Advances in the Identification of Novel Factors Required in Soybean Nodulation, a Process Critical to Sustainable Agriculture and Food Security

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Abstract

Nodulation is a process of organogenesis that results from a symbiotic relationship between legume plants and soil-dwelling, nitrogen-fixing bacteria, called rhizobia. The rhizobia are housed in newly formed structures on the host roots, called nodules. Within nodules, the rhizobia fix atmospheric N_2 into useable forms of nitrogen for the plant. This process is highly important to agriculture, as nitrogen is critical for plant growth and development and is typically the main component of fertilizers. Although fertilizers are effective, they are expensive and often pollute, making biological alternatives, such as legume nodulation, attractive for use in agriculture. Nodulation is regulated by the auto regulation of nodulation (AON) pathway, which enables the host plant to balance its needs between nitrogen acquisition and energy expenditure. Current research is elucidating the nodule development and AON signalling networks. Recent technological advances, such as RNA-sequencing, are revolutionizing the discovery of genes that are critical to nodulation. The discovery of such genes not only enhances our knowledge of the nodulation signalling network, but may help to underpin future work to isolate superior legume crops via modern breeding and engineering practices. Here, recent advances using the cutting-edge technique of RNA sequencing to identify new nodulation genes in soybean are discussed.

Global Use of Nitrogen Fertiliser

Approximately half of the world's population is directly reliant upon nitrogen fertiliser use in agriculture for their food supply [1-3]. Taking into account nitrogen fertiliser manufacture, transport and application, the fossil fuel consumed accounts for 50% of fossil fuel use in agriculture, and 5% of the global natural gas consumption annually [4,5]. With the rising cost of fossil fuels, the use of nitrogen fertiliser is becoming increasingly costly for farmers and is often too expensive in developing regions of the world [6]. Not only are nitrogen fertilisers expensive, they are inefficient, with 30-50% of nitrogen fertiliser typically lost to leaching. This run off can cause the eutrophication of waterways and other significant environmental problems [7]. Nitrogen contaminated drinking water can also cause methemoglobinaemia, or "Blue-baby syndrome", a potentially fatal condition in infants [8,9].

The global use of nitrogen fertiliser has been steadily increasing in most continents (Figure 1). Worryingly, this also means an increase in NO_x gases, which are released when nitrogen fertiliser is broken down. These gases contribute to the formation of ground-level ozone, which causes yield reductions. Nitrous oxide (N_2O) is also emitted by breakdown of nitrogen fertilisers [7,10] and is 292 times more active as a greenhouse gas than CO_2 [4]. Agriculture was the main source of anthropogenic N_2O emissions in 2005, making up 60% of the global total [11]. The majority of these emissions resulted from the application of nitrogen fertiliser [11]. By 2050 it is estimated that global nitrogen fertiliser use will increase by 50% in an attempt to boost food production and support a rising population [10]. These numbers have experts calling for agricultural reform to diminish nitrogen fertiliser use.

Legume Crops as a Safe Alternative to Nitrogen Fertiliser

One safe alternative to the use of nitrogen-based fertilisers is to take advantage of biologically-fixed nitrogen. Legumes are able to form a relationship with specialised nitrogen-fixing soil bacteria, called rhizobia. The rhizobia convert atmospheric di-nitrogen into usable forms of nitrogen for the plant, whilst being housed in novel root organs, called nodules. The use of legumes as rotation crops is an important agricultural practice that many experts argue must be increased to help curb nitrogen fertiliser use [2,6,10]. Optimizing biological nitrogen fixation processes, such as nodulation, has the potential to increase crop yields and enhance soil fertility whilst simultaneously reducing farming costs and harmful environmental impacts [1,5,6,12]. However, it is only with an increase in our knowledge of nodulation processes and its genetic basis that we can fully reach this goal.

Nodule Organogenesis

The most common entry point for rhizobia invasion is the region of root where the root hairs are developing, called the Zone of Nodulation (ZON) [13-16]. Rhizobia attach to the root hair, triggering root hair deformation and curling [13,14,17]. This process involves the rearrangement of underlying microtubules which allow bacterial entry and the establishment of tubular structures called the infection threads (IT) [18,19].

