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Gene editing to improve legume-rhizobia symbiosis in a changing climate

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Abstract

In the last three years, several gene editing techniques have been developed for both model and crop legumes. CRISPR-Cas9-based tools, in particular, are outpacing other comparable gene editing technologies used in legume hosts and their microbial symbionts to understand the molecular basis of symbiotic nitrogen-fixation. Gene editing has helped identify new gene functions, validate genetic screens, resolve gene redundancy, examine the role of tandemly duplicated genes, and investigate symbiotic signaling networks in non-model plants. In this review, we discuss the advances made in understanding the legume-rhizobia symbiosis through the use of gene editing and highlight studies conducted under varying environmental conditions. We reason that future climate-hardy legumes must be able to better integrate environmental signals with nitrogen fixation by fine-tuning long distance signaling, continuing to select efficient rhizobial partners, and adjusting their molecular circuitry to function optimally under variable light and nutrient availability and rising atmospheric carbon dioxide.

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Introduction

Crop production is expected to shift geographically as a result of climate change [1]. Although precipitation levels and extreme weather events introduce unpredictability, optimal regions for crop growth are shifting to higher latitudes and altitudes as temperatures rise. Historically, the yield of economically important legume plants such as soybeans has decreased in areas with high temperatures and low precipitation [1,2]. At the same time, increasing atmospheric carbon dioxide (CO_2) concentrations seemingly promote increased photosynthetic carbon assimilation and yields, but grain nitrogen (N) content varies [3–5]. The partnership between legumes and their compatible N-fixing bacterial symbionts called rhizobia, known as "root nodule symbiosis," is central to legume production [6]. Climate-related factors that endanger the legume-rhizobia symbiosis threaten worldwide legume output since profitable legume production relies on soil rhizobia to avoid using expensive, synthetic N-fertilizer [2,7]. Climate-hardy legumes of the future, therefore, must be able to adapt more readily to weather-variables by better integrating environmental signals with N-fixation, selecting beneficial rhizobial partners for optimal N-fixation and fine-tuning their molecular circuitry to function optimally under stress.

Selection of legume varieties that are well-adapted to a given environment has utilized conventional breeding, the introduction of transgenes, and more recently, gene editing, both independently and in combination. Gene editing is the process of optimizing endogenous gene function by precise modification of genomic sequences and has become an invaluable tool for diversifying breeding pools as well as for functional genetics and basic research. Gene editing has been demonstrated to develop crops that are frost tolerant, heat tolerant, and resilient to other abiotic stresses [8–11]. A popular and accurate gene editing technology is mediated by CRISPR-Cas9 (Clusters of Regularly Interspaced Short Palindromic Repeats-CRISPR associated protein 9). The Cas9 enzyme operates as a pair of "molecular scissors" to cut two strands of genomic DNA at a specific location for insertion-deletion mutagenesis with the help of guide RNAs. The high precision and efficiency of CRISPR technology make it a preferred genome-editing technology, largely replacing earlier technologies such as ZFNs (zinc finger nucleases) or TALENs (transcription activator-like effector nucleases). All three technologies use targeted double-stranded DNA breaks and repair damage through homologous recombination or non-homologous end joining. Imperfect cellular repairs ensure the genetic material is mutated or

"edited." RNA-DNA interactions in the CRISPR-Cas9 system regulate recognition of the DNA target. This affords CRISPR-Cas9 multiple benefits over ZFNs and TALENs, including ease of design for any genomic targets, easier prediction for off-target sites, and the capacity to edit multiple genomic sites simultaneously [12]. Readers are directed to other excellent reviews for more information on the mechanisms of CRISPR, TALEN, and ZFN activities [13–15]. With the rapid increase in the number of CRISPR-Cas9 edited crops available, countries such as the USA, Japan, Australia, Russia, and more recently, India, have modified their regulations to classify gene edited crops as non-genetically modified [16,17]. To some extent, these regulatory modifications have resulted from the capacity to generate transgene-free genome edited crops using preassembled CRISPR-Cas ribonucleoproteins, backcrossing edited progenies to their WT parental plants to be transgene-free, or using viral transfection techniques wherein the viral DNA is not inherited [16,17].

