Functional genomics dissection of the nodulation autoregulation pathway (AON) in soybean (Glycine max)

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Abstract

The combination of mutation-based genetics and functional genomics allowed a detailed dissection of the nodulation-induction and Autoregulation of Nodulation (AON) pathways of soybean. Applicable to all legumes, nodulation was induced by *Rhizobium/Bradyrhizobium* produced lipopolysaccharides (Nod factors), perceived by Nod factor receptors (NFR1/NFR5 dimers), leading to cortical and pericycle cell divisions. These induce the production of CLE (Clavata3-like) peptides, that travel in the xylem to the shoot, where they are perceived by a receptor complex including a LRR receptor kinase, encoded by GmNARK, LjHAR1, MtSUNN and closely related receptors in other legumes like *Phaseolus vulgaris* (common bean), *Pisum sativum* (pea) and *Glycine soja*. This activated receptor complex negatively regulates by phosphorylation the constitutive synthesis of miR2111 in the shoot, that normally is translocated via the phloem to the entire plant body, initiating suppression of root-expressed receptor kinase ‘Too Much Love (TML)’, which in turn suppresses the nodule initiation cascade. Nodulation thus is permitted during a developmental window between the induction and progress of the nodulation/cell division/infection cascade during the first few days after inoculation and the functional ‘readiness’ of the AON cascade, delayed by root-shoot-root loop. Loss-of-function mutations in *GmNARK* and *TML* result in excessive nodulation (supernodulation/hypernodulation/supernummary nodulation) as well as localised tolerance to externally applied nitrate. Recent analyses indicate interaction with gibberellin signalling, plant immunity as well as lateral root formation. Further details of the parallel functions of key points in this regulatory loop remain to be elucidated.

Introduction

Things in ‘Life’, and therefore ‘Science’, often develop unexpectedly, but also because of long-term effort combining ideas, technologies, insights and indeed some luck. One example is the analysis of early legume nodulation leading to agriculturally and environmentally important nitrogen fixation, and its control by both internal and external mechanisms. One regulatory process is by the common soil nutrient nitrate, which normally makes the essential element nitrogen available for plant growth and productivity; the other involves a complex internal control circuit, called Autoregulation of Nodulation (AON; Gresshoff and Delves, 1986; Caetano-Anollés and Gresshoff, 1991), which now has been dissected by a combination of plant mutation genetics, molecular physiology, and functional genomics.

We now have four decades stretching from a simple analysis of that plant physiological response (see Carroll and Gresshoff, 1983) to a cutting-edge insight into a complex, interorgan communication involving receptor proteins, transcription factors, peptides and microRNA as revealed by functional genomics and molecular physiology (see Zhang et al, 2021; Chu et al, 2022; Grundy et al, 2023). Our success is owed to the continued application of progressively precise technologies, international collaboration, and a decade-long continuum of talented researchers.

The processes to be addressed: Multiple legume nodulation control
Nitrate inhibition of nodulation

Nitrate (NO$_3^-$) is a common source of the nitrogen (N) element needed for growth of plants. Such plant nitrogen is mainly found in polypeptides (i.e., enzymes, peptides, structural, storage and regulatory proteins), nucleic acids (DNA and RNA) and their nucleotide components, and metabolites of various use. Nitrate is actively taken up by plants, before the subsequent reduction by nitrate and nitrite reductases to ammonium and further assimilation into glutamine, which serves as a central source for biologically available nitrogen in all relevant steps of metabolism.

Besides its function as a basic nutrient in plants, nitrate also has developmental signal properties, often at concentrations below those of the normal nutrient level. For example, the nitrate status of a legume plant regulates its ability to induce flowering (Jun et al, 2023). Likewise, legume plants, capable of symbiotic nitrogen fixation via nodule development induced by soil bacteria broadly called 'Rhizobium', regulate the symbiosis by external nitrate (Carroll and Gresshoff, 1983; Carroll et al, 1985a). Thus, nodule induction, (reflected in nodule number per plant), nodule growth (measured as nodule dry mass per nodule or per plant), and nodule symbiotic function (quantified by atmospheric nitrogen gas (N$_2$) being ‘fixed’ to form ammonia (NH$_3$)), is negatively controlled by the nitrate ion.

