LCO Receptors Involved in Arbuscular Mycorrhiza Are Functional for Rhizobia Perception in Legumes

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LCO Receptors Involved in Arbuscular Mycorrhiza Are Functional for Rhizobia Perception in Legumes

Highlights

- Mutants in Solanaceaous LysM receptors LYK10 are impaired in arbuscular mycorrhiza
- LYK10 proteins have a high affinity for lipo-chitooligosaccharidic signal molecules
- LYK10 promoter is expressed in arbuscule-containing cells in tomato roots
- Solanaceaous LYK10 can restore nodulation in legumes mutated in their orthologs

Authors

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In Brief

Soil rhizobial bacteria and arbuscular mycorrhizal (AM) fungi produce lipo-chitooligosaccharidic (LCO) signal molecules. Girardin et al. show that plant LCO receptors are involved in establishment of the ancient AM symbiosis and have been recruited during evolution for establishment of the nitrogen-fixing root nodule symbiosis with rhizobia.
LCO Receptors Involved in Arbuscular Mycorrhiza Are Functional for Rhizobia Perception in Legumes

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SUMMARY

Bacterial lipo-chitooligosaccharides (LCOs) are key mediators of the nitrogen-fixing root nodule symbiosis (RNS) in legumes. The isolation of LCOs from arbuscular mycorrhizal fungi suggested that LCOs are also signaling molecules in arbuscular mycorrhiza (AM). However, the corresponding plant receptors have remained uncharacterized. Here we show that petunia and tomato mutants in the LysM receptor-like kinases LYK10 are impaired in AM formation. Petunia and tomato LYK10 proteins have a high affinity for LCOs (Kd in the nM range) comparable to that previously reported for a legume LCO receptor essential for the RNS. Interestingly, the tomato and petunia LYK10 promoters, when introduced into a legume, were active in nodules similarly to the promoter of the legume orthologous gene. Moreover, tomato and petunia LYK10 coding sequences restored nodulation in legumes mutated in their orthologs. This combination of genetic and biochemical data clearly pinpoints Solanaceous LYK10 as part of an ancestral LCO perception system involved in AM establishment, which has been directly recruited during evolution of the RNS in legumes.

INTRODUCTION

Arbuscular mycorrhiza (AM) is an ancient mutualistic symbiosis between Glomeromycota fungi and the majority of land plants, in which fungi provide plants with nutrients acquired from the soil in exchange for carbohydrates and lipids [1, 2]. To colonize plant roots, arbuscular mycorrhizal fungi (AMFs) first cross the epidermal and outer cortical cells and then spread inter- or intra-cellularly within roots. Inside inner root cortical cells, AMFs form highly branched structures called arbuscules, across which most nutrient exchange takes place. In the more recent nitrogen-fixing root nodule symbiosis (RNS) that occurs between legumes and rhizobia, the bacteria can fix gaseous nitrogen inside the root nodules. Although the microorganisms are different between these two endosymbioses, the RNS is thought to have evolved through recruitment of genes implicated in the more ancient AM [3].

Nodule organogenesis and bacterial colonization rely on the secretion of lipo-chitooligosaccharide (LCO) signaling molecules by rhizobia [4]. All the rhizobial LCOs have a core structure of 4/5 N-acetyl glucosamine (GlcNAc) units of which the terminal non-reducing sugar is substituted with an acyl chain. Additional substitutions, which are important for host specificity, are characteristic of each bacterial strain [5]. Rhizobial LCOs are perceived by Lysin motif receptor-like kinases (LysM-RLKs) that are encoded by a multigene family, some of which have the ability to bind LCOs [6–8]. Members of the LysM-RLK LYR1A phylogenetic group (Figure S1A) [9], such as Medicago truncatula NFP (MtNFP) or Lotus japonicus NFR5 (LjNFR5), are required for activation of a signaling pathway leading to oscillations of the nuclear Ca2+ concentration (Ca2+ spiking), nodule organogenesis, and bacterial colonization [10–12].

Two lines of evidence suggest that AM establishment also involves LCO-mediated signaling. The first line is the identification of LCOs from AMFs, and the second is the identification of potential plant LCO receptors. LCOs isolated from AMFs by Maillet...
et al. (hereafter collectively referred to as Myc-LCOs) have a core structure similar to the rhizobial LCOs and can be sulfated or not on the reducing sugar [13]. Exogenous application of these Myc-LCOs both increases the level of AMF root colonization [13] and activates Ca^{2+} spiking in various plant species [14, 15]. Short-chain chitooligosaccharides (COs) produced by AMFs can also activate Ca^{2+} spiking [16], indicating that both LCOs and short-chain COs have the potential to be involved in partner recognition during AM. However, whether Myc-LCOs and/or short-chain COs are indeed involved in AM establishment is not known.

Several LysM-RLKs (Parasponia andersonii PanNFP1 and/or PanNFP2, tomato SILYK10 and SILYK12, Medicago truncatula MtLYK9, and rice OsCERK1) have been shown to be involved in AM [17–22], but their LCO/CO binding properties have not been determined so far. SILYK12, MtLYK9, and OsCERK1 belong to the LYKI phylogenetic group (Figure S1B [9]). These LysM-RLKs are likely co-receptors, since MtLYK9 and OsCERK1 have a dual function in AM and defense [19, 20, 23], and OsCERK1 is involved in perception of various ligands including short-chain COs, chitin, and peptidoglycan [24–26], the latter two being components of fungal and bacterial cell walls, respectively, known as plant defense elicitors. The other LysM-RLKs known to control AM belong to the LYRIA group that contains members only in plant species that establish AM and/or RNS [27, 28]. In tomato, virus-induced silencing of the unique LYRIA gene (SILYK10) resulted in significantly lower levels of AM colonization [21].

