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Forum

Unlocking the role of *NSP1* and *NSP2*: nutrient-responsive regulators in nodulation

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Symbiotic nitrogen fixation represents a crucial yet energy-demanding strategy for legumes to survive in nutrient-poor soils. We highlight the multifaceted roles of *NSP1* and *NSP2* in this symbiosis and propose their function as 'nutrient-responsive regulators', integrating environmental signals, physiological status, and nutrient availability, to ensure nodulation occurs only under favorable conditions.

Plants require nutrients from variable environments to sustain growth and survival. Most plant species form mutualistic associations with **arbuscular mycorrhizal (AM) fungi** (see Glossary), facilitating phosphorus (P) capture from the soil [1]. Legumes also establish endosymbiosis with **rhizobial bacteria**, which colonize specialized root structures called nodules, where they fix atmospheric nitrogen (N_2) to ammonia [2]. In exchange for enhancing nutrient uptake, microbial associations receive substantial sources of carbon (C) from their host plants. However, the trade-off between the cost of providing carbon and nutrient uptake can restrict plant growth, requiring tight regulation of these symbiotic engagements. Our understanding of how plants regulate these microbial associations has been an area of intense study for several decades [1,2].

Plant-specific GRAS transcriptional regulators, named after its three members: *Gibberellic acid-insensitive* (*GAI*), *Repressor of GAI*, and *Scarecrow*, play crucial roles in development and signaling and in particular facilitate environmental regulation of plant development [3]. Two GRAS proteins, *NODULATION SIGNALING PATHWAY 1* (*NSP1*) and *NSP2*, that form a heterodimer, were initially identified for their essential role in rhizobial symbiosis, but later found to be essential also for AM symbiosis [4–6]. Here, we summarize the multifaceted roles of *NSP1/NSP2* in root endosymbiosis and discuss their potential roles as 'nutrient checkpoints' in the regulation of nodulation in response to complex environmental conditions.

NSP1 and *NSP2* regulate small molecule biosynthesis for plant development and symbiosis

During the mycorrhizal symbiosis, *NSP1/NSP2* coordinate symbiotic signal production and fungal recognition, as a function of nutrient limitation [4]. *NSP1/NSP2* are transcriptionally upregulated by nutrient starvation and promote gene expression associated with the biosynthesis of small molecules, such as **strigolactones (SLs)** [4]. SLs are released into the soil to activate hyphal branching of AM fungi and stimulate fungal production of chitin-derived signals, particularly chitooligosaccharides (COs) and lipo-chitooligosaccharides (LCOs), which are essential signaling molecules for the plant [5]. *NSP1/NSP2* are predicted to regulate **karrikin** molecules for the DWARF14-LIKE (D14L) receptor, which promotes the expression of CO/LCO receptors and several symbiotic signaling genes, facilitating fungal recognition by the plant [4,7]. This P/N-regulated SL production by *NSP1/NSP2* is conserved across multiple plant species [4] and this regulation was shown to also modulate shoot and root architecture in rice (*Oryza sativa*) [8]. This suggests the primary role of *NSP1/NSP2* is regulation of genes involved in secondary metabolism.

Glossary

Arbuscular mycorrhizal (AM) fungi: a group of soil fungi (*Glomeromycota*) that form symbiotic relationships with most land plants. They help plants absorb water, phosphate, and other nutrients from the soil, while receiving photosynthetic carbohydrates, including lipids, in return.

Karrikin: a group of butenolide compounds found from plant-derived smoke, which act as signaling molecules to stimulate seed germination and regulate seedling growth. Karrikin signaling also affects abiotic stress responses and arbuscular mycorrhizal symbiosis.

Nod factors: a class of lipo-chitooligosaccharide signaling molecules produced by rhizobia, which play a crucial role in initiating and establishing the legume–*Rhizobium* symbiosis. Their species-specific structural variations mediate host recognition, ensuring symbiotic specificity.

Rhizobial bacteria: a group of soil bacteria (including *Rhizobium*, *Bradyrhizobium*, and *Sinorhizobium*) that form nitrogen-fixing symbioses with legumes (e.g., peas and beans) and non-legume *Parasponia* plants. They convert atmospheric nitrogen into ammonia within root nodules, while receiving carbon nutrients from their host plants.

