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REVIEW

Nod factor perception: an integrative view of molecular communication during legume symbiosis

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Abstract

Key message **Compatible interaction between rhizobial Nod factors and host receptors enables initial recognition and signaling events during legume-rhizobia symbiosis.**

Abstract Molecular communication is a new paradigm of information relay, which uses chemical signals or molecules as dialogues for communication and has been witnessed in prokaryotes, plants as well as in animal kingdom. Understanding this fascinating relay of signals between plants and rhizobia during the establishment of a synergistic relationship for biological nitrogen fixation represents one of the hotspots in plant biology research. Predominantly, their interaction is initiated by flavonoids exuding from plant roots, which provokes changes in the expression profile of rhizobial genes. Compatible interactions promote the secretion of Nod factors (NFs) from rhizobia, which are recognised by cognate host receptors. Perception of NFs by host receptors initiates the symbiosis and ultimately leads to the accommodation of rhizobia within root nodules via a series of mutual exchange of signals. This review elucidates the bacterial and plant perspectives during the early stages of symbiosis, explicitly emphasizing the significance of NFs and their cognate NF receptors.

Keywords Defence · Legumes · Nod factors · Nodulation · Receptors · Rhizobia · Symbiosis

Introduction

Nitrogen, being one of the essential elements of life, exists abundantly in an unavailable form in the atmosphere. Chemical as well as biological processes overcome the energy barrier required to reduce nitrogen and facilitate the formation of assimilable forms, such as nitrates and nitrites. Predominantly, leguminous plants have developed a mechanism to attract and accommodate a group of gram-negative bacteria called rhizobia within their roots to form a mutually beneficial relationship for biological nitrogen fixation. The flavonoids exuded by the roots of legumes are sensed by the cognate rhizobia in the rhizosphere. Flavonoid perception initiates host-specific chemotaxis and induces the expression of rhizobial *nod* genes essential for establishing a symbiotic relationship with its host plant (Cooper 2007). Upregulation of a repertoire of *nod* genes leads to the expression of enzymes that catalyse the synthesis of lipo-chito-oligosaccharides (LCOs), called nodulation factors or Nod factors (NFs). These are low molecular weight, diffusible compounds, semi-hydrophobic, thermostable and found to be protease-resistant (Tikhonovich and Provorov 2007; Oldroyd 2013). Once secreted, NFs are recognised by cognate host receptors like Nod Factor Receptor (NFRs) or lysin motif receptor-like kinases (LYKs/LYRs) (Broghammer et al. 2012). Recognition of a compatible interaction permits the commencement of infection.

Rhizobia may ingress the host intracellularly or intercellularly. During intracellular invasion, rhizobia usually attach to the root hairs and colonize the plant via infection thread (IT) formation. The root hairs curl to trap the bacteria and enable bacterial entry via plasma membrane invaginations, known as infection threads (Oldroyd 2013). In case of intercellular invasion, initiation of infection may occur between two intact epidermal cells, at the base of the root hair, wounds or fissures at the point where lateral roots emerge (crack entry) or the middle lamella between two root hair cells (Ibáñez et al. 2017). Apart from crack entry, other

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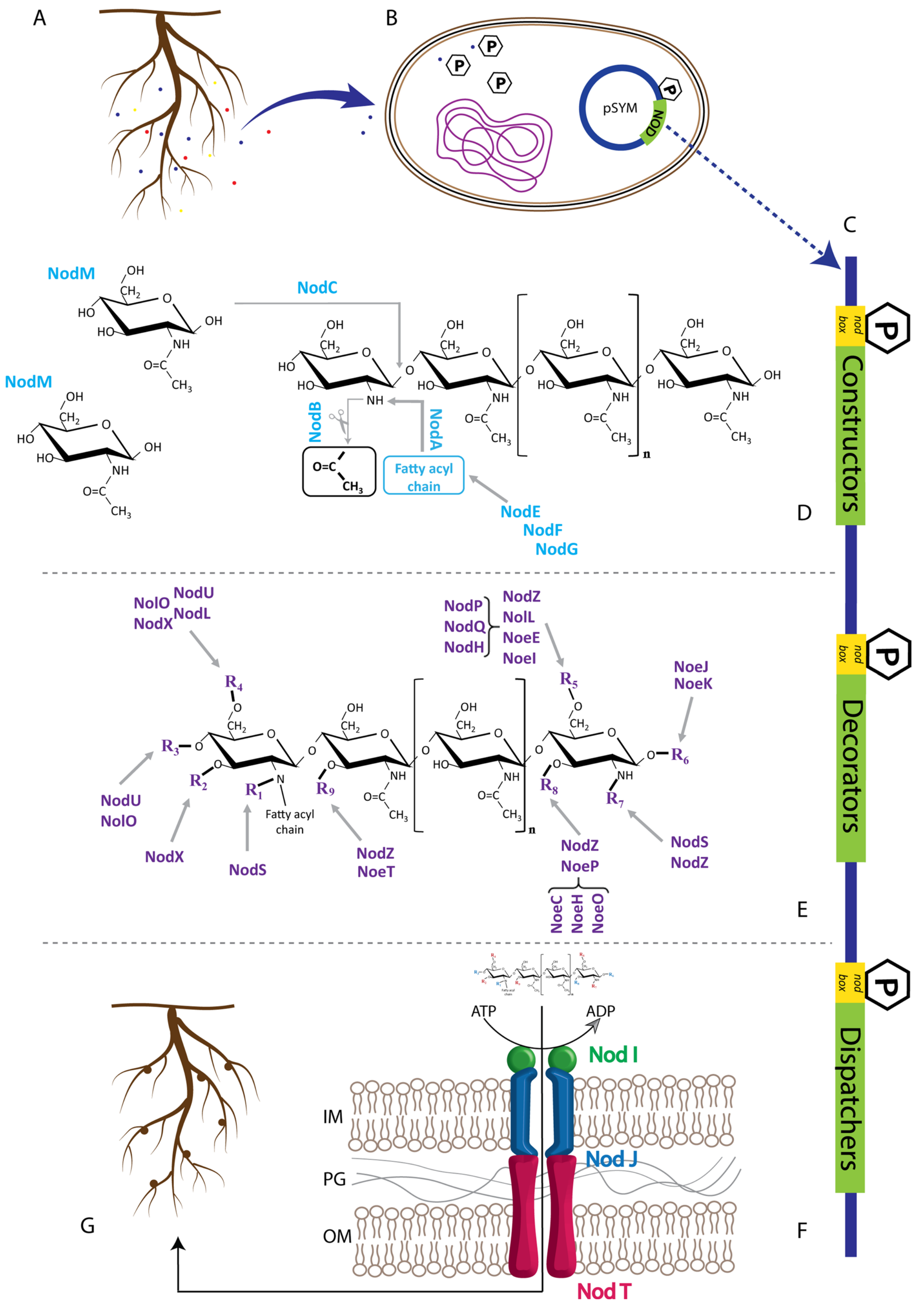
A
B
P
pSYM
NOD
C
nod box
P
Constructors
NodM
NodM
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NodB
NodA
Fatty acyl chain
NodE
NodF
NodG
n
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Decorators
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nod box
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Dispatchers
ATP
ADP
Nod I
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◂**Fig. 1** The Bacterial Perspective: General outline of nod factor induction, biosynthesis, and transport during legume-rhizobia symbiosis. (A) Plant roots exude flavonoids in nitrogen-deficient soils, (B) Appropriate flavonoids are perceived by patrollers [P] in the rhizobial cytoplasm, followed by binding to the *nod box* in their symbiotic plasmid, (C) Activation of the expression of constructors, decorators and dispatchers, (D) Construction of the NF backbone, (E) Ornamentation of the core structure with appropriate substituent groups, (F) Ferry of the assembled NF across the bacterial cell wall, (G) Downstream signaling events leading to the formation of nodules in the roots of the host plant. *P* patroller, *pSYM* symbiotic plasmid, *NOD* nod operon, *IM* inner membrane, *PG* peptidoglycan, *OM* outer membrane

modes of invasion into host require solubilisation of cell wall material, which is achieved by the secretion of cellulolytic enzymes like cellulase, polygalacturonase and pectinase by rhizobia (Mateos et al. 1992; Menéndez et al. 2019). In addition, it is also observed that the model plants *Lotus japonicus* and *Medicago truncatula* encode pectate lyase upon the perception of NFs, which is essential for host cell-wall degradation and infection thread initiation for bacterial entry (Xie et al. 2012; Liu et al. 2019a). Thus, it is likely that involvement of cell-wall degrading enzymes from both the symbionts is crucial for bacterial invasion.

Upon entry into the host, several host and rhizobial genes are induced, which eventually lead to the development of nodules to confine the bacteria from spreading further and provide a conducive environment for nitrogen fixation (Jin et al. 2016; Wong et al. 2019). Rhizobial transcriptomic changes occur in genes essential for nitrogen fixation, respiration and endoreduplication (Kondorosi et al. 2013; Jiménez-Guerrero et al. 2017), while host activates the expression of phytohormone biosynthesis genes for nodule organogenesis (Ding et al. 2008; Jin et al. 2016; Reid et al. 2018; Buhian and Bensmihen 2018; Dolgikh et al. 2020; Jarzyniak et al. 2021) as well as bacterial release from infection threads (Tsyganova et al. 2021), nodule-cysteine rich peptides (in IRLC and Dalbergioid clade of legumes) for imposing a typical terminal differentiation of bacteroids (Czernic et al. 2015; Wang et al. 2017, 2018b; Lamouche et al. 2019; Nicoud et al. 2021).

