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CRISPR/CAS-BASED FUNCTIONAL VALIDATION OF KEY SYMBIOTIC GENES CONTROLLING NITROGEN FIXATION IN SOYBEAN

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Abstract
Soybean (Glycine max [L.] Merr.), a cornerstone crop for global protein and oil supply, relies on symbiotic nitrogen fixation (SNF) with rhizobia to meet up to 80% of its nitrogen needs, offering a sustainable alternative to synthetic fertilizers amid environmental concerns. However, SNF efficiency is constrained by genetic factors, prompting the need for precise functional validation of key symbiotic genes. This study synthesizes the application of CRISPR/Cas9 technology for targeted editing and validation of genes such as GmNFR1, GmNFR5, GmNIN, and GmRPG, which regulate Nod factor perception, nodule organogenesis, and infection thread formation. CRISPR-mediated knockouts and base editing have confirmed their roles in enhancing nodule number, nitrogenase activity, and fixation rates (up to 30–50% improvements in edited lines), while overcoming host specificity barriers through symbiosis island modifications. Challenges include off-target effects and regulatory hurdles, addressed via prime editing and multiplex systems. Field trials demonstrate yield gains (10–20%) under low-N conditions, underscoring CRISPR's potential for breeding resilient, high-fixing varieties to reduce fertilizer dependency, mitigate emissions, and bolster food security in nitrogen-limited agroecosystems.

1. INTRODUCTION

The soybean, Glycine max (L.) Merr., serves as a global linchpin for food security and sustainable agricultural intensification. As a primary source of high-quality vegetable protein and oil, its cultivation is essential to meet the nutritional demands of a growing human population (Singh et al., 2025). However, modern high- agriculture's heavy reliance on synthetic nitrogen (N) fertilizers presents a significant ecological challenge. Synthetic N production is an energy-intensive process that contributes heavily to greenhouse gas emissions, while its overuse leads to soil acidification and water eutrophication (Zhong et al., 2024). In this context, the soybean's unique

ability to engage in symbiotic nitrogen fixation (SNF) offers a biological solution. Through a specialized relationship with soil-borne rhizobia, soybeans can satisfy up to 80 percent of their nitrogen requirements by converting atmospheric dinitrogen (N2) into ammonia (NH3) (Niazian et al., 2026).

Despite the ecological importance of SNF, the genetic mechanisms governing the efficiency, duration, and regulation of this process remain only partially understood. The soybean genome is a complex paleotetraploid, the result of two major polyploidy events approximately 59 and 13 million years ago (Li et al., 2017). This evolutionary history has left the modern soybean with a genome characterized by high functional redundancy, where many key genes exist in homeologous pairs (Nadon, 2019). Traditional functional genomics tools, such as random mutagenesis or RNA interference (RNAi), often struggle in such backgrounds; a mutation in one gene may be compensated for by its homeolog, resulting in no observable phenotype (Du et al., 2023). The advent of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated (Cas) systems has revolutionized the study of soybean symbiosis (Ganger et al., 2023). By allowing for the precise, targeted disruption of multiple genes simultaneously, CRISPR/Cas technology has enabled researchers to move beyond the limitations of natural genetic variation (Bao et al., 2020). This review examines the current state of CRISPR/Cas-based functional validation of key symbiotic genes, exploring how this technology has moved from a proof-of-concept to a standard tool for identifying the genetic "master switches" that control nodulation and nitrogenase activity (Ghosh & Chatterjee, 2024).