Although the current hypothesis is that the RNS evolved by coopting genes involved in the AM [3], it is unclear how LCO receptors may have evolved to become key players in RNS establishment.

Here, we functionally characterize LCO receptors from Solanaceae, a plant family that establishes AM but not RNS. We use heterologous expression in legumes to infer an evolutionary scenario of LCO receptor recruitment for RNS. Our data suggest that non-legume LYRIA genes encode LCO receptors involved in AM and that the transcriptional regulation required for LCO receptor function in RNS has been directly co-opted from AM.

### RESULTS

The Petunia and Tomato LYRIA Genes Are Involved in AM Establishment

We have previously shown that knockdown of the LYRIA gene in tomato (SILYK10) resulted in impaired AM establishment [21]. Because of the limitations of gene silencing, we screened an EMS-mutagenized tomato population and identified a line carrying a missense mutation in SILYK10 affecting the second LysM (E^{154}K) (Figure 1A). Segregants of this line with a homozygous mutation (SILYK10-1) displayed reduced numbers of AM colonization sites, root-length colonization, and expression of AM-marker genes in AM and that the transcriptional regulation required for LCO receptor function in RNS has been directly co-opted from AM.

![Figure 1. SILYK10-1 Is Affected in AMF Colonization](image)

(A) Schematic representation of SILYK10. The thick line represents the single exon. Arrowhead indicates the position of the mutation in SILYK10-1.
(B) Number of AMF colonization sites per root system. Boxplots represent the distribution between individuals from one experiment.
(C) Root-length colonization. Boxplots represent the distribution between root systems from three independent experiments.
(D) Relative expression of the plant AM-marker genes in SILYK10-1 versus control roots measured by qRT-PCR. RNAs were extracted from pools of four root systems. The line represents the mean, and the dots represent each replicate.

Statistical differences were calculated using a Kruskal Wallis test in (B) and (C). See also Figures S1 and S2.

![Figure 2.](image)
in dTPh1 close to the start codon of PhLYK10, suggests that PhLYK10 function is sensitive to gene dosage.

LCO Binding by LYRIA Proteins Predates the Evolution of RNS

LCO-binding in legume LYRIA proteins may have originated from ancestral LCO-binding proteins, or it may have been gained in legumes as a key property in the evolution of the LCO. To discriminate between these two possibilities, we determined the LCO-binding properties of SLYK10 and PhLYK10. We used Agrobacterium tumefaciens-mediated transient expression to produce SLYK10-YFP and PhLYK10-YFP in leaves of Nicotiana benthamiana, a plant protein expression system which allows the formation of disulfide bridges essential for LysM-RLK function [30, 31]. SLYK10-YFP was localized in undefined cytoplasmic structures in N. benthamiana leaf cells, although the protein was properly localized at the plasma membrane (PM) in transgenic tomato roots (Figures S3A–S3D). We previously observed that a chimeric LysM-RLK was well localized at the PM in N. benthamiana leaves and had LCO-binding properties similar to the corresponding full-length protein [6]. We thus generated a chimera (hereafter referred to as SLYK10c) composed of SLYK10 extracellular region (ECR) and MtNFP intracellular region. Although a fraction of SLYK10c-YFP was localized to the endoplasmic reticulum (ER) of N. benthamiana leaf cells (Figure 3A), both co-localization with a PM marker and the analysis of N-glycan maturation indicated that a significant fraction of the proteins reached the PM (Figures S4B–S4D). Subcellular localization of PhLYK10 and a PhLYK10 chimera (PhLYK10c) was similar (Figures 3A and S4B). SLYK10c-YFP, PhLYK10c-YFP, and SLYK10c-YFP were all immunodetected in the membrane fractions extracted from N. benthamiana leaves (Figure 3B). Their affinity for LCOs was determined by radio-ligand binding assays using LCO-V(C18:1,NMe) labeled with 35S. Specific binding of LCOs to membrane fractions was detected in extracts of leaves expressing PhLYK10-YFP, PhLYK10c-YFP, and SLYK10c-YFP but not in extracts of untransformed leaves (Figure 3C).

The affinity of PhLYK10-YFP and SLYK10c-YFP for LCO-V(C18:1,NMe,S) was determined by a cold saturation experiment. Scatchard plot analysis revealed single class of binding sites (Figure 3D) with dissociation constants ($K_d$) of 2 ± 5 nM (n = 3) and 19 ± 4 nM (n = 3), for PhLYK10 and SLYK10c, respectively, showing that both proteins exhibit high-affinity binding to this LCO. Their selectivity toward COs was then determined through competition assays between the 35S-LCO and an excess (1 nM) of unlabeled COs. CO4 and CO8 were much less efficient competitors of 35S-LCO binding (Figure 3E) with inhibitory constants ($K_i$) higher than 1 nM.
showing that the LCO-binding site of PhLYK10 and SILYK10c exhibits a low affinity for COs. We also determined the binding selectivity of SILYK10c-YFP toward Myc-LCOs. All Myc-LCOs were able to compete the binding of the 35S-LCO (Figure 3F). The affinities of SILYK10c-YFP for the sulfated and non-sulfated Myc-LCOs were further determined by competition assays. $K_i$ of 192 nM ± 52 nM (n = 3) and 354 nM ± 60 nM (n = 3) were obtained for LCO-IV(C16:0,S) or LCO-IV(C16:0), respectively (Figure 3G).
Finally, we found that affinity of PhLYK10c for LCOs (Kd of 60 nM ± 18 nM [n = 2]) and selectivity for LCOs versus COs were similar to that of PhLYK10 (Figures S5A and S5B), confirming that the LCO-binding properties of full-length proteins are conserved in our chimeric LysM-RLK.

