



# Innovations in two genes kickstarted the evolution of nitrogen-fixing nodules

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## Abstract

The root nodule symbiosis between plants and nitrogen-fixing bacteria is a fascinating trait limited to several plant species. Given the agronomic potential of transferring this symbiosis to nonleguminous crops, the symbiosis has attracted researchers' attention for over a century. The origins of this symbiosis can be traced back to a single ancestor, around 110 million years ago. Recent findings have uncovered that adaptations in a receptor complex and the recruitment of the transcription factor Nodule Inception (NIN) are among the first genetic adaptations that allowed this ancestor to respond to its microsymbiont. Understanding the consequences of recruiting these genes provides insights into the start of this complex genetic trait.

## Addresses

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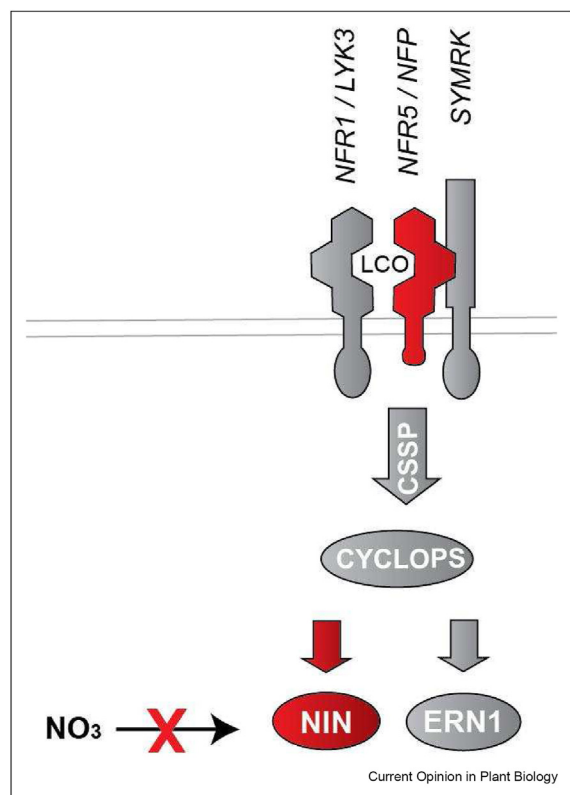
Nitrogen-fixing nodule symbiosis occurs in plant species spread across ten taxonomic lineages of the related orders Fabales, Fagales, Cucurbitales, and Rosales, known as the nitrogen-fixing clade [1]. The microbial partners are either diazotrophic Proteobacteria (collectively called rhizobia) or Actinobacteria of the genus *Frankia*. Within the nitrogen-fixing clade, nodulating species are interspersed with nonnodulating species. Given the common requirement of a core set of nodulation genes that have been pseudogenized in nonnodulating relatives, all nodulating plants are currently believed to be derived from a single ancestor that acquired the ability to establish a symbiosis with nitrogen-

fixing bacteria [2–4]. Subsequent lineage-specific inventions resulted in a substantial variation in nodule ontogeny, diazotrophic microsymbionts, and infection mechanisms [4,5]. Despite this variation, the evolution of nodulation relied on two key innovations that allowed plants to initiate specific transcriptional responses when recognizing a potential nitrogen-fixing microsymbiont (Figure 1). First, the ancestor of nodulating plants gained increased sensitivity to lipo-chitooligosaccharides (LCOs, also named Nod factors) secreted by its nitrogen-fixing microsymbiont [6,7]. Increasing sensitivity to LCOs required adaptations in a specific LysM-type receptor named Nod Factor Receptor5 (LjNFR5) and Nod Factor Perception (MtNFP) in the legume models *Lotus japonicus* (lotus) and *Medicago truncatula* (medicago) [8,9]. This receptor is part of a larger family with other members involved in the more generic symbiosis between plants and arbuscular mycorrhizal (AM) fungi [10–12]. Adaptations in NFR5/NFP allow plants to activate the ancient arbuscular mycorrhizal signaling cascade when recognizing nitrogen-fixing microsymbionts. Second, nodulating plants acquired adaptations in the transcription factor Nodule Inception (NIN). These adaptations rewired this member of nitrate-responsive NIN-like proteins (NLPs) to be responsive to lipo-chitooligosaccharide (LCO) signaling (Figure 1). NFR5/NFP and NIN are critical for nodulation in both legumes and nonlegumes, whereas these genes are pseudogenized in nonnodulating relatives [2,3,13–17]. It suggests that in the nitrogen-fixing clade, both genes lost their original function to become committed to nodulation. Therefore, understanding how NFR5/NFP and NIN have been adapted to function in nodulation is crucial for our understanding of the evolution of this trait.

## Recruitment of lipo-chitooligosaccharide signaling

In plant species not part of the nitrogen-fixing clade, the occurrence of NFR5/NFP orthologs correlates with the capacity to establish an arbuscular mycorrhizal (AM) symbiosis, even though mutant phenotypes in AM symbiosis are generally weak [10–12,16]. AM fungi use a mixture of chitin oligosaccharides and LCOs to trigger symbiotic responses [18], and such LCOs can also be perceived by plant species outside the nitrogen-fixing clade [7]. Biochemical studies with tomato (*Solanum lycopersicum*) and *Petunia hybrida* NFR5/NFP orthologs

Figure 1



#### Schematic representation of the rhizobium LCO signaling pathway.

Two innovations (marked in red) occurred in the common ancestor of all nodulating plants. The arbuscular mycorrhizal symbiosis pathway was recruited by an increased sensitivity to LCOs associated with subneofunctionalization of the LysM-type receptor NFR5/NFP. The transcription factor NIN was rewired to be regulated by this now-common symbiosis signaling pathway (CSSP) instead of nitrate.

(SILYK10 and PhLYK10) reveal that the encoded receptors can indeed bind LCOs [19]. Furthermore, the petunia receptor can trans-complement the medicago *Mtnfp* and lotus *Ljnfr5* knockout mutants when over-expressed [19]. These findings demonstrate that the ancestral NFR5/NFP receptor already functioned as an LCO receptor, although the sensitivity of plant species outside the nitrogen-fixing clade to LCOs might be several magnitudes lower when compared to legumes, as illustrated in barley (*Hordeum vulgare*) [7].