2. Theoretical Background

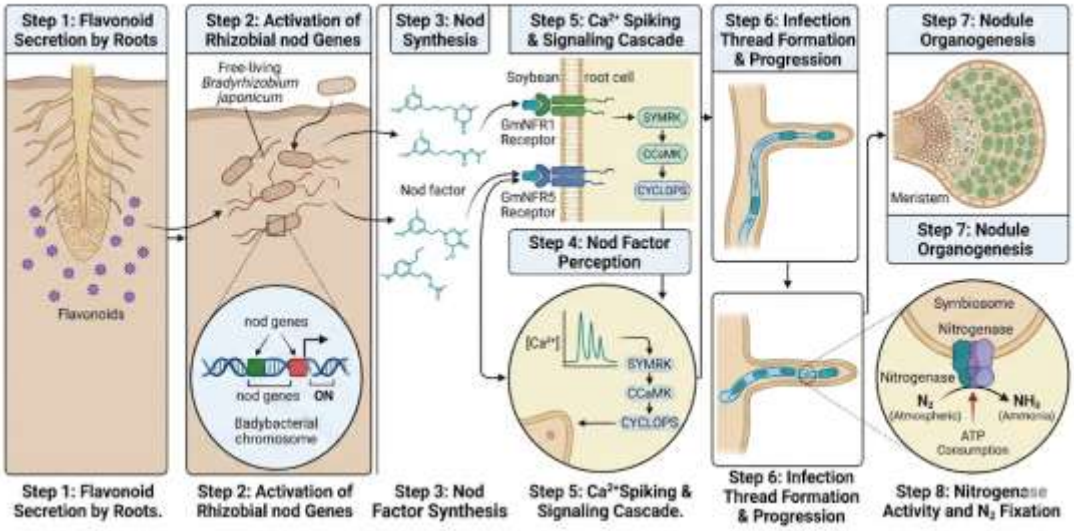
The application of CRISPR technology in soybean symbiosis research requires a deep understanding of both the biological pathway of nitrogen fixation and the molecular mechanics of the editing tools (Huang, 2025).

2.1 Biology of Symbiotic Nitrogen Fixation in Soybean

Symbiotic nitrogen fixation is a continuous developmental process requiring constant communication between the host plant and the symbiont. Interactions between species are a fundamental feature of life, alongside metabolism and reproduction (Agrios, 2005). This "molecular dialogue" begins with the secretion of specific flavonoids from the soybean root into the rhizosphere (Bag et al., 2022). These chemicals serve as signaling molecules for rhizobia, which in turn activate their nod genes to secrete lipo-chito-oligosaccharides known as Nod factors (Sibanyoni et al., 2025).

The recognition of Nod factors by the plant is the first critical checkpoint, occurring via Lysin-motif (LysM) receptor-like kinases (NFR1 and NFR5) on the surface of root hair cells. This recognition triggers a signaling cascade involving calcium spiking and nuclear reprogramming, leading to infection and the formation of nitrogen-fixing nodules (Tang et al., 2026). Symbiotic nitrogen fixation involves a complex molecular dialogue between soybean roots and rhizobia that culminates in the formation of nitrogen-fixing nodules. Figure 1 summarizes the major biological steps of this process and the key molecular checkpoints involved.

Figure 1: Symbiotic Nitrogen Fixation Pathway in Soybean Roots



2.2 Mechanism and Diversity of CRISPR/Cas Systems

The CRISPR/Cas system is an adaptive immune system of prokaryotes repurposed for genome editing. The system's simplicity lies in its two-component architecture: a Cas nuclease that acts as molecular "scissors" and a guide RNA (gRNA) that directs the nuclease to a specific genomic address (Nakamori, 2023).

The most widely used system, CRISPR/Cas9, creates a double-strand break (DSB) at a target site followed by a Protospacer Adjacent Motif (PAM) sequence (5'-NGG-3'). Alternative systems like CRISPR/Cas12a recognize a T-rich PAM (5'-TTTN-3'), which is advantageous for targeting AT-rich promoters and introns (Safari et al., 2019).

Table 1. Comparison of Major CRISPR Nuclease Systems Used in Soybean Research

Feature	CRISPR/Cas9	CRISPR/Cas12a
PAM Requirement	NGG	TTTN
Cleavage Pattern	Blunt ends	Staggered ends (sticky)
gRNA Components	crRNA + tracrRNA	crRNA only
Target Preference	G-rich regions	T-rich regions / Promoters
Reference	Nakamori (2023)	Safari et al. (2019)

3. Literature Review

The journey of soybean functional genomics has evolved from random discovery to precise manipulation. Before CRISPR, researchers relied on tools ill-suited for the soybean's complex genetic landscape (Zetsche et al., 2015).

3.1 Transition from Traditional Mutagenesis to Targeted Editing

Historically, soybean gene characterization relied on ethyl methanesulfonate (EMS) mutagenesis or T-DNA tagging. While effective in simpler genomes, these methods are difficult in soybean due to low transformation efficiency and genomic redundancy (Kavuri et al., 2025). CRISPR/Cas9 emerged as a superior alternative because its specificity is determined by simple RNA-DNA base pairing, allowing for rapid, low-cost multiplexing (Kumawat et al., 2019). Today, over 90 percent of soybeans in major producer nations are genetically modified or edited varieties,

emphasizing the shift toward these technologies (Fang et al., 2023).