Identification of optimized endonucleases, plant transformation, availability of reference genomes, and molecular analyses are all necessary foundation tools for genome editing. Plant transformation is, admittedly, a bottleneck in gene editing, and most efforts to date have focused on developing legume transformation protocols. Nonetheless, gene editing pipelines for soybeans (*Glycine max*), pea, alfalfa, chickpea, cowpea, and model legumes such as *Lotus japonicus* and *Medicago truncatula* have been developed successfully using Agrobacterium mediated transformation and particle bombardment [18,18,19,19,20,20,21,21,22,22,23,23,24,24–26]. Functionally validated gRNAs in legumes are 15–22 base pairs long (20 bps is the most common), with a GC content of 35–70% (average 45%); the most common edit is deletion of 1–60 bps, with 1 bp insertions being the most frequent, but deletions of up to 19.2 kbps and insertions of up to 98 bps have also been reported (readers are encouraged to access the meta-analysis presented in Supplementary Table 1). Guides normally target exons 1 or 2, and more efficient gene editing is achieved with two or more guide RNAs targeting the same gene [27]. Off-target mutations are typically not detected [28]. In this article, we review research performed to date using gene editing technologies to study root nodule symbiosis (RNS) in legumes and in rhizobia. While several traits are targets for improving legume resiliency in a changing climate [29], we highlight two for future research that will help legumes integrate changes in climatic factors with root nodule symbiosis, namely rhizobial partner selection and long distance signaling (Figure 1).

Gene editing is a powerful tool to conduct basic symbiosis research

The development of gene editing has made it possible to quickly validate the results of extensive screens, resolve gene redundancy, examine the function of expanded families of tandemly duplicated genes, and investigate basic signaling networks in non-model legumes, as detailed below. There is a large body of work that shows how one environmental component (such as high CO_2, light availability, or soil N) influences legume-rhizobia interactions under artificially-controlled conditions (Figure 1), but fewer studies have investigated how multiple environmental factors affect plant responses [30,31]. Future studies exploring the responses of mutants to interactions between two or more RNS-influencing environmental-variables, i.e., low-N with heat, drought, variable light, or CO_2, will be vital for understanding how fundamental symbiosis signaling circuits will rewire under a changing climate [6,30,31]. Below, we discuss the various uses of gene editing:

For generating mutant populations: CRISPR-mediated gene editing is highly efficient; nevertheless, crops with paleoploid genomes, such as soybean, have many gene copies and restricted transformation ability, and so are not always able to benefit from it. One solution is to target multiple genes of interest in a single experiment, requiring only one transformation round. Bai et al., targeted 102 genes in *G. max* that included 74 nodulation genes in 16 sgRNA subpools in the pGES201 vector with a soybean optimized cas9 protein [28]. The multiplex editing strategy also included sgRNAs targeting homologous regions of paralogous copies throughout the genome. This strategy helped characterize two AON pathway components: a double CLE peptide mutant with an increased nodule number phenotype and a triple soybean *ROOT DETERMINED NODULATION1* (*gmrdn1-1*, *gmrdn1-1*, and *gmrdn1-3*) mutant identified with defects in vegetative growth and an increased nodule number [28].

