Nitrogen Systemic Signaling: from symbiotic nodulation to root acquisition

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Keywords

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Highlights

- N nutrition relies on root acquisition of mineral resources, as well as on symbiotic N₂ fixation by soil bacteria in legume plants
- Both processes are regulated by systemic signaling pathways aiming to adjust N acquisition depending on plant N needs and assimilation capacities
- Similar effectors acting in systemic signaling pathways are shared between both N nutrition modes, including hormones such as cytokinins and signaling peptides as well as related NIN-Like Protein (NLP) transcription factors
- Recent advances highlighted that systemic signaling pathways linked to N fixation and acquisition are tightly related to phosphate systemic signaling
- Light and/or CO₂ shoot environmental factors impact N systemic signaling, suggesting mechanisms allowing integration of N and C signaling and metabolism

Abstract

Plant nutrient acquisition is tightly regulated by resources availability and metabolic needs, implying the existence of communication between roots and shoots to ensure their integration at the whole plant level. Here, we focus on systemic signaling pathways controlling nitrogen (N) nutrition, achieved both by the root import of mineral N and in legume plants through atmospheric N fixation by symbiotic bacteria inside dedicated root nodules. We explore conserved features between systemic pathways repressing or enhancing symbiotic N fixation and the regulation of mineral N acquisition by roots, as well as their integration with other environmental factors, such as phosphate, light and CO₂ availability.

Root-Shoot-Root communication is an essential circuit to regulate plant nitrogen acquisition

Plant metabolism combines light energy capture and carbon (C) fixation by photosynthetic shoots with water and nutrient acquisition by roots. Both organs are challenged with very different local environments, and yet, they need to cooperate to optimize nutrient supply to ensure plant growth. To do so, in addition to the local perception of different nutritional-related cues in the environment, dedicated systemic signaling networks integrating nutrient availability/needs with the growth/developmental status operate at the whole plant level [1]. Plant vascular tissues play an essential role in this long-distance communication between roots and shoots, with a directionality of nutrient and mobile signals exchanges achieved by xylem and phloem vessels [2]. Exhaustive analyses of xylem and phloem saps revealed, beside nutrients and metabolic product of their assimilation, a diversity of molecules ranging from hormones, small and long RNAs, signaling peptides and proteins [3-5]. Understanding the role in systemic root and shoot regulations of signals moving within this complex sap composition, as well as their origins and targets, is a challenging but crucial objective for improving the efficiency of nutrient use and homeostasis at the whole plant level.

Plant nitrogen (N) acquisition by roots is achieved by importing different mineral N sources from the soil as well as, in some specific plants such as legumes, by the fixation of atmospheric N_2 through a symbiotic interaction with soil bacteria, collectively referred to as rhizobia, within a dedicated root lateral organ, the nodule,. Both root and nodule N acquisition is under the control of a long-distance homeostatic signaling, which identification is described in Box 1 for nodules and in Box 2 for roots. These regulatory pathways share similarities as they both aim to regulate the plant N nutrition from different sources (atmospheric N_2 , soil mineral NO_3^- or NH_4^+) depending on i) environmental N availability, and ii) plant N needs and assimilation capacities that are notably driven by the C metabolism. Symbiotic N_2 fixation in root nodules is a unique model to study this cost-benefit control of N acquisition by long-distance signals. Indeed, a new organ only dedicated to N acquisition is formed, the nodule, that is non-essential when mineral N is available, in contrast to roots that achieve many other functions in addition to N nutrition. This specificity allowed performing efficient genetic screens to unravel the genetic basis of

symbiotic root nodulation [6]. In this review, the systemic pathways regulating N fixing root nodules in legumes will be used as a core to intertwine them with the knowledge gained on N acquisition in roots of the non-nodulating reference plant *Arabidopsis thaliana*. We will successively explore i) mechanisms ensuring the homeostatic repression of N acquisition, ii) mechanisms promoting N acquisition when low N is available and plants N needs are high, and iii), how shoot environmental conditions and C metabolism capacities may impact N-systemic signaling.

Repressing root nitrogen fixation and acquisition by long-distance signaling

The AON (Autoregulation Of Nodulation) homeostatic systemic signaling pathway restricts the number of N fixing nodules that form on legume roots dependent on rhizobial infections and nodules that were previously formed (Box 1). In nodulated roots, the AON signaling initiates with the production of specific SSP (Small Secreted Peptides) of the CLE (CLAVATA3/EMBRYO-SURROUNDING REGION RELATED) family (Figure 1A). Those 12-amino acid long peptides are conserved among legumes, and their expression is induced when roots interact with rhizobia and by high mineral N availability. These AON-related CLE peptides are encoded by the rhizobium-induced *MtCLE12* and *MtCLE13* genes in *Medicago truncatula* [7, 8]; the rhizobium-induced *LjCLE-RS1* (*CLE-Root Signal1*), *LjCLE-RS2* and *LjCLE-RS3* in *Lotus japonicus*, the two latter genes being also induced by NO_3^- [9, 10]; and in soybean the rhizobium-induced *GmRIC1* (*Rhizobia-Induced CLE1*) and *GmRIC2* genes and the NO_3^- -induced *GmNIC1* (*Nitrate-Induced-CLE1*) gene [11]. SSP activity and/or stability frequently rely on post-translational modifications, and accordingly, some of those CLE peptides were reported to be hydroxyprolinated and tri-arabinosylated in roots, by the MtRDN1 (Root Determined Nodulation1) enzyme for *MtCLE12* [12, 13] and by LjPLENTY for *LjCLE-RS2* and *LjCLE-RS3* [14-16]. Those SSPs can be detected in the xylem sap and are thus proposed to be root-to-shoot mobile signals, perceived in shoots by the CLV1-like (CLAVATA1-like) LRR-RLKs (Leucine Rich Repeats - Receptor Like Kinases) MtSUNN [7, 17], LjHAR1 [15, 18-21], and GmNARK [11, 22] (Figure 1A). A direct binding of *LjCLE-RS2* to LjHAR1 was demonstrated [15], and several co-receptors (LjKLAVIER, MtCLAVATA2, MtCORYNE) were shown to interact with MtSUNN/LjHAR1 [23-25].

