Dinitrogen constitutes about 80% of the earth’s atmosphere, but it is inert and metabolically inaccessible to most organisms. Some prokaryotes, are able to catalyze the enzymatic reduction of $N_2$ to ammonia. It is catalyzed by the bacterial enzyme nitrogenase. Nitrogenase and some of the proteins that supply it with the reductant are sensitive to oxygen.

**Enzymology of $N_2$ fixation:** The biological reduction of dinitrogen to ammonia is carried out by two enzymes, dinitrogenase and dinitrogenase reductase. Together, these enzymes are referred to as nitrogenase. Dinitrogenase is a 240kD heterotetrameric protein, which binds nitrogen and holds it while being reduced. Dinitrogenase reductase is a 64kD homodimeric protein, that provides dinitrogenase with high-energy electrons. For nitrogenase to function, additional proteins are needed to synthesize its unique metal-containing cofactors and to transfer low-potential electrons to nitrogenase reductase. Dinitrogenase is an $\alpha_2\beta_2$ heterotetramer, containing two pairs of unusual metal clusters. The Fe-protein, encoded by *nifH*, accepts electrons from a carrier, e.g., ferredoxin, flavodoxin. The Fe-protein transfers single electrons at very low potential to the MoFe-protein accompanied by net hydrolysis of ATP. The MoFe-protein, $\alpha_2\beta_2$ heterotetramer of subunits encoded by *nifD* and *nifK* accepts electrons and bind H$^+$ ions and N$_2$ gas in a stepwise cycle ultimately leading to the production of H$_2$ and ammonia (Fig 16.6). Dihydrogenase reductase (Fe-protein) is a homodimer that contains a single 4Fe-4S cluster held cooperatively by the two monomers at the protein’s surface. Dinitrogenase reductase transfers single electrons sequentially to dinitrogenase. As dinitrogenase is reduced, the complex acquires as many as four hydrogens before dinitrogen binding and reduction are initiated.

**Symbiotic Nitrogen fixation.** While many nitrogen-fixing bacteria are free living, there are those that form a symbiotic relationship with plants. Three major types of nitrogen-fixing symbioses:

i. Legume-Rhizobium symbiosis

ii. Actinomycete interaction with diverse group of dicots, generally trees

iii. Symbiosis between cyanobacteria and a diverse array of plants, dicots, cycads, ferns and liverworts.

**Legume-Rhizobium symbiosis:** Bacteria, of the group rhizobia, are able to establish an endosymbiotic relationship with legumes. Plant and bacterial activities create a nodular environment conducive to nitrogen fixation and microaerobic synthesis of ATP by the bacteria. Plant metabolism in the nodule generates organic acids that feed the bacteria and provide C-skeletons for N-transport compounds. In exchange, the bacteria fixes nitrogen and releases the resulting ammonia to the plant. Legume-rhizobia interaction is very specific. While alfalfa forms the symbiotic relationship with *Sinorhizobium*
*meliloti*, the symbiont for soybean is *Bradyrhizobium japonicus* (Table 16.4). Under nitrogen-limiting conditions, the leguminous plants can form root nodules, in which the rhizobia are hosted intracellularly. The interaction of rhizobia and legumes begin with signal exchange and recognition of the symbiotic partners, followed by the attachment of the rhizobia to the root hairs. The root hairs deform and the bacteria invade the roots by forming **infection threads**. Along with infection thread formation the cortical cells are activated giving rise to the nodule primordium. Infection threads go towards the primordium and the bacteria are released into the cytoplasm but are surrounded by host derived **peribacteroid membrane (PBM)**. The nodule primordium from here on develops into a mature nodule while the bacteria undergo differentiation to become the **bacteroids**. Bacteroids surrounded by the PBM are called **symbiosomes**. At this stage, the bacteria synthesize nitrogenase that catalyzes the conversion of $N_2$ into ammonia, which is then utilized by host derived enzymes.

