การวิเคราะห์หน้าที่ของยีน *nod* และระบบ Type 3 secretion ใน *Bradyrhizobium* สายพันธุ์ DOA9 ที่เกี่ยวข้องกับการเข้าสร้างปมกับพืชตระกูลถั่ว

นางสาวผ่องพรรณ ทรงวัฒนา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

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ปีการศึกษา 2559

FUNCTIONAL ANALYSES OF NOD GENES AND TYPE 3 SECRETION SYSTEMS IN *BRADYRHIZOBIUM* SP. DOA9 INVOLVED IN LEGUME NODULATION

Pongpan Songwattana

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biotechnology

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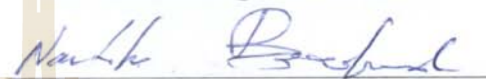
FUNCTIONAL ANALYSES OF NOD GENES AND TYPE 3 SECRETION SYSTEM IN *BRADYRHIZOBIUM* SP. DOA9 INVOLVED IN LEGUME NODULATION

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

Thesis Examining Committee



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แบคทีเรีย *Bradyrhizobium* sp. DOA9 ที่แยกได้จากปมรากของพืชตระกูลถั่ว *Aeschynomene americana* นำไปทดสอบการสร้างปม และการตรึงไนโตรเจนกับพืชตระกูลถั่ว ในวงศ์ Palbergioids Millitioid และ Genistioids พบว่าสามารถสร้างปม และตรึงไนโตรเจนได้ทั้งหมด เมื่อทำการวิเคราะห์ชุดยีนที่ควบคุมการสร้างปม (*nod*) โดยใช้เทคนิค Southern blot hybridization พบว่า DOA9 มีพลาสมิดขนาดใหญ่ 736,085 bp ที่มีชุดยีนควบคุมการอยู่ร่วมกันแบบพึ่งพาอาศัยกันและกัน โดยมียีน *nodA* จำนวน 2 ยีนที่แตกต่างกัน ($nodA_1$ และ $nodA_2$) และยีนเดี่ยวของยีน *nodB* *nodC* และ *nifH* และยังพบว่ามี *nifH* อีกหนึ่งชุดยีนอยู่บนโครโมโซมอีกด้วย ยีน $nodA_1$ พบในบริเวณใกล้กับยีน *nodBC* ส่วนยีน $nodA_2$ พบที่ด้านหน้าของยีน *nodIJ* สายพันธุ์กลายของยีน $nodA_2$ พบว่าสูญเสียความสามารถในการชักนำให้เกิดปมในทุกพืชที่ใช้ทดสอบอย่างสิ้นเชิง โดยลักษณะที่แสดงออกดังกล่าว มีลักษณะเช่นเดียวกับเชื้อสายพันธุ์กลายของยีน *nodB* ในทางตรงกันข้าม เมื่อทำให้ $nodA_1$ กลายพันธุ์ พบว่ามีผลต่อลักษณะที่แสดงออกแตกต่างกันไปตามชนิดของพืชเจ้าบ้าน กลุ่มแรก สายพันธุ์กลายของยีน $nodA_1$ ไม่ส่งผลกระทบต่อการสร้างปมกับถั่ว *Aeschynomene* (*A. americana A. afraspera*) *Indigofera tinctoria* และ *Desmodium tortuosum* กลุ่มที่สอง พบว่าลดความสามารถการสร้างปมกับถั่ว *Arachis hypogaea* cv. Thai Nan *Macroptilium atropurpureum* และ *Stylosanthes hamata* และเมื่อนำสายพันธุ์กลายมาเติมเต็มด้วยยีน *nodA1* (pMG103-nodA1) พบว่า สามารถคืนประสิทธิภาพการสร้างปมกับ *M. atropurpureum* และ *S. hamata* ได้ ผลเหล่านี้แสดงให้เห็นว่ายีน $nodA_1$ และ $nodA_2$ ทำหน้าที่แตกต่างกัน และอาจส่งในการเพิ่มความหลากหลายของ nod-factors acyl chain ซึ่งอาจทำให้เกิดความหลากหลายในการเข้าอาศัยในพืชให้มากขึ้น

สายพันธุ์กลายของระบบ Type 3 secretion (Δ*rhcN*) ส่งผลกระทบเล็กน้อยต่อการสร้างปมในถั่วต่าง ๆ ยกเว้นการสร้างปมกับถั่ว *Vigna radiata* cv. SUT4 และ *Crotalaria juncea* โดยพบว่าจำนวน และลักษณะปมดีขึ้นจากสายพันธุ์กลาย Δ*rhcN* ผลทั้งหมดนี้ชี้ให้เห็นว่า การที่ DOA9 มียีน

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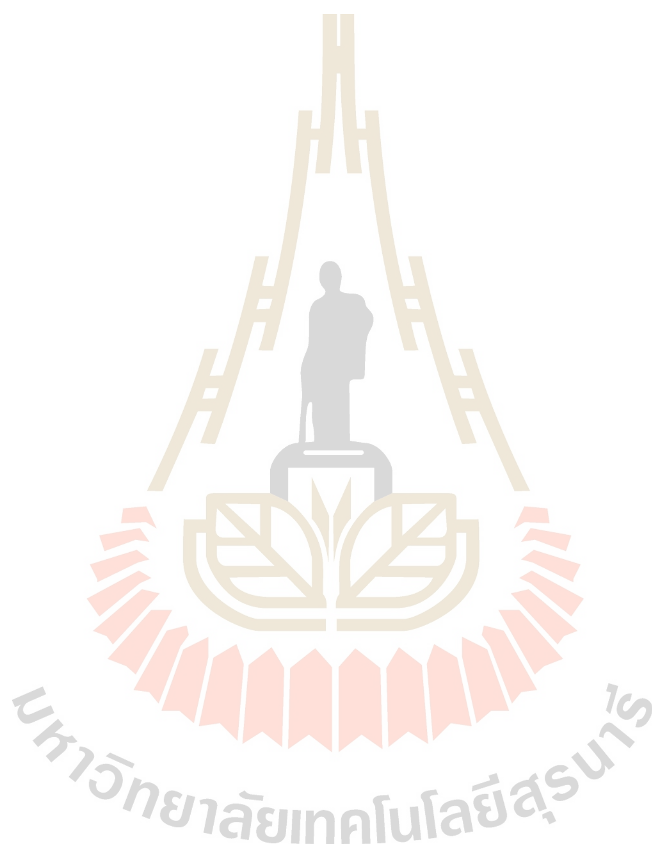
nodA 2 ชุด ที่แตกต่างกัน สนับสนุนให้แบคทีเรียสามารถเข้าสร้างปมกับพืชอาศัยที่หลากหลาย ในขณะที่ T3SS อาจจะกระตุ้นระบบภูมิคุ้มกันของพืชอาศัย โดยขึ้นกับสายพันธุ์ของพืชนั้นๆ



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ลายมือชื่ออาจารย์ที่ปรึกษาร่วม______________
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม______________

PONGPAN SONGWATTANA : FUNCTIONAL ANALYSES OF NOD GENES AND TYPE 3 SECRETION SYSTEMS IN *BRADYRHIZOBIUM* SP. DOA9 INVOLVED IN LEGUME NODULATION. THESIS ADVISOR : PROF. NEUNG TEAUMROONG, Dr.rer.nat. 81 PP.

NODULATION/NOD GENES/TYPE 3 SECRETION SYSTEM

The *Bradyrhizobium* sp. DOA9 was originally isolated from the root nodules of the *Aeschynomene americana*, which is able to induce nitrogen-fixing nodules on legumes belonging to Dalbergioid, Milletioid and Genistioids tribes. In order to analyze the symbiotic genes in DOA9, Southern blot hybridization was employed. The unique property of DOA9 DNA was observed. DOA9 contains megaplasmid (736,085-bp) which comprises two different copies of *nodA* genes ($nodA_1$ and $nodA_2$), a single of *nodB* and *nodC* genes, and a single copy of *nifH*. Another one copy of *nifH* was found on the chromosome. $NodA_1$ is found at the vicinity of the *nodBC* genes, and $nodA_2$ was found just upstream the *nodIJ* genes. Mutation in $nodA_2$ gene completely lost the ability to induce nodulation in all plants tested. These phenotypes were also observed in *nodB* mutant strain. In contrast, mutation in $nodA_1$ led to distinct phenotypes according to the host plant species. Firstly, no effect of the $nodA_1$ mutation on nodulations of *Aeschynomene* (*A. americana* and *A. afraspera*), *Indigofera tinctoria* and *Desmodium tortuosum* was found. Secondly, $nodA_1$ mutation drastically decreased the ability to form nodules on *Arachis hypogaea* cv. Thai Nan, *Macroptilium atropurpureum* and *Stylosanthes hamata.* The complementation of $nodA_1$ strain (pMG103-nodA1) was able to restore the nodulation ability in *M.*
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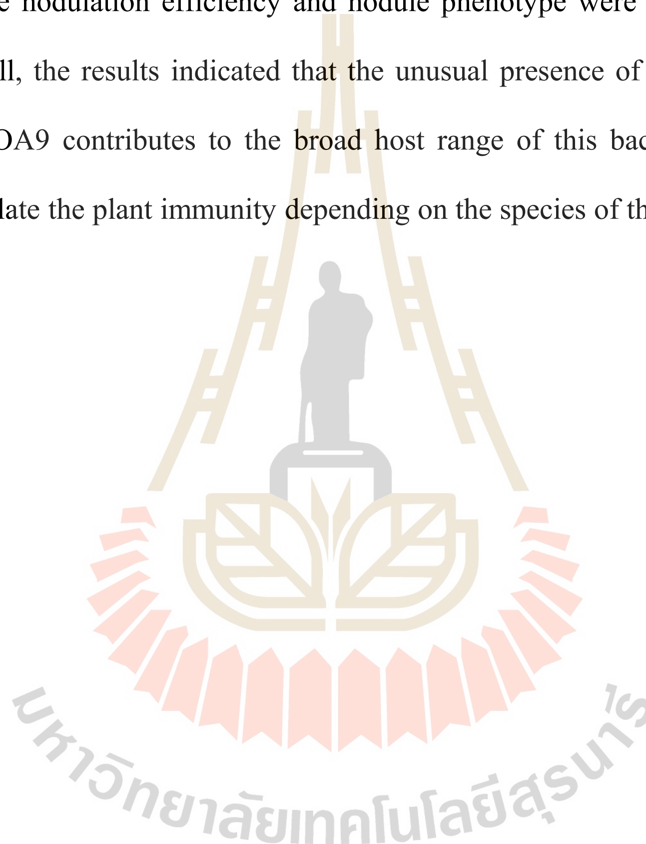
atropurpureum and *S. hamata* inoculation. These results implied that the divergent *nodA*$_1$ and *nodA*$_2$ were likely to expand the diversity of Nod-factor acyl chains, and might broaden the host range of the DOA9.

The Type 3 secretion system (T3SS) mutation (Δ*rhcN*) slightly affected the nodule formation except for those nodulating *Vigna radiata* cv. SUT4 and *Crotalaria juncea*. The nodulation efficiency and nodule phenotype were improved by Δ*rhcN* strain. In all, the results indicated that the unusual presence of two divergent *nodA* genes in DOA9 contributes to the broad host range of this bacterium, while T3SS might stimulate the plant immunity depending on the species of the plant.



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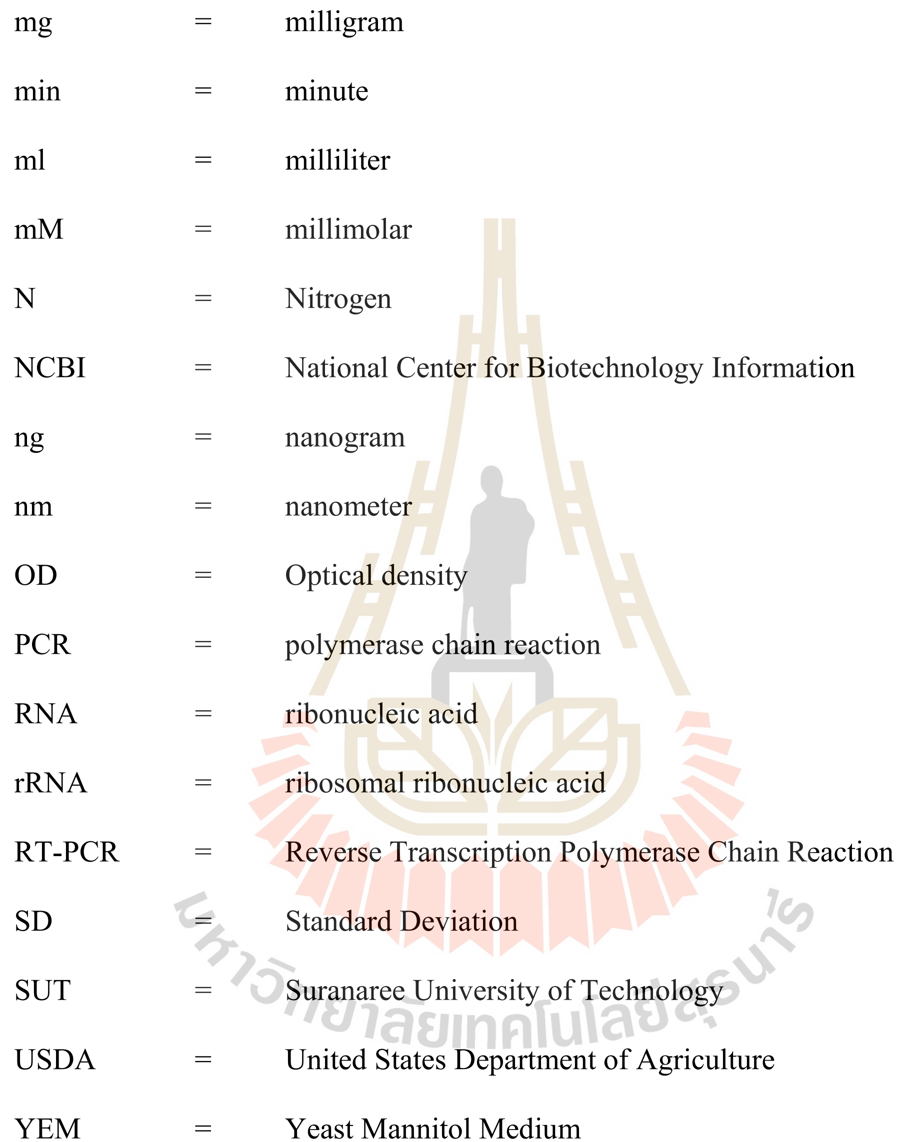
LIST OF ABBREVIATIONS

