

GENES AND MOLECULAR SIGNALS INVOLVED IN LEGUMES-RHIZOBIA SYMBIOSIS

Omar FARSSI^{1&2}, Mohammed MOURADI¹, Cherki GHOUAM³, Abdelaziz BOUIZGAREN⁴, Hicham BERROUGUI², Mohamed FARISSI^{1*}

¹Laboratory of Biotechnology and Sustainable Development of Natural Resources “B2DRN”, Polydisciplinary Faculty, Mghila PO Box: 592 Beni-Mellal, Morocco.

²Polyvalent Laboratory on Research & Development, Polydisciplinary Faculty, Beni-Mellal, Morocco.

³Unit of Plant Biotechnology and Symbiosis Agro-physiology, Faculty of Sciences and Techniques, Marrakesh, Morocco.

⁴Unit of Plant Breeding, National Institute for Agronomic Research (INRA), Marrakesh, Morocco.

*Corresponding author e-mail: farissimohamed@gmail.com / m.farissi@usms.ma

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ABSTRACT

Legumes are plants able to engage in root nodule symbiosis with nitrogen-fixing soil bacteria, called rhizobia. This symbiotic interaction is highly specific, such that each rhizobial species interacts with only a specific group of legumes, and vice versa. The rhizobia and the host plant develop a dialogue system based on an exchange of chemical molecules. Many studies reported that the flavonoids, in combination with the rhizobial NodD transcriptional activator, stimulate the expression of the genes required in the perception and signal transduction of Nod factors and the genes of nodule formation. This review seeks to highlight the genes and molecular signals involved in legume-rhizobia symbiosis. Recent knowledge leading to bacterial access and nodule formation was focused and discussed.

KEY WORDS: *legumes; rhizobia; nod factors, genes, signal transduction.*

Legumes are an important source of protein and vegetable oils. They are widely cultivated throughout the world (Graham & Vance, 2003). In Mediterranean area, these plants have an important position due to their agro-economic and environmental interests. Indeed, leguminous plants have a positive effect on soil fertility by contributing to the incorporation of nitrogen in ecosystems offering thus beneficial, ecological and economical impacts, helping to reduce or limit the use of chemical fertilizers by nitrogen-fixing symbiosis involving rhizobial strains (Farissi *et al.*, 2013; Latrach *et al.*, 2014).

One striking feature of the legume-rhizobia symbiosis is its high level of specificity. Such specificity can occur both at the early stages associated with bacterial infection and nodule development as well as at the late stages related to nitrogen (Graham & Vance, 2003; Wang *et al.*, 2012). Therefore, symbiotic specificity has long constituted a crucial research topic in the scientific community (Ilanovici, 2010).

The most important strategies employed in the last few years to maximize and improve the symbiotic nitrogen fixation, particularly under the stressful conditions, have been focused on a selection of legume-rhizobia symbiotic combinations efficient for symbiotic nitrogen fixation

(SNF). However, the understanding of molecular signals involving in the perception and signal transduction of Nod factors and the roles of genes required for nodule formation and consequently the proteins of NOD signaling pathways constitute the first step for all programs regarding the SNF improvement. In this context, the present work constitutes a review discussing the recent knowledge focusing on legumes-rhizobia symbiosis. Much information related to the molecular dialogue leading to bacterial entry and nodule formation was developed.

OVERVIEW ON LEGUMES

Fabaceae or legumes are classified as angiosperms, Eudicot. They are sisters of *Polygalaceae*, constituting with the families of *Quillajaceae* and *Surianaceae* the Fabales (Judd *et al.*, 2002). *Fabaceae* is the third largest family of flowering plants in terms of number of species (after *Orchidaceae* and *Asteraceae*), with 727 genera and nearly 20 000 species (Cronk *et al.*, 2006). The species range from arctic dwarf grass and mountains to immense trees of the tropical forests (Judd *et al.*, 2002). The tree forms predominate in hot countries and the herbaceous forms in temperate regions (Guignard & Dupont, 2005). They are extremely diverse, but they have one thing in common, their fruit is a pod (Caratini, 1984).

Based on the floral form, this family is divided into three (Guignard & Dupont, 2005), two are monophyletic (*Papilionoideae* and *Mimosoideae*) and third paraphyletic (*Caesalpinoideae*). They are by far the largest group of plants involved in nitrogen fixation with symbiotic bacteria (Raven *et al.*, 2000). However, there are still 40% of legumes that have never been examined for nodulation (Sprent, 1999).