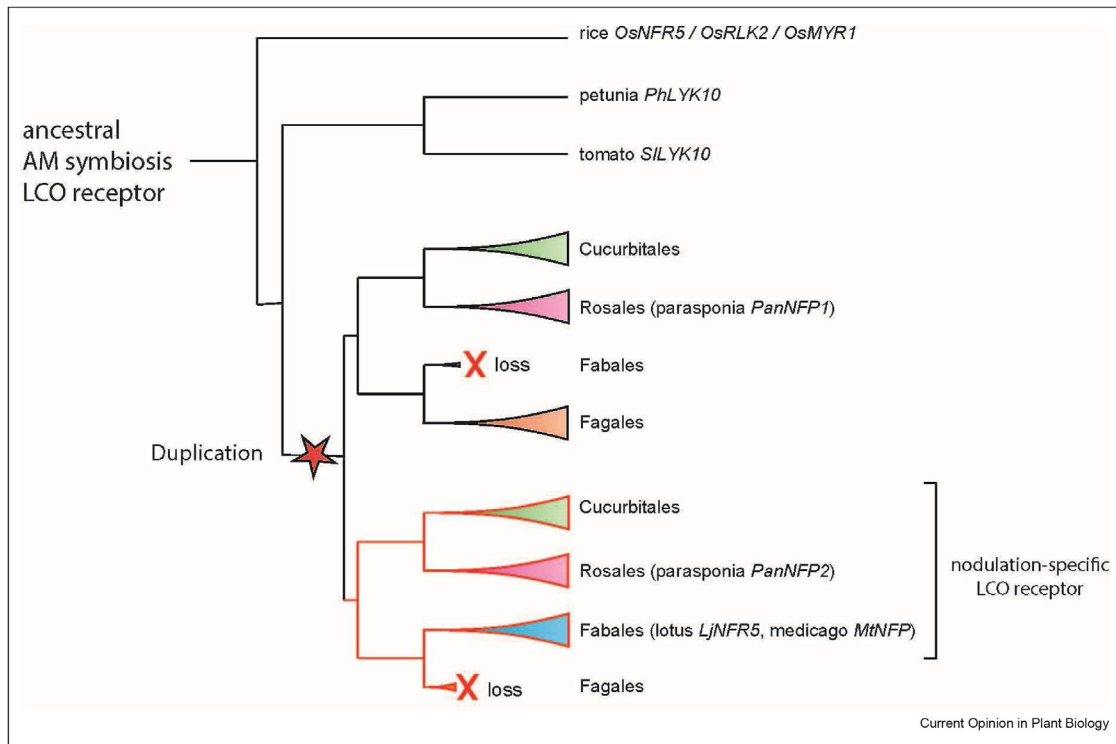
The ancestral NFR5/NFP gene experienced a duplication event in the common ancestor of the nitrogen-fixing clade (Figure 2) [16]. This provided freedom for subneofunctionalization, thereby shaping cis-regulatory transcriptional regulation and/or enhancing the LCO specificity of NFR5/NFP. Studies in *Parasponia andersonii* (parasponia), a nonlegume species that nodulates with rhizobium, on both *NFP* gene copies reveal that only a single gene -PanNFP2, is essential for nodulation, and a knockout mutation in this gene cannot be

complemented by introducing its paralog under the control of the PanNFP2 promoter [16]. Taken together, it shows that specific adaptations occurred in the protein after its duplication.

NFR5/NFP encodes a transmembrane receptor possessing three lysin motifs (LysM) in its extracellular domain and a cytoplasmic non-functional pseudokinase [8,9]. Biochemical and structural characterization of the MtNFP extracellular receptor domain revealed a compact cloverleaf structure where the three LysM domains are tightly interconnected and stabilized by disulfide bridges, a structural characteristic similar to other LysM-type receptor ectodomains [20]. Unique to NFR5/NFP is the presence of a hydrophobic patch in LysM2. This hydrophobic patch functions as a docking place for the acyl moiety of rhizobium-exuded LCOs, thereby enhancing the dwelling time of the LCO-receptor complex [20]. Binding experiments showed that MtNFP specifically binds LCOs that mimic those of *Sinorhizobium meliloti*, a rhizobial microsymbiont of medicago. These studies, together with genetic experiments in which lotus and medicago receptor variants were either altered or swapped [21], underline that NFR5/NFP LysM2 determines the recognition specificity of rhizobium LCOs.

NFR5/NFP acts in a heteromeric complex to enable LCO-induced cellular signaling. Two interacting transmembrane receptors have been identified: Symbiosis Receptor-like Kinase (SYMRK) and Nod Factor Receptor-like Kinase3 NFR1/LYK3 (Figure 1) [22–24]. The leucine rich repeats (LRR)-type SYMRK is critical for both nodulation and AM symbiosis, and SYMRK mutants are blocked in rhizobium-induced LCO signaling [25,26]. *NFR1/LYK3* encodes a LysM-type transmembrane receptor possessing a canonical intracellular kinase domain [8]. Functional studies in parasponia showed that this receptor also acts in chitin perception and AM symbiosis [16], revealing functional conservation with CERK-type receptors from plant species outside the nitrogen-fixing clade, such as rice (*Oryza sativa*) and tomato [27–29]. In legumes, *LjNFR1/MtLYK3* evolved upon tandem duplication of the ancestral CERK-type chitin elicitor receptor kinase and subsequent subneofunctionalization [30,31]. Structural and biochemical characterization of *LjNFR1/MtLYK3* and the CERK analogs *LjCERK6/MtLYK9* uncovered adaptations in the LysM1 domain of *LjNFR1/MtLYK3* as the determinant of ligand specificity [32]. CERK LysM1 is highly conserved and binds chitin oligomers, whereas LysM1 of the NFR1/LYK3 receptor is more variable between different legume species. Such variations may have evolved to allow the recognition of structural LCO variants [32]. Since the divergence of the CERK chitin receptor and the NFR1/LYK3 LCO receptor seems unique to legumes, adaptations in this receptor may not have contributed to the

Figure 2



**Nodulation-specific LCO receptors NFR5/NFP evolved upon gene duplication in the common ancestor of nodulating plants.** NFR5/NFP homologous receptors of species outside the nitrogen-fixing clade can perceive LCOs and function in AM symbiosis [10–12,19]. In the common ancestor of nodulating plants, this receptor duplicated (red asterisk), of which one gene copy evolved into a nodulation-specific LCO receptor [16]. Note the absence of NFR5/NFP LCO receptors in nodulating Fagales species that nodulate with Frankia bacteria that do not produce LCOs. Furthermore, legumes (Fabales) lost the closest *NFR5/NFP* paralogous gene.

initial recruitment of LCO signaling but are a legume-specific adaptation instead.

