About how nitrate controls nodulation: will soybean spill the bean?

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Abstract

Legumes have the beneficial capacity to establish symbiotic interactions with rhizobia, which provide their host plants with fixed nitrogen. However, in the presence of nitrogen, this process is rapidly repressed to avoid unnecessary investments of carbon in the symbiosis. Several players involved in regulating nodulation in response to nitrate availability have been identified, including peptide hormones, microRNAs and transcription factors. Nevertheless, how these molecular players are linked to each other and what underlying molecular mechanisms are at play to inhibit nodulation remain unresolved. Nitrate-mediated control of nodulation seems to differ between model legumes, such as *Medicago* and *Lotus*, compared to legume crops such as soybean. A deeper understanding of these regulatory processes, particularly in soybean, is expected to contribute to establishing increased nodulation efficiency in modern agricultural systems, hence improving sustainability by reducing the need for environmentally hazardous nitrogen fertilizers. This review describes the state of the art of nitrate-regulated nodulation in soybean, while drawing parallels with molecular mechanisms described in other legumes and addressing knowledge gaps that require future study.

Keywords

Soybean, nodulation, nitrate, sustainability, CLE peptide, CEP peptide, NLP transcription factors, microRNA.

1. Introduction

Nitrogen is one of the essential elements for plant growth, as it is required for the synthesis of macromolecules such as proteins, nucleic acids, hormones and vitamins. Plants are autotrophic and obtain their nitrogen via the uptake of mainly nitrate and ammonium, molecules that are often limiting in soils (Masclaux-Daubresse et al., 2010). As a result, farmers manure their field to optimize plant productivity and increase crop yield. The development of nitrogen fertilizers through the energy-consuming Haber-Bosch process, in which dinitrogen (N_2) gas is converted to ammonia, fuelled this practice since the green revolution. This caused a huge increase in crop yield, reinforcing our ability to nourish cattle and the increasing human population (Udvardi, Brodie, Riley, Kaeppler, & Lynch, 2015). It is now clear that this invention has a considerable environmental cost. The ample application of nitrogen fertilizers, consisting of nitrate, ammonia or urea, impacts the microbial community diversity and thus the nitrogen cycle in soil (Chen et al., 2019; Y. Li et al., 2023; Swify, Mažeika, Baltrusaitis, Drapanauskaitė, & Barčauskaitė, 2024) (Figure 1). Soil bacteria have the capacity to interconvert different forms of nitrogen, for example nitrifying bacteria are able to convert ammonia into nitrate (Bernhard, 2010). However, excess of nitrate leaches into groundwater and pollutes water streams, causing algae blooms, negatively affecting the aquatic biodiversity (Padilla, Gallardo, & Manzano-Agugliaro, 2018). Furthermore, denitrifying bacteria can convert nitrate in N_2 gas and during this process nitrous oxide (N_2O) – a potent greenhouse gas – is released. In short, up to 50% of applied nitrogen fertilizer is lost, either via leaching or release to the atmosphere as greenhouse gases (Ferguson et al., 2019).

Improving sustainability in agriculture involves reducing the use of nitrogen fertilizers, and developing and applying more environmentally friendly nitrogen inputs (Ferguson et al., 2010). Legumes have the capacity to establish symbiotic interactions with diazotrophic rhizobia inside newly formed root organs, the nodules. In these nodules, rhizobia convert atmospheric N_2 into ammonium, which serves as a nitrogen source for the host plant (Figure 1). In return, the host plant provides the bacteria with reduced carbon sources, mainly malate, to provide energy for nitrogen fixation (Banasiak, Jamruszka, Murray, & Jasiński, 2021). Insights into root nodule symbiosis (RNS) have extensively been gained through studies on model legumes such as *Medicago truncatula* (*M. truncatula*) and *Lotus japonicus* (*L. japonicus).* This review however focuses on soybean, which is an important legume crop for food, feed and oil production and thus holds potential for applications in the field. Currently, soybean is the most commonly cultivated legume, with a global production of 349 million tonnes in 2022 (Ritchie, 2021). The annual amount of nitrogen fixed by soybean amounts to 16.4 Tg, representing 77% of the nitrogen fixed by legume crops (Herridge, Peoples, & Boddey, 2008). Furthermore, soybean seeds are characterized by their high protein content (35-45%) and rich amino acid composition, indicative of their significant nutritional quality (Kudełka, Kowalska, & Popis, 2021; Sharma, Kaur, Goyal, & Gill, 2014). For the production of seed proteins, high nitrogen requirements need to be fulfilled. Therefore, enhanced nodulation efficiency in combination with optimized transport of nitrogen compounds from nodule to shoot could contribute to increased seed yield in a sustainable manner (Lu, Carter, & Tegeder, 2022).

1.1 The legume–rhizobia symbiosis

The establishment of RNS is initiated by the attraction of rhizobia to plant roots, followed by reciprocal signal exchange and recognition. Rhizobia recognize root exudates, such as flavonoids, and in response they produce strain-specific lipo-chitooligosaccharides, known as nodulation factors (NFs) (Caetano-Anollés & Gresshoff, 1991; Dénarié, Debellé, & Promé, 1996; Ferguson et al., 2019; Spaink, 2000). NFs are perceived by plant receptor-like kinases and induce calcium (Ca^{2+}) spiking. Subsequently, a Ca^{2+} and calmodulin-dependent protein kinase (CCaMK) activates several transcription factors (TFs), including NODULATION SIGNALLING PATHWAY (NSP) 1 (Smit et al., 2005), NSP2 (Kaló et al., 2005), ETHYLENE RESPONSE FACTOR (ERF) REQUIRED FOR NODULATION (ERN) (Middleton et al., 2007) and NODULE INCEPTION (NIN) (Borisov et al., 2003; Schauser, Roussis, Stiller, & Stougaard, 1999). These TFs induce transcriptional changes in a spatiotemporal way to elicit nodule primordium formation via the re-initiation of cortical cell divisions and to stimulate bacterial infection (Cervantes-Pérez et al., 2024; Ferguson et al., 2010; Gage, 2004; Pereira et al., 2024).

A key TF involved in nodule initiation, development and functioning is NODULE INCEPTION (NIN) (Fu, Sun, Li, Guan, & Xie, 2022; Schauser et al., 1999; Schiessl et al., 2019; Shen & Feng, 2024). The NIN-like protein (NLP) TFs are associated with nitrate-regulated gene expression and are presumably present in all land plants (M Konishi & Yanagisawa, 2013; Suzuki, Konishi, & Yanagisawa, 2013). However, in contrast to NLPs, the N-terminal domain of NIN is not responsive to nitrate, which might have played a role in the establishment of RNS in legumes during evolution (Suzuki et al., 2013). The spatiotemporal expression of NIN is tightly controlled because of its indispensable role in all steps of the nodulation process (J. Liu & Bisseling, 2020). NIN-regulated genes include early nodulation genes, such as genes encoding NUCLEAR FACTOR-Y SUBUNIT A1 (NF-YA1), NF-YB1, NODULATION PECTATE LYASE (NPL) and CYTOKININ RESPONSE 1 (CRE1), as well as late nodulation genes, including those coding for leghaemoglobins, thioredoxins, nodule-specific cysteine-rich (NCR) peptides and glycine-rich peptides (Shen & Feng, 2024). Among the early nodulation genes, *NF-YA1* is an important factor that promotes nodulation through facilitation of rhizobium infection and induction of *EARLY NODULIN 40* (*ENOD40*), a symbiotic signaling gene involved in primordium formation (Combier et al., 2006; Kereszt, Mergaert, Montiel, Endre, & Kondorosi, 2018). *NF-YA1* levels are also post-transcriptionally controlled by miRNA169, which represents an important regulatory module in nodule number control (Combier et al., 2006; Xu et al., 2021).

During infection, root hairs will curl to encapsulate the bacteria, which will penetrate the cell wall and induce invagination of the cell membrane. This results in the formation of the infection thread (IT), through which the bacteria will move to the root cortex, where they are released in the newly formed cells by an endocytosis-like process. During the uptake, the bacteria are surrounded by a plant-derived membrane, the peribacteroid membrane, forming the symbiosome. Then the bacteria differentiate into bacteroids and start to fix nitrogen. Alternatively, in some interactions, bacteria enter plant roots through cracks in the epidermal tissue, although this is a less common infection mechanism (Ferguson et al., 2010; Oldroyd & Downie, 2008). Interestingly, some bacteria do not use NFs, but the type III secretion system and accompanying effectors to enter the host plant (Camuel et al., 2023; Jiménez-Guerrero, Medina, Vinardell, Ollero, & López-Baena, 2022; Ratu, Amelia, & Okazaki, 2023).

Clearly, the establishment of RNS is achieved by a complex interplay involving reciprocal exchange and recognition of signaling molecules and phytohormone action. Cytokinins (CKs) are major players in nodule inception, regulation of rhizobium infection and nodule organogenesis (Gamas, Brault, Jardinaud, & Frugier, 2017). The activation of the NF signaling pathway rapidly induces CK accumulation and together with the NIN-dependent induction of the CRE1 CK receptor, this leads to cortical cell divisions for nodule primordia formation. At the same time, CK is also a negative regulator of epidermal infection, putatively by enhancing ethylene biosynthesis (Velandia, Reid, & Foo, 2022). Ethylene is generally known as a negative regulator of nodulation, inhibiting IT growth (Guinel, 2015; Prayitno, Rolfe, & Mathesius, 2006). Furthermore, ethylene controls the radial positioning of nodule primordia formation (Heidstra et al., 1997). Auxin is generally known as a nodulation-promoting phytohormone, it acts synergistically with CK to promote primordium formation. In the epidermis, auxin positively influences infection progression, acting antagonistically with CK (Jieshun Lin, Frank, & Reid, 2020).

