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Review

The NMN Module Conducts Nodule Number Orchestra

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Legumes control nodule number through nodulation and autoregulation of nodulation (AON) pathways. Nodule Inception (NIN) is essential for rhizobial infection and nodule organogenesis in legumes. The GmNINa-miR172c-NNC1 (NMN) module, which consists of two positive regulators, GmNINa and miR172c, and a suppressor, NNC1, integrates both pathways. GmNINa activates miR172c to downregulate *NNC1*, leading to nodulation, while NNC1 inhibits miR172c expression, forming a negative feedback loop. GmNINa and NNC1 interact with each other and antagonistically fine-tune *GmRIC1/RIC2* expression, turning AON on and off. Conversely, activation of AON inhibits *GmNINa* and miR172c expression, thereby reducing their inhibitory effects on NNC1 to attenuate both nodulation signaling and AON. The NMN module functions not only as an "accelerator" of the nodulation signal to promote nodulation but also as a "brake" on the signal by activating AON to orchestrate nodule number.

The nodule is a unique root organ of legumes and several nonlegume plants that hosts soil-borne rhizobia or Frankia bacteria to construct mutually beneficial relations. Nodule formation is initiated by the molecular communication between rhizobia and the host plant through the nodulation signaling pathway. Because nodulation and symbiotic nitrogen fixation are highly energy-demanding processes that are tightly linked to photoassimilate consumption of the host, nodule number is also dynamically and precisely controlled by a long-distance feedback loop, named autoregulation of nodulation (AON). Coordination of nodule formation and systemic nodulation inhibition underpins the growth of the host plant. To achieve maximum gains, plants have evolved a molecular machinery that integrates nodulation and AON signaling pathways to coordinate downstream cellular responses. In soybean, the NMN regulatory module, which consists of soybean Nodule inception (GmNINa), MicroRNA172c (miR172c) and Nodule number control 1 (NNC1), has been shown to control nodule number by orchestrating dynamic cross talk between nodulation and long-distance feedback signaling in soybean. In this study, we provide a review of the NMN module control of nodule number and discuss how it functions and whether its function is conserved, thereby helping to elucidate the mechanistic control of nodule number.

NIN IS A CENTRAL REGULATOR OF NF SIGNALING IN LEGUMES

Nodule formation is initiated by the host plant. Under low nitrogen conditions, the plant exudes flavonoid signals from its roots, which are perceived by compatible nitrogen-fixing bacteria rhizobia to induce the production of Nod factor, specialized lipochitooligosaccharide signals (Dakora, 2003; Downie, 2014). Perception of the Nod factor molecules (NFs) by the LysM-type receptor kinases (NF receptors) of the host plant triggers a signaling cascade resulting in cell responses of the root to NFs, rhizobial infection and the formation of root nodules (Limpens and Bisseling, 2003; Limpens et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003; Arrighi et al., 2006; Indrasumunar and Gresshoff, 2010; Downie, 2014).

Regulation of rhizobial infection and nodule organogenesis relies on the action of nodulation-promoting regulators. *Nodule Inception* (*NIN*) in *Lotus japonicus* is an essential component of symbiotic nodulation and is the founder gene for the NIN-like protein (NLP) transcription factor family in plants, which contains conserved Phox and Bem1 (PB1) and RWP-RK domains (Schauser et al., 1999; Konishi and Yanagisawa, 2013; Griesmann et al., 2018; van Velzen et al., 2018). There is extensive evidence showing that *NIN* in legumes regulates nearly all the processes of rhizobial infection and nodule organogenesis (Marsh et al., 2007; Heckmann et al., 2011; Kosuta et al., 2011; Popp and Ott, 2011; Yoro et al., 2014). For example, NIN plays a key role in rhizobial infection by activating expression of infection-specific genes, such as *ENOD11* encoding a putative repetitive proline-rich cell wall protein in *Medicago truncatula* (Andriankaja et al., 2007; Journet et al., 2001; Vernié et al., 2015), *NODULATION PECTATE LYASE* (*NPL*) involved in pectate degradation in the cell wall (Xie et al., 2012), *SCAR-Nodulation* (*SCARN*) responsible for actin

