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MINI-REVIEW

Lipo-chitooligosaccharidic nodulation factors and their perception by plant receptors

Judith Fliegmann[1,2] · **Jean-Jacques Bono**[1,2]

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Abstract Lipo-chitooligosaccharides produced by nitrogen-fixing rhizobia are signaling molecules involved in the establishment of an important agronomical and ecological symbiosis with plants. These compounds, known as Nod factors, are biologically active on plant roots at very low concentrations indicating that they are perceived by specific receptors. This article summarizes the main strategies developed for the syntheses of bioactive Nod factors and their derivatives in order to better understand their mode of perception. Different Nod factor receptors and LCO-binding proteins identified by genetic or biochemical approaches are also presented, indicating perception mechanisms that seem to be more complicated than expected, probably involving multi-component receptor complexes.

Keywords Lipo-chitooligosaccharides · Symbiosis · Receptor · Plant · LysM

Introduction

Lipo-chitooligosaccharides (LCOs) define a class of signaling molecules that play important roles in plant-microbe interactions. LCOs consist of a chitin backbone made of 3 or 4 β-1-4-linked *N*-acetylglucosamine (GlcNAc) units with a non-reducing terminal glucosamine unit *N*-acylated by a fatty acid. LCOs were first identified as nodulation factors (Nod factors), that are secreted by the bacterium *Sinorhizobium meliloti* and that are essential for establishment of the nitrogen-fixing root nodule symbiosis with legume plants of the Genus *Medicago* [1].

Nod factors result from the activity of bacterial *nod* genes encoding enzymes involved in the synthesis of the chitin backbone, the acylation reaction and the decoration with chemical substitutions at both ends of the molecule. Since this founding work, Nod factors isolated from many bacteria which nodulate legume plants, collectively known as rhizobia, have all been shown to have the same generic LCO structure but may differ in the number of GlcNAc units (2 to 4), in the length and degree of unsaturation of the fatty acid chain, as well as the presence of various substitutions on the oligosaccharide backbone [2]. These variations are characteristic for each rhizobium and are involved in the specific recognition between the legume plant and its symbiont [3]. Nod factors are responsible for nodule organogenesis and controlled infection, leading to the formation of root nodules where the bacteria fix atmospheric dinitrogen. At sub-nanomolar concentrations, purified Nod factors mimic early symbiotic plant responses to rhizobia and, in certain species, induce the morphogenesis of nodule primordia [4].

Recently, LCOs have also been isolated from arbuscular mycorrhizal (AM) fungi of the Glomeromycota family that establish another agronomically and ecologically important root endosymbiosis with plants [5]. This symbiosis is not restricted to plants of the legume family since it concerns approximately 80 % of terrestrial plants. These LCOs, named Myc-LCOs, which have been isolated so far from only one AM fungus (*Rhizophagus irregularis*, formerly *Glomus intraradices*), are less diverse than Nod factors. Myc-LCOs are sulfated or not at the reducing end and the acyl chain is

✉ Jean-Jacques Bono
jean-jacques.bono@toulouse.inra.fr

[1] INRA, Laboratoire des Interactions Plantes-Microorganismes (LIPM), UMR441, 31326 Castanet-Tolosan, France

[2] CNRS, Laboratoire des Interactions Plantes-Microorganismes (LIPM), UMR2594, 31326 Castanet-Tolosan, France

either a palmitic acid (C16:0) or an oleic acid (C18:1). Our knowledge of the biological function of Myc-LCOs is less advanced compared to Nod factors and will not be presented in detail in this mini-review. The use of bacterial mutants affected in *nod* genes has been instrumental to demonstrate the crucial role of Nod factors in the rhizobial symbiosis. Such an approach, exploiting genetic loss or gain of function, is not directly amenable to AM fungi because of their inability to be genetically manipulated. However, root treatment with exogenous Myc-LCOs stimulates mycorrhization in legumes and nonlegumes indicating that Myc-LCOs are biologically active on a variety of plants. Moreover, Myc-LCOs, just like Nod factors, stimulate root development and induce calcium spiking and transcriptional changes in *Medicago truncatula* [5–8].