Promoters from LYRIA Genes Did Not Neo-functionalize to Support RNS

Evolutionary genetics in various eukaryotic models indicates that recruitment of existing pathways to new traits often involves the gain or loss of cis-regulatory elements in promoter regions [32, 33]. We tested whether change in the transcriptional regulation for the LYRIA gene occurred for advent of the RNS by analyzing the expression patterns of Solanaceae LYRIA promoters in AM and RNS. In un-inoculated transgenic tomato roots, a 1.8 kbp sequence of the SILYK10 promoter region (ProSILYK10) drove the expression of the GUS reporter primarily in lateral roots (Figure 4A), the preferred site for AMF penetration [34]. Transverse and longitudinal sections revealed GUS activity in the epidermis and outer cortex (Figures 4B and 4C). In transgenic roots maintained as root organ cultures (ROCs) and inoculated with AMF, GUS staining was observed in arbuscule-containing cells (Figures 4D and 4E). Strongest GUS expression was observed in cells at the border of colonization units. Interestingly, this is the site where young arbuscules develop [35].

During nodulation, the M. truncatula LYRIA gene Mtnfp is expressed in nodule primordia and later in the infection zone of mature nodules [10]. We analyzed the activity of the petunia and tomato LYRIA promoters during nodulation in M. truncatula. ProSILYK10 and ProPhLYK10 exhibited an activity similar to ProMtNFP with GUS staining in the nodule primordia and in the apex of mature nodules (Figure 5A). This shows that the promoters of the two Solanaceae LYRIA genes contain all the information required for expression in legume nodules. We also compared the expression patterns of the three promoters in M. truncatula mycorrhizal roots. ProMtNFP showed a weak non-specific expression, while ProSILYK10 and ProPhLYK10 were mostly active in arbuscule-containing cells (Figure 5A). These results suggest that ProSILYK10 and ProPhLYK10 have the full symbiotic capacity required for expression during AM and RNS and that ProMtNFP has lost the ability to drive expression in mycorrhizal roots. In legumes, a whole-genome duplication at the base of the Papilionoideae gave rise to two paralogous LYRIA genes in Medicago, MtNFP, and MtLYR1. In contrast to MtNFP, MtLYR1 is expressed in mycorrhizal roots [36], but not in nodules (M. truncatula Gene Expression Atlas). The absence of ProMtNFP expression in mycorrhizal roots may reflect either a modification of the ancestral gene promoter required for its recruitment for RNS or the sub-functionalization following the gene duplication in the Papilionoideae. To test these possibilities, we analyzed the expression pattern of Mpnfp, the LYRIA gene from Mimosa pudica, a legume from the Mimosoideae clade that did not undergo whole genome duplication [37]. We found that in M. truncatula, ProMpnfp drives a similar expression pattern to ProSILYK10 and ProPhLYK10, with activity detected both in nodules and in arbuscule-containing cells (Figure 5A). This indicates that the evolution of RNS did not require the loss of LYRIA gene activation during AM. To determine whether Solanaceae LYRIA promoters are sufficient to provide LYRIA protein activity for RNS, we expressed the MtNFP coding sequence (CDS) under the control of ProSILYK10 in a mtnfp mutant line unable to form nodules. We observed a similar number of nodules in roots containing either the ProSILYK10:MtNFP-YFP construct or the ProMtNFP:MtNFP-YFP construct (Figure 5B). These results suggest that cis-regulatory elements essential for expression in nodules are conserved between ProSILYK10, ProPhLYK10, ProMtNFP, and ProMpnfp. To identify the region that contains these cis-regulatory elements, we first cloned a shorter version of the MtNFP promoter (240 bp before the start codon) and tested its activation during RNS. Similar to the 1.5 kb sequence, this shorter promoter was sufficient to drive expression of the GUS reporter in young nodules (Figure 5C). Through scanning the promoter region of orthologous LYRIA genes from nodulating and non-nodulating dicotyledonous species, we identified the AAAGCTANNAGACA consensus sequence in the promoters of at least one LYRIA gene in 60% of 71 investigated species (Figure S6). This consensus sequence is located in the proximal region of MtNFP and SILYK10 promoters (Figure 5D). A SILYK10 promoter region starting 10 bp upstream of...
this consensus sequence (185 bp before the start codon) also exhibited activity in young nodules (Figure 5C).

Taken together, our results indicate that the recruitment of \textit{LYRIA} genes for RNS did not require modification in the regulation of their expression.

**PhLYK10 Partially Complements the Lack of Nodules in Legume Mutants**

Besides modifications in \textit{cis}-regulatory elements, recruitment of a gene into a new trait may result from neo-functionalization of the encoded protein [32, 38]. To test whether the recruitment of \textit{LYRIA} genes for RNS involved neo-functionalization, we performed complementation assays of \textit{Mtnfp} and \textit{Ljnfr5} mutants with the CDS of \textit{PhLYK10} and \textit{SlLYK10}.