°C	=	degree Celsius
µm	=	micrometer
µg	=	microgram
µl	=	microlitre
ANOVA	=	Analysis of variance
ARA	=	Acethylene Reduction Assay
NFs	=	Nod-factors
NFR	=	Nod-factors receptor
bp	=	base pair
CFU	=	Colony-forming unit
dai	=	Days after inoculation
DNA	=	deoxyribonucleic acid
dNTP	=	deoxynucleotide 5' triphosphate
DOA	=	Department of Agricultural
et al.	=	Et alia (and other)
g	=	gram
h	=	hour
kb	=	kilobases
l	=	litre
LB	=	Luria Bertani broth
M	=	molarity

LIST OF ABBREVIATIONS (Continued)



mg	=	milligram
min	=	minute
ml	=	milliliter
mM	=	millimolar
N	=	Nitrogen
NCBI	=	National Center for Biotechnology Information
ng	=	nanogram
nm	=	nanometer
OD	=	Optical density
PCR	=	polymerase chain reaction
RNA	=	ribonucleic acid
rRNA	=	ribosomal ribonucleic acid
RT-PCR	=	Reverse Transcription Polymerase Chain Reaction
SD	=	Standard Deviation
SUT	=	Suranaree University of Technology
USDA	=	United States Department of Agriculture
YEM	=	Yeast Mannitol Medium

CHAPTER I

INTRODUCTION

1.1 Significances of this study

The nitrogen-fixing rhizobia are able to induce the symbiotic association with roots of legumes. The symbiotic interaction is initiated by the activation in response to rhizobial signaling molecules called nodulation factors (NFs). The NFs perception by receptor-like kinase in the plant epidermis of root hair induces several early symbiotic responses, such as calcium spiking, root hair curing deformation, cortical cell division and formation of an infection thread that guides the bacteria to the emerging primordium (Masson-Boivin et al., 2009). NFs consist of an oligomerlic backbone of β1-4 linked N-acetylglucosamine residues with N-linked acyl groups at the non-reducing terminal residue and other host specific decoration. The production of the NFs backbone is controlled under the function of common nodulation (*nod*) genes, *nodA* (acyl treansferase), *nodB* (deacetylase), and *nodC* (N-acetylglucosaminyl transferase). The additional modification of different chemical groups to the core structure is encoded by other host-specificity *nod* genes (e.g. *nodH*, *nodG*, *nodEF*, *nodPQ*, *nodRL*) (Janczarek et al., 2015). The expressions of genes in *nod* cluster are controlled by transcriptional regulatory genes (such as *nod*) in the present of suitable plant flavonoids (del Cerro et al., 2015; Gillette and Elkan, 1996; Hungria et al., 1992). Moreover, the compatible between plant receptors (known as nod-factors

receptor; NFR) and NFs from rhizobium are also important for stimulating symbiotic respond on early state of rhizobium-legume interaction.

The symbiotic relationship between *Bradyrhizabium* and *Aeschynomene* species have been classified into three groups based on their cross-inoculation (CI) ability to form stem and root nodules. Members in CI group I (e.g. *A. americana*) are nodulated by non-photosynthetic bradyrhizobia only on their roots. While, either non- or photosynthetic bradyrhizobia are able to form stem nodules with members of CI group II (e.g. *A.afraspera*) and III (e.g. *A. indica*). In case of photosynthetic bradyrhizobia (BTAi1 and ORS278), which have a narrow host range with only member CI group III because they do not contain classical *nod* genes required for the early stages of symbiotic nodule formation, but they used crack entry or nod-independent interaction instead (Miche et al., 2010). Whereas, *nod*-dependent interaction is a classical symbiotic model and it is also used by *Bradyrhizobium* nodulating *Aeschynomene* CI group I and II (Bonaldi et al., 2010 and Miche et al., 2010). But not all, Type III secretion system (T3SS) in *B. elkanii* strain USDA61 is a key factor for both of *nod*-dependent and -independent *Aeschynomene* invasion and also used as alternative way for soybean infection (Okazaki et al., 2013 and 2015). T3SS in *Sinorrhizobium fredii* HH103 induced rhizobial infection in the soybean (Francisco Javier López-Baena, 2008). *B. diazoefficiens* T3SS plays a crucial role for initiation of infection in siratro and soybean (Krause et al., 2002). While, T3SS of *S. mellioti* NGR234 impacted the nodulation ability in many tropical legumes (Viprey et al., 1998).

Bradyrhizobium sp. DOA9 was originally isolated from *A. americana* nodules from Thailand. It has a highly divergence of nodulaion (*nod*) genes (Noisangiam et

al., 2012) when compared with other members of the genus. Moreover, DOA9 could establish symbiosis with several plants of Papilionoideae and also showed the endophytic ability in rice (Teamtisong et al., 2014). Unlike other Bradyrhizobial strains, DOA9 lacks the ability to nodulate soybean roots. The genome of DOA9 consists of a single chromosome (7.1 Mb) and a symbiotic megaplasmid, pDOA9 (0.7 Mb). This is the first discovery of the symbiotic-gene localization on bradyrhizobial plasmid. The genome analysis of strain DOA9 showed highest similarity with that the chromosome of the non-photosynthetic *B. diazoefficiens* USDA110 in term of gene content. While, pDOA9 component is mostly belong to photosynthetic *Bradyrhizobium* sp. pBTAi1. Unlike in other bradyrhizobia, the plasmid encoded genes with symbiosis-related functions including roles in nodulation (*nod*), nitrogen fixation (*nif* and *fix* genes), and type III/IV protein-secretion systems (T3SS and T4SS). Interestingly, pDOA9 contains two copies of *nodA* genes. The $nodA_1$ gene is classically located upstream the *nodBC* genes and $nodA_2$ gene is found just upstream of the *nodIJ* genes.

Therefore, the acquisition of divergent *nod* genes and secretion systems may broaden the host range of DOA9. The analysis of symbiotic genes involved in infection process of divergent-*nod Bradyrhizobium* sp. DOA9 could be attractive. This study, we demonstrated the function of divergent-*nod* genes and other infection-related genes such as T3SS to provide more understanding of infection pathways as well as broad host range characteristics in leguminous plants.

1.2 Hypothesis

A. The divergent of *nod* genes in *Bradyrhizobium* strain DOA9 may broaden the host range in leguminous hosts

B. The T3SS in DOA9 may play an important role in nodulation process by trigger the plant defense response

1.3 Objective

1.3.1 General objectives

A. To demonstrate the localization and copy number of nodulation genes (*nodA*, *nodB* and *nodC*) and nitrogen fixation (*nifH*) on megaplasmid (pDOA9) and chromosome (cDOA9) of *Bradyrhizobium* strain DOA9

B. To understand the nodulation phenotype of *Bradyrhizobium* strain DOA9 in all leguminous hosts under the respond of the lacking of nod-factor (NFs) and type 3 secretion system (T3SS).

1.3.2 Specific objectives

A. To demonstrate the localization and copy numbers of common *nod* genes (*nodA*, *nodB* and *nodC*) and *nifH* on pDOA9 and cDOA9 by using Southern blot hybridization

B. To evaluate the nodulation phenotypes of DOA9 with leguminous hosts by inoculated with *nod* (*nodA*$_1$, *nodA*$_2$ or *nodB*) and T3SS (*rhcN*) mutant strains.

C. To understand the nodulation mechanisms by observation the gene expression of common *nod* genes and T3SS in respond to flavonoids and root exudates induction

CHAPTER II

LITERATURE REVIEWS

2.1 *Bradyrhizobium* spp. and their host range

Bradyrhizobium spp. is a nitrogen-fixing bacteria belonging to α-proteobacteria and establishing a nitrogen-fixing symbiosis in roots or stems of leguminous plants. This infection causes the formation of a new organ (nodule). The host range of this strain has been defined, such as soybean (Rumjanek et al., 1993), cowpea (Thies et al., 1991), and groundnut (Gronemeyer et al., 2014; Somasegaran et al., 1990). Some *Bradyrhizobium* strain can grow as heterotrophs, autrothrops, phototrophs and grow symbiotically with stem of the plant genus *Aeschynomene* (Giraud et al., 2007).

The host specificity of *Bradyrhizobium* within different *Aeschynomene* species has been identified into three different cross-inoculation (CI) groups (Figure 2.1). *Aeschynomene* species belonging to CI-group 1 (e.g. *A. elaphroxylon* or *A. americana*) are nodulated only on their roots by non-photosynthetic bradyrhizobia. Stem nodulation is restricted to CI-groups 2 (e.g. *A. afraspera*) and 3 (e.g. *A. indica*) by both non- and photosynthetic *Bradyrhizobium*. For example, photosynthetic *Bradyrhizobium* strain ORS278 and BTAi1 are able to nodulate *Aeschynomene* belonging to the CI-group 3 but impair the nodulation ability to plants from CI-groups 1 and 2. Whereas, photosynthetic bradyrhizobia (ORS285) have a rather wide host

range which nodulate on roots or stems of *Aeschynomene* belonging to CI-groups 2 and 3.

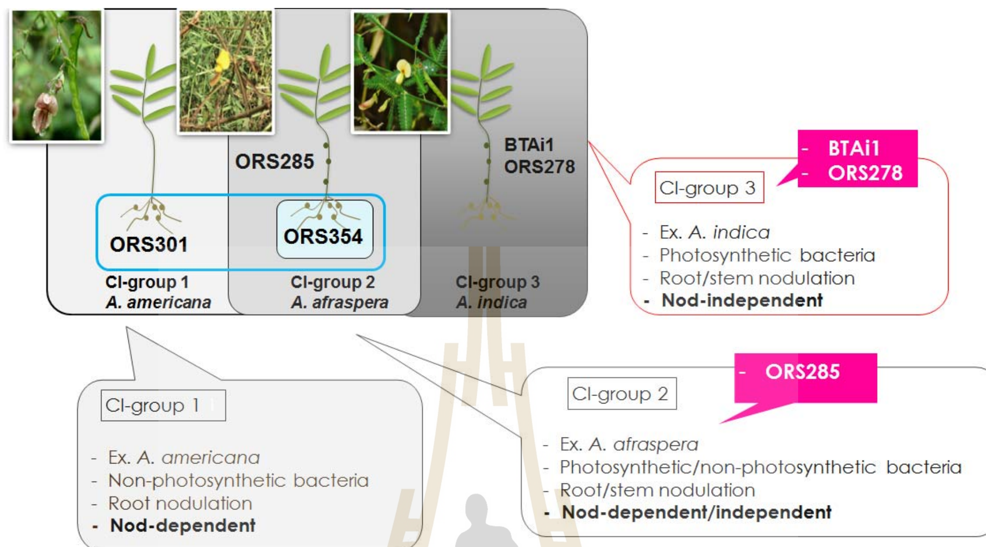


Figure 2.1 *Aeschynomene-Bradyrhizobium* spp. cross-inoculation (CI) group properties (modified from Miche et al. (2010))

Nod factors are produced by the bacteria related to CI-group 1 and 2 (indicated as *nod*-dependent strains) and it is used to control initiation process of infection. Meanwhile, bacteria in CI-group 3 do not have detected any *nod* genes on their genome (BTAi1 and ORS278). Thus, the infection mode and cell entry strategy of these bacteria are different (Giraud et al., 2007). However, a new evolution or adaptation of bacteria in environment is possible to establish a new strategy of infection and cross inoculated between groups. This situation may contribute to the new bacterial strain that have self-mutagenesis. The acceptation of exogenous genes is often seen in environment and cause to evolution of new bacterial strains. Their own symbiosis genes (such as infection genes) may be changed to the new gene

arrangement and their infection ability. Thus, this evolution is an important role for microbial adaptation in new habitat. The study of evolutionary novelty can understand by genome sequencing and comprehensive analysis of gene function and evolution (Markmann and Parniske, 2009; Menna and Hungria, 2011 and Piromyou et al., 2015). However, evolution of symbiosis genes that related to infection strategy in rhizobium is still unclear and need more study. The divergent gene related to infection process may enhance the bacterial infection which has no study in the past.

2.2 Nodulation mechanisms

2.2.1 Nod-dependent strategy

Nod-factor biosynthesis

Nod-factors (NFs) are β-1,4-acetylglucosamine with N-acyl chains at the non-reducing terminal and other host specific decoration (Figure 2.2A). NFs biosynthesis is encoded by nodulation genes (*nod*, *nol*, and *noe* genes), which are expressed specifically in response to the plant flavonoid compounds. The central of the regulation of the *nod* genes is NodD which activates the expression of other key members of *nod* genes that control the core structure synthesis, *nodA* (acyl treansferase), *nodB* (deacetylase), and *nodC* (N-acetylglucosaminyl transferase). The additional modification to the NFs core structure is encoded by other host-specificity *nod* genes (e.g. *nodH*, *nodG*, *nodEF*, *nodPQ*, *nodRL*) by adding the different chemical groups (Janczarek et al., 2015). The more varieties of NFs may govern symbiotic interaction between plant-rhizobia partner, as indicated in a promiscuously *Sinorhizobium fredii* NGR234 (Relic et al., 1993).

Normally, the regulation of transcriptional regulator NodD may respond to different or specific groups of flavonoids. However, some rhizobia contain a multiple copies of regulators that expand host specificity or redundancy allows the bacterium to infect hosts secreting a wide range of flavonoids (del Cerro et al., 2015; Gillette and Elkan, 1996). Additionally, in *B. japonicum* contains the NodVW which provide an alternative pathway for *nod* gene activation (Loh and Stacey, 2003). Originally, NodVW regulators have identified as a host-specific gene by M Göttfert et al. (1990). NodVW is essential for symbiotic intereaction of cowpea, siratro, and mungbean, but not soybean. Thus, NodVW is possible to be a host-specific function that specifically recognizes plant-released flavonoids produced by cowpea, siratro, and mungbean, but not soybean.

Nod-factor perception and transduction signaling cascades

The perception of specific NFs structure by plant NFs receptors triggers a transduction signaling cascade that leads the establishment of nodulation and nitrogen fixing symbiosis. The first genetic analyses NFs receptors were identified in *Lotus japonicus*, Receptor-Like Kinases (RLKs), LjNFR1 and LjNFR5. LjNFR1 and LjNFR5 belong to the lysine motif class of RLKs (LysM-RLKs) which localized in the plasma membrane. LysM-RLKs consist of an intracellular region of serine/threonine kinase, a single transmembrane region and an extracellular region containing three LysM domains separated by Cysteine-any amino acid-Cysteine motifs (CXC) (Madsen et al., 2003). The active kinase domain was demonstrated in LjNFR1 and its orthologs LYK3 of *Medicago truncatula* and SYM37 of *Pisum sativum*. While, the inactive kinase was found in LjNFR5 and its orthologs MtNFP and PsSYM10. LysM domains are protein motifs of about 44–65 amino acids which

are implicated in the binding of peptidoglycan and chitin, most likely recognizing the N-acetylglucosamine moiety. Interestingly, LysM domain sequences within RLKs are considerably diverge. A specific recognition of *R. leguminosarum* DZL NFs was identified at a single leucine (Leu118) in *L. japonicus* and Leu 154 in *M. truncatula* within LysM2 residue of NFR5 (Radutoiu et al., 2003; Radutoiu et al., 2007). The NFs perception most likely distinguishes the different NFs decorations synthesized by different bacteria. The specificity of perception by plant receptors explains why a limited number of rhizobial strains can nodulate to the particular legume (host specificity). Subsequently, NFs activate nuclear-associated calcium spiking via a signaling transduction cascade that required the perception of LysM-receptor kinases, a leucine-rich repeat receptor-like kinase in the plasma membrane, nucleoporins and ion channels in the nuclear membrane. The subsequent activation of a calcium and calmodulin-dependent kinase (CCaMK) then activates transcription factors required for the induction of nodulation and infection genes (Figure 2.2A).

