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# Modified to be Symbiotic; Events after Rhizobial Entry into Plant Cells

**Choong-Ill Cheon*, Hye-Sook Oh and Jong-Yoon Chun[1]** 천종일

*Department of Life Science, Sookmyung Women's University, Seoul 140-742, Korea*
*[1]Kumho Life and Environmental Science Laboratory, Kwangju 506-203, Korea*

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Nitrogen is one of the essential elements for biological compounds in plant cells. Although nitrogen supply is very critical to plant growth and agricultural productivity, most nitrogen exists in the air as a form difficult for higher plants to use. The endosymbiotic association between legumes and *Rhizobium* makes plants auxotrophic for external nitrogen, one of the most limiting factor for plant growth. Legumes contain specific sets of proteins in root nodules (nodulins), and the nitrogen-fixing bacteria fix nitrogen inside plant cells and supply it to plants (Verma, 1992). Symbiosis between legume and *Rhizobium* is not only a good example of biological nitrogen fixation but also a good model system for studying the interaction between prokaryotes and eukaryotes.

This unique association between *Rhizobium* and legume plant involves a series of events; the early signal exchange between the two partners, rhizobial infection into root hair cells and cortical cells, release of bacteria into host cytoplasm, nitrogen fixation and assimilation and ultimately senescence of the nodule (Long, 1996). Specific signals are exchanged during these interactions (Verma, 1992). It has been known that host-specificity between symbiotic partners is determined at the stage of early signal exchange. As nodule development advances, sequential induction of both bacteria-encoded (bacteroidins) and host-encoded (nodulins) nodule-specific proteins occur in a temporal manner. Several bacterial genes involved in nodulation and fixation of nitrogen, and host genes encoding nodulins that are essential for development of root nodules, have been characterized (Long, 1996). Attention has been currently focused on the successful biogenesis of the subcellular compartment, which may bring about pathogenic reaction in the host in all endosymbiosis.

*Corresponding author.
Phone) +82-2-710-9396, Fax) +82-2-718-2337
E-mail) ccheon@sookmyung.ac.kr

## Early Signal Exchange

The early interaction between two symbiotic partners can be characterized as a two-way molecular conversation. Leguminous plants synthesize specific flavonoids, which are products of the phenylpropanoid pathway and a few other phenolic compounds and release these compounds into the rhizosphere (see Peters and Verma, 1990). These compounds interact with a common nodulation gene product (NodD) of *Rhizobium*. NodD binds to promoter region (nod boxes) of *nod* operons, activating the gene expression of *nod* genes. The *nod* genes encode enzymes involved in the synthesis of Nod factor, a substituted oligosaccharide which acts as a return signal to legumes. This molecule alone can induce nodule morphogenesis, completing the early round of molecular conversation between the two specific symbiotic partners (Table 1) (Fisher and Long, 1992).

The Nod factors from bacteria are all oligomers of N-acetyl glucosamine (Lerouge et al., 1990). Precursor sugars of Nod factors are polymerized by several enzymes including the product of *nodC*, one of the common *nod* genes (Bulawa and Wasco, 1991). In the case of NodRm-IV

**Table 1.** Symbiotic relationships between rhizobia and legumes

| Bacterial species | Plant hosts |
|---|---|
| *Rhizobium meliloti* | Alfalfa (*Medicago sativa*) |
| *Rhizobium leguminosarum* | |
| biovar *viciae* | Pea (*Pisum sativum*), vetch (*Vicia sativa*) |
| biovar *trifolii* | clovers (*Trifolium* species) |
| biovar *phaseoli* | *Phaseolus* bean |
| *Rhizobium loti* | Lotus (*Lotus corniculatus*) |
| *Rhizobium* sp. NGR234 | Broad host range; tropical legumes, Parasponia |
| *Bradyrhizobium japonicum* | Soybean (*Glycine max*), cowpea, mungbean, siratro, pigeonpea |
| *Frankia* | alder (*Alnus glutinosa*) |

(C16:2), a Nod factor from *Rhizobium meliloti*, the *nodH* and *nodQ* genes were shown to determine changes in host range. Their products participate in the addition of sulfur to the Nod factor, which requires the action of *nodF* and *nodE* products. The functions and properties of the other *nod* genes have been studied actively (Fisher and Long, 1992). The signal molecule from *Bradyrhizobium japonicum*, which nodulates tropical legumes, is very similar to NodRm-IV (16:2) with some modifications.