In Arabidopsis, local functions of CLE peptides in meristems were extensively described, but only few links with nutrient and systemic regulations were reported [26, 27]. A role for the CLE-CLV1 signaling pathway in the regulation of lateral root development by N provision was however shown [28]. Indeed, the *AtCLE1*, 3, 4 and 7 genes are expressed in pericycle cells and are induced by N-limited conditions. Corresponding peptides would then be translocated into root phloem companion cells to repress the emergence of lateral root primordia in a CLV1-dependent manner [28]. This signaling module may thus also negatively regulate N acquisition locally in roots and would be the target of an unknown N-deficiency shoot-derived signal (Figure 1B). Interestingly in *L. japonicus*, the *har1* mutant, affected in the LRR-RLK most closely related to AtCLV1, has also a root architecture phenotype, consisting in shorter roots and an increased number of lateral roots [18]. The existence of a root-to-shoot CLE-related signaling pathway regulating root architecture and N acquisition remains to be explored further in Arabidopsis, as well as a function in shoots of the AtCLV1 receptor in relation to N-related CLE peptides.

In legumes, the induction of *CLE* genes expression by NO_3^- and rhizobia relies on transcription factors of the NODULE INCEPTION (NIN) family. Indeed, both in *M. truncatula* and *L. japonicus*, the induction of *MtCLE13* and *LjCLE-RS1/2* expression by rhizobia depends on NIN [8, 29], which binds *LjCLE-RS1/2* promoters [29]. Interestingly, cytokinin (CK) hormones, that are essential for the establishment of nodules, activate NIN and *MtCLE13* or *LjCLE-RS1/2* expression [7, 29-32] (Figure 1A). A subset of NIN-LIKE PROTEIN (NLP) similarly mediates the CLE-dependent NO_3^- inhibition of nodulation both in *L. japonicus* and *M. truncatula* [33, 34] (Figure 1A). Indeed, *Nitrate Unresponsive Symbiosis 1 (NRSYM1)/LjNLP4* binds the *LjCLE-RS2* promoter and activates its expression in response to NO_3^- , as well as the NITRITE REDUCTASE 1 (NIR1) encoding gene involved in NO_3^- assimilation but not nodulation, highlighting shared symbiotic and non-symbiotic root functions of this N/NLP/CLE module [33]. In *M. truncatula*, *MtNLP1* and *MtNLP4* redundantly accumulates in the nucleus in response to NO_3^- and interact with NIN to potentially inhibits its function, including hampering the activation of the CK receptor *MtCRE1* expression that is required for nodule initiation [34].

In Arabidopsis, regulators of CLE peptides expression in response to N provision are yet unknown. Given that AtNLP7 is an important hub for the NO_3^- root response [35-38], its involvement may be speculated, even though no CLE peptide linked to NO_3^- provision was yet identified in Arabidopsis. Transcriptomic analyses however did not identify any NLP7-dependent gene encoding a CLE peptide [37, 39]. It remains to be explored if other NLPs could nevertheless regulate the expression of *CLE* genes linked to N signaling. In addition, several NLPs interact together via their PB1 (Phox and Bem1) domain [40, 41], as shown for MtNIN and MtNLP1, or with other transcription factors, which may allow coordinating the root response to different N environments. AtNLP7 notably interacts with AtTCP20 (Teosinte branched1/Cycloidea/Proliferating cell factor1-20) to regulate the response of NO_3^- responsive genes and root meristem growth depending on N-availability [41]. Interestingly, AtTCP20 is a target in roots of the N-demand systemic signaling [42], indicating that the transcriptional complex AtNLP7/AtTCP20 may integrate local and systemic N signaling pathways (Figure 1B).

Downstream of the AON-related CLE receptor activation in legume shoots, shoot-to-root systemic effectors inhibiting root nodulation were identified. First, as the shoot expression of the *ISOPENTENYLTRANSFERASE3* (*IPT3*) gene increases in response to rhizobium inoculation depending on LjCLE-RS/LjHAR1 signaling pathway, as well as an intermediate form of CKs (N^6 -(Δ^2 -isopentenyl) adenine ribosides 5'-phosphates, iPRPs), and as CKs applied to shoots are transported through the phloem towards roots and inhibit nodulation independently of LjHAR1, it was proposed that CK could be a shoot-to-root signal inhibiting root nodulation, in addition to its role as a NIN/CLE activator in roots [43] (Figure 1A). In soybean, a *LjIPT3* ortholog, *GmIPT5*, is also shoot-induced by symbiotic conditions but independently of GmNARK, and CK applications on either shoots or roots similarly promote or inhibit nodulation, depending on the low or high concentration used, respectively [44]. Thus, the negative role of shoot CKs on root nodulation downstream of the AON pathway proposed in *L. japonicus*, implying a potential systemic action of CKs, seems not a general feature of AON in all legumes.

