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The Development and Regulation of Soybean Nodules

Brett James Ferguson

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http://dx.doi.org/10. 5772/52573

1. Introduction

1.1. Legumes: Environmentally and agronomically important plant species

Legumes represent the third largest family of angiosperms, with > 18,000 species worldwide (*Leguminosae* or *Fabaceae*). Due to their high nutritional value, they have been cultivated by many cultures for use in agriculture. Indeed, legumes currently represent some of the most important food, feed and fuel crops grown around the world, second only to cereals. They have a global-production value of > \$200 billion per year and are cultivated on 12–15% of the world's available, arable land (Graham and Vance 2003; Peoples et al., 2009; Jensen et al., 2012). Collectively, ~247 million tons of legumes are produced each year, and represent > 25% of the world's primary crop production (Graham and Vance 2003; European Association for Grain Legume Research 2009). Some key legume crop species include: soybean, bean, pea, chickpea, cowpea, pigionpea and alfalfa.

Legumes play a significant role in both agriculture and in natural ecosystems by having a unique influence on the nitrogen cycle. They do so via a highly-specialized symbiotic relationship with soil bacteria commonly called rhizobia. Via a sophisticated signaling exchange, the bacteria infect the plant root and induce the formation of novel organs, termed nodules (Ferguson et al., 2010). The nodule is colonized by the bacteria and provides them with an ideal habitat to convert or 'fix' atmospheric di-nitrogen into other nitrogen-based compounds, such as ammonia, which can be used by the plant. This benefits the plant directly, as nitrogen is essential for growth. It also benefits the rhizosphere as the soil nitrogen content is replenished once the plant dies and subsequently decomposes (referred to as 'green manure'). As a result, legumes are often used in crop rotations in agriculture. In addi-

tion, some legumes are being exploited as a source of sustainable biofuel. Species such as soybean, and the tree legume *Pongamia pinnata*, offer the most promise due to their high seed oil content (Scott et al., 2008).

2. Nitrogen fertilizer: An unsustainable reliance in modern agriculture

Current agriculture practices are heavily reliant on nitrogen-based fertilizers to achieve high yields (Peoples *et al.*, 2009; Jensen et al., 2012). Indeed, it is estimated that half of the world's population is fed as a direct result of nitrogen fertilizer use (Erisman et al., 2008). Although this approach works well to maximize crop production, it is a very risky dependence that is not sustainable, and is inefficient, expensive, and often pollutes. Hence, there are a number of very sound reasons to lessen these nitrogen inputs and alleviate the current reliance.

The production of industrial nitrogen fertilizers requires a great deal of fossil fuel, involving what is known as the Haber-Bosch process. Once manufactured, additional fossil fuel is needed to transport the fertilizer to its place of sale, and then even more fossil fuel is needed to apply it in the field. Collectively, this accounts for a staggering 50% of fossil fuel use in modern agriculture and 5% of the world's annual consumption of natural gas (Crutzen et al., 2007; Canfield et al., 2010). As a direct result, the purchase and application of nitrogen fertilizer can be quite expensive, preventing many farmers in developing regions of the world from being able to use it. Moreover, as the cost of fossil fuel continues to rise, so too does the cost of nitrogen fertilizer. This can considerably cut into farmer profits and often leads to costs being passed on to the consumer.

In addition to the monetary costs associated with nitrogen fertilizer use, there are often considerable environmental costs. It is reported that industrial manipulation of the nitrogen cycle has already exceeded safe boundaries for global environmental change (Rockström et al., 2009; Canfield et al., 2010; Charpentier and Oldroyd 2010; Beatty and Good 2011). Due to the combustion of fossil fuel, the production and application of nitrogen fertilizer results in large quantities of carbon dioxide (CO_2) being released into the atmosphere. This contributes to the greenhouse effect, as does the release of nitrous oxide (N_2O), which is produced during the decomposition of nitrogen fertilizer in the soil and is estimated to be 292 times more active as a greenhouse gas than CO_2 (Crutzen et al., 2007).

Each year >100 million tonnes of nitrogen are applied to crops worldwide (Glass 2003). This excessive use of nitrogen reduces the levels of trace nutrients in the soil and increases soil acidity. Moreover, the application of nitrogen-based fertilizers is a largely inefficient process, with as much as 30 - 50% lost to leaching. This nitrogen run-off can cause significant environmental damage, including the eutrophication of waterways resulting from associated algal blooms, *etc*. (Vance 2001). Recent reports indicate that the cost to fix this environmental damage will significantly outweigh the economic benefit gained from nitrogen fertilizer use (Sutton et al., 2011).