In indeterminate nodule forming temperate legumes (*Medicago truncatula, Pisum sativum*), inner cortical cells form the primordium and the infection thread penetrates at the base of the primordium, while the apex retains its meristematic activity for an indefinite time. In the case of determinate nodule forming legumes (*Arachis hypogaea, Glycine max*), the outer cortical cells form the primordia, and their meristematic activity is lost within 10 days post-infection (Heidstra and Bisseling 1996; Kohlen et al. 2018). Within the nodule, rhizobia transform into non-mobile, non-dividing, nitrogen-fixing bacteroids, which reduce atmospheric dinitrogen to ammonia and exchange it with the host plant for dicarboxylic acids (Watson et al. 1988; Mueller and González 2011). The balance between nitrogen influx and carbon efflux is maintained by the autoregulation of nodulation (AON) system in legumes, which involves a long-distance communication between the roots and the shoot (Okamoto et al. 2009; Soyano et al. 2014; Tsikou et al. 2018b; Suzaki and Nishida 2019). This review provides insights into the bacterial and plant perspectives during the early events of rhizobium-legume symbiosis, highlighting the significance of NF mediated signaling.

Early events in the rhizosphere

During nitrogen limiting conditions, host roots produce (iso) flavonoids in nanomolar range and secrete them into the rhizosphere, which are perceived by compatible rhizobia (Lea et al. 2007; Liu and Murray 2016) (Fig. 1A-B). In addition to activating the biosynthesis of NFs, flavonoids also induce nitrogen fixation genes, synthesizers and modifiers of exopolysaccharides, and transcriptional regulators involved in Type III secretion system (T3SS) (Kobayashi et al. 2004; Jiménez-Guerrero et al. 2017). Plants secrete a mixture of (iso) flavonoids and only a few of them (like genistein, naringenin, hesperitin, eriodictyol, luteolin, and daidzein) are essential for rhizobial recognition, while the others (like acetovanillin, acetosyringone, sinapic acid) act as phytoalexins and inhibit non-compatible bacteria. Interestingly, flavonoids that induce *nod* gene expression in *Bradyrhizobium sp.* (like genistein and daidzein) act as antagonists against *Rhizobium leguminosarum* (Firmin et al. 1986), thus, reinforcing stringency in specificity.

Then, how do compatible rhizobia endure the toxicity of its host's flavonoids? Rhizobia prevent intracellular accumulation and subsequent toxicity of flavonoids through the activation of efflux pumps. In the absence of flavonoid inducers, TetR type of regulators, like EmrR, FrrA and BdtR in *Sinorhizobium meliloti* and *Bradyrhizobium diazoefficiens,* repress an adjacent multidrug efflux pump essential for conferring resistance to rhizobia against various toxins (Wenzel et al. 2012; Han et al. 2020). Compatible flavonoids derepress the efflux system by interfering with BdtR-DNA (genistein), FrrA-DNA (genistein and daidzein) and EmrR-DNA (luteolin) interactions (Wenzel et al. 2012; Rossbach et al. 2014; Han et al. 2020). Derepression of the multidrug efflux pump alleviates the toxicity of flavonoids and prevents its accumulation in rhizobia (Wenzel et al. 2012; Rossbach et al. 2014; Han et al. 2020). In addition to luteolin, related flavonoids like apigenin, naringenin, galangenin and quercetin (a non-inducer of *S. meliloti*) can also interrupt EmrR-DNA interactions (Rossbach et al. 2014). This raises the question whether multi-host colonizing rhizobia possess multiple repressors for the efflux pumps or a single repressor that can be targeted by multiple flavonoids.

Why focus on NFs?

NFs are the prime signaling molecules secreted by rhizobia in response to the host's substandard nutrient availability. They are the key factors that intimate the presence of compatible microsymbionts in the vicinity of the beneficiary. NFs are continuously perceived by their cognate host receptors that modulate appropriate signaling circuits. For example, epidermal perception of NFs is essential for the entry of rhizobia into plant cell (Smit et al. 2007), while nodular perception facilitates the release of rhizobia from infection thread into the host cytoplasm (Moling et al. 2014). Perception of NF in the infection thread is required for the synchronization of bacterial invasion with nodule organogenesis and also for the expression of proteins required for elongation and progression of the infection thread, like flotillins and rhizobium directed polar growth (RPG) (Den Herder et al. 2007; Haney et al. 2011). In addition, nod factors also provoke early responses of symbiosis, like calcium spiking and cortical cell activation (Wais et al. 2002; Ibáñez and Fabra 2011). As the plant genetic reprogramming commences after NF perception, they can be considered as the crucial pioneers to establish symbiosis in most legumes.

Nonetheless, it is also important to acknowledge the role of other polymeric substances produced by the rhizobia, which also assist in the establishment of symbiosis. For instance, surface polysaccharides like lipopolysaccharides (LPS), capsular polysaccharides (CPS), exopolysaccharides (EPS), neutral polysaccharides (NPS), gel-forming polysaccharides (GPS), cyclic β-glucans and cellulose fibrils aid in symbiotic efficiency by contributing at different stages of interaction like attachment to host roots, biofilm formation, suppression of defence response and during infection thread initiation (Fraysse et al. 2003; Marczak et al. 2017; Castellani et al. 2021). However, they are accessory molecules that improve the efficiency of symbiosis in most of the rhizobia investigated till date and are absolutely crucial only for those rhizobia that exhibit narrow host range, like *S. meliloti, M. loti, R. leguminosarum* (Kelly et al. 2013; Rodríguez-Navarro et al. 2014; Wang et al. 2018a; Maillet et al. 2020; Acosta-Jurado et al. 2021), emphasizing that NFs are the major impetus behind the success of root nodule symbiosis. Thus, our review focusses on NFs for their pivotal role in mediating the inter-kingdom communication.

Structure of Nod factors

The backbone of NF constitutes an oligomer of N-acetyl D-glucosamine units (Fig. 1D). Since their monomer units resemble that of chitin monomers, these compounds are also commonly called lipo-chitooligosaccharides (LCOs) (Mergaert et al. 1997; Liang et al. 2014). *Mesorhizobium loti* NZP2213 and *Rhizobium* sp. GRH2 are the only strains reported to produce dimeric and hexameric NFs, respectively, while all other rhizobia predominantly produce tetramers or pentamers (Spaink 2000; D'Haeze and Holsters 2002). The N-acetyl group in the non-reducing end of the oligomer is replaced by an acyl chain of length ranging from C15-C22 and variable number of double bonds, wherein the most widely documented are vaccenic acid and stearic acid (Spaink 2000; D'Haeze and Holsters 2002).

Apart from these differences, there are nine crucial regions in the oligomer (R1–R9), which are substituted by variable groups (Fig. 1E). Predominantly, the R groups in the non-reducing end (R1–R4) are hydrogen, N-methyl, O-carbamoyl or O-acetyl, while such a generalization cannot be traced in the reducing end (R5-R7) (Spaink 2000; D'Haeze and Holsters 2002). The R9 region, which is proximal to the non-reducing end, also has similar groups as that of R1–R4. Sugars such as mannose and the triglyceride glycerol have been detected in the R6 position of *R. tropici* and *B. elkanii*, respectively. These groups are unique to the position as well as the organism. Similarly, the hydroxymethyl (CH_2OH) group is present only at the R7 position of *M. huakuii* (Spaink, 2000; D'Haeze and Holsters, 2002). Perhaps, the structural differences among LCOs are the major determinants of host specificity. It is also well known that the NFs produced by the same strain of bacteria may contain a mixture of NFs that vary in the number of monomers, acyl chain length, double-bonds and unique R groups (Wang et al. 2018a). Thus, secretion of a mixture of diverse LCOs can enable rhizobia to be recognised by more than one host.

The nod operon

In general, the rhizobial genome consists of one or two chromosomes and multiple plasmids. The genes essential for nodulation (*nod*, *nol*, *noe*) and nitrogen fixation (*nif* and *fix*) are organised into symbiotic genome compartments (SGCs), which includes episomes like symbiotic plasmids (pSym) and symbiotic islands on the chromosome (González et al. 2003). All the nodulation genes and most of the nitrogen fixation genes are localised on pSym and often flanked by transposases or insertion elements, and can be considered as candidates amenable for horizontal transfer. These genetic drive aid rhizobia to establish symbiosis with legumes that were non-hosts previously (Mergaert et al. 1997).

Most of the *nod* genes exist as operons, but apparently, there is a lack of synteny even among closely related species of rhizobia, suggesting independent genetic rearrangements and transposition (González et al. 2003). Upstream of each of these operons is a 47-bp consensus motif, termed as *nod*-box, which is the binding site for regulatory proteins that modulate the expression of the corresponding genes in the operon (Rostas et al. 1986; Kobayashi et al. 2004). In addition, a putative *nod*-box has been reported upstream of *nifA* gene, which encodes the major regulator of *nif-fix* operons (González et al. 2003). Here, the ensemble of nodulation genes has been classified into the patrollers, constructors, decorators and dispatchers (Fig. 1B-F).

Patrollers

Patrollers are the transcriptional regulators that respond to plant inducers and modulate the nodulation genes, both positively as well as negatively, by binding to *nod* box (Fisher and Long 1993; Loh and Stacey 2003; Peck et al. 2006; del Cerro et al. 2015, 2017). NodD is the central regulator, belonging to the LysR-type family of transcription factors and is auto-regulated as well as constitutively expressed, except in *B. diazoefficiens* (Zsófia et al. 1988; Nouwen et al. 2016). The GroESL chaperonin system is crucial for the accurate folding of NodD, essential for binding and bending DNA at the *nod* box (Yeh et al. 2002). Complementation of *S. meliloti nodD* mutants with functional NodD from diverse rhizobia upregulated the expression of nodulation genes in *S. meliloti* only in the presence of the flavonoids recognized by the recombinant NodD (Peck et al. 2006), suggesting that NodD plays a crucial role in conferring host specificity to rhizobia. Rhizobia possess one to five allelic variants of the regulatory protein; single copy in *R. leguminosarum,* two in *R. galegae,* three in *S. meliloti*, four in *Rhizobium* sp. strain BR816 and five copies in *R. tropici* and these isoforms show less conservedness in the putative flavonoid binding cleft and thus, interact with distinct plant inducers (Honma et al. 1990; van Rhijn et al. 1993, 1994; Lindstrom et al. 1995; del Cerro et al. 2015; Kelly et al. 2018). For example, NodD1 of *S. meliloti* perceives luteolin and methoxychalcone, while NodD2 responds to betaines (Hartwig et al. 1990). Recent research in *M. loti* has revealed the spatio-temporal regulation of nodulation genes by NodD, in which NodD1 is crucial for induction of *nod* genes in the infection threads and NodD2 in the rhizosphere and nodules (Kelly et al. 2018), thus, emphasizing that isoforms of NodD are not mere redundant copies, but might be essential for enhancing compatibility between symbionts by responding to distinct flavonoids at different stages of infection.

