the potential to function as receptors for *M. loti* LCOs, but none could fully transcomplement a *Ljnfr1-1;Ljnfr5-2* double mutant for nodulation.

**Figure 1.** (Continued.)

signaling are indicated. Bootstrap values indicate IQ-tree UF-bootstrap support%; values >98 are not shown. The scale bar represents substitutions per site. A complete list of species and accession numbers can be found in Supplemental Data Set S1.
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 (Supplemental Fig. S4). In order to determine whether the Pannfp1 infection phenotype is strain dependent, we nodulated plants also with S. fredii NGR234. This strain was shown to be less optimal under the chosen conditions (Agroperlite supplemented with EKM medium [see “Materials and Methods”]) 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 wpi. Under these conditions, no difference between Pannfp1 and the EV control was observed. Nodules formed on Pannfp1 mutant plants were infected normally (Supplemental Fig. S4).

Similarly to Pannfp1 mutant plants inoculated with M. plurifarium BOR2, we found a reduced nodulation efficiency in P. andersonii Panlyk3 knockout mutants but not in Panlyk3.1 and Panlyk3.2 variant-specific mutant alleles, nor in Panlyk1 mutants (Fig. 5; Supplemental Fig. S4). To determine whether nodules formed on Panlyk1 and Panlyk3 mutants have an infection phenotype, we analyzed thin sections. In contrast to legumes, P. andersonii does not guide rhizobia in infection threads toward 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 with one in infection threads, and possess a thinner cell wall (Lancelle and Torrey, 1984, 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 the wild type, 15 contained only infection threads but no fixation threads, and eight showed an intermediate phenotype with few infected cells (Fig. 5, F–I; Supplemental Fig. 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 six nodules with an intermediate phenotype. Next, we determined whether...
Figure 3. Phylogeny reconstruction of the LYR-IA 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). *P. andersonii*PanNFP1 and PanNFP2 are in boldface. Deduced pseudoproteins are depicted in gray/strike-through. Values indicate IQ-tree UF-bootstrap.
this infection phenotype is controlled specifically by either *PanLYK3.1* or *PanLYK3.2*, which was shown not to be the case (Supplemental Fig. S4). As ~50% of the nodules formed on the *P. andersonii* *Panlyk3* mutant plants displayed a wild-type phenotype, this suggests redundancy in gene functioning. Interestingly, *S. fredii* NGR234 could not nodulate *Panlyk3* mutants, which suggests that this strain is fully dependent on PanLYK3-controlled signal transduction (Supplemental Fig. S4).

As *P. andersonii* 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 cases 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 retransformation of the *Panlyk3* mutant (line a2). We obtained three independent *Panlyk1;Panlyk3* mutants (Supplemental Data Set S3). *M. plurifarium* BOR2 inoculation experiments revealed that all *Panlyk1;Panlyk3* double mutant lines were unable to form any nodule or nodule-like structure (Fig. 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 (Fig. 2), we used the *LjNFR1* promoter, as well as the constitutive *AtUBQ10* and CaMV35S promoters, to drive a CRISPR-resistant allele of *PanLYK3* (PanLYK3cr). Compound plants carrying transgenic roots expressing PanLYK3cr could be nodulated by *M. plurifarium* BOR2 (Supplemental Fig. S5). Together, this showed that in *P. andersonii*, *PanLYK1* and *PanLYK3* act redundantly in root nodule formation. (For complementation studies of *Panf2*, see below.)

The results demonstrate that *P. andersonii* *PanLYK1*, *PanLYK3*, *PanNPFP1*, and *PanNPFP2* function in rhizobium LCO-driven nodulation. *PanLYK3* and *PanNPFP2* 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, this suggests that LCO signaling is an ancestral function of these LYK-I receptors.

**A Repaired *Trema levigata* NFP2 Pseudogene, But Not *PanNPFP1*, Can Functionally Complement a *P. andersonii* nfp2 Mutant**

*PanNPFP1* and *PanNPFP2* differ in expression pattern. Whereas both genes are expressed in root tissue, only *PanNPFP2* is up-regulated in nodules (Fig. 2B; van Velzen et al., 2018). 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 *PanNPFP2*. *A. tumefaciens*-mediated transformation showed that a 2.75-kb *PanNPFP2* upstream region can be used to functionally complement the *P. andersonii* *Panfnp2* mutant when using a *PanNPFP2* CRISPR-resistant allele (*PanNPFP2cr*). Two independent lines formed 7 ± 7 and 4 ± 1 nodules 5 wpi with *M. plurifarium* BOR2 (Fig. 7). However, when we used *PanNPFP1* driven by the *PanNPFP2* promoter, no transcomplementation of the *P. andersonii* nfp2 mutant phenotype was observed. This suggests that there is a functional difference in the encoded *PanNPFP1* and *PanNPFP2* receptors.