INTEREST OF SYMBIOTIC NITROGEN FIXATION

The inorganic nitrogen deficiency in the soil is a limiting factor for plant growth. It was estimated that the biological reduction of atmospheric nitrogen N₂ into ammonium provides about 65% of the available nitrogen in the biosphere (Lodwig *et al.*, 2003). The majority of this nitrogen is provided by the rhizobia-legume symbiosis (Zahran, 1999; Table 1).

According to Danso (1995), SNF has a greater contribution to the growth of plants comparatively to the nitrogen fertilizer applied in agriculture in developing countries. The fixed nitrogen in the atmosphere contributes to 50-60% of N grain legumes, 55 to 60% of the N fixing trees, and 70 to 80% N forage legumes (Table 1).

This nitrogen reserve is stored in the leaves, the nodules or other organs, remains longer available in the soil compared to the strongly leached mineral nitrogen by water.

TABLE 1. Estimated amounts of nitrogen fixed by different leguminous crops (Soltner, 1999).

Species	Fixed nitrogen kg / ha
Alfalfa	200 (56-463)
Clovers	183(45-673)
Lupins	176 (145-208)
Horse bean	210(45-552)
Pea	65(52-77)
Lentil	101(88-114)
Soybean	75(1-168)

Extreme values are in parentheses.

RHIZOBIA

The second symbiont of the SNF is bacteria commonly called “rhizobia”. Rhizobia were characterized by their growth rate. The genus *Rhizobium* contains the fast-growing strains. This genus was divided into several genera (*Rhizobium*, *Ensifer*/*Sinorhizobium*, *Allorhizobium*, *Mesorhizobium*) and *Bradyrhizobium* containing slow-growing strains (Jordan, 1982). Currently, the functional group of rhizobia includes more than 100 species distributed in 9 families and 15 genera alpha and beta Proteobacteria. Thanks to the appearance of new molecular taxonomy tools and a wider exploration of diversity in high biodiversity areas, this number has increased steadily since about fifteen years.

SYMBIOTIC SPECIFICITY

One of the major characteristics of legume - rhizobia associations is their host specificity (Table 2). Indeed, a particular species of rhizobia is able, in general, to establish an effective symbiotic relationship with a limited number of plant partners. Similarly, a species of legume may be nodulated by a number of rhizobial species (Tilak *et al.*, 2005).

TABLE 2. Rhizobia and their corresponding host plants.

Bacterial species	Host plants	References
<i>Sinorhizobium meliloti</i>	<i>Medicago</i> , <i>Melilotus</i> , <i>Trigonella</i>	(Jordan, 1982)
<i>Rhizobium etli</i>	<i>Phaseolus</i>	(Vázquez <i>et al.</i> , 1993)
<i>Rhizobium loti</i>	<i>Lotus</i>	(Jordan, 1982)
<i>Rhizobium tropici</i>	<i>Phaseolus</i> , <i>Leucaena...etc.</i>	(Martínez-Romero <i>et al.</i> , 1991)
<i>Rhizobium fredii</i>	<i>Phaseolus</i> , <i>Glycine...etc.</i>	(Bec-Ferte <i>et al.</i> 1994)
<i>Bradyrhizobium japonicum</i>	<i>Glycine</i> , <i>Macroptilium</i>	(Elkan GH, 1992)
<i>Bradyrhizobium elkanii</i>	<i>Glycine</i> , <i>Macroptilium</i>	(Elkan GH, 1992)
<i>Rhizobium meliloti</i>	<i>Medicago</i> , <i>Melilotus</i> , <i>Trigonella</i>	(Jordan, 1982)
<i>Rhizobium etli</i>	<i>Phaseolus</i>	(Vázquez <i>et al.</i> , 1993)
<i>Rhizobium loti</i>	<i>Lotus</i>	(Jordan, 1982)

LEGUME-RHIZOBIA INTERACTION

Legume-rhizobia interaction occurs in the rhizosphere due to the release of carbon molecules (sugars, organic acids, hormones, vitamins and phenolics) by exudation, secretion, or autolysis of old root cells (Seneviratne & Jayasinghearachchi, 2003). Many organic substances released by the roots have a low molecular weight and are easily decomposed by microorganisms. This leads to the existence of a large microbial community around the root.

