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Identification of Key Signaling Genes in Soybean-Rhizobium Interaction

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Abstract The soybean-rhizobium symbiosis plays a crucial role in sustainable agriculture, promoting biological nitrogen fixation and reducing dependence on synthetic fertilizers. This study focuses on the molecular mechanisms underlying this symbiotic relationship, with particular emphasis on the identification and characterization of key signaling genes involved in nodulation. We explore the role of nodulation factors and their perception by LysM receptor-like kinases (e.g., GmNFR1 and GmNFR5) and downstream signaling components, including calcium/calmodulin-dependent kinases and transcription factors, such as NIN and ERN1. We further discuss the functional characteristics of these genes, drawing on evidence from gene knockout, overexpression, RNAi, and CRISPR-Cas-based studies. We also highlight the integration of transcriptomics and proteomics approaches in identifying new candidate genes. Furthermore, this study explores the interplay between symbiotic signaling and other regulatory pathways, including plant hormone signaling, defense responses, and environmental cues. Using GmNARK, a key regulator of nodulation autoregulation, as an example, we delve into its negative feedback mechanism and its impact on enhancing nodulation efficiency. Finally, the biotechnological applications of these signaling genes in breeding strategies aimed at enhancing nitrogen fixation and increasing soybean yield are discussed. This study aims to comprehensively understand the signaling networks in the soybean-rhizobium symbiosis system and outline future directions for sustainable improvement of legumes using advanced genomics and synthetic biology tools.

Keywords Soybean-rhizobium symbiosis; Nodulation signaling genes; LysM receptors; GmNARK; Nitrogen fixation

1 Introduction

In agricultural production, soybeans (*Glycine max*) have a very special ability - they can coexist with rhizobia. This cooperative relationship is no simple matter. Rhizobia live in the roots of soybeans to form root nodules, helping to "convert" nitrogen in the air into ammonia that plants can use. This matter may seem minor, but it is actually remarkable: not only does it save the trouble of nitrogen fertilizer, but it also helps to cultivate the land well. However, such "cooperation" is not something that everyone can participate in; it requires matching and communication. Without that set of precise communication at the molecular level, soybeans and rhizobia would have no connection at all (Ratu et al., 2021).

Interestingly, behind this mutualistic symbiosis lies a complex signal network in operation. For instance, soybeans have to first recognize the signal molecules released by rhizobia with their own receptors (such as NFR1, NFR5), namely the so-called nodular factors, before initiating subsequent physiological responses and gradually developing into the shape of rhizobia (Okazaki et al., 2013; Ma et al., 2021). However, the matter goes far beyond this. Hormones within plants, isoflavones secreted by roots, and the type III secretion system (T3SS) of rhizobia themselves are all involved, regulating the efficiency and specificity of symbiosis (Darwish et al., 2022; Chen et al., 2023). Sometimes, these factors also "fight" or "collaborate" with each other. For instance, the interaction among hormone signals, transcription factors and bacterial effector proteins is one of the key links that determine the success or failure of symbiosis (Lu et al., 2023).

Based on these phenomena, we intend to review the current research progress on the key signaling genes in the interaction between soybeans and rhizobia. We do not only focus on the signal recognition step, but also discuss the effector molecules released by plants and bacteria, hormone effects, environmental interference factors, as well as the genetic regulatory mechanisms behind root tumor development. This study hopes to find some new directions for enhancing the nitrogen fixation capacity of soybeans through these reviews and also provide some references for the future green transformation of agriculture.

2 Molecular Basis of Soybean-Rhizobium Symbiosis

2.1 Role of Nod factors and their perception by soybean root cells

To start symbiosis, soybeans and rhizobia need to greet each other first, and the "tumor-forming factor" is precisely the first signal sent by rhizobia. These lipid shell oligosaccharide structure signal molecules can be "heard" by the NFR1 and NFR5 receptors on soybean root hairs. Once identified successfully, these two recipients will team up to form a complex and transmit the signal in. During this process, guanine nucleotide exchange factors such as GmGEF2 are phosphorylated, small GTPases (such as GmROP9) become active, and scaffold proteins such as GmRACK1 are added. Finally, a multi-protein complex specifically for processing symbiotic signals is formed (Figure 1) (Gao et al., 2021; Yuan et al., 2023). Of course, not all plants can "communicate" like this. The matching degree between signal molecules and receptors determines whether this relationship can hold and how efficient it is (Kidaj et al., 2020; Kronauer and Radutoiu, 2021).