Strigolactones (SLs): a class of carotenoid-derived plant hormones that regulate both shoot and root architecture. These compounds are exuded by plant roots as important chemical signals to establish symbiotic relationships with arbuscular mycorrhizal fungi, particularly under phosphorus- and/or nitrogen-deficient conditions.

SLs and the D14L pathway appear to have no effect on nodulation in *Medicago truncatula* [4]. However, the recently demonstrated functions of *NSP1/NSP2* during mycorrhization may shed light on their role in nodulation, although the molecules they regulate during nodulation are likely different. Indeed, many of the secondary metabolite genes regulated by *NSP1/NSP2* are, as yet, uncharacterized [4] and hence this represents an opportunity for enhancing our understanding of small molecules potentially involved in plant development and microbial symbiosis.

NSP1 and *NSP2* are multifaceted gatekeepers in nodulation

During nodulation, the perception of rhizobial LCOs (also known as **Nod factors**) activates the symbiosis signaling pathway [5]. This activation induces calcium oscillations in the nuclei of root epidermal cells, which in turn trigger transcriptional networks that support bacterial infection and nodule organogenesis (Figure 1A). Initially, *NSP1/NSP2* were demonstrated to promote the expression of early nodulation genes downstream of symbiosis signaling [5]. Such a function for *NSP1/NSP2* was further confirmed using gain-of-function mutations in *Calcium/CaM-dependent protein kinase* (*CCaMK*), the calcium decoder in symbiosis signaling. The complete abolition of *CCaMK*-induced spontaneous nodulation in *nsp1/nsp2* mutants [9] provides genetic evidence that *NSP1/NSP2* are integral components of the symbiosis signaling pathway. *NSP1/NSP2* also play a role in the activation of cytokinin signaling in cortical cells below the sites of infection [9,10], which induces their division and leads to nodule formation (Figure 1A). Furthermore, *NSP1/NSP2* are expressed in the nitrogen-fixation zone of nodules [11], indicating a potential role in maintaining nodulation at later stages (Figure 1B). Collectively, these findings indicate that *NSP1/NSP2* function at multiple stages during nodulation, with distinct functions that again highlight their importance in the regulation of both nodulation and the closely related AM symbiosis.