Other compounds found in root exudates exert selective pressures on the microbial community. The early plant host signals secreted into the rhizosphere can be (iso)flavonoids, stachydrines, or aldonic acids. Many studies reported that the flavonoids, in combination with the rhizobial NodD transcriptional activator, induce expression of the *nod* gene regulon (Stougaard, 2000). These compounds are the most important of symbiotic perspective and can passively diffuse through the bacterial membrane (Begum *et al.*, 2001; Wang *et al.*, 2012).

NODULE FORMATION

The rhizobia and the host plant establish a dialogue system based on an exchange of chemical molecules. First, the roots excrete flavonoids (Hirsch *et al.*, 2001; Graham, 2008). These flavonoids (Fig. 1) attract rhizobia in the vicinity of the root and activate the bacterial *nod* genes, which encode for Nod factors (Ramos & Bisseling, 2005; Downie, 2005). These factors secreted by the rhizobia stimulate cell division of the cortical part of the roots resulting in the formation of a primary meristem (Heller *et al.*, 2000).

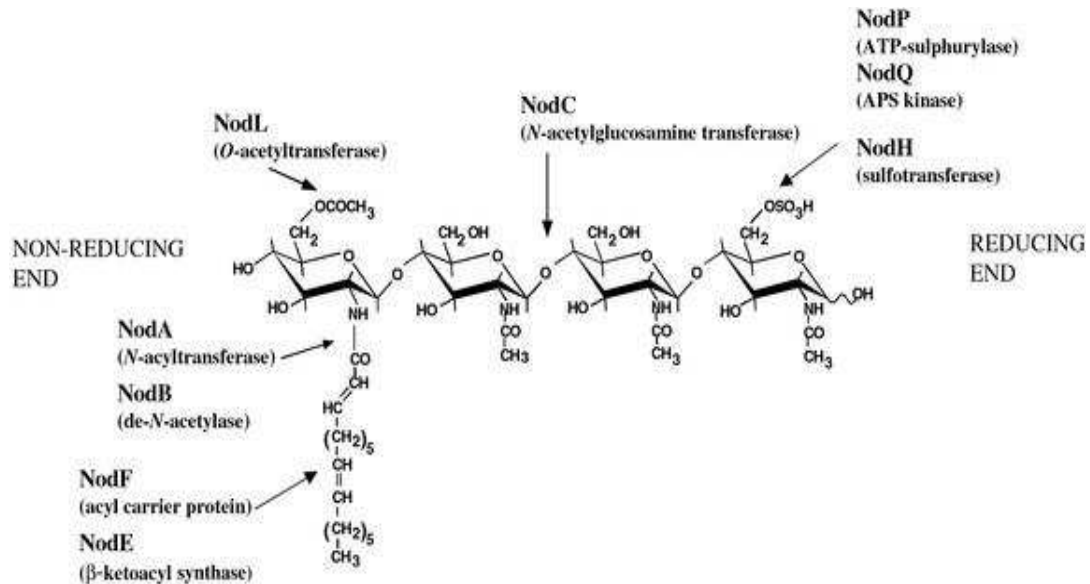


FIG. 1. *Sinorhizobium meliloti* Nod factor structure and Nod protein function (Wais *et al.*, 2002). This figure is copyrighted by the American Society of Plant Biologists and is reprinted with permission.

The bacteria attach to the roots by means of a specific adhesion molecule, rhicadhesin, localized at the surface of rhizobia cells. The rhicadhesin is a calcium binding protein. It allows adherence by complexing the calcium present in the root surface. The accession phase results in a retraction of the roots in response to secretion of molecules and the bacterium penetrates the cells by an invagination mechanism. Growth and movement of the bacteria in the root cause the formation of an infection thread. The infection is gradually spreading to the cells located near the site of infection. Rapid division of infected cortical cells results in the formation of the nodule (Madigan & Martinko, 2007; Fig. 2).

Bacteria proliferate quickly inside plant cells or they take forms more or less globular, becoming bacteroids. The bacteroids are locked in vesicles limited by a membrane derived from the plant cell to form a symbiosome (Parniske, 2000; Werner, 2007).

