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Plant Genetic Control of Nodulation and its Utilization in Nitrogen Fixation - A Review

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A B S T R A C T

Nitrogen is one of the most important major limiting nutrients for most crops and other plant species. Nitrogen fertilizers affect the balance of the global nitrogen cycle, pollute groundwater and increase atmospheric nitrous oxide (N_2O), a potent "greenhouse" gas. The production of nitrogen fertilizer by industrial nitrogen fixation not only depletes our finite reserves of fossil fuels but also generates large quantities of carbon dioxide, contributing to global warming. The process of biological nitrogen fixation offers an economically attractive and ecologically sound means of reducing external nitrogen input and improving the quality and quantity of internal resources. Biological Nitrogen Fixation (BNF) is an ecologically important phenomenon that can support an amount of nitrogen to compensate for the deficiencies of this element and legumes are mostly involved in the BNF process. Legumes can form a symbiotic relationship with nitrogen-fixing soil bacteria called rhizobia. The result of this symbiosis is to form nodules on the plant root, within which the bacteria can convert atmospheric nitrogen into ammonia that can be used by the plant. The establishment of a successful symbiosis requires the two symbiotic partners to be compatible with each other throughout the process of symbiotic development. However, incompatibility frequently occurs, such that a bacterial strain is unable to nodulate a particular host plant or form nodules that are incapable of fixing nitrogen. Genetic and molecular mechanisms that regulate symbiotic specificity are diverse, involving a wide range of host and bacterial genes signals with various modes of action. More work is needed on the genes responsible for rhizobia and legumes, the structural chemical bases of rhizobia legume communication, and signal transduction pathways responsible for the symbiosis-specific genes involved in nodule development and nitrogen fixation.

Introduction

Nitrogen fertilizers today are an indispensable part of modern agricultural practices and rank first among the external inputs to maximize output in agriculture. There is now little doubt that the world will face severe food shortages in the not too distant future, in part due to excessive population growth and negative environmental impacts associated with the increase of population. Thus, emphasis should be laid on developing new production methods that are sustainable both agronomically and economically. Biological

Nitrogen Fixation (BNF) is an ecologically important phenomenon that can support an amount of nitrogen to compensate for the deficiencies of this element. It can act as a renewable and environmentally sustainable source of nitrogen and can complement or replace fertilizer inputs (Peoples *et al*., 1995). BNF is a kind of beneficial plant-microbe (legume-rhizobia) interaction that provides a restricted range of plants with the often-limiting macronutrient-nitrogen. The legume-rhizobial symbiosis starts with a signal exchange between the host plant and its micro-symbiont (Oldroyd, 2013).The symbiosis of rhizobium and its host requires recognition of the bacteria and the plant root.

The rhizobium bacteria associate with the host's epidermal root hairs, and usually penetrate by deformation of the hair and subsequent formation of a specialized invasion structure, the "infection thread." Mitosis and cell growth in the plant root cortex lead to the formation of a root nodule, in which bacteria infect host cells and differentiate into "bacteroids" that fix nitrogen. This is of considerable physiological benefit to the host plant in nitrogen-limited conditions. The most studied nodules are of two types: indeterminate, generally elicited on temperate legumes, such as *Medicagosativa*, *Viciahirsuta*, and *Pisum* sativum; and *determinate*, generally found on tropical legumes, such as *Glycine max*, *Lotus japonicus* and *Phaseolus vulgaris*, the type and size being determined by the host plant (Rhijn and Vanderleyden, 1995).

Medicago truncatula and *L. japonicus* are being used as a model system to study indeterminate-type and determinate-type nodules, respectively (Stougaard, 2001). This type of symbiosis evolved some 60 million years ago and is an archetypal example of a monospecific association (Hirsch,2004).In agricultural settings, perhaps 80% of this biologically fixed N_2 comes from symbiosis involving leguminous plants and α-proteo bacteria, order Rhizobiales, family Rhizobiaceae, including species of *Rhizobium, Bradyrhizobium, Sinorhizobium, Azorhizobium* and *Mesorhizobium* (Farrand *et al*., 2003). Recently, it has been shown that β-proteo bacteria may also participate in this kind of relationship (Sawada *et al.,* 2003). Knowledge of the genetic basis of symbiotic specificity is important for developing tools for genetic manipulation of the host or bacteria in order to enhance nitrogen fixation efficiency. In this review article, we also highlight the discovering of new symbiotic genes, their roles in nitrogen fixation and symbiotic nitrogen fixation in cereals and other non-legume crops. Our main target in this review is the genetic mechanism involved in the nodulation process and its role in symbiotic fixing nitrogen.