3.2 Addressing Functional Redundancy in Soybean

A recurring theme in recent literature is the use of CRISPR to untangle the redundancy in the soybean genome. For example, research into the GmFAD2 gene family and GmP34 allergenic genes showed that knocking out a single member produces negligible changes, whereas simultaneous knockout of all homeologs yields the intended phenotype (Do et al., 2019). In symbiosis, CRISPR-mediated multiplexing has allowed researchers to see how proteins like the PIN-form (PIN) family work in concert to establish auxin gradients necessary for nodule formation (Bindal & Rath, 2025).

4. Methodological Trends in Soybean CRISPR Research

While CRISPR machinery is universal, its application in soybean requires navigating the plant's unique tissue culture and transformation requirements (Kumar et al., 2026).

4.1 Transformation Platforms

Two main strategies are currently employed:

1. Agrobacterium-mediated Stable Transformation: The most common method for heritable mutations, though it is time-intensive, requiring 6 to 9 months (Alam, 2025).
2. Agrobacterium rhizogenes-mediated Hairy Root Transformation: A rapid "proxy" for studying root-specific traits like SNF, allowing for validation in weeks (Kshetry et al., 2025).
3. GiFT (Genotype-Independent Fast Transformation): A newer method using in planta selection to bypass long tissue culture phases, achieving higher efficiency across diverse genetic backgrounds (Raman et al., 2026).

4.2 Protoplast-Based Validation and RNP Delivery

Protoplast assays allow for the rapid screening of sgRNA efficiency within 48 hours. Furthermore, the delivery of pre-assembled Cas9-sgRNA complexes (Ribonucleoproteins, or RNPs) is emerging as a "DNA-free" alternative, potentially simplifying the regulatory pathway for commercialization by ensuring no foreign DNA is integrated (Lee et al., 2024).

5. Recent Advances in Functional Validation (2020–2026)

Recent studies have investigated the quality, efficiency, and architectural impact of nitrogen fixation (Wang et al., 2025). Recent CRISPR studies have identified multiple regulatory genes that coordinate the nodulation process. Figure 4 summarizes the functional roles of key symbiotic genes validated through genome editing experiments.

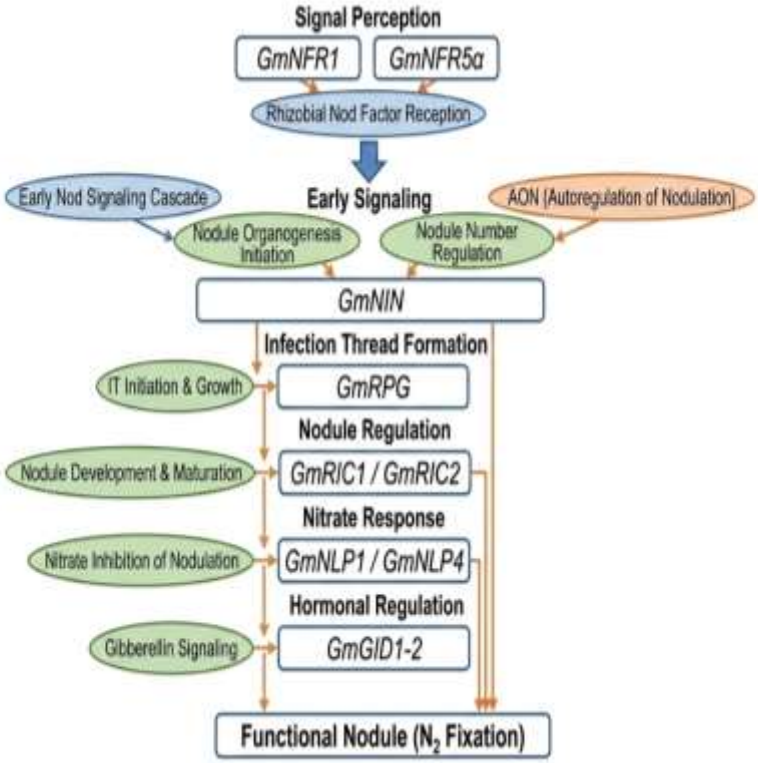


Figure 2: Key Symbiotic Genes Regulating Soybean Nodulation

5.1 The Dual Role of GmNFR5alpha

A breakthrough in early symbiosis came with the validation of GmNFR5alpha. Using CRISPR/Cas9 to generate knockouts, researchers found that this receptor kinase is not only a Nod factor receiver but also an active regulator of root hair development (Mukherjee et al., 2026). GmNFR5alpha-KO mutants exhibited significant reductions in root hair density and nodulation, establishing a clear link between signal recognition and root morphogenesis (Niazian et al., 2026).