Occurring in parallel to rhizobia invasion are inner cellular changes which lead to nodule primordia formation [19]. The ITs full of rhizobia progress towards the nodule primordia. The convergence of the rhizobia in the ITs and the nodule primordia is essential for successful nodule formation. Once the rhizobia reach the developing nodule, they are released from the ITs into specialised structures called symbiosomes, in which they differentiate into bacteroids. Using
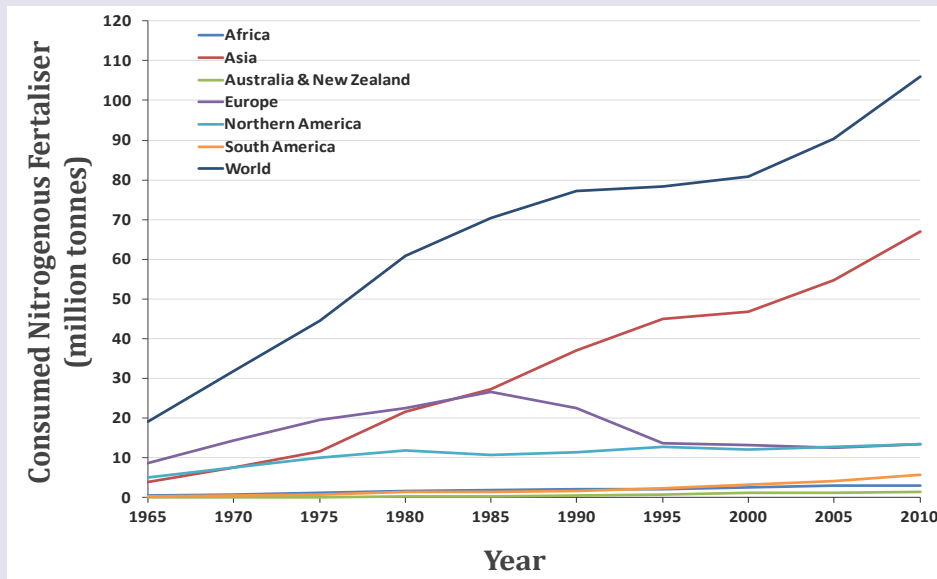
Figure 1: Global consumption of nitrogen fertiliser displayed per continent over a forty five year period, 1965-2010 (consumption in nutrients; tonnes; FAOSTAT, 2013). Data obtained from FAOSTAT.

their nitrogenase enzyme complex, the rhizobia bacteroids catalyses atmospheric N_2 into ammonia, which can be used by the plant [20].

There are two different morphological types of legume nodules: indeterminate and determinate. Indeterminate nodules are initiated by inner cortical cell divisions, followed by divisions in the endodermis and pericycle, and develop persistent meristems [21-24]. Determinate nodules initially arise from cell division of the outer cortex and have transient meristems [15,24-26].

Signalling in Nodulation

Nodule formation is initiated via a highly-specific signal exchange between compatible rhizobia bacteria and legume plants [19,27,28]. Flavonoids are released into the soil by the plant, attracting compatible rhizobia species to the host plant. They also trigger the expression of rhizobia nodulation (*Nod*) genes, which leads to the production of novel Nod Factor (NF) signals that are recognized by the host plant [29]. Additional rhizobia-produced factors, such as exopolysaccharides (EPS) and lipopolysaccharides (LPS), are also known to be important for nodulation and in determining rhizobia-plant specificity.

Following perception of compatible partners, two main pathways are triggered within the plant: one involved in bacterial entry and infection and the other involved in cell divisions that lead to the formation of the nodule primordia [19]. Formation of a functional nodule requires synchronisation between these different signalling pathways. Many of the genes known to act in early nodulation are conserved between different legume species, but in many cases these orthologous genes have different names.

At the plant root periphery, LysM receptor kinases (*Lotus japonicus* Nod Factor Receptor 1 and 5; LjNFR1 and LjNFR5; soybean, *Glycine max* NFR1α/β and GmNFR5α/β; *Medicago truncatula* MtLYK3, MtLYK4 and MtNFP; and pea, *Pisum sativum* SYM2A and PsSYM10), perceive NF from compatible rhizobia [30-

35]. These receptors are reported to associate with remorin proteins (MtSYMREM1), which may help in assembling a specialised NF receptor complex [36]. Other factors, such as LjROP6, a Rho-like small GTPase, have also been found to interact with LjNFR5 [37].

An additional receptor kinase (MsNORK/LjSYMRK/MtDMI2/PsSYM19) also associates with SYMREM1 and appears to be involved in downstream signalling and possibly also the perception of NF [36]. This receptor interacts with additional nodulation factors, including a coiled-coil protein (MtRPG; [38]), a transcription factor (LjSIP1; [39]) and a 3-hydroxy-3-methylglutaryl coenzyme reductase (MtHMGR1), which is involved in isoprenoid synthesis [40]. However, the precise roles for these factors in nodulation are not yet known.