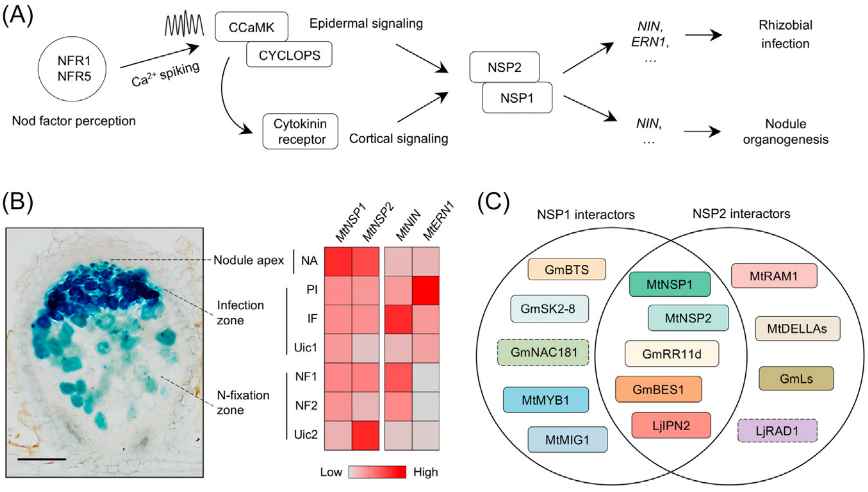

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Figure 1. *NSP1* and *NSP2* play multiple roles during microbial symbiosis. (A) A model describing the symbiosis signaling pathways for rhizobial infection and nodule organogenesis both require NSP1 and NSP2. (B) The left panel shows a nodule section of *Medicago truncatula*, with rhizobia stained in blue. Scale bar, 200 µm. The right panel displays relative expression levels of *MtNSP1*, *MtNSP2*, *MtNIN*, and *MtERN1* in 14-day post-inoculation nodules of *M. truncatula*, based on the single-cell transcriptome data [11]. (C) Known interactors of NSP1 and NSP2. The two broken-line proteins indicate interactions that were tested only with either NSP1 or NSP2 in the original literature. GmBTS is an iron sensor and E3 ligase known as BRUTUS. GmSK2-8, a shaggy-like kinase, mediates salt stress responses. GmNAC181, an NAC transcription factor, enhances salt tolerance during nodulation. MtMYB1, a MYB-like transcription factor, regulates arbuscule degeneration. MtMIG1, MtRAM1, LjRAD1, GRAS transcription factors, are required for arbuscular mycorrhizal symbiosis. GmRR11d, a cytokinin response regulator, mediates the systemic inhibition of symbiotic nodulation. LjIPN2, a MYB-like protein, regulates vascular identity and nodulation. MtDELLAs, members of the GRAS protein family, are key components of gibberellin signaling and the common symbiosis signaling pathway. GmBES1 is a transcription factor associated with brassinosteroid signaling. GmLs, Lateral suppressor, is a GRAS protein that negatively regulates nodulation. Abbreviations: CCaMK, calcium/CaM-dependent protein kinase; ERN1, ethylene response factor required for nodulation; Gm, *Glycine max* (soybean); IF, infection; Lj, *Lotus japonicas*; NF, nitrogen fixation; NFR, Nod factor receptor; NIN, nodule inception; PI, pre-infection; Uic, un-infected cell.

Currently, it is difficult to rationalize the seemingly disparate functions of *NSP1/NSP2* at different stages of symbiosis. The distinct roles of *NSP1/NSP2* in different cell types during nodulation may be attributed to their differential requirements in specific cell layers. Supporting this idea, *NSP1/NSP2* have been shown to interact with multiple proteins of diverse functions (Figure 1C). The integration of multi-omics approaches, such as chromatin immunoprecipitation and single-cell spatial transcriptomics, has potential to unravel the detailed functions of *NSP1/NSP2* and their individual contributions to the regulation of symbiotic interactions.

NSP1 and *NSP2* are environmentally sensitive regulators of symbiosis

A key feature of *NSP1/NSP2* function during mycorrhization is their regulation of symbiosis signaling based on P/N-availability [4]. This regulatory role may also be important during nodulation. Notably, the expression of *NSP2* is negatively regulated by microRNA171h, which is a key component of P-dependent regulation in AM symbiosis [6]. C-availability, determined by photosynthetic capacity, is crucial for plant establishment of symbiosis, because the microbial partners, and the nutrient services they provide, are energy-intensive [1]. Strikingly, the expression of *NSP1* was shown to respond to changes in light quality during nodulation [12], likely due to regulation by light signals and C-availability. The C-related regulatory mechanism is further supported by the circadian rhythmicity of *NSP2* expression [13]. This suggests that *NSP1/NSP2* may regulate symbiosis not only as a function of N/P-availability, but also C-availability. Such a role would be extremely interesting, as the effects of C supply on nodulation are relatively understudied.

It was shown that the PHOSPHATE STARVATION RESPONSE transcription factor binds and activates *NSP1/NSP2* promoters in rice [8], underscoring their link with P homeostasis. However, whether such direct links exist with regulators of N/C metabolism remains unclear.

Moreover, emerging evidence indicates that environmental factors beyond nutrient availability, such as salt stress and iron levels, also influence symbiotic nitrogen fixation through the post-transcriptional modification of NSP1 [14,15] (Figure 1C). Together, these suggest that expression and protein activity of *NSP1/NSP2* are tightly and rapidly regulated by the plant in response to its external environment (Figure 2). However, current knowledge primarily stems from controlled laboratory experiments that typically expose plants to isolated environmental stressors. A critical research direction involves elucidating the mechanisms by which *NSP1/NSP2* perceive and integrate multiple signaling cues from dynamic abiotic and biotic factors in natural environment.

Understanding ‘nutrient-responsive regulators’ for sustainable agriculture

Plants are able to measure multiple facets of nutrient availability, coordinating microbial associations to optimize nutrient capture and overall growth [1]. To date, how the C:N:P ratio is measured and balanced during symbiotic nutrient acquisition remains undefined, but *NSP1/NSP2* have emerged as strong candidate regulators in this process. We hypothesize that *NSP1/NSP2* not only serve as indicators of the plant’s need for N/P but also act as sensors for available C. In this framework, *NSP1/NSP2* could act as gatekeepers of symbiosis, only allowing engagement with symbiotic microorganisms when N and/or P are limited and when photosynthetic capacity is sufficient to sustain the increased C-demand. Such a gatekeeping function would enable a precise and rapid regulation within the molecular pathways of symbiosis [5], allowing the plant to respond dynamically to changing nutrient conditions and optimize its symbiotic interactions accordingly.