Next, we questioned whether the *nfp2* pseudogene as present in several nonnodulating Rosales species may have encoded a functional symbiosis receptor. To test this, we focused on the *nfp2* pseudogene of *T. levigata*, as it has only three mutations that cause a disturbance of the open reading frame (Fig. 7). We repaired these three mutations, using *PanNPFP2* as a template, resulting in an engineered CRISPR-resistant *TleNFP2cr* that encodes for a LysM-type receptor protein of 582 amino acids, similar to *PanNPFP2* of *P. andersonii*. We tested whether *TleNPFP2cr* driven by the *PanNPFP2* promoter can transcomplement the *P. andersonii* *Panfnp2* mutant. *A. tumefaciens* transformation resulted in two lines that can form functional root nodules 5 wpi with *M. plurifarium* BOR2. This supports the hypothesis that *T. levigata* *nfp2* encoded a functional symbiosis receptor prior to the pseudogenization of this gene.

**P. andersonii PanLYK3 Is Essential for Chitin-Triggered Immune Responses and Controls AM Symbiosis in Coherence with PanLYK1**

Next, we aimed to determine whether the *P. andersonii* 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 AM symbiosis: *L. japonicus* *LjCERK6*, *M. truncatula* MtLYK9/MtCERK1, *Arabidopsis* AtCERK1, tomato *SILYK1*, and rice OsCERK1 (Fig. 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., 2019; He et al., 2019). Similarly, some experimental evidence using transient silencing assays indicated that LysM-type receptors of the LVR-IA clade function in mycorrhization, including *P. andersonii*
PanNFP1 (Op den Camp et al., 2011). In line with this, we aimed to confirm this phenotype in stable Pannfp1 knock- out mutants and determine whether other P. andersonii symbiotic LysM-type receptors may also function in AM symbiosis and/or chitin-induced innate immunity signaling.

First, we investigated whether the P. andersonii LysM-type receptor mutants are affected in chitin-triggered immunity responses. To do so, two complementary assays were used: a chitin-induced reactive oxygen species (ROS)-burst production and a MITOGEN-ACTIVATED PROTEIN KINASE3 (MAPK3)/MAPK6 phosphorylation assay. Chitin heptamers (CO7) effectively induced a ROS burst in P. andersonii root segments at concentrations of 1 μM or greater when incubated at 28°C, the regular growth temperature of Parasponia spp. (Fig. 8A; Supplemental Fig. 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 P. andersonii but produce structurally different LCOs (López-Lara et al., 1995; Folch-Mallol et al., 1996).

However, neither triggered a ROS burst in P. andersonii roots (Supplemental Fig. S6A). To determine whether CO7-induced ROS bursts were associated with phosphorylation of P. andersonii 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). P. andersonii possesses a single PanMAPK3 gene and a single PanMAPK6 gene, each of which encodes a protein with a conserved Thr-202/Tyr-204 phosphorylation site (Supplemental Fig. 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 R7A (10−9 M). Numbers denote spiking roots versus the number of roots analyzed. The numbers in parentheses denote the total numbers of spiking nuclei observed. The y axis is the relative fluorescence intensity compared with the defined baseline in arbitrary units (a.u.). The scale bar = 10 min.