2.2 Early signaling events in nodule formation

After perceiving the Nod factor, the root's response is also very fast. First, there is a fluctuation in membrane potential, followed by a rapid increase in calcium ion concentration, and receptor-like kinases and G protein signaling pathways are also successively awakened. Take NFR1 as an example. It interacts with RGS proteins and causes them to phosphorylate, thereby regulating G protein activity - which is crucial in controlling root tumor formation (Choudhury and Pandey, 2024). Meanwhile, early response genes, such as *ENOD40*, as well as transcription factors like NIN and NSP1, also began to be upregulated, promoting root hair deformation, infection filament entry, and cortical cell division (Singh et al., 2021). However, things do not progress so linearly - the REDOX state and hormone signals within plants, such as the interweaving of auxin and cytokinin, can also interfere with or regulate these early steps, ensuring that root nodules grow where and at the right time (Buhian and Bensmihen, 2018).

2.3 Cellular reprogramming in response to symbiotic cues

The behavior of cells undergoes a series of changes under the influence of signals. For instance, the structure of the root cytoskeleton will be rearranged, transcription factors such as WRKY17 that are specifically active in root nodules will be activated, and micrornas (like miR172c, miR167) will also begin to participate in regulating those important genes related to nodules (Wang et al., 2014; Wang et al., 2015). This reprogramming is not limited to the surface - the root hairs will deform, the infected filaments will drill in all the way, and the cortical cells will start to divide, eventually forming the root nodule primordia. Subsequently, complex processes such as the formation of symbiotic membranes and cell functional differentiation unfolded one after another, establishing channels for nutrient and signal exchange between plants and bacteria (Geurts et al., 2005). However, this process is not entirely autonomous - it is simultaneously regulated by local signals, whole-plant feedback, and even the external environment. For instance, the plant's own "nodule regulation circuit" sometimes presses the pause button to control the number and distribution of root nodules (Fernandez-Gobel et al., 2019).

3 Key Classes of Signaling Genes Involved in Soybean-Rhizobium Interaction

3.1 LysM receptor-like kinases (e.g., GmNFR1, GmNFR5) and their recognition roles

Whether soybeans can "understand" the signals of rhizobia depends on a type of protein called LysM receptor-like kinases, especially GmNFR1 and GmNFR5. From the very beginning, it was they who were responsible for identifying the nodular factors released by rhizobia. However, this recognition is not merely a single dialogue; it is more like a "linkage" involving multiple parties - these receptors will form complexes together with other proteins such as GmREM1a and may also be regulated by the rhizobia effector factor NopL, which can enhance the recruitment ability of GmNFR5, thereby promoting the amplification of symbiotic signals. The expression level of receptors will increase rapidly after rhizobia inoculation, and without them, the subsequent formation of root nodules is basically difficult to carry out. So, although they are "sentries at the door", their status is by no means low.