Pro\textit{LjNFR5}:\textit{SlLYK10} did not restore nodulation in \textit{Ljnfr5} mutant. This is similar to what was observed in \textit{Mtnfp} mutant with the CDS of \textit{MtNFP} ortholog in pea, \textit{PsSYM10}, under the control of \textit{ProMtNFP} [39]. However, we found that \textit{PsSYM10} under the control of the strong \textit{35S} promoter was able to complement \textit{Mtnfp} for nodule formation and rhizobial colonization (Figures S7A and S7B). Strikingly, \textit{Pro35S:SlLYK10} and \textit{Pro35S:PhLYK10} were also able to restore the formation of nodules in \textit{Mtnfp} (Figure 6A) while \textit{Mtnfp} roots expressing \textit{AtCERK1}, an \textit{A. thaliana} LysM-RLK from the \textit{LYKI} group (Figure S1B) did not produce any nodules. The nodules formed in roots expressing \textit{SlLYK10} were fully colonized by rhizobia, similarly to roots expressing \textit{MtNFP}, while only a very weak rhizobial staining was observed in roots expressing \textit{PhLYK10} (Figure 6B). Immunodetection of proteins in \textit{Mtnfp} roots revealed that \textit{MtNFP} was expressed at the highest level (Figures 6C and S7C), whereas \textit{PhLYK10} was below the

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**Figure 5.** \textit{ProSlLYK10:GUS} and \textit{ProPhLYK10:GUS} Are Expressed in Nodules and in Arbuscule-Containing Cells of \textit{M. truncatula} Roots

(A) GUS staining (magenta) in young and mature nodules of \textit{M. truncatula} transgenic roots containing the indicated constructs or the empty vector (EV) and inoculated with \textit{S. meliloti} (Sm). GUS staining (blue) in arbuscule-containing cells (green) of \textit{M. truncatula} transgenic roots inoculated with \textit{R. irregularis}. Arbuscule-containing cells are marked by an asterisk.

(B) Number of nodules in \textit{Mtnfp} roots complemented by the indicated constructs and inoculated with \textit{S. meliloti}. Numbers in brackets indicate the numbers of root systems carrying nodules/root systems analyzed. Boxplots represent the distribution between individuals from at least two independent experiments. Scale bars represent 100 \textmu m in the nodule sections and 20 \textmu m the right panels.

(C) The GUS reporter (blue) under the control of a minimal \textit{MtNFP} (ProminNFP, 240 bp before the start codon) or \textit{SlLYK10} (ProminSLYK10, 185 bp before the start codon) promoters is expressed in young nodules of \textit{M. truncatula} roots inoculated with \textit{S. meliloti}. Scale bars represent 100 \textmu m.

(D) The putative \textit{cis}-regulating element in \textit{MtNFP} and \textit{SLYK10} promoters is highlighted in red in the 200 bp sequences before the start codons. The most conserved positions are in bold. The logo shows the degree of conservation of the putative \textit{cis}-regulating element among 71 dicotyledonous \textit{LYRIA} genes. See also Figure S6 and Table S2.
detection limit despite its ability to partially complement nodulation in Mtnfp. This may reflect differences in the stability of the orthologs in M. truncatula, which in turn may explain the different levels of complementation by the different LYRIA proteins. Nodulation was also restored in Ljnfr5 roots expressing PhLYK10 (Figure 6D), although, as in Mtnfp roots, fewer nodules were formed compared with complementation with the endogenous LYRIA gene. In this case, the nodules were fully colonized by rhizobia (Figure 6E). ProLjUBI:PhLYK10-mOrange also triggered spontaneous nodule formation in L. japonicus in the absence of rhizobia (Figures S7D and S7E) like overexpression of LjnFR5 [40].

**DISCUSSION**

Myc-LCOs can induce gene transcription, Ca²⁺ spiking, and root branching [13–15, 41, 42]. However, until now it was not clear whether they are involved in AM establishment. Here, we demonstrate high-affinity LCO-binding properties of PhLYK10 and SILYK10, which, together with the mycorrhizal phenotype of the Phlyk10-1 and Silyk10-1 mutant lines, provide the strongest evidence to date that Myc-LCOs are directly involved in AM establishment.

Detailed characterization of PhLYK10 and SILYK10 revealed that they are high-affinity LCO-binding proteins that discriminate LCOs versus COs; their affinity for LCOs being as high as that of the previously characterized legume LYRIA protein, LjnFR5, expressed in the same heterologous system [8]. SILYK10 recognized the Myc-LCO structures described in [13] with similar affinity for sulfated and non-sulfated Myc-LCOs. However, SILYK10 exhibited a higher affinity for LCO-V(C18:1,NMe,S) compared with the published Myc-LCO structures, indicating that such LCOs or related structures could potentially represent additional Myc-LCOs.

The similarity of the AM phenotype in the petunia line knockout for PhLYK10, the tomato line bearing a point mutation in SILYK10, and the tomato SILYK10-silenced plants [21] provides compelling evidence that the LYRIA gene is involved in AM establishment in Solanaceae. Reduction in the number of colonization sites in the above-mentioned plants suggests a role at early stages for AMF penetration in roots. Moreover, the aberrant arbuscule development observed in Phlyk10-1 and SILYK10-silenced plants suggests an additional role in arbuscule development. The activity of the SILYK10 promoter in tomato roots initially in the epidermis and upon colonization in arbuscule-containing cells further supports a role of the LYRIA gene at several steps of AM establishment in Solanaceae.

Although Phlyk10-1, Silyk10-1, and the SILYK10-silenced plants are affected in AM establishment, AMFs can still colonize roots and form arbuscules. In a mutant of the rice LYRIA gene OsNFR5, AM-marker gene expression was decreased, but the number of AMF colonization sites was not affected [18]. Mutants in Mtnfp are also colonized normally by AMFs [19, 43] despite an almost complete block of symbiosis-related responses to both rhizobial LCOs and Myc-LCOs [13, 14, 43, 44]. Moreover, a double mutant in the two LYRIA genes LjnFR5 and LjLys11 was not affected in AM establishment [45]. Altogether, this suggests redundancy at the level of LCO perception or that other signals could activate the LCO-mediated signaling pathway.
Indeed, Ca\(^2+\) spiking can be measured in an Mtnfp mutant after treatment with CO4 [16], suggesting that short-chain CO receptors are also involved in AM establishment. Other signals such as kainkin-like molecules and effector proteins produced by AMFs are known to play important roles in plant-AMF communication [46], but the connection of their perception and/or mode of action to LCO-mediated signaling remains elusive.