Most of the biological responses induced by LCOs require a very low concentration (in the nanomolar range), suggesting that these molecules are perceived by receptors in the host plant root. This mini-review will sum up our current knowledge on the perception of Nod factors and how the chemical synthesis of LCOs and their derivatives have provided important tools to better understand their perception, their mode of action and their discrimination from chitooligosaccharides (COs).

Chemical synthesis of Nod factors

A rhizobial strain, grown in axenic conditions in liquid medium, generally secretes a mixture of differently decorated Nod factors, with usually one major component. For example, the major Nod factor of *Sinorhizobium meliloti*, the rhizobial symbiont of *Medicago* plants, represents 60 to 70 % of the mixture. Termed NodSm-IV(Ac,S,C16:2Δ2,9), it consists of four sugar units, is O6-sulfated on the reducing residue and the terminal non-reducing glucosamine unit is *N*-acylated with a C16:2 fatty acid and partially O6-acetylated (Fig. 1). The other NodSm factors are non-acetylated, with five sugar units or *N*-acylated with a C16:0 or C16:1 fatty acid. For broad host range rhizobia, the mixture is more complex [9]. Thus, obtaining pure Nod factors from the supernatant of rhizobial cultures, by using extraction procedures followed by HPLC purification, has often been difficult and sometimes impossible, due to the close chemical structures of these amphiphilic compounds, and has limited the studies of structure-activity relationships of LCOs. Therefore, the chemical synthesis of Nod factors has given the opportunity to perform such studies with chemically pure and characterized compounds and also to obtain derivatives used to characterize or visualize perception events in plants.

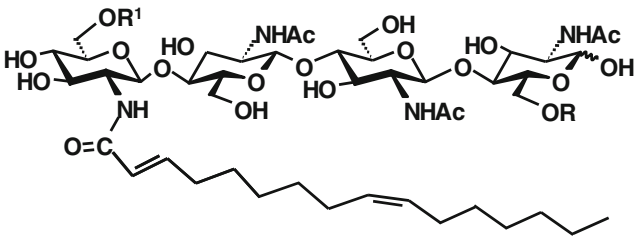

Fig. 1 Chemical structure of the major Nod factor produced by *Sinorhizobium meliloti* (NodSm-IV (Ac,S,C16:2)): R=SO_3^-, R1=Ac; lipid chain=C16:2Δ2E,9Z. Minor NodSm factors: R1=H; lipid chain= C16:0 or C16:1

The first total synthesis of the NodSm factor was achieved by Nicolaou and co-workers [10]. The synthetic route also enabled the incorporation of four tritium atoms in the C16:2 fatty acid chain, providing a ligand identical to the genuine NodSm factor, with a specific radioactivity high enough to perform radioligand binding experiments [11]. Other groups further proposed alternative strategies for the synthesis of the NodSm factor [12–14] or the Nod factors produced by *Bradyrhizobium japonicum,* the symbiont of soybean [15]. The procedure established by Tailler *et al.* [13] enabled the synthesis of a series of tetrameric sulfated LCOs differing for the structure of the fatty acid (length, degree and position of unsaturations) to perform structure-function studies [16]. Later on, large scale production of LCOs was achieved by chemo-enzymatic synthesis [17, 18]. This method used recombinant *Escherichia coli* strains harboring the rhizobial genes *nodC*, *nodB* and *nodH*, which encode a chitin synthase, a *N*-deacetylase, specific for the terminal non-reduing sugar, and a sulfotransferase, respectively, to produce large amounts of sulfated tetrameric and pentameric chitooligosaccharides [19, 20]. The chemical acylation of the free amino group at the nonreducing unit by different methods further led to the synthesis of Nod factors and Myc-LCOs [5, 17, 18]. More recently, a new access to synthetic LCOs was achieved by the controlled depolymerisation of chitin by acetolysis to produce peracetylated chitooligomers, which were then chemoselectively *N*-acylated [21]. However, Nod factors with complex structures, such as those produced by *Bradyrhizobium* sp. (*Lotus*) or *Mesorhizobium loti* which differ by the position of carbamoyl groups on the nonreducing end [22], will still be difficult to obtain by any method because of the need of a strict regioselectivity to incorporate this substitution.