Physiological, developmental and anatomical responses to nitrate have been published for a large number of legumes over the last 100 years. It was clear that legumes make a choice for nitrogen acquisition, either by root-nodule symbiosis or uptake of external sources (such as soil or nutrient solution). Whilst nitrate is the most common and stable form of nitrogen-containing molecules in soil and water, urea (CO(NH$_2$)$_2$), and ammonium (NH$_4^+$) are agricultural alternatives as fertilisers. Plants possess uptake systems for these for direct utilisation, though in most agricultural circumstances, these alternative N sources are converted by soil microbes to nitrate prior to plant uptake.

Nitrate reduction, via the nitrate reductase enzyme complex found in almost all plants, can occur in either root or shoot, or both. The resultant product, nitrite (NO$_2^-$), is quickly converted by the enzyme nitrite reductase to form ammonia. In aqueous solution (such as the plant cytoplasm), this is in the form of the cation ammonium (NH$_4^+$).

The genetic and molecular make-up of these regulatory systems, critical for plant growth and development, have been subject of research for the last half century. This article will not go further into any detail of the physiology and biochemistry of nitrate utilisation. However, it will focus on (a) the analysis of nitrate effects on nodulation as related to the internal process called ‘Autoregulation of Nodulation’, and (b) its molecular physiological and functional genomic dissection, for which the critical components such as receptors, signals, and reaction cascades are now known through mutation and functional genomics studies.

Additionally, new developmental concepts were developed; for example, (a) nitrate susceptibility and response of the soybean root is similar (actually identical) in all legumes (see Carroll et al, 1985;
Ferguson et al, 2010), (b) nitrate responses are related directly to nodule number control through regulation of initiated meristems in the root cortex, and (c) lateral root development is directly linked to root nodule number control (originally noted at a descriptive level by Nutman (1948) in red clover).

Autoregulation of Nodulation (AON)

Legume plants develop root nodules after stimulation from signals derived from bacterial symbionts such as *Rhizobium* and *Bradyrhizobium* (and few other) species. The interaction leads to a nitrogen-fixing symbiosis, important globally in both economic and environmental terms. The amounts of nitrogen fixed generally by most commercially grown legumes is around 100 kg per hectare per year, though Rochester et al (2001) reported soybean yielding as much as 488 kg N/ha/annum. An excellent up-to-date analysis of nitrogen inputs by agricultural legumes is given by Herridge et al (2022).

This symbiosis is regulated in the number and mass of nodules developing after inoculation though a process called ‘Autoregulation of Nodulation (AON)’ (Caetano-Anollés and Gresshoff, 1991), also called ‘Feedback regulation of nodulation’. It was discovered by Bhuvaneswari et al (1981), who demonstrated that prior inoculation of soybean roots inhibited further nodulation in lower root portions (*i.e.*, that developed later). Similarly, Kossak and Bohlool (1984) as well as Olsson et al (1989) showed that AON in soybean works systemically, so that one side of a physically separated split root system communicates the AON inhibition signal to the other side of the root system. Thus, the shoot is involved!

The inhibition of nodulation by nitrate lead Carroll et al (1985a,b) to use chemical mutagenesis of soybean cultivar Bragg with EMS (ethyl methyl sulphonate) to isolate homozygous recessive mutants with substantially (up to 20 fold) increased nodule number per plant in the presence of potassium nitrate (5 mM). Unexpectedly, the zero nitrate control treatments also had increased nodule number, leading to the concept of ‘Autoregulation of Nodulation’. These mutants nodulated more in the presence of nitrate, because they nodulated more *per se!* Increased nodulation in the presence of nitrate came from an absence of a naturally functional regulatory system controlling extended nodulation in root regions normally regulated by AON. This was confirmed in split root systems of soybean (Olsson et al, 1989).