Intriguingly, *nodD*$_1$ mutants of *B. japonicum* were found to be still capable of nodulating their host, suggesting the existence of other regulatory proteins. NodVW two-component regulator perform the alternate pathway for *nod* gene transcription (Göttfert et al. 1990). NodV is a sensor kinase that undergoes autophosphorylation upon perceiving appropriate flavonoids and phosphorylates its response regulator NodW, which in turn, induces the expression of *nod* genes (Loh and Stacey 2003). There are yet other regulators like nolA (MerR type), which activates NodD2 and inhibits subsequent *nod* gene expression in *B. japonicum* (Garcia et al. 1996), and SyrM (LysR type) which promotes the expression of NodD$_3$ as well as SyrA in *S. meliloti* to modulate the *nod* regulon (Barnett and Long 2015). NolR is a transcriptional repressor documented in *R. tropici, S. meliloti* and *S. fredii,* which interferes with the binding of NodD to the *nod*-box. Their expression is high when the rhizobia are in the free-living or bacteroid stage but is reduced in the presence of flavonoid inducers (Chen et al. 2005; López-Baena et al. 2008; Ormeño-Orrillo et al. 2012; Lee et al. 2014). Another regulator NrcR (ArsR type), with homologous DNA-binding domain of NolR was identified in *Rhizobium tropici*. Mutation of *nrcR* led to the reduction in number and decoration of NFs, enhanced motility, delayed nodulation, reduced nodule number and had reduced capacity to induce *nodC*, in comparison to the wild type rhizobia (Del Cerro et al. 2016). Therefore, these results reinforce that there is stringency and alternative routes in patrolling the expression of nodulation genes.

Apart from flavonoid inducers, changes in pH, salt and non-ionic osmolyte concentrations have also been reported to initiate NF biosynthesis and nitrogen fixation genes in *R. tropici* CIAT899, in a flavonoid-independent but NodD2 dependent manner (Morón et al. 2005; Estévez et al. 2009; Guasch-Vidal et al. 2013; del Cerro et al. 2017, 2019). In addition to the regulation of nodulation genes, NodD2 also controls the expression of genes involved in protein folding and secretion, chemotaxis, synthesis of polysaccharides and nitrogen fixation, under salt and osmolyte stress (Pérez-Montaño et al. 2016; Del Cerro et al. 2019) probably to improve the efficiency of symbiosis during abiotic stress.

Constructors

Constructors are the core enzymes involved in the construction of the general backbone of NFs (Fig. 1D). These genes are present in all genera of rhizobia, except the photosynthetic *Bradyrhizobia* of the *Aeschynomene* plant (Gully et al. 2018). *nodC* codes for N-acetyl-glucosaminyl (NAG) transferase that catenates the monomeric NAG units synthesised by glucosamine synthase, the product of *nodM.* The NAG transferase catalyses the β-1→4 linkage between the monomers and decides the degree of polymerisation of the chitin oligosaccharide (CO) (Ormeño-Orrillo et al. 2012). The *nodB* gene is a deacetylase, which removes the acetyl moiety from the nitrogen attached to the monomer at the non-reducing end and the framework of the fatty acyl chain to be added in this region is designed by *nodF* (acyl carrier protein) and *nodE* (ketoacetyl synthase) in most rhizobia (Peters 1997; Gomes et al. 2019). Isoforms of these proteins

Table 1 Role of decorators in rhizobia

Gene	Protein encoded	Function(s)	Rhizobia	References
nodS	Methyltransferase	Addition of methyl group	*A. caulinodans* *R. tropici* *R. etli*	Geelen et al. (1993), Waelkens et al. (1995), and González et al. (2003)
noeI	Methyltransferase	Fucose methylation	*Rhizobium* sp., NGR234 *Sinorhizobium fredii*	Jabbouri et al. (1998) and Vinardell et al. (2015)
nodL,	Acetyltransferase	Acetylation of C6 in non-reducing end	*R. leguminosarum*	López-Lara et al. (2001)
nolL	Acetyltransferase	Fucose acetylation	*Rhizobium* sp., NGR234	Berck et al. (1999)
nodX	Acetyltransferase	Double acetylation (at R2, R4)	*R. leguminosarum*	Tikhonovich and Provorov (2007)
noeT	Acetyltransferase	Unusual acetylation at R9	*R. galegae*	Österman et al. (2014)
noeL,	GDP-mannose dehydratase	Synthesis of GDP-L-fucose	*S. fredii*	Lamrabet et al. (1999) and Vinardell et al. (2015)
nodU, nolO,	Carbamoyl transferase	Carbamoylation	*R. tropici*	Jabbouri et al. (1998) and D'Haeze et al. (1999)
nodP, nodQ	Subunits of ATP sulfurylase and kinase	Produces PAPS (3'-phospho-adenosine-5'-phosphosulphate), an activated sulphate compound	*R. tropici*	Folch-Mallol et al. (1996)
nodH	Sulphotransferase	Transfers PAPS to reducing end of NF	*R. tropici*	Folch-Mallol et al. (1996)
noeE	Sulphotransferase	Sulphation of methylfucose	*Sinorhizobium* NGR234	Hanin et al. (1997)
nolK	Sugar epimerase	Synthesis of GDP-L-fucose	*S. fredii*	Lamrabet et al. (1999) and Vinardell et al. (2015)
nodZ	Fucosyltransferase	Fucose transfer	*A. caulinodans* *S. fredii*	Mergaert et al. (1996) and Vinardell et al. (2015)
noeJ	Mannose-6-phosphate isomerase	Synthesis of GDP-D-mannose	*S. fredii*	Wang et al. (2018a)
noeK	Phosphomannomutase	Synthesis of GDP-D-mannose	*S. fredii*	Wang et al. (2018a)
noeC,, noeH, noeO	NoeC, NoeH, NoeO	Synthesis of arabinosyl group	*A. caulinodans*	Poinsot et al. (2016)
noeP	Arabinosyl transferase	Arabinosylation of R8	*A. caulinodans*	Poinsot et al. (2016)

make acyl chain with variable length, number and position of unsaturation(s), which is coupled to the NF by an acyl transferase, encoded by *nodA* (Yang et al. 1999; Igolkina et al. 2019). In some rhizobia (*R. leguminosarum, S. meliloti, Mesorhizobium* sp.), *nodG*, which encodes for 3-oxoacyl-acyl carrier protein reductase is required for elongation of the fatty acyl chain (López-Lara and Geiger 2001; Mao et al. 2016). As discussed in the previous section, the patrollers confer the first line of specificity to the rhizobia, which is followed by the backbone constructors. For instance, there are two additional *nodA* genes in *R. tropici* CIAT899, apart from the one adjacent to *nodBC.* These are not redundant copies of the first, but instead add an alternative acyl chain and have been speculated to expand the host range of the rhizobium (Ormeño-Orrillo et al. 2012). In addition, when comparing the nucleotide sequence of constructors amongst different *Sinorhizobium* and *Bradyrhizobium* strains that nodulate soybean, *nodA* from all the soybean nodulating strains of the two genera were more closely related, while the phylogeny of *nodB* and *nodC* was specific to the genus of rhizobia (Wang et al. 2018a), emphasizing that *nodA* might be responsible for conferring host specificity towards soybean.

Decorators

Decorators are species-specific genes that ornament the backbone of NF and assign additional host-specificity. The diversity in substituent groups at R1 to R9 regions of NF owes to these modifiers (Fig. 1E). Among them, few genes are required to synthesise the side group, while the others are necessary to incorporate it into the core structure of NF (Table 1). Thus, the genetic composition of decorators is highly dissimilar amongst rhizobia. For example, *A. caulinodans* encompasses the *nodSUIJ* operon, in which the *nodS* encodes a methyl transferase required for the methylation of R1 and R7, while *R. tropici,* harbours the genes *nodH, nodP* and *nodQ,* essential for sulphation at R5 region, in addition to the *nodS* that monitors methylation (Geelen et al. 1993; Folch-Mallol et al. 1996). The role of decorators in different rhizobia are listed in Table 1. Genetic manipulation of decorators has pronounced effects on symbiotic specificity. For instance, *nodZ* and *nolL* deletion mutants of *Mesorhizobium loti* R7A lead to variable nodulation efficiencies on four different species of *L. japonicus*. Both mutants exhibited drastic impairment of nodulation on *L. filicaulis*, while *nodZ* mutant had a higher impact on *L. corniculatus* than *nolL* mutant. The mutants did not affect the nodulation of *L. japonicus* and *L. burttii*, except for a delay in the appearance of the first nodule (Rodpothong et al. 2009). Also, heterologous expression of *nodZ* into *R. leguminosarum* bv. *viciae* RBL5560 enables it to infect *L. japonicus*, a non-host (Pacios Bras et al. 2000). Thus, diverse decorations on NF confer host-range specificity to rhizobia.