Nod factor derivatives

The amphiphilic character of Nod factors raises questions about their physico-chemical behavior notably in the presence of membranes. Do they form micelles at physiological concentrations, do they insert spontaneously into membranes? To answer these questions, fluorescent derivatives of Nod factors consisting of a chitin backbone, acylated with BODIPY-tagged fatty acids of different lengths, were synthesized [23, 24]. Fluorescence spectroscopic and microscopic techniques

have thus shown that Nod factors are water soluble at physiological concentrations and that they insert into artificial membranes. Nod factors were also shown to be able to transfer rapidly from artificial vesicles to root hair cell walls suggesting that they can be easily released from rhizobia to reach plant root hairs where they act. However, no flip-flop between membrane leaflets was observed. These fluorescent derivatives, which were biologically active in the root hair deformation assay, were shown to accumulate and to be immobilized and concentrated in the cell wall, up to 50-fold with respect to the concentration which was initially applied [25]. This accumulation was identical for sulfated and nonsulfated Nod factors, either on host or on nonhost plants, suggesting a role in increasing the efficiency of Nod factor perception by plasma membrane-localized receptors [26].

Studies of Nod factor responses in *Medicago spp.* using *Sinorhizobium meliloti* mutants producing different Nod factor structures have highlighted the importance of certain chemical decorations at the reducing and the nonreducing end. While the presence of the sulfate group is essential for all biological responses, the structure of the fatty acid also plays an important role. By using synthetic LCOs differing by the length of the fatty acid (C8, C12, C16, C18) and the number or the position of the unsaturations, it was shown that the optimal chain length was C16 and LCO-IV(S,C16:2Δ2, 9) was more active than LCO-IV(S,C16:1Δ9) [16]. The role of the different substitutions on the oligochitin backbone or the structure of the lipid chain in Nod factor recognition was then examined by conformational studies of natural Nod factors and synthetic analogs, notably benzamide analogs, designed to mimic the geometry of the conjugated double bond in natural NodSm factors [27]. It was shown that the carbohydrate moiety displayed stable dynamic behavior indicating a rigid conformation, whereas the lipid moiety exhibited a high degree of freedom and sometimes a parallel orientation to the oligosaccharidic backbone. However, since the conformational changes depend on the solvent, LCOs may adopt specific shapes for recognition by their receptors in the context of the plant membrane.

The quest for Nod factor receptors

Candidate Nod factor receptors identified by genetic approaches

The development of genetic tools for two legume models, *M. truncatula* and *Lotus japonicus*, enabled the quest for the isolation of Nod factor receptors. At first, plant mutant collections were generated by chemical or fast neutron mutagenesis, and used in forward genetic screens. *M. truncatula* mutants called *Nod factor perception*, *nfp*, showed the most stringent phenotype among the isolated nodulation-defective mutants: *nfp* roots treated with Nod factors are completely deficient for any of the responses tested including the rapid calcium influx, which is one of the earliest Nod factor responses [28]. In *L. japonicus*, mutants in two independent loci, generated by T-DNA insertional mutagenesis, each showed a phenotype comparable to *nfp*, *i.e.,* lack of all Nod factor-dependent plant responses. Map-based cloning resulted in the isolation of the affected genes in *L. japonicus*, which were named *Nod factor receptor kinase 1* and *5* (*Nfr1*, *Nfr5*) [29, 30]. These genes encode transmembrane serine/threonine receptor-like kinases with extracellular domains composed of lysin motifs (LysM-RLKs) (Fig. 2). Functional complementation of the *nfp-1* mutant with a *M. truncatula* gene selected by virtue of its high similarity with SYM10 of pea and NFR5 of *L. japonicus* proved the educated guess that *NFP* encoded a LysM-RLK as well [31]. Since LysM domains were first described in bacterial peptidoglycan-binding proteins and mediate binding to GlcNAc-containing glycans [32], NFP and NFRs have been considered as Nod factor receptors. LysM-RLKs are only found in plants [33] and they are encoded by roughly 20