The interaction of plant and rhizobia occurs on a subset of elongating root hairs cells which are a susceptible zone. The root tips of individual root hairs curl to form a niche for a rhizobial microcolony that later colonize the infection thread (IT) reaching into the root cortex. Rhizobia are separated by the plant derived symbiosome membrane and developed into bacteriods metabolically adapted to N_2-fixation. Legume nodules can be categorized into two major groups base on the persistence of their apical meristem. First, indeterminate elongated nodules from *Medicago* have a permanent meristematic activity, while the determinate spherical nodules of soybean, common bean and *Lotus* have a short-lived meristem (Markmann and Parniske, 2009)

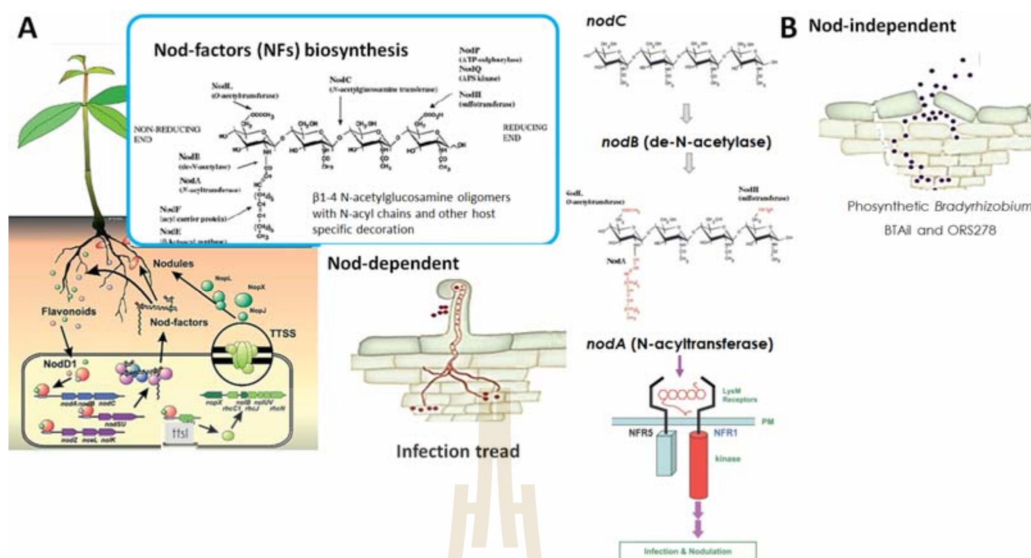


Figure 2.2 Nodulation strategies in rhizobia. A: Rhizobia induce the formation of nodules on legumes using a *nod*-dependent model. Nod-factor structure and biosynthesis are demonstrated. B: nod-independent model.

2.2.2 Nod-independent strategy

Some strain of photosynthetic bradyrhizobia associating *Aeschynomene* (strains BTAi1 and ORS278) are nod-lacking strain. However, they can nodulate a subset of *Aeschynomene,* including *A. sensitiva* and *A. indica* (CI-group 3) by using the crack-entry or nod-independent strategy (Giraud et al., 2007). Crack entry is initiated at the emergence sites of lateral roots then become internalized in plant cells via an endocytosis-like process that remains largely unknown. A hypothetical scheme is proposed for the *nod*-independent process is shown in figure 2.2B. The need for one initial plant signal remains to be demonstrated. Accumulation in these infection zones of plant hormone-like compounds or some signaling molecules synthesized by the bacteria might use as bypass the early nod factor signaling pathway and induce

nodule organogenesis. Interestingly, *nod* deletion mutant of strain ORS285 retains the ability to nodulate *A. sensitiva* and *A. indica* but not the other species (Giraud et al., 2007). This result suggested that a group of photosynthetic bradyrhizobia uses a nod-independent strategy for interacting symbiosis with some *Aeschynomene* species (CI-group 3). In addition, some of non-photosynthetic bradyrhizobia strains used T3SS as a main strategy for symbiotic infection to the *Aeschynomene* CI-group 3 (Okazaki et al., 2015).

However, initiation of infection in bacteria may require another mechanism such as secretion system and cell-wall degradation using bacterial-derived enzymes (pectinase or cellulase). These mechanisms play a crucial role in early symbiotic events, such as root-hair deformation, initiation of cortical-cell division, bacterial cell infection (Lomovatskaya et al., 2015; Xie et al., 2012).

2.2.3 Secretion system

Many secretion systems indicated in pathogenic bacteria are used for contributing to their virulence. This system is grouped into several classes base on their mechanism, composition, and evolutionary relationship the key structural features (Gophna et al., 2003; Tampakaki et al., 2004). Secretion system in bacterial-associated plant can be classified into two categories. First is secretion system of general use that consists of type I, type II and type V secretion systems (T1SS, T2SS and T5SS, respectively). These membrane transporters have previously been shown to mediate microbe-host interactions and also implicated in processes of organelle biogenesis and nutrient acquisition (Deakin and Broughton, 2009). A second category is specialized secretion systems which consist of type III, type IV and type VI secretion systems (T3SS, T4SS and T6SS, respectively). These secretion systems

seem to have specialized in mediating microbe-host interactions which have all been implicated in the *Rhizobium*-legume symbiosis as well as pathogens such as *Pseudomonas aeruginosa* and *Salmonella enteric* (Gazi et al., 2012 and Tseng et al., 2009). However, T3SS have been indicated as the important system for rhizobium infection on early stage (Okazaki et al., 2013 and 2015).

Type 3 secretion system (T3SS)

T3SS are the system of the injecting tube which ancestrally related to the flagellar basal body of bacteria and have been well characterized in several pathogenic bacteria such as *Yersinia species*, *Shigella species*, *Salmonella enterica* and *Escherichia coli*. They have also found in several plant pathogens which they are required for infection to both pathogenesis on susceptible hosts. This injection system can induce the reaction of a hypersensitive response on resistant hosts. T3SS have also been found in *Rhizobium* strain NGR234 (Marie et al., 2003), in *Mesorhizobium loti* MAFF303099 (Kaneko et al., 2000), *Sinorhizobium fredii* USDA257 (Kovacs et al., 1995; Krishnan et al., 2003) and in *Bradyrhizobium diazoefficiens* 110 (Göttfert et al., 2001). The T3SS genes in rhizobial strians are highly conserved with the genes that contains in other pathogenic bacteria. Core components of the secretion apparatus (rhizobia-conserved proteins [Rhc]) in rhizobia are clustered. This T3SS involved gene cluster also encodes secreted proteins cells nodulation outer proteins (Nop), such as NopB and NopL. *nopB* is the first gene of a highly conserved operon containing several *rhc* genes. NopB was found *Sinorhizobium fredii* USDA 257 and *Rhizobium* sp. strain NGR234 that was secreted by T3SS and associated with extracellular structures of the secretion apparatus. *nopL* is presented in NGR234, *S. fredii*, and *B. japonicum* which probably acts as an effector protein in plants (Marie et

al., 2003). One of the conserved genes in the cluster is *ttsI* which has high similarity to the two-component regulators. The expression of *ttsI* regulator is controlled by flavonoids-nodD regulation as demonstrated in nod genes expression. The expression of ttsI protein is essential for induction of other genes in T3SS cluster by binding to a conserved *tts* box motif before gene located (Zehner et al., 2008). The expression of T3SS involved genes are detected at early stages of plant infection and in mature nodules. Therefore, to demonstrate T3SS mediates symbiotic plants, mutation of core component and regulator gene were mostly investigated. The core component (*rhcJ*) and regulator gene (*ttsI*) mutants of *B. japonicum* USDA122 for the T3SS failed to secrete typical effector proteins and impaired the ability to nodulate original soybean plants. However, T3SS mutants are capable of nodulating *Rj2* soybean plant, which are symbiotically incompatible with wild-type USDA122. This suggested that effectors secreted via the T3SS might trigger compatibility with plant immunity in *Rj2* plant and prevent the nodulation by *B. japonicum* USDA122 (Tsukui *et al*., 2013). However, T3SS is possible to suppress the plant defend mechanisms by contributing the suppression of one or more components of PTI or ETI. For example NopL, which is a substrate for plant protein kinases that blocks inducible plant defense responses proteins (Bartsev et al., 2004). In case of *B. elkanii* strain USDA61, T3SS was used as alternative way for soybean infection and also plays a role for both of the *nod*-dependent and -independent *Aeschynomene* invasions (Okazaki et al., 2013 and 2015).

2.3 The evolutionary novelty in symbiosis rhizobium

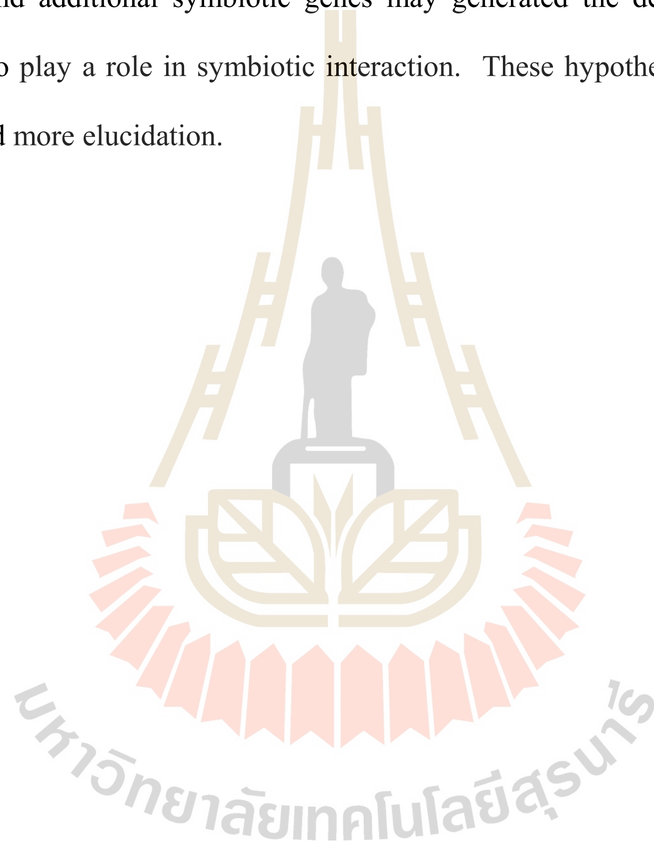
The multiphasic lifestyle adopted by rhizobia has influenced the size, complexity and also the content of their genomes. This appears to be a common trend

among other members of the α-proteobacteria that have a varied lifestyle, including *Rickettsia, Brucella* (animal pathogen) and *Agrobacterium* (plant phatogen). Comparative genomic analyses of members the α-proteobacteria have shown that these phylogenetically diverse bacteria share many similarities in their methabolism, physiology, and processes required for interaction with their cognate host. The ability of *Rhizobium* species to establish a symbiotic relationship with a host plant imposes an additional genetic requirement upon rhizobia via gene transfer and gene duplication events. Symbiotically relevant genes in rhizobia are often clustered on large plasmids (pSym), or within genomic islands (referred to as symbiosis islands [SIs]), emphasizing the accessory nature of the genes and their ability to be acquired via horizontal gene transfer. The symbiotic genes involved in the nodulation and nitrogenase structural (Klinger et al.) and nitrogenase structural genes (*nifHDK*) genes of *R. leguminosarum*, *S. meliloti*, *S. fredii* and *M. loti* were located on large plasmids varying in size from 100-300 Mb, calls megaplasmid (Galibert et al., 2001 and Janga et al., 2006). For genome database of bradyrhizobia (slow growing bacteria); they are composed of a unique chromosome, except strain *Bradyrhizobium* sp. BTAi1 which also harbors a plasmid in a size of 228 kb.

Rhizobial genomes have presence many of insertion sequence (IS) elements, transposases, and related genes, within regions encoding symbiotic functions. This situation demonstrated that, bacteria may receive the new symbiotic genes through lateral gene transfer from their ancestor. Not surprisingly that, evolutionary novelty in a symbiosis rhizobium has been discovered annually. However, the genome analyses can well explain gene alteration and evolution of their genomes. Traditionally, studies involving rhizobia have focused upon a subset of genes involved in root nodulation,

nitrogen fixation, and other symbiotic processes. However, the recent completion of genome sequencing projects in several rhizobial species permits a more comprehensive analysis of gene function and evolution (Klinger et al., 2016 and Remigi et al., 2016).

In case of *Bradyrhizobium* sp. DOA9, the symbiotic megaplasmid containing divergent and additional symbiotic genes may generated the determinant signaling molecules to play a role in symbiotic interaction. These hypotheses are still unclear and required more elucidation.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CHAPTER III

MATERIALS AND METHODS

3.1 Localization of symbiotic genes on megaplasmid of DOA9

3.1.1 DNA extraction and megaplasmid detection

Bradyrhizobium sp. DOA9 total DNA was prepared as described previously (Manassila et al., 2007). Megaplasmids were isolated by electrophoresis (Argandoña et al., 2003; Thomas et al., 1994) with the modifications described here. Bacteria were cultured on HM broth medium (Somasegaran and Hoben, 1994) with 0.05% (w/v) L-arabinose, 0.05% (w/v) yeast extract for *B. diazoefficiens* USDA110 and *M. loti* MAFF303099, and no L-arabinose for *Bradyrhizobium* strain DOA9, to reduce polysaccharide production. USDA110 and DOA9 were cultured at 30°C for 3 to 5 days on a rotary shaker at 200 rpm until the late-log phase was reached. Then 1% (v/v) of these pre-cultures was inoculated into new tubes containing HM broth medium. The cultures were incubated for 3 days until the exponential growth phase (the mid-log phase) was reached. MAFF303099 was incubated for 2 days and inoculated into a fresh tube containing HM medium and then cultured under the same conditions for 24–36 h. After that, the cell pellets were harvested by centrifugation at 3000 × *g* for 10 min. The cells were resuspended in 0.85% (w/v) NaCl to OD_{600}=1. The cells were harvested from 1 mL of cell suspension and washed with M9 salts (Joseph Sambrook et al., 1989) containing 0.5 M NaCl, and then with 1 mL 0.1% (w/v) Sarcosyl. The supernatant was removed immediately and the sediment was

resuspended in 50 µL of lysis buffer (1 mg/mL lysozyme, 1 mg/mL RNase A (first dissolved in 0.4 M sodium acetate, pH 4.0, and then boiled for 10 min (Plazinski et al., 1985)), 0.1% (w/v) bromophenol blue in Tris-borate buffer (pH 8.2, 89 mM Tris base, 12.5 mM disodium EDTA, and 8.9 mM boric acid) (Eckhardt, 1978), and 40% (v/v) glycerol).