Figure 4. P. andersonii PanNFP1, PanNFP2, PanLYK1, and PanLYK3 complement a L. japonicus Ljnfr1;Ljnfr5 mutant for rhizobium-induced Ca2+ oscillation. A, Schematic representation of the T-DNA region of the binary construct used for A. rhizogenes-based root transformation of a L. japonicus Ljnfr1;Ljnfr5 double mutant. cDNA clones of LYK-1 (marked blue) or LYR-1a (marked red) type genes were cloned in identical fashion. cDNA clones were inserted between native promoter (marked black) plLjNFR1 (4,171 bp) or plLjNFR5 (1,314 bp) 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 Ca2+ oscillation, as observed in different combinations of LYK-1 (red) and LYR-1a (blue) type receptors introduced in a L. japonicus Ljnfr1;Ljnfr5 double mutant. Note that the receptor combinations PanLYK1;LjNFR5 and LjNFR1;PanNFP2 did not complement for Ca2+ oscillations. Traces were recorded ~10 min postapplication of LCOs extracted from M. loti R7A (~10−9 M). Numbers denote spiking roots versus the number of roots analyzed. The numbers in parentheses denote the total numbers of spiking nuclei observed. The y axis is the relative fluorescence intensity compared with the defined baseline in arbitrary units (a.u.). The scale bar = 10 min.
Figure 5. *P. andersonii Panlp1, Panlp2, and Panlyk3* mutants are affected in nodulation. Data are represented as means ± se, and circles represent individual data points. Lowercase letters denote statistical significance based on one-way ANOVA and Tukey’s post hoc contrast (P > 0.05). A. Nodule numbers in *P. andersonii* 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) at 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 *P. andersonii* CRISPR-Cas9 mutant lines *Panlyk1* a1 (n = 19) and b1 (n = 20) at 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) represents untransformed plantlets. C. Nodule numbers in *P. andersonii* CRISPR-Cas9 mutant lines *Panlyk3* a2 (n = 21), c3 (n = 21), and e1 (n = 19) at 5 wpi with *M. plurifarium* BOR2. EV Control 1 (n = 14). EV Control 1 represents a positive control line transformed with a binary vector not containing sgRNAs. D to I, Toluidine Blue-stained sections of representative nodules grown with *M. plurifarium* BOR2. D, Wild-type *P. andersonii* transformed with an EV-1 construct expressing Cas9. E, Infected nodule cells containing fixation threads formed on EV-1 plants. F, Infected nodule of *Panlyk3* line e2. Note patches of infected cells. G, Infected nodule cells of *Panlyk3* line e2 containing fixation threads. H, Empty nodule of *Panlyk3* line e2. Note the absence of fully infected cells. I, Nodule cells of *Panlyk3* line e2 containing infection threads but no fixation threads. Bars = 100 μm (D, F, and H) and 20 μm (E, G, and I).
MAPK3/6 phosphorylation profile, as did wild-type root segments (Supplemental Fig. S6, E and F). In contrast, *P. andersonii* PanLYK3 mutant lines lacked a chitin-triggered ROS burst and showed no p44/42 MAPK phosphorylation (Fig. 8). Individual exon-knockout Panlyk3 line a2 (*n* = 12) and Panlyk1;Panlyk3 double mutant lines b3 (*n* = 10), b7 (*n* = 5), and b13 (*n* = 10) at 5 wpi with *M. plurifarium* BOR2. Data are represented as means ± se, and circles represent individual data points. Lowercase letters denote statistical significance based on one-way ANOVA and Tukey’s posthoc contrast (*P* > 0.05). B, Roots with nodules of EV Control 1 at 5 wpi with *M. plurifarium* BOR2. C, Roots without nodules of the Panlyk1;Panlyk3 double mutant (line b3) at 5 wpi with *M. plurifarium* BOR2. Bars = 5 mm.

We conducted an RNA sequencing experiment on *P. andersonii* roots mycorrhized by *Rhizophagus irregularis* strain DOAM197198. Several marker genes for mycorrhization were shown to be enhanced in expression in mycorrhized *P. andersonii* root samples, including PanSTR1, PanSTR2, PanPT4, PanVPY, PanD27, PanRAD1, and PanRAM1 (Supplemental Fig. S7). Also, this suggested that PanNFP1 is expressed at a higher level than PanNFP2 under these conditions (Supplemental Fig. 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 (Supplemental Fig. S7). To determine whether *P. andersonii* 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 wpi. These experiments revealed substantial variation in mycorrhization efficiency between replicates, although 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 (Supplemental Fig. S8, A–C). Analyzing both double mutants, PanNfp1;PanNfp2 and Panlyk1;Panlyk3, revealed a strong AM symbiosis phenotype only in the latter (Fig. 9; Supplemental Fig. S8). The fungal colonization of the Panlyk1;Panlyk3 mutant was severely affected, with only a few infections observed. Confocal imaging of wheat germ agglutinin conjugated to Alexa Fluor 488 (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 (Fig. 9). As both Panlyk1 and Panlyk3 single mutant plants do not show this 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), AM symbiosis (PanLYK1 and PanLYK3), and chitin innate immune signaling (PanLYK3). This suggests that no subfunctionalization of these receptors is required to allow functioning in the rhizobium nitrogen-fixing mycorrhization trait.