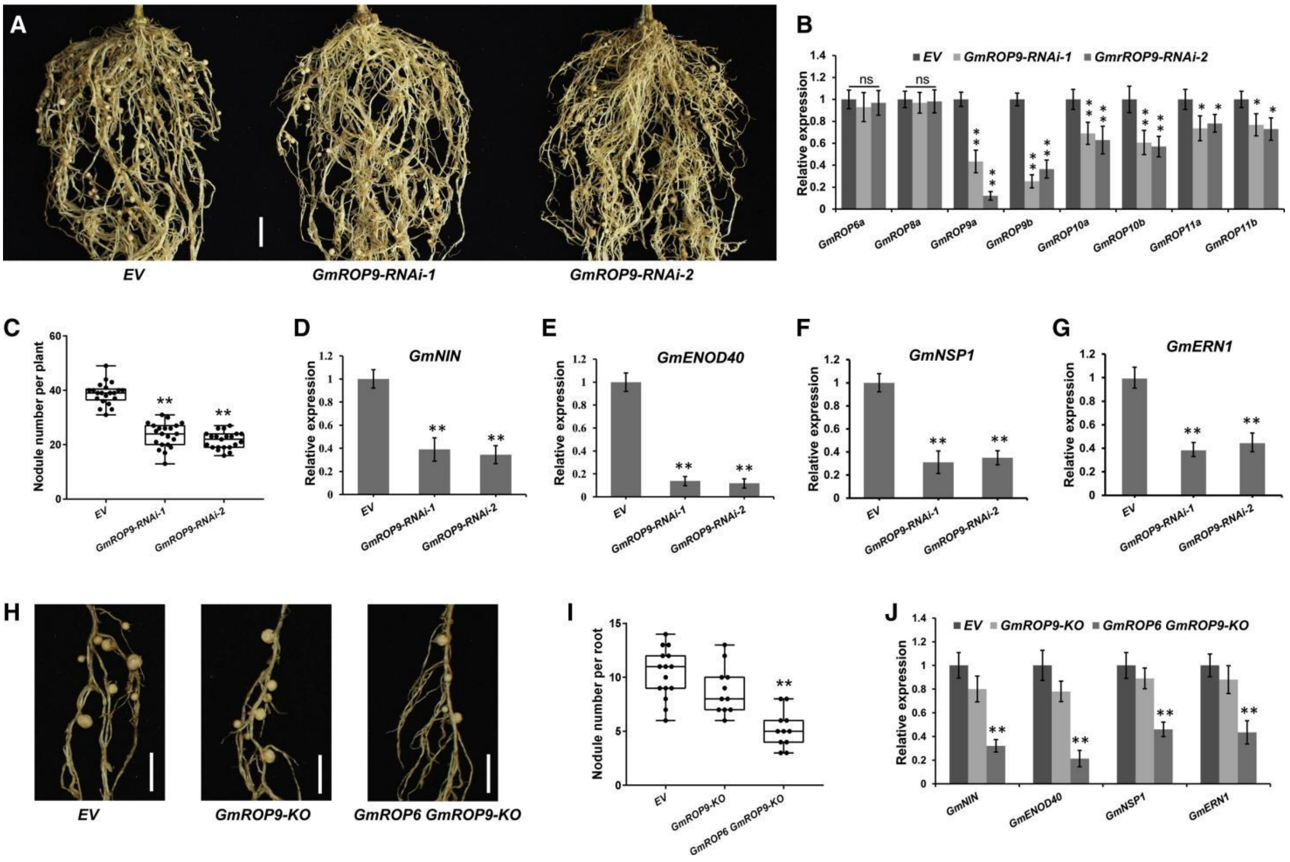

Figure 1 GmROP9 is involved in root nodule symbiosis (Adopted from Gao et al., 2021)