They showed that in split roots of wild-type soybean Bragg that prior inoculation of one side reduced nodule number in the other, physically separated, root portion. If the experiment was done with the recessive *nts382* (*Gmnark*; supernodulating) mutant prior inoculation of one side did not have any effect on nodule number in the second half. Interestingly, when split roots were used with nitrate inoculation, Carroll and Gresshoff (1983) already found that nitrate inhibited nodulation in a localised fashion, *i.e.*, exposure of one root portion to nodule number inhibitory levels of nitrate did not inhibit nodule formation in the other (unexposed) root. Furthermore, they discovered that although nodule induction and subsequent symbiotic nitrogen fixation are both regulated by external nitrate, their regulation is completely independent.

The chemical mutagenesis of soybean (genotype Bragg) involved treatment of some 50,000 wild-type seed, followed by raising the treated M1 survivors, and their single plant harvest (Carroll et al, 1985a).
These M2 seed were screened in pot trials for the ‘nodulation tolerant to nitrate’ phenotype; thus separately detected M2 families derived from separate M1 plants and thus seed, meant that independent mutant lines were detected. Multiple supernodulating and hypernodulating mutants were isolated, which turned out to be allelic as studied by complementation analysis (Delves et al, 1987). Parallel studies were done using fast neutron mutagenesis in collaboration with the IAEA research laboratory at Seibersdorf (Austria). The smaller seeded *Glycine soja* was used as the treatment chamber was small, allowing a limited sample size. M2 selection resulted in a stable supernodulation mutant, called *FN5*, shown to be deleted for large region around and including *GmNARK* (Men et al, 2002).

The same chemical mutagenesis also produced soybean non-nodulation mutants (Carroll et al 1986), which were later defined as being in the two Nod factor receptor genes *GmNFR1* (*GmNod49*) and *GmNFR5* (*GmNod139*) (Indrasumunar et al, 2011; 2012). An interesting aspect of cloning soybean genes, that if mutated act recessively, comes from the fact that the allotetraploid soybean genome is largely duplicated (Schmutz et al, 2000). For example, *GmSymRK* has two nearly identical copies (96%) in the genome; thus a mutant in any one of the genes is most likely to have a phenotype (Indrasumunar et al, 2015). However, use of RNAi, targeting both copies resulted in clear phenotypes of nodulation and mycorrhizal associations, confirming the mutational phenotype of *SymRK* found in *Lotus japonicus* and *Medicago truncatula*. Thus, duplicate copies of the nodulation facilitating genes and also the *GmNARK* gene controlling AON were discovered.

In the case of *GmNFR1* and *GmNFR5* both duplicate genes were inactive because of internal mutations. The duplicated *GmNARK* gene was called *GmClv1a*, as it has highest sequence homology to the *Clavata1* gene of *Arabidopsis*. It is characterised by a 12-nucleotide deletion relative to *GmNARK*. Mirzaei et al (2017) investigated the phenotype of the *GmClv1a* mutant and found that it is altered in stem, pod and fruiting morphology. For example, opposing as compared to alternate trifoliate leaves and highly abnormal seed pod were observed (Fig. 1). Mutations in such gene do not result in supernodulation, (as the duplicated *GmNARK* gene exists in a functionally dominant configuration), but severe developmental /structural changes (Mirzaei et al, 2017). It appears that in soybean the ancestral *Clavata1* gene, involved in broad architectural symmetry and structure, was duplicated to yield two copies, namely *GmClv1a* and *GmClv1b*, the latter maintaining the ancestral architectural function, while the duplicated version became specialised in Autoregulation of Nodulation (AON). The question remains as to what had happened in the other legumes as species like *L. japonicus* are true diploids and appear to possess only one *Clavata1* gene copy, which, if mutated, results in hypernodulation and reduced root growth (the *HAR1* phenotype (Krusell et al, 2002; Wopereis et al, 2000). Indeed, Lotus plants grown symbiotically on agar plates are very crippled but recover vegetative growth only when fed nitrate.

Similarly, the Bragg M2 seed population was screened by multiple-well plate assay for alterations in enzymatic activity for constitutive nitrate reductase (Carroll et al, 1987), illustrating that EMS mutation analysis of soybean, though showing a partially duplicated genome, is possible for both developmental and biochemical traits.
Extensive growth studies revealed that such AON mutants tended to grow slower than their wild-type segregants or wild-type Bragg (Day et al, 1986). All mutant lines were able to utilise nitrate, were able to fix nitrogen in the developed nodules, grew less rapidly (Day et al, 1986) and flowered about 4 to 7 days earlier (Gresshoff, P.M., unpublished results). Cytological analysis of the roots revealed that an early nodule cell division stage (about 3 days after inoculation) was negatively regulated by functional AON, but absent or weakened in the mutant lines (Mathews et al, 1980; 1982). Additionally, it was noted that whilst young seedlings of nts382 (7 days after inoculation) showed an increased number of lateral roots (Mathews et al, 1982), while more mature plants (4 weeks after inoculation) had decreased lateral roots.