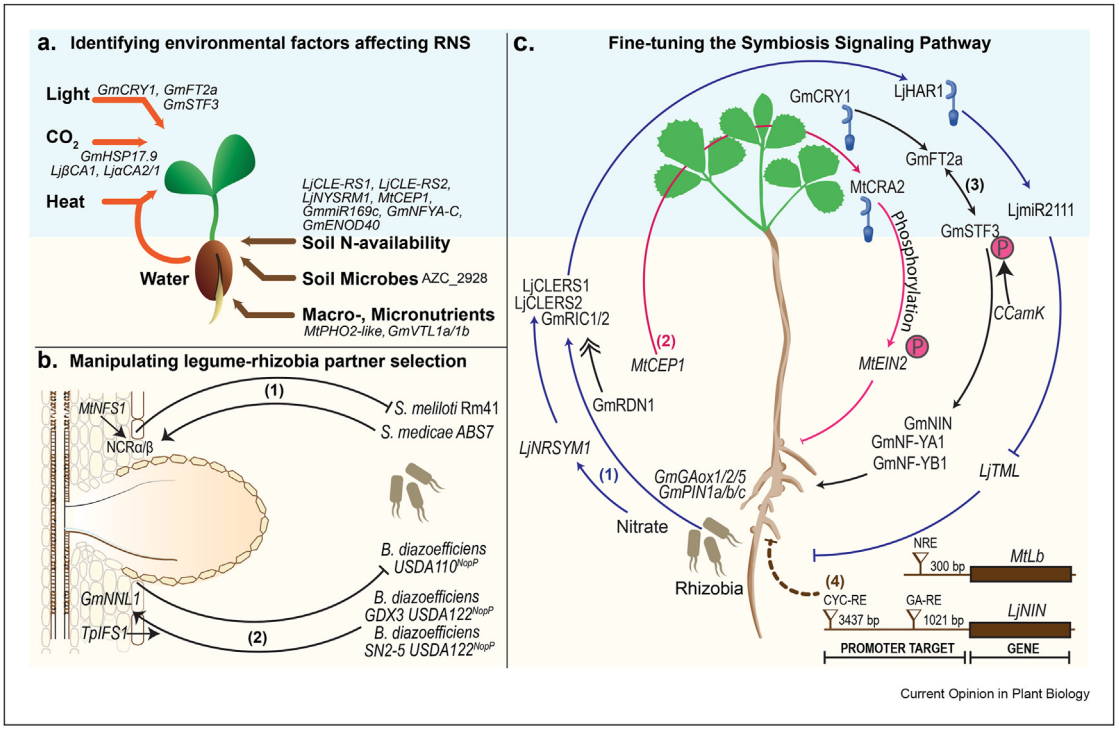
As a tool for validating genetic screens: Despite the widespread use of insertional mutagenesis, such as *Tnt1* in Medicago and *LOTUS RETROTRANSPOSON 1* (*LORE1)* in Lotus, no mutant lines for a specific gene are often identified, or background insertions can hide the gene's true phenotype. Precise gene editing can help functionally validate the roles of candidate genes chosen solely by *in silico* data analyses, such as in genome wide association studies. Generation of CRISPR mutants is a quick validation strategy and phenotypes can be determined with at least two independent alleles, such as in *UBIQUITIN CONJUGATE24* LIKE (*pho2-like)*, *PARTNER OF NOB1-LIKE* (*pno1-like)* and *PENETRATION3-LIKE* (*pen3-like)* that display reduced nodule numbers compared to control, reported in a GWA study to identify novel loci associated with the nodulation trait [32].

For gene discovery using transgenic hairy root lines: Transgenic hairy roots contain both transformed and untransformed roots, as well as chimeric roots. Recessive mutations

require biallelic edits for observable phenotypes, making gene editing for gene discovery in hairy roots possible but challenging (Supplementary Table 1) [28,33–45]. Nevertheless, integrating stable mutant line data with gene-edited hairy root lines may be a viable method to support conclusions on gene function. For example, soybean fast neutron (FN) mutant lines with mutated URICASE1 *(GmUOX)* and CRISPR-Cas9 mutant XANTHINE DEHYDROGENASE *(GmXDH)*, which are two important enzymes for ureide production, display prematurely senescent nodules with impaired nitrogenase activity (Fix$^-$) [40]. Additional CRISPR-cas9 knockout hairy root lines of *GmUOX* tested for FN mutant-like characteristics required DNA isolation from roots with white fix$^-$ nodules, PCR amplifying the targeted gene and screening band shifts for substantial deletions or insertions, and/or sequencing amplicons for edits. Despite the extra steps, 54–69 percent of hairy roots facilitated the development of fix-nodules that resembled FN mutant lines [40].