It has been postulated that RNS has evolved through recruitment of genes implicated in AM, but it is unclear how the LCO perception machinery may have been affected by the evolution of RNS. Our data are compatible with a scenario in which an ancestral LYRIA gene involved in LCO perception in AM was directly recruited for LCO perception in RNS in legumes (Figure 7). Because both symbiotic interfaces are intracellular, it can be proposed that LYRIA genes participate in these conserved accommodation mechanisms [47]. The promoters of the single LYRIA gene from the Solanaceae or from the legume *M. pudica* have the ability to drive dual expression both in mycorrhizal roots and in nodules of *M. truncatula*. In contrast, the LYRIA gene pairs in the legumes *Medicago* and *Lotus*, Mtnfp/Ljnfr5, and MtLYR1/ LjLYS11 have retained transcriptional regulation only during nodulation or AM, respectively [10, 36, 45]. This is indicative of promoter sub-functionalization following the whole genome duplication that predated the radiation of the Papilionoideae, the legume clade to which *Lotus japonicus* belongs (Figure 7). Interestingly, RNS is evolutionarily more stable in Papilionoideae than in any other clade of RNS-forming plants, including the Mimosoideae to which *Mimosa pudica* belongs [48]. In other words, the probability for a given species in the Papilionoideae to lose RNS is much lower than in other clades. Although the reason for this greater stability remains unknown, one possibility is that duplication and sub-functionalization of genes with a dual function in AM and RNS such as the LYRIA genes, for separated functions in AM and RNS, may have allowed stabilized symbiotic associations.

The AAAACTANNGACA sequence conserved in LYRIA promoters could represent an ancestral cis-regulatory element involved in transcriptional regulation during AM that has been recruited for transcriptional regulation during RNS. This putative cis-regulatory element is, however, conserved in the promoters of both paralogous LYRIA genes from the Papilionoideae, suggesting that sub-functionalization of the LYRIA promoter pairs has not occurred through divergence in this sequence. Further studies are required to validate the function of this putative cis-regulatory element and to identify the mechanism of LYRIA promoter sub-functionalization in Papilionoideae.

Strikingly, the Solanaceae *LYRIA* proteins PhLYK10 and SILYK10 can restore the full nodulation program in the legume *LYRIA* mutants Mtnfp and Ljnfr5, although with lower efficiency than the respective endogenous *LYRIA* genes Mtnfp and Ljnfr5. This suggests that the legume and non-legume *LYRIA* proteins can fulfill the function of endogenous *LYRIA* proteins for both nodule formation and rhizobial colonization. Lower complementation efficiency of SILYK10, PhLYK10, and PsSYM10 compared with Mtnfp correlated with lower levels of protein detected in complemented *Mtnfp* roots. However, lower complementation efficiency of heterologous *LYRIA* proteins in Mtnfp and Ljnfr5 may also be due to inefficient interactions with the respective co-receptors MtLYK3 and LjNFR1, two LysM-RLKs belonging the *LYKI* group. It has been suggested that evolution of the *LYRIA* gene for a new role in RNS may have involved a tandem gene duplication (preceding the advent of RNS) followed by neofunctionalization of one copy for RNS and loss of other copy in the species that acquired the RNS [49]. However, our results suggest that both the promoter and the CDS of the ancestral non-duplicated *LYRIA* gene were already fully competent for both symbioses.

Intriguingly, our results raise the question of how signal specificity in AM and RNS may be encoded. The fact that PhLYK10 can complement both Mtnfp and Ljnfr5 for nodule formation while *M. truncatula* and *L. japonicus* can specifically recognize the respective major LCOs produced by *Sinorhizobium meliloti* (LCO-IV(C16:2,S) [50] and *Mesorhizobium loti* (LCO-IV(C16:1,Cb,Fuc,Ac) [51] argues for limited LCO selectivity of LYRIA proteins PhLYK10 and Ljnfr5, although with lower efficiency than the respective endogenous *LYRIA* proteins PhLYK10 and Ljnfr5. This questions the hypothesis that Mtnfp and Ljnfr5 recognize specific LCO structures and suggests that co-receptors such as MtLYK3/Ljnfr1, or yet unidentified proteins, may interact with Mtnfp and Ljnfr5 to confer LCO binding specificity to LCO receptor complexes. Consistent with such a scenario, the number of LysM-RLKs in the *LYKI* group has dramatically increased in legumes compared with non-legumes and contains a legume-specific subgroup to which MtLYK3 and Ljnfr5 belong [52].

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- LEAD CONTACT AND MATERIALS AVAILABILITY
<table>
<thead>
<tr>
<th>EXPERIMENTAL MODEL AND SUBJECT DETAILS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cloning</td>
</tr>
<tr>
<td>o S. lycopersicum and P. hybrida mutant identification and genotyping</td>
</tr>
<tr>
<td>o Agrobacterium rhizogenes mediated transformation</td>
</tr>
<tr>
<td>o Inoculation with AMF</td>
</tr>
<tr>
<td>o Inoculation with rhizobia and spontaneous nodulation</td>
</tr>
<tr>
<td>o Transient Expression in N. benthamiana</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>METHOD DETAILS</th>
</tr>
</thead>
<tbody>
<tr>
<td>o Microscopy</td>
</tr>
<tr>
<td>o Western blotting and membrane fraction preparation</td>
</tr>
<tr>
<td>o LCO binding assays</td>
</tr>
<tr>
<td>o PNGaseF treatment and immunoblotting</td>
</tr>
<tr>
<td>o qRT-PCR</td>
</tr>
<tr>
<td>o Promoter investigation</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>QUANTIFICATION AND STATISTICAL ANALYSIS</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>DATA AND CODE AVAILABILITY</th>
</tr>
</thead>
</table>

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.jcb.2019.11.038.