Delves et al (1985) demonstrated using mutant and wild-type reciprocal grafts that the presence of AON required the shoot portion of the graft to be of the non-mutant (wild-type) nature. In other words, the gene mutated to give what was first called supernodulation in soybean (or hypernodulation in the model legume L. japonicus (Krusell et al, 2002; Wopereis et al, 2000) or supernumery nodule number in the temperate model legume M. truncatula (Schnabel et al, 2005) acted in the shoot, receiving some, then unknown, systemic signal from the early stages of nodule development in the root cortex/pericycle. The shoot then transmits a then unknown signal to the entire root system, dictating a cessation of further nodule development. This model was proposed by Gresshoff and Delves (1976), even then including the parallel involvement of the system controlling lateral root growth as the AON circuit was based on a pre-existing analogous system involving the root tip and its meristematic primordium.

Major questions arose quickly: (a) what is the mutated gene, (b) what is its function and regulation, (c) what are the 'upfront' and 'downward' signals, and (d) how does the AON circuit fit developmentally into the known regulation of lateral root growth and number? Though at present (2023), with billions of DNA datapoints, sophisticated amplification techniques, and a strong input from the model legume species L. japonicus and M. truncatula, our insights have expanded, indeed 'closing the circuit' in broad terms, but leaving critical steps totally unresolved (i.e., how does GmNARK phosphorylation control synthesis of miR2111? And how is lateral root and nodule formation inversely connected? Through gene action or hormonal control? Or both?)

**Functional genomics as a tool for AON mutant gene discovery**

Two questions became clear quickly; (a) what was the gene that was mutated, and (b) what were the signals exchanged between root and shoot allowing the AON regulation?

Positional cloning, or what was called 'map-based cloning' was chosen to attempt the isolation and characterisation of the mutated soybean gene. The Shoemaker laboratory in Iowa (USA) (Keim et al, 1989) had just developed the first molecular map of soybean using RFLP markers. Representative marker clones were provided to our laboratory to determined possible linkage and thus preliminary linkage position. Landau-Ellis et al (1991) found that RFLP marker pA132 (or more precisely its component region pA132a) was closely linked (0.3 centiMorgans in a small sample segregant population. Further markers were developed by arbitrary primer PCR (DAF; Caetano-Anollés et al, 1991)
and DNA silver staining in PAGE gels (Bassam et al, 1991). This allowed the first discovery of an AON-associated gene in soybean (Searle et al, 2003), though the work was done concurrently and independently from that is L. japonicus (Krusell et al, 2002). The homologous genes from the model legumes L. japonicus (LjHAR1; Krussell et al, 2002) and M. truncatula (MtSUNN; Schnabel et al, 2005) were isolated and shown to be identical LRR receptor kinases with identical function in the shoot and the proposed long-distance circuit.

Early phenotypic analysis of all mutants in either GmNARK, LjHAR1 or MtSUNN illustrated broadly common properties. Firstly, their nodule number per plant fresh (or dry) weight is higher than the parent's; secondly, they all have reduced root growth (LjHAR1 mutants in its name have the mention of altered root growth), and thirdly, all are somewhat resistant/tolerant to the inhibitory effect of nitrate on nodule initiation and growth. Mechanistically they are also identical, namely all are mutated in the same LRR receptor kinase, all are shoot-controlled, and all respond to the bacterial activation and subsequent root-to-shoot translocation of short peptides similar to the Arabidopsis Clavata3 peptide. One can suspect that control of root growth (e.g., lateral root primordia and their extension into lateral, then tertiary roots) developed on the ancestral forms of the here-stated control of nodule number; hence it is likely that these developmental steps still exist and are seen in alterations of GmNARK and its homologues in seemingly all legumes.