For resolving gene redundancy: Gene editing has been particularly useful for understanding lineage specific gene family expansion in legumes, many of which emerge as a result of tandem duplications [46]. Due to the essential nature of phytohormones, duplication and

Figure 1



An overview of the current research initiatives aimed at enhancing the legumes -rhizobia symbiosis using gene editing. a. Identifying environmental factors influencing root nodule symbiosis. Optimal symbiotic nitrogen fixation requires adaptation and efficient responses to external environmental factors such as variable light [67], soil nutrient availability [35,39], and rising atmospheric CO_2 [70]. b. Manipulating legume-rhizobial partner selection. (1) MtNFS1 encodes NCRα/β peptides that negatively affect the symbiotic performance of *S. meliloti* strain Rm41 in a host genotype and strain specific manner to prevent rhizobial infection and consequently, organogenesis and nitrogen fixation. In *M. truncatula* Jemalong A17, *S. meliloti* Rm41 strains form defective Fix– nodules while *S. medicae* strain ABS7 forms fully functional Fix + nodules [41]. (2) GmNNL1 directly interacts with Bradyrhizobium USDA 110 produced effector molecule, NopP and triggers strain specific immune responses to govern partner selection and symbiosis specificity. This NopP triggered effect is bacterial strain specific as GmNNL1 could only inhibit rhizobial infection for Bradyrhizobium diazoefficiens strain USDA110 and not USDA122 [61]. Phenylpropanoid metabolite production downstream of *Trifolium pratense* IFS1 deters associations with undesirable soil microbes [58]. c. Fine tuning the symbiosis signaling pathway. A diagrammatic representation of the three long distance signaling pathways integrating abiotic signals with root nodule symbiosis. (1) Rhizobia induced CLE peptides (GmRIC1/2, LjCLERS1/2) are arabinosylated by RDN1, and transported to the shoot [28]. Perception by receptor GmNARK/LjHAR1 triggers movement of miR2111 to repress LjTML expression, thereby regulating nodule number [32,71]. (2) Perception of light by GmCRY1 receptors triggers shoot to root movement of GmSTF3 and GmFT2a. Phosphorylation of GmSTF3 by GmCCaMK triggers GmSTF3-GmFT2a complex formation, promoting nodulation by activating transcription factors GmNIN and NF-YA1/NF-YB1 [67]. (3) Under low N-availability, root induced MtCEP1 moves shootward and binds to the receptor MtCRA2. MtCRA2 transphophotylates MtEIN2, a negative regulator of rhizobial infection and nodule organogenesis, resulting in repression of ethylene response, ensuring root susceptibility to rhizobia [53]. (4) CRISPR-Cas9 mutations in cis-regulatory elements of symbiosis related genes such as NIN and Leghemoglobin repress nodulation [34,73]. Triangular insert shows position of edit. Distance from start codon indicated in bps. Dashed lines indicate the phenotype of lines mutated in their promoter regions. P, phosphate group. Double arrow indicates interaction between gene products. See Supplementary Table 1 for gene names mentioned herein. Gm- *Glycine max*, Mt- *Medicago truncatula*, Lj- *Lotus japonicus*, Sm- *Sinorhizobium meliloti*, S. medicae- *Sinorhizobium medicae*.

significant redundancy in gene families mediating their response have hampered studies thus far in hormone biology. Using CRISPR-Cas9 to knock down all three homoeologous copies of PIN-FORMED transporter (*GmPIN1a*, *GmPIN1b*, *GmPIN1c*) it was recently established that even in determinate nodule-forming legumes such as soybean root nodule organogenesis requires a local auxin maximum at the primordia [47]. Similarly, disrupting the activity of a nodule-expressed MADS box transcription factor, *GmNMHC5*, that maintains an optimal Gibberellic acid concentration is essential for optimal nodule organogenesis. In *Gmnmhc5* mutants, an increase in expression of GA synthesis genes *GIBBERELLIN 3-OXIDASE* (*GmGA3ox1, GmGA3ox2, and GmGA3ox5*) correlated with a decrease in nodule number [48]. In indeterminate nodule-forming *M. truncatula*, *MtGA3ox1* mutants had reduced nodule number but displayed no effect on nodule density and nitrogenase activity, while *MtGA3ox2* had no effect on nitrogenase activity, nodule number, or nodule density [49].