NOD GENES

During the early stages of symbiosis, the substances released from the legume induce the activation or repression of expression of nodulation genes in rhizobia (Soussou, 2013). The nodulation genes involved in the biosynthesis of Nod factors, which act as signal molecules and induce the formation of nodules (Masson-Boivin *et al.*, 2009). *Nod* genes may be functionally divided into three classes: the regulator genes, common *nod* genes and specific genes (Wais *et al.*, 2002). Important role in the development of nodules belongs to *nod* genes of rhizobia, which are organized in several operons and located either in the chromosome or in Symplasmids (Masson-Boivin *et al.*, 2009). The expression of many genes is controlled by the NodD transcription factor of nodulation (Györgypal *et al.*, 1991). Its activation occurs in response to the appearance of a plant flavonoid in bacterial cell (Hassan & Mathesius, 2012). The common *nod* genes, *nodABC*, are highly conserved and present in different species of

rhizobia, with the exception of some *Bradyrhizobium* and are responsible for the synthesis of skeletal Nod factors (Giraud *et al.*, 2007). The specific *nod* genes are responsible for substitutions occurring on basic skeleton of Nod factors. These accessory genes are not found in all rhizobia and they are needed for nodulation of some plants (Soussou, 2013). Each strain has its own set of specific *nod* genes which allow the production of a cocktail of Nod factors (Wais *et al.*, 2002).

PERCEPTION AND SIGNAL TRANSDUCTION OF NOD FACTORS

Among the legumes such as *Medicago*, it appears that the responses to all Nod factors are dependent on the presence of O-sulfate group at the reducing end of Nod factors produced by *Sinorhizobium meliloti* (Mbengue, 2010). The infection initiation by the rhizobia is dependent on both the reducing and non-reducing ends (acetate and fatty acid). The first receptor, essential for all the responses, perceives the sulfate group of the reducing end. The second, essential for infection and the formation of the nodule, perceives the structures on the side of non-reducing end.

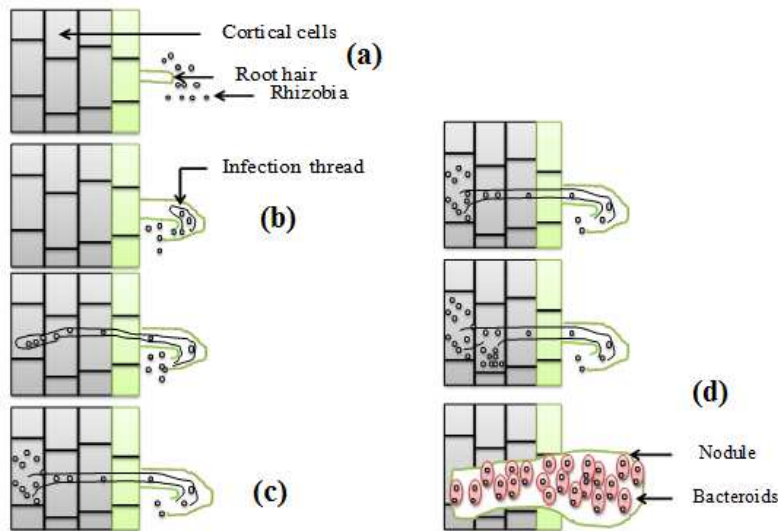


FIG. 2. Steps of root nodule formation in a legume infected with rhizobia. This figure is inspired from the work of Perry *et al.* (2004)

- a) Root hairs release chemical signals that attract rhizobia.
- b) Rhizobia proliferate and cause an infection thread to form.
- c) The infection thread grows into the cortex cell.
- d) The infection thread releases bacterial cells, which become bacteroids in the root cells. Nod factors from bacteria cause cortical cells to divide.

CALCIUM AND NOD SIGNALING

A rapid calcium influx followed by an output of Cl^- , K^+ and basification of the cytoplasm was first observed with selective microelectrodes (Debellé *et al.*, 2007). These ionic movements causing membrane depolarization which was originally described as the

first event associated with the perception of Nod factors (Oldroyd & Downie, 2004). The calcium spiking was observed in response to treatment with Nod factors to ten times lower concentrations (1 nM) than those causing the rapid influx (Fig.3). They are located in the perinuclear and nuclear areas, and spread towards the apex. The signal is divided into a rapid elevation phase of the Ca²⁺ concentration, followed by a gradual return to baseline (Pingret *et al.*, 1998 ; Engstrom *et al.*, 2002).