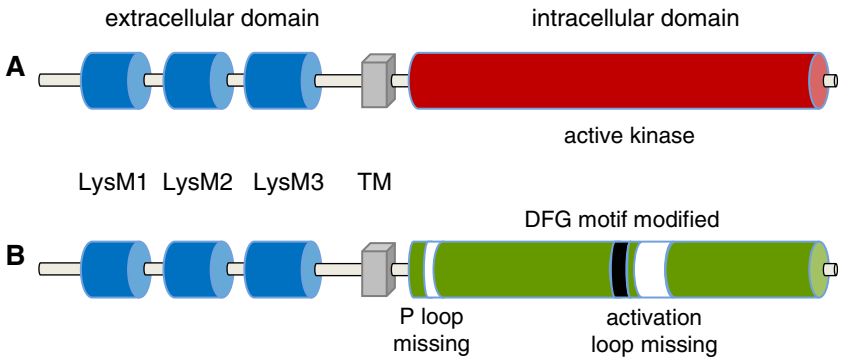

Fig. 2 Schematic representation of two types of LysM-RLKs showing the extracellular domain consisting of 3 lysin motifs (LysM) linked by a transmembrane (TM) domain to either an enzymatically active cytosolic serine/threonine kinase domain (**a**), as for example in LYK3, or to a dead kinase domain (**b**), as in NFP, which is lacking the P- and the activation loop, and carries a modification of an essential motif (NFG instead of DFG)

genes in legumes, in contrast to less than 10 in *Arabidopsis thaliana* and rice (*Oryza sativa*) [31, 34, 35] thus suggesting diverse functions.

A different class of mutants in *M. truncatula* is characterized by aberrant root hair curling and these mutants were therefore named *hcl* (hair curling). The *hcl* mutants are blocked in the formation of infection threads, the structures which allow rhizobia to reach the root cortex by traveling through root hairs [36]. Nod factor signaling leading to root hair deformation, epidermal marker gene activation, and cortical cell divisions (CCD) in *Medicago* spp. depends on the presence of a sulfate group at the reducing glucosamine residue on the NodSm factor, whereas additional modifications at the nonreducing end of the backbone (*O*-acetylation and the nature of the acyl chain) are essential only for the infection-related events, including formation of shepherd's crooks, infection threads and repolarization of the cytoskeleton of root hairs [37]. This strict dependency on Nod factor structure is also seen for the formation of infection threads and nodule development from cortical cell division foci in pea (*Pisum sativum*) [38]. It was therefore hypothesized that, in *Medicago spp*. and pea, two complementary perception systems operate, one triggering a signaling cascade which leads to the formation of CCDs ("signaling"), and the second controlling the intracellular accommodation of rhizobia ("entry") [37].

Due to the specific role of *HCL* in rhizobial infection and the Nod factor structure-dependent phenotype of one of the *hcl* mutants, the corresponding gene could encode an "entry receptor" while *NFP* could correspond to a less stringent "signaling receptor" [36, 37]. Synteny between the geneticall well characterized *SYM2* locus, which controls the formation of infection threads in pea, and chromosome 5 of *M. truncatula* led to the identification of an orthologous genomic region that contains, amongst others, 7 genes encoding LysM-RLKs, representing prime candidates for the entry receptor of *M. truncatula* [38, 39]. Reverse genetics identified two of these genes, *LYK3* and *LYK4*, as candidates to control infection thread formation [39]. Sequencing of the gene region revealed that the four different *hcl* alleles contained mutations in *LYK3*, which very likely interfere with the functionality of the predicted protein [40]. NFP and LYK3 belong to the two subtypes of LysM-RLKs, which differ by the predicted activity of the intracellular kinase domains (Fig. 2). NFP, as its ortholog NFR5, lacks important regions of the normally well conserved domain (Fig. 2b), and neither of these proteins are able to autophosphorylate [29, 31, 41]. In contrast, LYK3 and its ortholog NFR1 are active kinases [41–43].