It is noteworthy that the actual order of *nod* genes that perform their roles in NF synthesis is not always the constructors followed by the decorators. This general assumption is due to the existence of *nodABC* genes as a single entity (Vinardell et al. 2015; Paudel et al. 2020). But mass spectrometric analyses of NFs in *Rhizobium* sp. IRBG74 has revealed that *nodZ* can act prior to *nodB,* while products of *nodS* and *nodU* may precede the action of that of *nodA*. This is corroborated by the fact that *nodB* mutants were able to produce fucosylated COs, but not deacetylated COs nor LCOs. Similarly, *nodA* mutants could produce substituted dCOs, which included methyl, fucosyl and carbamoyl groups at the non-reducing end (Poinsot et al. 2016). In addition, there are many more decorators to be annotated; for instance, *noeA, noeB* have been identified in the same operon as *nodL* in *S. meliloti*. Although *noeA* has been reported to share 32% homology with the methyltransferase of *Mesorhizobium*, there has not been any experimental evidence to determine its function, since *noeA* mutants did not show a difference in the structure of NFs (Du et al. 2005). All of these suggest the possibility of redundancy in the nodulation genes through gene duplication, which might serve the purpose of supporting the conventional gene or substituting it in its absence.

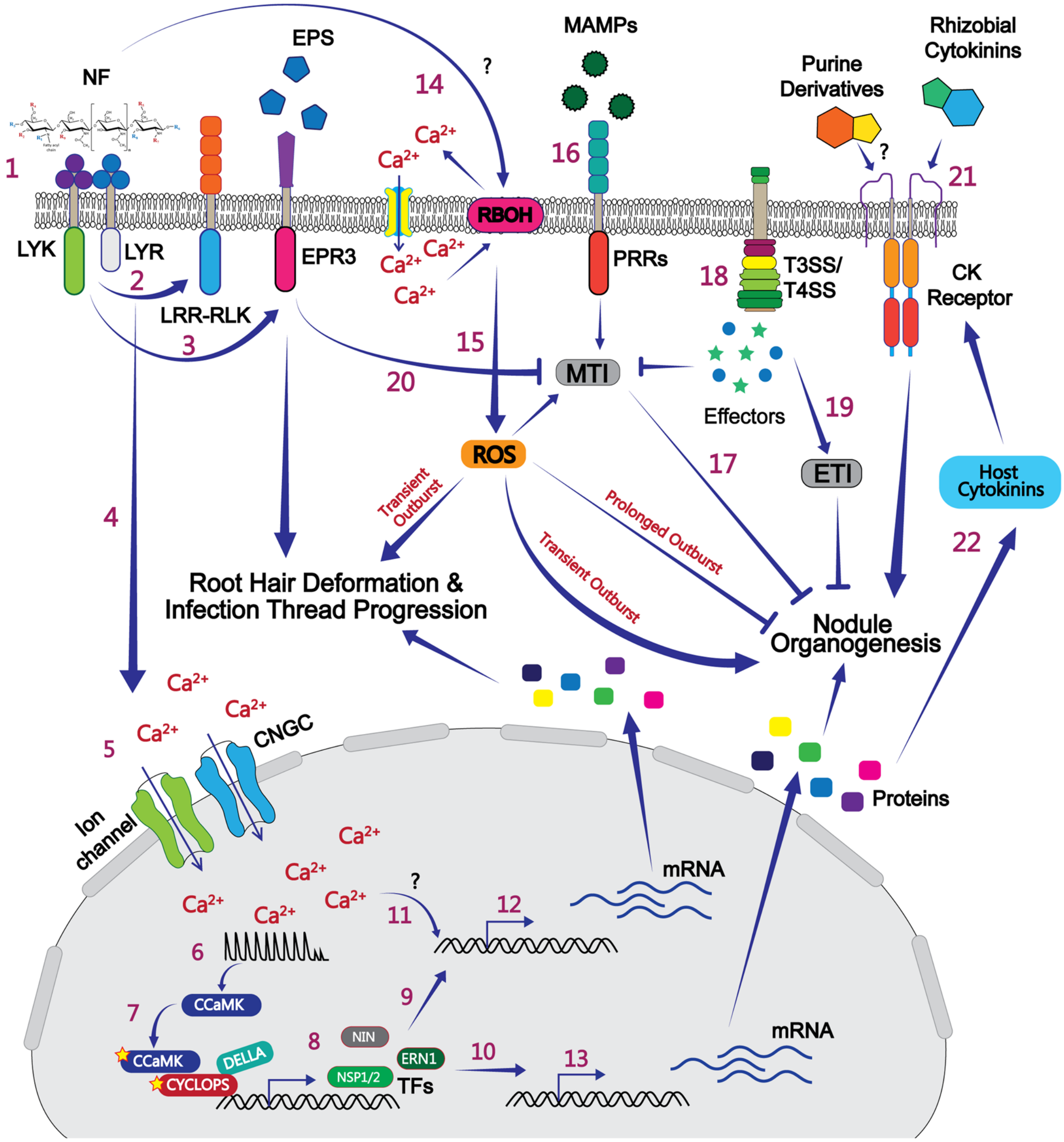
Decorating the backbone of NF with different substituents also offers protection against bond cleavage by plant chitinases (Ovtsyna et al. 2000; Staehelin et al. 2000). Since different plant chitinases have different affinities towards NFs, rhizobia that produce more than one type of NFs might be at an advantage due to the probability for at least one of them to be resistant against the hydrolytic activity of chitinases.

Dispatchers

Dispatchers are the proteins devoted towards the ferry of NF across the plasma membrane. The most commonly encountered dispatchers are the products of *nodI* and *nodJ,* which encode for the subunits of ATP-Binding Cassette (ABC) transporters (Fig. 1F). *nodJ* encodes for a hydrophobic transmembrane domain, while *nodI* encodes for the cytoplasmic ATP-binding domain, required for the active transport of the synthesised NFs across the PM of the gram-negative bacterium (López-Lara et al. 1996; Aoki et al. 2013). *nod*T encodes for an outer membrane protein that forms a transport complex by interacting with inner membrane proteins like *nodI* and *nodJ* (Downie, 1994; Hernández-Mendoza et al. 2007) and might be secreted directly outside the cell in a single step. Interestingly, *nodIJ* mutants of *R. leguminosarum* bv. *viciae* were able to secrete their NFs at usual levels, indicating the presence of alternative dispatchers in their genome (Spaink et al. 1991). Identification of genes like *nolF, nolG, nolH* and *nolI* that encode similar transport proteins and their organisation as an operon *nolFGHI,* has led to the speculation that these proteins complement for the ferry of NFs in *nodIJ* mutants of *R. leguminosarum* bv. *viciae* (Downie, 1994). Other proteins like *nolT, nolW* and *nolE* have also been identified that might act as transporters, but their functionality is questionable due to lack of experimental evidence (Davis and Johnston 1990; Meinhardt et al. 1993).

How does the host determine its cognate micro-symbiont?

Initial perception and interaction between the rhizobia and the host plant is the most critical step in symbiosis. On the root epidermis, NFs are perceived by LysM-RLKs, which form homo- or heteromeric complexes upon ligand perception (Geurts et al. 2005, 2016; Moling et al. 2014; Gao et al. 2021). They consist of an extracellular ligand-binding receptor domain containing three LysM modules, a transmembrane domain and an intracellular kinase domain connected to the transmembrane domain via a juxtamembrane domain (Mesnage et al. 2014; Bozsoki et al. 2020; Jose et al. 2020) (Fig. 2). The three LysM modules acquire a compact clover leaf arrangement with three βααβ folds, which are bridged by conserved disulphide bonds (Lefebvre et al. 2012). The intracellular domain may comprise of a typical kinase or a pseudokinase and is crucial for the signal transduction (Arrighi et al. 2006; Lefebvre et al. 2012; Bozsoki et al. 2020). The ligand-binding ectodomains of the NF receptors are always under a selective pressure due to the higher frequency of evolution encountered in the prokaryotic kingdom (Sulima et al. 2017). Therefore, the NF perceiving receptors from various legumes exhibit explicit diversity in their ectodomains while the intracellular domains are much identical (Radutoiu et al. 2007; Zhukov et al. 2008; Nakagawa et al. 2011; Sulima et al. 2017, 2019; Bozsoki et al. 2020). Receptors with typical kinase domain (LYK class) undergo autophosphorylation upon binding to NFs and activate downstream signaling components via transphosphorylation. On the other hand, receptors with atypical kinases (LYR



class) lack conserved residues required for ATP positioning, binding as well as catalytic activity of the kinase and are in turn devoid of both autophosphorylation as well as transphosphorylation abilities, and hence, act as co-receptors (Madsen et al. 2011; Palaka et al. 2021).

The signal is initiated by the host receptors through phosphorylation of downstream target proteins, per se, receptor-like cytoplasmic kinases, ubiquitin ligases, Rho related protein from plants (ROP) etc., (Mbengue et al. 2010; Ke et al. 2012; Choudhury and Pandey 2015; Tsikou et al. 2018a; Wong et al. 2019; Gao et al. 2021), which ultimately lead to the spiking of nuclear calcium levels, mediated by ion channels like cyclic nucleotide gated channels (CNGC) (Sieberer et al. 2009; Charpentier et al. 2016; Tsikou et al. 2018a). The spiking pattern is decoded by CCaMK, a Calcium-Calmodulin dependent kinase, whose activation is facilitated by autophosphorylation (Sathyanarayanan et al. 2001; Tirichine et al. 2006; Sinharoy and DasGupta 2009; Capoen et al. 2009; Hayashi et al. 2010). CCaMK forms a complex with CYCLOPS, a transcription factor, and phosphorylates it. The complex formation and phosphorylation are enhanced by DELLA proteins (Jin et al. 2016; Fonouni-Farde et al. 2016). The complex induces the expression of several other downstream transcriptional regulators, which further enhance the expression of proteins essential for infection thread progression as well as nodule organogenesis (Heckmann et al. 2006; Yano et al. 2008; Eckardt 2009; Ovchinnikova et al. 2011; Singh et al. 2014; Cerri et al. 2017; Lin et al. 2018; Liu et al.