PROTEINS OF NOD SIGNALING PATHWAYS

The genetic approach identified six genes in *Medicago truncatula* (*DMI1*, *DMI2*, *DMI3*, *NFP*, *NFR*, and *NSP1-NSP2*), involved in the early stages of signaling by Nod factors and necessary for symbiotic responses such as: deformation of root hairs, the expression of genes nodulins and division of cortical cells (Catoira *et al.*, 2000; Stacey *et al.*, 2006).

DMI genes

The *DMI1*, *DMI2*, and *DMI3* genes of control early steps of Nod factor signal transduction in *Medicago truncatula* (Ané *et al.*, 2002). *DMI1* encodes a membrane protein with low homology to a cation channel, *DMI2* encodes for an extracellular domain kinase receptor LRR (leucine rich repeat) and *DMI3* for a calcium and calmodulin dependent protein kinases (Stacey *et al.*, 2006). The *DMI1* gene of the model legume *Medicago truncatula* plays a major role both in the early steps of Nod factor signaling and in the establishment of mycorrhizal symbiosis. *DMI1* mutants do not exhibit many of the early responses to Nod factors and are incapable to form nodules (Ané *et al.*, 2002). Peiter *et al.* (2007) reported that *DMI1* can regulate the activity of calcium channels, the origin of the calcium spiking. A subsequent study showed that calcium oscillations are no longer present in *dmi1*, *dmi2* mutants and, unlike *dmi3* mutant that is not affected. *DMI1* and *DMI2*, unlike *DMI3*, are required to generate the calcium oscillations (Wais *et al.*, 2000). According to Bersoult *et al.* (2005), the study of the expression of *DMI2* by promoter::GUS fusion showed that this gene is expressed in the epidermis and cortex of the root before inoculation. After inoculation, it was strongly induced in nodule primordia before contact with the infection thread and in zone II of infection of mature nodule.

NFR genes (Nod-factor receptor genes)

According to Madsen *et al.* (2003) and Radutoiu *et al.* (2003), the genes *NFR1* and *NFR5*, isolated by positional cloning in *Lotus japonicus* L., are essential for the earliest physiological and cellular responses to Nod factors, as mutations in them lead to plants that either no longer respond to Nod factors or show attenuated responses. *NFR1* and *NFR5*, like most genes that control Nod factor signaling, also control root hair curling, the first step of rhizobial infection, preceding infection thread formation.

NFP genes (Nod Factor Perception), the signaling receptor

NFP is a kinase receptor with an extracellular domain having LysM motif known to interact with glycans (Stacey *et al.*, 2006). The *NFP* gene expression observed in root hairs before inoculation with *Sinorhizobium meliloti* is consistent with the role of *NFP* in controlling a rapid calcium flux, calcium spiking, and inhibition of reactive oxygen efflux within minutes of Nod factor addition to root hairs (Amor *et al.*, 2003). After rhizobial inoculation, *NFP* expression was strongly linked to nodule primordia development in the root cortex and to infection in root hairs and underlying outer cortical cells (Gough, 2003).

LYK genes (LysM Domain Receptor Kinases), the entry receptor

LYK genes were identified in *Medicago truncatula* as candidate Nod factor receptor genes (Fig. 3). They are guessed to contain a signal peptide, extracellular LysM domains, a transmembrane segment and an intracellular serine/threonine kinase domain. Using reverse genetics in *Medicago truncatula*, Limpens *et al.* (2003) showed that two *LYK* genes are specifically concerned in the infection thread formation. This, as well as the properties of the LysM domains, strongly suggests that they are Nod factor entry receptors. The study of the syntenic region SYM2 of pea in *Medicago truncatula* has allowed the identification of a group of 7 genes coding LysM RLKs with three lysin motifs in their extracellular domains. These LysM-RLKs appointed *LYK1* to *LYK7* only *LYK3*, *LYK6* and *LYK7* are expressed in the roots of *Medicago truncatula* (Limpens *et al.*, 2003). Selective silencing of *LYK3*, *LYK6* and *LYK7* by interfering RNA has shown a particular symbiotic role for *LYK3* gene. The silencing of *LYK3* gene in *Medicago truncatula* caused no nodulation phenotype in the presence of *Sinorhizobium meliloti*. In contrast, no nodules are formed after inoculation with mutant strain of *Sinorhizobium meliloti* nodFE (Limpens *et al.*, 2003).