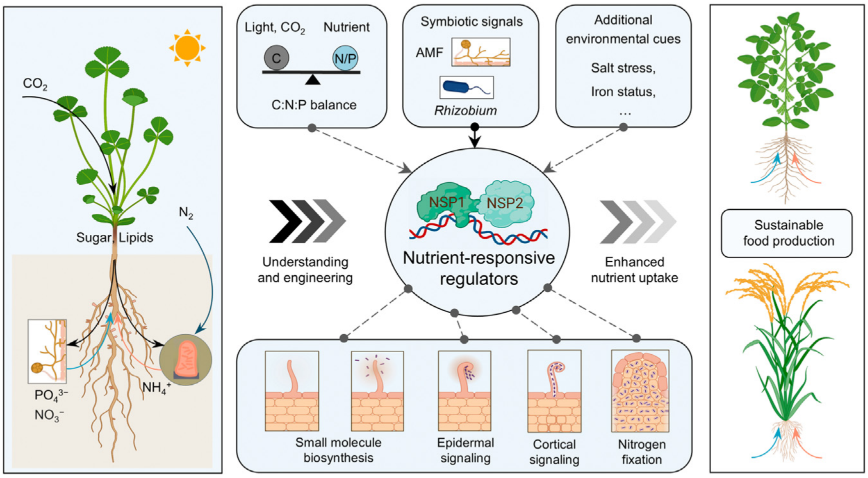

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Figure 2. *NSP1* and *NSP2* may serve as ‘nutrient-responsive regulators’, integrating environmental signals and nutrient availability to ensure nodulation under optimal conditions. The left panel illustrates the mutualistic symbiotic relationships between the model legume *Medicago truncatula*, arbuscular mycorrhizal fungi (AMF), and nitrogen-fixing rhizobia. The plant absorbs CO_2 through photosynthesis and transfers photosynthetic products (sugars and lipids) to the symbiotic partners, while AMF acquires phosphate (PO_4^{3-}) and nitrate (NO_3^-) from the soil, and the rhizobia fix atmospheric N_2 and provide ammonia (NH_4^+) to the plant. The middle panel shows that *NSP1* and *NSP2* function as ‘nutrient-responsive regulators’, integrating diverse environmental signals and nutrient conditions to regulate multiple stages of nodulation processes. The unbroken black lines indicate associations that have been extensively supported by studies. The gray broken lines indicate associations that are supported by emerging evidence discussed in this study, yet require further validation. Understanding and engineering these molecular mechanisms can significantly advance sustainable agricultural practices. The right panel illustrates the application of microbial symbiosis in crop improvement, with soybean and rice as representative examples. This innovative approach offers a promising strategy for enhancing crop productivity while reducing dependence on chemical fertilizers. Figure created with biorender.com.

We propose a novel concept, termed ‘nutrient-responsive regulators’, which ensures that symbiosis only occurs during suitable environmental conditions (Figure 2). This concept is consistent with the recently defined roles of *NSP1/NSP2* during AM symbiosis [4,8] and provides a valuable platform for studying *NSP1/NSP2* function during nodulation and for understanding their broader roles in plants. As *NSP1/NSP2* are widely conserved in plants [3,5], studying their evolutionary shift from plant development and AM symbiosis to nodulation will provide critical insights for genetic engineering.

Understanding how such ‘nutrient-responsive regulators’ integrate environmental signals and nutrient availability to modulate nodulation could enhance N-fixation in leguminous crops. By deciphering the environmental regulation of symbiosis, we can harness these microbial partnerships, particularly the widespread AM interactions, for agricultural applications. This knowledge has the potential to improve sustainable food production by optimizing beneficial plant–microbe relationships.

Author contributions

J-P.G. and G.E.D.O wrote the manuscript with input from C.H.C. All authors read and approved the final version of the manuscript.

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Declaration of interests

The authors declare no competing interests.

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