**DISCUSSION**

We used *P. andersonii* as a comparative system to legumes to obtain insight into the evolutionary trajectory
of LysM-type rhizobium LCO receptors. By conducting phylogenetic analysis, transcomplementation studies in a *L. japonicus* LCO receptor double mutant, and CRISPR-Cas9 mutagenesis in *P. andersonii*, we identified four LysM-type receptors that function in LCO-driven nodulation in a nonlegume. Two of these, *PanLYK3* and *PanNFP2*, are putative orthologs to known legume rhizobium LCO receptors *LjNFR1/MtLYK3* and *LjNFR5/MtNFP*, respectively. As the *Parasponia* spp and legume lineages diverged early in the nitrogen-fixing clade (more than 100 million years ago), 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 the genus *Parasponia* did not experience recent duplication events. Instead, the *Parasponia* spp. symbiotic LysM-type LCO receptors evolved following two ancient duplications. We hypothesize that the *PanNFP1*-*PanNFP2* duplication associates with the origin of the nitrogen-fixing clade, whereas in the case of *PanLYK1* and *PanLYK3*, the duplication occurred prior to 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.

Presently, the NFP1-NFP2 duplication cannot be precisely dated because legumes do not possess an
NFP-I-type gene. This can be explained in two scenarios. (1) 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. (2) 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 ec-topic 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 M. truncatula and L. japonicus 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 SILYK10 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 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 is sister to Fagales (Koenen et al., 2020). The phylogenetic analysis presented here (Fig. 3) suggests that the first scenario is most probable (aSH-aLRT/UF-Bootstrap/approximation 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 nonlegume NFP-II-type proteins that is distinct from NFP-I (Supplemental Fig. 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.

Transcomplementation studies in a L. japonicus Linfr1;Ljnfr5 double mutant showed that P. andersonii LCO receptors can only partially restore LCO signaling. This only partial complementation we did not anticipate, because of the shared microsymbiont M. loti that can nodulate P. andersonii as well as L. japonicus. One explanation for this limited functionality may be that such receptors function in larger multiprotein membrane domain complexes. In such a case, the P. andersonii LCO receptors are not adapted to interact with associated L. japonicus proteins. Additionally, legumes and Parasponia spp. have diverged in the mode of rhizobium
infection. Whereas rhizobium penetrates *Parasponia* spp. 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 receptor (Ardourel et al., 1994). Such entry receptors have not yet been fully characterized, but *MtLYK3* may 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 genus *Parasponia* LYK3 ortholog.

We showed that an engineered *TleNFP2* receptor can functionally complement the *P. andersonii* PanNFP2 mutant, whereas PanNFP1 cannot. This suggests that the NFP1 and NFP2 receptor proteins have functionally diverged. Based on the finding that NFP-orthologous proteins of *P. hybrida* (PhLYK10) and tomato (SILYK10) can complement *L. japonicus* *Ljnfr5* and *M. truncatula* *Mtnfp* mutants, it can be hypothesized that, in *P. andersonii* especially, PanNFP1 has experienced protein adaptations. However, it should be noted that the transcomplementation studies presented here were conducted using the native PanNFP2 promoter, whereas studies conducted with PhLYK10 and SILYK10 were...
conducted with CAMV35S (Girardin et al., 2019). Such overexpression may mask differences in substrate affinity and/or specificity, under which native transcriptional regulation is biologically relevant. Our data demonstrate that the ancestor of T. leviigata possessed an NFP2 receptor that can function in nodulation.