Structure and function of flavonoids and the flavonoid-nodD recognition

Flavonoids are secondary metabolic products of the central phenyl propanoid pathway and the acetate- malonate pathway of plants. They are polycyclic aromatic compounds, released by plants into the rhizosphere (Barbour *et al.,* 1991; Kape *et al.,* 1991). These are 2-phenyl-1,4-benzopyrone derivatives. Their structure is defined by two aromatic rings, A, B and a heterocyclic pyran or pyrone ring the C ring. Specific modifications of this basic structure produce different classes of flavonoids including chalcones, flavanones, flavones, flavonols, isoflavonoids, coumestans, and antho cyanidins (Harborne and Williams, 2000). So far more than 4000 different flavonoids have been identified in vascular plants (Perret *et al.,* 2000). Not all of them, however, are active as inducers of the nodulation genes. A comparison of the structure of different nod-inducing flavonoids revealed that hydroxylation at the C-7 and C-

4 positions are important for nod-inducing activity (Cunningham *et al.,* 1991). Host legumes are thought to be discriminated from non-hosts partly based on the specific flavonoids that they release (Parniske and Downie, 2003).Under nitrogen-limiting conditions, legume roots secrete a cocktail of flavonoid compounds into the rhizosphere, and they serve to activate the expression of a group of bacterial nodulation (nod) genes, leading to the synthes is of the Nod factor, a lipochitooligosaccharidic signal that is essential for initiating symbiotic development in most legumes (Oldroyd *et al.,* 2011). Induction of nod gene expression is mediated by the flavonoid activated NodD proteins, which are LysR-type transcription regulators (Long, 1996). NodDs activate nod gene expression through binding to the conserved DNA motifs (nod boxes) upstream of the nod operons (Fisher *et al.,* 1988). NodD proteins from different rhizobia are adapted to recognizing different flavonoids secreted by different legumes, and this recognition specificity defines an early checkpoint of the symbiosis (Peck *et al.,* 2006). Despite the absence of direct evidence for physical interaction between the two molecules, flavonoids can stimulate the binding of NodD to nod gene promoters in Sinorhizobium meliloti (Peck *et al.,* 2006). It is well documented that inter-strain exchange of nodD genes can alter the response of the recipient strain to a different set of flavonoid inducers and hence the host range (Perret *et al.,* 2000).

The evidence for the importance of flavonoids in determining the host range primarily comes from bacterial genetics, and the plant genes involved are less studied. Since legume roots secrete a complex mixture of flavonoid compounds, it is difficult to find out which flavonoids play a more critical role, and when and where they are produced. Recent studies in soybeans and the *Medicago truncatula* have highlighted key flavonoids required for rhizobial infection (reviewed in Liu and Murray, 2016). These so-called "infection flavonoids" are strong inducers of nod genes, secreted by roots, highly accumulated at the infection sites, and show increased biosynthesis in response to infection by compatible rhizobia. Although luteolin was the first flavonoid identified that can induce nod gene expression across a wide range of rhizobial strains, it is not legume-specific, mainly produced in germinating seeds, and has not been detected in root exudates or nodules. In contrast, methoxychalconeis one of the strong host infection signals from Medicago and closely related legumes that form indeterminate nodules, while genistein and daidzein are crucial signals from soybeans that form determinate nodules. Part of the flavonoid compounds may also function as phytoalexins, acting to reinforce symbiosis specificity (Liu and Murray, 2016). For example, *Bradyrhizobium japonicum* and *Mesorhizobium loti*, but not the Medicagosymbiont S.meliloti, are susceptible to the flavonoid medicarpin produced by *Medicago spp*. (Breakspear *et al.,* 2014), and the soybean symbionts *B.japonicum* and *Sinorhizobiumfredii* are resistant to glyceoll in when exposed to genistein and daidzein (Parniske *etal.,*1991).