Although the RNS benefits the host plant through providing plant-available nitrogen, nodule development is linked to a high energy cost, payed in the form of photosynthates. The nitrogen fixation process, executed by the bacterial nitrogenase enzyme, is energy intensive, requiring 16 moles of ATP and eight moles of electrons to fix one mole of N_2 (Hoffman, Lukoyanov, Yang, Dean, & Seefeldt, 2014; Seefeldt, Hoffman, & Dean, 2009). Furthermore, the engagement with a symbiont represents a risk in terms of distinguishing it from pathogenic organisms. Hence, nodulation is tightly controlled, internal and external stimuli are integrated for an optimal balance between benefits and costs. The central regulator of nodulation, NIN, is involved in regulating nodule numbers by coordinating the expression of the antagonistic peptide hormones C-TERMINALLY ENCODED PEPTIDE (CEP) and CLAVATA3/EMBRYO SURROUNDING REGION-RELATED (CLE), which respectively promote and inhibit nodulation (Laffont et al., 2020). These peptides are secreted in the apoplast and function non-cell autonomously through interaction with their respective membrane-associated receptors (Gautrat, Laffont, & Frugier, 2020; Lim, Lee, Lee, & Hwang, 2014; Reid, Ferguson, & Gresshoff, 2011). An important regulatory mechanism, called autoregulation of nodulation (AON), represents systemic negative feedback signaling in response to the presence of rhizobia. Rhizobia induce, via NIN, the production of RHIZOBIA-INDUCED CLE (RIC) peptides, activating AON and thereby limiting the establishment of new interactions. Besides this internal control, the presence of nitrogen in the soil or environmental stresses, such as phosphate deficiency and drought stress, also reduce nodulation (Chaudhary et al., 2008; Ferguson et al., 2019; Lira Jr, Nascimento, & Fracetto, 2015; Qin et al., 2012; Zahran, 1999).

Eventually, after a period of functioning during which nutrient exchanges are taking place, the nodule will enter a senescence process. This is characterized by a declined N_2 fixation, element recycling and the coordinated death of both bacteria and plant cells. The senescence process is either resulting from nodule aging (developmental senescence), or triggered prematurely by adverse environmental conditions, for example darkness, drought, salt or excess in nitrogen (Kazmierczak et al., 2020; S. Zhou et al., 2021).

1.2 Choosing is losing: direct nitrogen uptake versus symbiotic engagement

Three forms of nitrogen are commonly used in fertilizers, namely nitrate, ammonium and urea, and they are all directly available for plants through uptake via specific transporter families. For nitrate, low-affinity transporters are generally member of the NITRATE TRANSPORTER (NRT) 1 family, whereas NRT2s represent high-affinity transporters (Fan et al., 2017). Similarly for urea, the plant MAJOR INTRINSIC PROTEINS (MIPs) and DEGRADATION OF UREA 3 (DUR3) orthologs represent low- and high-affinity transporters respectively (W.-H. Wang, Köhler, Cao, & Liu, 2008). Ammonium uptake by plants is regulated by the AMMONIUM TRANSPORTER (AMT) family, which can be subdivided into two subfamilies based on sequence similarity: AMT1 and AMT2 (von Wittgenstein, Le, Hawkins, & Ehlting, 2014). Nitrogen uptake and symbiosis are closely linked to fulfil the plant's nitrogen needs. NRTs are regulated by CEP peptides (Paragraph 2.2) and NLP TFs (Paragraph 4.1), allowing them to regulate nodulation under different nitrogen availabilities (Luo et al., 2023). In soybean, the majority of the GmAMT members are regulated by GmNINa, suggesting a role in symbiosis (Yang et al., 2023).

Because the energy cost for nitrogen uptake is minimal compared to the cost for RNS, the presence of high nitrogen levels in the soil inhibits nodulation (Nishida et al., 2021; Nishida & Suzaki, 2018a; Ryle G. J. A., 1979). Hence, deeper insights into how nitrogen affects nodulation in soybean will contribute to the development of strategies to reduce nitrogen fertilizer use and improve sustainability in agriculture. Interestingly, the response depends on the source of nitrogen. In general, nitrate and ammonia have strong inhibitory effects on nodulation, while urea does not (Ferguson et al., 2019). Additionally, downstream molecular effects differ between nitrate- and ammonium-induced inhibition of nodulation. For example, although NLPs inhibit nodulation in response to nitrate, they are not responsive to ammonium (J.-s. Lin et al., 2018; Nishida et al., 2018). For soybean specifically, a milder effect was reported for ammonium, urea and glutamine as compared to nitrate (Yamashita et al., 2019). Therefore, this review addresses primarily how nitrate impacts nodulation.

The effect of nitrate on RNS depends on its concentration; low nitrate concentrations generally promote nodulation, while high nitrate levels are inhibitory (Barbulova, Rogato, D'Apuzzo, Omrane, & Chiurazzi, 2007; Bollman & Vessey, 2006; Ferguson et al., 2019; Gan, Stulen, van Keulen, & Kuiper, 2004). The enhancement of nodulation by low levels of nitrate likely occurs by promoting plant health without being sufficient to make symbiotic nitrogen fixation redundant (Ferguson et al., 2019). Both soil nitrogen and symbiotic nitrogen are required for optimal soybean production, since poor growth and a reduced yield were observed when soybean was solely dependent on either nitrogen source (Harper, 1974; Luo et al., 2023). Especially during early seedling growth, the uptake of inorganic nitrogen is crucial for plant development. Besides external nitrogen sources, downstream nitrogen metabolites were also reported to represent a signal for nitrogen demand, regulating nodulation (Lepetit & Brouquisse, 2023; Pervent et al., 2021).

The inhibitory effects of nitrate on nodulation are observed very rapidly and at different phases of the nodulation process (Cabeza et al., 2014; Ferguson et al., 2019; Nishida & Suzaki, 2018a). For example, nitrate affects flavonoid biosynthesis, which has an impact on nodule initiation (C.-W. Liu & Murray, 2016). Nodule functioning is impaired by nitrate because of altered leghaemoglobin production and variations in sucrose allocation to active nodules (Du, Gao, Li, & Liao, 2020; Lambert et al., 2020; Lepetit & Brouquisse, 2023; Van Noorden et al., 2016). In particular, excessive nitrate inhibits nitrogen fixation in mature nodules presumably by carbon-deprivation of nodules (Li et al., 2024), feedback inhibition by the accumulation of products of nitrate metabolism such as ureides (X. Wang et al., 2024) or decreased oxygen diffusion into nodules, which restricts the respiration of bacteroids (Yamashita et al., 2019). Furthermore, nodule senescence is accelerated in the presence of high nitrate levels. Recently, zinc was identified as a secondary messenger that connects environmental nitrogen conditions to transcription factor control of metabolic activity in root nodules and nodule senescence (J. Lin et al., 2024). In addition to rapid activation of nitrate inhibitory effects, these effects are also quickly reversible when nitrate is removed (Ferguson et al., 2019; Saito et al., 2014; X. Wang et al., 2023; Yamashita et al., 2019). This allows legume plants to respond and adapt immediately to changed environmental conditions.

Nitrate-regulated inhibition of nodulation can result from local or systemic signaling. Local signaling directly affects roots near the nitrate signal, in contrast, systemic signaling involves long-distance communication between the root and the shoot and affects distant parts of the root system. Split root and two-layered pot experiments allow to distinguish between the two different signaling mechanisms. In *L. japonicus* and *M. truncatula* both local and systemic effects are involved (M. Lebedeva, Azarakhsh, Yashenkova, & Lutova, 2020; Nishida & Suzaki, 2018b; Okamoto & Kawaguchi, 2015), whereas in soybean some studies reported only local signaling (Lyu et al., 2019; Reid et al., 2011; Xia, Ma, Dong, Xu, & Gong, 2017), while others described systemic effects of nitrate on nodulation (S. Li et al., 2023; Lyu et al., 2022). Specifically, it appears that nodule initiation is locally inhibited by nitrate, while nodule growth and functioning are controlled both locally and systemically (Lim et al., 2014).

Despite the extensive regulatory role of nitrate on RNS, underlying molecular mechanisms are only partially understood. In general, nitrate-regulated nodulation encompasses both the promoting effects at low nitrate levels and the inhibitory effects at high levels. Nitrate-mediated control of nodulation seems to differ between model legumes, such as *Medicago* and *Lotus*, compared to legume crops such as soybean. Research on the link between nitrate and nodulation in soybean is required to develop strategies for a sustainable use of nitrogen in agriculture. This review describes the state of the art of nitrate-regulated nodulation in soybean, while drawing parallels with molecular mechanisms described in other legumes and addressing knowledge gaps that require future study.