Image caption: (A) Representative hairy roots expressing empty vector (EV), *GmROP9-RNAi-2-1*, or *GmROP9-RNAi-2-2* construct at 21 days post-inoculation (dpi) with rhizobia. Scale bar represents 1 cm. (B) Transcript levels of *GmROP* genes in EV and *GmROP9-RNAi-2* transgenic roots. Data are normalized to *GmELF1b* and are mean ± SD from three replicates. Statistically significant differences were detected by Student's t test; ns indicates not statistically significant, $*p < 0.05$, and $**p < 0.01$. (C) Nodule numbers per plant transformed with EV and two independent *GmROP9-RNAi-2* constructs (*GmROP9-RNAi-2-1* and *GmROP9-RNAi-2-2*) at 21 dpi. Three biological replicates were performed, and at least 20 independent transgenic hairy roots per construct were analyzed and used for statistical analysis. Boxes show the first quartile, median, and third quartile; whiskers show minimum and maximum values; and dots show data points. Statistically significant *differences* were detected by Student's t test; $**p < 0.01$. (D–G) Transcript levels of *GmNIN* (D), *GmENOD40* (E), *GmNSP1* (F), and *GmERN1* (G) in EV and *GmROP9-RNAi-2* transgenic roots. Data are normalized to *GmELF1b* and are mean ± SD from three replicates. Statistically significant differences were detected by Student's *t* test; $**p < 0.01$. (H) Representative hairy root expressing EV, *GmRO9-KO*, or *GmROP6 GmROP9-KO* construct at 21 dpi. Scale bars represent 5 mm. (I) Nodule numbers per independent roots transformed with EV, *GmROP9-KO*, or *GmROP6 GmROP9-KO* construct at 21 dpi. Three replicates were performed, and at least 10 independent transgenic roots per construct were analyzed and used for statistical analysis. Boxes show the first quartile, median, and third quartile; whiskers show minimum and maximum values; and dots show data points. Statistically significant differences were detected by Student's *t* test; $**p < 0.01$. (J) Transcript levels of *GmNIN*, *GmENOD40*, *GmNSP1*, and *GmERN1* in EV, *GmROP9-KO*, or *GmROP6 GmROP9-KO* transgenic roots. Data are normalized to *GmELF1b* and are mean ± SD from three replicates. Statistically significant differences were detected by Student's *t* test; $**p < 0.01$ (Adopted from Gao et al., 2021)

3.2 Calcium/calmodulin-dependent kinases and nodulation signaling pathways

Once the Nod factor is perceived, the next step is to "translate" the signal into a language that the cells can understand. Here, the calcium signal comes into play. Calcium/calmodulin-dependent kinases (CCaMKs), such as GmDMI3, are key tools responsible for reading calcium oscillations in the cell nucleus. It not only serves as a bridge but also cooperates with many signal proteins and transcription factors to integrate the changes in the external environment with the plant's own developmental signals. There are also some other kinases, such as GmSK2-8, which are quite "busy". They link the stress response of plants with nodule signals by phosphorylating transcription factors like GmNSP1 (He et al., 2020; Wang et al., 2021). Interestingly, these kinases do not act alone. They often interact with plant hormone signals (such as strigolactone, brassinolactone), which makes the regulation of the tumor formation process more flexible and detailed (Ahmad et al., 2019).

3.3 Transcription factors regulating downstream symbiotic gene expression (e.g., NIN, ERN1)

Ultimately, root nodules still rely on gene expression to complete their specific construction, and it is a group of transcription factors that truly "direct" the expression of these genes. NIN (nodule initiation factor) is a typical representative. Its activity can drive the expression of downstream genes such as *ENOD40* and CLE peptide, thereby affecting the formation process and quantity control of root nodules (Zheng et al., 2023). But NIN is not the only "dispatcher". There are also ERN1, NSP1, NSP2, WRKY17 and some members of the ERF family, which are respectively involved in the extension of infected filaments, the structural development of root nodules, and even the transmission of hormone signals (Zhao et al., 2025). Of course, their "superiors" are often those kinases mentioned earlier, and environmental factors also interfere from time to time, making the entire regulatory process both precise and full of variations (Zhang et al., 2023).

4 Functional Characterization of Signaling Genes in Soybean-Rhizobium Interaction

4.1 Gene knockout and overexpression studies in soybean

Not all genes are "reticent"; some, once their expression is enhanced, will bring about significant changes. Genes like *GmPHT1-4*, *GsIMaT2* and *GmD27c*, once overexpressed in soybeans, can be observed to have an increase in the number of root nodules and more vigorous root growth. This indicates that they play a promoting role in nodular signaling or related metabolic pathways (Rehman et al., 2022a; Zhu et al., 2023). Conversely, for those genes that originally had restrictive effects, such as GmD53a and GmSK2-8, when they were knocked down or silenced, the number of root nodules increased instead, and the sensitivity of plants to stress decreased (Rehman et al., 2022b). Therefore, through this "addition and subtraction" operation, it can basically be determined that these signal genes are indeed directly involved in the regulation of tumor formation efficiency and the process of signal transmission.