Function of nod-factor

The key event in nodule formation is the synthesis and release by the bacteria of small molecules that are detected by the plant and that trigger the formation of the nodule. These molecules are called Nod factors. Detection of Nod factors by a legume host induces major developmental changes in the plant, which are required for entry of the rhizobia into the host (Geurts and Bisseling, 2002). The tip of a root hair, to which rhizobia are bound, curls back on itself, trapping the bacteria within a pocket, from which they are taken up into a

plant made intra cellular infection thread. Nod factors also induce cell division and gene expression in the root cortex and pericycle, where they initiate the development of the nodule (Cullimore *et al*., 2001). The structure of Nod factors was first determined in 1990 for *Sinorhizobium meliloti* (Lerouge *et al.*, 1990). Nod factors usually comprise four or five β-1-4-linked N-acetyl glucosamine residues with a long acyl chain that is attached to the terminal glucosamine. Many Nod factors from different rhizobia species have been identified and shown to differ concerning the number of glucosamine residues, the length and saturation of acylchain and the nature of modifications on this basic backbone (Denarie *et al.,* 1996; Downie, 1998). These host specific modifications include the addition of sulphuryl, methyl, carbamoyl, acetyl, fucosyl, arabinose and other groups to different positions on the backbone, as well as differences in the structure of the acyl chain. These variations define much of the species specificity that are observed in the symbiosis (Perret *et al.,* 2000). Proteins encoded by bacterial genes nodA, nodB, and nodC are involved in the biosynthesis of the basic Lithium Cobalt Oxide(LCO) structure (Brencic and Winans, 2005). Many different nod genes are involved in modifying the basic LCO structure specifically for different rhizobia. For instance, nodH encodes a sulfotransferase that transfers a sulfate group to the reducing end of Nod factors of *Rhizobium meliloti* (Ehrhardt *et al.,* 1995).

Perception of rhizobial exo polysaccharides

The exo polysaccharides have been studied in detail by a large number of rhizobial strains (*Sinorhizobium meliloti)*; two types of ESP forms could be discriminated against, ESP as succino-glucan and ESPII with thousands of saccharide units and a low molecular weight class with 8 to 40 saccharide units. All genes involved in the biosynthesis of repeating units have been identified. Exo polysaccharides play a major role in the primary stage of the infection of the host plant. These surface components are proposed to be able to suppress plant defense, but their active roles in promoting bacterial infection and nodulation remain elusive and are dependent on the specific interactions studied. Exo polysaccharides are required for rhizobial infection in multiple symbiotic interactions. This has been best illustrated in the Sinorhizobium-medicago symbiosis, in which succino-glycan, a major EPS produced by S. meliloti, is required for the initiation and elongation of infection threads, and increased succino-glycan production enhances nodulation capacity (Jones, 2012). However, the symbiotic role of EPS is very complicated in the Mesorhizobium-Lotus interaction (Kelly *et al.,* 2013).

For instance, a subset of EPS mutants of M. loti R7A displayed severe nodulation deficiencies on L. japonicus and L. corniculatus, whereas other mutants formed effective nodules (Kelly *et al.,* 2013). In particular, R7A mutants deficient in the production of an acidic octasaccharide EPS were able to normally nodulate L. japonicus, while ExoU mutants producing a truncated penta saccharide EPS failed to invade the host. It was proposed that full-length EPS serves as a signal to compatible hosts to modulate plant defense responses and allow bacterial infection, and R7A mutants that make no EPS could avoid or suppress the plant surveillance system and therefore retain the ability to form nodules. In contrast, strains that produce modified or truncated EPS trigger plant defense responses resulting in a block of infection (Kelly *et al.,* 2013). EPS production is common in rhizobial bacteria, and the composition of EPS produced by different species varies widely (Skorupska *et al.,* 2006). Several studies have suggested the

involvement of the EPS structures in determining infective specificity (Kelly *et al.,* 2013). Recently, an EPS receptor (EPR3) has been identified in L. japonicus, which is a cell surface-localized protein containing three extracellular LysM domains and an intracellular kinase domain (Kawaharada *et al.,* 2015). EPR3 binds rhizobial EPS in a structurally specific manner. Interestingly, Epr3 gene expression is contingent on Nod-factor signaling, suggesting that the bacterial entry to the host is controlled by two successive steps of receptor-mediated recognition of Nod factor and EPS signals (Kawaharada *et al.,* 2015, 2017). The receptor-ligand interaction supports the notion that EPS recognition plays a role in the regulation of symbiosis specificity.