Mutant analysis in legumes demonstrated that rhizobium nodule coopted elements of an AM signaling pathway, including the Leucine-rich repeat-type transmembrane receptor kinase L. japonicus SYMBOITIC RECEPTOR KINASE/ M. truncatula DOES NOT MAKE INFECTIONS2 (MtDM2), the nuclear envelope-located cation ion channels LjCASTOR, LjpOLLUX/MtDM11, the nucleus-localized CALCIUM CALMODULIN KINASE LjCcmK/MtDM13, and the transcription factor LjCYCLOPS/M. truncatula INTERACTING PROTEIN OF DMI3 (Geurts et al., 2012). However, in legumes, rhizobium and AM fungi were shown to have independent perception mechanisms to activate this common symbiosis signaling pathway. In L. japonicus and M. truncatula, 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., 2019). MtLYK3 and MtLYK9/MtCERK1 both belong to the LYK-1b clade and evolved following legume-specific duplication events (Fig. 1; De Mita et al., 2014). The strong phenotype in AM and nodule symbioses of the P. andersonii Panlyk1;Panlyk3 knockout mutant demonstrates that such subfunctionalization is not causal for the evolution of rhizobium LCO receptors. In P. andersonii, both receptors function in conjuction to control AM and rhizobium noduleation. Additionally, PanLYK3 acts as a chitin innate immune receptor. Such multifunctionality has also been reported for MtLYK9/MtCERK1 in M. truncatula and OsCERK1 in rice, which function both in AM symbiosis and chitin innate immune signaling (Miyata et al., 2014; Carotenuto et al., 2017; Feng et al., 2019; Gibelin-Viala et al., 2019). As monocots did not experience the LYK-1a/LYK-1b duplication, this demonstrates that performing multiple functions in symbioses 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-1A orthogroup) in species outside of the nitrogen-fixing clade is associated with the ability to establish an AM symbiosis (Fig. 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 P. andersonii PanNFP1 and PanNFP2 receptors can partially complement LCO-induced Ca2+ oscillation in the L. japonicus Ljnfr1;Ljnfr5 double mutant, this 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 coopted 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. L. japonicus Ljnfr5, M. truncatula Mtnfr, and pea Psymib10) and the genus Parasponia (P. andersonii Pannfp2; Madsen et al., 2003; Arrighi et al., 2006). As the genus Parasponia and legumes diverged at the root of the nitrogen-fixing clade, this 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 European beech or Chinese chestnut, the NFP-I-type gene pseudogenized. However, phenotypic studies in stable P. andersonii 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 RNAi construct used, which may have off-target effects (van Velzen et al., 2018). 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-type RLKs, including PanLYK1 and PanLYK3, which may explain the reported mycorrhization phenotype on PanNFP1 RNAi roots (Supplemental Fig. S10). The studies presented here using CRISPR-Cas9 knockout mutant lines revealed substantial biological variation in mycorrhization efficiency of P. andersonii 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 analyzed a Pannfp1;Pannfp2 double mutant. Also, these lines were shown to be effectively mycorrhized. Therefore, we conclude that our current mutant phenotype analysis does not find support for essential functioning of P. andersonii PanNFP1 and PanNFP2 in AM symbiosis.

The study presented here provides insight into the evolutionary trajectory of symbiotic LCO LysM-type receptors. By using P. andersonii 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 coopted to function specifically in this symbiosis, making NFP2 a target in approaches to engineer LCO-driven nodulation in nonleguminous plants.
MATERIALS AND METHODS

LysM-Type Receptor Phylogeny Reconstructions

Orthologs containing LysM-type receptor kinases of *Parasponia andersonii*, generated in a previous study (van Velzen et al., 2018), were combined and realigned into a single alignment using MAFFTv7.017 (Katuzaka and Schindler, 2013). MrBayes2.3.2 (Ronquist and Huelsenbeck, 2003; Ronquist et al., 2012) was used to calculate phylogenetic relationships under default parameters in Geneious R8.1.9 (Biomatters). Clades were named as published previously (Buendia et al., 2018). For clades LK-1 and LRY-IA, additional putative orthologs were collected from the Phytolome and National Center for Biotechnology Information databases using BLAST with AtCERK and MtNFP protein sequences as queries (Supplemental Table S1; Altschul et al., 1997). Available genomes from Fabales, Fagales, Cucurbitales, and Rosales species were downloaded, and local BLAST analysis was conducted using Geneious R8.1.9 (Biomatters) to search for additional unannotated LK-1 and LRY-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 P. andersonii protein. Phylogenetic analysis was performed using IQ-tree (Nguyen et al., 2015; Trifinopoulos et al., 2016), running the modeller extension to find the best substitution models (Kalyaanamoorthy et al., 2017). Branch support analysis was done using Sh.aLRT 1,000 replicates, UF-BOOTSTRAP support 1,000 iterations (Kalyaanamoorthy et al., 2017; Hoang et al., 2018), and approximate Bayesian support. Branch supports shown are UF-Bootstrap support%. The best generations (Kalyaanamoorthy et al., 2017; Hoang et al., 2018), and approximate Bayes support. Branch supports shown are UF-Bootstrap support%. The best G4. Resulting tree 98 were omitted for visual clarity. Gene names, accession numbers, and alignment files of identified homologs can be found in Supplemental Data Set S1 for LK-1, Supplemental Data Set S2 for LRY-IA, and Supplemental Table S1 for *P. andersonii*.