2. Peptide signaling in nitrate-regulated nodulation

2.1 CLE peptide signaling inhibits nodulation

CLE peptides belong to a family of signaling peptides in plants, which are involved in several aspects of plant development, such as the homeostasis of the shoot apical meristem and root apical meristem and vascular development (Fletcher, Brand, Running, Simon, & Meyerowitz, 1999; Rojo, Sharma, Kovaleva, Raikhel, & Fletcher, 2002; Song, Hou, & Liu, 2021; G. Wang & Fiers, 2010). They are recognized by a family of the leucine-rich repeat receptor-like kinases (LRR-RLK) from which CLAVATA1 (CLV1) was the first identified one. In general, CLE prepropeptides show a tripartite domain structure, consisting of an N-terminal signal peptide, a central variable domain and a highly conserved and functional CLE peptide domain (Hastwell, Gresshoff, & Ferguson, 2015a; Matsubayashi, 2014). This prepropeptide is post-translationally cleaved into the final ligand, which is 12-13 amino acids in length. Subsequent post-translational modification, involving hydroxylation of proline residues and arabinosylation, was demonstrated to be important for CLE peptide functionality (Matsubayashi, 2014; Ohyama, Shinohara, Ogawa-Ohnishi, & Matsubayashi, 2009; Shinohara & Matsubayashi, 2013). Mature CLE peptides are secreted and act non-cell autonomously (Kucukoglu & Nilsson, 2015). CLV1 can form a functional homomer, although it was also described to form heteromeric receptor complexes with the receptor-like protein CLV2, the pseudokinase CORYNE (CRN) and the receptor-like kinase RECEPTOR-LIKE PROTEIN KINASE 2 (RPK2) for downstream signal transduction (Jeong, Trotochaud, & Clark, 1999; Kinoshita et al., 2010; Müller, Bleckmann, & Simon, 2008; Somssich, Bleckmann, & Simon, 2016; Y. Zhu, Wan, & Lin, 2010). Although BARELY ANY MERISTEM (BAM) receptors were also reported to be involved in CLE–CLV signaling in Arabidopsis, they seem to have an antagonistic role as compared to CLV1 (B. J. DeYoung et al., 2006; Brody J DeYoung & Clark, 2008; Hazak & Hardtke, 2016). Detailed downstream CLV1 signaling mechanisms remain uncharacterized.

In addition to CLV1 signaling that regulates stem cell niche maintenance in apical meristems, legume plants carry an extra copy of the CLV1 receptor, which evolved independently and acquired a function in the regulation of nodulation. In soybean, CLV1a regulates apical meristems whereas CLV1b (or NODULE AUTOREGULATION RECEPTOR KINASE, NARK) is involved in the regulation of nodulation. This divergent evolution explains why single mutants of NARK only have a nodulation phenotype (Carroll, McNeil, & Gresshoff, 1985; Mirzaei et al., 2017; Searle et al., 2003). To inhibit nodulation, nitrate-responsive CLE peptides are perceived by their respective CLV1-like receptors, NARK in soybean, SUPER NUMERIC NODULES (SUNN) in *M. truncatula* and HYPERNODULATION AND ABERRANT ROOT FORMATION 1 (HAR1) in *L. japonicus*.

2.1.1 Nitrate-induced CLE peptides inhibit nodulation locally in soybean

In soybean, the CLE peptide family consists of 84 members (Hastwell et al., 2015a), in particular the NITRATE-INDUCED CLE (NIC) 1a and NIC1b are involved in nodulation regulation in response to nitrate. NIC1a and NIC1b are solely induced by nitrate and act locally in the roots, where they are recognized by NARK to inhibit nodulation (Lim et al., 2014; Reid et al., 2011). *NIC1a* gene expression was observed in soybean primary root tissues, with a significant upregulation 8 h after nitrate treatment and a further increase until 24 h (Reid et al., 2011). The overall tripartite structure characteristic of CLE prepropeptides is observed in soybean NIC prepropeptides. Furthermore, multiple sequence alignment indicates sequence similarity with CLE peptides in *Medicago* and *Lotus*, especially in the C-terminal CLE domain (M. Lebedeva et al., 2020). In contrast, the C-terminal extension domain is absent in the NIC peptides. This domain is thought to act as a protective mechanism from degradative protease enzymes in the xylem, supporting the local role of NIC peptides (Hastwell, Gresshoff, & Ferguson, 2015b). Post-translational hydroxyproline arabinosylation has been demonstrated for CLE peptides in *M. truncatula, L. japonicus* and for soybean RIC peptides (Hastwell et al., 2019; Imin, Patel, Corcilius, Payne, & Djordjevic, 2018; Okamoto, Shinohara, Mori, Matsubayashi, & Kawaguchi, 2013). Further research is required to indicate whether this modification is also present on and required for the function of soybean NIC peptides.

In soybean, two different CLE signaling pathways can be activated to control nodule numbers, initiated by RIC or NIC peptides. Although they are perceived by the same receptor, NARK, the site of ligand perception differs, as well as the stimulus for induction of the peptide. RIC is perceived by NARK in the leaves to activate AON, a systemic negative feedback pathway. Secretion of RIC peptides to the extracellular space is crucial for its function, hence root–shoot transport is suggested to occur via these extracellular spaces (Lim, Lee, & Hwang, 2011). Whereas NIC is recognized locally in the roots by NARK to activate the nitrate-regulated pathway, secretion of the peptide is also expected. Further, RIC peptides are induced solely by rhizobia and not by nitrate, while the opposite holds for NIC peptides. Interestingly, in contrast to the local response in soybean, in *M. truncatula*, the CLE peptide MtCLE35 is induced both by nitrate and rhizobia and acts systemically via SUNN, the ortholog of NARK, in the shoot (Mens et al., 2021; Moreau, Gautrat, & Frugier, 2021). MtCLE34 is another nitrate-responsive CLE peptide, but contains a mutation that generates a premature stop codon in the *M. truncatula* reference genome of the A17 line, suggesting that it is a pseudogene (Hastwell, de Bang, Gresshoff, & Ferguson, 2017; M. Lebedeva, Dvornikova, & Lutova, 2022). Other *M. truncatula* accession lines, such as the R108 line, do not have this mutation, however overexpression did not inhibit nodulation (M. Lebedeva et al., 2022). Similarly, in *L. japonicus*, LjCLE-RS2, LjCLE-RS3 and LjCLE40 are induced by rhizobia and nitrate and act systemically via the CLV1-like receptor kinase HAR1 to inhibit nodulation (Nishida, Handa, Tanaka, Suzaki, & Kawaguchi, 2016; Okamoto & Kawaguchi, 2015; Okamoto et al., 2009).

In summary, it seems that the rhizobia- and nitrate-induced control of nodulation is separated in soybean in contrast to other legumes. Another possibility might be that alternative, still uncharacterized members of the soybean CLE peptide family are induced by both nitrate and rhizobia to regulate nodulation in response to both triggers.

2.1.2 CLE peptide receptors and putative interactors

NARK and its orthologs in other legumes have a similar structure as the CLV1 receptor in Arabidopsis: an extracellular LRR domain to perceive the CLE peptide ligands, a transmembrane domain and a cytoplasmic kinase domain to transmit the signal (Searle et al., 2003). Loss-of-function mutations in the CLE-receptor genes lead to increased nodulation, called hypernodulation, and insensitivity to nitrate inhibition of nodulation (Krusell et al., 2002; Nishimura et al., 2002; E. Schnabel, Journet, de Carvalho-Niebel, Duc, & Frugoli, 2005; Searle et al., 2003). In soybean, *GmNARK* is mainly expressed in the phloem parenchyma of vascular bundles (Nontachaiyapoom et al., 2007). Accordingly, a single-cell transcriptomics study in *Medicago* indicated that CLE–SUNN signaling takes place in the stele, more specifically in the pericycle cells (Pereira et al., 2024).

Drawing parallels with the CLV1 signaling pathway in Arabidopsis, CLV1 coreceptors were studied in legumes to verify their function in the regulation of nodulation (Figure 2). Coreceptors of MtSUNN and LjHAR1 have been reported in *Medicago* and *Lotus*, respectively. MtCLV2 and MtCRN interact with MtSUNN and a mutation in *MtCRN* results in a hypernodulation phenotype (Crook, Schnabel, & Frugoli, 2016). Furthermore, the BAM2 receptor was recently reported to be involved in nodulation control in *Medicago* via specific root interactions with MtSUNN (Thomas & Frugoli, 2024). Mutation of *bam2* could rescue the *sunn* hypernodulation phenotype, suggesting that the loss of *BAM2* can compensate for the lack of the SUNN signal from the shoots. In *Lotus*, interaction between KLAVIER (KLV), which is an ortholog of AtRPK2, and LjHAR1 has been shown and mutations in *LjCLV2* and *LjKLV* result in hypernodulation (Krusell et al., 2011; Miyazawa et al., 2010). Soybean homologs for the CLV2, CRN and RPK2/KLV CLE receptors have been identified in the genome: at least two paralogs for each coreceptor were reported (Guo et al., 2015). Most receptors are expressed in roots and some in the vascular tissues, overlapping with *NARK* gene expression and hence the putative localization for NARK signaling. However, whether they interact with NARK and function in the regulation of nodulation is unclear. Besides CLV2, CRN and RPK2 homologs, two additional putative CLE peptide receptor pairs have been described in soybean, with an expression pattern responsive to nodulation, namely GmRLK1–GmRLK2 and GmRLK3–GmRLK4 (Mortier, Fenta, Kunert, Holsters, & Goormachtig, 2011). For GmRLK3–GmRLK4, the temporal and tissue-specific expression patterns suggest possible interaction with NARK. Functional and biochemical analysis are needed to confirm this hypothesis and whether they are involved in nitrate-regulated nodulation. All above-mentioned data indicate that CLV2, CRN, BAM and KLV coreceptors are important players in the regulation of nodulation, however, their biological function in nitrate-induced signaling needs further research.

In addition to CLV1 coreceptors, the KINASE-ASOCIATED PROTEIN PHOSPHATASE (KAPP) has been shown to interact with CLV1 and to negatively regulate CLV1 signaling in Arabidopsis (Stone, Trotochaud, Walker, & Clark, 1998; Williams, Wilson, & Meyerowitz, 1997). Based on this knowledge, the soybean KAPP1 and KAPP2 were tested *in vitro* for interaction with NARK (Miyahara et al., 2008). Not only was the interaction between NARK and KAPP1/2 confirmed, but KAPP proteins were also shown to be phosphorylated by NARK and subsequently dephosphorylate NARK *in vitro.* Similar as in Arabidopsis, this could represent a negative feedback mechanism to deactivate NARK. However, the involvement of this phosphatase in *in vivo* NARK signaling has not yet been investigated and since these studies were performed *in vitro*, it is possible that KAPP is involved in both nitrate- and rhizobia-induced signaling.