Forced heterodimerization of lotus *LjNFR5* and *LjNFR1* (or medicago *MtNFP* and *MtLYK3*) in legume roots using nanobody technology is sufficient to activate the nodule organogenesis program [33]. Strikingly, nodule formation can also be initiated upon forced heterodimerization of barley *NFR5/NFP* - CERK homologous receptors when expressed in lotus, underlining that the intracellular cytoplasmic domains of these receptors are functionally conserved in nodulating and nonnodulating species [33].

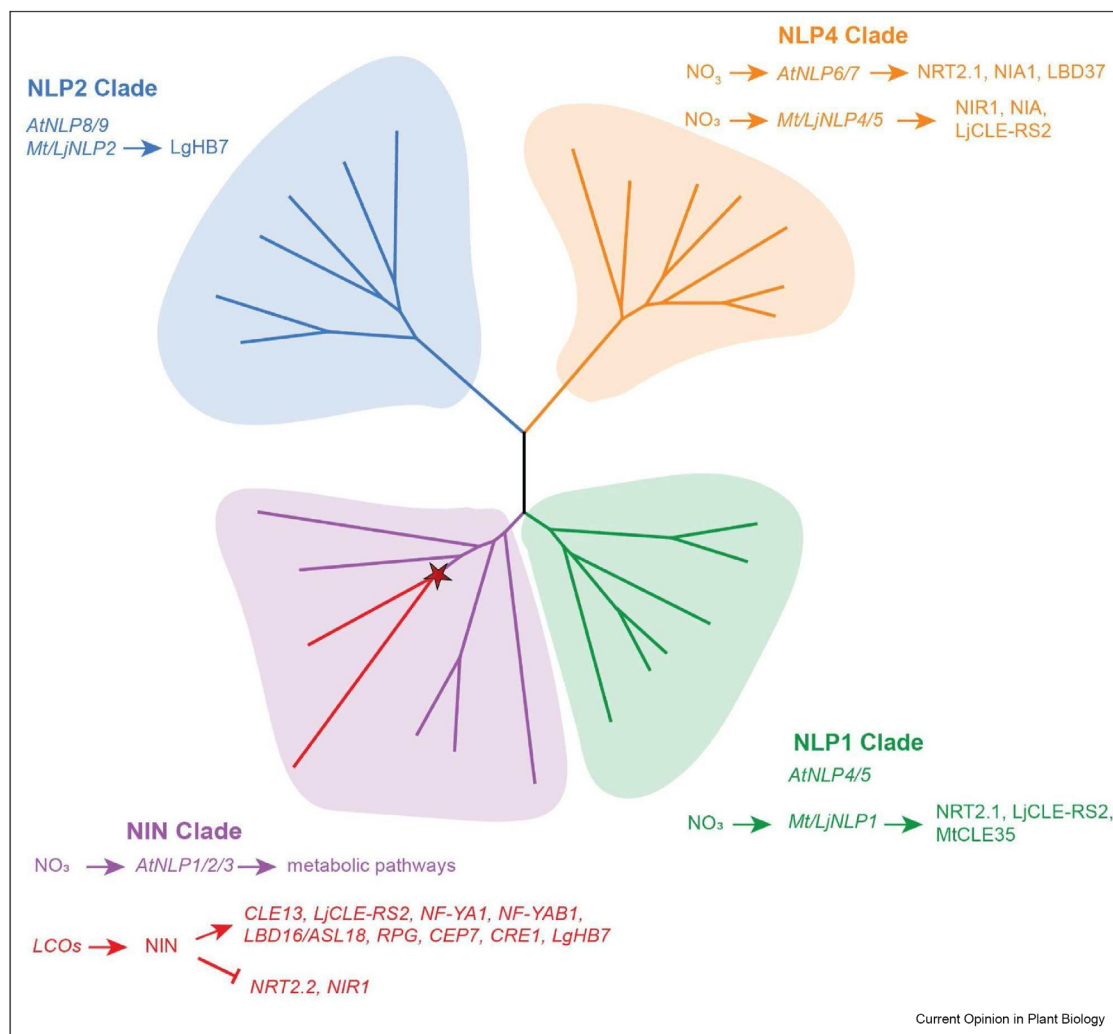
### Recruitment of NIN in nodulation

The second key event in the evolution of nodulation is the recruitment of the transcription factor NIN in a common ancestor of all nodulating plants. NIN is part of a small family of NLPs, of which most members are involved in primary responses towards nitrate [34–36] (Figure 3). The recruitment of NIN in symbiosis required adaptations to its promoter and functional changes to the protein. Most NLP genes are broadly expressed, and the protein is continuously exported from

the nucleus in the absence of nitrate [36,37]. At high nitrate concentrations, NLP proteins relocate to the nucleus [35,36,38,39]. As a result, NLPs are only active at elevated nitrate concentrations. The recruitment of NIN in nodulation coincided with adaptations in its N-terminus, resulting in its constitutive nuclear localization and activity irrespective of nitrate concentration [34,40,41]. Instead, the activity of symbiotic *NIN* depends on its transcriptional regulation and protein processing [17].

The evolution of the symbiosis-specific transcription of *NIN* comprises at least two adaptations in its promoter. First, it gained a cyclops-responsive element (CYC-RE or PACE) that is directly bound and activated by cyclops, the most downstream transcription factor of the signaling pathway shared between AM fungi and nodulating bacteria (known as the common symbiosis signaling pathway or CSSP) (Figure 1) [42,43]. The CYC-RE in the *NIN* promoter is conserved in nodulating species and may even have evolved before nodulation, as a putative CYC-RE was reported to be present in one *NIN* ortholog of poplar (*Populus* sp.) [44]. The CYC-RE controls LCO-dependent *NIN* expression in epidermal

Figure 3



**Recruitment of an NLP family member into nodulation.** The NLP family consists of 4 orthogroups in dicotyledonous plants. The subcellular localization of most NLP proteins is regulated by nitrate [35,36,67]. The recruitment of NIN, marked by red asterisks, involves a switch from this nitrate-controlled regulation to transcriptional regulation by LCO signaling. The targets that are directly and positively regulated by NIN [50,61,67–71] are markedly different from the direct targets that are positively regulated by other NLPs [37,67,72,73]. Therefore, the recruitment of NIN may have coincided with a switch in its target genes or an inversion of the direction in which they are regulated.