The existence of two signaling pathways controlled by a signaling and an entry receptor exhibiting different stringencies towards the Nod factor structure was also suggested by the existence of two different Nod factor-induced calcium responses: a calcium influx at the tip of root hairs and nuclear calcium oscillations associated respectively with the infection process [44] and nodule organogenesis [45]. These calcium responses differ in terms of the required Nod factor concentrations and structures, in agreement with the signaling and entry receptor concept. However, both calcium responses depend on NFP [46], indicating that for the control of two biological issues (organogenesis and infection) NFP needs to form a receptor complex with different specificity and signaling properties upon ligand binding [46]. Since a *lyk3* mutant is not affected in the calcium influx, LYK3 is not the partner of NFP for this Nod factor response [46]. Therefore, the rapid calcium influx related to the infection process is not directly linked to LYK3 but to NFP acting with another component within a receptor complex.

Complementation studies of the *nfp* mutant, using a tissue-specific promoter construct that restricted the expression of *NFP* to the root epidermis, showed that epidermal expression of *NFP* suffices for cortical activation of cell divisions [47]. However, root hair infection thread formation was not observed, suggesting a role for NFP in the "entry" process as well [47]. Likewise, expression of a chimeric version of NFP in the *nfp* mutant, in which the ECD was exchanged with the ECD of SYM10 (the ortholog or NFP in pea) complemented signaling but not infection [48]. Since the rhizobial symbionts of pea belong to a different cross-inoculation group and produce nonsulfated Nod factors, the complementation of Nod factor signaling by the chimeric SYM10-NFP protein in *nfp* also implied the existence of an additional component, in *M. truncatula*, which would recognize the sulfate decoration on the NodSm factor [48], which is absolutely essential for nodule organogenesis and infection [1].

In *L. japonicus*, both Nod factor receptors are equally necessary for Nod factor signaling and infection [29, 30]. Interestingly, complementation of *nfr1* or *nfr5* with epidermis-specific constructs of *NFR1* or *NFR5*, respectively, revealed different capacities for the restoration of nodule development, indicating a specific role only of NFR1 (the LYK3 ortholog) in infection [49]. Hence, in conflict with the first hypothesis of division of labor by LYK3 and NFP in Nod factor perception by *M. truncatula* (infection *vs* nodule organogenesis), they now seem to have partially overlapping roles, whereas NFR1 and NFR5, which were first described to be equally involved in nodulation, now show nevertheless some distinctive roles in the control of rhizobial infection. Additional receptor components have been postulated for *L. japonicus* as well. In a study which made use of spontaneously nodulating *L. japonicus* mutants, the cortical activity of ancillary, less selective Nod factor receptors was suggested, since the generation of trans-cellular infection threads in infected nodules after root hair-independent entry of rhizobia depended on the presence of Nod factors in the absence of both NFR1 and NFR5 [50].

Direct binding of Nod factors to NFR1 and NFR5 has been reported, showing similar and high affinity binding in the

nanomolar range for each protein [51]. In *M. truncatula,* the early responses elicited by Nod factors depend mainly on NFP. However, after many attempts by various approaches, no high affinity binding to Nod factors has been found for NFP (J-J. Bono *et al.*, unpublished data). As hypothesized above, NFP could be associated to another partner in a signaling receptor complex independent of LYK3. This partner has not yet been identified and could be either one of the remaining 19 members of the LysM-RLK family of *M. truncatula*, or another protein with carbohydrate binding properties.

