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## Post Genomic Approaches to Nodulation in Soybean

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**An interaction between Legumes and Rhizobia establishes a symbiotic new organ, the nodule that supports atmospheric nitrogen fixation. The specific communications between the microbes and legume plants are necessary for both nodulation and nitrogen fixation. Through genetic and biochemical analyses several genes playing pivotal roles in nodulation had been identified to be a receptor kinase like CALVATA1 involved signal transduction for development. This emphasizes peptides as signals to be transmitted for a short or long distance transport for nodulation. In addition, a quorum sensing in rhizobia has become a focus as counterpart signal. In an attempt to reveal proteins factors and signaling molecules acting on nodulation, proteome analyses of nodule and the proteins in apoplast upon communication between Legumes and Rhizobia were performed.**

Nitrogen is one of the major elements to be supplied as a form of major fertilizer since nitrogen is demanded in a large quantity for crop production in order to enhance quality as well as yield. However the plants belonged to Legume family are able to establish a symbiont relationship with Rhizobium and to fix atmospheric nitrogen into the organic forms so that they can survive without any supply of nitrogen. The nitrogen fixation is started with an activation of Nod genes in Rhizobium by flavones released from Legumes and then an infection thread is formed through hairy root reaching to cortex cells where a nodule is formed. Within the nodule, Rhizobium is transformed into a bacteroid, an enlarged cell with thick cell wall and fixes nitrogen into ammonia. Nodulation is known to be under the control of the previously formed nodule such that a signal from nodule in the older root is transmitted to shoot where an inhibitor is released and suppresses nodule

emergence in the younger root. Grafting experiments had proved the mechanism of autoregulation by showing the presence of transmitting inhibitor working regardless of differences in species. The hypernodulation mutant was isolated based on nitrate tolerance in nodule formation and turned out to be a lack of such inhibitor synthesized in the shoot (Carroll et al., 1985; Lee et al., 1997; Szczyglowski et al., 1998; Wopereis et al., 2000). The genes involved in or responsible for nodulation were studied by genetic approaches using various mutants unable to form nodule, differential screening of nodule specific cDNAs, analyses of ESTs and proteome in nodulation. There have been many nodulins isolated but many of them remain to be identified. The genes responsible for signaling of nodulation had isolated from hypernodulating mutants and turned out to be a receptor kinase (Nishimura et al., 2002; Searle et al., 2003). The growing number of observations indicate an involvement of peptides as a signaling molecule in a short of intercellular transport and in a long distance between shoot and root to control development of the new organ, nodule. Quorum molecules regulating the size of microbe population was also effective on infection of Rhizobia and some Legume plants were shown to produce quorum molecules to regulate the symbiotic microbe (Loh et al., 2001; Gao et al., 2003). All these together may feature the significances of peptides as signal molecule within plant tissue and in between microbe and plant.

### In Search of the Genes Involved in Nodulation

The bacterial Nod factors consisting of beta-1,4-linked N-acetyl-D-glucosamine (GlcNAc) backbone of four or five residues are known to initiate nodulation in legume hosts by deforming of the root hair to be curled. Biochemical attempts to isolate a Nod factor receptor, Nod factor binding proteins were purified. A lectin-nucleotide phosphohydrolase (LNP) was shown to bind Nod factors and the antibody against LNP blocked root hair

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deformation (Etzler et al., 1999). In additions, cytoplasmic alkalinization and calcium spiking at the root hair tip were marked and will provide some clues to decipher what is going on at early stage of nodulation (Shaw and Long, 2003). Mastoporan, an agonist of G proteins system was known to mimic Nod factors by inducing root hair deformation. This indicates a possibility that Nod factors transmit their signal through G protein complexes. In fact PLC and PLD antagonists were known to stop root hair deformation (Den Hartog et al., 2001).

There had been several mutants reported to show malfunction in each step in nodulation of the legumes in response to Nod factors. Mutants called as Does not Make Infections (DMI1, 2, 3) had been isolated from *Medicago truncatula* and among them a DMI2 gene was shown to encode for Nodulation ReceptorLike Kinase (NORK) in alfalfa and pea and Symbiosis Receptor-Like Kinase (SYMRK) in Lotus. NORK consists of a typical Leucine-Rich Repeats (LRRs) and Ser/Thr kinase domain. Based on the structure, a NORK may involve in signal perception and signal transduction with regard to early response in nodulation (Limpens and Bisseling, 2003). Another mutant, Nodule-Signal Pathway (*nsp1*, 2) showing a reduced activation of cortical cells and infection in response to Nod factor and mutants, Hair Curling (*hc1*) and Nodule Inception (*nin*) also had been isolated by Ac transposon tagging to Lotus. NIN encodes for a transcription factor containing a typical dimerization domain as well as a DNA binding domain (Limpens and Bisseling, 2003).