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We thank Marie Cumener, Fabienne Mailet, Mireille Chabaud, and Vérona Poinot for technical help, Sébastien Fort for CO4 an CO8 production, and Julie Cullimore and Malick Mbengue for critical reading of the manuscript. This work was supported by the ANR “WHEATSYM” (ANR-16-CE20-0025-01), the “Laboratoire d’Excellence (LABEX) TULIP (ANR-10-LABX-41), and the research project Engineering Nitrogen Symbiosis for Africa (ENSA), which is funded through a National Science Foundation (31003A_169732), and the research project Environment Occitanie and INRA/C19. Work in M.P.’s lab was supported by the ANR “WHEATSYM” (ANR-16-CE20-0025-01), and the research project “Stress in legumes and rice.” Plant Cell 27, 823–838.

AUTHOR CONTRIBUTIONS

DECLARATION OF INTERESTS
The authors declare no competing interests.

REFERENCES


# STAR★METHODS

## KEY RESOURCES TABLE

<table>
<thead>
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### Oligonucleotides

- **ProSILYK10** for **GGTCTCTAAATGGGTTATAGAGCTGTAATGC**: This work N/A
- **ProSILYK10 rev** **GGTCTCATTTGCGATGCAAAGCTTAGATAAC**: This work N/A
- **ProPhLYK10** for **ATCGGTCTCCTTTGTCGAAAGCTCAGATGGGC**: This work N/A
- **ProPhLYK10 rev** **ATCGGTCTCCTTTGCTAATGAGAGTTTAGCAGAGG**: This work N/A
- **ProMpnPf for** **ATCGGTCTCCTTTGCTAATGAGAGTTTAGCAGAGG**: This work N/A
- **ProMpnPf rev**: **ATCGGTCTCCTTTGCTAATGAGAGTTTAGCAGAGG**: This work N/A
- **PhLYK10ECR for**: **GGTCTCCCCAAATGGAAGGCTCCTTCTTCTCCTCT**: This work N/A
- **PhLYK10ECR rev**: **GGTCTCTCCTAAATGGAAGGCTCCTTCTTCTCCTCT**: This work N/A
- **SILYK10ECR rev**: **GACAGACAGACTTTTCGCTCT**: This work N/A
- **PhLYK10 Genotyping rev**: **ACAGCTCTGGTACCAACTGTC**: This work N/A
- **PhLYK10 Genotyping for**: **ACAGCTCTGGTACCAACTGTC**: This work N/A

### Recombinant DNA

- **Pcambia-Pro35S:PhLYK10-YFP**: This work N/A
- **PcambiaGG-Pro35S:PhLYK10c-YFP**: This work N/A
- **PcambiaGG-Pro35S:SlLYK10-YFP**: This work N/A
- **PbinGW-Pro35S:SlLYK10-YFP**: This work N/A
- **PcambiaGG-ProSlLYK10c-YFP**: This work N/A
- **PcambiaGG-ProminSlLYK10:GUS**: This work N/A
- **PcambiaGG-ProPhLYK10:GUS**: This work N/A
- **PcambiaGG-ProMtNFP:GUS**: This work N/A
- **Pbin-ProMtNFP:GUS**: [16] N/A
- **PcambiaGG-ProMtNFP:GUS**: This work N/A
- **Phn-ProMtNFP:Mont-PFP**: This work N/A
- **PcambiaGG-ProSlLYK10:Mont-PFP**: This work N/A
- **Pcambia-35S:SlNFR3:YFP**: [62] N/A
- **Pcambia-ProUJUB1:UNFR5-mOrange**: This work N/A
- **Pcambia-ProUJUB1:PhLYK10-mOrange**: This work N/A
- **Pbin-PsSYM10-YFP**: [63] N/A
- **Pbin-pro35S:PsMA4-GFP**: [64] N/A
- **Pbin-pro35S:PsHDEL-GFP**: [64] N/A

### Software and Algorithms

- **LASX**: Leica N/A
- **Zen**: Leica N/A
- **ImageJ**: [http://imagej.nih.gov/ij](http://imagej.nih.gov/ij) N/A
- **R**: [http://r-project.org](http://r-project.org) N/A
- **tBLASTn**: v2.9.0+ [65] N/A
- **MAFFT**: v7.407 [66] N/A
- **TrimAl**: v1.4 [67] N/A
- **ModelFinder**: [68] N/A
- **IQ-TREE**: v1.6.1 [69] N/A
- **SH-airt**: [70] N/A
- **iTOL platform**: v4.4.2 [71] N/A

(Continued on next page)
**Cloning**

1.8, 1.5 and 1.9 kbp corresponding to the non-coding region between SlLYK10, PhLYK10, and MpNFP and the preceding genes, including the 5′ UTR were amplified by PCR (with the primers listed in the key resources table) from genomic DNA isolated from S. lycopersicum, P. hybrid and P. pudica, respectively, and cloned in transcriptional fusion with a GUS reporter containing a plant intron, in a pCambia 2200 modified for Golden gate cloning and containing a ProUbi:DsRed reporter as in [73]. Note that the PhLYK10 sequence in P. hybrid originates from the P. axillaris parent. 240 and 185 bp sequences preceding the start codons were synthesized and cloned as described previously. ProSILYK10: MtNFP-YFP was made by Golden gate cloning in a pCambia 2200 modified for Golden gate cloning in [6]. ProMtNFP: MtNFP-YFP was made as in [30] excepted that MtNFP was in translation fusion with YFP instead that of FLAG.