Perception of NF triggers Ca^{2+} fluxes, followed by the Ca^{2+} spiking, in root hair cells. The oscillation of Ca^{2+} in these cells is thought to initiate downstream signalling events [41-43]. Ca^{2+} spiking events require putative potassium ion-channels (MtDMI1, LjCASTOR and LjPOLLUX; [44-46]), and two nucleoporins (LjNUP133 and LjNUP85; [47,48]). Acting downstream of the Ca^{2+} spiking, and likely perceiving the Ca^{2+} signal, is a calcium and calmodulin-dependent protein kinase (CCaMK; MtDMI3/PsSYM9; [49-52]). Novel proteins shown to interact with CCaMK include LjCIP73, which belongs to the ubiquitin superfamily [53], and MtIPD3/LjCYCLOPS, which regulates expression of *NSP1* [54-56]. Following activation of CCaMK, a number of transcription factors, including Nodulation Signalling Pathways 1 (MtNSP1) and MtNSP2 [54,57], Ets2 repressor factor (ERF), ERF required for nodulation (MtERN; [58]), and Nodule Inception (Lj/PsNIN; [59,60]) are activated. These transcription factors work in combination to activate the expression of the early nodulation (*ENOD*) genes in the epidermis (e.g. *MtENOD11*; [61]).

CCaMK activation is also believed to trigger the increase in cytokinin level in these cells. Hormonal changes are detected by the cytokinin receptor, LjLHK1/MtCRE1, on the cortical cell membrane [62,63]. Activation of LjLHK1/MtCRE1 is thought to activate

signalling within the cortical cells to initiate the cortical cell divisions required for the formation of the nodule [62,63].

Other components that are essential for nodule development include SCAR/WAVE proteins that appear to have roles in root hair deformation and rhizobia infection (LjNAP1/MtRIT1 and LjPIR1; [64,65]) and flotillin proteins that initiate the production of ITs for bacterial progression in the root (MtFLOT 2 and 4; [66]). An ankyrin protein that may have a role in IT development (MtVAPYRIN) is also required [67], in addition to a number of transcription factors (e.g. MtERF1 and EFD), U-box proteins (e.g. LjCERBERUS/MtLIN and MtPUB1) and early nodulin proteins of unknown function (e.g. ENOD11 and ENOD40 (reviewed in Ferguson et al. [19]). Further, a subunit of a signal peptidase complex (MtDNF1) that has a role in the processing of nodule specific cysteine-rich (NCR) proteins is an essential factor for rhizobia differentiation into nitrogen-fixing bacteroids in *M. truncatula* [68].

Autoregulation of Nodulation

The formation and maintenance of nodules is an energy-intensive process. As such, the plant strictly regulates the number of nodules it forms, regardless of rhizobia availability, through the Autoregulation of Nodulation (AON). AON acts systemically, following nodule development. Root-derived CLV3/ESR-related (CLE) peptide hormones are synthesised, called GmRIC1 and GmRIC2 in soybean, LjCLE-RS1 in *Lotus* and MtCLE12/13 in *Medicago* [69-73]. These signals are predicted to travel to the shoot, presumably via the xylem [72,74], where they are thought to be perceived by an LRR receptor kinase, GmNARK/LjHAR1/MtSUNN/PsSYM29 [75-78]. Mutants lacking a functional version of GmNARK/LjHAR1/MtSUNN/PsSYM29 are unable to regulate their nodule numbers and exhibit a super- or hyper-nodulating phenotype (Figure 2). It is possible that this receptor acts in conjunction with other receptor components, such as Lj/PsCLAVATA2 and/or LjKLAVIER [79,80]. Three additional factors, two Kinase-Associated Protein Phosphatases, GmKAPP1 and GmKAPP2 [81] and a putative Ubiquitin Fusion Degradation protein, GmUFD1a [82] have also been shown to possibly interact with GmNARK as part of the AON pathway.

Once the root-derived CLE peptide signal has been perceived, a novel Shoot-Derived Inhibitor (SDI) is produced which travels to the roots, presumably via the phloem, where it inhibits further nodulation [19,83]. Although SDI has yet to be identified, it has been shown to be NF dependent, heat stable, small (<1KDa) and unlikely to be a protein or RNA [84,85].
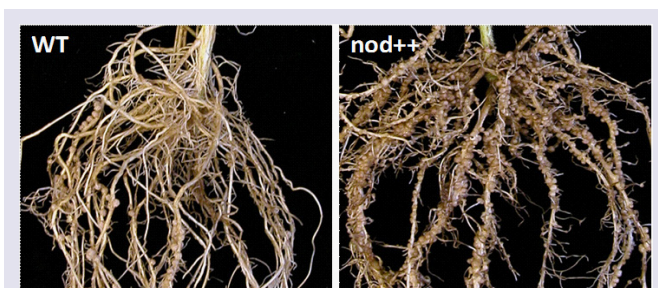

Figure 2: Root systems of wild-type (WT) and supernodulating mutant (nod++) soybean plants exhibiting mature nodule structures as a result of a symbiotic relationship with *Bradyrhizobium japonicum*.