The signal molecules elicit several plant responses including cortical cell division, root hair curling, infection thread initiation and early nodulin expression. (reviewed in Heidstra and Bisseling, 1996). In the case of NodRm-IV (16:2) and its acetylated form, the purified molecule at concentration in the micromolar to nanomolar range can induce cortical cell divisions in asceptically grown seedlings of alfalfa. When non-specific Nod factors were tested on *Vicia sativa*, they caused only hair deformation, while fully host-specific factors caused both root hair deformation and induction of nodule meristems. The comparison of the effects of different Nod metabolites with slight chemical modifications will be important for understanding the mechanism of morphogenesis by Nod factors.

Biochemical studies have characterized the activities of Nod factor in *Medicago* (Niebel et al., 1997) Since root epidermis contact with Nod factor directly, it has been the focus of various studies on Nod factor signal depolarization (Fig. 1) (Felle et al., 1995; Pingret et al., 1998) and rapid intracellular alkalinization (Felle et al., 1995) in alfalfa. It was demonstrated that intracellular cytosolic calcium oscillation was triggered by Nod factor in alfalfa root hairs, and plateau-like increases of intracellular calcium (Gehring et al., 1997) was observed in cowpea root hairs in response to Nod factors. However, it is not yet clear whether the phenomena are part of a signal transduction pathway (Fig. 1) leading to symbiotic downstream events, such as root hair curling or plant-specific gene expression.

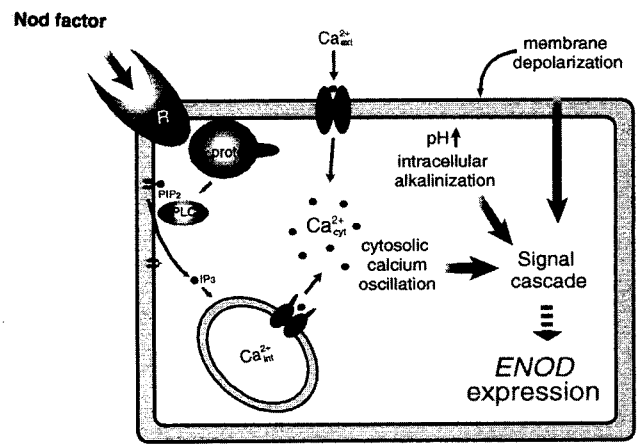


**Fig. 1.** Model for the signal transduction mediated by Nod factor at the early stage of nodulation. Modified from Pingret et al. (1998).

## Internalization of Bacteria into Host Cytoplasm

Successful infection requires internalization of rhizobia into cytoplasm of the host cells. Rhizobia in the infection thread are still outside of the host cells. Entry of the rhizobia occurs from unwalled regions of the infection thread by a process resembling endocytosis. In the infected cell, "unwalled droplets" are found at the tip or side of the infection thread (Newcomb, 1976; Verma and Long, 1983). The unwalled droplets appear to be formed by degradation of the infection thread wall mediated by cellulase and pectinase. Robertson and Lyttleton (1982) observed that the number of coated vesicles associated with the infection thread membrane near the tip of the thread was about 20-fold greater than that associated with the membranes towards the base of the thread.

Recent studies on the interaction of pathogenic bacteria with their animal hosts have revealed components of bacterial invasion and a possible mechanism of signal transduction (Bliska et al., 1993; Falkow, 1991). The enteropathogenic *Yersinia pseudotuberculosis* in its interaction with mammalian cells uses invasin (Monack et al., 1996) and YadA (an adhesin) as bacterial attachment and entry factors. YadA protein is encoded on the *Yersinia* and is solely responsible for the accumulation of actin and actin-associated proteins around the entering bacterium (Young et al., 1992). Latex beads coated with invasin become internalized, suggesting that invasin is sufficient for the entry process. Mammalian cell receptors are known to be integrins, which constitute a large family of α/β heterodimeric transmembrane proteins. Multiple members of the β1chain integrin family (α3β1, α4β1, α5β1, and α6β1) are bound by invasin. These proteins are involved in cell to cell interactions and cellular adhesions to Extracellular matrix (ECM) proteins (e.g. collagen, fibronectin and laminin) (Hynes, 1992). However, the signal transduction mechanism mediated by integrins is not known. Several outer membrane proteins of *Yersinia* (Yop) have been shown to have homologies to eukaryotic signal transduction proteins; YopH (protein tyrosine phosphatase; PTPase), YopM (glycoprotein Iba receptor), YpkA (Ser/Thr protein kinase) and YopP (effector) (Bliska et al., 1993; Mills et al., 1997). Studies on the interaction of these proteins may reveal the signal transduction mechanism for *Yersinia* internalization.