NSP1 and NSP2, GRAS family transcription factors

Nodulation signaling in legumes depends on an NSP1-NSP2 complex (Eckardt, 2009). The investigation of Hirsch *et al.*(2009) on the function of the GRAS domain proteins, nodulation signaling pathway1 (NSP1) and NSP2 showed that the two proteins interact to form a complex that binds directly to a specific promoter region of Nod factor-inducible genes. They showed that the interaction between NSP1 and NSP2 is enhanced by Nod factor perception and is necessary for proper development of nodules (Fig. 3). Same authors reported that NSP1 binds directly to ENOD promoters (early nodulins) through the novel cis-element AATTT. While NSP1 shows direct binding to the ENOD11 promoter in vitro, this association in vivo requires NSP2. The NSP1-NSP2 association with the ENOD11 promoter is enhanced following Nod factor elicitation.

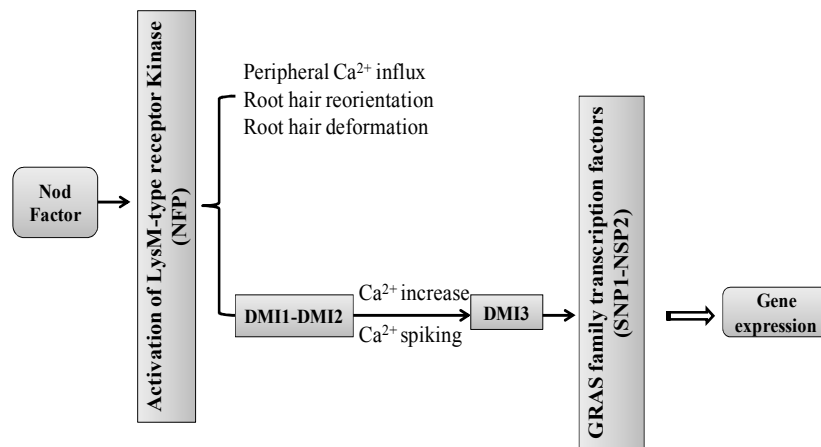


FIG. 3. Nod factor signaling pathway in the epiderm of *Medicago*.

ROLES OF PLANT HORMONES IN NODULATION

All of the classical plant hormones have been suggested to influence nodulation, including some that interact with the autoregulation of nodulation (AON) pathway (Table 3; Fig. 4). Leguminous plants strictly regulate the number of nodules formed through this AON pathway via a root-shoot-root loop that acts to suppress excessive nodulation (Foo *et al.*, 2014).

Genes related to auxin such as, GH3, AUX1, DR5, MtPIN2, MtCycA2 were differentially expressed during nodular initiation (Pacios-Bras *et al.*, 2003; Van Noorden *et al.*, 2007; Huo *et al.*, 2006; Roudier *et al.*, 2003). Van Noorden *et al.* (2006) documented that optimal concentration of auxin seems to stimulate nodulation. The auxin concentration is higher in nodules than in the roots in many species of legumes (Ferguson & Mathesius, 2003). The addition of auxin transport inhibitors lead to the appearance of pseudo-nodules (Wu *et al.*, 1996). In contrast, the addition of exogenous auxin restores nodulation (Fukuhara *et al.*, 1994). In the same sense, the rhizobia deficient for the synthesis of auxin induce fewer nodules in soybean. Mathesius *et al.* (1998) suggest that the induction of *enod40* (nodulin genes) expression is correlated with a local change of auxin concentration.

TABLE 3. Comparison of the influence of classical plant hormones on nodulation development (Foo *et al.*, 2014).

Bacterial species	Host plants	References
Auxin	+	(Van Noorden <i>et al.</i> , 2006 ; Deinum <i>et al.</i> , 2012)
Gibberellin	+ (optimal range)	(Ferguson <i>et al.</i> , 2005 ; Lievens <i>et al.</i> , 2005)
Ethylene	-	(Penmetsa, Cook, 1997 ; Oldroyd <i>et al.</i> , 2001)
Brassinosteroid	+ (optimal range)	(Ferguson <i>et al.</i> , 2005 ; Foo <i>et al.</i> , 2014)
Strigolactone	+	(Soto <i>et al.</i> , 2010 ; Foo <i>et al.</i> , 2013)
Cytokinin	+	(Plet <i>et al.</i> , 2011 ; Mortier <i>et al.</i> , 2014)
Abscisic acid	+/-	(Ding <i>et al.</i> , 2008 ; Tominaga <i>et al.</i> , 2009)
Jasmonic acid	+/-	(Sun <i>et al.</i> , 2006 ; Kinkema <i>et al.</i> , 2008)
Salicylic acid	-	(Van Spronsen <i>et al.</i> , 2003 ; Stacey <i>et al.</i> , 2006)

Hormones that positively influence colonisation are indicated by (+); those that do so only within an optimal range of hormone level are indicated), those that negatively affect colonization are indicated by a (-), those where there is evidence in both directions (such as at different developmental stages or using different experimental systems) are indicated with (+/-).