In Arabidopsis, *AtIPT3* expression is triggered by NO_3^- [45]. In roots, this ultimately leads to the accumulation of the CK precursor *tZR* (*trans*Zeatin Riboside) and of the

biologically active *tZ*, which are then translocated to the shoots through the ABCG14 transporter [46-48]. Both *tZR* and *tZ* act as systemic signals to promote shoot growth in response to NO_3^- [49-52]. The shoot apical meristem fate is notably likely modulated by the influence of *tZR* on *WUSCHEL* expression [50, 51] (Figure 1B), *WUSCHEL* being directly activated by a subset of type B Arabidopsis Response Regulators (see [53] for an overview). Moreover, in split-root experiments mimicking NO_3^- heterogeneity, the *tZ/tZR* integration in shoots is also required to enhance lateral root development and NO_3^- transport in NO_3^- -supplied roots, compensating for the partial N deficiency [54-56] (Figure 1B). As shown in legumes, CK are proposed to act at several levels of N-signaling since it also regulates locally the NO_3^- -dependent primary root growth [57], lateral root development and NO_3^- transport [58, 59].

In legumes, another shoot-to-root signal conserved between *L. japonicus* and *M. truncatula* was more recently proposed, corresponding to the microRNA miR2111, which expression is shoot-specific and repressed by rhizobium inoculation, dependent on LjHAR1/MtSUNN [60, 61] (Figure 1A). This miRNA regulates post-transcriptionally the accumulation of *TML* (*Too Much Love*) genes (*LjTML* in *L. japonicus* [60, 62, 63] and *MtTML1/MtTML2* in *M. truncatula* [61, 64]), which encode E3 ubiquitin ligases acting in roots to inhibit nodulation. Although TMLs targets remain unknown, one could anticipate that they regulate the stability of symbiotic regulators, and notably of NIN that is a homeostatic target of the NIN/CLE/AON pathway [29]. Overall, whereas the initial AON model was proposing a nodulation “Shoot-Derived Inhibitor” (SDI) signal, as could be CKs in *L. japonicus* (see above), the miR2111 shoot-to-root systemic effector behaves as a nodulation “Shoot-Derived Activator” (SDA) signal that is repressed by the AON pathway (Figure 1A).

In Arabidopsis, the miR2111/TML regulatory module is evolutionary conserved, suggesting a possible recruitment in the regulation of root responses to N availability beyond nodulation. Indeed, one of the two TML homologs (At3g27150) encoding an uncharacterized E3 ubiquitin ligase, is targeted by mir2111, which was detected in Arabidopsis phloem saps in agreement with a function as a systemic shoot-to-root effector [65]. However, miR2111 accumulation is induced under phosphate (Pi)-limited conditions and not by N-limitation [65], in contrast to legumes (Figure 1B).

Interestingly, others F-box proteins that are also regulated by systemic miRNAs control nutrient homeostasis depending on combined P and N availability. Indeed, the E3 ubiquitin ligase NLA (NITROGEN LIMITATION ADAPTATION), and the E2 ubiquitin conjugase PHO2 (PHOSPHATE2), are targeted by the shoot-to-root mobile mir827 and mir399 miRNAs, respectively, in Pi-limiting conditions to inhibit their activity and to favor Pi acquisition [66-68]. Furthermore, the phosphate starvation response (PSR) is conditional to the NO_3^- provision [69-71] and this feature depends on the reciprocal regulation of the transceptor NPF6.3/NRT1.1 and of PHO2 [69] (Figure 1B). This is achieved by integrating local and systemic signals, suggesting that the furniture of a N signal to forage for P acquisition is mandatory even at long-distance [69].

Promoting root nitrogen acquisition and fixation by long-distance signaling

As a mirror of the negative AON systemic pathway, a positive systemic pathway promoting nodulation was more recently evidenced in *M. truncatula*, involving another class of SSP, the CEP (C-terminally Encoded Peptide) family, which expression is generally enhanced in N-deficient roots [72] (Figure 1A). CEP peptides increase the number of nodules formed [72] through a systemic pathway involving the activity in shoots of the MtCRA2 (Compact Root Architecture2) LRR-RLK receptor [73, 74]. Under N deficiency conditions, this systemic MtCEP/MtCRA2 pathway upregulates in shoots the expression of the miR2111 shoot-to-root systemic effector, promoting in roots the cleavage of *MtTML* transcripts that encode an inhibitor of nodulation (see above) [61]. The MtCEP/MtCRA2 pathway thus actively maintains roots competent for nodulation in N deficiency conditions (Figure 1A). Interestingly, the previously described rhizobium- and N-induced MtSUNN AON pathway and the MtCRA2 pathways act independently [75] but modulate antagonistically the same mir2111/TML regulatory module, which is thus a central hub to regulate dynamically nodule number depending on environmental N-availability and the plant N-metabolic status [61]. Interestingly, the MtCEP/MtCRA2 pathway also regulates locally the root system architecture, inhibiting lateral root development and being required for primary root growth depending on a YUCCA-dependent local auxin biosynthesis pathway [72, 73, 75-77] (Figure 1A). *cra2* mutant roots perceive changes in N environments but have altered responses to these conditions [77]. Indeed, instead of repressing root growth and increasing lateral root

density, NO_3^- promotes *cra2* mutant root growth and represses its lateral root density. In addition, this pathway regulates systemically from shoots the gravitropic set-point angle of lateral roots by limiting shoot auxin transport to roots, ultimately allowing a wider foraging area of the root system [78] (Figure 1A).