2.1.3 Downstream NIC peptide signaling remains a knowledge gap

Common downstream signaling mechanisms might exist between nitrate-induced and rhizobia-induced nodulation inhibiting pathways, since they share the GmNARK/LjHAR1/MtSUNN receptor. In soybean, RIC peptides are transported from root to shoot where they are perceived by NARK (Lim et al., 2011). This results in secondary shoot-to-root signals to inhibit nodulation. Thus far the best described one is the downregulation of miR2111 in leaves (Zhang, Su, Gresshoff, & Ferguson, 2021), which allows the accumulation of the nodulation-inhibiting Kelch-repeat F-box protein TOO MUCH LOVE (TML) in the roots. Whether the miR2111–TML regulatory module is also implicated in local nitrate-induced signaling in soybean remains to be answered. In *Medicago* and *Lotus*, the miR2111–TML module seems to be involved in both rhizobia- and nitrate-regulated nodulation (Gautrat et al., 2020; Moreau et al., 2021; Okuma, Soyano, Suzaki, & Kawaguchi, 2020; Tsikou et al., 2018). In *Medicago*, high nitrogen levels or ectopic expression of *MtCLE35* repress the accumulation of miR2111, thus allowing TML accumulation and consequently inhibiting nodulation (Moreau et al., 2021). Conversely, ectopic expression of miR2111 partially bypasses the nitrate inhibitory effect on nodulation. The downregulation of miR2111 in the presence of nitrate, depending on MtSUNN/LjHAR1, was observed in both *Medicago* and *Lotus*. However, in *Medicago*, *TML* expression is observed in the roots of the *sunn* mutant, suggesting that alternative pathways might exist to control nodule numbers downstream of MtSUNN (Pereira et al., 2024; E. L. Schnabel et al., 2023). Nevertheless, in contrast to soybean, these legumes systemically control nodulation in response to nitrate, which might explain differences in downstream signaling. Originally, miR2111 was identified as a phosphate (Pi)-responsive miRNA in Arabidopsis (Hsieh et al., 2009; Pant et al., 2009). In *Phaseolis vulgaris* (common bean), Pi deficiency triggers a systemic NARK-dependent mechanism that negatively regulates nodule development, although the involvement of miR2111 was not described (Isidra-Arellano et al., 2020).

Besides TML, the downstream CLE–CLV signaling module regulating nodulation in response to nitrate still represents a knowledge gap. In *Medicago*, overexpression of the gene encoding the nitrate-responsive MtCLE35 peptide locally induces the expression of *MtD53* and thioredoxin (*MtTRX*) in roots (M. A. Lebedeva, Dobychkina, Yashenkova, Romanyuk, & Lutova, 2023). DWARF 53 (D53) is a known repressor of strigolactone signaling (L. Jiang et al., 2013). In soybean, downregulation of GmD53a results in increased nodule numbers, which might indicate a suppressive role in nodulation for D53 and makes it a good potential downstream component of nitrate-regulated nodulation (Rehman et al., 2022). Nevertheless, the role of D53 in this pathway remains to be determined. *MtTRX* was previously reported to be upregulated by arbuscular mycorrhiza in a SUNN-dependent manner, suggesting that control of cellular redox state by TRX could be a common mechanism to negatively control plant–microbe interactions (Karlo et al., 2020). In soybean, TRX was reported to reduce reactive oxygen species levels in roots, an essential process for nodule development (Lee et al., 2005). However, whether these candidate genes are involved in nitrate-regulated nodulation and what role they play need further investigation.

Several research questions with regard to nitrate-induced CLE signaling in soybean remain to be elucidated. Does nitrate only induce local signaling via NIC? Is the miR2111–TML module involved in NIC signaling or in parallel nitrate-induced signaling pathways? What is the role of KAPP in downstream signal transduction, and if it does have other targets than NARK, which ones? Which NARK coreceptors are crucial for NIC peptide perception? Moreover, what happens upstream of the NARK module also represents a knowledge gap. It is not clear how nitrate sensing is linked to NIC induction, in which cells it happens and how the signal is translocated. Also, how downstream nodulation inhibiting signals are translocated back to stop infection in the epidermis, to impede nodule primordium growth or block the functioning of mature nodules are questions to be addressed in future research.

2.2 CEP peptide signaling promotes nodulation

CEP peptides are secreted 15-amino acid peptide hormones that act systemically through CEP receptors (CEPR) to control nitrogen-demand signaling, nodulation and lateral root development, allowing plants to adapt to varying nutrient availabilities in the soil (Taleski, Imin, & Djordjevic, 2018). CEP propeptides are characterized by an N-terminal secretion signal, a variable domain and one or more CEP domains (Aggarwal et al., 2020). Mature CEP peptides contain one or more (arabinosylated) hydroxyproline residues, and similarly as for CLE peptides, this post-translational modification is essential for their biological activity (Aggarwal et al., 2020; Patel et al., 2018). In Arabidopsis, CEP–CEPR interaction in the shoot triggers translocation of the shoot-derived mobile signals, namely CEP DOWNSTREAM (CEPD) 1 and CEPD2 to modulate expression of nitrogen transporters, such as NRT1.1, NRT2.1 and NRT3.1, in the roots and to negatively regulate primary root growth as well as lateral root density and elongation (Ohkubo, Tanaka, Tabata, Ogawa-Ohnishi, & Matsubayashi, 2017; Tabata et al., 2014; Taleski et al., 2024).

2.2.1 Medicago CEP–CRA2 signaling acts via miR2111, NRT2.1 and phytohormones

The role of CEP peptides in nodulation control has mainly been studied in *M. truncatula* (Figure 3). The genome of *Medicago* contains 11 *CEP* family members with a structure similar to that of the *AtCEP* genes (Imin, Mohd-Radzman, Ogilvie, & Djordjevic, 2013). *MtCEP1* encodes two CEP domains and post-translational proline hydroxylation is also crucial for its function. *MtCEP1* is mainly expressed in the root tip region and vascular tissues, where it is upregulated in low-nitrate conditions and downregulated in nodules as they mature and in the adjacent root tissue. This induction of *MtCEP1* in conditions of low soil nitrogen positively influences nodule number, while inhibiting lateral root formation (Imin et al., 2013; Mohd-Radzman et al., 2016; Patel et al., 2018; Taleski et al., 2024). In agreement, overexpression of *MtCEP1* increases the nodule number, nodule size and level of N_2 fixation, even under high-nitrate conditions, which normally inhibit nodulation (Imin et al., 2013). MtCEP1 acts systemically through the COMPACT ROOT ARCHITECTURE 2 (CRA2) receptor in the shoot, increasing the production of the shoot-to-root mobile miR2111, which affects TML accumulation in the roots, consequently promoting nodulation (Gautrat et al., 2020). This antagonistic regulation of the miR2111–TML module by CEP and CLE peptides allows to integrate environmental cues for the fine-tuning of nodule numbers. *MtCEP1* induction also promotes *MtNRT2.1* expression in a CRA2-dependent manner (Luo et al., 2023). MtNRT2.1 has both low- and high-affinity nitrate uptake transport activity and plays a dual role in the nitrate-regulated nodulation. Under low-nitrogen conditions, MtNRT2.1 is essential for nitrate uptake and stimulation of nodulation, whereas in high-nitrogen conditions, *MtNRT2.1* expression promotes nitrate uptake but inhibits nodulation. Evidence from *Mtnrt2.1* mutant studies supports this, as this mutant develops less nodules in low-nitrogen conditions and is partially nitrate tolerant. Furthermore, the expression of CEPD homologs *MtCEPD1/2* is strongly induced under low-nitrogen conditions in a CRA2-dependent manner (Gautrat et al., 2020). Further research is required to elucidate their biological function: are they involved in regulating nitrate transporters as well? Is miR2111 accumulation dependent on signaling via CEPD?

MtCEP1–MtCRA2 signaling also controls nodulation by modulating hormone levels. MtCEP1-activated MtCRA2 interacts locally in the roots with ETHYLENE INSENSITIVE PROTEIN 2 (MtEIN2), a key player in ethylene signaling. This interaction results in the repression of ethylene signaling and promotes root susceptibility to rhizobia (F. G. Zhu et al., 2020). Supporting this, the low nodulation phenotype of the *cra2* mutant can be rescued by a mutation in the *EIN2* gene. Further, resistance to nitrate-mediated inhibition of nodulation was reported for the ethylene-insensitive mutant *Mtein2/sickle*, suggesting the requirement for ethylene biosynthesis and signaling in the inhibition of nodule development under high-nitrate conditions (Gühl et al., 2021; F. G. Zhu et al., 2020). Ethylene is also known to control the radial position of nodules. Interestingly, they do not develop at phloem poles, where SUNN/NARK signaling is located (Nontachaiyapoom et al., 2007; Pereira et al., 2024; Velandia et al., 2022), hence it is possible that ethylene signaling is controlled by both CLE and CEP peptides, although further research should examine this hypothesis. In addition to its effect on ethylene, MtCEP1–CRA2 signaling represses *MtYUCCA*, an auxin biosynthesis gene, affecting auxin responses in the root and root architecture (F. G. Zhu et al., 2020). In soybean, elevated root auxin levels are observed in the presence of rhizobia, but this increase is inhibited under high-nitrate conditions (Caba, Centeno, Fernández, Gresshoff, & Ligero, 2000).