Before sample loading, electrophoresis was performed in 0.7% agarose gel, which leveled off with 0.5×TBE buffer (4°C) until it touched the gel. The wells were then filled with 50 µL Sodium Dodecyl Sulfate (SDS; 10% w/v) mixed with xylene cyanol (1mg/mL). The current was run for 10–15 min at 100 V with reversed polarity until the SDS was 1 cm above the wells. After that, 50 µL of sample mixed with lysis solution was directly loaded and left for 15 min before 15 µL of Protinase K (5 mg/mL) in 40% (w/v) glycerol was overlaid. The wells were sealed with melted agarose gel, and 0.5×TBE buffer was then added to cover the gel. After 1 h, electrophoresis was carried out in a cold chamber at 4°C. The current was run at 10 mA for 14 h and at 50 mA for a further 10 h. The DNA in the gel was stained for 30 min in ethidium bromide (0.5 µg/mL) and washed with distilled water before being viewed under UV light.

3.1.2 Southern blot hybridization

The megaplasmid and chromosomal DNAs separated in the gel were used for Southern blot hybridization of nodulation *(nod)* and nitrogen fixation (*nifH*) genes as described (Noisangiam et al., 2012). In brief, probes for *nodA* (550 bp), *nodB* (530 bp), and *nodC* (1 kb) were obtained through PCR amplification using genomic DNA of *B. yuanmingense* SUTN6-2, *B. canariense* SUTN7-2, and *B. diazoefficiens* USDA110, respectively. DNA fragments of the respective strains were

amplified with the primer pairs nodAYF46/nodAYR595(*nodA*), nodB26/nodB625 (*nodB*), and nodC195/nodCI (*nodC*) (Noisangiam et al., 2012). The probe for *nifH* was derived from *B. yuanmingense* using nifHF/nifHI primer pairs (Laguerre et al., 2001). DNA probes were labeled overnight at 37°C by random priming, and hybridized with the Digoxigenin (DIG) High Prime DNA Labeling and Detection Starter Kit I (Roche, Switzerland). The DNA was capillary-transferred to a Hybond-N^+ nylon membrane (Amersham, Cardiff, UK) as described previously (Joseph Sambrook et al., 1989). Low-stringency conditions were used for hybridization: membranes were hybridized at 40°C (*nodA* and *nodC* genes) or 42°C (*nodB* and *nifH*) for 18 h and then washed twice in 2× SSC + 0.1% (w/v) SDS at 25°C for 15 min and in 0.5× SSC + 0.1% (w/v) SDS at 62°C for 15 min.

DOA9 genomic DNA was digested with *Eco*RI, *Eco*RV, *Hind*III, or *Not*I for evaluating the copy number of *nodB, nodC* and *nifH*; with *Eco*RI, *Hind*III, *Bgl*II, or *Not*I for *nodA*. Fragments were separated in 1% (w/v) agarose gels before hybridization as described above.

3.2 The function of nodulation (*nod*) genes and Type 3 secretion system (T3SS) in DOA9

3.2.1 Bacterial strains and culture conditions

The bacterial strains used this study were listed in Table 3.1. *Bradyrhizobium* sp. strain DOA9 (Noisangiam et al., 2012) and its derivatives were grown in modified YM medium (Giraud et al., 2000) at 28°C under aerobic condition for further analyses. *Escherichia coli* strains were cultured at 37°C in LB medium (Sambrook et al., 2001). Antibiotics were supplemented with medium at the flowing

concentrations when appropriate: 200 µg/ml streptomycin (Sm), 20 µg/ml cefotaxime (cefo), 20 µg/ml nalidixic acid (nal) and 50 µg/ml Kanamycin (Km).

3.2.2 Construction of the reporter, mutant and complementation strains

The single cross-homologous recombination technique was used for constructing the insertion mutant of nodulation genes ($nodA_1$, $nodA_2$ and *nodB*) and structural type III secretion system gene (*rhcN*). All primers used for cloning partial genes are listed in Table 3.2. The internal fragment of independent gene was cloned into the plasmid pVO155-nptII-GFP-nptII-Sm/Sp at *xba*I and *Sal*I sites. The recombinant plasmids were introduced into *Bradyrhizobium* sp. DOA9 by bi-parental mating as described by Noisangiam et al. (2012). The transconjugants were spread on YM medium supplemented with 200 µg/ml streptomycin (Sm), 50 µg/ml Kanamycin (Km) to select for the single homologous recombination events (annotated as $\Delta nodA_1$, $\Delta nodA_2$, Δ*nodB*, and Δ*rhcN*). The mutant strains were verified by PCR using external primers paired with specific primers of internal GUS and Sm/Sp resistance genes to determine the insertion recombinant (Figure 3.1).

Complementation of the $\Delta nodA_1$ mutant strains were constructed by the introducing of the carried plasmid pMG103-nptII-cefo including full length of the amplified $nodA_1$ gene with its promoter (1320-bp in total length). The fragment of $nodA_1$ gene including the 800-bp upstream promoter region were amplified and directly cloned into pMG103-nptII-cefo at *Xba*I/*Eco*RI site (Figure 3.1). The constructed plasmid was transformed into the $\Delta nodA_1$ mutant by electroporation using 17.5 kV/cm field strength, 5-ms pulse length and 0.2-cm cuvette supplied by the manufacturer (BTX GeminiX2, Havard Bioscience, Inc.) (Hattermann and Stacey,

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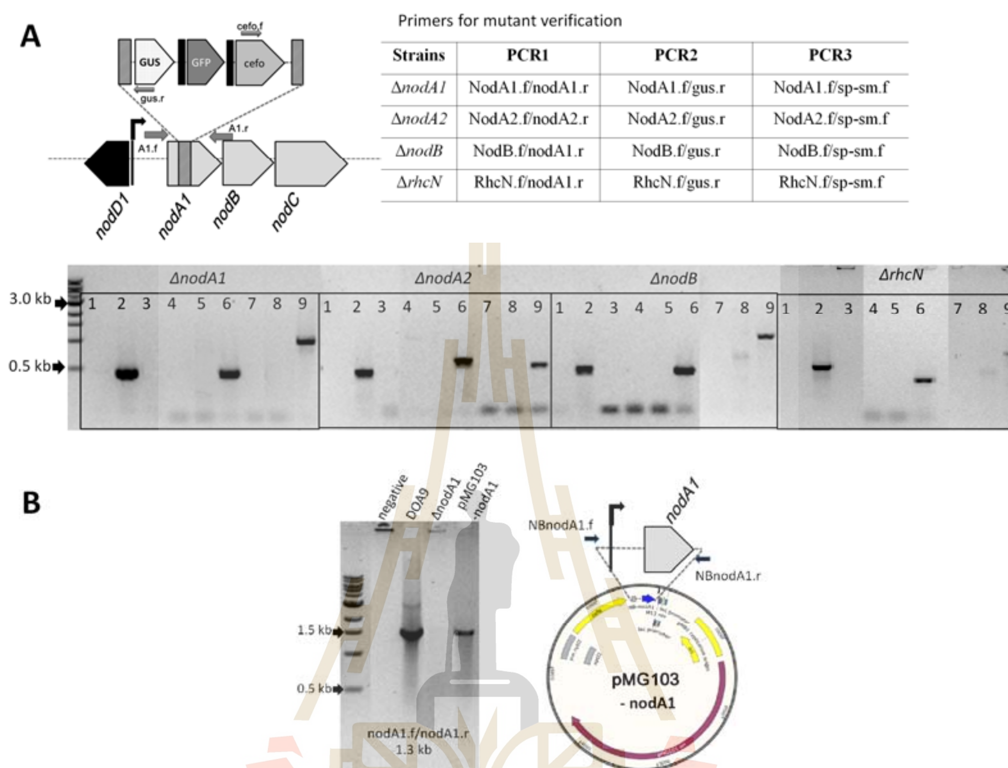
1990). The transformant was selected on YM medium supplemented with 20 µg/ml cefotaxime and 200 µg/ml streptomycin, annotated as pMG103-nodA1.



Primers for mutant verification

Strains	PCR1	PCR2	PCR3
Δ*nodA1*	NodA1.f/nodA1.r	NodA1.f/gus.r	NodA1.f/sp-sm.f
Δ*nodA2*	NodA2.f/nodA2.r	NodA2.f/gus.r	NodA2.f/sp-sm.f
Δ*nodB*	NodB.f/nodA1.r	NodB.f/gus.r	NodB.f/sp-sm.f
Δ*rhcN*	RhcN.f/nodA1.r	RhcN.f/gus.r	RhcN.f/sp-sm.f

Figure 3.1 The verification of mutant and construction. Mutant construction and verification were showed in gel (A). PCR primers for verification were indicated in table. The results of PCR1 were indicated in lane 1-3, PCR2 were indicated in lane 4-6, PCR1 were indicated in lane 7-9. Lane 1, 4 and 7 were negative control (no template), lane 2, 5 and 8 were DNA of DOA9 (wild-type) and lane 3, 6 and 9 indicated PCR product from each mutant strains. PCR verification of complement *nodA₁* strain (pGM103-nodA1) (B) was indicated in gel. Full length of *nodA₁* including nod-box promoter were amplified by NBnodA1.f and NBnodA1.r primers, 1.3 kb target band was detected in DOA9 and pMG103-nodA1.

GFP-labeling DOA9 was constructed by the introducing of plasmid pMG103-nptII-Sm/Sp harboring a constitutive GFP gene (pMG103-nptII-Sm/Sp-nptII-GFP). The derivative strains and plasmids used in this study are described in Table 3.1.

3.2.3 Plant nodulation and symbiosis analysis

DOA9 and its derivatives were grown for 5 days as described above and used as inoculants. Seeds sterilization and germination of all tested plants were as described by Teamtisong et al., 2014. After germination, symbiotic abilities of DOA9 and mutant strains were inoculated into several plant species in Genistoids, Dalbergioids and Milletioid tribes, including *Crotalaria juncea* from Genistoids tribe, *Aeschynomene americana* (Thailand), *A. afraspera* (provided by LSTM, France), *Arachis hypogaea* cv. Thai Nan, and *Stylosanthes hamata* (from Dalbergioids). The plant in Milletioid includes *Vigna radiata* cv. SUT4, *Macroptilium atropurpureum* (siratro), *Desmodium tortuosum*, and *Indigofera tinctoria*. Plants were sown in Leonard's jar which filled with sterilized vermiculite (Somasegaran and Hoben, 1994). All plants were maintained by BNM medium (Ehrhardt et al., 1992) and grown under controlled environmental condition of 28 ± 2°C on 16-h-light/8-h-dark cycle at light intensities 300 $\mu E/m^2S$ and 50% humidity. Five days after planting, each seedling was inoculated with 1 ml of a 5-day-old inoculum after washing and adjusting the optical density at 600 nm to 1. For nodulation and nitrogen fixation abilities were measured after at least 21 day-post inoculation (dpi). Five plants were taken and analyzed for the number of nodules and nitrogenase activity using acetylene reduction assay (ARA) assay as previously described (Somasegaran and Hoben, 1994). Briefly, all nodules were collected from each plant and placed in headspace

bottles with 10% acethylene, and incubated at 28°C for 1 hr. Gas chromatography was conducted to measure peak height of ethylene and acetylene with 1 ml gas samples from the bottles by using PE-alumina packed column with 150˚C of injector, 200˚C of oven, and 50˚C of Flame Ionization Detector (FID) (Somasegaran and Hoben, 1994).

3.2.4 Microscope observation

Histochemical GUS staining of GUS-labeling DOA9 in nodules was performed as described by Teamtisong *et al.*, 2014. An Olympus Fluoview FV1000 confocal laser scanning microscope was used for investigating the symbiotic cell development and bacteroid differentiation in nodule sections. Specimens were co-stained with 0.01% of calcofluor (plant staining dye) and Propidium iodide (PI) to determine dead cell. Green florescence protein (GFP) was excited with the 488-nm laser line and the barrier filter had a 520-nm cutoff. Propidium iodide (PI) was excited with the 568-nm laser line, and the barrier filter had a 590-nm long-pass cutoff. The excitation/emission maxima for calcofluor stain were about 395/440 nm. Confocal images were reconstructed with NIM Image software, and images were colorized and prepared for publication with Adobe Photoshop software.

3.2.5 Preparation of root exudates and bacterial induction

The sterilized legume seeds were germinated and transferred into tubes containing BNM medium (50 mg seeds/ml). Plants were maintained in controlled environmental condition as mentioned above for 5 days. The root exudates were obtained from plant medium after filtration by using 0.2 μm filter syringe. Root exudates were stored at -20˚C for future use.

The mid-log phage culture bacteria of DOA9 and derivatives were washed and adjusted the OD600 of approximately 0.4 with YM supplemented with 1/3 (vol/vol) of the root exudates or purified flavonoids (20µM of Naringenin and Genistein in final concentration). The sterilized BMN medium and DMSO were used as negative induction. The inducted bacteria were cultured at 28°C for 12 h and 24 h. Bacterial cells were collected by centrifugation (4,000×g for 10 min, 4˚C) and immediately frozen in liquid nitrogen and stored at –80°C for further total RNA isolation.

3.2.6 RNA isolation and reverse transcript PCR amplification (RT-PCR)

Total bacterial RNA was extracted from induced cells using the RNeasy® Mini Kit (QIAGEN, USA) according to the manufacturer's protocol. Total RNA was treated with RNase-free DNase I (NEB) for 30 min at 37°C. cDNA was synthesized from 500 ng total RNA using High Capacity cDNA Reverse Transcription Kits (The Applied Biosystems™) according to the manufacturers' protocols. Thirty ng of cDNA was subjected to PCR amplification using specific primers of nodulation genes ($nodA_1$, *nodB* and *nodC*). All primer sets used in the expression analysis were listed in Table 3.2. The PCR amplification was performed under cycling condition (Thermal cycler BIO-RAD T100TM) following; an initial denaturation step at 95°C for 2 min; 35 cycles at 95°C for 30 s, 52˚C for 30 s and at 72°C for 1.5 min; and a final extension step at 72°C for 10 min. PCR products were visualized in 1% agarose gel stained with 0.5 µg mL^{-1} ethidium bromide. The intensity of bands was analyzed using a Gel documentation and analysis system (Gel Doc XR+system, BIO-RAD). The relative expression levels of genes were normalized to the house keeping 16S ribosomal gene.