All of the steps of nodule development is accompanied by the expression of nodule-specific plant genes called the **nodulin** genes. The early nodulins are involved in the infection and in the establishment of the nodule, while the late nodulins are involved in the interaction with the symbiont and in the metabolic specialization of the nodule.

Two kinds of nodules:

- **Determinate** (or spherical) (Model system – *Lotus japonicus*)
- **Indeterminate** (or meristematic) (Model system - *Medicago truncatula*)

Nodule initiation involves changes in three root tissues, epidermis, cortex and pericycle. While rhizobia are still on the root surface, they induce morphological changes in the epidermis. This is preceded by the induction of some early nodulin genes: **ENOD11**, and **ENOD12**, encoding proline-rich proteins. They have been used as markers to monitor signal transduction events in the epidermis. Root hair deformation, takes place following inoculation - formation of shepherd’s crook. During root hair curling, the bacteria become entrapped in the pocket of the curl. The plasma membrane invaginates and forms a tube like structure, the infection thread.

Even before the infection thread has crossed the epidermis, cortical and pericycle cells respond. In the pericycle, a rapid induction of **ENOD40** takes place opposite a protoxylem pole followed by a limited number of cell division. Following activation of the pericycle, cells in the inner cortex dedifferentiate by entering the cell cycle, to form the nodule primordium.

Outer cortical cells, through which the infection threads will grow, form radially oriented, conical cytoplasmic columns. This organization of the cytoplasm takes place before the infection threads enter these cells and are therefore called the pre-infection threads. The pre-infection threads facilitate infection thread growth and direct them towards the nodule primordium. The infection threads ramify followed by the release of the bacteria into the primordial cells. Upon release, the bacteria remain surrounded by a membrane of plant origin (PBM).
The Rhizobium signal molecules that play a key role in the induction of the initial stages of nodulation are **lipochitooligosaccharides** known as **Nod factors**. The rhizobial genes, *nod, noe* and *nol*, are involved in the synthesis of the Nod factors. The expression of these genes is activated when the bacteria perceive certain plant signals (**flavonoid**) (Fig 16.23). Flavonoids activate the transcriptional regulator NodD that in turn induces transcription of the other nodulation genes (Fig 16.22).

Nod factors (NFs) consist of a backbone of three to five β-1,4-linked N-acetylglucosamines bearing a fatty acid on the nonreducing sugar residue. In addition, they can have various substitutions on both the reducing and nonreducing terminal sugar residues (Fig 16.24). The synthesis of the Nod factor backbone is catalyzed by the products of *NodABC* loci. The backbone is further modified by the action of other Nod proteins that synthesize or add various substituents. These substituents determine host specificity (Fig 16.21). In general, rhizobia have the ability to interact with only a limited number of host plants. However, some rhizobia have a more promiscuous nature. One rhizobium which can nodulate various tropical legumes excretes 18 different Nod factors. Purified Nod factors induce the deformation of the root hairs and can also induce the expression of some nodulin genes. The early nodulin genes *ENOD5* and *ENOD12* that encode proline rich proteins, and *Mtrip1*, which encodes a peroxidase.

**Nod Factor Binding.** Very low concentrations of Nod factor (10⁻⁹ M or less) can provoke profound morphological changes in the host. In the first minutes of exposure to the Nod factor, the plasma membrane of alfalfa root hairs depolarize, which is accompanied by fluxes of specific ions. Within 10 min, cells display periodic spikes in calcium. Cytoskeletal rearrangements and further reorientation of cell calcium gradients are seen in root hairs. Cell division in the cortex is detected within 18 to 30 hours and follows a pattern that is characteristic for each plant. The earliest divisions are sometimes strikingly located in the cortical region on a radius extending outward from each xylem pole, implying the role for an endogenous host factor that comes out from the stele. The signals are not known but plant hormones, like auxin, cytokinin and ethylene have been implicated to have a role.

