CHEMICAL COMMUNICATION IN NODULE FORMATION FOR ATMOSPHERIC NITROGEN FIXATION IN LEGUMES

(KOMUNIKASI KIMIA DALAM PEMBENTUKAN BINTIL AKAR UNTUK MEREPUT NITROGEN DARI UDARA PADA TANAMAN LEGUM)

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ABSTRAK

Nitrogen yang direput dari udara oleh bintil akar tanaman legum merupakan sumber unsur hara penting dalam pertanian berkelanjutan. Bintil akar merupakan wujud simbiosis antara tanaman legum dengan Rhizobia (nama umum untuk spesies bakteri yang mampu membentuk bintil akar dan mengikat N dari udara). Keeberhasilan simbiosis ditentukan oleh keberhasilan komunikasi kimiawi timbal-balik antara kedua makro- dan mikro-simbion tersebut. Akar tanaman legum mengeluarkan senyawa flavonoid atau isoflavonoid ke dalam lingkungan rhizosfer; sementara itu, spesies dan strain rhizobia yang sesuai merespon keberadaan senyawa-senyawa ini dengan mengeluarkan senyawa lipochito-oligosaccharida yang sesuai pula. Keseuaian melekul-molekul kimia ini yang diproduksi oleh kedua makro- dan mikro-simbion inilah yang menyebabkan spesifikasi dan keluasan cakupan simbiosis. Molekul flavonoid yang sesuai mengakibatkan sel-sel Rhizobia bergerak ke arah dan menempel pada dinding rambut akar bagian ujung, menginfeksi akar dan masuk ke dalam jaringan meristem akar, membentuk bakteroid yang diselaputi oleh jaringan meristem, dan berkembang menjadi bintir akar tanaman legum yang berfungsi mereput nitrogen dari udara tanah. Makalah ini mereview proses pertukaran molekul sinyal, termasuk genetika pembentukan lipochito-oligosaccharida, dalam simbiosis untuk mereput nitrogen secara biologis, terutama pada simbiosis antara tanaman kedelai dan *Bradyrhizobium japonicum*.

ABSTRACT

Atmospheric nitrogens which are biologically fixed by root nodule of leguminous plants are essential nutrient source for sustainable agriculture. Root nodules is a realization of symbioses between legumious plants and Rhizobia (common name of bacteria which are capable of forming root nodule and fixing atmospheric N_2), which success is determined by precise exchange chemical communication between both macro- and micro-symbionts. Roots of legume plants excreeted flavonoid or isoflavonoid compounds into the rhizosphere; whereas species and strains rhizobia, in response to these signal compounds, produce and excrete lipochito-oligosaccharides. Matching between the molecules produced by these macro- and micro-symbionts determine the specificity and broadness of symbioses. The presence of suitable flavonoids attrachs rhizobia cells to attach in the hairy root tip zone, infect root and enter into meristematic cells, form bacteroids surrounded by root meristem which then develop to become functional root nodule which fix atmospheric nitrogen. This paper reviews process of molecular signal exchange, including the genetics of lipochito-oligosaccharide production, in the biological nitrogen fixation symbiosis, especially those occur between soybean and *Bradyrhizobium japonicum*.

1. Introduction

Farmers around the world replaced legume rotations and other traditional sources of nitrogen (N) fertilizer with synthetic N fertilizers produced by using Haber-Bosch process during the 20th century. A sizable percentage of farmers are now depending on synthetic N fertilizers for planting their crops. However, in recent decades, N fertilizers have been linked to numerous environmental hazards including marine eutrophication, global warming, groundwater contamination, and stratospheric ozone destruction. Some researchers suggest that legumes, which can support biological N_2 fixation, offer a more environmentally sound and sustainable source of N to cropping systems. The success of biological N_2 fixation relies upon matching between legume species planted and existing soil rhizobia (common name of bacteria, including those in the genus Rhizobium, Azorhizobium, Allorhizobium, Bradyrhizobium, and Mesorhizobium, which are capable of forming root nodule and fixing atmospheric N_2). Some existing rhizobia strains are often not a good nitrogen fixer. For this reason, inoculation with effective N_2-fixing Rhizobia sometimes needed for legume production.