The cytokinins play an important role in the nodulation process. The *enod2* and *enod12* genes are induced by cytokinins (Hirsch *et al.*, 1997) and also *enod40* was suggested to be induced in the protoxylem poles and surrounding cell layers upon accumulation of cytokinin (Hirsch *et al.*, 1997). Heckmann *et al.* (2001) reported that the expression of *nodulin* genes was up-regulated upon cytokinin treatment, suggesting that the genuine nodulation program was indeed activated in *Lotus japonicus*. Same authors documented that the external cytokinin application induced expression of the Nin::GUS reporter gene within the root cortex but not in the root epidermis.

Gibberellins are reported to be involved in the formation and maturation of legume nodules, highlighted by recent transcriptional analyses of early soybean symbiotic

steps (Hayashi *et al.*, 2014). Ferguson *et al.* (2011) demonstrated that gibberellin deficient mutants of pea developed fewer nodules than wild-type plants.

The hormone ethylene plays a role as a negative regulator of nodulation, and acts on different processes during nodule formation, including regulation of total nodule numbers, infection thread formation, nodule morphology, and nodule positioning (Guinel & Geil, 2002). Suganuma *et al.* (1995) observed the induction of ethylene hormone during nodule initiation in *Glycine max* L. This increase was due to a defense response of the plant in response to the invading bacteria (Ferguson & Mathesius, 2003). Heidstra *et al.* (1997) showed that ACC oxidase, last enzyme involved in ethylene biosynthesis, was activated by Nod factors in the inner cortex.

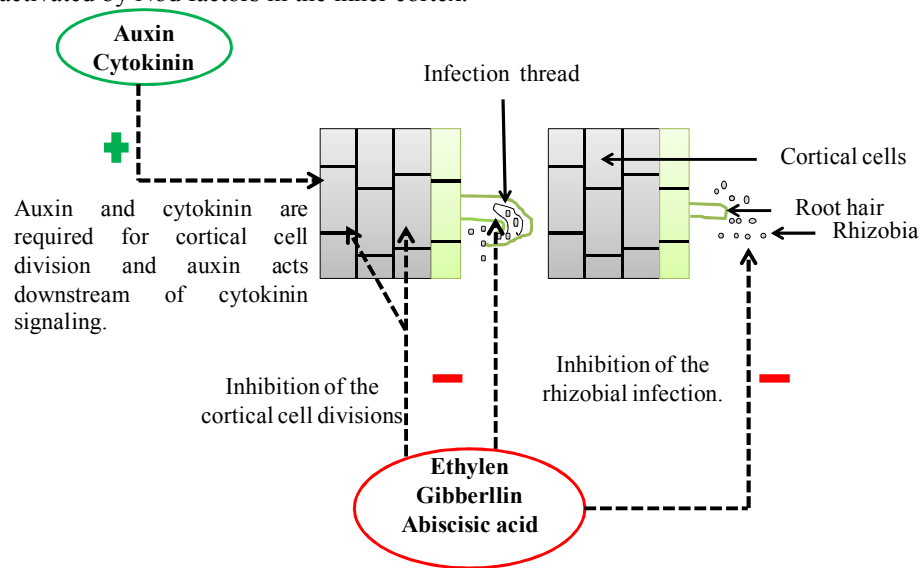


FIG. 4. Effects of plant hormones on nodulation process in legume-rhizobia interaction.

CONCLUSIONS

The genetic approach has helped to identify key genes involved in Nod signaling pathway. The studies of the perception and Nod factor signal transduction pathway led to the discovery of many essential genes in model legumes. It is now possible to study this pathway at functional level, particularly in relation to calcium signaling. The discovery of specific mutant alleles has helped to discover new essential genes. The understanding of molecular signals involving in the perception and signal transduction of Nod factors and the roles of genes required for nodule formation and consequently the proteins of NOD signaling pathways constitute a promising approach the SNF improvement.

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