The nodule generates a microaerobic reducing environment that can supply aerobic ATP synthesis. The microaerobic environment is generated by the interaction between the two symbionts (Fig 16.30). Three factors are important for maintaining low oxygen concentrations in the nodule: i. entry of oxygen into nodules is controlled by a variable-permeability barrier in the nodule parenchyma; ii. **Leghemoglobin**, an oxygen binding plant protein, plays an active role in regulating and delivering oxygen in the infected cells. A monomer with a single heme moiety, leghemoglobin is transcribed primarily in the infected cells of the nodule and is produced in high abundance (millimolar level). Leghemoglobin increases the flux of oxygen moving through the plant cytoplasm to the bacteroids while controlling the concentration of free oxygen. iii. Bacterial respiration constitutes a major oxygen sink. Whereas the free-living rhizobia typically have a cytochrome oxidase with a Km for oxygen of around 50nM, the bacteroid cytochrome oxidase has a Km of only 8nM. Expressed only in the nodules, this cytochrome oxidase is required for nitrogen fixation. The peripheral location of plant mitochondria in
infected cells may give them better access to oxygen. The nodular oxygen concentration maintained by the plant support submaximal nitrogenase activity, a factor that might contribute to the stability of symbiotic nitrogen fixation.

The low oxygen concentration within the nodule is a key element in regulating bacteroid nitrogenase activity. In some rhizobial species, an oxygen-sensitive hemoprotein kinase, FixL, control a regulatory cascade that activates transcription of nitrogen-fixation genes (Fig 16.31). FixL, part of a two component regulatory system, phosphorylates its partner FixJ. Once phosphorylated, FixJ, controls expression of other regulatory proteins. Two of these, NifA and FixK, control expression of diverse nif and fix genes.

The nodule obtains the photosynthate in the form of sucrose. The sucrose is converted into organic acids before it can be used. There is evidence indicating that phosphoenolpyruvate is converted into oxaloacetate by PEP carboxylase and NAD⁺ is generated by the reduction of oxaloacetate to malate. The malate is transported into the symbiosome. Dicarboxylic acids in the nodules also provide carbon skeletons for nitrogen containing transport compounds like glutamine and asparagine.

Plant glutamine synthetase (GS) and glutamate synthase (GOGAT) are responsible for the initial assimilation of ammonia into organic acids. The fate of glutamine differs based on the nitrogenous transport compounds. In one class (alfalfa) nodules export ammonia as amides, namely, glutamine and asparagine. These nodules contain aspartate amino transferase, asparagine synthetase (Fig 16.32). Nodule-specific isoforms of both the enzymes are found in many plants.

A second major group of legumes, including soybeans, exports nitrogen from the legumes as the ureides, allantoin and allantoic acid, compounds produced by synthesizing purines in the infected cells and then oxidizing them into in the neighboring uninfected cells (Fig 16.33).

Nod factors are recognized by a high affinity receptor. The amphiphilic nature of Nod factors, with their hydrophobic lipid tail and hydrophilic sugar backbone, suggests that Nod factor receptors are located in the plasma membrane.

**Perception of microbial signals**

Approaches to identify Nod Factor receptor: analysis of protein extracts and “candidate gene” approach. Putative NF receptors have been identified in many legumes:

NFR1 and NFR5 from *L. japonicus* and *Glycine max*

LYK3 and NFP from *M. truncatula*

SYM37 and SYM10 from *P. sativum*

**Structure:** It has a single-pass transmembrane domain anchored to an extracellular lysine motif (LysM) receptor domain and an intracellular kinase domain and are thus termed LysM receptor-like kinase (LysM-RKs). The LysM domain binds to peptidoglycan and/or structurally related molecules, such as chitin oligosaccharides. In *L. japonicus*, ...
NFR1 and NFR5 are thought to form a receptor complex that recognizes the NF secreted by *Mesorhizobium loti*. LjNRF5 lacks the intracellular kinase domain, LjNFR1 could be crucial for transmitting the intracellular signal to downstream symbiotic signaling pathway. In *L. japonicus*, a single receptor complex composed of NFR1 and NFR5 appears to be responsible for both activation of infection and the nodulation processes. Legume plants have a large number of LysM-RKs compared with nonlegumes raising the possibility that NF perception and subsequent intracellular signaling are mediated by complex combinations of LysM-RKs including those other than NFR1 and NFR5. NFR1 has been shown to retain the ability to activate transiently defense-related genes in response to purified NFs. It is interesting to find out how LysM-RKs induce apparently opposite biological responses in their host plants – one induces endosymbiotic association and the other induces defense reaction.

**Early symbiotic signaling**

Two receptor-like kinases (RLK) located on the epidermal cells that are involved in NF binding: in *L. japonicus* LjNFR1 and LjNFR5, in *P. sativum* PsSYM2a and PsSYM10, in *M. truncatula* MtLYK3/MtLYK4 and MtNFP. These NF receptors have an intracellular kinase domain, a transmembrane domain and an extracellular LysM domain. Another RLK involved in NF signaling has leucine rich repeat (LRR) and Ser/Thr kinase domains and is encoded by *LjSYMRE/MtDM12*. It is located in the plasma membrane and on the infection thread membrane and is predicted to function in both NF perception and downstream signal transduction since it is required for the earliest root hair responses. Activation of the LysMRLK is seen as a prerequisite for the activation of this LRR RLK. LysRLK may have a specific role in the NF signaling cascade whereas the LRR RLK may function more in initiating bacterial infection events.

NF perception initiates a downstream signal transduction cascade. This involves potassium ion-channel proteins localized in the nuclear membrane, encoded by *MtDM1, LjCASTOR* and *LjPOLLUX*, two nucleoporins encoded by *LjNUP133* and *LjNUP85* and a calcium and calmodulin dependent protein kinase (CCaMK) encoded by *MtDM13/PsSYM9*. As quickly as 1 min after application, Ca$^{2+}$ fluxes denoted by a rapid influx of Ca$^{2+}$, followed by membrane depolarization, efflux of Cl$^{-}$ and K$^{+}$ occur in root hairs. Ca$^{2+}$ spiking (oscillation in Ca$^{2+}$ concentrations) are subsequently induced in the same cells some minutes after the induction of Ca$^{2+}$ fluxes. The ion channel proteins and the nucleoporins are required for these Ca$^{2+}$ spiking events and CCaMK may act as to perceive the Ca$^{2+}$ spiking signals.

Several transcription factors are activated downstream of CCaMK, including nodulation signaling pathway1 (NSP1) NSP2, ETs2 repressor factor (ERF) required for nodulation (*ERN*) and nodule inception (*NIN*). In the epidermal cells, NSP1 and NSP2 are thought to be colocalized with CCaMK in the nucleus. ERN1 and NSP1 bind to the promoter of *ENOD11* (expressed in the epidermal cells), where the binding of NSP1 to the *ENOD* promoter requires NSP2. NSP1 has also been shown to bind to the promoters of *ERN1* and *NIN*. This suggests that NSP1, NSP2, ERN1 and NIN all work in combination to regulate the expression of *ENOD* genes in the epidermis.
The signaling components required for bacterial infection events are triggered by the activation of the NF LRR RLK. One such component is 3-hydroxy-3-methylglutarylCoA reductase in *M. truncatula* (MtHMGR) which may be involved in the synthesis of isoprenoid-derived phytohormones, such as cytokinins and brassinosteroids. SymRK interacting protein of *L. japonicus* (LjSIP1) and Rhizobium-directed polar growth of *M. truncatula* (MtRPG) have also been shown to interact with the LRR RLK.