However, natural variation in host-range specificity that results from specific recognition between host receptors and strain-specific EPS has not been demonstrated in any legume-rhizobial interactions. It is noteworthy that acidic EPS of bacterial pathogens also promotes infection to cause plant disease (Beattie, 2011). Thus, rhizobial EPS might also be recognized by host immune receptors to induce defense responses that negatively regulate symbiosis development.

Specificity mediated by host innate immunity

Symbiotic and pathogenic bacteria often produce similar signalling molecules to facilitate their invasion of the host (Deakin and Broughton, 2009). These molecules include conserved microbe-associated molecular patterns (MAMPs) and secreted effectors (Okazaki *et al.,* 2013). The host has evolved recognition mechanisms to distinguish between, and respond differently to pathogens and symbionts (Bozsoki *et al.,* 2017; Zipfel and Oldroyd, 2017). However, this discrimination is not always successful; as a result, recognition specificity frequently occurs in both pathogenic and symbiotic interactions. In the legume-rhizobial interaction, effect or MAMP - triggered plant immunity mediated by host receptors also plays an important role in regulating the host range of rhizobia (Tang *et.al.,* 2016). Several dominant genes have been cloned in soybeans (e.g., Rj2, Rfg1, and Rj4) that restrict nodulation by specific rhizobial strains. In these cases, restriction of nodulation is controlled similarly as 'gene-for-gene' resistance against plant pathogens. Rj2 and Rfg1 are allelic genes that encode a typical TIRNBS-LRR resistance protein conferring resistance to multiple *Bradyrhizobium japonicum* and *Sinorhizobium fredii* strains (Fan *et al.,* 2017). Rj4 encodes a thaumatin-like defense-related protein that restricts nodulation by specific strains of *Bradyrhizobium elkanii* (Tang *et al.,* 2016).

The function of these genes is dependent on the bacterial type III secretion system and its secreted effectors (Tsurumaru *et al.,* 2015; Tang *et al.,* 2016; Yasuda *et al.,* 2016). These studies indicate an important role of effector-triggered immunity in the regulation of nodulation specificity in soybeans. As discussed earlier, rhizobial Nod factors and surface polysaccharides could play a role in suppression of defense responses (Cao *et al.,* 2017), but these signaling events are not strong enough to evade effector-trigged immunity in incompatible interactions. Many rhizobial bacteria use the type III secretion system to deliver effectors into host cells to promote infection, and in certain situations, the delivered effector(s) are required for Nod-factor independent nodulation as demonstrated in the soybean-*Bradyrhizobiu melkanii* symbiosis (Okazaki *et al.,* 2013, 2016). On the other hand, however, recognition of the effectors by host resistance genes triggers immune response store strict

rhizobial infection. The nodulation resistance genes occur frequently in natural populations, raising a question of why hosts evolve and maintain such seemingly unfavourable alleles. This could happen because of balancing selection, as the same alleles may also contribute to disease resistance against pathogens, considering that some rhizobial effectors are homologous to those secreted by bacterial pathogens (Kambara *et al.*, 2009). Alternatively, legume may take advantage of Rgenes to exclude nodulation with less efficient nitrogen-fixing strains and selectively interact with strains with high nitrogen fixation efficiency, which is the case of the soybean Rj4allele. A single dominant locus, called NS1 was also identified in the *Medicago truncatula* that restricts nodulation by *S.melilotis* train Rm41 (Liu *et al.*, 2014). Unlike R gene-controlled host specificity in soybeans, which depends on bacterial type III secretion system, Rm41 strain lacks genes encoding such a system. It will be interesting to know what the host gene (s) controls this specificity and what bacterial signals are involved.