4.2 RNA interference and CRISPR-Cas technologies for functional validation

If one only relies on expression changes to guess the function of genes, it will always be inaccurate. Thus, RNA interference (RNAi) and CRISPR-Cas have become more reliable "proven tools". For instance, after knocking down GmD53a or GmSK2-8 by RNAi, or removing miR169c with CRISPR-Cas9, the results showed that the number of root nodules, nitrogenase activity, and the expression of some symbiotic related genes were significantly affected (Xu et al., 2020). This targeted approach not only verified the actual role of these genes in tumor formation but also enabled researchers to more clearly observe their positions and functions within the entire regulatory network.

4.3 Use of transcriptomic and proteomic profiling to identify candidate genes

Of course, not all functions need to be tested one by one. Sometimes, researchers tend to use high-throughput methods for screening. Rna-level analysis methods such as transcriptome sequencing can quickly identify genes that are upregulated during root tumor formation, such as *GmNIN*2b, *GmENOD40*, *GmNFR1*, *GmNFR5*, *GmDRR1*, etc. (Shi et al., 2020). But merely looking at transcription is not enough; proteomics is also crucial - after all, it is the proteins that do the work in the end. These analyses revealed some changes in protein abundance and post-translational modifications, thereby further supporting the importance of these candidate genes in root tumor development and information exchange between soybeans and rhizobia.

5 Cross-Talk with Other Signaling Pathways in Soybean-Rhizobium Interaction

5.1 Interaction between symbiotic signaling and plant hormone pathways

Nodulation cannot be accomplished merely by rhizobia signals; the intervention of plant hormones often alters the entire process. For instance, brassinolide (BR) is a "disruptor", which can interfere with the signals of tumor-forming factors and exert an inhibitory effect. The transcriptional regulatory factor GmBES1-1 will bind to *GmNSP1* and GmNSP2, thereby rendering them "ineffective" and ultimately inhibiting the tumor formation process. On the contrary, some hormones are "helpful", such as strigolactone (SL) and karigin (KAR), which indirectly affect multiple signals including auxin, jasmonic acid and abscisic acid by regulating genes like *GmMAX2a*, *GmD14s* and *GmKAI*. This signal series connection makes nodules more likely to occur (Rehman et al., 2018). It is worth mentioning that the phosphorus transporter GmPHT1-4 has also been found to be present in multiple hormone signaling pathways and may even be a key node for integration effect signaling.

5.2 Overlap between defense and symbiotic gene regulatory networks

The process of symbiosis is actually not completely "peaceful", and the boundary between it and the defense responses of plants is often blurred. RIN4, a familiar immune regulatory factor, is rapidly phosphorylated shortly after rhizobia inoculation. Studies have found that it is not only involved in defense but also promotes nodule formation (Toth et al., 2025). However, rhizobia do not just sit and wait to die. The type III effector molecules they release, such as NopL, NopT, NopP, NopAA, and NopD, will "communicate" with proteins like GmREM1a, GmNFR5, and GmPBS1 in the host, thereby influencing the plant's existing defense mechanisms. It also rewrites the tumor formation process (Li et al., 2023; Ma et al., 2024). From the transcriptome perspective, these changes are not just individual phenomena. Many genes regulated by these effector factors occur simultaneously in pathogen responses and hormone signals, indicating that defense and symbiosis are not completely separate paths (Wang et al., 2023).

5.3 Role of environmental cues in modulating signaling

Internal signals alone are far from enough; sometimes the influence of the external environment is more direct. Take nitrogen as an example. Under high nitrogen conditions, miR169c will be activated, thereby inhibiting the expression of GmNFYA-C. The result is a reduction in the number of root nodules - this phenomenon has occurred in various experiments. However, when GmNFYA-C is forcibly overexpressed, even if there is more nitrogen, it can still retain some of its tumorigenesis ability. As for salt stress, it blocks the subsequent steps of tumor formation by activating the kinase GmSK2-8, which limits the function of *GmNSP1*. Another factor that is often overlooked is light, not the light directly shining on the roots, but the light signals perceived by the above-ground parts. Mobile factors such as GmSTF3/4 and GmFTs from the stem are transmitted to the roots to participate in regulation, bringing the "opinion of the sun" to the symbiotic mechanism. That is to say, environmental signals are not just observing from the periphery; they are actually involved and can even determine the extent to which tumor formation progresses.