Other factors having a role in nodule development include LjCERBERUS, ethylene responsive factor1 (LjERF1) and ethylene response factor required for nodule differentiation (MtEFD).

A cytokinin receptor functions in the root cortex and is required for cell division events. The receptor has a histidine kinase domain and is encoded by *MtCRE1/LjLHK1*. Downregulation of this receptor results in a dramatic decrease in nodule numbers caused by the plant’s inability to form nodule primordia. CCaMK appears to be required for events occurring in both epidermis and the cortex, yet involving entirely different pathways. NSP1 and NSP2 act downstream of CCaMK in the epidermis as a part of the NF signaling pathway, are also required for cell division in the root cortex. Cytokinin is a key component of nodule organogenesis. It seems plausible that cytokinin could be the mobile signal that communicates epidermal perception of NF to the inner root. Abscisic acid is considered to be negative regulator of nodule development and appears to have a role in both the epidermis and cortex. Auxin, gibberellins and brassinosteroids are positive regulators while reactive oxygen radicals, jasmonic acid and ethylene are considered to be negative regulators of nodulation.

Many of the non-nodulating legume mutants are also defective in mycorrhizal symbiosis implying that the genes responsible for those mutants are required for both RN and AM symbioses. The signal transduction pathway mediated by those genes are termed ‘common symbiosis pathway’ (CSP). In *L. japonicus*, seven genes so far have been positioned in the CSP. Following perception of NFs through LysM-RKs, biphasing Ca$^{2+}$ signaling is induced in the root hair cells, i.e. a rapid influx of Ca$^{2+}$ into the root hair cells and then periodical oscillation of cytosolic Ca$^{2+}$ concentrations at perinuclear region (Ca$^{2+}$ spiking). Ca$^{2+}$ spiking is also induced in response to AM symbiosis and is critical for both RN and AM symbioses. In *L. japonicus*, five out of seven CSP genes: SYMRK, CASTOR, POLLUX, NUP85 and NUP133 are required for Ca$^{2+}$ spiking, whereas CCaMK and CYCLOPS are positioned downstream of Ca$^{2+}$ spiking and thus may play a role in transmitting symbiotic signals mediated by Ca$^{2+}$ signals to downstream RN- and AM-specific pathways.

*SYMRK* and *DMI2*, which encode LRR receptor kinases, have been isolated from *L. japonicus* and *M. truncatula*, respectively. SYMRK/DMI2 are believed to be the starting point of CSP although the ligand(s) of their extracellular receptor domains are still not known. *CASTOR* and *POLLUX*, both of which encode Ca$^{2+}$-gated cation channel proteins. NUP85 and NUP133 are postulated to be involved in transport or localization of the factors needed for the induction of Ca$^{2+}$ spiking.

Ca$^{2+}$ and calmodulin-dependent protein kinase, CCaMK consists of 3 functional domains, i.e., a Ser/Thr-kinase domain, a CaM-Binding (autoinhibitory) domain (CaMBD) and
domain of three EF hands, which interact with Ca\(^{2+}\) ions. Pivotal role of CCaMK is in nodule organogenesis.

The RN symbiosis is presumed to have its evolutionary origins in the more ancient AM symbiosis and to have evolved by recruiting the preexisting CSP genes.

**Infection process and nodule organogenesis**

Initiation of infection threads requires several sequential steps, attachment of bacteria on root hairs, root hair curling and bacterial colonization at the tip of curled root hairs. Purified NFs can induce a nodule meristem but no root hair curling, indicating that the latter requires the presence of bacteria at the surface of a root hair tip.

Concomitantly with IT formation in the root hair, differentiated cells at the root cortex (and then pericycle) start to divide to develop a nodule meristem. This coordinated development between infection process and nodule organogenesis is believed to be crucial for successful nitrogen-fixing nodule formation. So far 11 genes have been cloned and reported to be essential for coordinative progression of IT growth and nodule organogenesis.