2.2.2 Which CEP peptides are involved in nitrate-regulated nodulation in soybean?

Recently, 22 *GmCEP* family members were identified *in silico* in the soybean genome. Proteomic analyses of soybean xylem sap suggested CEP transport via xylem to the shoot, where they presumably interact with a CEP receptor to positively control nodulation (Okamoto, Suzuki, Kawaguchi, Higashiyama, & Matsubayashi, 2015; Taleski et al., 2024; S. Wu et al., 2024). In soybean, the closest homolog of MtCEP1 is GmCEP20, according to the phylogenetic analysis of S. Wu et al. (2024). However, because this CEP peptide has not yet been functionally characterized, its role in nitrate regulation of nodulation remains to be elucidated. Among the identified *GmCEP* genes, *GmCEP6* expression is the highest in root nodules and changes significantly in response to both rhizobia infection and nitrate treatment (S. Wu et al., 2024). Mutagenesis of *GmCEP6* decreases, while *GmCEP6* overexpression increases nodule number, even in high-nitrate conditions, pointing towards a positive role in nodulation control. GmCEP6 likely regulates nodulation by modulating the expression of symbiosis-related genes, including *GmNINa*, *GmENOD40* and *GmNSP1*. Analysis of the soybean xylem-associated peptidome focused on stress-responsive mobile peptides indicated upregulated translocation of XYLEM-SAP ASSOCIATED PEPTIDE (GmXAP) 6a/GmCEP3 and GmCEP-XYLEM SAP PEPTIDE (XSP) 1/GmCEP12 peptides in xylem sap under nitrogen deficiency and an effect on primary root growth (Okamoto et al., 2015; Sin, Lam, & Ngai, 2022). Furthermore, although upregulated gene expression was observed for *GmXAP6a/GmCEP3*, *GmXAP6b/GmCEP7* and *GmCEP-XSP1/GmCEP12* in roots during nitrogen deficiency, no link with nodulation was reported thus far (Figure 3).

CEP signaling in soybean remains elusive. The specific CEP peptides involved in the regulation of nodulation in response to nitrate are yet to be identified. Besides, further research is needed to identify potential soybean CEP receptor(s) or CEPD homologs and to understand the mechanisms of

CEP perception and downstream signaling. Questions remain about whether CEP signaling affects the miR2111–TML module in soybean and if hormone signaling is involved, similarly as in *Medicago*.

3. miRNA regulatory modules in nitrate-regulated nodulation

3.1 MiR169c modulates inhibitory effects of nitrogen on nodulation in soybean

MicroRNAs are noncoding RNAs which act as master regulators modulating various biological processes by post-transcriptionally repressing their target genes. During nodulation, multiple miRNAs regulate important TFs to control nodulation at different levels. For example, miR166 and miR169 were identified in *Medicago* to play a role in nodule organogenesis (Boualem et al., 2008; Combier et al., 2006), whereas miR171 and miR397 in *Lotus* are involved in nodule function (De Luis et al., 2012). The regulation of nodulation also involves miRNA regulatory modules, such as NIN1a–miR172–NODULE NUMBER CONTROL 1 (NNC1) and miR2111–TML, as previously mentioned (Y. Wang et al., 2014; Z. Wang, Wang, Wang, & Li, 2020; Zhang et al., 2021). The miR2111–TML module is antagonistically regulated by CLE and CEP peptide signaling to fine-tune nodule numbers in response to internal and external cues. Next to its role in nodule number control, miR172 also coordinates the regulation of flowering time by nodulation (Yun et al., 2023). In soybean, the miR169c–NFYA–C–ENOD40 module was characterized as nitrogen-responsive (Xu et al., 2021). MiR169c is induced by high nitrogen levels and is mainly expressed in the vascular bundle of primary roots and leaves. It inhibits nodulation by targeting NUCLEAR FACTOR-Y subunit A-C (NFYA-C), which normally promotes nodulation by inducing *GmENOD40*. Overexpression of miR169c reduces expression of not only *GmENOD40* but also *GmERN1*, an ethylene responsive gene necessary for nodulation, which functions in early NF signaling. The miR169c responses to nitrogen conditions have been reported in other miR169 family members across different plant species, suggesting that the miR169 family retains conserved functions related to nitrogen nutrition (M. Zhao, Ding, Zhu, Zhang, & Li, 2011; M. Zhao et al., 2012). In *Medicago*, miR169 targets HEME ACTIVATOR PROTEIN (MtHAP) 2-1/MtNF-YA1, a TF with a key role during nodule development, although its link to nitrate-induced signaling has not been studied (Combier et al., 2006).

3.2 Beyond the known, other nitrate-responsive miRNAs as candidates for nodulation regulation

Multiple other nitrate-responsive miRNAs have been characterized in plants, and this knowledge could contribute to the identification of those that regulate nodulation in legumes (Islam, Tauqeer, Waheed, & Zeng, 2022). For example, in Arabidopsis miR393 targets the *AUXIN SIGNALLING F-BOX 3* (*AFB3*) receptor gene and regulates root system architecture in response to nitrate (Vidal et al., 2010). In soybean, the miR393 family targets *TRANSPORT INHIBITOR RESPONSE* (*TIR*) *1/AFB3* genes, negatively regulating rhizobial infection and nodule development (Cai et al., 2017). Downregulation of the miR393 family members or overexpression of *GmTIR1* genes significantly increases nodule number. A particular member of the soybean miR393 family, miR393j-3p, targets a nodulin gene, *ENOD93,* and has been described as a key control point during nodule formation (Yan et al., 2015). Strong ectopic expression of this miRNA or downregulation of its target gene *ENOD93* reduces nodule number. However, a role of the miR393 family in nitrate signaling in legumes has not been reported. It would be valuable to elucidate whether miR393 plays a role in nitrate-regulated nodulation in soybean. Similarly, the nitrogen-responsive miR167–AUXIN RESPONSE FACTOR 8 (ARF8) module is known to regulate lateral root growth in Arabidopsis (Gifford, Dean, Gutierrez, Coruzzi, & Birnbaum, 2008; L. Zhao, Liu, Crawford, & Wang, 2018). In soybean, the miR167–ARF8 module not only regulates lateral root development, but is also required for nodulation (Y. Wang et al., 2015). The regulatory role of miR167 in nodulation is dependent on the NF receptor GmNFR1a and it acts upstream of the nodulation genes *NIN, NSP1, ENOD40-1* and *NF-YA1.* Although MiR167 expression is induced by rhizobia inoculation, its responsiveness to nitrate has not been studied yet.

Clearly, miRNA regulatory modules play important roles in nodulation control. While the orthologs of some of these miRNAs are reported to be nitrate-responsive in Arabidopsis*,* further investigation is needed to determine whether they function in nitrate-regulated nodulation in legumes. Additional characterization of the role of miR169c in nodulation control is required as well. Does this module act downstream of CEP/CLE peptide hormone signaling? Moreover, is miR169c also involved in nitrogen-induced nodulation control in other legumes, or is this function specific to soybean?

4. Transcriptional control of nitrate-regulated nodulation

4.1 NIN-like proteins are conserved nitrogen-responsive TFs

NLPs are plant-specific TFs known for their involvement in nitrate signaling. NLPs harbor both RWP-RK and PHOX AND BEM1 (PB1) domains, a DNA-binding and protein–protein interaction domain, respectively (Mu & Luo, 2019; Sakuraba, Zhuo, & Yanagisawa, 2022). Furthermore, the N-terminal region of NLPs is nitrate-responsive (Mineko Konishi & Yanagisawa, 2014; Mu & Luo, 2019; Suzuki et al., 2013). NLPs are involved in the nitrogen-starvation response, legume nodule formation, N and P interactions and root cap release in higher plants. In Arabidopsis, AtNLP6/AtNLP7 play central roles in nitrogen signaling and their activities and nuclear localization are fine-tuned by phosphorylation. The founding member of the NLP family is NIN, which was first identified in *L. japonicus* and is crucial for nodule development, yet does not respond to nitrate (Schauser et al., 1999).

4.1.1 Legume NLPs initiate transcriptional changes to inhibit nodulation in response to nitrate

In legumes, although NLPs play a key role in nitrate-induced inhibition of rhizobial infection, nodule development and nitrogenase activity, they are not responsive to ammonium (J.-s. Lin et al., 2018; Nishida et al., 2018). High concentrations of nitrate trigger nuclear accumulation of NLPs and subsequent suppression of *NIN* target genes. This suppression could occur either by binding NIN proteins or by competitive binding to promotors of symbiosis-responsive genes (J.-s. Lin et al., 2018). Furthermore, NLPs are able to regulate *CLE* and *NRT2.1* gene expression to inhibit nodulation and to control nitrate uptake (J.-s. Lin et al., 2018; Misawa et al., 2022; Nishida et al., 2018) (Figure 4).