For qRT-PCR, 30 ng of cDNA of each sample was mix with SYBR™ Select Master following the manufacturer's protocol and performed the cycling condition by QuantStudio 3 Real-Time PCR System Mix (Applied Biosystems™). The relative gene expression was analyzed by comparative Ct method (-ΔΔCT) that normalized to the endogenous housekeeping gene (16S rRNA).

Three biological replicates were pooled and analyzed. At least triplicate PCR amplifications were performed for each sample.

3.2.7 Quantitative GUS assays

Bacteria were grown to mid-exponential phase in YM. Cells were harvested and resuspended in BNM medium containing 10 mM succinic acid, 6 mM Glutamate, 2 mM $CaCl_2$, 5 mM Fe-EDTA and 1X Gibco® MEM Vitamin Solution. Cell suspensions (Asb_{600} 0.1) were induced at 28°C for 24 h with flavonoids of different functional classes, including isoflavones (formononetic, genistein and daiazein), flavanones (liquiritigenin, naringenin, naringenin-7-O-glucoside and eriodictyol), flavones (7,4'-dihydroxyflavone, apigenin, luteolin, and acacetin) and flavonols (Quercetin). The negative control was DMSO and 50% ethanol. For the β-glucuronidase (Gust et al.) assays, 200 µl of culture was measured the cell density at OD 600. Then, a 200 µl aliquot of a culture was mixed with 680 µl of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$ and 0.05 M β–mercaptoethanol) and incubated in 450 µl of substrate solution (1.35 mg/ml of 4-nitrophenyl-beta-D-glucuronid (4-NPG) in Z-buffer) at 30°C for 15 min. After sufficient color has developed, 700 µl of stop solution (1 M Na_2CO_3) was added and a clear solution was collected by centrifugation. The colorization level was measured

OD 420 and OD 550. The GUS-activity was calculated as Miller Units using the formula:

$$\text{Miller Units} = 1000 \times [(OD_{420} - 1.75 \times OD_{550})] / (T \times V \times OD_{600})$$

- OD_{420} and OD_{550} are read from the reaction mixture.
- OD_{600} reflects cell density in the washed cell suspension.
- T = time of the reaction in minutes.
- V = volume of culture used in the assay in mL.

3.2.8 Amplification and analysis of Nod factor receptor (NFR)

The genomic DNA of all tested plants were extracted from leaf tissue using manually protocol (J. Sambrook et al., 2001). NFR fragments were amplified using AhNFP-F and AhNFP-R designed from Ibáñez et al. (2015). A PCR product of the expected size (509 bp) was cloned into TA cloning vector (invitrogen) and sequenced with M13 primers by Macrogen (Korea). Homologous sequences were identified using BLASTN and BLASTX algorithms (Altschul et al., 1997). For sequence analysis, orthologous NFR5 sequence was searched from the NCBI database and aligned using ClustalW. Evolutionary analyses were conducted in MEGA6 software (Tamura et al., 2013) by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980).

Table 3.1 bacterial strains, plasmids used and plants tested in this study

Strains	Relevant characteristics	Source or reference
Bradyrhizobium sp. DOA9	*A. americana* type strain	(Noisangiam *et al.*, 2012)
Δ*nodA*$_1$	Insertional *nodA*$_1$ of DOA9 by integration of pVO155. Sm/Sp.gusA.nptII.gfp at Sal1/XbaI; Sm^r Sp^r Km^r	This study
Δ*nodA*$_2$	Insertional *nodA*$_2$ of DOA9 by integration of pVO155. Sm/Sp.gusA.nptII.gfp at Sal1/XbaI; Sm^r Sp^r Km^r	This study
Δ*nodB*	Insertional *nodB* of DOA9 by integration of pVO155. Sm/Sp.gusA.nptII.gfp at Sal1/XbaI; Sm^r Sp^r Km^r	This study
Δ*rhcN*	Insertional *rhcN* of DOA9 by integration of pVO155. Sm/Sp.gusA.nptII.gfp at Sal1/XbaI; Sm^r Sp^r Km^r	This study
pMG103-nodA1	Δ*nodA*$_1$ harbored pMG103-nptII-Sm/Sp-nptII-GFP	This study
Escherichia coli S17-1	hsdR pro thi (RP4-2 km::Tn7 tc::Mu	
Plasmid	**Relevant characteristics**	**Source or reference**
pVO155-nptII-Sm/Sp-gusA-nptII-gfp	pUC119-derived suicide vector with GusA gene, GFP and Sm/Sp cassette, Km^R Sm^r Sp^r	Giraud E, IRD, France
pMG103-nptII-Sm/Sp-nptII-GFP	Cloning vector harbors a GFP gene under the control of the constitutive *nptII* promoter; Sm^r Sp^r Km^r	(Bonaldi *et al.*, 2010)
pMG103-nptII-cefo	Complementation vector harbors a cefotaxime resistance gene under the control of the constitutive *nptII* promoter; cefo^r	Giraud E, IRD, France

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Table 3.1 Bacterial strains, plasmids used and plants tested in this study (cont.)

Plasmid	Relevant characteristics	Source or reference
pMG103-nptII-cefo-nodA1	Fragment of nod-box to *nodA1* (1320-bp) cloned in pMG103-nptII-cefo plasmid	This study
Tribes	**Plants tested**	**Source or reference**
Gienistioids	*Crotalaria juncea*	Nakhon Rathasima, Thailand
Derbergioids	*Aeschynomene americana*	Nakhon Rathasima, Thailand
	Aeschynomene afraspera	Giraud E, IRD, France
	Arachis hypogaea cv. Thai Nan	Nakhon Rathasima, Thailand
	Stylosanthes hamata	Nakhon Rathasima, Thailand
Millitioid	*Macroptilium atropurpureum*	Nakhon Rathasima, Thailand
	Indigofera tinctoria	Nakhon Rathasima, Thailand
	Vigna radiata cv. SUT4	Nakhon Rathasima, Thailand
	Desmodium tortuosum	Nakhon Rathasima, Thailand

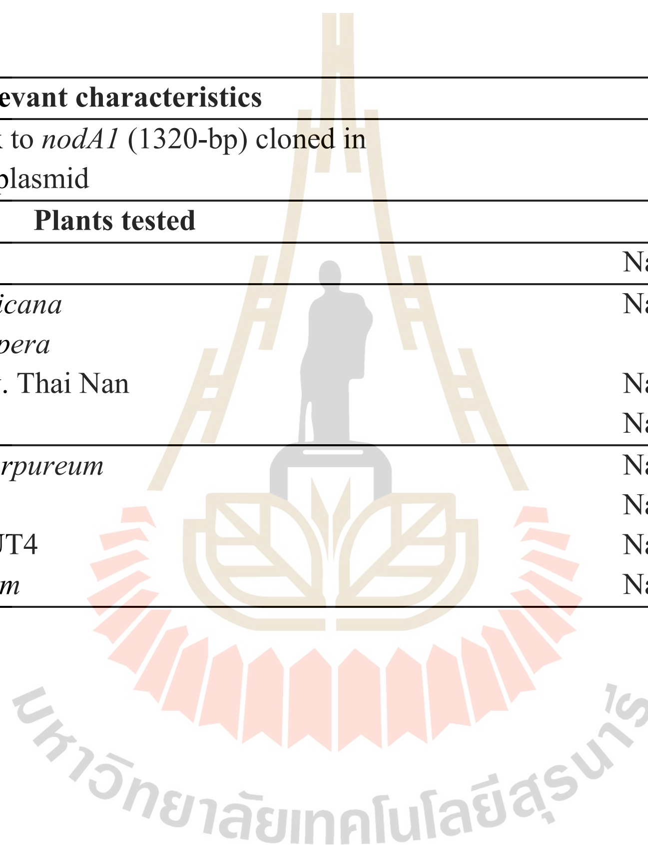

Table 3.2 List of primers used in this study for southern blot hybridization, construction of mutant and complement strains, PCR verification and RT-PCR.

Name of primers	Sequence 5'-->3'	Description
nodAYF46	GCTCAAGTGCAGTGGAGCCTTC	*nodA* probe amplification (Noisangiam *et al*., 2012)
nodAYR595	CCGGCCATTCGCTTATCGAGCG	
nodB26	CTGTCCGCTGCGACTACGC	*nodB* probe amplification (Noisangiam *et al*., 2012)
nodB625	CGCGCCGTTGTAGTGCTGG	
nodC195	CGCCGAATGTCTGGAGTCG	*nodC* probe amplification (Noisangiam *et al*., 2012)
nodCI	CGYGACAGCCANTCKCTATTG	
nifHF	TACGGNAARGGSGGNATCGGCAA	*nifH* probe amplification (Laguerre *et al*. 2001)
nifHI	AGCATGTCYTCSAGYTCNTCCA	
NodA1.D9p.int.f	ATCGAGGTCGACCTGCTCTATACCCACCGACGCT	*ΔnodA1* construction
NodA1.D9p.int.r	GCCGCATCTAGACGGGTACATTGTGCGCTCATTG	
NodA2.D9p.int.f	GGCCGAGTCGACCGTTCAATGCAAGACCTTTTG	*ΔnodA2* construction
NodA2.D9p.int.r	TGCGAGTCTAGATGTGCCGAACCCGAAGGGAAC	
NodB.D9p.int.f	TGACTCGTCGACGACGCCGCACCTCTTGGATGTTTTAG	*ΔnodB* construction
NodB.D9p.int.r	GTTCCGTCTAGAGCCCATAGGGCGCACGTATATG	
RhcN.D9p.int.f	CATCTCGTCGACTCGCAGCAAAGGATGTCGATAC	*ΔrhcN* construction
RhcN.D9p.int.r	GAGCAGTCTAGACCCGACTGACACTTCCTGCATG	
NodA1.f	CGACCATGCGGGGAGAACAAAG	*ΔnodA1* verification
NodA1.r	GTGTGGCAGGCAATGTGGGATAG	
NodA2.f	CGGCTTCAGTGGAGAACGTGCTG	*ΔnodA2* verification
NodA2.r	CATTCCGGGCGATTTGCGACTTC	

Table 3.2 (Continued).

Name of primers	Sequence 5'-->3'	Description
NodB.f	GGCCTGCCGAGCGTCCATCTAAC	*ΔnodB* verification and RT-PCR
NodB.r	GGTGGACAGCCGTCGTGCAATAG	
RhcN.f	GCAACAATCAGGCCATAATCAAG	*ΔrhcN* verification
RhcN.r	AGGGCATCTCCAATATGACGTTC	
Sm.Sp.f	CCAGTATCAGCCCGTCATACTTG	specific in pVO155-npt2-Sm/Sp-npt2-GFP, mutant verification
gus.r	GCACAGCAATTGCCCGGCTTTCTTG	
NBnodA1.f	GCGTTTTCTAGATAGCGCATCAAGTGCAACGA	pMG103-nodA1 construction and verification
NBnodA1.r	GTCACGGAATTCGGCCGTTCCGCTCAATTGTT	
nodA1-doa9/291F	CCACGAGGGTCTCGTAATGT	RT-PCR for *nodA1* gene
nodA1-doa9/407R	CCGTCTTGTGTGCTCTCTGA	
nodA2-doa9/373F	TTCGGCTCGCACTTAAAGAT	RT-PCR for *nodA1* gene
nodA2-doa9/528R	TGGGAAAATCACGATTAGGG	
AhNFP-F	TCTCCAAAYYTTYTGAGYCTRWCHAAYATATC	NFR5 analysis (Ibáñez *et al.*, 2015)
AhNFP-R	TGGGATYARAAYTGGAAGGTTGKYTGC	

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CHAPTER IV

RESULTS

4.1 Localization of symbiotic genes in megaplasmid of DOA9

Both chromosomal and megaplasmid DNAs of DOA9 were successfully extracted. The megaplasmid size was estimated from the plasmid profile of *M. loti* MAFF303099, which contains megaplasmids of 208 and 352 kb (Kaneko et al., 2000) (Figure 4.1A lane 1). DOA9 is contained one megaplasmid, which was larger than 352 kb (Figure 4.1A lane 2). The megaplasmid was hybridized with all the probes for *nodA*, *nodB*, *nodC*, and *nifH* (Figure 4.1B-E). The *nifH* probe was also hybridized with the chromosomal DNAs (Figure 4.1E).

By using the probe for *nodA*, two fragments were detected (Figure 4.2A). This finding suggested that *nodA* on the megaplasmid were two copies. For *nodB* and *nodC*, single fragment was detected on each lanes suggesting that these two genes were single copy on the megaplasmid (Figure 4.1C-D and Figure 4.2B-C). At least two fragments were detectable probed by *nifH*. The result supported that two *nifH* genes were separately located both on the megaplasmid and on the chromosome (Figure. 4.1E and 4.2 D). Therefore, this finding was the first report of Bradyrhizobia symbiotic genes located on a megaplasmid. The broad host range properties were also reported in Teamtisong et al. (2014) that the DOA9 was able to nodulate many of the dalbergioids, millettioids, and robinoids. As DOA9 contains divergent *nod*-genes, these genes might facilitate the broad host range nodulation ability. Moreover,

infection tread formation was not detected in any infected plant.

Therefore, the ability of DOA9 to invade roots via the cracks through which lateral roots emerge might allow it to infect a wide variety of legumes (Noisangiam et al., 2012).

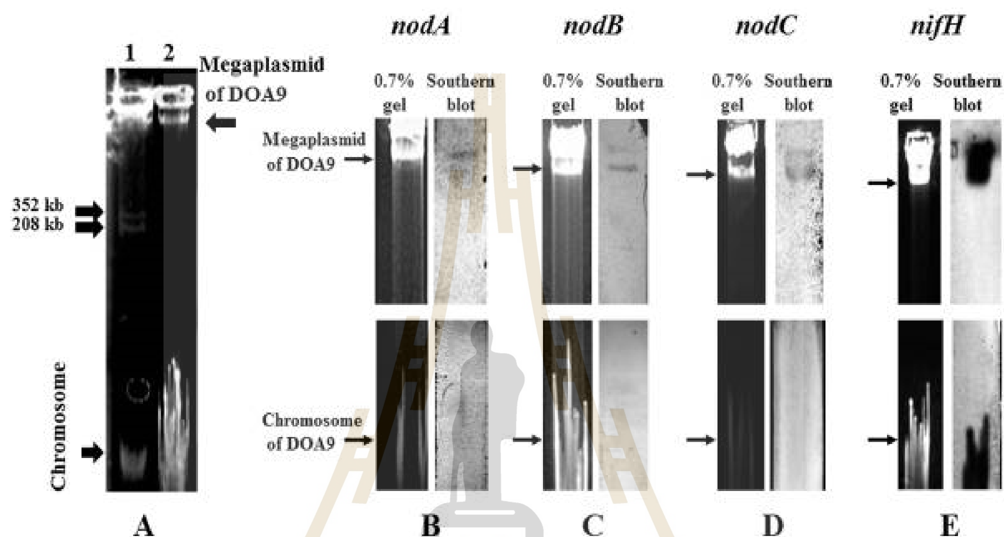

Figure 4.1 Determination of *Bradyrhizobium* sp. DOA9 replicons and symbiosis genes. (A) Megaplasmid profiles: lane 1, *Mesorhizobium loti* MAFF303099 (208 and 352 kb); lane 2, DOA9; (B–E) Southern blot hybridization signals of nodulation and N-fixation genes on megaplasmid and chromosome from DOA9 under low stringency conditions: (B) *nodA*; (C) *nodB*; (D) *nodC*; (E) *nifH*.