During the progress of nodulation, up-regulated proteins were described as nodulins and a group of nodulins induced in early stage of nodulation are called as ENODs that are involved in cell division and production of particular cell wall components (Govers et al., 1987). However the structure and location of many ENODs remain to be elucidated and they are not related to any mutant known to be defective in nodulation.

### Autoregulation of Nodulation

The interaction between rhizobia microbes and legume plants result in development of a new organ called nodule capable of nitrogen fixation. A reprogramming in development of the root cortical cells upon the interaction is shown first with swelling and the curling of the root hair tip following entrapping of the microbes. The entrapped microbes form an infection thread and construct a nodule in a close communication to plant host. The molecular dialogue between two species leads to build up a symbiosis but to suppress any possible host defense response. The formation of nodule in most of leguminous plants are only active in a specific physiological state lacking of nitrogen

sources supplied or of nodules formed in the older part of the same root. The suppression of the nodule emergence from younger root tissue by previously formed in the older parts is called as autoregulation which limit the number of nodule per a root (Carroll et al., 1985; Lee et al., 1997; Szczyglowski et al., 1998; Wopereis et al., 2000). It appears that the formation of nodules is energetically so expensive that the strict control of the number of nodules is essential for legume to survive. It is hypothesized that a substance formed during nodulation is released from root and transported to shoot where it is converted into the shoot-derived inhibitor (SDI) (Caetano-Anolles and Gresshoff, 1991). The SDI moved back to root is thought to prevent new formation of nodule thus limiting the number of nodules in a root. The inhibition is known to be very rapid (Bhuvaneswari et al., 1980) and systemic (Caetano-Anolles and Gresshoff, 1991). The split-root system showed a state of strong autoregulation in a branch of root is apparent in 30 hours after inoculation of the bacteria in other branch of the same root (van Brussel et al., 2002). The autoregulation maintains a fixed number of nodules formed in a plant but hyper and supernodulating mutants were shown to be released from the regulation. The grafting and split root experiments indicated that the autoregulation is determined mainly by shoot not by root where the nodulation occurs (Abd-Alla, 1999; van Brussel et al., 2002). There must be long distance signals between shoot and root involved in the autoregulation but no relevant molecule was found yet.

Recently, the genes responsible for the autoregulation were isolated from several legumes including Soybean (GmNARK), Pea (PsNARK, Sym29) and Lotus japonicus (HAR1) by chromosome walking (Nishimura et al., 2002; Searle et al., 2002). They turned out to be a receptor kinase resembling of CLAVATA1 (CLV1) in Arabidopsis known to determine development of shoot meristem. It appears that these proteins receive a signal from the root and generate shoot derived inhibitor that is an autoregulation signal leading to suppress any new set of nodule formed (Van Brussel, et al., 2002). The GmNARK/HAR1 is a typical receptor like kinase (RLK) consisting of extracellular LRRs (19 in GmNARK and 21 in HAR1) and an intracellular serine/threonine kinase domain. Like CLAVATA1, the GmNARK has roles in regulation of dividing and differentiating cells, especially in nodulation in a long distance signaling from shoot to root. In the light of that GmNARK/HAR1-1 mutants showed an altered root phenotype without an inoculation of *Rhizobium*, the NARKs may have role in lateral root development (Gresshoff, 2003; Searle, 2003). It has been shown that the lateral root can be replaced by nodule, a switch in development of root hair to nodule is a key role of NARK. Based on analogy to CLAVATA working together with

CLV2 and CLV3, the NARK may have two other partners to interact for generation of a signal for autoregulation. Therefore the next focus to reveal the regulatory process in nodulation must be a small peptide such as CLV3 that assembling CLV1 and CLV2 together to generate the signal for development. The CLV3 gene encodes a small polypeptide of 96 amino acids expressed in stem cells of shoot and floral organs (Fletcher, 1999). The CLV3 control the fate of stem cells in meristemic center by form a ligand binding to CLV1 and CLV2 complex (Trochaud et al., 2000). Recent immunological assay revealed that CLV3 is localized in the extracellular space where acts as a ligand to CLV1/CLV2 receptor complex (Rojo et al., 2002). In facts, there are many peptides identified to play crucial roles in development in plants (Lindsey, 2002).