NSP2 encodes a GRAS family transcription factor and is required for cortical cell division (CCD) and root hair curling. NSP2 is localized in the nucleus and interacts with another GRAS family TF, NSP1. This interaction is necessary for nodule organogenesis and NSP1 associates with the promoters of early nodulin genes, such as *ENOD11*, *NIN* and *ERN1*. NIN is a putative TF that is required for CCD and controlling root hair curling. NIN expression is drastically induced in the epidermis soon after NF perception and is confined to the infection zone. This induction requires NSP2 and CERBERUS. *CEREBUS* and its *Medicago* ortholog *LIN*, encode a U-box protein with WD-40 repeats which is postulated to be an E3 ubiquitin. *CEREBRUS* is necessary for IT initiation in addition to *CYCLOPS* and *ERN1*. *ERN1* encodes an ERF TF that contains an AP2 DNA-binding domain. ERN1 is essential for spontaneous nodulation by gain-of-function CCaMK. CYCLOPS is a nuclear localized protein and has been shown to be phosphorylated by CCaMK in vitro. Since loss of function mutants of CCaMK display neither root hair curling nor CCD, CYCLOPS is thought to be important for the function of CCaMK in the rhizobial infection process.

(GrAS family of putative transcription regulators is found throughout the plant kingdom and these proteins have diverse roles in plant development including root development, axillary shoot development and maintenance of the shoot apical meristem).

Housing within the nodule cells and differentiation into bacteroids is essential for nitrogen fixation. Therefore, bacterial differentiation and their nitrogen fixation are under strict control with complex interactions between the host cells and the intracellular bacteria. In indeterminate nodules, the rhizobial proliferation is terminated by DNA endoreduplication triggered by host plant factors, which have been suggested to be nodule-infected cell-specific **cysteine-rich peptides** and **BacA** protein in rhizobia is shown to be involved in the uptake of host-derived peptides. In *M. truncatula*, nodule-specific cysteine rich (NCR) peptides were shown to be host plant factors which direct
rhizobia into terminal bacteroid differentiation. The NCR peptides are most similar to defensin-type antimicrobial proteins, and have signal peptides that targets them into secretory pathway. The host plants control the differentiation of rhizobia into bacteroids by targeting NCR peptides through the nodule-specific protein secretory system in indeterminate nodules.

A number of nodulin genes are specifically expressed during the nodulation process. Many genes are specifically or highly induced in nodules. Leghemoglobin functions in keeping oxygen low and in facilitating $O_2$ to the bacteroids. Nodule enhanced sucrose synthase is crucial for nitrogen fixation. Nodule enhanced PEP carboxylase and glutamine synthetase are involved in the C and N flux and is essential for nitrogen fixation. Putative transporters have been identified, their exact function is not known. Transport of dicarboxylates and some amino acids to bacteroids from the host cells is essential for nitrogen fixation/ nodule persistence.

A symbiotic sulfate transporter ($SST1$) gene was found by map-based cloning from $L. japonicus$ Fix- mutant of $sst1$. SST1 is located on the PBM. Sulfur has special importance in bacteroids as a component of metal-sulfur clusters within the nitrogenase complex and the related electron transfer proteins.

**Homocitrate synthase** (HCS), is expressed specifically in nodule-infected cells. Homocitrate is a component of iron-molybdenum cofactor (FeMo-co) in the nitrogenase complex on which nitrogen fixation is though to occur. $NIFV$, which encodes HCS has been identified in many daizotrophs and shown to be essential for their nitrogenase activity. However, $NIFV$ orthologs are not found in Rhizobium species. These findings highlight the complementary and indispensable partnership between legumes and rhizobia in symbiotic nitrogen fixation.

$IGN1$, encoding ankyrin-repeat membrane protein, is required for preventing the host plant cells from inappropriately invoking a defense system against compatible microsymbionts, thus being essential for differentiation and /or persistence of bacteroids and symbiosomes, although the exact function $IGN1$ is still to be elucidated.