The gene was identified to be highly structurally similar to an Arabidopsis thaliana gene controlling apical shoot architecture, name Clavata1, a gene encoding a leucine-rich receptor kinase. Multiple mutant alleles were sequenced and found to be mainly non-sense mutations leading to a major truncation of the gene product (Searle et al, 2003). Highly conserved genes and mutant alleles were found in other legumes such as L. japonicus (Krusell et al, 2002; Wopereis et al, 2000), G. soja (Men et al, 2002), Pisum sativum (Sagan and Duc, 1996), Phaseolus vulgaris (Ferguson et al, 2000) and M. truncatula (Schnabel et al, 2005) showing the supernodulation / hypernodulation / SUNN phenotype. It is concluded that the legume CLV1 gene is commonly used in AON.

What is the root-to-shoot AON signal in soybean?

The Clavata1 polypeptide of Arabidopsis is associated with Clavata2, forming the transmembrane receptor complex whose extracellular domain interacts with the 13 amino acid long Clavata3 peptide. This peptide belongs to the CLE (CLAVATA3/Endosperm Surrounding Region-related) family of peptides characterised by a typical spacing of internal proline residues (.PnPxxP.). This central proline cluster, and the presence of a small side-chain amino acid like glycine between the .PxxP. is thought to be essential for the functionally-important bending of the peptide. Likewise, the CLE peptide is tri-arabinosylated, most likely essential for translocation via the xylem system (Corcelius et al, 2018). The prepeptide is usually around 100–110 amino acids long, with a moderately conserved N terminal domain, an interstitial highly variable region, and a terminal or near-terminal CLE peptide domain (Fig. 2). Soybean contains 63 such peptide genes (Hastwell et al, 2017). They synthesised these and treated soybean seedlings in the absence of B. japonicum (the normal symbiont of soybean (Hastwell et al, 2015, 2016, 2017). Such
peptides were fed to the soybean plant by petiole feeding (Lin et al, 2010, 2011). Figure 2 gives the post-translationally modified amino acid structure of GmRIC1a.

Oelkers et al (2008) already speculated that the most likely ligand of GmNARK was related the Arabidopsis CLV3 peptide. This led to the discovery of two Rhizobium-induced CLE (RIC) peptides in soybean (Reid et al, 2001, 2004). Feeding these peptides in the presence of Bradyrhizobium results in complete suppression of nodulation. An additional peptide gene was found to be specifically involved in nitrate inhibition involving the GmNARK receptor functioning in the root. This localisation explains the root specific function of nitrate as compared to the systemic action of AON involving the shoot of the plant. Such control circuits exist in all tested legume species so far (see Ferguson et al, 2017).

RIC peptides in L. japonicus interact directly with LjHAR1, being the equivalent to GmNARK, suggesting that GmRIC1 and GmRIC2 interact with the receptor domain of the soybean gene product.

**What is the shoot-derived signal in soybean?**

As shown above, legumes control their nodule numbers through the Autoregulation of Nodulation (AON). Rhizobia infection stimulates the production of root-derived and tri-arabinosylated CLE peptide hormones (Corcelius et al, 2018) that are translocated via the xylem to the shoot where they regulate a new signal. Early models suggested the name ‘Shoot-derived Inhibitor’ (SDI), which was incorrect as GmNARK produces a positive signal (phosphorylation; Miyahara et al, 2008), that inhibits an inhibitor (see Fig. 3). In soybean this shoot-derived signal is miR2111 (Zhang et al, 2021), which is transported via phloem to the root, where it targets transcripts of the Too Much Love (TML) gene, a negative regulator of nodulation. Shoot perception of rhizobia-induced CLE peptides GmRIC1 and RIC2 suppresses miR2111 expression, resulting in TML accumulation in roots and subsequent inhibition of nodule organogenesis. Feeding synthetic mature miR2111 RNA via the petiole (Lin et al, 2011) increased nodule numbers per plant. Likewise, elevating miR2111 availability by over-expression promoted nodulation, while target mimicry of TML induced the opposite effect on nodule development in wild-type plants and alleviated the supernodulating and stunted root growth phenotypes of AON-defective mutants. Additionally, in non-nodulating wild-type plants, ectopic expression of miR2111 significantly enhanced lateral root emergence with a decrease in lateral root length and average root diameter. In contrast, hairy roots constitutively expressing the target mimic construct exhibited reduced lateral root density. Overall, these findings demonstrated that miR2111 RNA is the critical shoot-to-root factor that positively regulates both root nodule development and also acts to shape root system architecture (i.e., lateral root formation and apical meristem vigour). Indeed, one wonders whether the involvement of the microRNA and its regulation via the shoot-located LRR receptor kinase with lateral root formation were ancestrally the prior activity, which then was evolutionary hijacked (gene duplication, neodiversification, etc) for the evolving nodulation process.