◀**Fig. 2** The Plant Perspective: General outline of plant signaling mechanisms involved in establishing plant-rhizobial symbiosis. (1) NFs are perceived by transmembrane LysM-RLK complexes comprising of LYK (typical kinase) and LYR (pseudokinase) (2) Transmembrane LRR-RLKs are also activated, either by the LysM-RLK complex or by unidentified ligands (3) The complex also activates directly/indirectly another LysM-RLK, EPR3, which recognizes rhizobial exopolysaccharides (4) Downstream signal activation results in an influx of calcium near the cell periphery as well as around the nuclear membrane (5) Increase in calcium ion concentration activates nuclear membrane-bound ion channels and CNGC, (6) which further result in calcium spiking within the nucleus (7) Calcium signature is decoded by calcium/calmodulin-dependent kinases (CCaMK), leading to its autophosphorylation as well as transphosphorylation of CYCLOPS. The complex is stabilized by DELLA (8) CCaMK-CYCLOPS-DELLA recruit several transcription factors that activate the expression of proteins involved in [9, 12] root hair deformation and infection thread progression as well as [10, 13] nodule organogenesis (11, 12) Calcium influx within the nucleus is also known to activate proteins involved in root hair deformation, in a CCaMK-independent mechanism via unknown factors (14) NFs trigger ROS production, which increases calcium influx into the cell via calcium channels and the calcium ions, in turn, activate the RBOH (15) Transient ROS production promotes root hair deformation and nodule organogenesis, while prolonged production results in the inhibition of nodule organogenesis (16) Other molecular determinants like MAMPs are perceived by plant recognition receptors (PRRs) and activates MTI (Zipfel 2014) (17) MTI prevents the process of nodule organogenesis (Lopez-Gomez et al. 2012) (18) In addition to MAMPs, rhizobia introduce effector molecules into the plant cytoplasm via T3SS/T4SS (Okazaki et al. 2013) (19) Some of these effectors activate ETI, which blocks nodule organogenesis (Yang et al. 2010), while other effectors inhibit MTI and promote nodule organogenesis (Kambara et al. 2009) (20) MTI is also negatively regulated by EPR3 signaling cascade, which is initially activated by the perception of compatible exopolysaccharides (EPS) from rhizobia (21) Rhizobial cytokinins (Podlešáková et al. 2013) and probably purine derivatives (Giraud et al. 2007) are known to contribute towards nodule organogenesis by activating the host cytokinin receptor (22) The symbiotic signaling within the host results in accumulation of CK, which also activates the host CK receptor to regulate the nodule organogenesis, thus forming a positive feedback loop (van Zeijl et al. 2015). *NF* Nod factor, *LRR-RLK* Leucine-rich repeat containing receptor like kinases; RBOH: respiratory burst oxidase homolog, *MAMP* microbe-associated molecular pattern, *PRR* pattern recognition receptor, *EPS* exopolysaccharide, *EPR* EPS receptor, *T3SS/T4SS* type III/IV secretion system, *CK* cytokinin, *MTI* MAMP-triggered immunity, *ETI* effector-triggered immunity, *ROS* reactive oxygen species, *CNGC* cyclic nucleotide gated channel, *TFs* transcription factors, *NIN* nodule inception, *NSP* nodulation signaling pathway, *ERN* ERF required for nodulation

2019c, 2021). On the other hand, a parallel CCaMK-independent pathway also activates proteins involved in the root hair responses (Madsen et al. 2010; Murray 2011) (Fig. 2).

In addition to the perception of NFs at the epidermis and transducing the signal to the cell layers beneath, NF receptors also continue perceiving the microsymbiont's signal in the infection thread as well as the nodule cells (Arrighi et al. 2006; Moling et al. 2014). Their transient expression in the IT membrane enables synchronization of bacterial entry with that of nodular accommodation by equipping the plant with the genetic machinery required for symbiosome formation. NF receptors have been reported to accumulate in a narrow zone within the nodules, pertaining to the infection zone. Subsequently, NFRs are removed from the plasma membrane of nodule cells soon after bacterial release from the IT, suggesting possible roles of NFRs in triggering bacterial release into the nodule cells (Arrighi et al. 2006; Moling et al. 2014). Therefore, NF-NF receptor interaction is crucial during the commencement as well as the later events during root nodule symbiosis.

Are there nuances in the NF perception and signaling mechanism amongst legumes?

LjNFR1 (LYK) and LjNFR5 (LYR) are the known LysM-RLKs that are indispensable for nodulation of the model plant *Lotus japonicus*. It is corroborated by the lack of crucial physiological and molecular changes like root hair deformation, Ca^{2+} spiking and nodulin gene expression in *nfr1* and *nfr5* mutants of *L. japonicus*, in the presence of *M. loti* (Madsen et al. 2003; Radutoiu et al. 2003; Miwa et al. 2006). LjNFR1 and LjNFR5 directly bind to nanomolar range of *M. loti* NFs and form a receptor complex in which LjNFR1 undergoes autophosphorylation and activates LjNFR5 by transphosphorylation, thereby initiating the signaling cascade for rhizobial symbiosis (Broghammer et al. 2012). Interestingly, overexpression of *LjNFR1* and *LjNFR5* under *Ubiquitin* promoter results in spontaneous nodule formation, even in the absence of rhizobia, suggesting their importance in the plant signaling cascade during symbiosis (Ried et al. 2014).

Alternatively, the other model legume *Medicago truncatula* showcases the two-receptor model proposed by Ardourel et al. (Ardourel et al. 1994), in which one of the receptors acts as a signaling receptor to initiate early events of infection while the other acts as an entry receptor. MtNFP (LYR) acts as the signaling receptor and MtLYK3 is the entry receptor, affirmed by the respective mutant studies. *lyk3* mutants undergo root hair deformation, calcium spiking and cortical cell activation upon interaction with *S. meliloti* but can neither undergo tight root hair curling nor produce infection threads. On the other hand, *nfp* mutants are entirely insensitive to the NFs from *S. meliloti* (Amor et al. 2003; Haney et al. 2011). Transcriptional activation of GUS by the promoter of *NFP* (p*NFP*::GUS) led to the detection of NFP expression *M. truncatula* at different stages post rhizobial inoculation. GUS activity was observed in the inner cortical cells around nodule primordia, in the outer cortical cells beneath infected root hairs, and also in the infection zone of nodules, suggesting possible roles of NFP in the infection process as well (Arrighi et al. 2006; Rival et al. 2012). Corroboratingly, rare Nod$^+$ RNAi lines of *NFP,* which showed weak expression of *NFP,* had multiple abortive infection threads that ended up in sac-like structures and failed to reach the nodule primordia, suggesting the requirement of optimal level of NFP for infection thread morphology as well as proliferation (Arrighi et al. 2006). Interestingly, epidermal specific expression of NFP in *Mtnfp* mutants led to the restoration of symbiotic events in the cortex, i.e., the cortical cell divisions. Though NFP expression was detected in both the epidermis and the cortex, epidermal expression alone is sufficient to restore the nodule organogenesis signaling (Rival et al. 2012).

Studies of *P. sativum* NF receptor, PsSYM37 (LYK), are also in line with that of the two-receptor model. Inactivation of *PsSym37* does not affect the root hair deformation nor cortical cell activation but drastically lowers the number of primordia formed 15 days post inoculation and ultimately, the plant fails to produce nodules. The defect in nodulation is due to the impairment in initiation of infection thread formation (Zhukov et al. 2008). Although MtNFP and PsSYM10 are inactive kinases (LYRs) and require an active kinase for relaying the signal downstream after NF perception, their counterparts in the heteromeric complex cannot be MtLYK3 or PsSYM37, because *mtlyk3* and *pssym37* mutants do not inhibit the early responses of symbiosis (Smit et al. 2007; Zhukov et al. 2008; Bensmihen et al. 2011). Irrespective of the type of nodules formed (indeterminate in *M. truncatula* and *P. sativum*; determinate in *L. japonicus* and *G. max*), the NF mediated signal transduction is non-linear and involves two parallel pathways; one for the early cell division response and the other for the rhizobial invasion response (Esseling et al. 2004; Zhukov et al. 2008; Madsen et al. 2010). Though the existence of an entry receptor in *L. japonicus* has not yet been identified, the demarcation between the two aforementioned processes has been demonstrated (Madsen et al. 2010).

MtLYK3 is essential for initiation of infection threads as well as for its polar growth, the latter requiring stringency in the NF structure and complex signaling than the former. To elaborate, *M. truncatula hcl-4* mutant, a weak allele mutant that encodes for reduced levels of LYK3, when infected with a *nodFnodL* double mutant exhibits root hair curling, but the rhizobial microcolonies end up in a sac-like structure due to the absence of an infection thread to guide them towards the developing nodule. In contrast, wild type *S. meliloti* succeed in nodulating the *hcl-4* mutant, corroborating with the NF structure dependence of LYK3 for efficient nodulation. Perception of wild type NFs by LYK3 downregulates prolonged root-hair branching, which is evident with the production of multitude of branches on a single root hair in the absence of LYK3 (Smit et al. 2007). A similar NF structure-stringency has also been observed in *P. sativum* Afghanistan accession containing a putative LysM encoding SYM2 locus (Firmin et al. 1993). The allelic state of the *PsSym2* gene determines the selectivity towards rhizobium and *Sym2^A* (Afghanistan cv.) being more selective than *Sym2^E/Sym2^C* (European/ Cultivated). The two proteins differ in their amino acid composition at three sites in the LysM1 module (Sulima et al. 2019). Sym2^A in the Afghan cultivar recognizes the *nodX* mediated acetylation at the reducing end of NFs from *R. leguminosarum* bv. *viciae* strains isolated from the middle east soils. Plants infected with European strains of *R. leguminosarum* bv. *viciae* that do not encode for *nodX,* resist nodulation and end up inducing defective infection threads that stall in the epidermis (Geurts et al. 1997; Hogg et al. 2002). On the other hand, European pea cultivars possessing Sym2^E receptor have broad specificity and can recognize as well as permit the entry of both *nodX*$^+$ and *nodX*$^-$ rhizobial types. Recently, another allelic state of *PsLykX* proposed to be the candidate for the elusive *PsSym2* gene has been discovered and termed *Sym2^T*, named after its origin from a Tajikistan cv., which differs from Sym2^E protein in one amino acid residue (Sulima et al. 2019). However, further genetic studies are required to confirm the nomenclature and to delineate its function.