The whole genome of DOA9 was published by Okazaki et al. (2015). A single chromosome (cDOA9) and megaplasmid (pDOA9) were 7.1 Mb and 0.7 Mb in size. The sequencing of pDOA9 demonstrated that pDOA9 was also contained many symbiotic genes such as type 3 secretion system (T3SS) that have been reported as the

determinant for bradyrhizobia association in legumes (Krause et al., 2002; Okazaki et al., 2009). To understand the host range determinant in DOA9, nodulation genes and T3SS in DOA9 were inactivated by insertional mutation method.

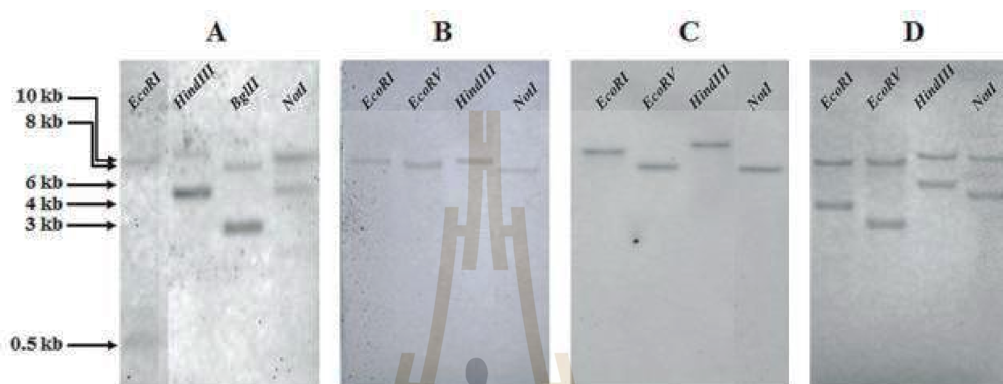

Figure 4.2 Determination of copy numbers of (A) *nodA*, (B) *nodB*, (C) *nodC*, and (D) *nifH* by Southern blot hybridization. *Bradyrhizobium* sp. DOA9 genomic DNA was digested with the restriction enzymes shown, and the blot was hybridized with probes for *nodA* (from *B. yuanmingense* SUTN6-2), *nodB* (from *B. canariense* SUTN7-2), *nodC* (from *B. japonicum* USDA110), and *nifH* (from *B. yuanmingense* SUTN6-2).

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4.2 The function of nodulation (*nod*) genes in DOA9

The whole genome of DOA9 demonstrated a single *nod* cluster on megaplasmid which consists of 2 copies of *nodA* ($nodA_1$ and $nodA_2$) and a single copy of *nodB* and *nodC* (Okazaki et al., 2015 and Teamtisong et al., 2014). $nodA_1$ is classically found at the vicinity of *nodB* and *nodC*, and that $nodA_2$ is found just upstream of *nodIJ* genes. A nod-box promoter motif was detected on the upstream of

nodA$_1$, *nodA*$_2$ and other genes involved in NF biosynthesis and regulation, as in other rhizobia (Figure 4.3).

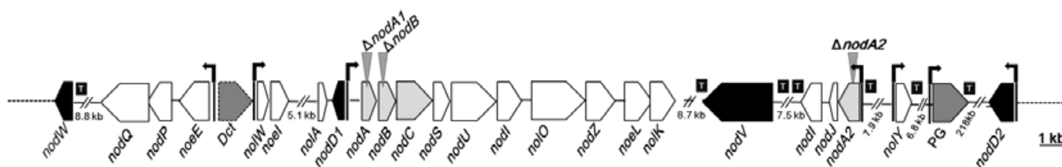

Figure 4.3 Genetic organization of *nod* gene cluster of *Bradyrhizobium* sp. DOA9. The orientations and sizes of the ORFs were indicated by arrows. Location and orientation of nod-box motifs are shown by black rectangular arrows. Mutant strains Δ*nodA*$_1$, Δ*nodA*$_2$ and Δ*nodB* were interrupted from cointegration of pVO155-npt2-Sm/Sp-gusA-nptII-gfp plasmid at the sites depicted by arrowheads.

BLASTP and phylogenetic tree analysis of the common *nod* genes were evaluated. Amino acid sequence of nodA$_1$ and nodA$_2$ shares only 38% identity each other. Although, nodA$_1$ shares very low similarity (32–36%) with the copies in other *Bradyrhizobium* strains, while nodA$_2$ shares 63–69% identity. nodB and nodC shared more than 60% identity with the corresponding genes in *B. japonicum*. Phylogenetic analyses of the *nodA*, *nodB* and *nodC* genes showed that common *nod* genes those of DOA9 were not placed on classified branches of the known *nod* gene-containing rhizobia strains (Figure 4.4).

To understand the function of *nod* genes in DOA9, *nodA*$_1$, *nodA*$_2$, and *nodB* genes were interrupted by cloning the internal fragment from that of genes into pVO155-nptII-Sm/Sp-nptII-GFP and used for single homologous recombination events. The mutant strains were verified and annotated as Δ*nodA*$_1$, Δ*nodA*$_2$ and Δ*nodB* (Figure 3.1A and Figure 4.3).

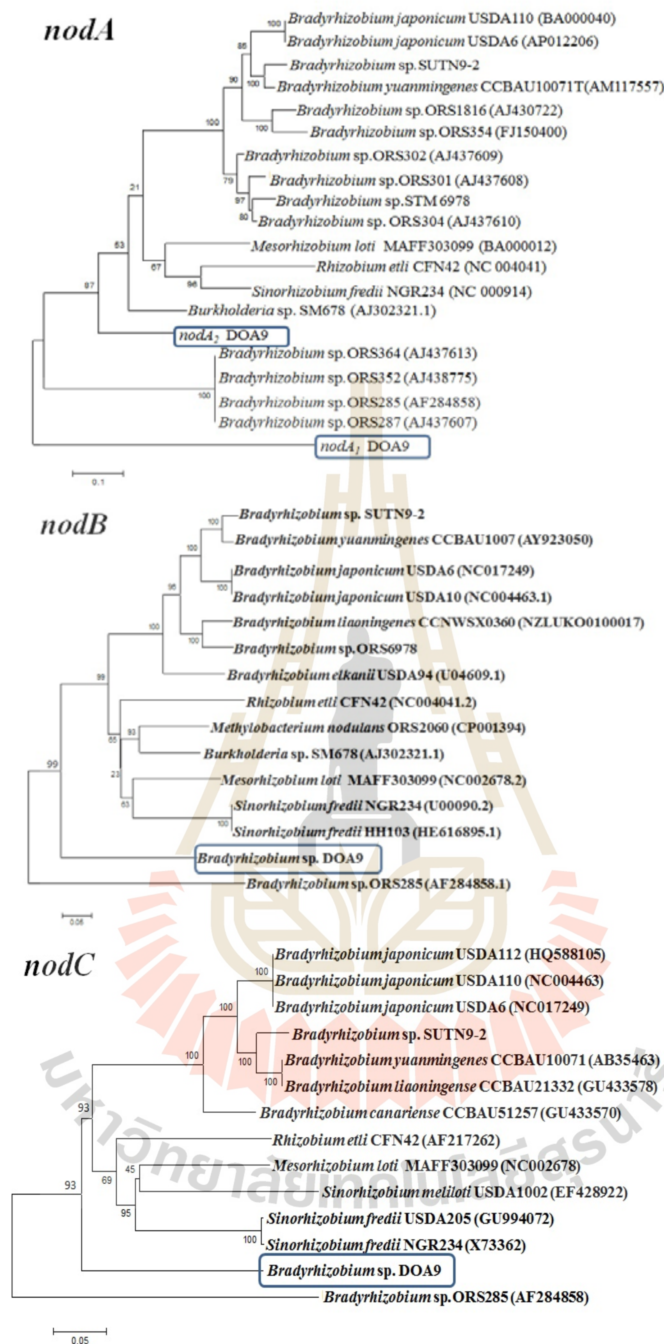

Figure 4.4 Molecular phylogenetic analysis of *Bradyrhizobium* sp. DOA9 *nodA, nodB* and *nodC* genes by Maximum Likelihood (ML) method based on the Hasegawa-Kishino-Yano model. Bootstrap percentages out of 1,000 replicates are shown on branches.

All of the *nod* mutant strains and DOA9 wild type were inoculated on leguminous plants in Dalbergioids and Milletioids tribes as listed in Table 3.1. The results revealed that Δ*nodA*$_2$ and Δ*nodB* mutant strains lost their ability to induce nodules on all the plants tested. These results indicated that NFs were absolutely required for the establishment of the symbiotic interaction of DOA9 with these host plants and that *nodA*$_2$ and *nodB* were essential for NFs synthesis (Figure 4.5 and 4.6).

In case of Δ*nodA*$_1$ mutant strain, the nodulation effects on plants tested were categorized into 2 groups including *nodA*$_1$-independent and *nodA*$_1$-dependent phenotypes. The plants in *nodA*$_1$-independent group displayed a nodulation and a nitrogenase activity similar to that of the wild-type strain. *nodA*$_1$-independent plants were *A. americana* (Figure 4.5A-E) and *A. afraspera* (Figure 4.5F-J) from Dalbergioids tribe, and *Indigofera tinctoria* (Figure 4.5K-O) and *D. tortuosum* (Figure 4.5P-T) from Milletioids tribe. In contrast, *nodA*$_1$-dependent plants were strongly affected in their ability to form nodules and to fix nitrogen with Δ*nodA*$_1$ mutant strain. *S. hamata* (Figure 4.6H-N) and *M. atropurpureum* (Figure 4.6O-U) displayed a significant reduction in nodule numbers and nitrogenase activity, while nodulation was completely aborted in *A. hypogaea* (Figure 4.6A-G).

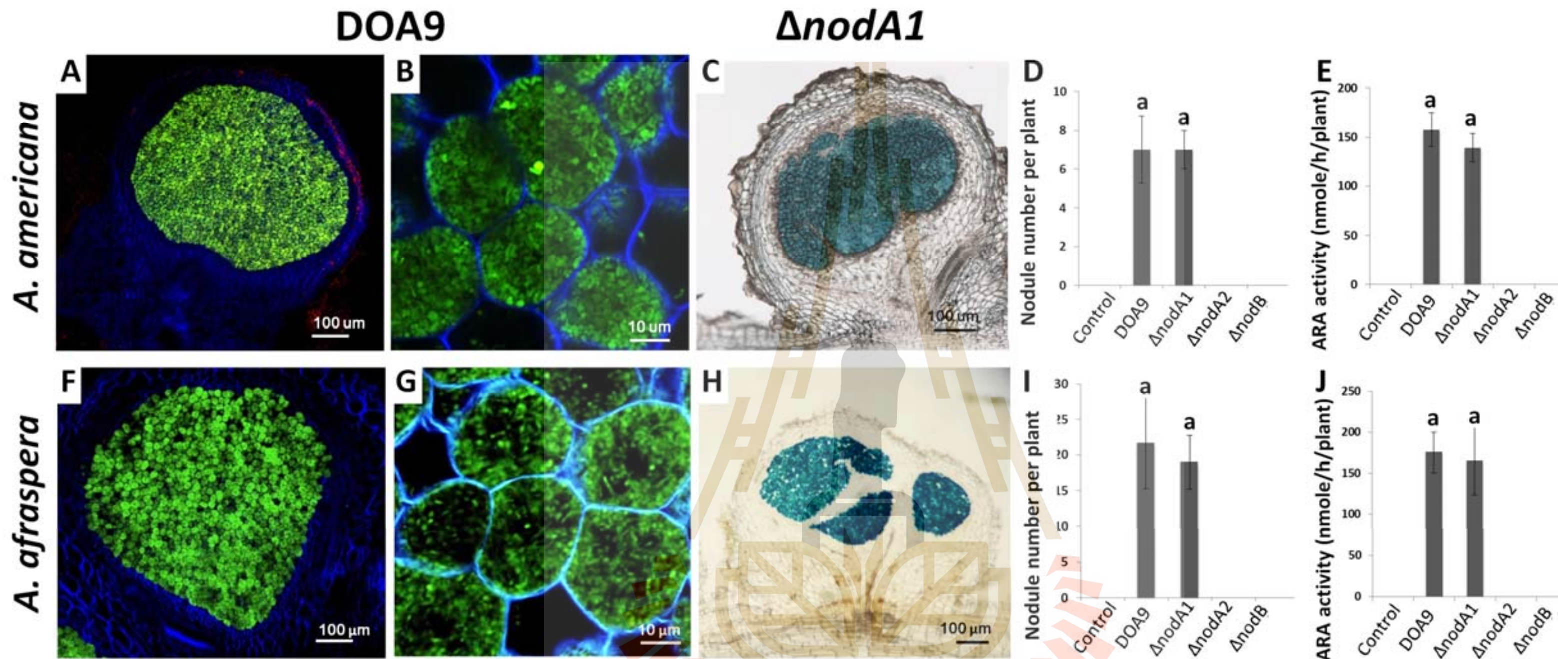

Figure 4.5 Symbiotic interaction of *nodA$_1$*-independent group. Nodule phenotype of *A. americana, A afraspera, I. tinctoria* and *D. tortuosum* after inoculated with *Bradyrhizobium* sp. strain DOA9 wild-type (A-B, F-G, K-L and P-Q), and Δ*nodA$_1$* mutant strain (C, H, M and R). Transcriptional GUS fusion in Δ*nodA$_1$* was detected by staining with GUS substrate (C, H, M and R). Nodule numbers and nitrogenase activity also demonstrated (D-E, I-J, N-O and S-T).