Over the last century inoculants have been improved through selection of better carriers (both type and sterility), better strains of bacteria and application of higher cell numbers (Brockwell and Bottomley 1995; Deaker et al. 2004). Recent advances in understanding signaling between bacteria and plants offer further opportunities in inucation technology for improving N fixation symbiosis. Rhizobia-legume symbiotic interaction is among the better understood plant-microbe interactions. This interaction leads to organogenesis of the nitrogen fixing nodules. It is a highly specialized process and involves sophisticated molecular communication between the two symbiotic partners.

2. Plant-to-Rhizobia Signals - Flavonoids

During the initial events of Rhizobia-legume symbiosis, the host legume plant exuded low concentrations of plant-to-bacteria signal molecules which trigger the transcription of bacterial nodulation genes (Zaat et al. 1987), which subsequently produce lipochito-oligosaccagaride (LCO - *see below*). Flavonoids are the best understood group of *nod* gene inducer molecules in rhizobia-legume symbioses, and their role in *nod* gene induction has been widely studied. These *nod* gene inducing compounds have been shown to attract rhizobia (Currier and Strobel 1976; Caetano-Anolles et al. 1988). When these compounds are bound by rhizobial cells they induce transcription of rhizobial *nod* genes. Rhizobia, in turn, produce Nod factors – lipo-chiooligosaccharides (LCOs) – which act as bacteria-to-plant signal molecules.

Flavonoids are a diverse group of phenolic compounds, ubiquitously found in plants that serve a variety of ecological and physiological functions (Stafford, 1990; Mathesius et al. 1998). The term flavonoid applies to a general group comprised of the following subgroups: flavanols, flavonols, flavones, flavanones, and isoflavonoids.

In plants, flavonoids are biosynthesized from the precursor amino acid phenylalanine, which is a product of the shikimic acid pathway. Phenylalanine is converted into trans-cinnamic acid in the presence of PAL, the first committed step in this pathway. Cinnamic acid is the starting point of many secondary metabolites, leading to the production of condensed tannins, flavonoids, lignins, and simple phenolic compounds. The biosynthesis of flavonoids from the precursor L-phyenylalanine can be viewed as occurring via 3 sub-pathways: 1) the core phenylproponoid pathway, consisting of enzymatic reactions from phenylalanine to 4-coumaroyl coenzyme A, (CoA); 2) the flavonoid synthesis pathway comprised of steps leading from the conversion of 4-coumaroyl CoA and malonyl CoA to a flavanone intermediate; and 3) the isoflavonoid-specific branch pathway leading to the biosynthesis of isoflavonoids from the flavanone intermediate molecules. The enzyme isoflavone synthase catalyzes the first reaction specific to isoflavonoid biosynthesis (Yu et al. 2000; Broughton et al. 2003). The isoflavone biosynthetic pathway is a specialized branch pathway restricted to legumes (Perret et al. 2000).

Flavonoids are stored inside the plant cell vacuoles after glycosylation or malonylation. Before exudation into the rhizosphere, the non-flavonoid moieties are removed through hydrolysis; however, glycoside forms of flavonoids are also found in the rhizosphere. The flavonoid moiety is the active *nod* gene inducer (Hungria et al. 1991) while the glycoside form of flavonoids sometimes looses *nod* gene induction activity. For instance, the alfalfa seed coat contains high levels of luteolin-7-O-glucoside, which is not active in *nod* gene induction, however, the aglycone form, luteolin, is active (Hartwig and Phillips 1991).