In *M. truncatula*, five MtNLPs have been identified and shown to interact with NIN through the PB1 domain (J.-s. Lin et al., 2018). Grafting experiments indicated that MtNLP1 functions locally in the roots (Luo et al., 2021). Downregulation of either *MtNLP1*, *MtNLP3*, *MtNLP4* or *MtNLP5* results in the formation of pink nodules in the presence of nitrate. This pink color originates from the presence of leghaemoglobin and is indicative of nitrogen fixing activity, hence suggesting that these genes play a role in nitrate-mediated inhibition of nodulation (J.-s. Lin et al., 2018). Conversely, downregulation of *MtNLP2* does not lead to pink nodules; instead significantly more white, and thus non-functional, nodules are formed compared to the wild type. Hence, MtNLP2 appears to exhibit a positive role in nodule function, which is also supported by its capacity to activate the expression of leghaemoglobins together with NIN (S. Jiang et al., 2021). MtNLP1 controls nodulation bivalently by activating *MtCLE35* and repressing *MtCEP1* expression, which are negative and positive regulators of nodulation, respectively (Luo et al., 2021; Luo et al., 2023; Moreau et al., 2021). MtNLP4 was demonstrated to function redundantly with MtNLP1 in nitrate-mediated inhibition of nodulation (J.-s. Lin et al., 2018). For MtNLP3 and MtNLP5, no in-depth functional characterization has been reported.

The genome of *L. japonicus* also harbors five *NLP* genes, of which mainly *NITRATE UNRESPONSIVE SYMBIOSIS 1* (*NRSYM1*)/*LjNLP4* and *NRSYM2*/*LjNLP1* have been characterized (Misawa et al., 2022; Nishida et al., 2021; Nishida & Suzaki, 2022; Nishida et al., 2018). Nuclear accumulation in response to nitrate was only reported for LjNLP4 (Misawa et al., 2022). Besides regulation of *LjCLE-RS2* expression, LjNLP4 also regulates the expression of other downstream target genes, which allows to achieve LjHAR1-independent regulation of nodulation (Nishida et al., 2018). Moreover, nitrate-mediated inhibition of CK biosynthesis and signaling requires LjNLP1/4, which act via interfering with NF signaling (Jieshun Lin, Roswanjaya, Kohlen, Stougaard, & Reid, 2021). In *Medicago*, rhizobia-induced accumulation of CK is also reduced under high-nitrate conditions, however whether this is linked to NLP activity remains unclear (Gühl et al., 2021). Although CK biosynthesis and signaling are regulated by nitrate, they do not seem critical to nitrate-induced inhibition of nodulation. Presumably, alternative pathways or downstream events of CK biosynthesis are targeted as well (Jieshun Lin et al., 2021).

4.1.2 Identification of soybean NLPs, yet what are their target genes?

In the soybean genome, 14 *GmNLP* genes were identified (X. Wu et al., 2023). Among them, *GmNLP7a* to *GmNLP7d* are the most closely related to *AtNLP6/7*. Their expression is induced by high-nitrate levels, but not by rhizobial infection. Functional characterization of *GmNLP7s* indicated their negative role in the regulation of nodule number in soybean in response to nitrate availability. At the molecular level, GmNLP7a was shown to interact with GmNIN1a via the PB1 domain and to suppress both *GmNIN1a* and *GmENOD40* expression, resulting in inhibition of nodulation. In contrast to the nitrate-triggered nuclear accumulation of NLPs in *Medicago* and *Lotus*, GmNLP7a seems to be localized in the nucleus regardless of nitrate availability. Another *GmNLP* gene was reported to have a nitrate-responsive expression profile (Kim et al., 2023). Knock-out and overexpression of this gene affects the nodulation phenotype under varying nitrate conditions. However, this *GmNLP* gene does not have the RWPK and PB1 domains, which are characteristic of NLP TFs, and it was not reported in the phylogenetic analysis of X. Wu et al. (2023). Instead, the phylogenetic analysis of X. Wu et al. (2023) pointed *Glyma04G017400* and *Glyma06G017800* as the most closely related *GmNLP* genes to *MtNLP1* and *LjNLP1*. These are also closely related to the Arabidopsis homologs AtNLP4/5, which were described to be key players in regulating rhizobia-induced changes in root system architecture in response to nitrate in non-legumes (Hernández-Reyes et al., 2022). Putatively, these two soybean candidate genes could be involved in nitrate-regulated nodulation, hence representing targets of interest for further study.

In conclusion, there is a need for more in-depth functional characterization of soybean *NLP* genes to fill research gaps with regard to their role in nitrate-regulated nodulation. Which soybean NLPs are involved in nodulation control? Do downstream NLP targets involve the peptide hormones NIC and CEP, or miRNA regulatory modules, such as miR169c? Do they regulate nitrate transport via control of *NRT* gene expression? The soybean NLP candidates identified by X. Wu et al. (2023) and Kim et al. (2023) were selected based on expression pattern, suggesting transcriptional control, while the activity of known NLPs is regulated by nitrate-triggered nuclear accumulation. Does this post-translational control also hold for soybean NLPs? Finally, future research is required to elucidate what happens upstream of NLP, which molecular players are involved and how they transmit the nitrate signal to affect NLP function.

4.1.3 TCP TFs as candidate NLP interactors

A soybean transcriptome study has identified *TEOSINTE-BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR1* (*GmTCP*) as a key candidate gene in nitrate-induced inhibition of nodulation (Kim et al., 2023). The TCP TFs are a family of plant-specific proteins that play critical roles in plant growth, development and stress response (Cubas, Lauter, Doebley, & Coen, 1999; Manassero, Viola, Welchen, & Gonzalez, 2013). In Arabidopsis, AtTCP20, a homolog of GmTCP, can bind the promotor of nitrate-induced genes, being a crucial factor in the nitrate signaling pathway. Furthermore, under nitrogen starvation conditions, AtTCP20–AtNLP6/7 heterodimers accumulate in the nucleus to upregulate nitrate assimilation genes and downregulate the G2/M cell cycle marker gene *CYCB1;1* (P. Guan et al., 2017; Peizhu Guan et al., 2014; Hervé et al., 2009). The expression of *GmTCP* has been observed in both roots and nodules and is upregulated in conditions with higher nitrate concentrations (Kim et al., 2023). Further study is required to unravel the molecular activities of GmTCP with regard to its role in nodulation control and its potential interactions with GmNLP7a-d or other GmNLPs in soybean.

4.2 What is the role of legume-specific TGA TFs and nodule senescence-related SNAP TFs?

Apart from NLPs, two families of TFs in soybean have recently been reported for their putative link to nitrate-responsive regulation of nodulation. The first is the TGACG-binding (TGA) TF family, which is known to play essential roles in plant defense in Arabidopsis (Després et al., 2003; Kesarwani, Yoo, & Dong, 2007; Ullah, Magdy, Wang, Liu, & Li, 2019). In the soybean genome, three members of a legume-specific clade of TGA genes have been reported, *GmTGA3/4/23* (Ullah et al., 2019). All three genes are upregulated by rhizobial infection, while high-nitrate levels induce *GmTGA4*, but suppress *GmTGA3/23* expression. Further functional characterization is needed to elucidate the specific roles of these TFs in soybean nodulation and nitrogen response and how they impinge on the nodule signaling network. In

L. japonicus, the TGA transcription factor FIXATION UNDER NITRATE (FUN) is involved in nitrate-induced control of nodule senescence. FUN activity is regulated by zinc, which acts as a secondary messenger and accumulates intracellularly depending on soil nitrate availability (J. Lin et al., 2024).

The second family of TFs is the NAM, ATAF1/2 and CUC2 (NAC) TF family, to which the *SOYBEAN NITROGEN ASSOCIATED NAP (SNAP) 1-11* genes belong. In particular, SNAP1/2/3/4 were described as central regulators mediating high-nitrogen responses in mature nodules, thereby regulating later stages of nodulation compared to NLP or TGA TFs (X. Wang et al., 2023). These SNAP TFs seem to regulate a subnetwork of senescence-associated genes and transcriptional regulators, including ERF, WRKY and NAC, in response to high nitrogen levels, leading to reduced nitrogenase activity and accelerated nodule senescence. ERFs are key components of ethylene signaling, which positively regulate nodule senescence in *Medicago* (Kazmierczak et al., 2020; X. Wang et al., 2023). WRKY TFs represent a large family of plant-specific TFs, characterized by the highly conserved amino acid sequence WRKYGQK. They have diverse biological functions, including involvement in plant disease resistance, abiotic stress responses, senescence and hormone-controlled processes (Bakshi & Oelmüller, 2014). In particular WRKY6, WRKY22 and WRKY33 have been shown to promote leaf senescence in Arabidopsis (Datta et al., 2015; Robatzek & Somssich, 2002; X. Zhou, Jiang, & Yu, 2011). NAP TFs are evolutionary conserved, which might suggest that NAP-mediated nitrate signaling in nodules could be a conserved mechanism among legume species. For example, in *Medicago*, MtNAC969, a homolog of GmSNAP10/11, is also induced by nitrate in symbiotic nodules and is involved in nodule senescence (de Zélicourt et al., 2012). In *Lotus,* LjNAC094 has been identified as a positive regulator in nitrate-induced senescence of nodules, acting downstream of LjNLP1/4 and FUN (J. Lin et al., 2024; L. Wang et al., 2023).

Conclusions

Legumes have the beneficial capacity to establish symbiotic interactions with rhizobia, which provide their host plants with fixed nitrogen. However, in the presence of nitrogen this process is rapidly repressed to avoid unnecessary investments of carbon in RNS. Several players involved in regulating nodulation in response to nitrate availability have been identified, including peptide hormones, miRNAs and TFs (Figure 5, Table 1). Nevertheless, how these molecular players are linked to each other and what underlying molecular mechanisms are at play to inhibit nodulation remain unresolved. Also the location of these interactions is still elusive, questions remain about how the nitrate signal is translocated to the regulatory modules, such as NARK or NLP, and how elicited responses are translocated back to adjust the nodulation phenotype. A deeper understanding of these regulatory processes, particularly in soybean, will contribute to increase the nodulation efficiency in modern agricultural systems, hence improving sustainability. Furthermore, an enhanced nodulation efficiency might also impact seed protein content, improving nutritional quality. However, it is important to validate this obtained knowledge through field trials, considering the highly variable environmental conditions that affect plant behavior and yield.