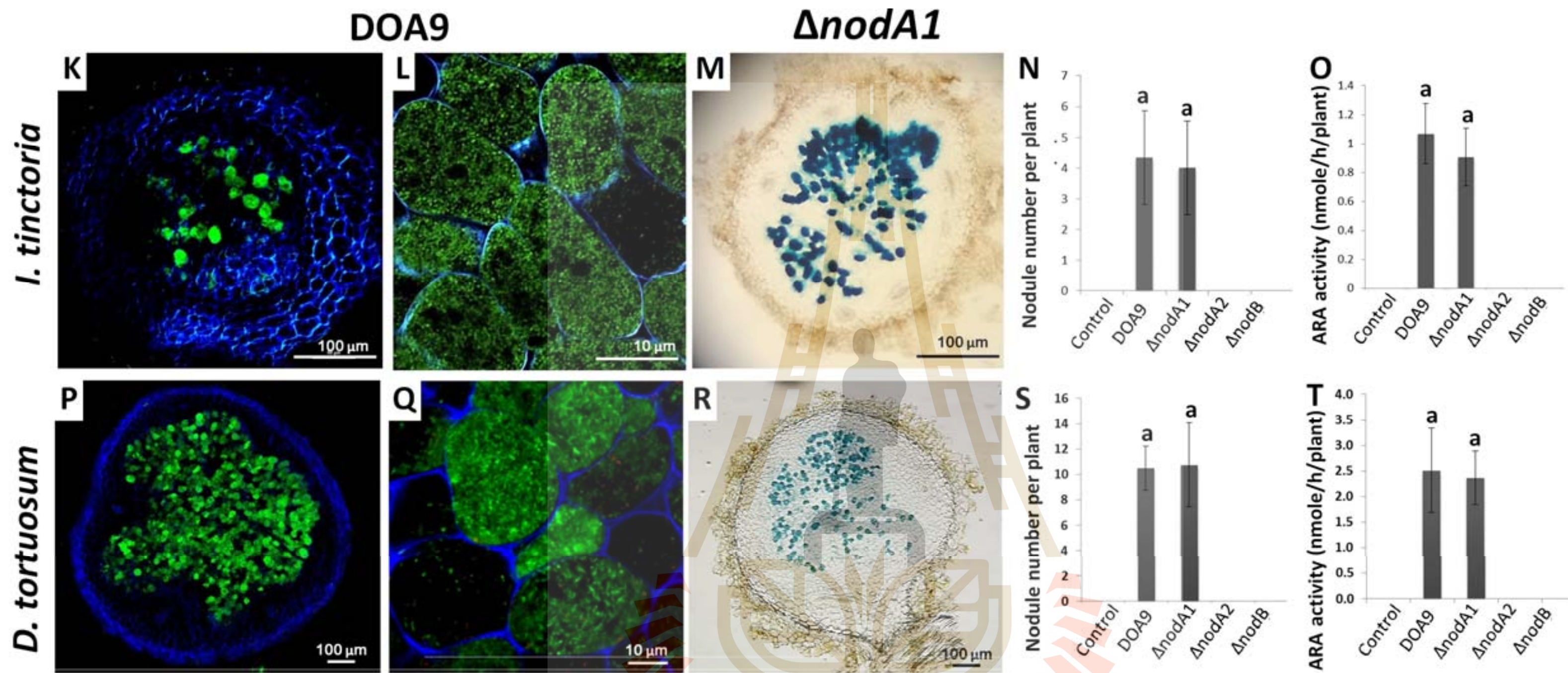

Figure 4.5 (Continued).

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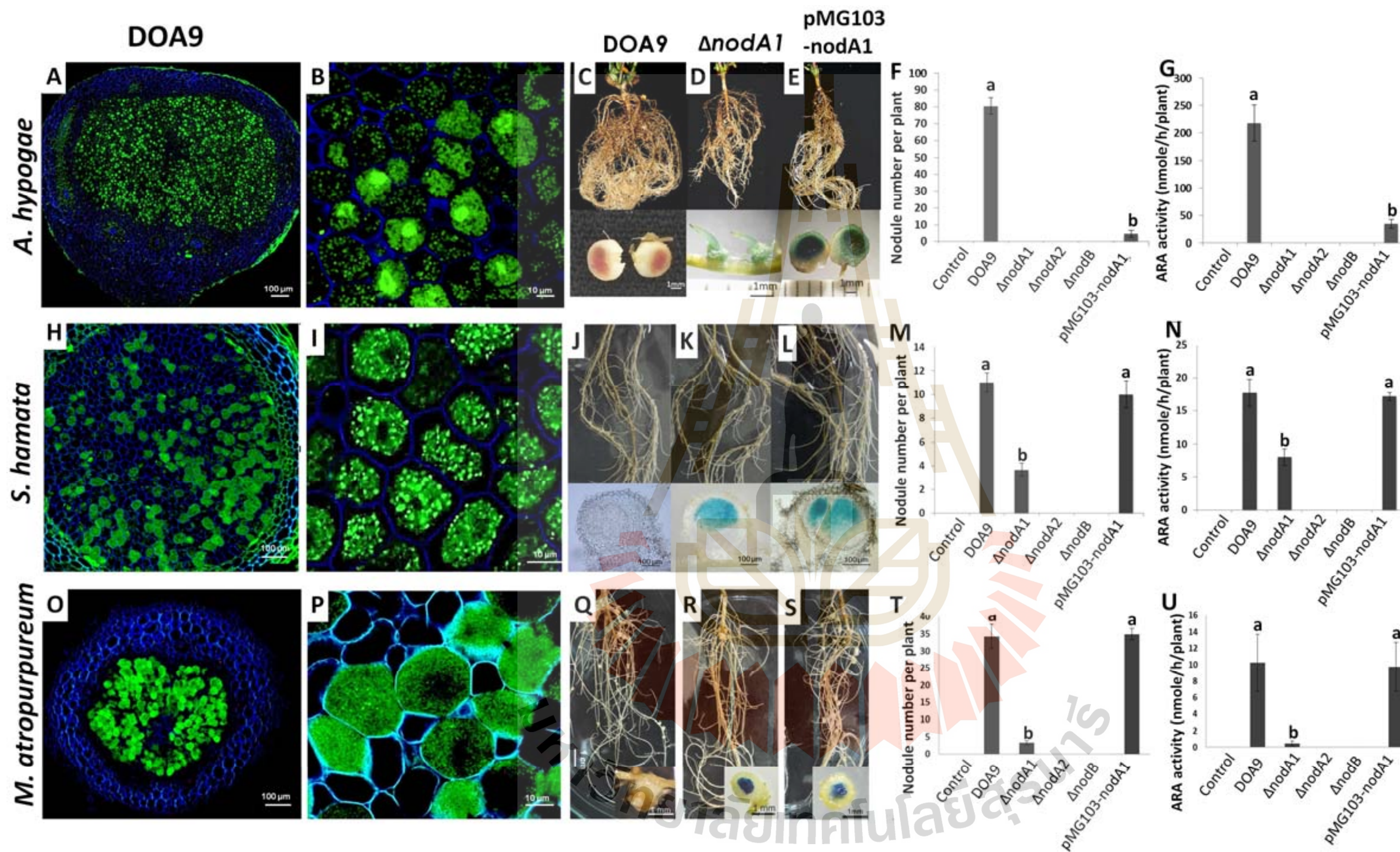


Figure 4.6 Symbiotic interaction of $nodA_1$-dependent group. Nodule phenotype of *A. hypogaea*, *S. hamata* and *M. atropurpureum*

(siratro) after inoculated with *Bradyrhizobium* sp. strain DOA9 wild-type, $\Delta nodA_1$ and

complement *nodA*$_1$ strain (pMG-nodA1). Transcriptional GUS fusion in Δ*nodA*$_1$ and pMG-nodA1 were detected by staining with GUS substrate (D-E, K-L and R-S). Nodule numbers and nitrogenase activity also demonstrated (F-G, M-N and T-U).

The interruption of *nodA*$_1$ by transcriptional fusion of pVO155 with promoterless-GUS reporter gene showed positive staining with X-Gluc substrate (Figure 4.5C, H, M, R and 4.5D, K, R). These results were elucidated that promoter belonging to *nodA*$_1$ was used and *nodA*$_1$ was transcribed. Although, *A. hypogaea* roots were absence of nodules, but GUS activity was also detected in the lateral root tissues (Figure 4.6D).

However, the interruption of either *nodB* or *nodA*$_2$ (*ΔnodB* and *ΔnodA*$_2$) could loss a first signaling molecule (NFs) that lead to a complete absence of nodules in all plants tested. Thus, NFs in DOA9 could play the major roles for symbiotic interaction between partners, whereas *nodA*$_1$ may expand a broad host range by adding acyl-chains on the NF backbone. To confirm the function of *nodA*$_1$, full length of *nodA*$_1$ and its promoter (1320-bp in length) was introduced into Δ*nodA*$_1$ (annotated as pMG103-nodA1) and inoculated into *nodA*$_1$-dependent plants. pMG103-nodA1 was able to restore the nodulation and nitrogen fixation capacity in *S. hamata* (Figure 4.6L-N) and *M. atropurpureum* (Figure 4.6S-U). In contrast, the complementation was slightly induced the nodule formation only a few number on *A. hypogaea* roots (Figure 4.6E-G). In case of *V. radiata*, *nodA*$_1$ also impacted to the nodule numbers (Table 4.1), small and senescence nodules were formed as that of wild-type. Thus, complementation test was disregarded in this plant. Overall, these results indicated that *nodA*$_1$ plays a role in the symbiotic interaction with *S. hamata* and *M. atropurpureum*, but not clear in the *A. hypogaea* symbiosis.

To observe *nod* genes responding to different functional classes of flavonoids, the ß-glucuronidase transcriptional fusion analyses were evaluated (Figure 4.7A). The results showed that the isoflavones genistein, formononetic and daiazein are good inducer for of $nodA_1$, $nodA_2$ and *nodB* gene. Some of the flavones (apigenin and 7,4'-dihydroxyflavone) and flavanones (naringenin and liquiritigenin) also activated the expression of *nod* genes but in different levels. In detail, *nodA2* and *nodB* were highly induced by genistein, formononetic, daiazein and apigenin, but slightly induced were found in the present of 7,4'-dihydroxyflavone, naringenin and liquiritigenin. The expression of *nodA1* was highly activated by daiazein while genistein, formononetic, daiazein apigenin, 7,4'-dihydroxyflavone, naringenin and liquiritigenin were less active in the induction It was likely that the *nod* genes expressions in DOA9 response well to the same classes of inducer (Figure 4.7A).

The relative expression of *nod* genes under induction of flavonoids and root exudates was also investigated by using RT-PCR and qRT-PCR. In relative quantification, up or down regulation were analyzed for the changing in gene expression in the induced sample relative to non-inducible control (such as non-induced cells (NI) or DMSO treatments). The expression of $nodA_1$, $nodA_2$ and *nodB* were highly up-regulated after addition of naringenin and genistine flavonoids and also with root exudates from individual legumes tested for 24 h while they were less responded in 12h (Figure 4.7A and B). Although, the expressions of $nodA_1$, $nodA_2$ under induction of *V. radiata* and *A. hypogaea* root exudates were not detected at 24h, but they were appeared after induced for 12h (Figure 4.7A).

Since DOA9 lack the ability to nodulate soybean roots (*Glycine max*). Thus, *nod* genes expression responding the soybean root exudates were not detected under

this treatment. The expression of *nodA*$_1$ and *nodA*$_2$ by using RT-PCR showed similar level of induction but *nodB* expression was more up-regulated than those of genes. In case of qRT-PCR, the expression of *nodA*$_2$ has less responded with root exudates inducers, especially under *A. hypogaea*, *S. hamata* and *M. atropurpureum* root exudates which belonging to *nodA*$_1$-dependent group (Figure 4.7 D). Although, *nodA*$_2$ expression under *A. americana* and *A. afrasfera* root exudates were less induced than *nodA*$_1$ but the ratio of expression kinetic were lower than that of the *S. hamata* and *M. atropurpureum* induction. These results implied that *nodA*$_1$ was likely play an important role for nodulation of legumes belonging to *nodA*$_1$-dependent (Figure 4.7 D).

The expression of *nodA* and *nodB* in Δ*nodA*$_1$ mutant strain was evaluated after induced with flavonoids (naringenin and genistine) for 24 h. The mutation of *nodA*$_1$ was completely abolished the expression of *nodA*$_1$ while complement *nodA*$_1$ strain (pMG103-nodA1) was able to restore *nodA*$_1$ expression. (Figure 4.8). Surprisingly, *nodB* was not affected by Δ*nodA*$_1$ mutant strain. This result indicated that *nodA*$_1$ mutation do not affect to the expression of downstream *nodB* genes. Moreover, non-polar effect of *nodB* could explain for the nodulation of Δ*nodA*$_1$ in *nodA*$_1$-independent group is that they were not required *nodA*$_1$ for their nodulation but might use *nodA*$_2$ instead of *nodA*$_1$.

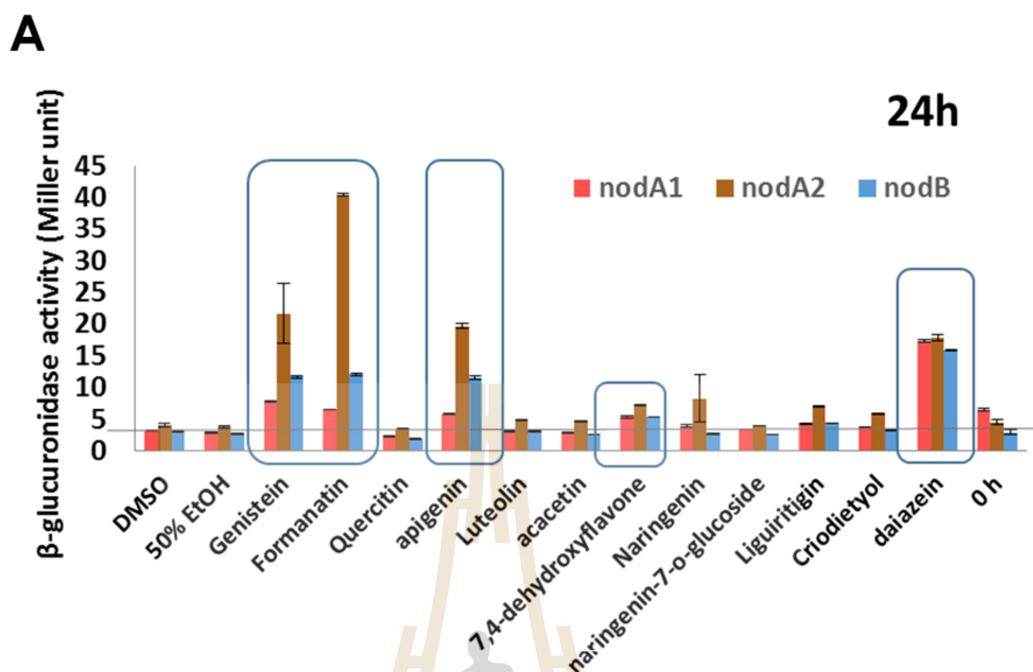


Figure 4.7 The relative expression of *nodA*$_1$, *nodA*$_2$ and *nodB* genes of *Bradyrhizobium* sp. DOA9 (wild-type) under induction of flavonoids (Nar = Naringenin, Gen= Genistein) and legume root exudates (A, B and C). A indicated ß-glucuronidase transcriptional fusion analyses of *nodA*$_1$, *nodA*$_2$ and *nodB* under induction of different functional classes of flavonoids. B and C indicated *nodA*$_1$, *nodA*$_2$ and *nodB* genes expressions in DOA9 (wild-type) under legumes root exudates were evaluated at 12h (B) and 24h (C) by using RT-PCR. For qRT-PCR of *nodA*$_1$, *nodA*$_2$ and *nodC* under legumes root exudates induction for 24 h indicated in D. The root exudates from legumes were define as AA = *A. amercana*, AF = *A. afraspera*, Sty = *S. hamata*, Ah = *A. hypogaea* cv. Thai Nan, MB = *V. radiate* cv. SUT4, Ig = *I. tinctoria*, Sir = *M. atropurpureum* (siratro), Des = *D. tortuosum*, Cj = *C. juncea* and SB = *Glycine max*. The control is non-induced cells (NI) and DMSO.