Genes involved in nodulation process

The first class involves genes whose protein products biosynthesize, modify, or transport the lipo-chitin nodulation signal. The lipo-chitin Nod signal is essential for nodulation and is the bacterial signal that triggers *de novo* organogenesis of the root nodule, which is intracellularly colonized by the bacterial symbiont. Core synthesis of the Nod signal involves the products of the *nodABCMFE* genes.

The products of the *nodIJ* genes have been implicated in the transport of the Nod signal to the exterior of the bacterial cell. NodT is a bacterial outer membrane protein. NodO is excreted and probably acts by inserting itself into the plant membrane. Some of the *nod* genes have counterparts involved in normal bacterial metabolism, e.g., *nodM* encoding glucosamine synthase, which is an ortholog of *glmS*. The only *nodM* is co-regulated with the other nodulation genes. The other nodulation genes in this first-class carry out a variety of biochemical reactions that modify the chemistry of the core Nod signal structure. These chemical modifications are important since they determine the host specificity of the signal. It should be stressed that not all of the *nod* genes listed in Table 1 are found in a single rhizobium. The specific complement of genes in an organism helps determine its host range.

Nitrogen fixation process and nif genes:

Environmental symbiotic nitrogen requires the coordinated interaction of two major classes of genes present in rhizobia, the nif genes and fix genes. The nif genes have structural and functional-relatedness to the N_2 fixation genes found in *Klebsiella* pneumonia. The structural nif genes from taxonomically diverse microbes are nearly identical and function in a similar manner to encode nitrogenase. A majority of the nif genes are plasmid-borne in the rhizobia but are located on chromosome in the Bradyrhizobium. Nitrogen fixation in symbionts and free-living microbes is catalyzed by nitrogenase, an enzyme complex encoded nifDK and nifH genes. Nitrogenase itself consists of a molybdenum-iron protein (MoFe), subunit I and an iron-containing protein (Fe) subunit II. The MoFeProtein subunits are encoded by nifK and nifDand a FeMo cofactor (FeMo-Coo) is required for activation of the MoFe protein. This is assembled from nifB, V, N and knife genes. The Fe subunit protein is encoded by the nifH gene. The organization and complexity of nif genes are organized in about 8 operons. In most systems, however, the regulation of all nif genes is controlled by

NifA (a positive activator of transcription) and NifL (the negative regular). Environmentally, nif gene expression is regulated by both oxygen and nitrogen levels. For example, elevated soil ammonia (NH_3 or NH_4) concentration allows NifL to act as a negative controller of gene expression by preventing NifA to act as an activator. Besides, elevated O_2 concentrations inhibit FixJ, which in turn prevents increases in nifA.

Since nifA is the transcriptional activator of the other nif genes elevated O_2 results in a net decrease in the synthesis of nitrogenase and a decrease in, or abolition of symbiotic N_2 fixation. In addition to nif genes, many other microbial genes are involved in symbiotic nitrogen fixation, these collectively referred to as fix genes. Moreover, several other genes have been reviewed that they play a direct or indirect role in nitrogen fixation such as exo polysaccharide, hydrogen uptake, glutamine synthase, dicarboxylate transport, nodulation efficiency, B-1,2 Glucans, and lipopolysaccharides. Different kinds of nif genes that have been identified and their functions are listed in (Table 2) and published by Klipp and co-workers (2014).

Other genes involved in nodulation and nitrogen fixation

A large number of bacterial genes that are playing a role in the formation of nodules on leguminous plants have been identified. Lately, there are more than 65 nodulation genes have been identified in rhizobia, each strain can carry one or more of these genes. Several investigators explained the possible function of the common genes involved in the nodulation process. There are different types of *nod* genes designated as nodA, nodB, and nodC. Collectively, they are responsible for the biosynthesis of the chitin backbone while nod is a regulator gene that activates the transcription of other inducible nod gens. Different kinds of other nodulation and nitrogen fixation genes that have been identified and their functions are listed in (Table 2) and published by Sadowsky and co-workers (2012).