and cortical cells that guide bacterial infection progression [43,45,46]. Although the presence of the CYC-RE is sufficient for infection-associated *NIN* expression, studies in lotus showed that this *cis*-regulatory promoter element is not essential [46]. In legumes, the CYC-RE may act redundantly with another legume-specific adaptation in the *NIN* promoter. Nodule organogenesis requires the induction of *NIN* in the pericycle and inner cortex, for which legume *NIN* gained a cytokinin-response element-containing region (CE) in its promoter [45]. Perception of rhizobium LCOs in the epidermis activates a mobile signaling loop, allowing CE-dependent *NIN* expression in a wider range of cell layers. Given the presence of cytokinin-responsive elements in the CE region and the requirement for

cytokinin receptors LjHK1/MtCRE1 for nodule organogenesis in legumes, this mobile signal could be cytokinin, though solid evidence for this is yet lacking [45,47,48]. The CE region in the *NIN* promoter is not present outside the legume family [41], and in contrast to most legumes, cytokinin treatment does not trigger cortical cell divisions in nonlegume nodulating plants [49]. This suggests that the initial recruitment of NIN occurred with the evolution of its CYC-RE, whereas its activation in deeper cell layers may have evolved later.

### What is the point in recruiting NFR5/NFP and NIN for nodulation?

Recruitment of NFR5/NFP and *NIN* during the early evolution of nodulation indicates the trait is built on two

pre-existing genetic networks: AM symbiosis signaling (CSSP) and the primary nitrate response pathway. Presumably, recruiting these two networks allowed the diazotrophic bacteria to enter living plant cells and manipulate the plant's nitrogen signaling networks. Despite commonalities in both symbioses, there are surprisingly few shared transcriptional responses towards nitrogen-fixing bacteria, AM fungi, and nitrate that explain the recruitment of these two networks [50–52].

The initial driving force behind the evolution of LCO-driven nodulation may lie in the co-option of the AM symbiosis-related gene network controlled by the CSSP. In particular, some infection-related genes are required for both AM and rhizobium symbiosis; for example, the major sperm domain- and ankyrin-repeat-containing protein *VAPYRIN* (*VPY*), *VAMP721**de*, and a splice variant of *SYP132*, which function in membrane trafficking and biogenesis [53–55]. Additionally, the AP2-type transcription factor *ERN1* is a direct transcriptional target of the CSSP. *ERN1* and its paralog *MtERN2* in medicago are required for bacterial infection [56–58]. In contrast to *NIN*, the CYC-RE in the *ERN1* promoter is highly conserved outside the nitrogen-fixing clade, indicating it represents an ancestral target of the CSSP involved in controlling the microbial infection process [43].

Although several targets of *NIN* have been uncovered in the past decade that are important for nodulation in legumes, it remains an open question which of these targets are ancestral to the recruitment of *NIN* into nodulation. One target that is critical to allowing rhizobium infection is the coiled-coil protein rhizobium directed polar growth (RPG), which interacts with *VPY* and a U-box E3 ligase to control cellular polarization and bacterial intracellular infection [59–61]. Interestingly, the occurrence of a functional RPG gene within the nitrogen-fixing clade is associated with the nodulation trait [2,3]. It suggests that RPG acquired adaptations such that it functions exclusively in nodulation. Further, it implies that the recruitment of RPG already occurred in the common ancestor of all nodulating plants, suggesting it may be an early *NIN* target. This contrasts with other *NIN* targets, such as *MtLBD16/LjASL18*, whose *NIN*-responsive cis-regulatory element is unique to legumes [51,62]. One strategy to predict what the primary *NIN* targets were when it was recruited into nodulation is to consider which genes are targeted by *NIN* orthologs in species outside the nitrogen-fixing clade. *Arabidopsis thaliana* *AtNLP2*, one of these *NIN* orthologs, regulates a wide range of genes involved in nitrogen assimilation and metabolic pathways, with no apparent orthologs of symbiosis genes [36]. However, this strategy has limitations, as the targets and functions of different NLP orthogroups show substantial variation between species [35,36,63] (Figure 3). Furthermore, it should be noted that in legumes, *NIN* and

nitrate-induced signaling act antagonistically [50]. Therefore, the recruitment of *NIN* may have involved additional mutations to the protein that invert the direction in which target genes are regulated. In such a scenario, the point of recruiting *NIN* is not the co-option of a pre-existing nitrate response network but rather its suppression.

### Future perspective

Current genomic, genetic, and biochemical studies provide insights into the evolutionary trajectory of the nodulation trait. However, several unanswered questions remain surrounding LCO perception and *NIN* functioning. It still remains elusive which adaptations in *NFR5/NFP* are critical to allow high-affinity LCO signaling, or whether they have co-evolved with proteins in the receptor complex. Furthermore, many Actinorhizal plant species and a few legumes bypass LCO signaling to initiate nodule formation. First insights into the molecular mechanism underlying LCO-independent nodulation have been obtained in the legume *Aeschynomene evenia*, showing that the CSSP can be activated by a *Bradyrhizobium* strain-specific effector [64–66]. However, whether similar microbial effector-driven activation of the CSSP functions occur in the *Frankia*-Actinorhizal nodule symbiosis remains elusive. Additionally, it remains puzzling how *NIN* expression in the root cortex may have led to the onset of nodule formation, as there are no clear links between NLPs and organogenesis. Together with the legume-specific evolution of the CE-region in the *NIN* promoter, it may suggest that nodule organogenesis evolved in a lineage-specific fashion after the initial recruitment of *NIN*. Studies in plants other than legume models may be relevant to obtaining more comprehensive insights into the evolutionary trajectory of the nodulation trait.

### Declaration of competing interest

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### Data availability

No data was used for the research described in the article.