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Figures and Tables

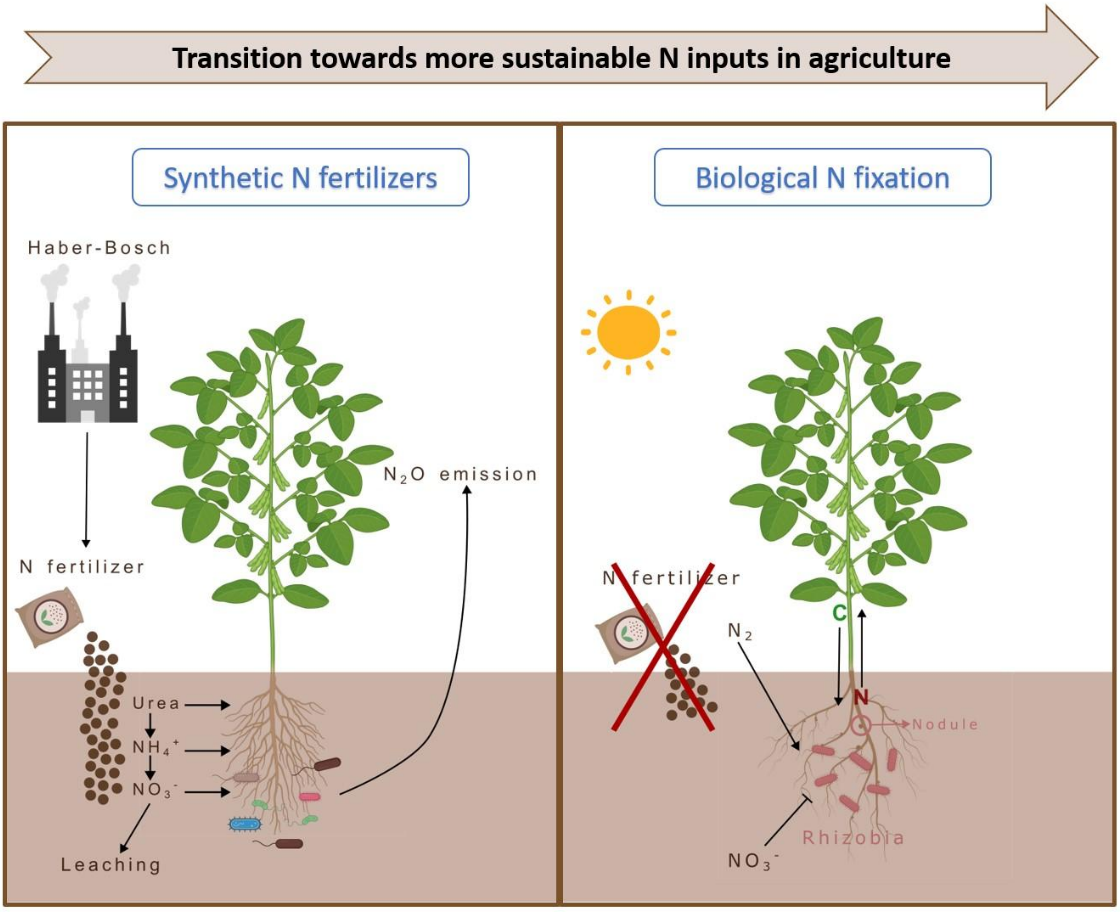

Figure 1. Transition towards more sustainable nitrogen inputs in agriculture. Left: synthetic nitrogen (N) fertilizers are produced by the energy-consuming Haber-Bosch process. These fertilizers contain different forms of nitrogen: urea, NH_4^+ and/or NO_3^-, which are directly available for plants, but can also be interconverted by soil microbiota. Urea is converted to NH_4^+, which can be converted to NO_3^- (nitrification). In turn, denitrification can generate the greenhouse gas N_2O from NO_3^-, which is emitted in the atmosphere. Excess NO_3^- leaches in the groundwater, causing pollution of water streams. Right: soybean can establish symbiotic interactions with rhizobia, which fix atmospheric N_2 into NH_3 for their host plant in return for carbon sources, eliminating the need for nitrogen fertilizer application. The presence of NO_3^- in the soil inhibits this interaction.

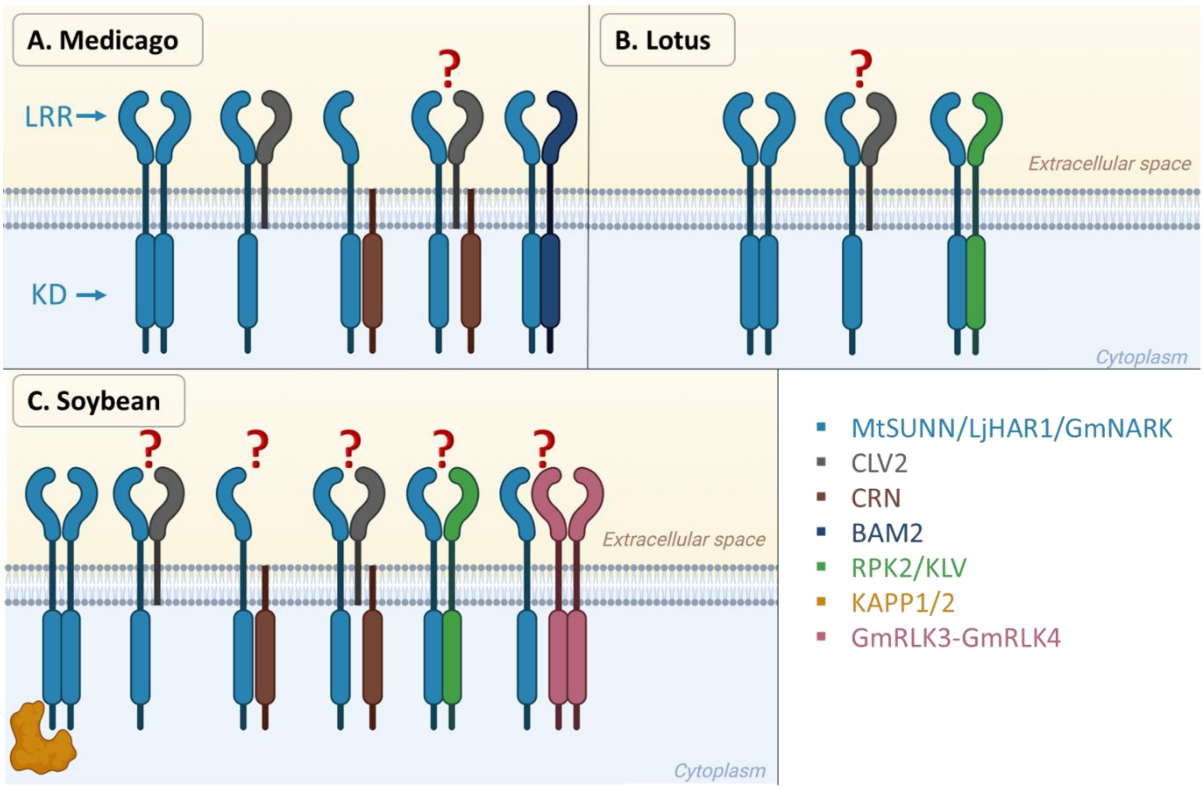

Figure 2. Known and putative receptor complexes for CLE peptides in *Medicago*, *Lotus* and soybean. The legume CLE peptide receptors can form homomeric or heteromeric receptor complexes, they have an extracellular leucine-rich repeat (LRR) domain for ligand perception and an intracellular kinase domain (KD) for signal transmission. In *Medicago*, MtSUNN interacts with MtCLV2 and MtCRN and specific root interaction between MtSUNN and MtBAM2 was reported. The formation of the trimeric complex with MtSUNN, MtCLV2 and MtCRN, which is known from Arabidopsis, is not confirmed. In *Lotus,* LjHAR1 interacts with KLV. Mutation in *LjCLV2* leads to hypernodulation, hence this receptor might be involved in CLE peptide perception together with LjHAR1. In soybean, putative NARK coreceptors have been identified, although direct interaction with NARK still needs experimental validation. Homologs of CLV2, CRN and KLV and the GmRLK3-GmRLK4 receptor pair are depicted. KAPP1/2 interacts with and dephosphorylates NARK *in vitro*. Question marks indicate that the suggested interaction needs further confirmation.