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https://doi.org/10.1016/j.isci.2020.100825

rearrangement during rhizobial infection in *L. japonicus* and *M. truncatula* (Qiu et al., 2015; Liu et al., 2019), and *Epr3* encoding a LysM-type receptor kinase perceiving rhizobial exopolysaccharides and facilitating rhizobial entry in *L. japonicus* (Kawaharada et al., 2015, 2017). During nodule formation, NIN can also target two *Nuclear Factor-Y* (*NF-Y*) subunit genes (*LjNF-YA1* and *LjNF-YB1*) and *ASYMMETRIC LEAVES 2-LIKE 18/LATERAL ORGAN BOUNDARIES DOMAIN 16a* (*ASL18/LBD16a*) that function in cortical cell division and subsequent root nodule organogenesis in *L. japonicus* and *M. truncatula* (Soyano et al. 2013, 2019). It has been shown that NIN can recruit the developmental program of lateral root for root nodule organogenesis through promoting LBD16, which stimulates auxin biosynthesis in *M. truncatula* (Schiessl et al., 2019). Further transcriptome data indicate that NIN regulates polar growth and cell wall modifications, hormonal responses, and nutrient (N, P, and S) uptake and assimilation in *L. japonicus* and *M. truncatula*, making it a central regulatory hub in rhizobial infection (Soyano et al., 2014; Liu et al., 2019). In addition to its role as a transcription activator of nodulation genes, NIN appears to have a positive role in onset of AON signaling and ultimate nodule number control (Soyano et al., 2014). It has been shown that NIN directly binds to the promoters of two genes encoding CLAVATA3/endosperm surrounding region (CLE)-related small peptides, CLE root signal1 (CLE-RS1), and CLE-RS2 and activates their transcription in *L. japonicus*, leading to activation of AON. Conversely, *NIN* gene expression is reduced by shoot-derived inhibitors (SDIs) in a hypernodulation aberrant root formation 1 (HAR1)-dependent manner. As a result, nodulation is suppressed and expression of *CLE-RS1* and *CLE-RS2* is attenuated. Thus, NIN is a common component of both symbiotic nodulation and autoregulation of nodulation signaling pathways that dynamically controls nodule number.

NMN MODULE CONTROLS NODULATION IN SOYBEAN

Comparative sequencing analysis results reveal that NIN is one of the genes that are essential for symbiotic root nodulation in legumes and non-legume plants (Griesmann et al., 2018; van Velzen et al., 2018). In soybean, GmNINa is a functional ortholog of NIN in *L. japonicus* and *M. truncatula* (Wang et al., 2019). GmNINa has a typical NLP protein structure and is highly similar to NIN in Lotus and Medicago. Most importantly, GmNINa has the same role as NIN in Lotus and Medicago during soybean nodulation (Wang et al., 2019). Knockdown or overexpression of *GmNINa* results in developmental defect in rhizobial infection and nodule organogenesis of soybean. Similarly, GmNINa promotes the transcription of the putative homologues of NIN target genes (e.g., *GmNF-YA1a*) during rhizobial infection. Thus, GmNINa may exert its function in rhizobial infection and symbiotic root nodule organogenesis through a conserved regulatory mechanism.

Interestingly, GmNINa has been shown to transcriptionally activate a noncoding small RNA miR172c (Wang et al., 2019). MiR172c is specifically induced by rhizobia in an NF receptor-dependent manner to promote soybean nodulation by mainly cleaving the mRNA of its target gene *NNC1* (*Nodule Number Control1*), which encodes an AP2 transcriptional repressor. Downregulation of *NNC1* reduces NNC1-mediated transcriptional repression of the early nodulin genes *ENOD40-1* and *ENOD40-2*, leading to nodule formation (Wang et al., 2014). NNC1 can also directly target miR172c to repress its transcription in a short feedback loop, and interestingly, NNC1 can interact with GmNINa to repress the transcriptional activation of GmNINa on miR172c with an unknown molecular mechanism. NNC1-mediated transcriptional feedback and antagonistic repression of GmNINa on miR172c may maintain the proper level of miR172c, which is crucial for optimal nodulation (Wang et al., 2019). Taken together, these findings indicate that GmNINa and miR172c, two components of the NMN module, act as key activators of nodule formation during nodulation through downregulating the third component NNC1, a nodulation suppressor in soybean. Reduced expression of *NNC1* by GmNINa-miR172c promotes nodule formation, and conversely, NNC1 represses miR172c expression to inhibit nodulation. Beyond its functional role in nodulation regulation, the NMN module forms a central signal integrator that allows dynamic cross talk of nodulation and AON signaling to define the optimal nodule number.