In Arabidopsis, the first CEP peptide, AtCEP1, was identified *in silico* and then by LC-MS (Liquid Chromatography-Mass Spectrometry) as a 15 amino acid long hydroxyprolined peptide. A subset of AtCEP peptides, including notably AtCEP3, 5 and 9 accumulates in N-deficient roots and inhibit primary root growth (AtCEP3 and 5), with a reduction of meristematic cell number and of the size of the meristem, as well as the emergence of lateral roots (AtCEP3 and 5), and/or the gravitropic set-point angle of lateral roots (AtCEP3) [78-84] (Figure 1B). In addition, AtCEP1 upregulates systemically the expression of the *AtNRT2.1* high affinity NO_3^- transporter depending on the LRR-RLKs AtCEPR1/AtCEPR2 (CEP Receptor 1 and 2) [85], closely related to MtCRA2 in *M. truncatula* (Figure 1B). Noteworthy, the binding of the AtCEP1 peptide to these two CEPR receptors was demonstrated [85]. A shoot-to-root systemic signal upregulating *AtNRT2.1* expression in roots downstream of the AtCEP/CEPR1 pathway was identified as belonging to the class III glutaredoxins family, and so called AtCEPD1 (CEP Downstream1) and AtCEPD2. Split-root experiments revealed that these shoot-produced AtCEPDs are translocated through the phloem in the root system to upregulate *AtNRT2.1* expression only in roots locally supplied with NO_3^- , through a yet unknown mechanism implying an interaction with local N conditions [86] (Figure 1B). Interestingly, it was previously shown that the Class III glutaredoxins AtGRX3/4/5/8, homologous to CEPDs, were regulated by NO_3^- and CK, and negatively controlled primary root growth [87], suggesting a complex involvement of GRXs in the NO_3^- -dependent root plasticity. Overall, the CEP/CEPR/CEPD pathway is a root-shoot-root circuit allowing plants to adapt to heterogeneous NO_3^- supply in soils.

In Medicago, CEPDs homologs were recently identified as upregulated by N deficiency conditions depending on the MtCRA2 receptor, both in shoots and in roots, in agreement with the role of MtCRA2 in shoots or roots to regulate nodule or lateral root development, respectively [61, 73, 75], but it remains to be established whether *MtNRT2.1* homologs as well as nodulation are targeted by a

MtCRA2/MtCEPD pathway. Conversely, a relationship between the Arabidopsis CEP/CEPR pathway and the conserved miR2111/TML-like module remains to be explored.

Finally, in Arabidopsis, another member of the class III GRX family expressed predominantly in shoots under N deficiency, and denominated AtCEDPlike2 (AtCEPDL2), was recently shown to regulate root NO_3^- uptake and root-to-shoot transport [88] (Figure 1B). AtCEPDL2 acts independently of AtCEPR1/2 but complements *cepd1/2* mutations for the NO_3^- uptake [88]. Interestingly, the maximal induction of *AtCEPDL2* expression in shoots requires *tZ* accumulation [88] (Figure 1B). As described in the previous part, *tZ/tZR* is a N-supply root-borne signal whose translocation in shoots has a positive effect on NO_3^- uptake, including *AtNRT2.1* upregulation, and on lateral root growth [55]. Altogether, these results show that multiple systemic signals coexist to positively regulate NO_3^- uptake in plants. This would allow integrating local N-deprivation pathways (*i.e.*, CEP/CEPR/CEPD) with global N-deprivation pathways (*i.e.*, CEPDL) and local NO_3^- supply (*i.e.*, *tZ/tZR*) to finely modulate N acquisition in NO_3^- heterogeneous environment (Figure 1B).

Influence of shoot environment on N-related long-distance signaling

The systemic regulation of root N acquisition and fixation relies on the perception of root-borne signals modulated by local environmental soil conditions, and by their integration in shoots. This suggests that the N acquisition may be modulated depending on the nutrient status of shoots, *i.e.* the N status but also C resources that can be provided to sustain root and nodule development as well as N assimilation, and thus on plant C fixation capacities and C allocation to roots. Aboveground atmospheric environmental conditions, including CO_2 levels as well as light quality and quantity, are thus anticipated to impact systemic signaling pathways targeting N acquisition. The increase of atmospheric CO_2 concentrations associated to the global climate change has various consequences depending on plant species (Box 3), notably impacting C allocation to roots and the different modes of N mineral vs symbiotic acquisition. Below, we expose emerging data indicating that C provision likely impacts N-systemic signaling effectors and pathways regulating N acquisition.