Background to symbiotic nitrogen fixation in cereals

The introduction of symbiotic biological nitrogen fixation into cereals and other major non-legume crops would be regarded as one of the most significant contributions that biotechnology could make to agriculture. However, this has been recognized for many years as a major research challenge (Conway and Doubly, 1997.) Currently, there are two strategic approaches used in attempts to achieve this long-standing aspiration. One is a long-term synthetic biology GM approach, engineering a nitrogen-fixing symbiosis from existing signaling and developmental mechanisms, to provide a suitable environment for rhizobial nitrogen as activity in the plant nodule (GED and Dixon, 2014).

The other, much shorter term and simpler approach builds on the discovery that a non-rhizobial, naturally occurring nitrogen-fixing bacterium that fixes nitrogen in sugarcane can intracellularly colonize the root systems of cereals and other major crops (Cocking *et al.*, 2006). In this approach, which is now at a field trial evaluation stage (Dent and Cocking in preparation), an adequate level of bacterial intracellular colonization and nitrogen fixation can be established throughout the plant without any need for nodulation. In such symbiotic nitrogen fixation, nitrogen-fixing bacteria establish an intracellular symbiosis with plants in which they fix nitrogen inside the cells of their host utilizing energy supplied by plant photosynthesis.

Table.1 Proposed functions of the known nodulation (*nod*, *nol*, *noe*) genes

Gene	Proposed function
Regulatory genes	
***nodD*$_1$**	Transcriptional activator
***nodD*$_{2,3}$**	Transcriptional regulator
nodV	Two-component regulator
nodW	Two-component regulator
nolA	Transcriptional regulator
nolR	Transcriptional repressor
syrM	Transcriptional regulator
Nod signal core synthesis	
nodA	Acetyl transferase
nodB	Deacetylase
nodC	Chitin synthase
nodM	D-glucosamine synthase
Node	β-Ketoacyl synthase
nodF	Acyl carrier protein
Nod signal modifications	
nodG	3-oxa acyl-acyl carrier protein reductase
nodH	Sulfo transferase
nodL	Acetyl transferase
nodS	Methyl transferase
nodU	Carbamoyl transferase
nodP	ATP-sulfurylase subunit
nodQ	ATP-sulfurylase subunit/APS kinase
nodX	Acetyl transferase
nodZ	Fucosyl transferase
nolK	NAD-dependent sugar epimerase
nolL	*O*-acetyltransferase activity
nolO	Carbamoyl transferase
nolXWBTUV	Cultivar-specific nodulation
nolYZ	Unknown
noeC	Arabinosylation
noeD	Genotype-specific nodulation
noeE	Sulfo transferase
noeI	2-*O*-methylation
noeJ	Phosphate guanylyl transferase
noeK	Phosphomannomutase
noeL	Dehydratase
Nod signal transport	
nodI	ATP-binding protein
nodJ	Integral membrane protein
nodT	Outer membrane protein
nodO	Calcium-binding, a pore-forming protein

(Source: Stacey *et al*., 2001)

Table.2 *nif* genes products and their role in Nitrogen fixation

Nif genes	Role in Nitrogen fixation
NifH	Dinitrogenase reductase
nifD	α-subunit of dinitrogenase
nifK	B subunits of dinitrogenase. B clusters are present at B subunit-interface
nifT	In *Klebsiellapneumoniae*, aids in the insertion of FeMo-co into apo dinitrogenase.
nifY	Unknown
nifE	Forms a_2B_2 tetramer with nifN. Required for FeMo-cosynthesis.
nifN	Required for FeMo-cosynthesis.
nifx	Involved in FeMo-cosynthesis.
nifx	Involved in the mobilization of Fe-S cluster synthesis andrepair.
nifU	Involved in the mobilization of S for Fe-S cluster synthesis andrepair.
nifV	Homocitrate synthesis involved in FeMo-cosynthesis.
nifW	Involved instability of dinitrogenase. Proposed to protect dinitrogenase from O_2inactivation.
nifZ	Unknown
nifM	Required for the maturation of *nifH*.
nifF	Flavodoxin, Physio logic electrondonorto*nifH*.
nifA	Positive regulatory element.
nifB	Required FeMo-co synthesis. Metabolic product. NifB-co is the specific Fe and S donor toFeMo-co.
fdxN	Ferredoxin serves as electron donor to nitrogenase.
nifQ	Involved in FeMo-co synthesis. Proposed to function in early $MoO4^2$processing
nifJ	Pyruvate flavodoxin (ferredoxin) oxido reductase involved in electron transport to nitrogenase.