The excess runoff from nitrogen fertilizer can also contaminate drinking water. High levels of nitrogen in drinking water can lead to methemoglobinaemia, commonly referred to as

Blue-baby syndrome because of the blue-grey skin colour displayed by affected infants (Murphy 1991; Knobeloch et al., 2000). Blue-baby syndrome is potentially fatal and occurs when the hemoglobin of an infant's red blood cells is oxidized to methemoglobin, which is unable to properly transport oxygen.

3. Biological nitrogen fixation: A sound alternative to nitrogen fertilizer

Attempts to reduce the use of nitrogen fertilizers need to incorporate responsible, cost effective and environmentally sound options; an improved use of legumes and an increased understanding of legume nodulation represent such options. Indeed, this symbiotic relationship between legumes and rhizobia represents the most important nitrogen-fixation association in the world, with an annual production of approximately 200 million tons of nitrogen (Peoples et al., 2009; Jensen *et al.,* 2012). Optimising this symbiosis can increase crop yields and enhance soil fertility, whilst reducing the negative monetary costs and environmental impacts associated with nitrogen fertilizer use (Hirel et al., 2007; Peoples et al., 2009; Canfield et al., 2010). Hence, increasing our use of legume crops and identifying critical factors required to control nodulation are seen as pivotal steps towards reducing our reliance on nitrogen fertilizers and improving agricultural sustainability (*e. g.* , Giller and Cadisch 1995; Vance 2001; Peoples et al., 2009; Rockstrøm *et al.,* 2009; Canfield et al., 2010; Jensen et al., 2012).

Soybean alone is estimated to produce up to 200 kg N ha^{-1} in aboveground biomass in a single growing season. Of the soybean nitrogen content, 58-68% is estimated to be derived from symbiotic nitrogen fixation (Salvagiotti et al., 2008; Peoples et al., 2009; Jensen et al., 2012). Following harvesting, the remaining portions of the plant, including roots and nodules which represent 30-60% of the nitrogen content, are left to replenish the nitrogen content of the surrounding soil (Mahieu et al., 2007; McNeill and Fillery 2008).

4. Legume nodules: The perfect environment for rhizobia nitrogen fixation

Nodulation is a complex process orchestrated by a multitude of bacteria and plant signals (reviewed in Ferguson and Mathesius 2003; Ferguson et al., 2010). The process is initiated by plant roots secreting flavonoid molecules into the soil. This attracts compatible rhizobia and concomitantly stimulates them to synthesize a highly specific signal molecule called Nod factor. The rhizobia strain that is compatible with soybean is *Bradyrhizobium japonicum*. The plant perceives Nod factor via LysM receptors on the root. In soybean, these receptors are called GmNFR1 and GmNFR5 (Indrasumunar et al., 2010, 2011). Nod factor perception triggers a subsequent signaling cascade that is required for proper nodule establishment. The known legume and rhizobia genes/signals that are involved in this signaling cascade have recently been thoroughly reviewed in Ferguson (2012).

The presence of the rhizobia together with their Nod factor signal molecule initiates the nodulation infection process. Root hair penetration is the most common form of rhizobia invasion. The bacteria attach to emerging root hairs, which begin to deform and eventually encapsulate some of the bacteria, which are continuously dividing (Callaham and Torrey 1981; Turgeon and Bauer 1985). This process happens in as little as 6 – 8 h post-inoculation (Yao and Vincent 1969; Bhuvaneswari et al., 1981; Bhuvaneswari and Solheim 1985; Turgeon and Bauer 1982, 1985). Specialized structures, called infection threads, begin to form and provide a passage way for the bacteria to enter the root (reviewed by Gage 2004). These infection threads are predominately comprised of plant cell wall components and they permit the bacteria to continue proliferating within the host plant.

As the process of rhizobia infection occurs, cortical cells in the root begin to divide and eventually give rise to the nodule primordium (Calvert et al., 1984; Mathews et al., 1989). The position of the nodule primordium is typically adjacent to the radial cells of the xylem, and away from the phloem. This positioning is thought to be largely dependent on plant hormone levels, namely gradients of the gaseous hormone, ethylene (Heidstra et al., 1997; Gresshoff et al., 2009; Lohar *et al.*, 2009). Additional tissues, including vascular tissues and central nodule tissues that are composed of both invaded and non-invaded cells, also develop to form the nodule structure (Newcomb et al., 1979; Calvert et al., 1984; Ferguson and Reid 2005).