In *M. truncatula*, additionally to their transcriptional upregulation in response to low NO_3^- , the expression of most *CEP* genes is cumulatively induced by low N and high CO_2 (800 ppm) conditions [72]. In Arabidopsis, the root architecture phenotype of the *cep3* mutant is observed not only under low N but also under low light conditions [80]. In addition, a subset of *AtCEP* genes (*AtCEP5-AtCEP9*) is upregulated by a treatment with metabolizable sugars, such as sucrose [89]. AtCEP5 notably restricts the promotion of lateral root growth in response to sucrose depending on an AtCEPR1 activity in both roots and shoots. An upregulation of sucrose responsive genes is detected in the *Atcepr1* transcriptome, suggesting a potential interaction with the SnRK1 (Sucrose non-fermenting 1-Related protein Kinase 1) C-signaling pathway [89]. This indicates that the Arabidopsis CEP/CEPR1 pathway, and thus likely by homology the *M. truncatula* CEP/CRA2 pathway, integrates N and C signals to modulate root system architecture and growth. Whether this coordination implies a regulation by C sources of already known systemic effectors (e.g. CEPDs and/or the miR2111), and/or of novel shoot-to-root signals, remain to be discovered (Figure 1B). In addition, the role of the AtCEPR1/MtCRA2 pathway into the regulation by C provision of root NO_3^- transport, assimilation, and corresponding genes should be investigated. Indeed, photosynthesis products stimulate NO_3^- uptake and NO_3^- transporter gene expression [90, 91]. To date, the regulation of *AtNRT2.1* and *AtNPF6.3/NRT1.1* seems not dependent on the SnRK1 pathway [92] but rather linked to the oxidative pentose phosphate pathway (OPPP) [93, 94]. In addition, glucose itself promotes NRT2.1 protein accumulation [94]. Interestingly, the redox metabolism, including the OPPP output, may actually regulate NO_3^- transport depending on N and C signaling (see [95] for a review). On this line, the CEPDs/CEPDLs shoot-born mobile signals, being class III GRX, may thus participate to such redox integration of C and N regulations.

In Arabidopsis, a systemic shoot-to-root function of the transcription factor AtHY5 (Elongated Hypocotyl5) was also proposed to control N-acquisition and shoot/root development, depending on the C status of shoots [96]. AtHY5 was initially described as a positive regulator of photomorphogenesis, modulating hypocotyl growth depending on light availability, but was also linked to various aspects of root development and to redox regulations [97-101]. Recently, under light illumination, AtHY5 was shown to up-regulate the expression of sucrose biosynthesis and efflux

genes in shoots (*AtTPS1* for Trehalose-6-Phosphate Synthase; *SWEET11* and *SWEET12*), and AtHY5 proteins was shown to be translocated from shoots-to-roots through the phloem [96]. In roots, AtHY5 binds its own promoter to generate an amplification loop, as well as the *AtNRT2.1* promoter to enhance its expression and root NO₃⁻ uptake [96] (Figure 1B). In *L. japonicus*, LjHY5/LjBzf (BZip ring-Finger) inhibits nodulation but not LR development [102]. However, it is not known if this occurs through a local and/or a systemic regulation.

Finally, hormones are anticipated to mediate the integration of shoot local environmental C-related signals with N-systemic signaling. For instance, CK is a positive regulator of chlorophyll synthesis as well as of chloroplast development [103, 104], and might potentially favor C allocation to roots and thus N acquisition. Conversely in Arabidopsis, photosynthesis-related sugars, which synthesis is favored by elevated CO₂ environments, induce the expression of the IPT3, CYP735A2 and ABCG14 CK biosynthesis enzymes and transporter, and consequently CK accumulation, promoting shoot growth [105]. By promoting CK biosynthesis in shoots, elevated CO₂ may thus interfere with the CK-dependent systemic signaling controlling NO₃⁻ acquisition in roots [55, 88] (Figure 1B). The regulation of CK biosynthesis may depend directly on sugars but also on derived metabolic signals [105], such as glutamine for example, which is also known to influence CK biosynthesis [106].

Interestingly, in Arabidopsis shoots, an overrepresented number of genes encoding enzymes involved in the glutamine metabolism show a differential expression depending on NO₃⁻ availability, and this regulation relies on tZ/tZR translocated from roots [55]. The role of amino acids as a nutrient status reporter of the whole plant level, and in particular of glutamine that is the first amino acid issued from C and N assimilation, was a long-standing hypothesis that could however never be validated experimentally [107].

Altogether, these studies shed light that CK-glutamine interaction in shoots may thus be a potential hub in systemic signaling controlling root responses and integrating C and N availability.

Concluding remarks and Future Perspectives

In legumes, a mechanistic framework of the N-related systemic control of nodulation recently emerged, involving antagonistic CEP/CRA2 and CLE/SUNN-HAR1 pathways differentially activated depending on N availability and/or the whole plant N status to regulate nodulation, and likely more globally root system architecture and N acquisition. This new knowledge allowed reconsidering the historical AON model, which is not only activated by previously established nodules but also by NO_3^- , indicating that rhizobium seems considered as an N source by the plant. In addition, the initially proposed shoot-to-root systemic signal inhibiting nodulation, which may involve CKs in some legume plants, also comprises the repression of a positive regulator of nodulation produced only in shoots, the miR2111 miRNA. Strikingly, the production of this miRNA is actively maintained under low N and/or N deficit conditions by the CEP/CRA2 pathway (Figure 1A). In addition to these two antagonistic systemic pathways regulating rhizobial infections at the onset of nodule initiation, AON-independent systemic signaling pathways also exist to regulate later nodulation stages including N fixation and assimilation (Box 1).