Multiplex editing for uncovering crosstalk between distinct signals: In addition to classical hormones, small signaling peptides or peptide hormones also act as key regulators of RNS [50,51]. The CEP (C-terminally Encoded Peptide) gene family is involved in root development, nodule organogenesis and nutrient uptake [27,52]. The triple mutant *Mtcep1/cep2/cep12* and quintuple mutant *Mtcep1/cep2/cep5/cep8/cep12* generated in the wildtype R108 and *cep5cep8* double mutant backgrounds have more lateral roots and fewer nodules but still cannot replicate the severity of the receptor mutant COMPACT ROOT ARCHITECTURE2 (*mtcra2)* which makes no nodules [53]. However, simultaneous deletion of the ethylene signal transducer MtEIN2/MtSICKLE generating the *cra2ein2* double mutant, partially rescues the *cra2* nod$^-$ phenotype, indicating MtCRA2 regulates nodulation via ethylene signaling [53]. The maximum number of guide RNAs that may be cloned for multiplex gene editing cassettes is normally five or six. This is sufficient to create specific higher-order mutants in a single transformation step, which was previously not feasible. Moreover, guide RNA selection is not constrained by sequence similarity requirements for the targeted genes and can be unrelated, as shown in the case above.

In non-model legumes and N-fixing non-legumes: While gene editing in non-model legumes is still in its infancy, it is undeniably a powerful tool for translating fundamental research findings into economically important crop species. One of the first steps of protocol development is to target genes that have easily identifiable phenotypes. The presence or absence of nodules due to missing early signaling components of the symbiosis signaling pathway is a prominent visual marker of effective gene editing in root nodule symbiosis. In the two legumes, *Vigna unguiculata* and *Arachis hypogea*, CRISPR-Cas9 protocols were set up to target the leucine-rich repeat receptor like kinase SYMBIOTIC RECEPTOR LIKE KINASE (*VuSYMRK*) and the LysM RLK NOD FACTOR RECEPTOR (*AhNFR1/AhNFR5*) [36]. The SYMRK LRR domain is involved in ligand-protein interaction, which controls SYMRK activation while NFRs are LysM RLKs that bind and recognize Nod factors [37]. Both studies report a complete lack of nodules in gene edited lines when infected with rhizobia, demonstrating a core conserved function of the receptors across legumes. These non nodulating lines are not available for many legume species, and going forward, can be a great tool for evaluating nitrogen fixation efficiency when developing new varieties. Similarly, in the N-fixing nonlegume *Parasponia andersonii*, four LysM-type receptors regulate nodulation and N-fixation: LYSM DOMAIN CONTAINING RECEPTOR KINASE (PanLYK1, PanLYK3) and NOD FACTOR PERCEPTION (PanNFP1, PanNFP2). Single mutants *Pannfp1, Pannfp2, Panlyk3* mutant plants all showed reduced nodulation efficiency [54]. Similarly, the downstream GRAS transcription factors NODULATION SIGNALLING PATHWAY 1 (PanNSP1, and PanNSP2) fulfilled identical roles during nodulation as their legume relatives but not the HISTIDINE KINASE (PanHK4) [55]. Through single, double, and higher order CRISPR-Cas9 knockout mutants, it was confirmed that the non-legume nodulating tree species *P. andersonii* uses NIN and NUCLEAR FACTOR Y (NFYA1) as important transcriptional regulators of symbiosis, just like its legume counterparts [56].

Editing legume and microbial genomes can develop efficient symbiotic relationships

In addition to facilitating functional genetics studies, gene editing can be used to alter agronomically valuable traits, such as rhizobial partner selection. Under increasing atmospheric CO_2, legumes, just like other C3 plants, which lack CO_2 concentrating mechanisms, favor the photosynthetic carbon reduction cycle over the photorespiratory cycle, resulting in higher rates of carbohydrate production, if other factors remain constant [5]. Unlike in non-legumes, marginal to no change in seed N or protein content is observed in legumes at elevated CO_2 concentrations in managed systems. This suggests that increased carbohydrate production due to enhanced photosynthesis does not alter interaction with compatible rhizobia [31]. However, the change in metabolic profiles in the host secretome could affect the natural root microbiome and change rhizobial access to plants through selective metabolites. A high C:N ratio is thought to regulate the phenylpropanoid metabolism pathway and increase production of flavonoids and isoflavonoids [4,57]. While the production of nod-gene inducing flavonoids is one of the first steps in root nodule symbiosis, the production of isoflavonoids that act as phytoalexins may deter incompatible rhizobial

partners [57]. Significant reductions in levels of isoflavones like formononetin, biochanin A, and genistein in a 9-bp deletion mutant line in the *ISOFLAVONE SYNTHASE* (*TpIFS1*) gene in red clover, had no effect on nodule number [58]; supporting a possible role in plant defense.