Infection threads initiating in the root hair eventually grow and extend towards the dividing nodule primordium located in the root cortex. Once there, rhizobia located at the tip of the infection threads are released into an infection droplet that separates and is released into the cytoplasm of the host cell. Within the cytoplasm, the rhizobia are encapsulated by a specialized plant-derived membrane, known as the peribacteroid membrane, making what is commonly referred to as the symbiosome (Udvardi and Day 1997).

Ultimately, the dividing bacteria differentiate into what are known as bacteroids, which are highly specialized and whose main purpose is to fix atmospheric di-nitrogen gas. Inside the mature nodule, the bacteroids use a nitrogenase enzyme complex to fix the di-nitrogen into forms of nitrogen that the plant can use, such as ammonia. The ammonia, which is toxic to the plant, is then quickly converted into compounds such as glutamate or ureides that are non-toxic and are safely transported throughout the plant. Legume nodules provide the ideal setting for this process as they establish a peripheral oxygen barrier, via physical and metabolic barriers, to create a low-oxygen environment that is essential for nitrogenase activity to occur.

The nodules formed on the roots of soybean plants are referred to as 'determinate' nodules. They are spherical and lack a persistent meristem, unlike indeterminate nodule structures that can form on other legume species, particularly those from temperate growing regions (Ferguson et al., 2010). The life-span of a soybean nodule is typically a few weeks, after which they senesce and are replaced by new nodule structures developing on the growing root system. Following nodule senescence, the bacteroids can re-differentiate and become new inoculum for the soil (Gresshoff and Rolfe 1978).