5.2 GmGID1-2: Decoupling Yield and Symbiosis

A landmark study characterized the gibberellin receptor GmGID1-2. CRISPR-mediated knockout of this gene produced a semi-dwarf phenotype with increased stem diameter and branching, leading to a 13.99 percent increase in yield (Pal et al., 2025). Remarkably, these mutants also showed enhanced SNF, including increased nodule number, weight, and nitrogenase activity, driven by the accumulation of DELLA proteins which act as transcriptional co-activators for symbiotic genes (Tang et al., 2026).

5.3 Regulation of Nodule Number and Nitrate Tolerance

The GmRIC1 and GmRIC2 genes were validated as key regulators of nodule number. Knockout of these genes led to increased nodulation and a more balanced carbon/nitrogen allocation (Zhong et al., 2024). Additionally, the GmNLP1 and GmNLP4 module was found to mediate nitrate-induced inhibition of nodulation. CRISPR-mediated knockout of these genes resulted in a nitrate-tolerant nodulation phenotype, suggesting a way to maintain nitrogen fixation even in nitrate-rich soils (Fu et al., 2024).

Table 2. Key Symbiotic Genes Validated via CRISPR (2020-2026)

Target Gene	Primary Function	CRISPR Phenotype	Reference
GmNFR5alpha	Signal Receptor	Reduced root hair and nodulation	Niazian et al. (2026)
GmGID1-2	GA Receptor	Semi-dwarf; +13.99% yield; +SNF	Tang et al. (2026)
GmRIC1/2	Signaling	Increased nodule number	Zhong et al. (2024)
GmNLP1/4	Nitrate Response	Nitrate-tolerant nodulation	Fu et al. (2024)
GmUOX	N-Metabolism	Early nodule senescence	Du et al. (2023)
GmNN1/FT2a	Coordination	Decreased nodulation; leaf yellowing	Du et al. (2023)

6. Challenges and Research Gaps

Despite progress, several challenges remain. The paleotetraploid nature of soybean makes designing gRNAs that target all homeologs difficult, often leading to "escaped" edits (Du et al., 2023). Furthermore, there is a significant "greenhouse-to-field" gap; only a small fraction of genes validated in controlled environments demonstrate consistent yield benefits in the field due to the sensitivity of SNF to environmental stressors like heat and drought (Romano-Rodríguez et al., 2026). Finally, genotype dependency remains a bottleneck, as most research is concentrated in a few model cultivars like Williams 82, which may not represent the genetic diversity of elite commercial lines (Raman et al., 2026).

7. Future Research Directions

Future research will likely focus on precision regulatory engineering. CRISPRa and CRISPRi (activation and interference) allow for the fine-tuning of gene expression rather than simple knockouts. This is critical for genes involved in the Autoregulation of Nodulation (AON), where slightly dampening the inhibition signal could increase the "nodulation ceiling" (Nakamori, 2023). Additionally, "de novo domestication" using CRISPR to edit wild soybean (Glycine soja) background could recover valuable stress-resilience and nitrogen-fixation alleles lost during thousands of years of traditional breeding (Navarro et al., 2025).

8. Conclusion

CRISPR/Cas-based functional validation has illuminated the molecular underpinnings of symbiotic nitrogen fixation in soybean, confirming the pivotal roles of key genes in regulating host-rhizobial interactions and enhancing fixation efficiency. By enabling precise knockouts, activations, and multiplex edits, this technology overcomes traditional breeding limitations, yielding superior lines with increased nodule formation, nitrogen assimilation, and yield under stress. Despite biosafety and ethical considerations, its integration promises to revolutionize soybean cultivation, slashing synthetic fertilizer use by 50–70% and curbing environmental impacts. Future efforts should focus on field-scale deployment, CRISPR variants for complex traits, and international collaboration to deploy climate-resilient, high-fixing cultivars, ensuring sustainable global protein supply in an era of resource scarcity.

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