Structural stringency is also observed in the semi-aquatic *Sesbania rostrata*, which switches between intracellular and intercellular modes for rhizobial entry during non-flooded and flooded conditions, respectively. The host is more compromised on the NF structure during intercellular entry while exhibits stringency during intracellular entry (Goormachtig et al. 2004). Identification and functional characterization of the respective NF receptors are necessary to determine the molecular mechanism behind such transitions. In *Glycine max*, which is a palaeoalloploid, there are two copies of *NFR1* and *NFR5*, namely *GmNFR1α, GmNFR1β, GmNFR5α* and *GmNFR5β*. Overexpression studies specify that *GmNFR1α, GmNFR5α* and *GmNFR5β* rescue the Nod$^-$ phenotype. However, the transcript level of *GmNFR5α* and *GmNFR5β* do not limit nodulation, while the transcript abundance of *GmNFR1α* dictates the nodule number in soybean (Indrasumunar et al. 2010, 2011; Gao et al. 2021).

What contributes to the ligand specificity within the ectodomain of NFRs?

Ectopic expression of *LjNFR1* + *LjNFR5* in *M. truncatula* resulted in extension of its symbiont range to that of *L. japonicus*, which includes *M. loti* as well as *R. leguminosarum* DZL strain. The transgenic *M. truncatula* facilitates rhizobial recognition and nodule organogenesis, but fails to establish symbiosomes (Radutoiu et al. 2007). *Lotus filicaulis* is a close relative of *L. japonicus* and is nodulated by *M. loti* but not the DZL strain of *R. leguminosarum*. Ectopic expression of *LjNFR1* + *LjNFR5* in *L. filicaulis* facilitated its nodulation by the DZL strain. When chimeric receptors containing LfNFR1_ED + LjNFR1_KD and LfNFR5_ED + LjNFR5_KD were transformed into *nfr1* and *nfr5* mutants of *L. japonicus* respectively, there was a reduction in the nodulation efficiency of plants complemented with *LfNFR5* but not with *LfNFR1* (Radutoiu et al. 2007). Thus, it is obvious that NF specificity and recognition are contributed by the LysM domains of NFR5 in *L. japonicus*. LjNFR5 and LfNFR5 differ in three amino acid residues. Hybrid receptors were created via site-directed mutagenesis, in which amino acid residues of LfNFR5 were individually substituted with corresponding residues from that of LjNFR5 (Q30K, K118L, K230E). Such hybrid receptors were functionally characterized for their ability to complement *ljnfr5* mutants. It was determined that the substitution from a basic hydrophilic Lys residue at position 118 with a hydrophobic Leu residue successfully complemented the *ljnfr5* mutants, suggesting that the difference in the Leu/Lys at 118th amino-acid residue of the protein in LysM2 modules of *L. japonicus* and *L. filicaulis* respectively, contributed to their difference in specificity towards the NFs of *R. leguminosarum* DZL strain (Radutoiu et al. 2007). A similar observation was detected through interaction studies between NFs and the individual LysM domains that were chemically synthesized (Sørensen et al. 2014). Also, homology modelling and molecular dynamics simulation study on the NFRs of chickpea reveals the significance of LysM2 domain in NF recognition (Palaka et al. 2021).

Homology models of the ectodomains of LjNFR5 and MtNFP had been designed more than a decade ago using prokaryotic LysM domains as reference (Arrighi et al. 2006; Radutoiu et al. 2007) and a putative hydrophobic cleft had been predicted to act as the NF binding groove (Radutoiu et al. 2007). However, the functional relevance of the cleft had not been determined until the recent breakthroughs of two independent experiments, in which the ectodomains of LjNFR1 and MtNFP were crystallized and their structures elucidated using AtCERK1 as reference (Bozsoki et al. 2020; Gysel et al. 2021). Electrostatic surface potential of the NFP crystals unveiled a hydrophobic patch in the LysM2 module in which the fatty acyl moiety of NF could be super positioned without steric hindrance. Homology modelling of the ectodomains of NFR5 class of receptors from other plants, like LjNFR5, PsSYM10 and GmNFR5α validated the presence of a hydrophobic patch at a corresponding location in LysM2 (Gysel et al. 2021). The functional significance of the hydrophobic patch was ingeniously unravelled by studying the ability of signal transduction of NFP variants whose residues from the hydrophobic patch were substituted. Corroboratingly, when site directed mutants of NFP whose residues outside the hydrophobic patch were substituted, the construct successfully complemented *nfp* mutants and exhibited similar nodulation phenotype as the wildtype plant, when inoculated with *S. meliloti* 1021. However, Leu to Asp substitution of two surface exposed leucine residues from the hydrophobic patch (L147 and L154) had a contrasting effect (Gysel et al. 2021). The double mutant (L147D/L154D) failed to complement *nfp* mutants and the plants were devoid of nodules when infected with *S. meliloti* 1021 and showed a drastic decline in the number of nodules when inoculated with *S. medicae*, a higher efficiency nodulating symbiont of *M. truncatula* (Terpolilli et al. 2008; Gysel et al. 2021). The significance of L154 in MtNFP had also been emphasized previously, in which Leu to Pro substitution (L154P) in the LysM2 module failed to complement *nfp* mutants of *M. truncatula* (Bensmihen et al. 2011). Biochemical experiments strengthen the functional relevance of the hydrophobic patch in the symbiotic signaling pathway. Biolayer Interferometry (BLI) results revealed a 13-fold lower affinity of mutant NFP (L147D/L154D) towards *S. meliloti* NFs, in comparison to the wildtype NFP. Also, the kinetics of association and more drastically, the dissociation were altered in the double mutant, emphasizing that the hydrophobic patch in LysM2 is essential for the stability of NF-NFR interaction (Gysel et al. 2021).

In contrast, recent investigations in the ectodomains of LjNFR1 from *L. japonicus* and PsLykX from *P. sativum* have attributed the LysM1 module as the major determinant of ligand recognition (Sulima et al. 2019; Bozsoki et al. 2020). Chimeric receptors containing different combinations of LysM modules from LjCERK6 and LjNFR1 were fused with LjNFR1 kinase domain and used for the complementation of *ljnfr1* mutants. Chimeras with LysM1 from NFR1 successfully rescued the non-nodulating phenotype of *ljnfr1* mutants, irrespective of the origin of the other two LysM modules, suggesting the significance of LysM1 in NF perception. However, as the results were disparate from the earlier prediction that LysM2 bears the LCO binding pocket, one amino acid residue from LysM1 and one residue from LysM2 of LjNFR1 was substituted individually with a bulky residue to study which of them perturbed the nodulation of *nfr* mutants. Corroborating with the chimeric receptor data, I78W substitution in LysM1 did not complement the *nfr* mutants whereas I140W substitution in LysM2 successfully complemented the non-nodulating phenotype and the plants produced similar number of nodules as that of the wildtype NFR1 transformed plants, reinforcing that LysM1 is the major determinant of NF specificity in LjNFR1(Bozsoki et al. 2020). Previous reports of non-nodulating pea mutant harbouring L77F substitution in LysM1 of PsSYM37 are in line with Bozsoki et al. 2020 (Zhukov et al. 2008; Bozsoki et al. 2020). These experiments highlight the significance of hydrophobic residues within the NF binding pocket of LysM1 modules. Also, P87S substitution in LysM1 of MtLYK3 led to defective root hair curling in *M. truncatula.* Although Pro lies outside the predicted binding pocket of NF, it might be essential for the ectodomain to attain the appropriate 3D conformation (Smit et al. 2007). Therefore, it is likely that LysM1 of LYK class of NF receptors and LysM2 of LYR class are the major determinants of NF specificity.

Do the kinase domains also contribute towards ligand affinity of the NF receptors?

Binding experiments using biolayer interferometry (BLI) revealed that purified ectodomain of MtNFP binds to immobilized LCO-V from *S. meliloti* in the micromolar range (Gysel et al. 2021). However, under physiological conditions, NFs are perceived by the plants even in nanomolar range (Broghammer et al. 2012). Does it imply that the cytoplasmic domain also contributes to ligand affinity? Such an enigma was resolved by performing binding experiments with full-length receptors using BLI (Gysel et al. 2021). Convincingly, full-length NFP exhibited a higher affinity for the LCO-IV in comparison to that of the isolated ectodomains. A similar pattern was observed when binding of LjNFR1 and LjNFR5 with *M. loti* LCOs was measured using surface plasmon resonance (SPR) and microscale thermophoresis (MST) (Broghammer et al. 2012). These results support the hypothesis that the presence of intracellular domain increases the receptor-ligand affinity, probably by receptor oligomerization. It subsequently results in more ligand binding pockets in the receptor complex, a phenomenon known as avidity (Gysel et al. 2021).

Are NF receptors required even for intercellular entry of rhizobia?

Aeschynomene spp. are (semi-) aquatic legumes that form nitrogen-fixing nodules on roots and stem by interacting with photosynthetic *Bradyrhizobia* (Alazard 1985; Giraud and Fleischman 2004; Zhang et al. 2019), with a few exceptions like *A. americana* which associates with non-photosynthetic *Bradyrhizobium* and *A. patula*, which is compatible with both photosynthetic as well as non-photosynthetic *Bradyrhizobium* (Brottier et al. 2018). The compatible rhizobia of *Aeschynomene* follow intercellular mode of entry (Ibáñez et al. 2017; Sprent et al. 2017) and may exhibit NF-dependent or NF-independent mode of nodulation (Giraud et al. 2007; Bonaldi et al. 2011; Brottier et al. 2018). As mentioned earlier in the review, strains of *Bradyrhizobia* like ORS278 and BTAi1 are devoid of *nodABC* genes and efficiently nodulate *A. indica* (Giraud et al. 2007), whereas *Bradyrhizobium* ORS285, which possesses *nodABC* infects *A. afraspera* in a NF-dependent and *A. indica* in a NF-independent manner (Chaintreuil et al. 2001; Renier et al. 2011; Nouwen et al. 2016). Root exudates of *A. afraspera* induced NodA expression in ORS285, while that of *A. indica* did not (Chaintreuil et al. 2001; Renier et al. 2011; Nouwen et al. 2016), affirming that the choice of NF-dependency is determined by the host. The transcriptome of *Aeschynomene evenia,* a representative of the NF-independent symbiosis, poorly expresses orthologue of LYK3, and no other *Aeschynomene*-specific LysM-RLK was identified. On the other hand, ten other NF-independent *Aeschynomene* spp. completely lack the expression of LYK3 and NFP (Quilbé et al. 2021). As the rhizobia enter via intercellular matrix, these plants probably do not require NF recognition. Then, how do they recognize the presence of compatible microsymbiont?