6 Case Study

6.1 Functional role of GmNARK as a negative regulator of nodulation

Root nodules cannot grow as many as you want. Plants also have their own "braking mechanism". GmNARK is precisely such a receptor kinase that plays a limiting role. It has a leucine repeat sequence and belongs to the receptor-like kinase family. Whenever rhizobia infect, plant roots produce some CLE signaling peptides, such as GmRIC1 and GmRIC2, which are transported to the above-ground parts and recognized by GmNARK. After recognition, the above-ground part will "reply" to generate some inhibitory signal, which is sent back to the root to prevent the further formation of new root nodules. This mechanism, simply put, is a feedback system to prevent the number of root nodules from getting out of control (Figure 2) (Gresshoff et al., 2025). It can help plants keep the number of root nodules within the range permitted by resources and the environment, avoiding the overall growth being hindered by too many root nodules.

6.2 Insights from mutant analysis and phenotypic characterization

What would happen if GmNARK broke down? Researchers discovered the answer through several mutants. Loss-of-function mutants like nts1007, SS2-2, and Sakukei 4 exhibit a state of "rampant root nodules". Even in environments that usually inhibit nodule formation, such as high nitrate nitrogen or phosphorus deficiency, these mutants still grow a large number of root nodules (Isidra-Arellano et al., 2020). Further analysis revealed that their expression patterns on CLE peptides and downstream related signals were also different from those of normal soybeans, indicating that GmNARK is indeed involved in systemic nodular regulation. Although these mutants have a stronger nitrogen fixation ability, problems also arise: the overall plant growth deteriorates (Lin et al., 2024). That is to say, a large number of root nodules does not necessarily mean a high yield; instead, it may lead to "neglecting one aspect for another".