LYK3 Alignment and Variant Detection

Genomic LYK3 regions of *P. andersonii*, *Parasponia rigida*, *Parasponia rugosa*, *Trema orientalis* RG16, *T. orientalis* RG53, and *T. levigata* were extracted from the respective assemblies (van Velzen et al., 2018) and aligned using MAFFTv7.017 implemented in Geneious R8.1. Coding sequences of *P. andersonii*, *P. rigida*, and *P. rugosa* LYK3 protein variants were translated and aligned using MAFFTv7.017 implemented in Geneious R8.1 (Supplemental 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). *P. andersonii* LysM-type receptor cDNA clones were sequence synthesized as level 0 modules, including silent mutations in golden gate 85al or Bpi restriction sites. Golden gate-compatible clones of LjNFR1 and LjNFR5 promoters, coding sequences, and terminators were obtained from Aarhus University. The calcium signaling reporter pLUBQR1-R-GECO1.2 was published previously (Kelner et al., 2018). The generation and assembly of *P. andersonii* 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. Lists of primers and constructs can be found in Supplemental Tables 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 by Doench et al. (2014). To be selected, guide RNAs must have no potential target sites in the genome with (1) less than three mismatches or (2) less than two indels. Known off-target locations in coding sequence 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 Supplemental Data Set S3. Primers used for the creation of sgRNAs and subsequent sequencing of mutants and off-targets are listed in Supplemental Table S4.

Bacterial Strains

We used *Mesorhizobium plurifarium* BOR2 (van Velzen et al., 2018) and *Sinorhizobium fredii* NGR234.pHc60 expressing GFP (Trimick and Galbraith, 1980; Cheng and Walker, 1996; Op den Camp et al., 2011) for *P. andersonii* inoculation experiments. *Mesorhizobium loti* R2A.pHc60 (Cheng and Walker, 1998; Sullivan et al., 2002) was used for *Lotus japonicus* inoculations. *M. loti* R7A and *Rhizobium tropici* CIAT899 (Martinez-Romero et al., 1991) containing plasmid pMP64 (Spanik et al., 1989) were used for LCO extraction. *Agrobacterium rhizogenes* strain AR10 (Hansen et al., 1989; Martinez-Romero et al., 1991) was used for *L. japonicus* root transformation. *Agrobacterium tumefaciens* strain AG-L-1 (Lazo et al., 1993) was used in *P. andersonii* transformation. *Agrobacterium sp*. MSU440 was used for *P. andersonii* hairy root transformation (Cao et al., 2012). The *Echerichia coli* strain DH5α was used to propagate plasmids and in all subsequent cloning steps.

Rhizobium LCO Isoolation

To isolate rhizobium LCOs, the plasmid pMP64 containing an autococyte NodD protein was introduced in *M. loti* R7A and *R. tropici* CIAT899 (Spanik et al., 1989; Lopez-Lara et al., 1995). LCOs were extracted from a 750-mL liquid culture of *OD600 = 0.5*, grown at 28°C in minimal medium (5.75 mM K2HPO4, 7.35 mM KH2PO4, 5.9 mM KNO3, 460 mM CaCl2, 37.5 μM FeCl3, 2.07 mM MgSO4, 20.5 mM biotin, 2.9 mM thiamine-HCl, 8.1 mM nicotinic acid, 4.8 mM pyridoxine-HCl, 2.8 mM myoinositol, 4.6 mM panthotenate, and 1% (w/v) Suc) by the addition of 150 mL of 1-butanol and 1 h of shaking. The butanol phase was transferred and subsequently evaporated (water bath at 40°C). The pellet was dissolved in 75 mL of 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 *L. japonicus* wild-type cv Gifu roots 3 h postapplication. The lowest active dilution was estimated to be ~10−10 M.

*L. japonicus A. rhizogenes* Root Transformation

*L. japonicus LMyf1-L,LMyf5-2* double mutants (Madsen et al., 2003; Radutoiu et al., 2003) were used for LysM complementation assays and cv Gifu wild type as control. Seedlings for *A. rhizogenes* root transformation were moved to fresh one-half-strength B5 medium and cocultivated for 1 week as described previously using *A. rhizogenes* strain AR10 (Stougaard et al., 1987; Hansen et al., 1989; Stougaard, 1995). During root emergence, plants were grown on 1% (v/v) agar plates containing one-half-strength B5 medium containing 0.33% (w/v) cefotaxime and 1% (w/v) Suc. Plants were screened for transformed roots using nucleus-localized RGECO1.2 fluorescence. Shoots with transformed roots were grown in Agroperlite (Maasmond-Westland) supplemented with modified one-half-strength Hoagland medium (Hoagland and Arnon, 1950) containing 0.56 mM NH4NO3 and inoculated with *M. loti* R7A.pHc60 (expressing GFP) at OD600 = 0.05. Plants were grown at 21°C under a 16-h-light/8-h-dark regime. For calcium oscillation analysis, transformed plants were grown on one-half-strength Hoagland plates with 1% agar containing 0.56 mM NH4NO3 for 1 week. Plants were moved to N-free one-half-strength Hoagland 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 40×/1.0. Transformed root segments expressing R-GECO1.2 were selected and incubated with 500× diluted LCO extract (estimated to represent ~10−9 M) in nitrate-free one-half-strength Hoagland medium (Hoagland and Arnon, 1950) on a glass slide with coverslip. Images were taken at 5-s intervals for a minimum of 20 min per sample using an excitation wavelength of 552 nm and emission spectrum of 585 to 620 nm. It is possible to monitor a large number of nuclei per root sample. However, only epidermal and especially root hairs were shown to be responsive. Therefore, total nuclei numbers vary largely between samples. Video recordings of imaged root samples were exported to