SILYK10 and PhLYK10 coding sequences were amplified by PCR from genomic DNA isolated from S. lycopersicum and P. hybrid and cloned in translational fusion with YFP under the control of Pro35S in a pbin vector modified for gateway cloning as in [74] for SILYK10 or in a pCambia 2200 modified for Golden gate cloning as in [6] for PhLYK10. For expression in M. truncatula, SILYK10 sequence was optimized with a M. truncatula codon usage and cloned in translational fusion with YFP under the control of Pro35S in a pCambia 2200 modified for Golden gate cloning as in [6]. For expression in L. japonicus, PhLYK10 coding sequence was amplified by PCR from genomic DNA isolated from P. hybrid and cloned in translational fusion with mOrange under the control of LjUbiquitin promoter into a pCambia-based Golden Gate expression vector [75]. For SILYK10c and PhLYK10c constructs, the sequences coding the extracellular region of SILYK10 or PhLYK10 were amplified by PCR (with the primers listed in the key resources table) and cloned in translational fusion with the sequences coding TM/ICR of MtNFP and YFP under the control of Pro35S in a pCambia 2200 modified for Golden gate cloning as in [6].

**S. lycopersicum and P. hybrid mutant identification and genotyping**

The Silky10-1 mutant allele (line 1051, G168A) was identified by sequencing (NGS) an amplicon (key resources table) obtained on tomato (cv M82) EMS-mutagenized lines. Homozygous mutant or WT SILYK10 alleles were identified by sequencing (Sanger) a similar amplicon on the progeny. The Phlyk10-1 mutant allele (line LY0882, dtPh1 insertion 116 bp from the start codon) was identified by BLAST-searching in a Petunia dtPh1 transposon flanking sequence database [29] with the full PhLYK10 coding sequence. This line was crossed with the stabilizer line W5 [60], to segregate out the activator locus required for dtPh1 transposition. Genotyping on different progenies was done by PCR with the primers listed in the key resources table.

**Agrobacterium rhizogenes mediated transformation**

Tomato (cv Marmande) seeds were surface sterilized and germinated in vitro for 7 to 10 days until cotyledons were fully expanded. Plantlets were cut at the hypocotyl level, immerged in a A. rhizogenes ARqua1 suspension at OD600nm = 0.3 and grown for 3 days at 25°C on MS, then on MS supplemented with 50 mg/l kanamycin and 200 mg/l cefotaxim until emergence of transgenic roots. Transgenic roots were selected by fluorescence microscopy. Plantlets were transferred in pots containing vermiculite as described in [21]. ROC lines derived from transformed roots were grown in dark, on MS medium supplemented with 50 mg/l kanamycin.

Chimeric M. truncatula A17 and Mtnfp-2 plants were produced as described in [73] for analysis of promoter expression pattern and for complementation experiment, respectively. Chimeric L. japonicus Gifu and Ljnfr5-2 plants were produced as described in [77].
Inoculation with AMF
For AM phenotyping, petunia seeds were germinated on a sterilized potting soil until cotyledons were fully expanded. Tomato seeds were surface sterilized and germinated in sterile water. Petunia and tomato plantlets were then transferred in 50 mL containers filled with attapulgite, watered with 20 mL of 0.5x modified Long ashton (7.5 μM NaH2PO4), and inoculated with 500 spores of R. irregularis DAOM 197198. Roots were harvested, washed and stained between 3 and 4 weeks post inoculation.

For analysis of GUS activity in tomato roots, sterilized Gigantea gigaspora spores, harvested from a leek nurse culture, were pre-germinated 5 days on M medium [78] in a 3% CO2 incubator at 32°C. Two spores and one fragment of a transgenic tomato ROC line were then co-cultured on a Petri dish containing M medium supplemented with 50 mg/l kanamycin. Petri dishes were placed vertically with ROC lines above the fungal spores for 4 weeks. For analysis of GUS activity in M. truncatula transgenic roots, chimeric plantlets were transferred in 50 mL containers filled with a mix 1:1 of attapulgite and sand, watered with 20 mL of 0.5x modified Long ashton medium and inoculated with 200 spores of R. irregularis DAOM 197198. Roots were harvested, washed and stained 2 weeks post inoculation.

Inoculation with rhizobia and spontaneous nodulation
M. truncatula chimeric plantlets were transferred in 250 mL containers filled with attapulgite, watered with 20 mL of Fahraeus medium supplemented with 1 mM NH4NO3. After 4 days, 2.5 mL of a suspension at OD600nm = 0.025 of a S. meliloti strains 2011 harboring the hemA-lacZ plasmid (pXLGD4) was added around the hypocotyl. Roots were harvested, washed and stained 4 weeks post inoculation.

For complementation experiments, L. japonicus chimeric plantlets were transferred to Weck jars containing 300 mL of a mix of sand and vermiculite and inoculated with 20 mL of a M. loti MAFF303099 DsRED suspension in FP medium (OD600 = 0.05). Plants were phenotyped 25 days post inoculation.

Spontaneous nodulation experiments on L. japonicus roots were performed as described previously [40]. L. japonicus chimeric plantlets were transferred to Fahraeus medium plates containing 0.1 μM of the ethylene biosynthesis inhibitor L-α-(2-aminoethoxyvinyl)-glycine 2.5 weeks after transformation. Root systems were analyzed 60 days post transformation.

Transient Expression in N. benthamiana
Leaves of N. benthamiana were infiltrated with A. tumefaciens LBA4404 virGN54D strains as described in [79]. Leaves were harvested 3 days after infiltration.