**Systemic regulation of symbiosis**

Legume plants have developed a specific mechanism to control the nodule number in response to internal and external cues. An important internal cue is a systemic feedback regulatory system involving long-distance root-shoot signaling termed **autoregulation of nodulation** (AON), which is shown to be closely linked with early symbiotic signaling triggered by NFs. AON is believed to consist of two presumptive long-distance signals, a root-derived and shoot-derived signals. The root-derived signal is generated in roots in response to rhizobial NFs and then translocated to the shoot, while the shoot-derived signal is generated in shoots and then translocated to the root to restrict further nodulation. Mutants defective in AON, display a so-called ‘**hypernodulation**’ phenotype. Grafting experiments indicated that these mutants are defective in the production of shoot derived signals. The responsive genes have been shown to encode an LRR receptor-like kinase ($LjHAR1$, and have a Ser/Thr kinase domain) and have similarity to CLAVATA1 ($CLV1$) of Arabidopsis and $FON1$ of rice. $CLV1$ and $FON1$
are specifically expressed in shoot and floral meristems and restrict their sizes by receiving a **CLE peptide** derived from the stem region. In contrast, the legume genes represented by *L. japonicus HAR1* are widely expressed in various organs but not in the shoot apex, suggesting that these genes are uniquely evolved in legumes to produce the shoot derived inhibitor of nodulation by receiving the root derived signal.

Together with HAR1 receptor-like kinase, KLAVIER (KLV) is also indispensable for AON signaling in *L. japonicus*. The mutation exhibits stem fasciation as well as hypernodulation phenotype. A double mutation analysis indicated that KLV functions in the same genetic pathways as HAR1. KLV encodes an LRR receptor-like kinase and is specifically expressed in the leaf vascular tissues as with the case of HAR1. Thus, KLV and HAR1 are likely to function coordinately to receive the root-derived signal in the shoot. Molecular identification of these genes might shed light on a common mechanism to connect RN- and legume-specific shoot apical meristem regulation.

HAR1 shows similarity to Arabidopsis CLV1. The extracellular domain of CLV1 binds to a modified CLE peptide. The **CLE** gene encodes a small secreted peptide composed of 12-13 amino acids. In *L. japonicus*, at least 39 potential **CLE** genes have been identified in its genome. Among them, small peptides derived form two **CLE** genes (*LjCLE-RS1* and *LjCLE-RS2*) have been proposed as strong candidates for the root-derived signal.

The perception of the root-derived signal by legume CLV-like receptor kinase is then presumed to initiate the production of the shoot-derived signal(s). Although the chemical nature of the shoot-derived signal is unknown, foliar application of plant hormones has produced results indicating that brassinolide and methyljasmonate may function in shoot-derived signal. Alternatively, polar auxin transport has been postulated to play an important role in long-distance control of nodulation.

Independently of AON, the role of ethylene is well characterized as a negative regulator of nodulation. Endogenous ABA is also a negative regulator of nodulation. Inhibition of ABA synthesis and signaling leads to a hypernodulation phenotype.

High concentrations of nitrogen as nitrate or ammonia abolish nodulation and hypernodulation mutants such as *nts, har1, klv* and *nod3* exhibit more or less nitrate tolerant phenotypes. Recently, it has been shown that *L. japonicus CLE-RS2* is strongly upregulated in roots in response to nitrate. As **CLE-RS2**-mediated suppression of nodulation is not observed in the *har-1* mutants, the nitrate induced **CLE-RS2** peptide is likely to suppress nodulation through HAR1. According to this model, nitrate tolerance of the hypernodulation mutants can be explained because the mutations in NTS and HAR1 lead to defective CLE peptide perception in the presence of nitrate. The **CLE-RS2** is most probably a key regulator in nitrogen signaling and developmental plasticity that adapts to environmental nitrogen conditions.

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