(Source: Klipp *et al*., (2004)

Table.3 Different genes involved in BNF (Sadowsky *et al.*, 2012)

Gene code	Function	References
hsn	Host specificity nodulation	**Horvath *et al*., 1986**
gsn	Genotypic specific nodulation	**Sadowsky *et al*., 1991**
exo	Exo polysaccharides	**Becker and Puhler 1998**
hup	Hydrogen uptake	**Maier 1986**
gln	Glutamine synthase	**Carlson *et al*., 1987**
dct	Dicarboxylate transport	**Finan *et al*., 1983**
nfe	Nodulation formation efficiency	**Sanjuan and Olivares 1989**
ndv	ß, 1,2 Glucans	**Breedveld and Miller 1998**
lps	Lipopolysaccharide	**Carlson *et al*., 1987**
bacA	Bacteroid development	**Glazebook *et al*., 1993**
tts	Type III secretion system	**Krause *et al*., 2002**
virB	Type IV secretion system	**Hubber *et al*., 2004**
acds, rtx	Inhibition of plant ethylene biosynthesis	**Ma *et al*., 2003**
pur	Purine biosynthesis	**Giraud *et al*., 2007**
rosR	Cell surface and competitiveness	**Bittinger and Handelsamn 2000**
iol	Inositol catabolism (competitivness)	**Kohler *et al*., 2010**
tfx	Trifolitoxin (competitivness)	**Robleto *et al*., 1998**
moc	Rhizopine catabolism (competitiveness)	**Murphy *et al*., 1995**
enod1, enod12 and enod40	Nodulin genes	**Van de Sande *et al*., 1997**
lectin	Interact with LCos	**Berwin and Kardailsky 1997**
Rj2* and *Rfg1	Responsible for host specificity with legumes	**Yang *et al*., 2012**
KnOx	Cytokinin hormone plays an important role in symbiotic nodule development and nodule organogenesis.	**Ariel *et al*., 2012**
ACC	Aminocyclopropane 1-carboxylate deaminase plays viotal role in ACC deaminase activity in legume-Rhizobium symbiosis and nodule senescence.	**Nukui *et al*., 2006**
ESN1	Contribute in nodule senescence and symbiotic nitrogen fixation	**Xi *et al*., 2013**

LHK1	Coding for Lotus Histidine Kinase this is important in nodule initiation and primordium	**Suzaki *et al*., 2013**
Cre1	Contribute in nodule formation, mutant strain of this gene cannot form nodules because it is defective in cytokinin response.	**Gonzalez-Rizzo *et al*., 2006**
MtNIN	MtNIN functions downstream of the early NF signaling pathway to coordinate and regulate the correct temporal and spatial formation of root nodules.	**Marsh *et al*., 2007**
CLE	CLE-RS glycopeptides are the long sought mobile signals responsible for the initial step of autoregulation of nodulation.	**Okamoto *et al*., 2013**
CelC2	development of the canonical nitrogen-fixing *R. leguminosarum*sv. *trifolii*-white clover symbiosis	**Robeldo *et al*., 2008**
nap and nos	**Genes involved in nitrate reductase plays vital role in nodule regulation**	**Sanchez *et al*., 2013**