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## References

Papers of particular interest, published within the period of review, have been highlighted as:

\* of special interest

\*\* of outstanding interest

- Soltis DE, Soltis PS, Morgan DR, Swensen SM, Mullin BC, Dowd JM, Martin PG: **Chloroplast gene sequence data suggest a single origin of the predisposition for symbiotic nitrogen fixation in angiosperms.** *Proc Natl Acad Sci U S A* 1995, **92**: 2647–2651.
- Griesmann M, Chang Y, Liu X, Song Y, Haberer G, Crook MB, Billault-Penneteau B, Lauressergues D, Keller J, Imanishi L, *et al.*: **Phylogenomics reveals multiple losses of nitrogen-fixing root nodule symbiosis.** *Science* 2018, **361**:eaat1743.
- van Velzen R, Holmer R, Bu F, Rutten L, van Zeijl A, Liu W, Santuari L, Cao Q, Sharma T, Shen D, *et al.*: **Comparative genomics of the nonlegume *Parasponia* reveals insights into evolution of nitrogen-fixing rhizobium symbioses.** In *Proceedings of the National Academy of Sciences*; 2018. E4700–E4709. 115.
- van Velzen R, Doyle JJ, Geurts R, Resurrected Scenario A: **Single gain and massive loss of nitrogen-fixing nodulation.** *Trends Plant Sci* 2019, **24**:49–57.
- Shen D, Xiao TT, van Velzen R, Kulikova O, Gong X, Geurts R, Pawlowski K, Bisseling T: **A homeotic mutation changes legume nodule ontogeny into Actinorhizal-type ontogeny.** *Plant Cell* 2020, **32**:1868–1885.
- Lerouge P, Roche P, Faucher C, Maillet F, Truchet G, Promé JC, Dénarié J: **Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal.** *Nature* 1990, **344**:781–784.
- Li X-R, Sun J, Albinsky D, Zarrabian D, Hull R, Lee T, Jarratt-Barnham E, Chiu CH, Jacobsen A, Soumpourou E, *et al.*: **Nutrient regulation of lipochitooligosaccharide recognition in plants via NSP1 and NSP2.** *Nat Commun* 2022, **13**:6421.
- This study shows that barley can perceive rhizobium LCOs, although with a lower affinity when compared to medicago. The signaling response is nutrient-regulated via two GRAS-type transcription factors, which are also critical for nodulation.
- Radutoiu S, Madsen LH, Madsen EB, Felle HH, Umehara Y, Grønlund M, Sato S, Nakamura Y, Tabata S, Sandal N, *et al.*: **Plant recognition of symbiotic bacteria requires two LysM receptor-like kinases.** *Nature* 2003, **425**:585–592.
- Arrighi J-F, Barre A, Ben Amor B, Bersoult A, Soriano LC, Mirabella R, de Carvalho-Niebel F, Journet E-P, Gherardi M, Huguet T, *et al.*: **The *Medicago truncatula* lysine motif-receptor-like kinase gene family includes *NFP* and new nodule-expressed genes.** *Plant Physiol* 2006, **142**:265–279.
- Buendia L, Wang T, Girardin A, Lefebvre B: **The LysM receptor-like kinase *SILYK10* regulates the arbuscular mycorrhizal symbiosis in tomato.** *New Phytol* 2016, **210**:184–195.
- Miyata K, Hayafune M, Kobae Y, Kaku H, Nishizawa Y, Masuda Y, Shibuya N, Nakagawa T: **Evaluation of the role of the LysM receptor-like kinase, *OsNFR5/OsRLK2* for AM symbiosis in rice.** *Plant Cell Physiol* 2016, **57**:2283–2290.
- He J, Zhang C, Dai H, Liu H, Zhang X, Yang J, Chen X, Zhu Y, Wang D, Qi X, *et al.*: **A LysM receptor heteromer mediates perception of arbuscular mycorrhizal symbiotic signal in rice.** *Mol Plant* 2019, **12**:1561–1576.
- Schauser L, Roussis A, Stiller J, Stougaard J: **A plant regulator controlling development of symbiotic root nodules.** *Nature* 1999, **402**:191–195.
- Clavijo F, Diedhiou I, Vaissayre V, Brottier L, Acolatse J, Moukouanga D, Crabos A, Auguy F, Franche C, Gherbi H, *et al.*: **The *Casuarina NIN* gene is transcriptionally activated throughout *Frankia* root infection as well as in response to bacterial diffusible signals.** *New Phytol* 2015, **208**:887–903.
- Bu F, Rutten L, Roswanjaya YP, Kulikova O, Rodriguez-Franco M, Ott T, Bisseling T, van Zeijl A, Geurts R: **Mutant analysis in the nonlegume *Parasponia andersonii* identifies *NIN* and *NF-YA1* transcription factors as a core genetic network in nitrogen-fixing nodule symbioses.** *New Phytol* 2020, **226**:541–554.
- Rutten L, Miyata K, Roswanjaya YP, Huisman R, Bu F, Hartog M, Linders S, van Velzen R, van Zeijl A, Bisseling T, *et al.*: **Duplication of symbiotic lysin motif receptors predates the evolution of nitrogen-fixing nodule symbiosis.** *Plant Physiol* 2020, **184**:1004–1023.
- Feng J, Lee T, Schiessl K, Oldroyd GED: **Processing of NODULE INCEPTION controls the transition to nitrogen fixation in root nodules.** *Science* 2021, **374**:629–632.
- Study on posttranslational processing of the medicago *NIN* protein and its functioning in late-stage nodule symbiosis.
- Maillet F, Poinsot V, André O, Puech-Pagès V, Haouy A, Gueunier M, Cromer L, Giraudet D, Formey D, Niebel A, *et al.*: **Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza.** *Nature* 2011, **469**:58–63.
- Girardin A, Wang T, Ding Y, Keller J, Buendia L, Gaston M, Ribeyre C, Gascioli V, Auriac M-C, Vernié T, *et al.*: **LCO receptors involved in arbuscular mycorrhiza are functional for rhizobia perception in legumes.** *Curr Biol* 2019, **29**: 4249–4259.e5.
- Gysel K, Laursen M, Thygesen MB, Lironi D, Bozsóki Z, Hjulér CT, Maolanon NN, Cheng J, Bjørk PK, Vinther M, *et al.*: **Kinetic proofreading of lipochitooligosaccharides determines signal activation of symbiotic plant receptors.** In *Proceedings of the National Academy of Sciences*; 2021:118.
- This study revealed the structural characteristics of the NFR5/NFP ectodomain, identifying a hydrophobic patch that functions as docking place LCOs.
- Radutoiu S, Madsen LH, Madsen EB, Jurkiewicz A, Fukai E, Quistgaard EMH, Albrektsen AS, James EK, Thirup S, Stougaard J: **LysM domains mediate lipochitin-oligosaccharide recognition and *Nfr* genes extend the symbiotic host range.** *EMBO J* 2007, **26**:3923–3935.
- Broghammer A, Krusell L, Blaise M, Sauer J, Sullivan JT, Maolanon N, Vinther M, Lorentzen A, Madsen EB, Jensen KJ, *et al.*: **Legume receptors perceive the rhizobial lipochitin oligosaccharide signal molecules by direct binding.** *Proc Natl Acad Sci U S A* 2012, **109**:13859–13864.
- Antolín-Llovera M, Ried MK, Parniske M: **Cleavage of the SYMBIOSIS RECEPTOR-LIKE KINASE ectodomain promotes complex formation with Nod factor receptor 5.** *Curr Biol* 2014, **24**:422–427.
- Moling S, Pietraszewska-Bogiel A, Postma M, Fedorova E, Hink MA, Limpens E, Gadella TWJ, Bisseling T: **Nod factor receptors form heteromeric complexes and are essential for intracellular infection in medicago nodules.** *Plant Cell* 2014, **26**:4188–4199.
- Stracke S, Kistner C, Yoshida S, Mulder L, Sato S, Kaneko T, Tabata S, Sandal N, Stougaard J, Szczyglowski K, *et al.*: **A plant receptor-like kinase required for both bacterial and fungal symbiosis.** *Nature* 2002, **417**:959–962.
- Endre G, Kereszt A, Kevei Z, Mihacea S, Kaló P, Kiss GB: **A receptor kinase gene regulating symbiotic nodule development.** *Nature* 2002, **417**:962–966.
- Miyata K, Kozaki T, Kouzai Y, Ozawa K, Ishii K, Asamizu E, Okabe Y, Umehara Y, Miyamoto A, Kobae Y, *et al.*: **The bifunctional plant receptor, *OscERK1*, regulates both chitin-triggered immunity and arbuscular mycorrhizal symbiosis in rice.** *Plant Cell Physiol* 2014, **55**:1864–1872.
- Carotenuto G, Chabaud M, Miyata K, Capozzi M, Takeda N, Kaku H, Shibuya N, Nakagawa T, Barker DG, Genre A: **The rice LysM receptor-like kinase *OscERK1* is required for the perception of short-chain chitin oligomers in arbuscular mycorrhizal signaling.** *New Phytol* 2017, **214**:1440–1446.