METHOD DETAILS

Microscopy
Tomato ROC expressing SLYK10-YFP were incubated at room temperature 5 min in water with 1 μg / ml DAPI or 20 μM FM4-64 before confocal imaging. For plasmolysis, ROC lines were incubated for 1 h in a 0.8 M mannitol. Tomato ROC and chimeric M. truncatula plants expressing the GUS reporter were stained with 0.1% X-Gluc or Magenta-Gluc (20 min under vacuum followed by incubation at 37°C). AMF were stained by treating root tissues with 100% ethanol for 4 h, then with 10% KOH for 8 min at 95°C (tomato ROC and P. hybrida roots) or 1,5 days at room temperature (M. truncatula roots) and finally with 0.2 M PBS pH 7.2, Triton X-100 0.01%, 1 μg/mL WGA CF488A conjugate overnight at room temperature. For analysis of subcellular localization, tomato ROC and N. benthamiana leaves were imaged using a SP8 confocal microscope. Arbuscules in P. hybrida were imaged with a SP2 confocal microscope. Overlay corresponds to merge of green fluorescence channel images with differential interference contrast images. GUS and WGA staining were imaged using an Axiozoom V16 microscope (Figure 4) or an Axioplan 2 microscope (Figure 5).

Automatic delimitation and drawing of cells strongly expressing GUS was performed with ImageJ (Figure 4).

Numbers of colonization sites and root length colonization were quantified on entire root systems using a S6E microscope after ink staining of the AMF as described in [21].

M. truncatula nodulated roots systems expressing the GUS reporter were stained with 0.1% mangenta-gluc and then fixed with glutaraldehyde 1.25% in 0.1 M PBS pH7.2 (30 min under vacuum). In case of Mtnfp-2 complementation, nodulated roots systems were first fixed with glutaraldehyde 1.25% and then stained with 2% X-Gal (30min under vacuum and followed by incubation at 28°C). Nodules were sectioned after incubation in 6% agarose low gelling temperature using a vribratome VT 1000S and sections were imaged with an Axiozoom V2 microscope. Nodules of M. truncatula roots expressing the GUS reporter under the control of the minimal promoters were stained 0.1% X-Gluc and directly imaged with an Axiozoom V16 microscope.

Western blotting and membrane fraction preparation
Immunoblotting of YFP fusions in M. truncatula roots was performed on 20 mg of a total extract of a pool of 10 root systems inoculated by S. meliloti. For LCO binding assays, approximately 20 g of leaves were homogenized at 4°C in a blender in the presence of 40 mL of extraction buffer (25 mM Tris, pH 8.5, 0.47 M sucrose, 5 mM EDTA, 10 mM DTT, 0.6% PVPP and protease inhibitors (0.1 mM AEBSF, and 1 mg/mL each of leupeptin, aprotinin, antipain, chymostatin, and pepstatin). Samples were centrifuged for 15 min at 3000 g, and then the supernatant was recentrifuged for 30 min at 45000 g. The pellet (membrane fraction) was first washed in 5 mL and then resuspended in 2 mL of binding buffer (25 mM Na-Cacodylate pH 6, 250 mM sucrose, 1 mM CaCl2, 1 mM MgCl2 and protease inhibitors). After each extraction, amount of fusion proteins was quantified by immunoblotting in 10 μg of membrane

Current Biology 29, 4249–4259.e1–e5, December 16, 2019 e4
fraction proteins. PhLYK10-YFP, PhLYK10c-YFP and SILYK10c-YFP have expected molecular masses of about 104, 102 and 102 kDa respectively (including 6 predicted N-glycans). For Figure S4D, after homogenization samples were centrifuged for 20 min at 100000 g and resuspended in the same volume of extraction buffer. Proportional volumes of total extract, resuspended pellet and supernatant were loaded on SDS-PAGE.

**LCO binding assays**

LCO-V(C18:1Δ11,NMe) and LCO-V(C18:1Δ11,NMe,S) were purified from the rhizobial strain *Rhizobium tropici*. Labeling of LCO-V(C18:1Δ11,NMe) was performed as described in [80]. LCO binding assays on membrane fractions containing 20 µg or 40 µg of proteins were performed as in [5] using between 1 and 2 nM of radiolabeled LCO and ranges of unlabeled LCO between 1 nM to 1 µM. Similar amount of membrane fraction from leaves expressing PhLYK10-YFP, PhLYK10c-YFPc, SILYK10c-YFP or from untransformed leaves were used in each experiment. Competition with COs were performed with 1 µM of unlabeled pure CO4 and CO8.

**PNGaseF treatment and immunoblotting**

PNGaseF treatment, SDS-PAGE, transfer to nitrocellulose membranes and western blotting were performed as described in [30].

**qRT-PCR**

RNA extraction, cDNA synthesis was performed as described in [21]. Relative expression levels were calculated using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference gene. Primers were as in [81] and [21].

**Promoter investigation**

*MtNFP* orthologs were retrieved from genomes of 71 dicotyledonous species (list in Table S2) using tBLASTn and an e-value threshold of $10^{-10}$. Putative orthologs were aligned with MAFFT with default parameters and aligned positions with more than 50% of gaps were removed using TrimAl. The best-fitting evolutionary model was tested using ModelFinder and according to the Bayesian Information Criteria. The model TVM+F+R5 was further used for Maximum Likelihood (ML) analysis using IQ-TREE. Branch support was tested using 10,000 replicates of SH-alrt. The resulting tree was annotated using the iTOL platform. For each ortholog, 600 bp promoter sequences were extracted upstream of the gene start using a custom Python script. Promoters were searched for enriched motif using MEME with following parameters: zero or one occurrence of motif per site, motif length comprises between 5 and 25 bp and a minimum of 2 sites by motifs.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Number of independent biological replicates and individuals analyzed, as well as the statistical tests used to analyze the data are indicated in the figure legends. All statistical analyses were performed using the R software (http://r-project.org).

**DATA AND CODE AVAILABILITY**

This study did not generate any unique datasets or code.