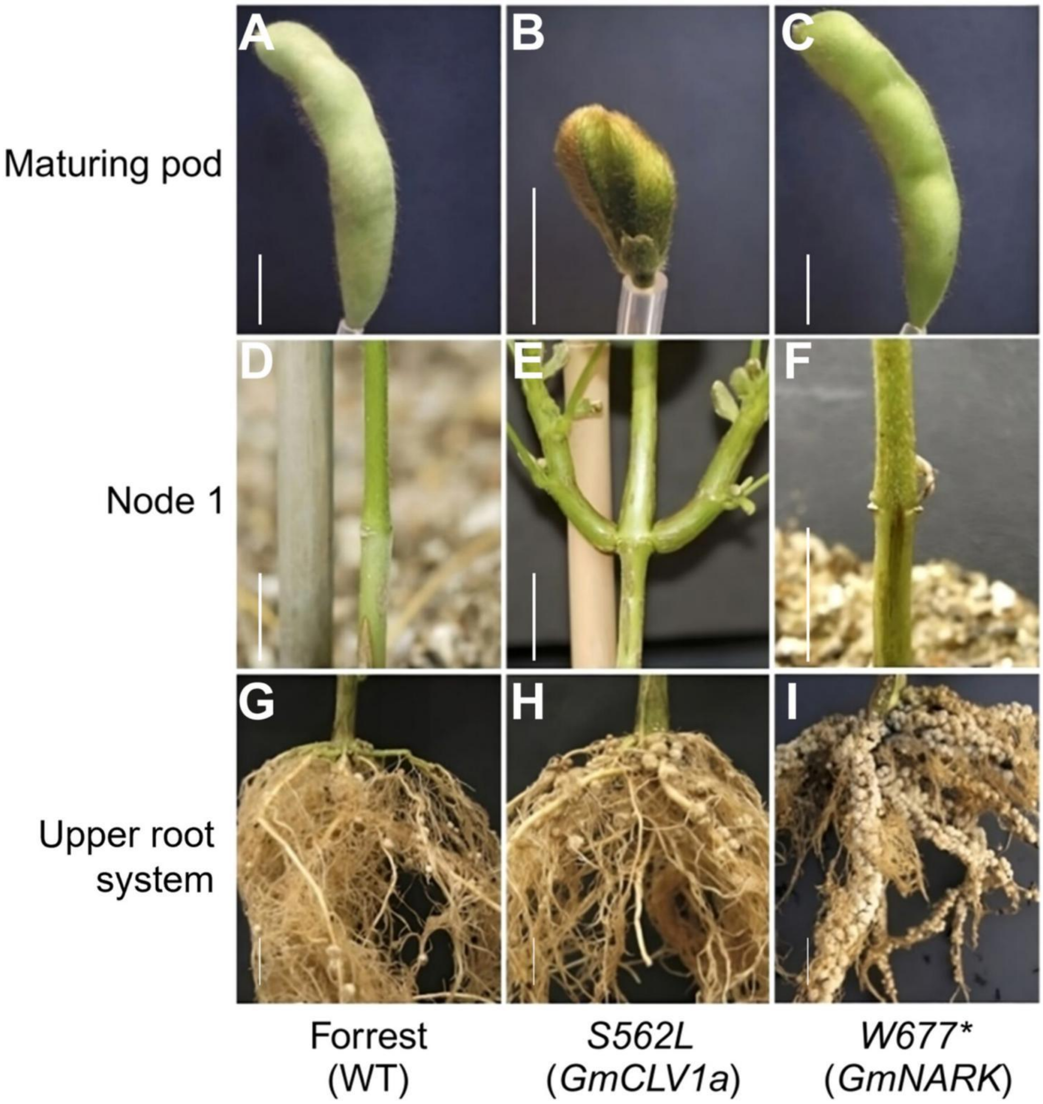

Figure 2 Phenotypes of wild-type, *GmCLV1A* and *GmNARK* soybean mutants (Adopted from Gresshoff et al., 2025)
Image caption: Note the abundant nodulation (normal-sized nodules!) for the near-isogenic *W677** mutant of soybean cultivar Forrest (*GmNARK* and *GmCLV1A*) inoculated with the *Bradyrhizobium diazoefficiens* strain USDA110 (previously named *B. japonicum* USDA 110) and its behavior normally in vegetative and fruit development (A, D, G). Mutant *S562L* is mutated in *GmCLV1A*, the duplicated copy of *GmNARK*, and shows normal nodulation (it is *GmNARK*), but is severely altered in its vegetative and fruit development (reminiscent of one of the *Arabidopsis Atclv1−* phenotypes) (B, E, H). Mutant *W677** is mutated in *GmNARK*. It shows supernodulation, but there was no strong effect on its vegetative and fruit development (*GmCLV1A*) (C, F, I). Scale bars =1 cm (Adopted from Gresshoff et al., 2025)

6.3 Implications for improving nodule efficiency in soybean cultivation

So, should "brake genes" like *GmNARK* be activated or not? The answer is probably not that simple. On the one hand, moderate knotting can enhance nitrogen fixation efficiency. However, if it gets out of control, a large amount of plant nutrients will flow into the root nodules, which will instead affect the yield. At this point, if the activity of *GmNARK* can be appropriately regulated or the CLE peptide signal can be intervened, it is possible to achieve the goal of "forming tumors when it should and stopping when it should not". This is crucial for breeding.

What's more interesting is that GmNARK not only manages root nodules, but also plays a role in coping with stresses such as salinization and drought. It can also interact with other signaling pathways (Cheng et al., 2018). So, from the perspective of regulation, it is more like a dispatching center rather than a simple switch.

7 Biotechnological Applications and Breeding Implications in Soybean-Rhizobium Interaction

7.1 Identification of gene targets for enhancing nitrogen fixation

Some genes related to nitrogen fixation were not so easy to discover from the very beginning. It was not until the development of quantitative trait loci (QTL) mapping and transcriptomics research that researchers gradually identified the positions of some of these "hidden heroes". For example, on chromosome 18, a QTL that is different from the known Rj/rj nodular gene was found. It contains a nucleotide-binding gene with leucine repeats, and the expression varies significantly among different varieties (Teraishi et al., 2025). This discovery offers us a new direction: perhaps in a low-nitrogen environment, such genes can help soybeans enhance symbiotic efficiency. In addition, there are some classic symbiotic genes that have long been noticed, such as *Nif*, *nodA*, *IAA*, *exoX*, *htrA*, etc., which are involved in flavonoid synthesis, signal sensing, and resilience mechanisms. They can all be found in the symbionts of soybeans and rhizobia, especially when facing environmental stress. Their existence is even more significant (Ni et al., 2024).