29. Liao D, Sun X, Wang N, Song F, Liang Y: **Tomato LysM receptor-like kinase SILYK12 is involved in arbuscular mycorrhizal symbiosis**. *Front Plant Sci* 2018, **9**:1004.
30. De Mita S, Streng A, Bisseling T, Geurts R: **Evolution of a symbiotic receptor through gene duplications in the legume-rhizobium mutualism**. *New Phytol* 2014, **201**:961–972.
31. Bozsoki Z, Cheng J, Feng F, Gysel K, Vinther M, Andersen KR, Oldroyd G, Blaise M, Radutoiu S, Stougaard J: **Receptor-mediated chitin perception in legume roots is functionally separable from Nod factor perception**. *Proc Natl Acad Sci U S A* 2017, **114**:E8118–E8127.
32. Bozsoki Z, Gysel K, Hansen SB, Lironi D, Krönauer C, Feng F, de Jong N, Vinther M, Kamble M, Thygesen MB, et al.: **Ligand-recognizing motifs in plant LysM receptors are major determinants of specificity**. *Science* 2020, **369**:663–670.
33. Rübsam H, Krönauer C, Abel NB, Ji H, Lironi D, Hansen SB, Nadzieja M, Kolte MV, Abel D, de Jong N, et al.: **Nanobody-driven signaling reveals the core receptor complex in root nodule symbiosis**. *Science* 2023, **379**:272–277.
- In legumes, it is shown that spontaneous nodulation can be induced upon 'forced' dimerization of NFR1/LYK3 and NFR5/NFP-type receptors. This response can also be obtained when exploring barley receptor homologs in lotus, indicating that downstream signaling doesn't require specific adaptations in the intracellular receptor domains.
34. Liu J, Bisseling T: **Evolution of NIN and NIN-like genes in relation to nodule symbiosis**. *Genes* 2020, **11**.
35. Liu K-H, Liu M, Lin Z, Wang Z-F, Chen B, Liu C, Guo A, Konishi M, Yanagisawa S, Wagner G, et al.: **NIN-like protein 7 transcription factor is a plant nitrate sensor**. *Science* 2022, **377**:1419–1425.
- The study shows that Arabidopsis NLP7 directly binds nitrate through residues in its N-terminus, a domain that is evolutionarily conserved in NLPs. By making chimaeric fusion proteins between LjNIN and AtNLP7, it is shown that, unlike the N-terminus of AtNLP7, the N-terminus of LjNIN is active in the absence of nitrate.
36. Durand M, Brehaut V, Clement G, Kelemen Z, Macé J, Feil R, Duville G, Launay-Avon A, Roux CP-L, Lunn JE, et al.: **The Arabidopsis transcription factor NLP2 regulates early nitrate responses and integrates nitrate assimilation with energy and carbon skeleton supply**. *Plant Cell* 2023, **35**:1429–1454.
- One of the Arabidopsis NIN orthologs, AtNLP2, is studied, and its function is compared to that of AtNLP7. Like *Atnlp7*, *Atnlp2* mutants show a severe growth defect when grown at high nitrate concentrations. A transcriptomics approach showed that AtNLP7 and AtNLP2 activate an overlapping and unique set of genes. The genes that are uniquely regulated by AtNLP2 include genes involved in metabolic pathways, suggesting that AtNLP2 links nitrate assimilation to carbon metabolism.
37. Marchive C, Roudier F, Castaings L, Bréhaut V, Blondet E, Colot V, Meyer C, Krapp A: **Nuclear retention of the transcription factor NLP7 orchestrates the early response to nitrate in plants**. *Nat Commun* 2013, **4**:1713.
38. Lin J-S, Li X, Luo Z, Mysore KS, Wen J, Xie F: **NIN interacts with NLPs to mediate nitrate inhibition of nodulation in Medicago truncatula**. *Nat Plants* 2018, **4**:942–952.
39. Liu J, Rasing M, Zeng T, Klein J, Kulikova O, Bisseling T: **NIN is essential for development of symbiosomes, suppression of defence and premature senescence in Medicago truncatula nodules**. *New Phytol* 2021, **230**:290–303.
- In this study, it is shown that MtNIN constitutively localizes to the nucleus of nodule cells. Weak NINmutant alleles are described that can form nodules. The lack of symbiosome differentiation and early senescence of these nodules demonstrate the role of NIN in the later stages of nodule development.
40. Suzuki W, Konishi M, Yanagisawa S: **The evolutionary events necessary for the emergence of symbiotic nitrogen fixation in legumes may involve a loss of nitrate responsiveness of the NIN transcription factor**. *Plant Signal Behav* 2013, **8**.
41. Zhang Y, Fu Y, Xian W, Li X, Feng Y, Bu F, Shi Y, Chen S, van Velzen R, Berry AM, et al.: **Comparative phylogenomics and phylotranscriptomics provide insights into the genetic complexity of nitrogen fixing root nodule symbiosis**. *bioRxiv* 2023, <https://doi.org/10.1101/2023.04.03.535273>.