Duplication in LysM Receptors Predates Nodulation

Plant Physiol. Vol. 184, 2020

1019
ImageJ. (Collins, 2007). The Geciquant ImageJ plugin was used for back-ground subtraction and region of interest selection (Srinivasan et al., 2015). The average pixel intensities of regions of interest (individual nuclei) were measured. Average pixel values (0–255) per nucleus were plotted, and a back-ground R-GECo1.2 fluorescence baseline of 2 × 1 min (two 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 1.3 times background were considered as positive.

**P. andersonii Growth Conditions for Propagation, Transformation, Mycorrhization, and Nodulation**

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

**P. andersonii Nodulation Assay and Analysis**

Rooted tissue culture plantlets for phenotyping assays were grown in crystal-clear polypropylene containers (1 L) with a gas-exchange filter (OS140BOX; Duchefa Biochemie). Pots were half-filled with Agromin (Maasmond-Westland) and watered with modified EKM medium (3 mM MES [CH₃H₂N⁺O³⁻], pH 6.6, 2.08 mM MgSO₄·7H₂O, 0.88 mM KH₂PO₄, 2.07 mM K₂HPO₄, 1.45 mM CaCl₂, 0.7 mM NaSO₄, 0.375 mM NH₂NO₃, 15 μM iron citrate, 6.6 μM MnSO₄, 1.5 μM ZnSO₄, 1.6 μM CuSO₄, 4 μM H₂BO₃, and 4.1 μM Na₂MoO₄·Hecking, 1983). For nodulation assays, modified EKM medium (Becking, 1983) was inoculated with rhizobia (OD₅₆₀ = 0.025) prior to planting the shoots. For inoculation with strain *S. fredii* NG234, pHC60, containers were half-filled with sterilized river sand and watered with modified EKM medium containing the bacteria at OD₅₆₀ = 0.05.

All nodules were fixed in buffer containing 4% (w/v) paraformaldehyde mixed with 3% (v/v) glutaraldehyde for 50 min phosphate (pH 7.4). A vacuum was applied for 2 h during a total 48-h incubation. Fixed nodules were embedded in polymer (Technovit 7100; Heraeus-Kulzer) according to the manufacturer's recommendations. Sections were made using an R2035 microtome (Leica Microsystems). Sections were stained using 0.05% (w/v) Toluidine Blue O. Images were taken with a DM5500B microscope equipped with a DFC425c camera (Leica Microsystems).

**P. andersonii Mycorrhization Assay**

For mycorrhization experiments, pots were half-filled with sterilized river sand and watered with modified one-half-strength Hoagland medium containing 20 μM potassium phosphate. Pots were inoculated with 250 spores of *Rhizopagus irregularis* (Agrotriton-DAOM1971918). 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 were harvested 6 weeks with *R. irregularis* (Agrotriton-DAOM1971918). Root segments were treated with 10% (w/v) KOH and incubated at 90°C for 20 min. The root samples were then rinsed six times with water and stained with Trypan Blue at 90°C for 5 min. For each mutant, 10 plants were assessed, and from each plant, 30 root segments (each segment approximately 1 cm long) were examined, and mycorrhizal structures (hyphae, vesicles, and arbuscules) were determined using the magnified line intersect method (Trousselet et al., 1986) using a Leica CTR6000 microscope. For staining with WGA-Alexa488 (Molecular Probes, Thermo Fisher Scientific), roots were incubated in 10% (w/v) KOH at 60°C for 3. Then, roots were washed three times in phosphate-buffered saline (150 mM NaCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄, pH 7.4) and incubated in 0.2 μM M₆. WGA-Alexa488 in phosphate-buffered saline at room temperature for 16 h. For RNA isolation, mock-inoculated plantlets were harvested as controls. Three independent biological replicates were taken per sample. Expression was determined using RNA sequencing. 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 were identified using Benjamini-Hochberg multiple testing correction (q ≤ 0.05).