NMN MODULE CONTROLS AON IN SOYBEAN

AON is a long-distance negative feedback loop that prevents the host plant from excessive nodulation. It has been known that the AON signaling pathway is initiated at the nodule primordia formation stage by root production of nodulation-specific CLAVATA/EAR-related (CLE) peptides (Fletcher et al., 1999; Brand et al., 2000; Penmetsa et al., 2003). These highly conserved nodulation-specific CLE peptides (e.g., MtCLE12 and MtCLE13 in *M. truncatula*, LjCLE-RS1 and LjCLE-RS2 in *L. japonicus*, and GmRIC1 and GmRIC2 in *G. max*) are transported from root to shoot, where they are perceived by their receptors, leucine-rich-repeat receptor-like kinases (e.g., MtSUNN in *M. truncatula*, LjHAR1 in *L. japonicus*, and

GmNARK in *G. max*) located in the parenchyma cell membrane of phloem in leaves to induce SDIs that can be transported to root to inhibit further nodulation (Krusell et al., 2002; Nishimura et al., 2002; Lim et al., 2011; Reid et al., 2011; Mortier et al., 2012). Recently, research found that miR2111 is involved in AON by repressing its target gene Too much love (TML) to keep the plant susceptible to nodulation (Tsikou et al., 2018). Autoregulation is a manifestation of systemic nodulation regulation and is the intrinsic ability of plants to maintain a constant nodule number for optimal growth under low nitrogen conditions.

The first step for turning on AON signaling is to activate these nodule-specific *CLE* genes; thus, the timing and expression levels of these *CLE* genes are crucial to the autoregulation of nodulation. Most recently, the NMN module has been shown to mediate the on-off switch for autoregulation of nodulation. For example, GmNINa has been shown to directly target *GmRIC1* and *GmRIC2* to activate the transcription of two genes (Wang et al., 2019; Suzaki and Nishida, 2019). These findings reveal a conserved mechanism of NIN-mediated transcriptional activation of these nodulation-specific *CLE* genes in nodule number regulation. In addition to direct transcriptional activation of *GmRIC1* and *GmRIC2*, GmNINa can also activate *GmRIC1* and *GmRIC2* by activating miR172c, which reduces the repression of NNC1 on *GmRIC1* and *GmRIC2*. It has been shown that miR172c acts as a positive regulator of *GmRIC1* and *GmRIC2*, whereas NNC1 functions as a repressor of these two genes. The double tuning system of *GmRIC1* and *GmRIC2* may be essential for the precise production of specific CLE peptides. Interestingly, NNC1 has stronger binding activity to the *cis* element of *GmRIC1* and *GmRIC2* than GmNINa to outcompete GmNINa from binding the same sequence, reducing the transcriptional activation of *GmRIC1* and *GmRIC2*. Moreover, NNC1 physically interacts with GmNINa, although the function of the NNC1-GmNINa interaction in antagonistically regulating GmNINa transcriptional activity on the expression of *GmRIC1* and *GmRIC2* has not been determined. It is apparent that NNC1 and GmNINa antagonistically regulate *GmRIC1* and *GmRIC2* transcription, leading to CLE production and switching on AON. Thus, the NMN module plays a central role in the activation and attenuation of AON signaling and responses that control nodule number (Figure 1).

REFINING NODULATION SIGNALS

Early developmental patterning and organ number usually involve a master transcriptional response to morphogenetic signals followed by refinement of this expression domain. One crucial mechanism that can initiate refinement is autoregulation, which can maintain a certain threshold for transcriptional activation. The NMN module is a central transcriptional activator for both nodulation and autoregulation. During rhizobial infection and nodule organogenesis, gene expression of *NIN* is induced (Schauser et al., 1999). NIN activates rhizobial infection and nodule formation, and it can also activate *LjCLE-RS1* and *LjCLE-RS2* and subsequent activation of AON that, in turn, suppresses expression of *NIN* through a long-distance negative feedback loop (Soyano et al., 2014). This autoregulatory circuit is able to refine the expression of *NIN* and maintain a well-defined optimal level for nodulation. The most recent data have shown that *GmNINa* is a downstream gene and that the expression of *GmNINa* is negatively regulated by the Glycine max nodule autoregulation receptor kinase (GmNARK) and AON signaling (Wang et al., 2019), indicating that the autoregulatory circuit for refining transcriptional activation of nodulation is conserved in legumes.