Whether it is through N_2 fixation in symbiotic nodules of legume plants, or NO_3^- import from the soil at the root surface of all plants, N acquisition is constantly modulated by a combination of local and long-distance systemic signals. The parallel made between the legume and Arabidopsis knowledge that is at the basis of this review allowed to highlight similarities and overlaps between N-related systemic pathways regulating N fixing nodules and root N acquisition. The respective role of the mir2111, CEPDs/CEPDLs, CK and HY5 shoot-to-root signals remains however to be better analyzed in parallel between Arabidopsis and legume plants, as well as the potential role of NLP transcription factors to regulate CLE and/or CEP signaling peptides related to N systemic pathways (see “Outstanding Questions”). Some differences exist, such as the regulation of root development by the CEP pathway, both systemically from shoots and locally from roots in Arabidopsis, at least in response to sucrose, or only locally in roots in *M. truncatula*, or such as the LjHAR1/MtSUNN and AtCLV1 CLE peptide receptor functions that, despite being the most closely related proteins, apparently diverged functionally regarding their functions in shoot and floral meristems or N related systemic signaling [108].

Regulatory mechanisms regulating inorganic N acquisition exist in all plants, and are thus expected to be likely conserved in legumes, including the regulation of NO_3^- transport (Box 2). The acquisition of nodulation, implying the co-option of local and systemic N- (or P-) related regulatory circuits, thus required an integration of the regulation of N-fixing nodules with root N-acquisition through related pathways. This may also allow ensuring a robustness of repressive mechanisms against detrimental effects of exaggerated root and nodule development resulting from an over C allocation to nodules, as exemplified by the phenotypes of peptide receptors mutants – either “supernodulating” as *sunh/har1* or “superrooting” as *cra2* (see “Outstanding Questions”).

This review also highlights that we know few about interactions between these different systemic signaling pathways and how they integrate the multiplicity of signals that plants continuously perceive in their different organs (e.g., rhizobium, N-limitation, NO_3^- provision in roots) (see “Outstanding Questions”). For example, in *Arabidopsis*, the study of the potential interactions between CK (tZ/tZR)- and CEPD/CEPDL-pathways in shoots remains in its infancy (Figure 1B). Interestingly, results obtained on the antagonistic regulation of the miR2111 by CRA2 and SUNN illustrates the integration of the balance between permissive and repressive nodulation states according to N provision (i.e., N-limitation and rhizobium) [61] (Figure 1A). Indeed, a specific *M. truncatula* CEP gene, *MtCEP7*, systemically promoting nodulation through the MtCRA2 receptor, was recently shown to be induced by rhizobium and CKs, depending on the MtCRE1 CK receptor and on the MtNIN transcription factor [109] (Figure 1A). Interestingly, MtNIN binds both *MtCLE13* and *MtCEP7* promoters and activate their expression through NIN binding sites required for their activation in response to rhizobium [29, 109]. MtNIN thus coordinates the regulation of specific SSPs from two different families regulating antagonistically nodulation, potentially to allow switching from a negative to a positive regulation of nodulation depending on changes in N availability and of the N plant status [109]. In addition to the co-regulation of specific CLE and CEP genes by a single transcription factor, SSP may also regulate each other’s expression. Indeed, AtCEP3 up-regulates the expression of *AtCLE3*, 4, 7, suggesting that the AtCEP3/AtCEPR1-dependent low N response may promote the low N-dependent AtCLV1/CLE signaling pathway [81] (Figure 1B). Finally, different types of CKs have

various roles in shoots vs roots, and locally vs systemically, but how these specificities are decoded depending on the N availability/whole plant N status remains to be explored.

It is likely critical that local and systemic signaling associated to the heterogeneous distribution of other nutrients than N also intersects with N signaling (see “Outstanding questions”). Herein, we covered interactions between C availability and N-systemic signaling, but other nutrients also have an influence. An implication of N in Pi-limitation signaling was reported [69-71], and one can anticipate a large integration between these two major nutrient signaling pathways. Interestingly, the low Pi regulations of nodulation and of the arbuscular endomycorrhiza symbiosis also require the CLE/SUNN systemic pathway, thus mirroring the AON pathway and so-called AOM for Autoregulation Of Mycorrhization [110-112]. The integration of these different systemic pathways ensuring an integrated plant nutrition through the root acquisition of various mineral nutrients and through mutualistic symbioses establishment is key to understand how plants adapt to fluctuating and heterogeneous environments (See “Outstanding Questions”).

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Figure 1.

Figure 1. Regulation of Nitrogen Fixation and Acquisition by Systemic Signaling Pathways in Legumes and in Arabidopsis.

(A) Systemic signaling repressing (left) and promoting (right) nodulation in legumes.

In legumes, the CLE (CLAVATA3/EMBRYO-SURROUNDING REGION RELATED)-dependent long-distance signaling represses the N fixing nodulation (Blue arrow). Symbiotic rhizobia infection triggers this systemic signaling partially through the CK (Cytokinin) signaling pathway involving the CK receptor CRE1 and the NIN (Nodule Inception) transcription factor activity that are also required for nodulation (+). Nitrate (NO_3^-) also triggers this CLE-systemic signaling through the activity of NLP (NIN-like Protein) transcription factors. CLE peptide signals are perceived in shoots by a CLV1 (CLAVATA1)-like receptor (LRR-RLK, Leucine Rich Repeats - Receptor Like Kinases; SUNN in *M. truncatula*). This induces downstream the expression of *IPT3* (*ISOPENTENYLTRANSFERASE 3*) and thus CK biosynthesis, a putative nodulation “Shoot-Derived Inhibitor” (SDI) signal.