### The Peptide signals and Transporters

Many of the peptides found in phloem may have roles in N- or C-suppliers or conjugating partner for phytohormones to be translocated. In additions, there are increasing observations of the peptide as signal molecules. The first peptide identified to act as a signal in plant is systemins isolated from tomato (Pearce et al., 1991). The systemin has a role in a signal transduction with regard to a systemic response to wounding and it transmits signal in order for wounded tissue to induce a sets of genes encoding for systemic wound response proteins (SWRPs) (Bergey et al., 1996). The systemins of 18 amino acids was generated by processing of the proproteins of 200 amino acids. One of the early nodulation protein, ENOD40 may have roles in nodulation due to its location for accumulation such as the pericycle of legume roots just after initiating nodulation and an enhanced nodulation observed with the transgenic alfalfa with ENOD40 gene. There are two open reading frames of 13 and 27 amino acids long found in ENOD40 and the short peptides may interact with other factors in nodule. CLV3, a member of CLAVATA complex analogous to a putative NARK system for nodulation, is a significant peptide acting as a signal in association with receptor kinase. The CLV3 of 96 amino acids was known to induce assembly of CLV1 and CLV2 within membrane to form a heterodimer and to transmit a signal through kinase associated proteins phosphatase (KAPP) and rho-like GTPase (ROP) and MAP kinase (Carles and Fletcher, 2003). The peptide may be a suitable candidate for short or long distance signaling transmitted intercellularly as well as between different tissues such as shoot and root in case of nodulation.

There are 16 times more peptide transporters expected to exist in plants comparing to prokaryotes and other eukaryotes (Stacey et al., 2002). This may indicate that a

translocation of peptides plays significant roles in many aspects of plant system and their roles include not only simple translocation of energy and nutrient but also signaling for intercellular communication in either hormone conjugated form or peptides as a specific signal molecule. There are three types of peptide transporters have been reported; the first one is a ABC type (ATP-binding cassette) requiring energy for the action, the second are di- and tripeptide transporter and the third are tetra- and pentapeptide oligopeptide transporter (OPT). The latter two transporters are working in costs of proton motive force (Stacey et al., 2002).

There must be a great chance of involvement of peptides in signaling for nodulation and the partner for the peptide could be either NARK or NORL, or others unidentified yet. Recently a novel family of more than 300 genes encoding for small secreted polypeptides with conserved cysteine motifs has been found in *Medicago truncatula* and provide possibilities of their involvement in signaling for nodulation (Mergaert et al., 2003).

### Quorum sensing and Nodulation

Quorum sensing is a way for the cells to communicate themselves using of the self-produced signal molecules to control a population density of the same species (Loh et al., 2002). As quorum signals, either AHL (N-acylhomoserine-lactones) or post-translational modified peptides are used for bacteria belonging to gram negative or positive group, respectively (Loh et al., 2001). The population dependant regulation of nodulation genes in *Bradyrhizobium japonicum* suggested that nod genes are not only induced by flavonoids released from root of legume but also affected by a quorum molecule, Nola. The fact that no nodules were developed with an addition of Nola to rhizobial inoculum suggests that the quorum sensing may play a major regulatory role in soybean nodulation (Loh et al., 2001).

Besides, some plant including a model plant of legume, *Medicago truncatula* had shown to produce substances affecting bacterial quorum sensing and suggested a possibility of quorum sensing as a clue to elucidate a molecular interaction between legume and rhizobia (Gao et al., 2003).

### Proteome Approaches in Search of the Proteins for Nodulation

A proteome analysis of whole proteins expressed in a specific tissue of under a particular condition is ideal to dissect nodulation initiated from an interaction between legume and microbe. Comparative proteome analyses of

various mutants in nodulation will lead to understandings of the proteins involved in nodulation. In addition, phosphor-proteomics can be used to point out the altered status of signal proteins in terms of phosphorylation (Xing et al., 2002). As attempts to use a tool of proteomics for nodulation, there were a comparative proteome analysis of free-living bacterium versus bacteroid isolated from nodules (Natera et al., 2000) and a time course analysis of proteins induced during nodulation was performed in *Medicago truncatula* and soybean (Bestel-Corre et al., 2002; Hwang et al., unpublished). Cultivar specific interactions between Legume and Rhizobium were analyzed in comparative proteomics (Morris and Djodjevic, 2001) and proteome of peribacterial membrane in soybean nodule was also investigated (Panter et al., 2000). All these results had provided basic information on what is going on during nodulation but no particular protein with a specific role in nodulation was found yet. Comparative analyses of proteome of root and nodule in between hypernodulating mutant and its wild parent showed some proteins up or down-regulated in mutant root and nodule (Hwang, unpublished). Reciprocal graftings in between hypernodulating mutant and the wild type provide ideal comparisons and some clue to the proteins under autoregulation by the shoot. The proteins and peptides are analyzed in apoplast of the stem and the root in Legume after an inoculation of Rhizobium and the differential display of the apoplastic proteome in both xylem and phloem of the wild type and the mutant will show a presence of tentative signal molecules transmitted through the grafted area. The facts that there was a 0.1 to 0.4 mg/ml of proteins found in phloem sap of lupin and more than a 5% of total nitrogen in xylem sap is derived from peptide, suggest the complexity in apoplastic peptides. The proteome analysis of small sized peptide will be helpful to find signal molecules for nodulation.

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