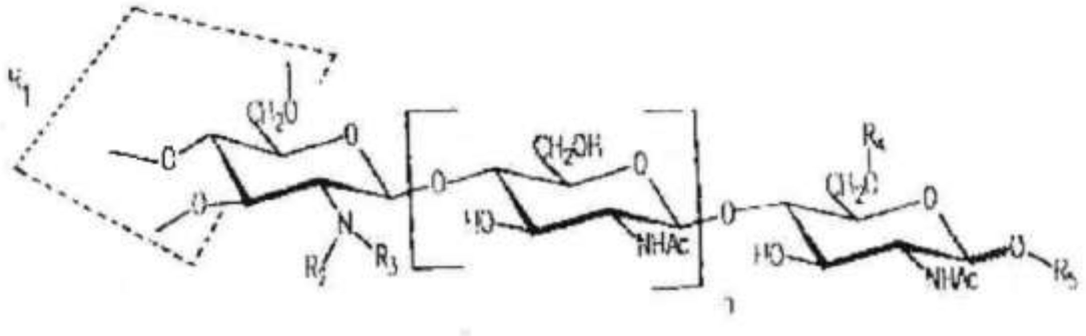
Not all flavonoids induce *nod* genes in all rhizobia; specific flavonoids induce *nod* genes in specific rhizobia. The exact nature of this specific interaction is not fully understood, however, it is generally accepted that the interaction between the flavonoid and the regulatory rhizobial Nod proteins determine this specificity. Soybean produces two major *nod* gene inducing isoflavones genistein and diadzein (Kosslak et al. 1987), which induce the transcription of *nod* genes in *Bradyrhizobium japonicum*. Alfalfa seeds release two major flavonoid *nod* gene inducer molecule, luteolin and chrysoeriol, which act as signals for *Sinorhizobium meliloti* (Hartwig et al. 1990). Common bean produces a wide range of *nod* gene inducer molecules, including anthocyanidins, flavonols, isoflavonoids, and flavanones. However genistein and naringenin are the best inducers of the *nod* genes in *Rhizobium leguminosarum* bv. Phaseoli (Hungria et al. 1992). Naringenin and hesperatin are potent *nod* gene inducers of *Rhizobium leguminosarum* bv. Viceae (Begum et al. 2001b).

3. Rhizobia-to-Plant Signals - Lipochito-oligosaccharides

Lipochito-oligosaccharides (LCOs), also known as Nod factors, are produced through the transcription and translation of *nod*, *nol* and *noe* genes, – collectively referred to as the *nod* genes. LCO biosynthesis begins with the constitutively expressed *nodD* genes. In the presence of appropriate flavonoids/isoflavonoids in the rhizosphere, the nodD protein binds the correct flavonoid and this binds to the *nod* box promoter, which induces DNA bending, leading to *nod* gene transcription (Spaink 2000). There are 7 to 9 *nod* boxes in *B. japonicum*, *Mesorhizobium loti* and *S. meliloti*, 15 in *Rhisobium etli*, and 19 in *Rhizobium sp.* NGR234 (Kaneko et al. 2000, 2002; Galibert et al. 2001; Kobayashi et al.

2004). The *nod* genes (*nod, noe, nol*) encode the synthesis of Nod factors, which are key signal molecules in symbiont recognition and nodule formation on legume roots. The chemical structure of a Nod factors was first identified by Larouge et al. (1990) from *S. meliloti* culture filtrate. Since then, Nod factors from other rhizobia have been characterized (see reviews by Hungria and Stacey 1997; Hanin et al. 1999). All Nod factors are composed of a backbone of three to five 1,4-β-linked N-acetylglucosamine units that is N-acylated at the terminal non-reducing glucosaminosyl residue; hence they are called lipo-chitooligosaccharides (LCOs). Variation among LCOs includes (1) length of the chitin backbone (3 to 6 N-acetylglucosamine units), (2) the nature of the lipid chain (saturated, unsaturated, α/β unsaturated or ω-1 hydroxylated fatty acids), (3) a number of specific components attached to the chitin backbone, including acetyl-, carbamoyl-, fucosyl-, glyceryl-, hydroxyl-, mannosyl-, and suphyl-groups (Hanin et al. 1999).

Figure 1. Summary of Nod factors produced by *B. japonicum and B. elkanii* (modified from Stacey et al. 1995). H = hydrogen, Ac = acetyl, Cb = carbamoil, Methyl, Fuc = Fucose, N = nitrogen, Gro = glycerol.