In the case of *Salmonella*-mammalian cell interactions, membrane ruffling occurs in the infected cells, accompanied by profound cytoskeletal rearrangements at bacteria-host cell contact point. A number of cytoskeletal proteins,

including actin, α-catinin, talin, tublin, tropomyosin, and ezrin, accumulate at these sites (Finlay et al., 1991). Membrane ruffling, cytoskeletal rearrangements and [$Ca^{++}$] influx caused by *Salmonella* attachment to the cell seems to occur as a consequence of the activation of a number of host cell surface receptors including the epidermal growth factor receptor (EGFR)(Moolenar et al., 1984; Rijken et al., 1991). Rac and Rho, small GTP-binding proteins, are also known to be involved in growth factor-induced membrane ruffling and cytoskeletal rearrangements in culture cells (Ridley and Hall, 1992; Tapon et al., 1997).

A study with ineffective rhizobial mutants (Morrison and Verma, 1987) provided insight into rhizobial internalization legumes. When the Tn5 mutant of *B. japonicum* was used to infect host plants, infection and nodule differentiation proceeded normally. However, bacteria were not released from the infection thread, and bacterial invasion was stopped. Nodules formed by the mutant were devoid of bacteria. The results suggest that the internalization process requires the involvement of both plant and rhizobial genes. cDNA's encoding legume homologs of mammalian Rab1 and Rab7 (Cheon et al., 1993) were isolated from soybean and *Vigna* (moth bean). Induction of *rab1* and *rab7* genes was examined by northern analysis. *rab7*, in particular, was found to be highly induced in young nodules. The antisense-*rab1* nodules had structurally unstable Peribacteroid membrane (PBM) compartments, resulting in frequent release of rhizobia into vacuoles. Nodules expressing antisense-*rab7* contained fewer PBM compartments and many late endosomal structures in the perinuclear region. Induction of Rab proteins in nodules which might be connected to the burst of cortical cell division may be shed a light into our understanding on the process of internalization of *Rhizobium*.

## Targeting of Nodulins to the PBM Compartment

Release of bacteria from the infection thread requires proliferation of PBM during early stages of the infection process and redirecting some of the plasma membrane protein and all of the PBM nodulins to this *de novo* formed subcellular compartment. Many differences were found in the protein profiles between the PBM harbors many new proteins and lacks some of the plasma membrane proteins to meet the requirements of symbiosis. According to Robertson and Lyttleton (1982), 20 times more vesicles fuse with the tip of the infection thread than with its base or with the plasma membrane. After the release of bacteria and their enclosure within the PBM, five times more vesicles fuse with the PBM than with the plasma membrane. Extensive membrane proliferation may be achieved by nodule-specific stimulation of many enzymes, including a new form of choline kinase (Mellor et al., 1986). Evidently the presence of bacteria redirects the transport of vesicles to their vicinity. In the same context, the subcellular localization of nodulin-24, nodulin-26 and other PBM nodulins indicates that they are targeted to the PBM following synthesis and are not incorporated in the plasma membranes (Cheon et al., 1994;

**Table 2.** Host-specificity found at the late stage of nodule development

| Bacteria | soybean | cowpea | siratro | pigeonpea | groudnut | *Lotus* tenius | *Lotus* pedunculatus |
|---|---|---|---|---|---|---|---|
| Wild type | | | | | | | |
| *R. loti* | | | | | | Nod$^+$ | Nod$^+$ |
| NZP2037 | | | | | Fix$^+$ | Fix$^+$ | |
| *R. loti* | | | | | Nod$^+$ | Nod$^+$ | |
| NZP2213 | | | | | Fix$^+$ | **Fix$^-$** | |
| *B. japonicum* | Nod$^+$ | Nod$^+$ | Nod$^+$ | | | | |
| USDA110 | Fix$^+$ | Fix$^+$ | Fix$^+$ | | | | |
| Mutants | | | | | | | |
| *B. japonicum* | Nod$^+$ | Nod$^+$ | Nod$^+$ | | | | |
| **NAD164** | Fix$^+$ | Fix$^+$ | Fix$^+$ | | | | |
| *B. japonicum* | Nod$^+$ | Nod$^+$ | Nod$^+$ | | | | |
| **NAD163** | Fix$^+$ | **Fix$^-$** | Fix$^+$ | | | | |
| *B. japonicum* | Nod$^+$ | Nod$^+$ | Nod$^+$ | | | | |
| AN218 | Fix$^+$ | **Fix$^-$** | Fix$^+$ | | | | |
| *B. japonicum* | Nod$^+$ | Nod$^+$ | Nod$^+$ | | | | |
| BjjC211 | Fix$^+$ | **Fix$^-$** | Fix$^+$ | | | | |
| *B. japonicum* | | | Nod$^+$ | Nod$^+$ | Nod$^+$ | | |
| Sp. NC92 | | | Fix$^+$ | **Fix$^-$** | Fix$^+$ | | |

Fortin et al., 1987; Miao et al., 1992).