Nodulation is not only regulated by the number of nodulation events, but also in response to environmental factors such as stress (e.g. ethylene), soil acidity and soil nitrate (e.g. [86-92]). This gives the host plant the ability to regulate nodule development in response to its surrounding environment, thus optimizing nodulation and nitrogen-fixation under a variety of growing conditions.

The New Generation of Gene Discovery: RNA-seq

The identification of factors acting in the development and control of legume nodules has considerably increased our understanding of these processes. Moreover, it has provided novel targets for breeding and engineering programs dedicated to generating superior crop species. Recent technological advances have significantly increased the speed and efficiency with which new molecular components can be discovered. This includes new, high-throughput sequencing technology that has enabled the genomes of many legume species to be assembled in recent years, including soybean, *L. japonicus, M. truncatula*, chickpea and pigeon pea [93-97]. Similarly, next-generation RNA-sequencing (RNA-seq) technology enables the complete transcriptome of a given plant sample to be determined. This includes establishing the expression of both known and unknown genes in a sample. This cannot be achieved using other techniques, such as microarrays.

Soybean is one legume species that has recently been subjected to a number of RNA-seq studies seeking to identify new factors required for nodulation within its transcriptome. Indeed, soybean is often used as a model legume species [92,98], as it has had its complete genome sequenced [94], with gene atlases and gene expression databases also being publically available [99-101]. It is also amenable to a number of molecular approaches, including *Agrobacterium rhizogenes*-mediated transformation (e.g., [102,103]), that are essential for follow-up research aimed at confirming and functionally characterising the role of candidate genes in nodulation. In addition, a number of mutant and TILLING populations are also available, which can considerably assist genetic studies [86,104-106]. Physiologically, soybeans are also excellent for scientific purposes, being fast growing, high yielding, amenable to grafting and of appropriate size for most field and laboratory studies [6].

Soybean research is highly applicable to other legume crops including pea, lentil, chickpea, bean, peanut, lucerne, clover and faba bean [107]. Soybean is also an important crop in its own right, with production of ~250 million tonnes globally in 2011, accounting for 50% of the world's oilseed production. It generates 200 kg N ha^{-1} in aboveground biomass each growing season with 58-68% of its nitrogen content resulting from symbiotic nitrogen fixation [1,2,108]. As a rotation crop, or "green manure", soybean can be ploughed back into the soil whether their seed has been harvested or not. This provides farmers with some flexibility and helps to replenish the soil nitrogen content, as in addition to the aboveground biomass, the roots and nodules contain 30-60% of the overall plant nitrogen content [109,110].

Three separate studies have reported using RNA-seq to identify differentially-expressed genes in the transcriptome of rhizobia-inoculated soybean roots [16,99,111]. The genes identified represent candidates required for nodule development. An additional study used RNA-seq to determine the transcriptome of soybean leaves to identify differentially-expressed gene candidates acting in AON [82].

The work of both Libault et al. [99] and Hayashi et al. [16] focused on the early stages of nodulation, with samples harvested 48 hours post inoculation. Libault et al. [99] focused on root hairs and stripped roots, enabling a tissue-specific analysis to be conducted. In contrast, Hayashi et al. [16] focused on the ZON of the tap root, enabling nodulation-specific transcripts to be concentrated by removing transcripts found throughout the remaining portion of the root system that are not specifically nodulation related. These studies both identified a number of new nodulation gene candidates. One such candidate, *GmNMNa*, has already been followed up and confirmed to be involved in the regulation of rhizobia infection [112].

Barros de Carvalho et al. [111] also investigated the transcriptome of soybean root tissue; however, these authors focused their study on whole root systems harvested 10 days after rhizobia inoculation. The expression data from these samples showcase genes involved later in the nodulation pathway, including those involved in nodule maturation and growth.

To identify novel components functioning in the AON pathway, Reid et al. [82] used RNA-seq to determine the transcriptome of soybean leaves. Leaf tissue was collected from soybean shoots fed with xylem sap taken from soybean plants that were either nodulating or non-nodulating. This led to the identification of the putative ubiquitin fusion degradation protein, *GmUFD1a*, whose product may interact with GmNARK in the regulation of legume nodulation.

Conclusion

Collectively, the four abovementioned datasets have led to the identification of numerous new gene candidates potentially having roles in the development and regulation of soybean nodules. The confirmation and subsequent functional characterization of these genes aids in the understanding of the signalling mechanisms involved in legume nodulation. Moreover, the identification of critical nodulation genes could one day help to benefit the isolation of superior cultivars for use in agriculture and help to reduce the over-application of nitrogen fertilisers in agriculture.

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