**Quantitative PCR Analysis of PanNFp1 cDNA Samples**

PanNFp1 cDNA samples were generated previously (Op den Camp et al., 2011). Quantitative PCR was performed in 10-μL reactions using 2× iQ SYBR Green Supermix (Bio-Rad). PCR was executed on a CFX Connect optical cycler according to the manufacturer’s protocol (Bio-Rad). Three technical replicates per cDNA sample were used. Data analysis and statistical analysis of biological replicates were performed using CFX Manager 3.0 software (Bio-Rad). Gene expression was normalized against reference genes PanACTIN and PanEF1α. Primers can be found in Supplemental Table S4.

**ROS Assay**

*P. andersonii* 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 1-cm pieces. Each well of a black 96-well flat-bottom polystyrene plate (Nunc) was filled with 10 root pieces. Ten replicates per line were analyzed. After filling the wells, the plate was kept 5 h 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 μM L-1 horseradish peroxidase (Sigma), and respective elicitors (CO7, ELICITYL) or LCOs extracted from *M. loti* or *R. tropici* at the described concentrations. As a mock treatment, 100 μL of water was added. The light emission was immediately measured at 30-s intervals for 30 min using a Clariostar multi-well plate reader. All data are averages of at least three independent biological replicates.

**Protein Extraction from *P. andersonii* and Western Blotting**

*P. andersonii* plantlets were grown on rooting medium (van Zeijl et al., 2018) for 4 weeks at 28°C before the treatment. About 200 mg of roots was cut while submerged in water and collected in a PCR tube. Root segments were incubated for 5 h 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), 150 mM KCl, 1 mM EDTA (pH 7.5), 0.1% (w/v) Triton X-100, 1 mM DTT, complete protease inhibitors (Roche), and phosphatase (Roche). Amounts of extracted protein were measured with Quibit (Thermo Fisher Scientific), and equal amounts of protein (~20 μg) were electrophoresed on Mini-PROTEAN TGX stain-free gels (Bio-Rad). 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 Signaling Technology). Anti-rabbit antibody (no. 7054; Cell Signaling Technology) was used as a secondary antibody. Equal loading was confirmed by Coomassie Brilliant Blue staining.

**Quantification and Statistical Analysis**

Node number was quantified as mean node number ± s.d. for all experiments. Replicate number is denoted in figures or figure legends. Addi- tionally, all individual data points were plotted for graphical visualization of variation. Graphical and statistical analysis were performed using R studio 1.1.456 for nodulation experiments. Statistical tests on node numbers was done using one-way ANOVA and Tukey’s posthoc test for multiple comparisons. Statistical significance was defined as P < 0.05. Levene’s test for homogeneity of variance was used prior to running one-way ANOVA. In cases where the normality assumption was violated, alternative tests such as the Mann-Whitney-Wilcoxon test 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 LSD of the knockout mutants with the wild-type control. The LSD with respect to the control was Benferroni adjusted to correct for multiple testing.

**Accession Numbers**

Sequence data from this article can be found in the GenBank/EMBL data libraries under the accession numbers mentioned in Supplemental Table S1.
**Data Supplement**

The following supplemental materials are available.

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

**Supplemental Figure S2.** Duplication of the LK3 first exon is conserved among *Parasponia* spp. and *Trena* subspecies.

**Supplemental Figure S3.** Complementation of a *L. japonicus* LjNfr1-LjNfr5 mutant for LCO-induced calcium oscillation.

**Supplemental Figure S4.** Nodulation is affected in *P. andersonii* PanNfr1, PanNfr2, and PanNfr3 CRISPR-Cas9 mutants.

**Supplemental Figure S5.** Complementation of the *P. andersonii* LjNfr1-LjNfr3 double mutant.

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

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

**Supplemental Figure S8.** *P. andersonii* LysM-type receptor mutants can establish AM symbiosis.

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

**Supplemental Figure S10.** The PanNFR RNAi construct has off-target activity on PanNFR2 and other LysM-type receptor kinases.

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

**Supplemental Table S2.** Transcomplementation of *L. japonicus* LjNfr1-LjNfr5 for nodulation.

**Supplemental Table S3.** Constructs 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-la-type receptors in fasta format.

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

**Supplemental Movie S1.** Calcium spiking in root hairs of the *L. japonicus* LjNfr1-LjNfr5-2 double mutant complemented with LjNFR1-LjNFR5.

**Supplemental Movie S2.** Calcium spiking in root hairs of the *L. japonicus* LjNfr1-LjNfr5-2 double mutant transcomplemented with PanLk31-LjNFR1.

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