Strain compatibility also depends on the genetic background of the host and is mediated by multiple loci [33,38,41,59]. The more nitrogen a strain provides in exchange for photosynthetic-C, the more efficient it is. Less efficient N-fixing rhizobia that form a symbiotic relationship with their legume hosts produce infected but white nodules that do not "fix" N. In *M. truncatula*, *NITROGEN FIXATION SPECIFICITY 1* (*MtNFS1*) encodes a nodule-specific cysteine-rich (NCR) peptide that negatively affects the symbiotic performance of *Sinorhizobium meliloti* Rm41 in a strain specific manner. For a genetic investigation of symbiotic specificity, two *M. truncatula* accessions, DZA315 which makes Fix^+ nodules, and A17 which develops Fix^- nodules upon infection with *S. meliloti* Rm41 were used [41]. Deletion of the NCR-α and NCR-β loci in the DZA315 background via CRISPR-Cas9 has no effect on the Fix + phenotype of nodules, but CRISPR-Cas9 mediated mutations in NCR-α allele of A17 rendered Fix^- nodules into functional, N-fixing nodules [59]. While some peptides have been shown to be induced by elevated CO_2 levels, a thorough analysis has not been performed and C-control of NCR peptide expression is unknown [60]. Additionally, the plant immune system has checkpoints that select compatible microorganisms while discouraging incompatible ones. The resistance gene NODULE NUMBER LOCUS 1 (GmNNL1) directly interacts with a Bradyrhizobium USDA 110 produced effector molecule, Nodulation outer protein P (NopP). This interaction elicits an immune response and blocks nodulation. Mutations in *gmnnl1* however, allow infection by NopP carrying USDA110 strains. Interestingly, gene edited *Nod Factor Receptor* (*Gmnfr 1α1*) lines also do not nodulate upon inoculation with *Bradyrhizobium* USDA110, indicating NopP effects are GmNFR1 dependent [61]. By precisely altering R genes and other compatibility variables like NCR peptides (Figure 1), gene editing might expand the number of rhizobia a host interacts with, increasing its adaptability and chances of survival despite irregular weather events.

Gene editing in microbial symbionts to modify N-fixing capabilities: Mutagenesis in N-fixing bacteria has traditionally relied on homologous recombination or transposon-mediated gene mutation. CRISPR-based gene editing in N-fixing bacteria has been demonstrated in *Azorhizobium caulinodans*, resulting in increased biofilm formation and chemotaxis with its legume host [62]. In *S. meliloti*, a species lacking native Cas enzymes, a Cas12k-CRISPR-transposon transposase system was recently developed as a homologous recombination-free strategy for precise integration of large DNA fragments of up to 10 kb [63]. In a separate study, a CRISPR-cas9-based base editing system was developed by fusing the Cas9 nickase to adenine or cytidine deaminases to make precise A-to-G or C-to-G conversions without double stranded breaks. This base editing technique was used to change the *nod* (nod factor biosynthesis) gene, and plants inoculated with edited *S. meliloti* strains failed to recover vegetative growth under limited-N as expected [64]. Newly evolving CRISPR-Cas systems have a significant promise to simplify and streamline rhizobial editing to enhance N-fixation. Targets of interest include *nif* and *fix* gene clusters for increased N-fixing activity and decreased oxygen sensitivity, or knockout of the *nif* gene cluster repressor *nifL*; *glnE*, *glnA*, and *ampB* transporters to direct more nitrogen to the plant and reduce bacterial N-assimilation; and *nod* gene clusters for improved host associations.