Nod factor-binding proteins identified by biochemical approaches

Using a biochemical approach, a lectin-nucleotide phosphohydrolase (LNP) was isolated from roots of the legume *Dolichos biflorus* and was found to bind Nod factors [52]. The biological role of LNP was then studied in the model legume *L. japonicus* by generating stable antisense transformants with reduced LNP levels [53]. Antisense inhibition of LNP blocked both nodulation and mycorrhization, suggesting a role of LNP in early stages of the establishment of these symbioses, either by presenting the LCOs (Nod factors and Myc-LCOs) to their respective receptors or by acting in signaling pathways that might be modulated by the changes in extracellular ATP/ADP-ratio as a result from the apyrase activity of LNP.

Binding experiments performed on plant extracts using radioactive NodSm factors labeled either with ^{3}H or with ^{35}S, led to the characterization of three Nod factor binding sites (termed NFBS) differing by their affinities [11, 17, 54]. The absence of discrimination of the sulfate group and the selective recognition of LCOs *vs* COs are common features of the three NFBSs. Interestingly, none of the sites correspond to NFP [54] or LYK3 (Bono *et al.*, unpublished) since they are still detected in mutants altered in the corresponding genes. NFBS1 and NFBS3 are associated to a high-density root fraction of *M. truncatula*, suggesting a cell wall localization, and bind the NodSm factor with an affinity (K_d) of 86 and 0.45 nM, respectively [11, 54]. NFBS1 is not restricted to legumes since a binding site with a similar affinity was detected in tomato roots [11]. Therefore, NFBS1 could be involved in Nod factor and Myc-LCO recognition in legumes and nonlegumes. The high affinity of NFBS3 suggests a role in Nod factor perception at low concentrations. However, NFBS3 does not discriminate the chemical substituents that are important for host specificity, suggesting that it could be involved in an initial non-stringent perception event of LCOs. NFBS2 was initially characterized in cell suspension cultures of *M. varia*, *Phaseolus vulgaris,* and *M. truncatula* [17, 54, 55] and differs from NFBS1 and NFBS3 by its ability to discriminate (i) the degree of polymerization of the oligochitin backbone of LCOs and (ii) the length of the fatty acid chain, which both play a role in the biological activity of Nod factors [16]. It is noteworthy that NFBS2 was more abundant in a cell culture line generated from a mutant affected in Nod factor responses compared to the *M. truncatula* wild-type line [54]. By combining photoaffinity labeling experiments using a photoactivatable and radioactive LCO with quantitative proteomic and transcriptomic analyses of these two cell lines, the LysM-RLK LYR3 was identified as the high affinity LCO-binding protein in NFBS2 [56]. LYR3 discriminates LCOs (Nod factors and Myc-LCOs) from COs but not the presence of the sulfate group. The biological context in which LYR3 intervenes is as yet unknown since no clear phenotypes have been observed so far for *lyr3* mutants (Fliegmann *et al.* unpublished data). Since the kinase domain of LYR3 is predicted to be inactive, it presumably needs a partner within a receptor complex for LCO signal transduction.

LCO and CO perception by plants: differences and similarities

LCOs (Nod factors and Myc-LCOs) and COs are structurally-related signals involved in symbiotic and pathogenic interactions. COs with a degree of polymerization higher than six are potent elicitors of plant defense whereas shorter COs, produced in addition to Myc-LCOs by endomycorrhizal fungi, are additional components of symbiotic signaling mechanisms in legumes and non-legumes [8, 57]. Since LCOs and COs are perceived by structurally-related LysM receptors, one of the main challenges for plant biologists is to identify the molecular mechanisms underlying ligand recognition and receptor activation for these signals.