**AON-related processes: flowering, immunity and lateral root formation**
Immunity and symbiosis

Plant roots are constantly exposed to a diverse microbiota of pathogens and mutualistic partners (Grundy et al, 2023). The host's immune system is an essential component for its survival, enabling it to monitor nearby microbes for potential threats and respond with a defence response when required. Current research suggests that the plant immune system has also been employed in the legume-rhizobia symbiosis as a means of monitoring different rhizobia strains, and that successful rhizobia have evolved to overcome this system to infect the roots and initiate nodulation.

With clear implications for host-specificity, the immune system has the potential to be an important target for engineering versatile crops for effective nodulation in the field (Grundy et al, 2023). However, current knowledge of the interacting components governing this pathway is limited, and further research is required to build on what is currently known to improve our understanding of microbe-associated molecular pattern-triggered immunity (MTI) and effector-triggered immunity (ETI).

Nodulation symbiosis and legume flowering

It is known that fixed nitrogen increases reproductive and thus seed yield success, but until now, the regulatory mechanism was unknown. Yun et al (2023) find a flowering pathway that couples symbiotic and nutrient signals to the flowering induction pathway in legumes. The symbiotically important microRNA (i.e., microRNA172c (miR172c)) and fixed nitrogen (nitrate) systemically and synergistically convey symbiotic and nutritional cues from roots to leaves to promote soybean (*Glycine max*) flowering (see Fig. 2). The combinations of symbiotic miR172c and locally active miR172c, elicited by fixed nitrogen and development in leaves, activate florigen-encoding *FLOWERING LOCUS T* (*FT*) homologs (*GmFT2a/5a*) by repressing *TARGET OF EAT1-like 4a* (*GmTOE4a*). Thus, FTs trigger reproductive development, which allows legumes to survive and reproduce under low-nitrogen conditions. Plant development, indeed, is a network of integrated cellular/tissue and biochemical pathways that we are just starting to understand by careful dissection using modern approaches like functional genomics, gene editing and biochemical genetics.

Nodulation and gibberellin regulation

Functional genomics opened up our ability to look more deeply at parallel regulation by classical plant hormones such as gibberellins (like GA₃), ethylene, auxins (like IAA) and cytokinins (like BAP and kinetin). Gene sequences, detected by RNA expression analysis of inoculated (*NodC*⁺ wild-type *B. japonicum*) and fake-inoculated (*NodC*⁻ mutant of *B. japonicum*) were analysed for transcription resulting from GA exposure during different nodulation development stages. Gene sequences were edited to test the influence of specific domains on gene expression. Modern techniques like a Förster resonance energy transfer-based GA biosensor allowed the monitoring of hormone changes relative to nodule development.

The plant hormone gibberellin (GA) is required at different stages of legume nodule development, with its spatiotemporal distribution tightly regulated. Transcriptomic and bioinformatic analyses established that
several key GA biosynthesis and catabolism enzyme encoding genes are critical to soybean (*Glycine max*) nodule formation.

We examined the expression of several GA oxidase genes and used a Förster resonance energy transfer-based GA biosensor to determine the bioactive GA content of roots inoculated with *DsRed*-labelled *B. diazoefficiens*. We manipulated the level of GA by genetically disrupting the expression of GA oxidase genes using CRISPR-Cas9 gene editing. Moreover, exogenous treatment of soybean roots with GA3 induced the expression of key nodulation genes and altered infection thread and nodule phenotypes.