42. Singh S, Katzer K, Lambert J, Cerri M, Parniske M: **CYCLOPS, a DNA-binding transcriptional activator, orchestrates symbiotic root nodule development**. *Cell Host Microbe* 2014, **15**:139–152.
43. Cathebras C, Gong X, Andrade RE, Vondenhoff K, Keller J, Delaux P-M, Hayashi M, Griesmann M, Parniske M: **A novel cis-element enabled bacterial uptake by plant cells**. *bioRxiv* 2022, <https://doi.org/10.1101/2022.03.28.486070>.
44. Irving TB, Chakraborty S, Maia LGS, Knaack S, Conde D, Schmidt HW, Triozzi PM, Simmons CH, Roy S, Kirst M, et al.: **An LCO-responsive homolog of NODULE INCEPTION positively regulates lateral root formation in Populus sp.** *Plant Physiol* 2022, **190**:1699–1714.
- The study focuses on the role of NIN orthologs in poplar, a plant species representing an outgroup of the nitrogen-fixing clade. A putative CYC-RE is identified in the *PtNIN1a* promoter region. Also, it is found that the expressions *PtNIN2a*, *PtNINb*, and *PtNINc* are LCO-responsive. Overexpression of *PtNIN2b* increases the lateral root density, linking LCO perception to lateral root organ formation prior to the evolution of nodulation.
45. Liu J, Rutten L, Limpens E, van der Molen T, van Velzen R, Chen R, Chen Y, Geurts R, Kohlen W, Kulikova O, et al.: **A remote cis-regulatory region is required for NIN expression in the pericycle to initiate nodule primordium formation in Medicago truncatula**. *Plant Cell* 2019, **31**:68–83.
46. Akamatsu A, Nagae M, Takeda N: **The CYCLOPS response element in the NIN promoter is important but not essential for infection thread formation during Lotus japonicus rhizobia symbiosis**. *Mol Plant Microbe Interact* 2022, **35**:650–658.
47. Tirichine L, Sandal N, Madsen LH, Radutoiu S, Albrechtsen AS, Sato S, Asamizu E, Tabata S, Stougaard J: **A gain-of-function mutation in a cytokinin receptor triggers spontaneous root nodule organogenesis**. *Science* 2007, **315**:104–107.
48. Gonzalez-Rizzo S, Crespi M, Frugier F: **The Medicago truncatula CRE1 cytokinin receptor regulates lateral root development and early symbiotic interaction with Sinorhizobium meliloti**. *Plant Cell* 2006, **18**:2680–2693.
49. Gauthier-Coles C, White RG, Mathesius U: **Nodulating legumes are distinguished by a sensitivity to cytokinin in the root cortex leading to pseudonodule development**. *Front Plant Sci* 2018, **9**:1901.
50. Soyano T, Shimoda Y, Hayashi M: **NODULE INCEPTION antagonistically regulates gene expression with nitrate in Lotus japonicus**. *Plant Cell Physiol* 2015, **56**:368–376.
51. Schiessl K, Lilley JLS, Lee T, Tamvakis I, Kohlen W, Bailey PC, Thomas A, Luptak J, Ramakrishnan K, Carpenter MD, et al.: **NODULE INCEPTION recruits the lateral root developmental program for symbiotic nodule organogenesis in medicago truncatula**. *Curr Biol* 2019, **29**:3657–3668.e5.
52. Libourel C, Keller J, Brichet L, Cazalé A-C, Carrère S, Vernié T, Couzigou J-M, Callot C, Dufau I, Cauet S, et al.: **Comparative phylotranscriptomics reveals ancestral and derived root nodule symbiosis programmes**. *Nat Plants* 2023, <https://doi.org/10.1038/s41477-023-01441-w>.
- Nodule expression data across the nitrogen-fixing clade are compared to reconstruct the nodule transcriptome of the common ancestor of nitrogen-fixing plants.
53. Murray JD, Muni RRD, Torres-Jerez I, Tang Y, Allen S, Andriankaja M, Li G, Laxmi A, Cheng X, Wen J, et al.: **Vapyrin, a gene essential for intracellular progression of arbuscular mycorrhizal symbiosis, is also essential for infection by rhizobia in the nodule symbiosis of Medicago truncatula**. *Plant J* 2011, **65**:244–252.
54. Ivanov S, Fedorova EE, Limpens E, De Mita S, Genre A, Bonfante P, Bisseling T: **Rhizobium-legume symbiosis shares an exocytotic pathway required for arbuscule formation**. In *Proceedings of the National Academy of Sciences*; 2012: 8316–8321. 109.
55. Huisman R, Hontelez J, Mysore KS, Wen J, Bisseling T, Limpens E: **A symbiosis-dedicated SYNTAXIN OF PLANTS 131l isoform controls the formation of a stable host-microbe interface in symbiosis**. *New Phytol* 2016, **211**:1338–1351.