In the *M. truncatula* legume, the CEP (C-Terminally Encoded Peptide)-dependent long-distance signaling promotes N fixing nodulation in low N conditions (Green arrow). CEP peptide signals are perceived in shoots by a LRR-RLK receptor, named CRA2 (Compact Root Architecture2). The MtCEP/MtCRA2 pathway regulates also locally in roots negatively LR (Lateral Root) development and promotes PR (Primary Root) growth. This pathway also systemically regulates LR GSA (Gravitropic Set Angle) by inhibiting shoot-root auxin transport.

These CLE and CEP-systemic signaling pathways respectively repress and induce the expression of the mobile microRNA mir2111, a nodulation “Shoot-Derived Activator” (SDA) signal, that targets the expression of the E3 Ubiquitin Ligase TML (Too Much Love) inhibiting nodulation. Thickness of arrows and lines represents the strength of the signaling pathway.

In addition, rhizobia promote through CK/CRE1 and NIN the expression of MtCEP7, promoting nodulation depending on MtCRA2, thus potentially allowing switching from a negative to a positive regulation of nodulation depending on changes in N availability and of the N plant status.

(B) N provision (left) and N deficiency (right) systemic signaling in Arabidopsis.

On the left (with the light-purple background), NO_3^- signaling requires the NRT1.1/NPF6.3 transceptor and NLP7 in roots. The TCP20 transcription factor (Teosinte branched1/Cycloidea/Proliferating cell factor1-20) interacts with NLP7 and

intersects with N-systemic signaling to control the PNR (Primary Nitrate Response) and LR development. In roots, NO_3^- induces *IPT3* expression and thus the biosynthesis of CK precursor (*tZR*, *trans*-Zeatin Riboside) and active (*tZ*, *trans*-Zeatin) forms, which is followed by their translocation into shoots. Their perception in shoots promotes shoot growth (*tZ*) and Shoot Apical Meristem development (SAM Dvt) (*tZR*), through the induction of the expression of the transcription factor *WUS* (*WUSCHEL*) related to the local CLV3/CLV1 peptide signaling. *tZ* and *tZR* shoot integration controls a shoot-to-root signaling ultimately enhancing PNR, LR development, and NO_3^- transport, also modulating in shoots the expression of glutamine (Gln) metabolism-related genes, which may have a role in N-systemic signaling.

On the right (with the light-green background), low N triggers a CEP/LRR-RLK pathway, similarly as in *M. truncatula*. The CEPR1 receptor homologous to MtCRA2 represses LR and PR development dependent on its activity in roots and in shoots. Repression of LR emergence is also controlled by a root CLE/CLV1 signaling pathway induced by a low N (shoot) status, and maybe also by CEP peptides. Low N availability also triggers in shoots the expression of class III Glutaredoxins, named CEPD1&2 (CEP-Downstream) acting downstream of the CEP/CEPR1 pathway, and of CEPD-like2 depending on the shoot low N status. These CEPD shoot-to-root mobile signals enhance the expression of the *AtNRT2.1* NO_3^- transport gene, and thus NO_3^- uptake. CEPDL enhances also *AtNRT1.5* expression and thus NO_3^- root-to-shoot transport (R→S). *tZ/tZR* being required for *CEPD-like2* expression, the interaction in shoots between the “ NO_3^- /CK” and the “CEP/CEPD/CEPD-like” systemic signaling may finely tune N acquisition depending on root NO_3^- availability and N-demand.

At the extreme left (with the yellow background), the integration between phosphate (P/Pi) or carbon (C) signaling with the N-systemic signaling network is depicted, *via* i) potentially the conserved miR2111/TML pathway, paralleling the mir399/PHO2 pathway known to control Pi acquisition (Acqu.) in relation to the regulation of *NRT1.1/NPF6.3*; ii) the shoot-to-root HY5 (Elongated Hypocotyl5) transcription factor controlling its own expression, *AtNRT2.1* and NO_3^- uptake, and iii) a possible direct or indirect regulation of the expression of the different shoot systemic effectors (arrow pointing on the grey dotted frame).

Box 1. Systemic control of the N₂ fixing nodule symbiosis in legume plants

Nodule organogenesis and maintenance are energetically costly, thus legumes must tightly control their number and activity to balance nutrient gain and loss, thanks to a dedicated network of signaling pathways [113]. First evidences of a systemic control of nodulation, in the early 50's, came from nodule excision, varieties or mutant grafting, and split-root experiments in soybean, which highlighted that mechanisms of host plants limiting the nodule number integrates shoot and root cues [114-116]. This systemic negative regulation of nodulation was named Autoregulation Of Nodulation (AON) [117], as it originally corresponded to a restriction of the nodule number following a first wave of rhizobial infection events. Later, the observation that the nodulation of super/hypernodulating mutants was also NO₃⁻ resistant expanded the delineation of AON to nodulation repressive conditions mediated by high NO₃⁻, which for the plant is a cheaper source of N than N fixing rhizobia are [17]. More recent results showed that the systemic AON restricts rhizobial infections, likely by impairing the perception of rhizobial signals [60, 64, 118]. N-repression involves not only "AON"-dependent mechanisms, but also independent local and/or systemic pathways controlling nodule number and rhizobial infections as well as later nodulation stages, including N fixation and assimilation in nodules [33, 119-121].

N-limitation is a mandatory factor for promoting root nodule symbiosis. Split-root experiments showed that new nodule formation and nodule expansion, rather than an increase in N-fixation activity in existing nodules, which seem already at their maximum capacity, is systemically compensated in distal non N-limited area [120, 122]. The stimulation of mature nodule metabolism and expansion would act through an independent pathway and rely on a rapid re-allocation of sucrose to sustain nodule metabolism along with a repression of nodule senescence and plant defense markers [123].