Three genes, *GmGA20ox1a*, *GmGA3ox1a*, and *GmGA2ox1a* (Hayashi et al 2012), are upregulated in soybean roots inoculated with compatible *B. diazoefficiens*. *GmGA20ox1a* expression is predominately localised to the transient meristem of soybean nodules and coincides with the spatiotemporal distribution of bioactive GA occurring throughout nodule organogenesis. *GmGA2ox1a* exhibits a nodule vasculature-specific expression pattern, whereas *GmGA3ox1a* can be detected throughout the nodule and root. Disruptions in the level of GA resulted in aberrant rhizobia infection and reduced nodule numbers. Collectively, these results establish a central role for GAs in root hair infection by symbiotic rhizobia and in nodule organogenesis.

**Conclusion**

The here-described isolation and characterisation of genes involved in Autoregulation of Nodulation (AON) shows the power of functional genomics in soybean. It also revealed the mechanism by which external nitrate inhibits nodulation in legumes. At first, mutants were isolated to have clear phenotypic differences. These supernodulation (altered in AON) and non-nodulation mutants (caused by lack of initial reception of the bacterial Nod factor) allowed the use of molecular markers, comprehensive marker maps and fast DNA sequencing methods to isolate and analyse the action of mutated genes (Chu et al, 2022; Hayashi et al, 2012; Mens et al, 2018).

It seems clear that the mutual interaction of diverse experimental approaches such a map-based cloning, DNA expression analysis, epigenetics, pan-genome analysis for critical targets in breeding, molecular physiology, mutational studies and functional genomics will improve our understanding of the thousands of plant processes controlling growth, survival and productivity. Whether such potential improvements of crop plants’ performance is sufficient to provide food, feed and fuel for the expanding global population of humans is a major and problematic challenge for the future decade.

**Declarations**

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**Author contribution** Peter M. Gresshoff wrote the manuscript, Huanan Su performed the *GmCLV1a* and *GmNARK* protein modelling and structure alignment, prepared the Fig. 1 and Fig. 2. Huanan Su and
Estelle B. Grundy made the Fig. 3. All authors read and edited the manuscript.

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**Competing interests** The authors declare no competing interests, no financial interests.

**References**


Figures

![Figure 1](image-url)
Phenotype of wild-type, \textit{GmCLV1a} and \textit{GmNARK} soybean mutants. Note the abundant nodulation (normal sized nodules!) for the near-isogenic \textit{W677*} mutant of soybean cultivar Forrest inoculated with \textit{Bradyrhizobium diazoefficiens} strain USDA110 (previously named \textit{B. japonicum} USDA 110). Mutant \textit{S562L} is mutated in \textit{GmCLV1a}, the duplicated copy of \textit{GmNARK} and shows normal nodulation (it is \textit{GmNARK}'), but severely altered in vegetative and fruit development (reminding one of the \textit{Arabidopsis} phenotype of \textit{Atclv1}). \textit{GmCLV1a} and \textit{GmNARK} protein domain structure alignments are shown on the right panel, 3D structure modelled by Phyre 2, visualised and aligned by PyMol. This figure is adapted from Mirzaei et al, 2017.

\textbf{Figure 2}

Post-translationally modified amino acid sequence of nodulation suppressing CLE peptide GmRIC1a. C-terminal is to the right side. \textit{R} represents the site of tri-arabinosylation, which is essential for functionality. GmRIC1a contains 12 amino acids.
Figure 3

The AON control circuit of soybean. Multiple combinations of receptor components are possible, with CLAVATA2 (CLV2), KLAVIER (KLV) and CORYNE (CRN) being the most likely candidates. This will give the complex different affinities to different peptide ligands. NARK signalling is also involved in flowering (Yun et al, 2023). Multiple function of activated GmNARK receptor complex suppresses synthesis of miR2111, but also increases cytokinin biosynthesis, supporting cell divisions in nodulation. miR172c also inhibits synthesis of GmNNC1 mRNA, which in turn suppresses GmENOD40, an essential gene for nodulation. NFR1 and NFR5 are Nod factor Receptor1 and 5. TML is ‘Too Much Love’. RIC1 and RIC2 are Rhizobium-induced CLE peptides.