Nod metabolite	R_1	R_2	R_3	R_4	R_5	n	$(M+H)^+$
***B. japonicum* strain 110**							
NodBj-V($C_{18:1}$, MeFuc)	**H**	$C_{18:1}$	**H**	**2-O-MeFuc**	**H**	**3**	**1416**
***B. japonicum* strain 135**							
NodBj-V($C_{18:1}$, MeFuc)	**H**	$C_{18:1}$	**H**	**2-O-MeFuc**	**H**	**3**	**1416**
NodBj-V(Ac,$C_{18:1}$,MeFuc)	**Ac**	$C_{18:1}$	**H**	**2-O-MeFuc**	**H**	**3**	**1458**
NodBj-V($C_{16:0}$, MeFuc)	**H**	$C_{16:0}$	**H**	**2-O-MeFuc**	**H**	**3**	**1390**

NodBj-V(Ac,$C_{16:0}$, MeFuc)	**Ac**	**$C_{16:0}$**	**H**	**2-O-MeFuc**	**H**	**3**	**1432**
NodBj-V($C_{16:1}$, MeFuc)	**H**	**$C_{16:1}$**	**H**	**2-O-MeFuc**	**H**	**3**	**1388**
***B. elkanii* strain 61**							
NodBj-V($C_{18:1}$MeFuc)	**H**	**$C_{18:1}$**	**H**	**2-O-MeFuc**	**H**	**3**	**1416**
NodBj-V(Ac,$C_{18:1}$,MeFuc)	**Ac**	**$C_{18:1}$**	**H**	**2-O-MeFuc**	**H**	**3**	**1458**
NodBj-V(Cb,$C_{18:1}$,NMe,MeFuc)	**Cb**	**$C_{18:1}$**	**Me**	**2-O-MeFuc**	**H**	**3**	**1473**
NodBj-V(Ac,Cb,$C_{18:1}$,MeFuc)	**Ac,Cb**	**$C_{18:1}$**	**H**	**2-O-MeFuc**	**H**	**3**	**1501**
NodBj-IV($C_{18:1}$, MeFuc)	**H**	**$C_{18:1}$**	**H**	**2-O-MeFuc**	**H**	**2**	**1213**
NodBj-IV(Cb,$C_{18:1}$,MeFuc)	**Cb**	**$C_{18:1}$**	**H**	**2-O-MeFuc**	**H**	**2**	**1256**
NodBj-IV($C_{18:1}$,Fuc,Gro)	**H**	**$C_{18:1}$**	**H**	**Fuc**	**Gro**	**2**	**1273**
NodBj-IV($C_{18:1}$,NMe,Fuc,Gro)	**H**	**$C_{18:1}$**	**Me**	**Fuc**	**Gro**	**2**	**1287**
NodBj-IV(Cb,$C_{18:1}$, Fuc,Gro)	**Cb**	**$C_{18:1}$**	**H**	**Fuc**	**Gro**	**2**	**1316**
NodBj-IV(Cb,$C_{18:1}$, NMe,Fuc,Gro)	**Cb**	**$C_{18:1}$**	**Me**	**Fuc**	**Gro**	**2**	**1330**

Each rhizobial strain produces a structurally diverse mixture of LCOs which play a role in determining its host-range. Broad host range *R. fredii* strain USDA257 can infect 72 legume genera, which are a subset of the 112 genera infected by *R. fredii* strain NGR234, which also nodulates the non-legume *Parasponia andersonii* (Puppeke and Broughton 1999). In addition, NGR234 produces 40 fold more LCO than USDA257 (Relić et al. 1994). Puppeke and Broughton (1999) suggest that total Nod factor level and the levels of various specific LCOs is responsible for the nodulation patterns of rhizobial species. Nod factors produced by *B. japonicum* strains USDA110 and USDA135 and *B. elkanii* USDA61 have substitutions in the non-reducing end consisting of an acetyl and/or carbamoyl groups (Stacey et al. 1995). The acetyl group of NodBj-V(Ac,$C_{18:1}$,MeFuc) is located at C-6, whereas the carbamoyl group can be at C-3, C-4 or C-6.