5. Autoregulation of nodulation: Too much of a good thing can be bad

A number of genes that are required for proper nodule formation have been elucidated (reviewed in Caetano-Anollés and Gresshoff 1991; Ferguson et al., 2010; Ferguson, 2012). The loss of any of these genes typically results in a reduced, or a complete lack of, nodule development. In addition to these positive regulators of nodule formation, there are also a number of external and internal factors that act as negative regulators of nodulation. Mutants unable to synthesise or perceive these factors exhibit increased nodule numbers, often referred to as a hyper- or super-nodulation phenotype (Figure 1). Many of these factors function in the Autoregulation of Nodulation (AON) pathway, which is a mechanism used by the host plant to regulate its nodule numbers (reviewed in Reid et al., 2011a). Indeed, less than 10% of all rhizobia infection events result in the establishment of a fully functional nodule, largely due to AON. By controlling nodule development in this way, the host plant can balance its need to acquire nitrogen against its ability to expend energy establishing and maintaining nodules. Supernodulating plants lacking AON are typically developmentally-stunted (when inoculated with a compatible rhizobia strain) and yield poorly as a result of this balance being disrupted (Figure 2).
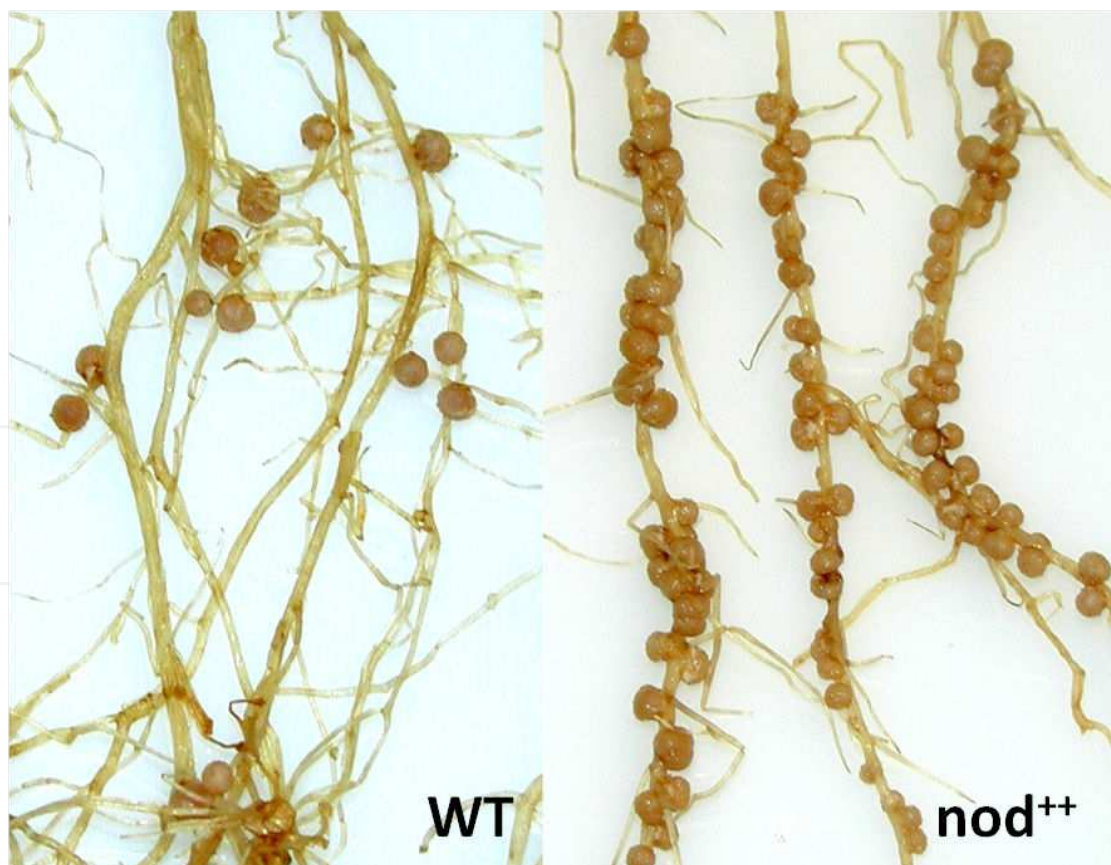
Figure 1. Roots of wild-type (WT) and supernodulating mutant (nod++) soybean plants exhibiting mature nodule structures as a result of a symbiotic interaction with *Bradyrhizobium japonicum*.
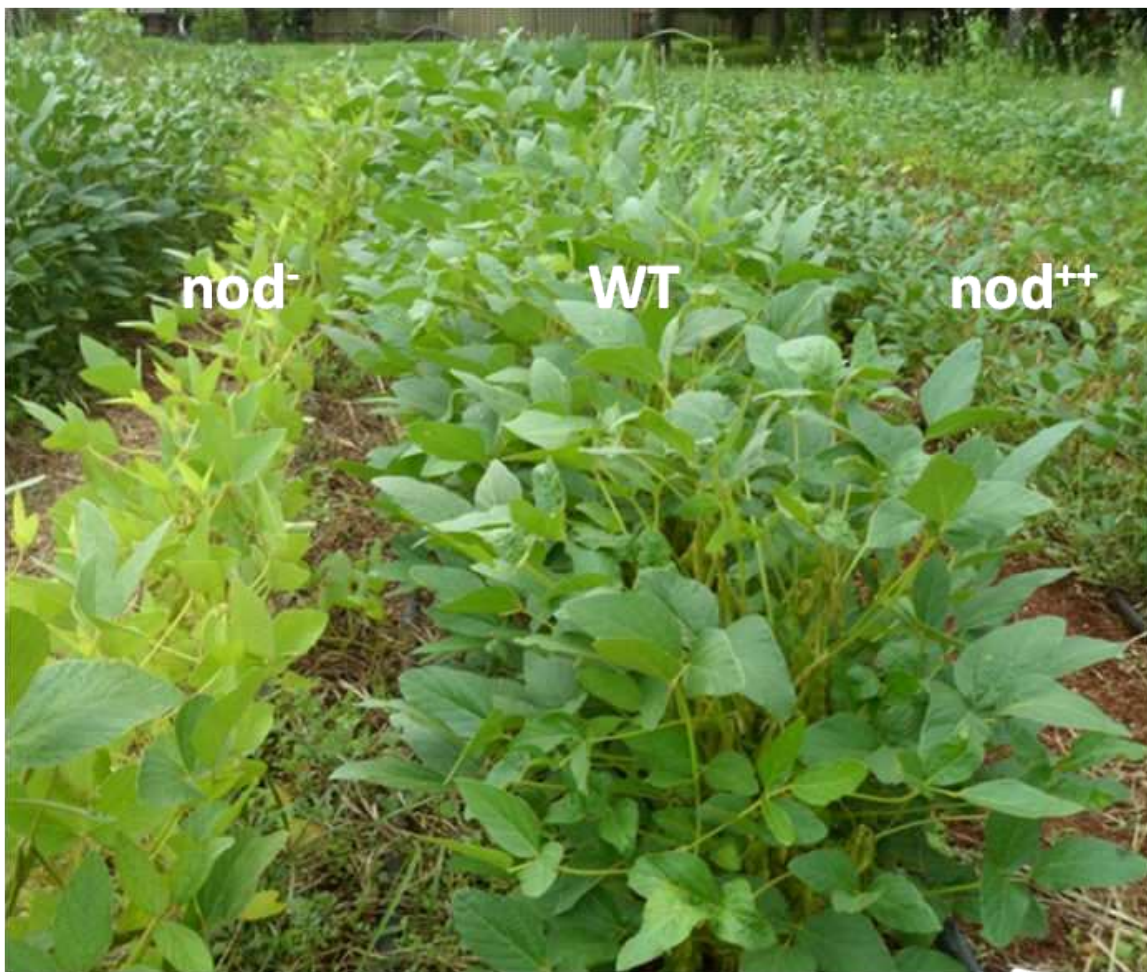
Figure 2. Soybean plants growing in a field in Toowoomba, Queensland, Australia. Mutants unable to form nodules (nod⁻) are stunted and pale compared with wild-type (WT) plants due to their inability to establish a symbiotic interaction with nitrogen-fixing *Bradyrhizobium japonicum*. Supernodulating mutants (nod⁺⁺) are significantly stunted in stature as a result of investing too much energy into forming nodule structures.