(Source: Sadowsky *et al.*, 2012

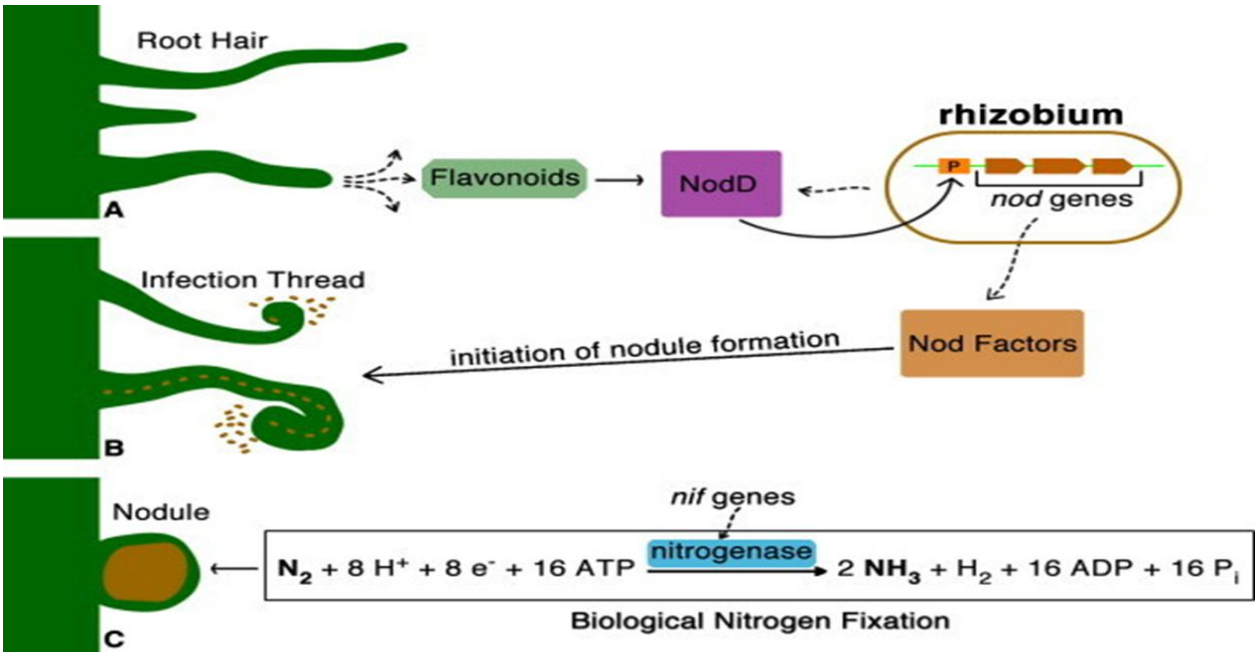

Fig.1 Schematic overview of the nodulation process and biological nitrogen fixation Laronja *et al.*, 2013

Legumes form novel plant organs, the "root nodules", in response to lipo-oligosaccharide signals, "Nod factors", delivered by specific soil bacteria called rhizobia. The adoption of model legumes for genetic analysis of nodulation has led to major advances in our understanding of initial steps in Nod signal recognition and subsequent signaling, however, a complete picture of the genetic interplay involved in rhizobial symbiosis is yet to appear. There are still several genes, with a role in Nod-factor signal transduction that remains to be cloned. Detangling of this system (Legume-Rhizobium symbiosis) would help in a better understanding of the molecular mechanisms governing nodule differentiation.

With a complete understanding of early signaling pathways, quest like which genes are responsible for nodule formation and which genes are missing from crop plants such as wheat and rice that do not form endosymbiosis with nitrogen-fixing bacteria will be answered. Recent studies have just begun to reveal the underlying molecular mechanisms that regulate this specificity, and many challenging questions are waiting to be answered. Effector-triggered immunity is an important factor in determining the host range of rhizobia in soybeans but the cognate effectors have not been defined. The need for an improved means of delivering nitrogen to cereals and other non-legume crops is crucial for the future of sustainable agriculture, including the reduction of ammonia, nitrate, and nitrous oxide pollution but ensuring food security (Lynas 2011). The promise of biological nitrogen fixation for cereals proffered in the 1980s may now be realized, however, not as originally envisaged through rhizobial association with genetically manipulated root nodules on wheat for instance, but rather through the appropriate application of the naturally occurring nitrogen-fixing endophyte Gd to the plant using products, such as NFix®. The development of a nitrogen-fixing endophytic bacterium that can be applied to all staple food crops and substitute for mineral nitrogen fertilizers, whilst delivering yield benefits, is a significant major development and heralds the prospect of a Greener Nitrogen Revolution.

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