4. Physiological Responses of Host Plants to LCOs

Flavonoid/isoflavonoid or jasmonate pre-induced rhizobial inoculants increase the production of LCOs leading to improved legume nodulation. Application of LCOs, at sub-micromolar concentrations, induce physiological and morphological changes in host roots, leading to the formation of a novel plant organ, the nodule, where nitrogen fixation occurs (see reviews Spaink 1996; Hungria and Stacey 1997; Gage 2004; Mulder et al. 2005). Physiological responses elicited by LCOs include increases in ion efflux (including H^+, Cl^-, and K^+) and Ca^{2+} influx across the root hair plasma membrane, within seconds of LCO application (Allen et al. 1994; Felle et al. 1998), depolarization of the cytoplasmic membrane potential within two minutes (Ehrhardt et al., 1992, Felle et al. 1995), and induction of cytoplamic streaming, deformation and reinitiation of polar tip growth (Heidstra et al. 1994). Morphological responses of host plants to LCOs are related either to rhizobial infection (e.g. hair deformation at concentrations as low as 10^{-12} M, Prithiviraj et al. 2000) or nodule organogenesis (e.g. cortical cell activation at concentrations as low as 10^{-9} M, Truchet et al. 1991). Vernoud et al. (1999) found that LCOs at concentrations as low as 10^{-11} M trigger early nodulin gene expression; MtENOD20 transcription (which is tightly linked to cortical cell activation) is induced within 12-24 h in the root cortex. Root swelling can be visible 7-10 days after rhizobial inoculation. We also found that application of LCOs into hydrophonic solution increases root Ca^{2+} uptake by soybean seedlings when observed in the leaves (Supanjani et al. 2006).

5. Agricultural Significances

Both flavonoids and LCOs have been exploited to improve the quality of Rhizobial innoculant. Flavonoids are important signal molecules, essential during the early stages of nodulation. They induce the transcription of rhizobial nodulation genes thereby inducing the biosynthesis of Nod factors, the bacteria-to-plant signal molecules. Genistein and diadzein are the major *nod* gene inducer isoflavonoid signal molecules and their availability in the rhizosphere may remain below threshold levels under certain stressful environmental conditions, for instance low root zone temperatures (RZT) (Zhang and Smith 1996a). Thus application of flavonoids to rhizobia might ameliorate the negative effect of environmental factors and promote legume nodulation and nitrogen fixation. Zhang and Smith (1995) reported that pre-incubation of *B. japonicum* cells with genistein increased soybean nodulation and nitrogen fixation when grown under low RZTs. Genistein applied onto seeds or in open furrows during seed sowing, also promoted soybean nodulation and seasonal nitrogen fixation under low soil temperature conditions (Zhang and Smith 1996b; 1997). Pre-incubation of *B. japonicum* with genistein accelerated the early stages of nodulation (infection, infection thread formation), nodule development and the onset of nitrogen fixation (Pan and Smith, 2000).

Soybean yield increases have been documented in Canada and USA (Leibovitch et al. 2001).

When rhizobial cultures induced with specific flavonoids are used to inoculate their legume hosts under low RZT conditions, it partially alleviates the low RZT inhibitory effects. For instance, when pea and lentil were inoculated with hesperetin induced *Rhizobium legumninosarum* bv. viceae, nodulation and early plant growth were increased under greenhouse conditions (Begum et al. 2001a). When *Rhizobium legumninosarum* bv. Viceae pre-incubated with naringenin or hesperetin and inoculated onto pea plants or naringenin and hesperetin directly applied to the soil at sowing promoted pea nodulation and plant growth under field conditions (Begum et al. 2001b). The role of naringenin induced *R. leguuminosarum* bv. viceae in promoting pea growth and development has also previously been documented (Bandyopadhyay et al. 1996; Novak et al. 2002). Lira Junior et al. (2003) reported that the promotive effect of addition of either hesperetin or naringenin was more pronounced when the pre-incubated inoculant was applied under saline or low root zone tempertature conditions.

On the other hand, LCOs have been exploited for improving inoculant quality. Nitragin Inc. has recently introduced a product, Optimize, that is comprised of *B. japonicum* cells and LCOs, demonstrating the potential for these signals in promotion of legume nitrogen fixation and in the promotion of plant growth in general (Smith 2005). A second company, Agribiotics Inc., is about to release products based on LCO stimulation of growth in a wide range of non-legume crops (McIver 2005). However, it is clear that there is much to learn regarding the regulation of their production and the full scope of their utility.

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