During nodulation, the level and activity of NIN determine the function of the NMN module in soybean. Thus, repression of *GmNINa* by AON would lead to downregulation of miR172c and upregulation of NNC1. Indeed, miR172c acts downstream of GmNARK, and the level of miR172c transcription is negatively regulated by GmNARK (Wang et al., 2014, 2019). In the loss-of-function mutant of *GmNARK*, the expression of miR172c was strongly increased; downregulation or overexpression of miR172c in the mutant of *gmnark* restored or exacerbated the hypernodulation phenotype of the mutant, respectively. Consequently, *NNC1* expression is also regulated by GmNARK and AON (Wang et al., 2014, 2019). Thus, the activation of AON by the NMN module can maintain the activity of the master regulatory module of NMN below a potentially harmful level to control nodulation and nodule number. The molecular mechanism of AON-mediated transcription maintenance of NIN transcription factors has not been determined.

REPROGRAMMING NODULE NUMBER UNDER HIGH NITRATE

The ability of legumes to reprogram nodulation in response to changing environment cues underpins the adaptation of legumes. Nitrogen availability is a primary environmental factor determining rhizobia-legume symbiosis of legumes. Nodule number is a characteristic feature of nitrogen-affected nodulation. With increasing levels of nitrogen, nodule number is gradually reduced and even completely abolished in the presence of sufficient nitrate (Mortier et al., 2011). It has been shown that AON positively regulates

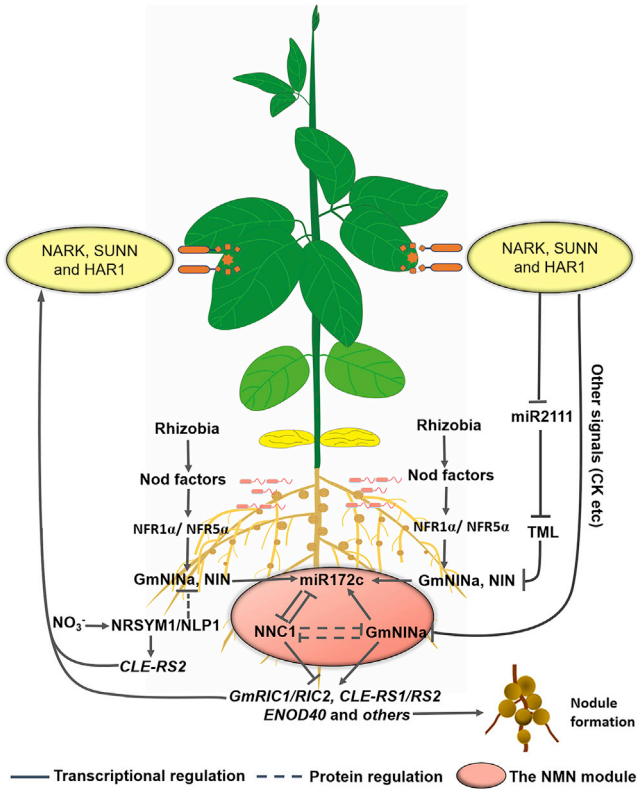

Figure 1. NMN Module Is a Fine Tuner of Nodule Number

NIN and miR172c are positive regulators of nodulation, and NNC1 is a repressor of nodulation. The NMN module integrates both nodulation signaling and AON signaling pathways to systemically control nodule number in legumes. In low-nitrogen conditions, plant exudes flavonoid signals from its root, which are perceived by rhizobia to induce the production of Nod factors. The Nod factors are perceived by the Nod factor receptors (NFR1α and NFR5α in soybean) localized in the plasma membrane of root hair and activate NF signaling pathway. The transcription factors, such as GmNINa in soybean, are induced by NF signaling to activate the expression of miR172c, which then represses *NNC1* expression by cleaving its mRNA. NNC1 in turn downregulates the expression of miR172c or interacts with GmNINa to repress its binding to miR172c to form a negative feedback loop. GmNINa and NNC1 activate or repress the expression of *GmRIC1* and *GmRIC2* in soybean. GmNINa can promote the transcription of *GmRIC1* and *GmRIC2* alone and/or through alleviating NNC1 inhibition on *GmRIC1* and *GmRIC2* by activating miR172c. The CLE peptides are transported to the shoot to activate the nodulation autoregulation receptor, GmNARK in soybean, SUNN in Medicago, and HAR1 in Lotus. The activation of AON produces the SDIs, including cytokinins, miR2111, or other factors, which are transported to the root to inhibit the expression of *NIN* and *GmNINa*, leading to suppression of the downstream nodulation genes (e.g., *EOND40s*) that regulate nodulation. In high-nitrogen conditions, nitrogen induces the subcellular localization of NLP1 in Medicago and NRSYM1 in Lotus from cytoplasm to nucleus. NLP1 interacts with NIN to repress its activation of downstream target genes. NRSYM1 promotes production of CLE peptides to activate AON.

nitrate-induced nodulation inhibition because loss of function mutations in *L. japonicus* HAR1, *M. truncatula* SUNN, and *glycine max* GmNARK reduce the sensitivity of nodulation to high nitrate (Carroll et al., 1985a; Carroll et al., 1985b; Magori and Kawaguchi, 2009; Wopereis et al., 2000; Mortier et al., 2011).