In *L. japonicus*, EPR3 (a LysM-RLK induced after NF perception) was involved in the recognition of exopolysaccharides (EPS) and permitted bacterial invasion during intracellular as well as intercellular entry, hinting that exopolysaccharide could have been the signals for communication (Fig. 2). However, even EPR3 orthologues were not identified in the transcriptome of *A. evenia* (Kawaharada et al. 2015; Wong et al. 2020); (Quilbé et al. 2021). Interestingly, determinants like *EXO70H4, LUMPY INFECTIONS (LIN), VAPYRIN (VPY)*, which are essential for infection thread growth and intracellular accommodation of rhizobia in *M. truncatula* (Liu et al. 2019b) were symbiotically upregulated in *A. evenia* (Quilbé et al. 2021). In addition, many other components of nod-signaling pathway like *AeSYMRK, AeLHK1*, *AePOLLUX, AeCCaMK, AeCYCLOPS, AeNSP2*, and *AeNIN* were symbiotically expressed during the root and stem nodulation of *A. evenia* (Fabre et al. 2015; Quilbé et al. 2021). Taken together, these results suggest the conservedness of the core Nod-signaling pathway and the intracellular accommodation machinery for rhizobial symbiosis, but does not provide clarity about the initial rhizobial recognition. Quilbe and group wanted to establish the link between the core Nod-signaling pathway and rhizobial perception, and identified *AeCRK* (cysteine-rich RLK) as an indispensable receptor for establishing nodulation in *A. evenia* (Quilbé et al. 2021). However, it is unclear how AeCRK mediates rhizobial perception. Further characterization via generation of *AeCRK* loss-of-function mutants and study of their symbiotic phenotypic is essential to determine their involvement as well as significance in the symbiosis signaling.

Arachis hypogaea, which paves way for *Bradyrhizobia* via intercellular cracks, never forms infection threads at any point during its symbiosis with rhizobia. *Bradyrhizobia* directly activate the cortical cells that they are in contact with and form nodules which are devoid of uninfected cells (Boogerd and van Rossum 1997; Fabre et al. 2015). Identification of CCaMK's significance in nodulation reveals the involvement of NF perception (Sinharoy and DasGupta 2009), which is further supported by the inability of NF mutants of *Bradyrhizobia* to activate cortical cell division despite being able to colonize the inner root tissues of the host (Ibáñez and Fabra 2011). These results imply that rhizobia entry into *A. hypogaea* is NF-mediated. The orthologues of LjNFR1 and LjNFR5 were identified in *A. hypogaea*, namely AhNFR1 and AhNFP. Tridimensional analysis of the LysM2 module of the putative AhNFP receptor revealed similarity with that of GmNFR5, and that both the receptors recognize similar NFs (Ibáñez et al. 2015). Later, using GmNFRs as the query sequences, four copies of AhNFR1 and two copies of AhNFR5 were identified in *A. hypogaea*, which had the structural similarities in the ectodomains like that of the canonical NFRs from the model legumes (Shu et al. 2020). Transcriptomic analysis and genetic screening of Nod^{-} and Nod^{+} hosts of peanut have indicated the possibility of AhNFR5 over AhNFR1 in recognizing NFs (Peng et al. 2017). In line with this, CRISPR/Cas9 knockout of AhNFR1A2 and AhNFR1B2 produced nodules on *A. hypogaea,* while that of AhNFR5A and AhNFR5B did not (Shu et al. 2020), indicating the requirement of AhNFR5 during rhizobial symbiosis. However, presence of nodules in AhNFR1A2 and AhNFR1B2 knock-out plants could have also been due to the functional complementation by its two other paralogues. In addition, about 35 NFR1 like genes had been identified from the PeanutBase database when the BLAST search was performed with much relaxed E value (Shu et al. 2020), all of which suggests that there is high divergence of NFR1s in *A. hypogaea* and identifying the one that's crucial for NF recognition would be challenging. Therefore, RNAi knockdown of all the AhNFR1s would be necessary to eliminate misinterpretations due to functional redundancy and characterization of the *A. hypogaea* receptors is essential to determine whether they have any significant role in rhizobial perception during intercellular mode of entry.

Are NF receptors indispensable only for rhizobial symbiosis?

LysM-RLKs are not restricted to rhizobial symbiosis and are well known to mediate other micro and macro symbiotic interactions as well as pathogens (Rasmussen et al. 2016; Jose et al. 2020). The downstream signaling of rhizobial and arbuscular mycorrhizal (AM) fungal perception adopt the common symbiotic signaling pathway (CSSP), constituting signaling components like the LRR-RLK, cation channels, nucleoporins, and the calcium/calmodulin dependent kinase CCaMK (Walker et al. 2000; Stracke et al. 2002; Lévy et al. 2004; Kanamori et al. 2006; Banba et al. 2008; Groth et al. 2010) (Fig. 2), and further bifurcate in their downstream responses due to altered signatures in their calcium spiking patterns (Kosuta et al. 2008). NFR5 homologues in plants other than the nitrogen-fixing legumes are involved in AM symbiosis. Majorly, the legumes have undergone tandem or segmental duplications, which led to the neofunctionalization of NFR5 towards rhizobial perception, while some of its other paralogues are devoted towards AM symbiosis (Arrighi et al. 2006; Lohmann et al. 2010; Rasmussen et al. 2016; Thiergart et al. 2019). For instance, SlLYK10, a homologue of MtNFP in tomato, is essential for AM fungal colonization (Buendia et al. 2016), while the *nfp* mutants of *M. truncatula* remain unaffected in AM symbiosis (Feng et al. 2019). However, there was a drastic reduction in the fungal colonization in the *nfp/cerk1* double mutants. The extent of reduction in fungal colonization was higher than that observed in the single mutants of *cerk1* (*lyk9*) (Feng et al. 2019), implicating the possible role of NFP in enhancing the signal transduction of CERK1 during AM symbiosis. Likewise, in *Parasponia andersonii*, PanNFP1 and PanNFP2 are essential for AM symbiosis, while the PanNFP2 alone is required for nodulation (Op den Camp et al. 2011; Rutten et al. 2020).

Therefore, NFR5 class of receptors seem to have an additional role in transducing the AM symbiosis signaling in some legumes, although they are not indispensable. The probable reason for NFR5 being dispensable in AM symbiosis could be due to the functional redundancy of the other paralogues, which requires further study with combinations of different receptor mutants. In rice, *Osnfr5* mutants show no conspicuous phenotypic differences from the wild-type rice plants and exhibit normal colonization by the AM fungi, suggesting the involvement of functionally redundant receptors (Miyata et al. 2016). However, there is also a possibility of the existence of NFR5-independent perception and signaling mechanism, which might be mediated by novel non-LCO type ligands.

In addition to their dispensable role during AM symbiosis in legumes, the NFR5 class of receptors are also involved in the defence signaling during biotic stress. MtNFP seems crucial for conferring resistance to *M. truncatula* against an oomycete and a fungus, evidenced by the significantly high expression (tenfold) of fungal tubulin in *nfr5* mutants compared to wild-type plants (Rey et al. 2013). The nature of the ligand perceived by the receptor might alter the heteromeric partner and the ultimate signaling output relayed. A similar role of altered response is observed in OsCERK1, orthologue of NFR1 in rice, which binds with Chitin Elicitor Binding Protein (CEBiP) to elicit an immune signal, while binding with Myc-factor receptor OsMYR1 initiates AM symbiosis signaling (Miyata et al. 2016; He et al. 2019).

How does the plant avoid eliciting an immune response despite the structural similarity between NFs and other chitinaceous signals?

Both NFs and chitin induce defence signaling in the host plants. The NF-induced defence signaling is mediated by NF receptors and not due to the perception by chitin receptors (Nakagawa et al. 2011). The perception of NFs and pathogen associated molecular patterns (PAMPs) occurs via distinct receptors per se, LjNFR1/MtLYK3 and LjNFR5/MtNFP for NF and LjLYS6/MtLYK9 and MtLYR4 for PAMPs, in *L. japonicus* and *M. truncatula* respectively. Insertional mutants of *Ljlys6*, *Mtlyk9* and *Mtlyr4* were highly susceptible to bacterial and fungal pathogens but were efficient in rhizobial colonization (Bozsoki et al. 2020), indicating independent signal activation by NF and PAMPs. Interestingly, a chimeric receptor encompassing LjNFR1_ED (ectodomain) + LjLYS6_KD (kinase domain) (RLK involved in chitin perception during immune-signaling) could partially restore the nodulation defect of *L. japonicus nfr1* mutants (Nakagawa et al. 2011). Similarly, chimeric receptors with LjNFR1_ED + LjCERK6_KD complemented the *nfr1* mutants of *L. japonicus* after *M. loti* infection, but couldn't trigger the production of ROS in *cerk6* mutants of *L. japonicus*, upon chitin octamer perception (Bozsoki et al. 2020). Also, a swap in the ectodomain of OsNFR5 with that of LjNFR5, *i.e.*, LjNFR5_ED + OsNFR5_KD restored the nodulation phenotype of *ljnfr5* mutant (Miyata et al. 2016). At the molecular level, the LysM modules of NF receptors exhibit a hydrophobic patch, which is lacking in the chitin receptors. In addition, the kinetics of chitin binding and dissociation to its receptor is very fast in comparison to the kinetics of binding and dissociation during NF-NF receptor interaction. A longer time of interaction between NF-NF receptor rather than very high affinity of the receptor towards the ligand is essential to initiate symbiotic signal (Gysel et al. 2021). Cumulatively, these studies also show higher conservedness in the intracellular domains of LysM-RLKs in comparison to their ectodomains, implying that the distinction between NFs and various chitinaceous molecules originates majorly from the ectodomains of the plant LysM-RLKs.