The molecular mechanisms of recognition are better documented for COs than LCOs. Indeed, the first crystal structure for a LysM domain of eukaryotic origin was solved for AtCERK1 (*A. thaliana* Chitin Elicitor Receptor Kinase 1) in complex with a chitin pentamer [58]. The ligand-binding site was located in the central LysM (LysM2) and the model proposes that a chitin octamer, which is a symmetric ligand, accommodates this binding site by inducing homodimerization of AtCERK1 that would lead to receptor activation. An alternative mode of chitin binding involving an intermolecular mechanism with multiple LysM domains has also been reported from structural studies on the bacterial *Thermus thermophilus* NlpC/P60 endopeptidase [59]. Other LysM proteins in *A. thaliana,* AtLYK4 and AtLYK5 acting in complex with AtCERK1, have been reported to bind chitin and are important for innate immunity [60, 61]. For AtLYK5 a binding site located between LysM1 and LysM3, similar to that identified for the Extracellular protein 6 (Ecp6) of the fungus *Cladosporium fulvum* [62], has been proposed. AtLYK5 exhibits a 200-fold higher affinity for COs than AtCERK1 and is suggested to be the primary CO receptor [61]. After binding to chitin, AtLYK5 could associate with AtCERK1 resulting in

dimerization, kinase activation and downstream signaling. In rice, where the Chitin Elicitor Binding Protein (OsCEBiP) was identified [63], a sandwich type mechanism seems to occur between two CEBiP monomers and long-chain COs [64]. Since OsCEBiP is a GPI-anchored protein without a kinase domain, the working model suggests a further interaction with OsCERK1 for signal transduction [65]. Interestingly, AtCERK1 also intervenes in a receptor complex with AtLYM1 and AtLYM3, two GPI-anchored LysM proteins, for peptidoglycan perception [66], and more recently OsCERK1 has been shown to play a role in arbuscular mycorrhizal symbiosis in rice [67]. Therefore the same protein can be part of different receptor complexes with different biological outputs.

In terms of LCO perception, no structural data exists for any of the identified LysM-RLKs and the ability of NFP or LYK3 to physically interact with Nod factors has not been demonstrated. Interestingly, besides its crucial role in all Nod factor responses, NFP also controls lateral root formation in response to Myc-LCOs but is dispensable for mycorrhization [5] and plays a role in the susceptibility of *M. truncatula* to pathogens [68]. To explain these multiple roles, NFP, like CERK1, could function within different receptor complexes, and conceivably within LysM receptor complexes, which might be formed *in situ* depending on the cell or tissue type, the developmental stage, or external stimuli. In contrast to COs, LCOs are not symmetric. Therefore the contribution of the binding partners to accommodate the chitin backbone and the lipid moiety within a receptor complex could be different. Considering this hypothesis and the fact that NFP could form multiple receptor complexes in symbiotic or pathogenic contexts, the specificity for the recognition of the decorations of LCOs (Nod factors and Myc-LCOs) might then depend on its partner within a complex. The mechanism of ligand binding for LYR3, which selectively binds LCOs, could be different. However LYR3, as NFP, is a dead kinase, and therefore probably requires a partner to transduce the signal, which could also provide some specificity in ligand recognition in a biological context that still needs to be identified.

In *L. japonicus*, NFR5 and NFR1 exhibit a similar high affinity to Nod factors [51]. Domain swaps between *Lotus spp*. NFRs, derived from plant species displaying differential host specificities [69], showed the importance of the ECDs for Nod factor recognition. Fine-tuning of these experiments led to the identification of a single amino acid position in the second LysM domain of NFR5 from *L. japonicus* and *L. filicaulis* respectively, which was responsible for the discrimination in complemented roots of the *L. japonicus nfr5* mutant of a bacterial strain producing Nod factors with modified decorations [69].

Comparing *L. japonicus* and *L. pedunculatus*, which are nodulated by *M. loti* and *Bradyrhizobium sp. (lotus),* producing Nod factors differing in the position of one carbamoyl residue at the nonreducing end of the Nod factor, respectively, showed that the precise structures of the Nod factors are not equally important at all steps of the symbiotic interaction [22]. No discrimination was observed in the initial epidermal perception events, whereas invasion of the cortex and nodule persistence depended on the respective position of the carbamoyl decoration [22]. Cross-complementation assays showed furthermore that NFR1 and NFR5 are not sufficient to restore cortical infection, suggesting the involvement of additional, most likely LysM-RLK-dependent recognition mechanisms [22], a scenario resembling the one described above for the discrimination of the sulfate group in *M. truncatula* [48].