As common components in nodulation and AON, the NMN module has recently been shown to mediate nitrate-induced nodulation inhibition. The nitrate signaling pathway seems to converge toward the NMN module to regulate nodulation. NIN-like protein 1 (NLP1) in *M. truncatula* can translocate into the nucleus under high nitrate to suppress the transcriptional activity of NIN on downstream target genes by directly interacting with NIN during nodulation (Lin et al., 2018). In addition, NITRATE UNRESPONSIVE SYMBIOSIS 1 (NRSYM1), an NLP family transcription factor in *L. japonicus*, enters the nucleus in response to high nitrate to directly activate *CLE-RS2* expression and production of CLE-RS2, resulting in activation of AON (Nishida et al., 2018). This finding, together with the antagonistic function of GmNINa and NNC1 in the expression and production of nodule-specific CLEs, supports the hypothesis that, during nodulation,

NRSYM1 interacts with NIN and/or NNC1 or their counterparts in legumes to regulate ON and OFF of AON in response to environmental nitrate change and to control nodule number for optimal growth of plants, whereas in uninoculated root, nitrate triggers NRSYM1 to activate *CLE* gene expression and inhibits nodulation independently of NIN (Nishida et al., 2018). Thus, the NMN module may also be a central integrator of environmentally modulated nodulation and nodule number regulation in legumes.

CONCLUDING REMARKS

In light of these recently discovered evidence, it is immediately apparent that the NMN module is a master integrator of nodulation and autoregulatory signaling pathways that determine the ultimate nodule number. The NMN module is a shared regulatory unit between nodulation and AON signaling pathways that connect two pathways together to act as one regulatory system. This arrangement may enable plants to systemically monitor incoming signals (e.g., hormones, nutrients, cellular energy levels) from nodulation and energy sensors to activate a cellular machinery that regulates nodulation and autoregulation of nodulation. The various interactions between its components and between the NMN module and other regulators enable the integration of different developmental and environmental pathways to provide a coordinated regulation of nodule formation. Ultimately, the functioning of this molecular regulatory hub ensures that nodule number is precisely and dynamically controlled under certain soil conditions, enabling adaptive growth.

These works have established a fundamental role for the NMN module in nodule number control in soybean. The apparent conservation of the components of the NMN module or the NMN module suggests that leguminous plants may have evolved this unique module to master-switch the nodule number during evolution of rhizobia-legume symbiosis. Thus, the presence of the NMN module as a master switch for nodule number control might be shared by a large number of legumes. The common thread that links the NMN module to various aspects of nodule number control is its transcriptional regulation of numerous genes during nodulation and autoregulation. It is important to determine how the NMN module interacts with other transcription factors through various *cis* elements, particularly how the NMN-mediated nodule number regulation is integrated spatially and temporally. Many outstanding questions in this research direction await to be answered. These include: what are the cellular cues that controls this master integrator? How does this master switch integrate with other regulators in the control of nodule number? The precise mechanisms by which the NMN module controls these biological processes are likely to vary among different leguminous plants. Thus, understanding the regulatory circuit of the NMN module in nodule formation and development in these species will help decipher the genetic basis and evolution of nodule number regulation and will facilitate genetic improvement of the symbiotic nitrogen fixation efficiency of leguminous crops.

In conclusion, unveiling how this NMN master switch spatially and temporally orchestrates the nodule number of legumes may help to elucidate the molecular mechanism of nodule number control. The integration of these intrinsic and extrinsic signaling pathways at the NMN module may help to elucidate how the number of symbiotic nodules is continuously optimized to respond to the changing environment.

ACKNOWLEDGMENTS

This work was supported by grants from the National Key Research and Development Program of China (2016YFA0500503), the National Natural Science Foundation of China (31730066), the Huazhong Agricultural University Scientific and Technological Self-innovation Foundation (2015RC014) and the Fundamental Research Funds for Central Universities (2662018PY075).

AUTHOR CONTRIBUTIONS

X.L. outlined the manuscript. All authors wrote the manuscript and prepared the figures. X.L. and Z.W. edited and provided feedback on the manuscript.

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