Interestingly, MtLYR3, an inactive kinase with dual phosphorylation sites, shows high affinity for NFs and its interaction with MtLYK3 is negatively regulated by NFs either by disrupting the stability of the complex or by causing a conformational change in the protein (Fliegmann et al. 2016). MtLYR3 has unique phosphosites, which are not conserved in either MtNFP or the homologues of MtLYR3 like MtLYR2, MtLYR4, which is the probable reason for higher specificity of transphosphorylation of MtLYR3 by MtLYK3. In addition, co-expression of MtLYR3 with MtLYK3 and MtNFP in the leaves of *Nicotiana benthamiana*, resulted in a reduction in the cell death response. It is most likely that MtLYR3 prevents interaction of MtLYK3 and MtNFP in the absence of NFs to prevent activation of defense-signaling. Also, legumes avoid the activation of defense signaling by accumulating the NFRs transiently in the infection threads and the nodules. NFRs are frequently engulfed by membrane vesicles and directed to the vacuoles for degradation (Smit et al. 2007), which is essential to increase the sustenance of nodules. Nodule cells that ectopically express NFP and LYK3 under Arabidopsis ubiquitin promoter had small size, fewer infected cells in the infection zone and seem to accumulate defense/senescence activated polyphenols (Moling et al. 2014).

Compatible NFs trigger plant NADPH oxidase/respiratory burst oxidase homolog (RBOHs), which generate a transient oxidative burst and increase the calcium influx (Cárdenas et al. 2008; Morieri et al. 2013). ROS acts as a signaling molecule and its transient outburst is essential for infection thread progression, nodule organogenesis and senescence (Lohar et al. 2007; Montiel et al. 2016; Fonseca-García et al. 2021; Tsyganova et al. 2021) (Fig. 2). Rhizobial or purified NF perception by GmNFR5 elicits a calcium-dependent redox signal within 30 min and later reduces, which is opposed to the prolonged sustenance of ROS levels upon the perception of a pathogen or an elicitor (Muñoz et al. 2012; Mu Oz et al. 2013; Gilroy et al. 2014; Fernandez-Göbel et al. 2019; Rey et al. 2019). In addition, pretreatment of soybean and Arabidopsis leaves with Nod factors (100 nM) from *B. japonicum* diminished the flg22-triggered ROS production by reducing MAP kinase phosphorylation (Liang et al. 2013). Cumulatively, these results suggest that cell-death response promoted by sustained ROS production is overcome during NF signaling by fine-tuning the ROS accumulation spatio-temporally as well as quantitatively.

In summary, defence-like reactions are induced in the host plant even during rhizobial perception, but the sustenance of the defence signaling is inhibited by involving additional proteins that enable the host to favour the symbiotic pathway over the defence pathway. As we are still unaware of many of the signaling networks governing the symbiosis process, we cannot assertively conclude that the host avoids eliciting the defence response via a particular pathway. However, we can affirm that the distinction between symbiosis and defence emanates from the ectodomains of the NF receptors. Further investigation on how ligand perception by the ectodomain induces a conformational change in the kinase domain to relay an alternative signaling output during symbiosis and defence would help us understand how the plant distinguishes between the two mechanisms.

What are the consequences of excess production of NFs?

Bradyrhizobium strain ORS285, which possesses *nodABC* genes nodulates *A. indica* in a NF-independent manner and the root exudates of *A. indica* do not induce the expression of *nod* genes. However, when *nod* genes were ectopically induced in *Bradyrhizobium* strain ORS285 using naringenin, it delayed the production of nodules in *A. indica,* implying that NF production negatively regulates symbiosis in NF-independent processes (Nouwen et al. 2016), and the mechanism behind is yet to be unravelled. Even in NF-dependent processes, maintenance of optimal concentration of NFs is crucial for establishing efficient symbiosis. For instance, excess of NFs delays infection thread formation due to uncontrolled curling or swelling of root hairs, thereby impairing the formation of a conventional infection chamber; promotes clustering of nodule primordia, nodule hypertrophy and nodule branching in *M. truncatula* (Cai et al. 2018)**,** while it arrests the transformation of infected primordia into nitrogen-fixing organs in *L. japonicus* (Malolepszy et al. 2018). Overexpression of NFs in *R. leguminosarum* strain TOM supresses nodulation by inhibiting initiation of infection threads in pea roots, by a phenomenon called "competitive nodulation blocking" (Hogg et al. 2002). On the other hand, sub-optimal levels of NFs are insufficient to establish effective symbiosis (Baev et al. 1991; Krishnan et al. 1999; Cai et al. 2018) and thus, maintenance of optimal levels of NFs is essential for the plant.

Due to their structural conformation, NFs can be hydrolysed by plant chitinases, which cleave the β-1→4 linkage between monomers and consequently inactivate the NF (Staehelin et al. 1994, 1995; Goormachtig et al. 1998; Ovtsyna et al. 2000, 2005). Recent studies have identified the recruitment of NF-specific chitinases such as Nod Factor Hydrolase1 (MtNFH1) and Chitinase5 (LjCHIT5) that hydrolyse NFs in a dosage-dependent manner, activated by Nod-signaling pathway (Tian et al. 2013; Cai et al. 2018; Malolepszy et al. 2018). Therefore, low levels of NFs would stimulate only less expression of the hydrolases and there will be lower hydrolysis of NFs (Ovtsyna et al. 2000; Cai et al. 2018). Pre-treatment of *M. truncatula* seedlings with NFs for 2 h was adequate enough to stimulate MtNFH1 production and NF hydrolysis (Cai et al. 2018), implicating the role of MtNFH1 during the early stages of symbiosis. However, the morphology of ITs and nodule primordia seem unaltered in *chit5* mutants in *L. japonicus*, and there might be additional CHIT5-independent enzymes governing these early processes. The authors propose that higher production of NFs within the primordial ITs (due to the swap from NodD1 to NodD2) might cause an imbalance in the defence vs symbiosis signaling pathways, and LjCHIT5 curtails the impact by hydrolysing excess NFs (Kelly et al. 2018; Malolepszy et al. 2018). Thus, maintenance of NF optima seems to be mediated by host chitinases and the involvement of more host factors has to be examined.

Conclusions

Communication during root nodule symbiosis constitutes a series of interactions, involving plant and rhizobial signal molecules, receptors from both of the symbionts, transcriptional regulators, biosynthetic enzymes, defence proteins/peptides, and hormones, which cooperatively pave way for bacterial entry and accommodation into the host system. Each step is governed stringently and is patrolled by alternative pathways as well to promote only favourable and synergistic interactions. NF-NFR interaction plays a pivotal role in initiating this complex process and also in ensuring specificity between the symbionts. The manoeuvre of patrollers, constructors, decorators and dispatchers is the impetus behind the establishment of such compatible interactions and have been studied since many years. Recent breakthroughs have been achieved in the structural and functional characterization of host receptors as well as the identification of its signaling intermediates. It has led us to uncouple rhizobial entry and nodule organogenesis and discover signaling intermediates that are specialized in one of the processes as well as identify intermediates that mediate both processes. The discovery of a hydrophobic signature in the LysM modules of NF receptors can be utilized to computationally identify probable LCO receptors of the LYR class from other host plants. It can also be applied for the identification of the functional receptors from polyploid plants which may have multiple paralogues of the receptor. With the current knowledge about the structural attributes of both the NF and the NFRs that contribute to symbiotic stringency, rhizobia may be engineered to produce NFs that bind to the NF receptor with higher stringency. As higher stringency might not always result in improved symbiotic efficiency, it must be ensured experimentally that there is enhanced symbiosis between the partners. This way, the nodulation efficiency of legumes can be improved by introducing the engineered rhizobia into soil containing underperformed rhizobia. Due to the stringency of the receptor and its higher specificity towards the NFs of the engineered rhizobia, the legumes might abolish symbiosis with the under-performed rhizobia.

Though much progress has been achieved towards understanding the inter-kingdom signaling, it is leading to more questions regarding several aspects involving both the bacterial and host perspectives. Specifically, the mechanism of choice of symbiosis over defence has remained a puzzle. In essence with the discovery of host chitinases that hydrolyze NFs in excess, 'whether the hydrolysed NFs act as secondary signals to activate autoregulation of nodulation in the host' would be an interesting question to be explored. Additionally, due to enormous investment of energy in the synthesis of NFs, excess production of NFs will be detrimental to the rhizobia. Thus, how the rhizobial system regulates the optimal quantity of NF biosynthesis, will be another interesting field to venture upon. Given the diversity in the number of NFs and the rhizobial modes of entry, our knowledge regarding their recognition and downstream signaling mechanisms in the host is still incomplete.

Previously, more attention was given towards the infection thread mode of entry. Recently, much research on the Dalbergioid clade of legumes, within which rhizobia follow intercellular entry has expanded our knowledge on the symbiosis signaling. Involvement of NF receptors even during crack entry makes one wonder the role of the receptors during such a signaling event. In addition, upregulation of various genes whose orthologues in the model plants were previously considered to be involved in infection thread progression, suggests specialized and/or additional roles for the genes in a crack-entry legume. Further functional characterization of the proteins would give us more insights into the signaling mechanism involved. Detailed structural, molecular and genetic studies will not just unravel the ligand-receptor interaction mechanisms but also help us comprehend the contribution of recognition specificity towards symbiosis signaling and plant immunity.

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