Conclusions and perspectives

Phylogenetic analyses indicate that whole genome duplications and local genomic rearrangements leading to the amplification of genes encoding LysM-RLKs might have been key events in the evolution of legumes to develop the rhizobial nitrogen-fixing symbiosis from the more ancient endomycorrhizal symbiosis [33–35, 70]. Studies in *Parasponia*, the only genus outside the legume family that associates with both rhizobia and AM fungi, argue in favor of a primordial function of LysM-RLKs in endomycorrhization [71]. In *Parasponia*, the knock-down of *NFP* impaired both symbiotic interactions, indicating an ancient origin of the gene, which must have participated in the establishment of endomycorrhization in the last common ancestor of *Parasponia* and legumes, which predates the evolution of the rhizobial symbiosis [71]. Gene duplication in legumes led to the liberation of one copy (*NFP*), which was recruited for the signaling of Nod factors. In *Medicago*, the paralogous gene, encoding LYR1, might be involved in Myc-LCO perception since it is induced during mycorrhization [72]. However, the Nod factor receptor NFP itself shows traces of this evolutionary history since it is still involved in Myc-LCO signaling [5, 6]. A comparable origin was recently retraced for LYK3 and NFR1, involving two rounds of gene duplications in legumes prior to the evolution of the rhizobial symbiosis leading to three paralogous gene copies, from which one copy evolved to encode host-specific Nod factor receptors, which, again, still possess remnants of mycorrhizal functions [73, 74]. Apparently, AtCERK1, the nonlegume LYK3/NFR1 homolog, shares the same symbiotic origin, since it clusters within a clade containing the other LysM-RLKs, except for *A. thaliana*, and has retained two motifs in the kinase domain that were shown to be required for symbiotic signaling [73, 75]. These data led to the hypothesis that AtCERK1 evolved into a major player in innate immunity after losing its symbiotic function in *A. thaliana* [73], unlike

OsCERK1, for which a bifunctionality in symbiosis and in chitin-triggered defense was recently observed [67].

Short-chain COs, derived from germinating fungal spores, were only recently proposed to represent signals in the communication between AM fungi and *M. truncatula* [57]. It is now tempting to speculate that these simple, nondecorated COs, might constitute the primordial symbiotic signals which were already perceived by the ancestor of LysM-RLKs in primitive host plants of AM fungi. Recently, components of the so-called "symbiotic toolkit", which are molecules which are required for the establishment of AM associations, have been identified in green algae [76], suggesting an even earlier emergence of endosymbiotic interactions in the predecessors of land plants. Alternatively, symbiotic LCO recognition could have evolved from LysM domains perceiving pathogen-derived COs in order to suppress defense activation [77].

Nowadays, plants are confronted to a great variety of Nod factor structures. This variety is not only due to their origin from different rhizobial species, and even strains, but also, as explained above, because rhizobia produce mixtures of Nod factors. Therefore, plants need to react appropriately to Nod factors from different rhizobia, and maybe even to the different structures produced by one rhizobial strain depending on the stage of the symbiotic process. Moreover, legume plants need to discriminate Nod factors from Myc-LCOs and also from COs. Hence, we need to better analyze the interplay of putative receptors, which might engage in homo- or heterodimers, with LCOs and COs. High-throughput screening of physical interactions, for example using a glycan microarray [78] could be a starting point to achieve a fine-tuned analysis of specific ligand-receptor relationships *in vitro* and *in vivo*. For this, tools will have to be developed to reliably produce receptor proteins or binding domains with high yield and purity for physical interaction and structural studies at the atomic level and a greater variety of structures of LCOs will also be needed.

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Conflict of interest The authors declare that they have no conflict of interest.

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