Mobile regulatory molecules integrate environmental signals with root nodule symbiosis

Although several traits have been identified as targets for developing more climate-resilient legumes [29], long-distance signaling is frequently overlooked despite being essential for integrating above- and below-ground processes. While mobile factors such as FLOWERING LOCUS T2a, small RNAs, and small signaling peptides are good examples of long-distance signals (Figure 1), the biological implications of spatial separation between the site of signal generation and the localization of their targets or receptors are poorly understood. Further research is needed on systemic signaling that helps integrate above-ground conditions with below-ground N-acquisition. This is important for developing improved cultivars that, for example, respond optimally to the increased N-demand due to the higher rates of photosynthesis projected for the coming decades [5,65].

Photosynthesis is driven by the availability of light, which controls the amount of carbon fixed into sugars. Because plants nourish rhizobia by supplying them with sugars, nodules are carbon sinks, and legumes grown in the shade do not fix-N optimally [66,67]. Local nodule C-assimilation depends on enzymes like sucrose synthase that catalyze the formation of UDP-glucose, a glycolysis pathway substrate entering the tricarboxylic acid cycle, which supplies a carbon source and energy for N-fixation in nodules [68]. Single mutations in *heat shock protein 17.9, a* sucrose synthase chaperone, are enough to reduce nodule number by 40−50%, decrease nitrogenase activity, compromise symbiosome membrane integrity, and alter nodule morphology [69]. The non-photosynthetic roles of *L. japonicus* nodule-enhanced carbonic anhydrases (LjβCA1, LjαCA2 and LjαCA1), enzymes that

catalyze bicarbonate to CO_2 conversion, previously thought to be involved in CO_2 concentrating mechanisms, are only beginning to be understood [70]. However, the importance of light in symbiosis is not limited to photosynthesis. Light, when perceived by shoot receptors called CRYPTOCHROME 1 (GmCRY1), triggers the movement of mobile signals FLOWERING LOCUS T2a (GmFT2a) and Soybean TGACG motif-binding Factor 3 (GmSTF3) to roots [67]. Upon rhizobial infection in roots, the symbiotic calcium sensor CALCIUM and CALMODULIN DEPENDENT PROTEIN KINASE phosphorylates GmSTF3, which promotes the transcriptional activity of the GmFT2a-GmSTF3 complex, thereby increasing nodule formation [67].

Nodulation is also influenced by soil edaphic factors such as nitrogen and iron availability [35,39,71]. In soybean, nitrogen inhibits nodulation through a regulatory pathway that includes the microRNA *miR169c*, Nuclear Factor-Y Subunit A (GmNFYA-C), and Early Nodulin (GmENOD40) [35]. Plants with sufficient-N induce expression of microRNA *miR169c*, which inhibits RNS by repressing nodulation-responsive *GmNFYA-C* and, consequently, its downstream target *GmENOD40*. CRISPR-Cas9 deletion mutants of *gmnfya-c* have 40% fewer nodules while those of *miR169c* have 35% more nodules [35]. In contrast, deletion of the microRNA *miR2111-5* locus in *L. japonicus*, which targets a nodulation suppressor in the CLAVATA3/EMBRYO SURROUNDING REGION-RELATED (CLE) peptide mediated autoregulation of nodulation pathway - *TOO MUCH LOVE* - results in a significant decrease in the number of infection threads and nodules [71].

Gene editing can be especially useful to investigate the regulation of key nodulation signaling genes by environment-responsive promoter elements [34,50,72,73]. Recent pioneering studies demonstrating precise deletion of nitrate responsive elements in promoters of *NODULE INCEPTION* and *NIN-Like PROTEIN 2* transcription factors have paved the way for future progress [34].

Outlook

Several economically significant legumes already have sequenced genomes and transformation protocols in place, and legume geneticists have adopted the fundamentals of gene editing. Beyond the application of this technology to create multiple gene knockouts and precise base-editing, the creation of gene insertions, or 'knock ins', used in gene regulation and RNA editing, has not yet seen widespread use. Rapid climate change action requires pursuit of bold research directions such as with gene editing to conduct future research on root nodule symbiosis in combination with one or more abiotic stress factors.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pbi.2022.102324.

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* of special interest
** of outstanding interest

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