7.2 Development of soybean lines with optimized nodulation signaling

In field experiments, sometimes the true capabilities of genes can be better revealed than in laboratories. The breeding team is now attempting to use natural variations and recombinant inbred lines to screen for soybean varieties that are more active in nodulation and have stronger nitrogen fixation capabilities. Moreover, when used in combination with good rhizobia strains, the performance of such varieties is usually more stable and the yield increase is more significant (Kolapo et al., 2025). Some regions have even improved farmers' income in this way. Of course, relying solely on the inoculation of a single strain may not necessarily cover the complex field environment. Thus, the strategy of using rhizobia together with other beneficial microorganisms, such as mycorrhizal fungi, also began to gain popularity. This "microbial mix" not only enhances nutrient absorption rate but also shows potential in improving stress resistance (Wangiyana et al., 2022).

7.3 Potential for gene editing and synthetic biology approaches in legume improvement

Traditional breeding relied on time and screening, but now there are some more "shortcut" methods. For instance, the CRISPR-Cas gene editing technology can directly "cut" on the target gene, bypassing the complex selection process in the middle. Verifying the functions of candidate genes through this approach is not only highly efficient but also accelerates the screening of strains that are more favorable for symbiosis, stress resistance, and strain compatibility (Ni et al., 2024). On the other hand, the development of synthetic biology has also opened up new paths. Some people have begun to attempt to "modify" the rhizosphere microbiome, and even design artificial microbial communities that are suitable for specific soybean varieties. This strategy, starting from the root ecosystem, aims to enhance nitrogen fixation capacity and optimize growth conditions, thereby driving an overall increase in yield.

8 Concluding Remarks

Regarding the interaction between soybeans and rhizobia, no one simply regards it as a matter of "who infects whom" anymore. This is a complex molecular exchange involving many signaling genes and regulatory pathways. It is not merely about controlling nodules and nitrogen fixation, but also related to plants' responses to various environmental changes. In this regard, host genes such as *NFRs*, *NSPs*, *NIN* and *Rj2*/*Rfg1* are research hotspots. On the microbial side, many effector molecules have also been proven to be directly related to compatibility, defense response and symbiotic efficiency. However, these effects are not universal. The rhizosphere environment, plant genotypes, and even seasonal changes can all influence the symbiotic effect. Especially the mutually beneficial relationship between rhizobia and mycorrhizal fungi, combined with the cooperation of other beneficial microorganisms, can truly bring about practical benefits such as nutrient absorption, enhanced resistance, and increased yield.

On the other hand, the advancement of technological means has also promoted the "breaking out of the circle" of research. Whether it is metagenomics, transcriptomics, proteomics, or high-throughput sequencing platforms, these tools have made the mining of candidate genes and regulatory networks faster and more accurate. And gene editing tools like CRISPR-Cas are no longer "cold technologies" in the laboratory; they have begun to be used in the actual breeding design of soybeans. Further attempts also include the artificial regulation of the microbiome, such as designing specific microbiota to adapt to certain soybean genotypes. This kind of "customized" operation, which is expected to simultaneously enhance tumor formation, nitrogen fixation and stress resistance, is not a theoretical assumption but is gradually becoming a reality.

Looking ahead, the focus may not be on how many new genes are discovered, but rather on how to integrate the existing multi-omics data, environmental information and variety responses. Especially under low-input or adverse conditions, to maximize the value of symbiotic efficiency, strategies such as co-inoculation of strains and microbiome remodeling are worth further attempts. In the long run, the approach that combines signal gene regulation, biome management and precision breeding may be the breakthrough for sustainable soybean production - using less chemical fertilizers and protecting the soil. This is not only the goal but also the direction of action.

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Conflict of Interest Disclosure

The authors affirm that this research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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