The AON pathway involves long-distance root-shoot signaling initiated during nodule development by the synthesis of a root-derived signal (Gresshoff and Delves, 1986; Delves et al, 1986; Reid et al., 2011a). Recent work has indicated that this signal is likely a CLV3/ESR-related (CLE) peptide(s) hormone (Okamoto et al., 2009; Mortier et al., 2010; Reid et al., 2011b; Lim et al., 2011). In soybean, these CLE peptides are called Rhizobia Induced CLE1 (RIC1) and RIC2 (Reid et al 2011b; Lim et al., 2011). Grafting and over-expression experiments have shown that these signals travel to the shoot (Delves et al., 1986; Reid et al., 2011b), likely via the xylem, where they, or a product of their action, are perceived by a LRR receptor kinase, called the Nodulation Autoregulation Receptor Kinase (NARK) in soybean (*e. g.* , Searle et al., 2003). NARK may act in a complex with other receptors, such as CLAVATA2 and KLAVIER (Miyazawa et al., 2010; Krusell et al., 2011). This perception results in the production of a novel Shoot-Derived Inhibitor (SDI). The SDI signal subsequently travels from the shoot back down to the roots, likely via the phloem, where it acts to inhibit further nodulation events (Reid et al 2011a). It has recently been established in soybean that SDI is

small (<1 kDa), Nod factor- and NARK-dependent, heat stable, and is unlikely to be an RNA or a protein (Lin et al., 2010, 2011a). Recent work using soybean has also identified a number of novel components that may interact with NARK directly, or that may function downstream of NARK to regulate the AON process. These include genes identified using site-directed mutagenesis that encode Kinase-Associated Protein Phosphatases, called *GmKAPP1* and *GmKAPP2* (Miyahara et al., 2008), and genes identified using complete transcriptome analyses (RNAseq), such as the putative Ubiquitin Fusion Degradation protein, *GmUFD1a* (Reid et al., 2012).

Additional genes and factors also regulate nodule numbers. Root-specific genes in pea (*PsNOD3*; Postma et al., 1988) and *L. Japonicas* (*LjRDH1*, Ishikawa et al., 2008; *LjTML*, Magori et al., 2009) may have a role in the biosynthesis or translocation of RIC1 and RIC2, or in the perception of SDI. However, the identity of these genes is not yet known. Loss of function of *LjASTRAY*, which encodes a bZIP transcription factor (Nishimura et al., 2002), or *MtEFD*, which encodes an ERF transcription factor (Vernié et al., 2008), also results in increased nodule numbers. Whether these genes function in the AON pathway remains to be determined.