Box 2. Systemic control of nitrogen acquisition in roots

Plants can acquire N from a range of mineral sources, NO_3^- and NH_4^+ , and from organic compounds (e.g., amino acids) [124-126]. The acquisition of mineral N relies on root transporters belonging to three main gene families: NRT2s and NPFs/NRT1s for NO_3^- , and AMTs for NH_4^+ [127, 128]. Their characterization advanced our understanding of N acquisition regulation by environmental cues, including the fluctuating availability of the ions themselves [129, 130]. Moreover, an intricate relationship has been established with a role of some transporters in the control of root development, independently of their transport activity [131-133]. The architecture of N acquisition (i.e., root transporters and development) is tightly regulated by a network of local but also systemic signaling, integrating N external availability with the global plant N need and in coordination with other nutrient resource pools [134, 135].

Early works using split-root experiments in rice, corn and barley revealed that a systemic compensation response to a N-deprivation exists in the distant root system consisting in an increased NO_3^- uptake and root development [136] [137]. Going along with these physiological responses, NO_3^- supplied roots display a rapid increase in the expression of genes involved in NO_3^- transport and assimilation, in split-root experiments performed on Arabidopsis and Medicago [54, 121, 138]. These physiological and molecular responses are controlled by a systemic N-demand signaling that likely includes several signaling pathways related to the heterogeneous NO_3^- availability at the root system level and/or to the NO_3^-/N -limited status of shoots [54, 85, 90, 138-141]. Local NH_4^+ availability is not compensated by an increase of NH_4^+ uptake [121, 138], but rather in Arabidopsis by an AMT1.3-dependent enhanced proliferation of lateral roots [132], indicating some specificity of the N-systemic signaling with respect to the N source.

On the other hand, all these N acquisition components are repressed by a systemic N supply [54, 121, 142], suggesting a lower specificity for the repressive mechanisms. For instance, as amino acid supply represses N acquisition, and as they can circulate inside plants, their role as a systemic inhibitory signal for N acquisition was proposed [143, 144].

Box 3. Differential plant N acquisition efficiencies to elevated CO₂

The “anthropocene” era is synonymous of increased greenhouse gas emissions. In the 1750-2011 period, CO₂ concentrations increased from 280 to 380 ppm, with a dramatic acceleration of emissions these last 40 years (+2ppm/year). Consequences of associated climate changes on plant responses have been evaluated these last years, notably with the FACE system (Free-Air CO₂ Enrichment) that allowed simulating the effect of CO₂-enriched atmosphere up to a whole ecosystem for several years [145]. Increasing CO₂ concentrations in the atmosphere is expected to increase plant biomass and yield due to a “CO₂ fertilization effect” increasing the photosynthesis rate, but several studies revealed a limited beneficial effect on the long-term in some plants, such as C3 grains (wheat, rice) and legumes (field pea, soybean), leading to lower yields, protein contents, and even nutritional food quality, because of a CO₂/photosynthesis acclimation [145-147]. Whether elevated CO₂ consequences on plant growth and protein content are also different depending on the N inorganic form remains under debate [148, 149]. Some studies point to an inhibition of photorespiration and consequently of NO₃⁻ assimilation [150, 151], whereas other studies report no differences in N assimilation and growth in C3 plants supplied with NO₃⁻ or NH₄⁺ [152].

CO₂/photosynthesis acclimation also strongly relies on the sink organ strength to consume photosynthesis C products, since a reduced sink strength negatively feedback on photosynthesis [145]. The N₂ fixing symbiosis is particularly interesting in this context since nodules constitute an important C sink, and since the nodule N₂ fixation capacity depends on C allocation. Recent studies suggest that the nodule N₂ fixation activity may allow avoidance of the negative feedback on photosynthesis provoked by elevated CO₂ conditions [153]. This way, symbiosis in N fixing legumes is anticipated to gain higher yields and biomass productivity under elevated CO₂, a condition that will be the future agricultural context to consider.

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OUTSTANDING QUESTIONS

- What are the respective role and relevance of evolutionary conserved regulatory modules, including the mir2111, CEPDs/CEPDLs, CK and HY5 mobile signals, in different plants? Combining evolutionary and functional approaches is needed to understand the origin of these systemic signaling pathways and how they have evolved in different plants to ensure a robust regulation of the different N acquisition modes.
- What are the roles and target overlaps of N-related NLP transcription factors, notably regarding the regulation of CLE and/or CEP signaling peptides?
- The co-existence of various systemic signals, such as peptide and hormones, questions how these molecular effectors interact and impact N-systemic signaling.
- How the various roles of different types of CKs, locally in shoots vs roots, and systemically, are decoded depending on the N availability/whole plant N status?
- How low N / N deficiency signals and high N / N satiety signals, as well as local and systemic regulations, integrate?
- How are integrated the multiplicity of N availability signals that plants continuously perceive in different parts of their body together with the whole plant metabolic status as well as in relation to other nutrients than N (e.g., C and P)? How shoot systemic effectors (mir2111, CEPDs/CEPDLs, CK...) are regulated by these other nutrient inputs? Such questions will require the use of integrated analyses.
- Some systemic signals, such as CKs, are known to regulate the vascular system development. This questions the possibility that systemic signals may by themselves modulate vascular channeling and thus long-distance communication.