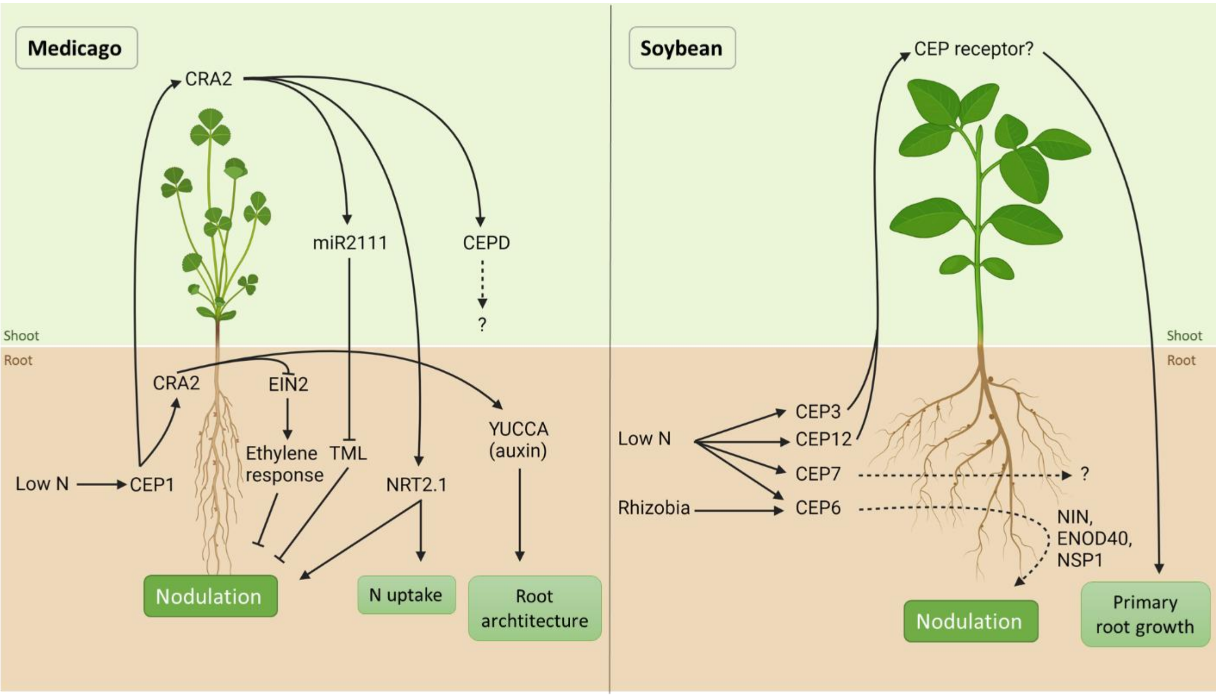

Figure 3. CEP peptide signaling in *Medicago* and soybean. Left: low nitrogen (N) levels induce the CEP1 peptide in *Medicago*, which is perceived by the CRA2 receptor locally in the roots or systemically in the shoots. Local signaling promotes nodulation by inhibiting ethylene signaling via interaction with EIN2. Root architecture is also affected by local interactions between CEP1-activated CRA2 and YUCCA, an auxin biosynthesis protein. Systemic CEP1–CRA2 signaling upregulates miR2111, which targets TML in the roots to promote nodulation, and induces NRT2.1, to promote nitrate uptake and nodule formation. Right: four soybean CEP peptides are induced under low-nitrogen conditions, CEP3 and CEP12 are shown to be transported to the shoot and to affect primary root growth. CEP6 is also induced in the presence of rhizobia and affects nodulation by regulating *NIN, ENOD40* and *NSP1*. Dashed lines indicate unknown signaling mechanisms, this can be local or systemic.

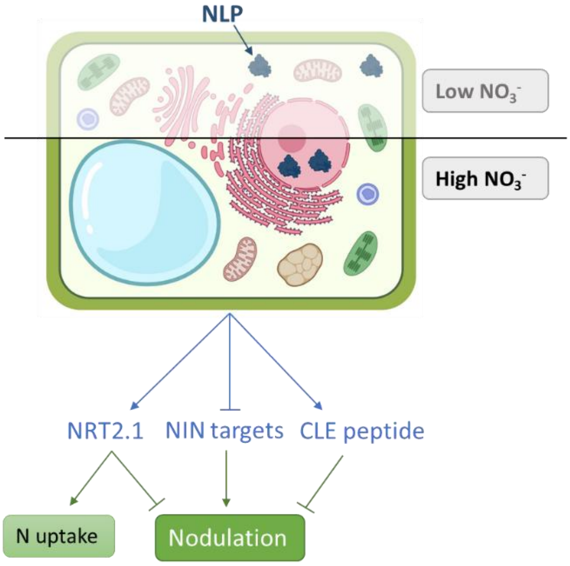

Figure 4. NLP accumulates in the nucleus under high-nitrate conditions to promote nitrate uptake and inhibit nodulation. High-nitrate conditions trigger the nuclear accumulation of NLP. This inhibits nodulation via *NRT2.1* and *CLE* upregulation, as well as via inhibition of NIN target genes. Moreover, *NRT2.1* induction also promotes nitrate uptake. This figure summarizes the knowledge about NLP function in *Lotus* and *Medicago*, while in soybean, NLP signaling remains a knowledge gap.

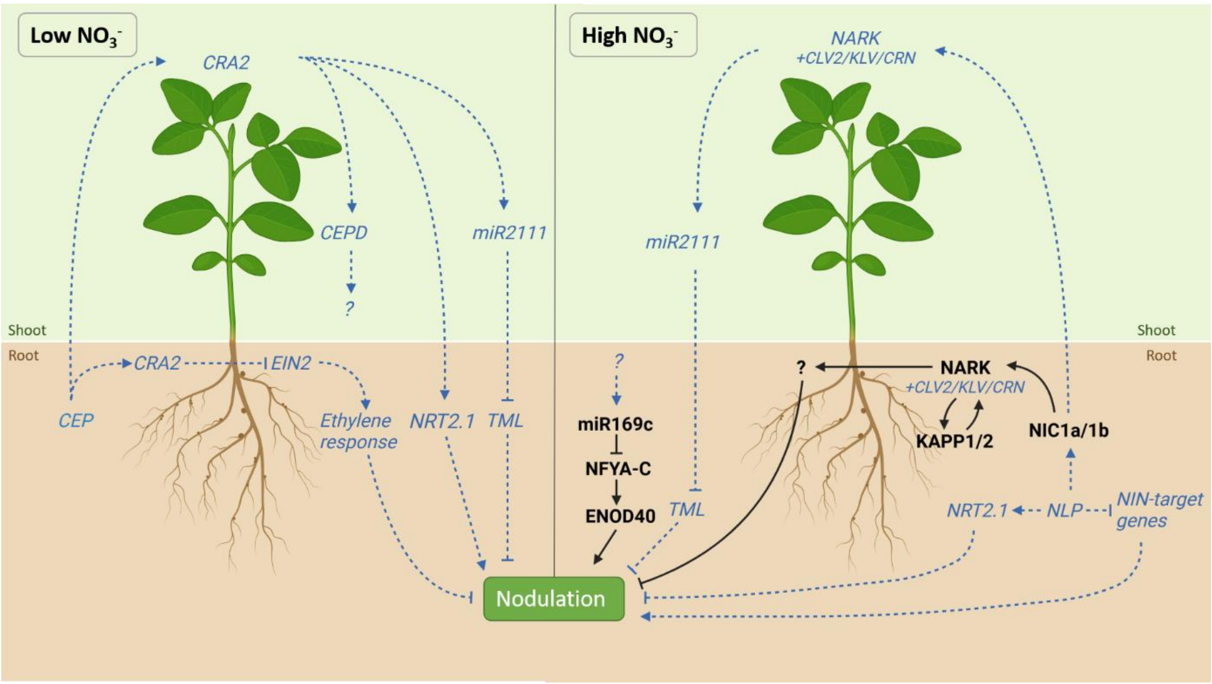

Figure 5. Molecular mechanisms involved in nitrate-regulated nodulation in soybean. Dashed lines and the blue color indicate signaling and molecular players known from other legumes, which have not (yet) been confirmed in soybean. Left: low-nitrate conditions. CEP peptides act systemically via the shoot CEP receptor CRA2 to promote nodulation, candidate downstream targets are CEPD, NRT2.1 and the miR2111–TML module. Local CEP–CRA2 signaling promotes nodulation by inhibiting ethylene signaling via interaction with EIN2. Right: high-nitrate conditions. NIC1a/1b peptides are perceived locally in the roots by NARK and putative co-receptors (CLV2/KLV/CRN) to inhibit nodulation. KAPP1/2 was shown to interact with and dephosphorylate NARK *in vitro.* In other legumes, CLE signalling was shown to act systemically and to activate the miR2111-TML module to inhibit nodulation. Also, NLP activates *CLE* expression and induces *NRT2.1*, while repressing NIN target genes. In soybean, miR169c is induced in high-nitrogen conditions and targets *NFYA-C*, inducing *ENOD40* expression.

Table 1. Molecular players involved in nitrate-regulated nodulation in *Medicago, Lotus* and soybean.

	Pathway component	***Medicago truncatula***	***Lotus japonicus***	***Glycine max***	**Function**
Peptides	**CLE peptide**	CLE35	CLE-RS2/3/40	NIC1a/NIC1b	Inhibition of nodulation
	CEP peptide	CEP1	-	*CEP3, CEP6, CEP7, CEP12, CEP20*	Promotion of nodulation
(Co)receptors	**CLE receptor**	SUNN	HAR1	NARK	Inhibition of nodulation
	Coreceptor	CLV2, CRN, BAM	CLV2, KLV	*Homologs of CLV2, CRN, KLV; GmRLK3-GmRLK4*	Interactions with CLE receptor
	CEP receptor	CRA2	-	-	Promotion of nodulation
	KAPP	-	-	KAPP1/2	Regulation of CLE receptor activation
microRNAs	**miR2111**	miR2111	miR2111	miR2111*	Targets *TML* to inhibit nodulation
	miR169c	miR169*	-	miR169c	Targets *GmNFYA-C* to inhibit nodulation
Transcription factors	**NLP**	NLP1/3/4/5	NLP1/4	*GmNLP7a-d, Glyma04G017400, Glyma06G017800*	Inhibition of nodulation
	TCP	-	-	*GmTCP*	Potential interactor of NLP
	TGA	-	-	*TGA3/4/23*	Unknown function, nitrate- and rhizobia-dependent gene expression
	SNAP	NAC969	NAC094	SNAP1-4	Regulation of nodule senescence

Italics indicate that the candidate genes still need further validation. - : no homologous gene linked to nodulation has been identified. *The role in nitrate signaling needs to be validated.