56. Cerri MR, Frances L, Kelner A, Fournier J, Middleton PH, Auriac M-C, Mysore KS, Wen J, Erard M, Barker DG, *et al.*: **The symbiosis-related ERN transcription factors act in concert to coordinate rhizobial host root infection.** *Plant Physiol* 2016, **171**, 00230. 2016.
57. Cerri MR, Wang Q, Stolz P, Folgmann J, Frances L, Katzer K, Li X, Heckmann AB, Wang TL, Downie JA, *et al.*: **The ERN1 transcription factor gene is a target of the CCaMK/CYCLOPS complex and controls rhizobial infection in Lotus japonicus.** *New Phytol* 2017, **215**:323–337.
58. Kawaharada Y, James EK, Kelly S, Sandal N, Stougaard J: **The ethylene responsive factor required for nodulation 1 (ERN1) transcription factor is required for infection-thread formation in Lotus japonicus.** *Mol Plant Microbe Interact* 2017, **30**: 194–204.
59. Liu C-W, Breakspear A, Stacey N, Findlay K, Nakashima J, Ramakrishnan K, Liu M, Xie F, Endre G, de Carvalho-Niebel F, *et al.*: **A protein complex required for polar growth of rhizobial infection threads.** *Nat Commun* 2019, **10**:2848.
60. Lace B, Su C, Invernout Perez D, Rodriguez-Franco M, Vernié T, Batzenschlager M, Egli S, Liu C-W, Ott T: **RPG acts as a central determinant for infectiousome formation and cellular polarization during intracellular rhizobial infections.** *Elife* 2023, **12**.
- In this study, the function of RPG in controlling focal infection thread growth in medicago is demonstrated. RPG co-localizes with other infectiousome components, VPY, LIN/(Lj)CERBERUS, and EXO70H4, at foci in the infection thread tip. It is shown that RPG is required for the localization of VPY to these foci, maintaining the cytoskeleton connection between the infection thread tip and nucleus, the polar secretion of NPL, and the lipid composition of the infection thread membrane. Altogether, this paper shows that RPG is a crucial regulator of polarized exocytosis at the infection thread tip.
61. Li X, Liu M, Cai M, Chiasson D, Groth M, Heckmann AB, Wang TL, Parniske M, Downie JA, Xie F: **RPG interacts with E3-ligase CERBERUS to mediate rhizobial infection in Lotus japonicus.** *PLoS Genet* 2023, **19**, e1010621.
- This study in lotus shows that the RPG gene is a direct target of NIN and that the RPG protein interacts with CERBERUS in the early endosome compartment, where it may manipulate the exocytotic pathway to facilitate infection thread growth.
62. Soyano T, Shimoda Y, Kawaguchi M, Hayashi M: **A shared gene drives lateral root development and root nodule symbiosis pathways in Lotus.** *Science* 2019, **366**:1021–1023.
63. Misawa F, Ito M, Nosaki S, Nishida H, Watanabe M, Suzuki T, Miura K, Kawaguchi M, Suzuki T: **Nitrate transport via NRT2.1 mediates NIN-LIKE PROTEIN-dependent suppression of root nodulation in Lotus japonicus.** *Plant Cell* 2022, **34**:1844–1862.
- Insight is provided into the overlapping functions of LjNLP1 and LjNLP4. The authors show that in lotus, LjNLP1 but not LjNLP4 is required for the induction of the nitrate transporter LjNRT2.1. Both LjNLP1 and LjNLP4 are positioned upstream of the nitrate regulation of LjNLP4.
64. Teulet A, Busset N, Fardoux J, Gully D, Chaintreuil C, Cartieaux F, Jauneau A, Comorge V, Okazaki S, Kaneko T, *et al.*: **The rhizobial type III effector ErnA confers the ability to form nodules in legumes.** In *Proceedings of the National Academy of Sciences U S A*; 2019, <https://doi.org/10.1073/pnas.1904456116>.
65. Quilbé J, Lamy L, Brottier L, Leleux P, Fardoux J, Rivallan R, Benichou T, Guyonnet R, Becana M, Villar I, *et al.*: **Genetics of nodulation in Aeschynomene evenia uncovers mechanisms of the rhizobium–legume symbiosis.** *Nat Commun* 2021, **12**: 1–14.
- A Comparative genomics study of the *Aeschynomene* genus to provide insight into the conserved elements in the symbiotic signaling network associated with LOC-independent nodulation.
66. Quilbé J, Nouwen N, Pervent M, Guyonnet R, Cullimore J, Gressent F, Araújo NH, Gully D, Klopp C, Giraud E, *et al.*: **A mutant-based analysis of the establishment of Nod-independent symbiosis in the legume Aeschynomene evenia.** *Plant Physiol* 2022, <https://doi.org/10.1093/plphys/kiac325>.
- Reverse genetic study in *Aschynomene evenia* to provide insights on the modus operandi of LCO-independent nodulation.
67. Jiang S, Jardinaud M-F, Gao J, Pecrix Y, Wen J, Mysore K, Xu P, Sanchez-Canizares C, Ruan Y, Li Q, *et al.*: **NIN-like protein transcription factors regulate leghemoglobin genes in legume nodules.** *Science* 2021, **374**:625–628.
- This study in medicago links MtNLP2 to nodulation. It is shown that *MtNLP2* is expressed in nodules, where it is, together with NIN, required for the expression of leghemoglobins. A double nitrate response cis-regulatory element (dNRE) is described, which is bound by both MtNIN and MtNLP2.
68. Soyano T, Kouchi H, Hirota A, Hayashi M: **Nodule inception directly targets NF-Y subunit genes to regulate essential processes of root nodule development in Lotus japonicus.** *PLoS Genet* 2013, **9**, e1003352.
69. Laffont C, Ivanovici A, Gautrat P, Brault M, Djordjevic MA, Frugier F: **The NIN transcription factor coordinates CEP and CLE signaling peptides that regulate nodulation antagonistically.** *Nat Commun* 2020, **11**:3167.
70. Soyano T, Hirakawa H, Sato S, Hayashi M, Kawaguchi M: **Nodule inception creates a long-distance negative feedback loop involved in homeostatic regulation of nodule organ production.** *Proc Natl Acad Sci U S A* 2014, **111**:14607–14612.
71. Vernié T, Kim J, Frances L, Ding Y, Sun J, Guan D, Niebel A, Gifford ML, de Carvalho-Niebel F, Oldroyd GED: **The NIN transcription factor coordinates diverse nodulation programs in different tissues of the Medicago truncatula root.** *Plant Cell* 2015, **27**, 00461. tpc.15.
72. Nishida H, Nosaki S, Suzuki T, Ito M, Miyakawa T, Nomoto M, Tada Y, Miura K, Tanokura M, Kawaguchi M, *et al.*: **Different DNA-binding specificities of NLP and NIN transcription factors underlie nitrate-induced control of root nodulation.** *Plant Cell* 2021, **33**:2340–2359.
73. Luo Z, Moreau C, Wang J, Frugier F, Xie F: **NLP1 binds the CEP1 signalling peptide promoter to repress its expression in response to nitrate.** *New Phytol* 2022, **234**:1547–1552.