Ethylene and nitrate are also known to inhibit nodule development (Carroll et al., 1985; Lorteau et al., 2001; Ferguson et al., 2005 a,b; Ferguson et al., 2011). Mutations to ethylene sensitivity or response genes, such as *LjETR1* and *LjEIN2/MtEIN2*, result in increased nodule formation (Penmetsa et al., 2008; Gresshoff et al., 2009; Lohar et al., 2009). Interestingly, an additional CLE peptide identified in soybean that negatively regulates nodule development, called Nitrogen-Induced CLE1 (NIC1) is highly similar to RIC1 and RIC2, but is induced by nitrate, not rhizobia (Reid et al., 2011b). Both the RICs and NIC1 appear to be perceived by GmNARK, only the nitrate-induced CLE exhibits little-to-no mobility and is perceived in the root, whereas the rhizobia-induced CLE undergoes long distance transport and is perceived in the shoot.

6. Soybean: A model species for legume research

Soybean has been the subject of a great deal of research in an effort to identify unique traits and to isolate superior cultivars offering improved growth and yields (Gresshoff 2012). It also represents an excellent model species for legumes in general (Ferguson and Gresshoff 2009), with outcomes frequently extrapolated to the other important food and feed legume crops, such as bean, pea, chickpea, faba bean, lentil, peanut, clover and lucerne (*e. g.* , Rispail et al., 2010).

Soybean represents one of the best characterized legumes species, both physiologically and biochemically. It grows quickly, is high yielding, and has a size and stature that are well suited for most field and laboratory studies (Figure 3). Its relatively large size enables the harvest of large quantities of tissues and it is ideal for studies involving grafting (*e. g.* , Delves et al., 1986; Reid et al., 2011b), xylem sap analyses (*e. g.* , Djordjevic et al., 2007), *Agrobacterium rhizogenes*-mediated transformation for gene over-expression and RNA interference (*e. g.* , Kereszt et al., 2007; Reid et al., 2011b; Lin et al., 2011b) and Virus-Induced Gene

Silencing (VIGS) for functional genomics approaches (*e. g.* , Zhang and Ghabrial, 2006). Furthermore, soybean has a large germplasm, including vast mutant (Figure 2; *e. g.* , Carroll et al., 1985; Bolon et al., 2011) and TILLING populations (*e. g.* , Cooper et al., 2008; Batley et al., 2012).

Figure 3. Glasshouse grown soybean plants 1 and 3 weeks after germination. The fast, uniform growth of soybean, together with the availability of its genome sequence and its amenability to most physiological, molecular and biochemical analyses makes it an ideal model species for legume research.

Recently, the soybean genome was sequenced (Schmutz et al., 2010) and complete transcriptome analyses have been performed, including the generation of transcriptome atlases (Libault et al., 2010a,b; Severin et al., 2010; Reid et al., 2012; Hayashi et al., 2012). Together, these resources provide an efficient non-targeted tool to identify new genes and patterns of gene expression. Analyses between the genome of soybean and those available for other legumes species, including *M. truncatula*, *L. japonicus*, pigionpea and bean, also provide an excellent opportunity for comparative legume genomics (Cannon et al., 2009).

Understanding the genes and genomes of legumes will help to establish elite cultivars that benefit sustainable farming practices. Integrating central regulators of nodulation is essential for such targeted legume crop improvement. Indeed, outcomes derived via soybean research could help to underpin future advances in managing the legume-rhizobia symbiosis (*e. g.* , Rispail et al., 2010). This could lead to improved nitrogen use efficiency and reduced nitrogen-fertilizer inputs, thus helping to reduce the monetary and environmental costs associated with nitrogen-fertilizer use.

7. Conclusions and future perspectives

The processes of nodulation and nitrogen fixation have long been recognized for their agricultural benefits and ability to improve soil-health. Recent work identifying the genes and signals involved in nodule formation and AON have significantly enhanced our understanding of the processes and could provide targets for plant breeding and engineering programs aiming to develop legume, and perhaps even non-legume, varieties with an improved ability to acquire nitrogen.

Abbreviations

AON (Autoregulation Of Nodulation);

CLE (CLV3/ESR-related peptide);

NARK (Nodulation Autoregulation Receptor Kinase);

NIC (Nitrogen-Induced CLE peptide);

Nod Factor (Nodulation Factor);

RIC (Rhizobia-Induced CLE peptide);

RNAseq (RNA sequencing);

SDI (Shoot Derived Inhibitor)

Acknowledgements

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