However, the sequence comparison of the three PBM nodulins (nodulin-23, nodulin-24, and nodulin-26) has not identified a common features, either at the primary or at the secondary structure levels. Nodulin-23 and nodulin-24 have a cleavable amino-terminal signal sequence, while nodulin-26 does not (Cheon et al., 1994; Miao et al., 1992), although both nodulin-24 and nodulin-26 were shown to be co-translationally translocated. Based on a hydropathy plot, nodulin-24 lacks a putative membrane-spanning domain. In addition, nodulin-26 is induced prior to the endocytosis of rhizobia (Fortin et al., 1987). Quite possibly, the mechanism of targeting of these nodulins to the PBM may differ. Nodulin-26 may have a sorting mechanism that is similar to other vacuolar membrane proteins, such as vacuolar $H^+$-ATPase, or tonoplast intrinsic protein (TIP; Ludevid et al., 1992). In contrast, the targeting mechanism for nodulin-24 may be connected to its post-translational modification which is responsible for the size increase from 20 kD to 33 kD and its membrane attachment. The PBM acquires strongly acidic phospholipids that are not found in other endomembrane systems (Mellor et al., 1986). Interaction of PBM nodulins with these PBM specific phospholipids at the TGN may be responsible for their sorting to the PBM.

## Host-Specificity in Nitrogen Fixation

Bacterial host specificity which affects symbiotic nitrogen-fixation was reported. In this case, rhizobial strains form effective ($Nod^+$ $Fix^+$) nodules on a subset of the legumes but they form ineffective ($Nod^+$ $Fix^-$) nodules on other hosts (Bromfield and Barran, 1990). Table 2 shows various cases of host-specificity found at the late stage of nodulation. For example, *Rhizobium loti* strain NZP2037 has $Nod^+$ $Fix^+$ phenotype on both *Lotus tenuis* and *Lotus pedunculatus,* but NZP2213, another strain, has $Nod^+$ $Fix^-$ relationship with *L. tenuis* (Pankhurst et al., 1979). Bromfield and Barran (1990) found that although most legume plants (*Trigonella foenum-graecum* and *Phaseolus vulgaris)* nodulated, thirty-three isolates of indigenous *Rhizobium meliloti* induced symbiotically ineffective nodules.

Additionally, Tn*5*-induced mutants with altered exopolysaccharide production (*Rhizobium* strain NGR234) exhibited host-specificity at the late stage on nodulation(Chen et al., 1985); $Fix^+$ on some hosts but $Fix^-$ on others. *Bradyrhizobium* sp. (Arachis) strain NC92 was mutated with Tn*5* and its host range became to be ineffective symbiosis with pigeonpea, but effective symbiosis with groundnut and siratro (Wilson et al., 1987). Another Tn*5*-induced mutants of *Rhizobium loti* strain NZP2037 were $Fix^-$ on *L. pedunculatus* but $Fix^+$ on *L. corniculatas* (Ward et al., 1989).

In above cases of host-specificity, the inability of the rhizobia to form an $Fix^+$ nodule with a particular host must be related to a step subsequent to nodule induction. The trait involved must not affect the intrinsic ability of the rhizobia to fix nitrogen because the same bacteria can make $Fix^+$ nodules on some hosts. Recent discovery (Chun et al., 1994) of host-specific nitrogen fixation (*hsfA*) gene provides an excellent model to study the regulation of bacteroid development on plant nodules. The *hsfA* mutant induced effective, nitrogen-fixing nodules on soybean but produced ineffective nodules on cowpea plants. The *hsf*A gene is expressed only in bacteroids and not under free-living conditions. Therefore, the *hsfA* gene may have an upstream activator responsible for bacteroid-specific expression. It was thought that this gene may be essential for events after bacterial entry into plant cells.

## Conclusion

Early stage of nodulation has been known to determine the host-specificity in legume-*Rhizobium* symbiosis. Nod factor-mediated signal transduction in root hairs is one of the devoted area for studying the mechanism on establishing symbiotic relationship. Once symbiosis is set up between legume and *Rhizobium*, new nodulins are successfully expressed and transported to PBM compartment. However, before nitrogen fixation occurs, the adjustment of host and bacterial symbionts is to be made immediately after endocytosis, as phenotypic and molecular biological evidences show. At this stage, *hsfA* is believed to be expressed and essential for determining the host-specificity. There must be a set of genes which are involved in modifying each symbiotic partner and *hsfA* should be one of the genes needed especially for establishing host-specific relationship. Studying host-specificity at late stage will give us better understanding on this transition stage of nodulation and on how the late stage of nodulation is regulated.

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