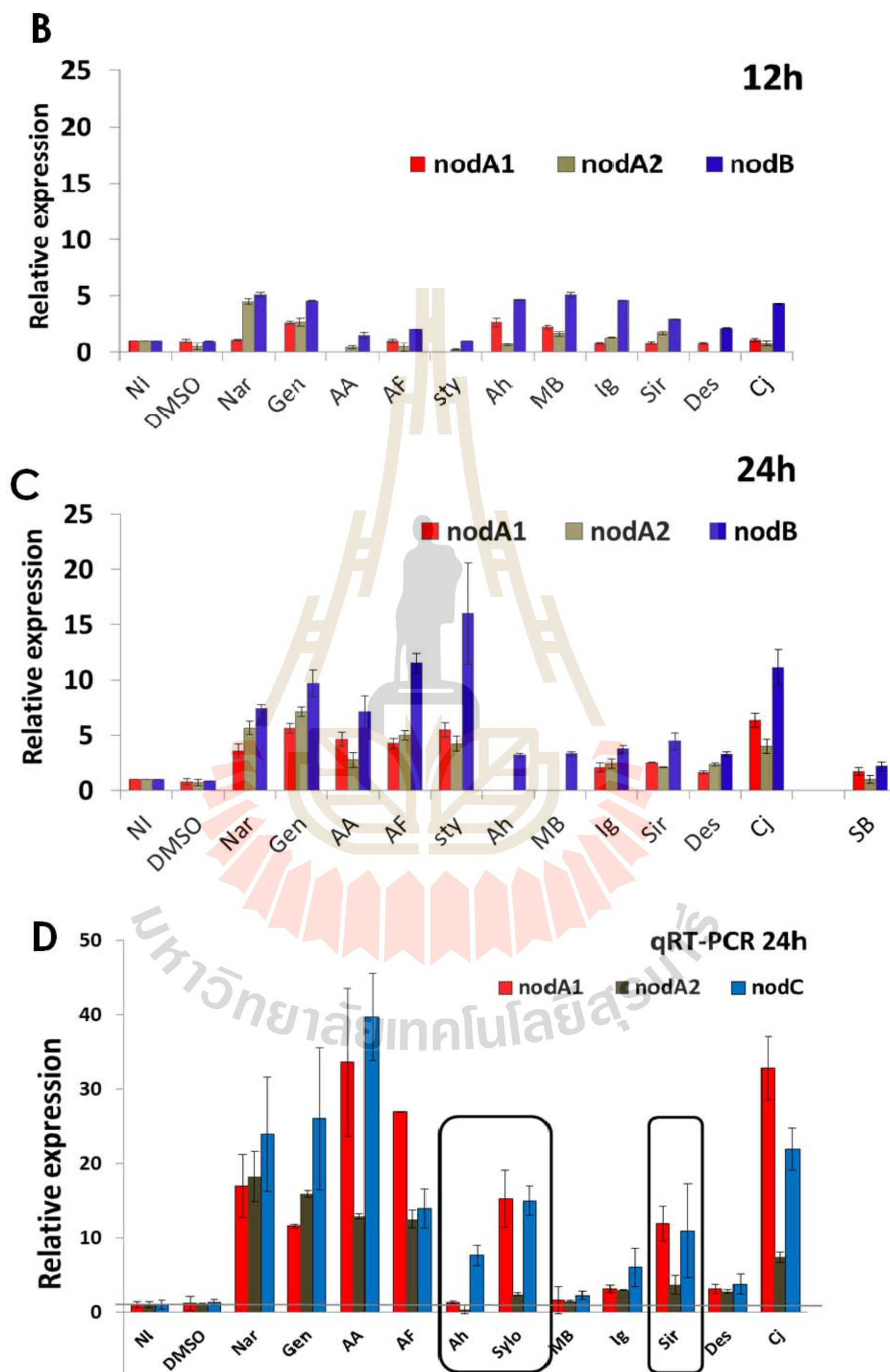


Figure 4.7 (Continued).

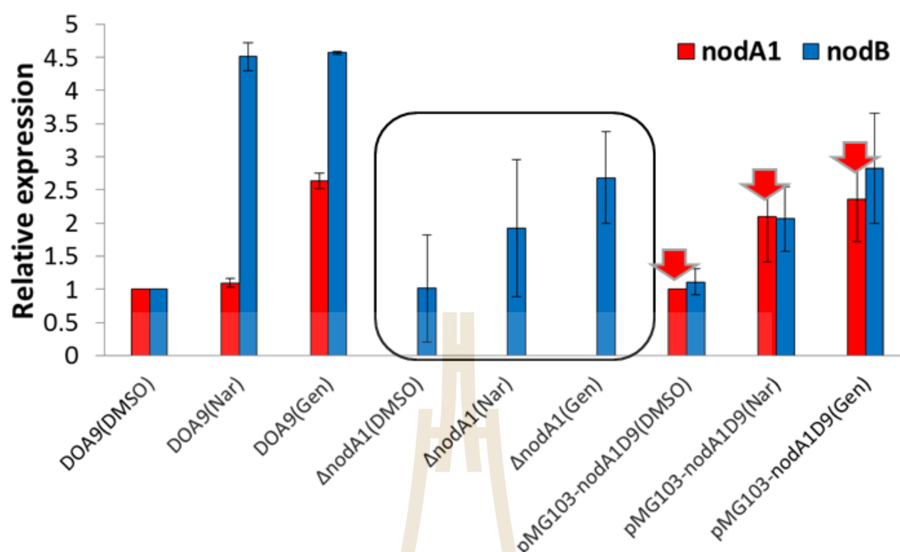


Figure 4.8 The relative expression of *nodA₁* and *nodB* genes of *Bradyrhizobium* sp. DOA9 (DOA9), Δ*nodA₁* and pMG103-nodA1 (*nodA₁* complement strain) after induced with Nar = Naringenin, Gen= Genistein for 12 h. The control is non-induced cells (NI) and DMSO.

4.3 Analysis of Nod-factor receptor 5 (NFR5) proteins in all plants tested

To investigate the determinants involved in early recognition events in DOA9-leguminous symbiosis, 510-bp of putative extracellular NFR5 orthologs in all plants tested were analyzed. Phylogenetic analysis revealed that the NFR5 protein sequences were clustered into 5 groups belonging to the legume subfamily.

Unlike *A. americana, A. afraspera* and *A. hypogaea*, *Stylosanthes* NFR5 was grouped within Milletioid and closely related with *M. atropurpureum*. According to the nodulation responding to *nodA₁* mutant strain, *nodA₁*-dependent (yellow box) and

independent (green box) were distributed to both Dalbergioids and Milletioid (Figure 4.9). Interestingly, *S. hamata* and *M. atropurpureum* NFR5 had high similarity and also strongly affect by *nodA₁* deficient. In addition, pMG103-nodA1 strain was able to restore the nodulation on these plants.

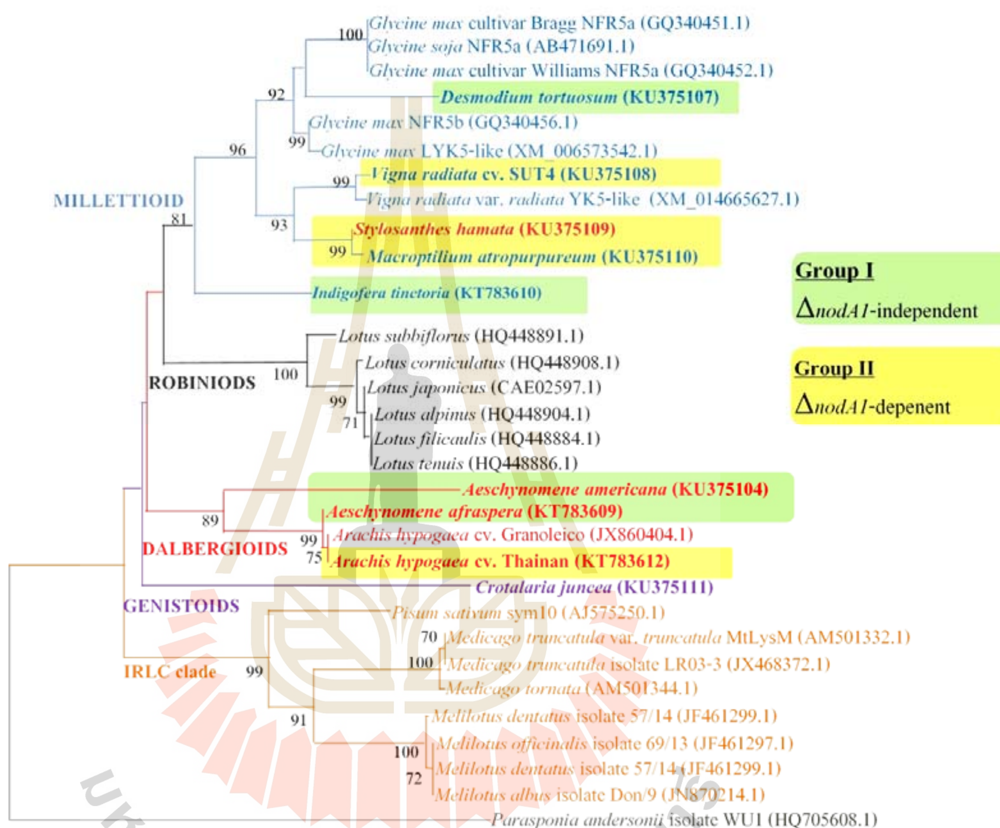


Figure 4.9 Molecular phylogenetic analysis of nod-factor receptor 5 (NFR5) from legumes tested. The evolutionary history was inferred by using the Maximum Likelihood (ML) method based on the Kimura 2-parameter model. Bootstrap percentages out of 1,000 replicates are shown on branches.

These results suggesting that *S. hamata* and *M. atropurpureum* NFR5 orthologs could recognize restrict to the NF associated with acyl-chains added by

nodA$_1$. Moreover, extracellular regions of LysM domains on NFR5 were not conserved within tribes but might modify or change depend on geographic adaptation or cultivar selection.

4.4 The effect of Type 3 secretion system (T3SS) on plant nodulation by DOA9

T3SS cluster of DOA9 was depicted in Figure 4.10. The nodulation of T3SS machinery mutant strain (Δ*rhcN*) showed some defects on plant nodulation, depending on the plant species (Figure 4.11 and 4.12).

Figure 4.10 Genetic organization of T3SS involved gene cluster of *Bradyrhizobium* sp. DOA9. The orientations and sizes of the ORFs were indicated by arrows. Location and orientation of tts-box motifs were rectangular open arrows, while nod-box motifs are shown by black rectangular arrows. Mutant strain Δ*rhcN* was interrupted from co-integration of pVO155-npt2-Sm/Sp-gusA-nptII-gfp plasmid at the sites depicted by arrowheads.

The nodulation phenotypes responding to T3SS deficiency were classified into 3 characteristics. First, non-responsive phenotype (indicated as T3SS-no effect group) the mutant showed the same nodule number and N_2 fixing capacity as wild-type formed. These plants were *A. americana*, *A. afraspera*, *I. tinctoria*, *D. tortuosum*

and *M. atropurpureum* (Figure 4.11). Nevertheless, some nodules of *M. atropurpureum* associated with wild-type DOA9 displayed brown necrotic areas in some part of the nodule while never detected in *rhcN* mutant nodules (Figure 4.12). The non-viable bacteroids or dead cells were detected by propidium iodide (PI), indicating on the stained red cells. Second group was T3SS-effect which positive or negative affected by the Δ*rhcN* mutant strain. The negative effect was found in *S. hamata*. The reduction of nodule numbers and nitrogenase activity were detected on *S. hamata* (Figure 4.13). In case of positive effect from Δ*rhcN*, improving of nodule phenotypes were observed in *V. radiata* (Figure 4.14M-Q) and *C. juncea* (Figure 4.14E-K). In detail, small nodules with necrotic symptom on *V. radiata* roots were reformed to be bigger and regular shapes (Figure 4.14M and N). For the *C. juncea* nodulation, the indeterminate nodules were formed by Δ*rhcN* instead of pseudonodules infected by wild-type DOA9 (Fig 4.14E and I). The inoculation of Δ*rhcN* induced a high number of nodules in *A. hypogea* but do not significantly increased N_2-fixation value per plant (Figure 4.14C). However, plants inoculated with neither DOA9 wild type nor Δ*rhcN* were not improved the plant dry weight, except plants in Dalbergioids (Table 4.1). In these findings suggested that defective nodules derived from Δ*rhcN* were not significantly induced N_2 fixating properties but clearly improved the nodule phenotypes, such as *V. radiata* and *C. juncea.* Furthermore, the effective nodule induced by DOA9 implied that DOA9 may originally symbiosis with Dalbergioids rather than Genistioids and Millettioid plants. Thus, the symbiotic genes in DOA9 might acquire the sequential evolution for expanding the host range in other leguminous plants.

The expression of *rhcN* was evaluated under induction of legumes root

exudates (Figure 4.15) by using qRT-PCR. The results found that flavonoids (naringenin and genistine) and legumes root exudates were able to up-regulated the expression of *rhcN* in DOA9. The low expression were detected in *S. hamata*, *A. hypogaea, I. tinctoria and D. tortuosum* treatments. These plants have no or less affected to nodulation and N_2 fixation kinetic after inoculated with *rhcN* mutation. Although, *A. americana* and *A. afraspera* were a good inducers for gene expression but they were T3SS-no effect phenomenon (Figure 4.11 and 4.15). Therefore, T3SS or effectors derived from T3SS of DOA9 may be not impact to the nodulation of those plants. However, T3SS had caused a large impact for some legumes, such as *C. juncea* and *V. radiata*. *rhcN* expressions were high after induction with root exudates from *C. juncea* and *V. radiate* (Figure 4.15).

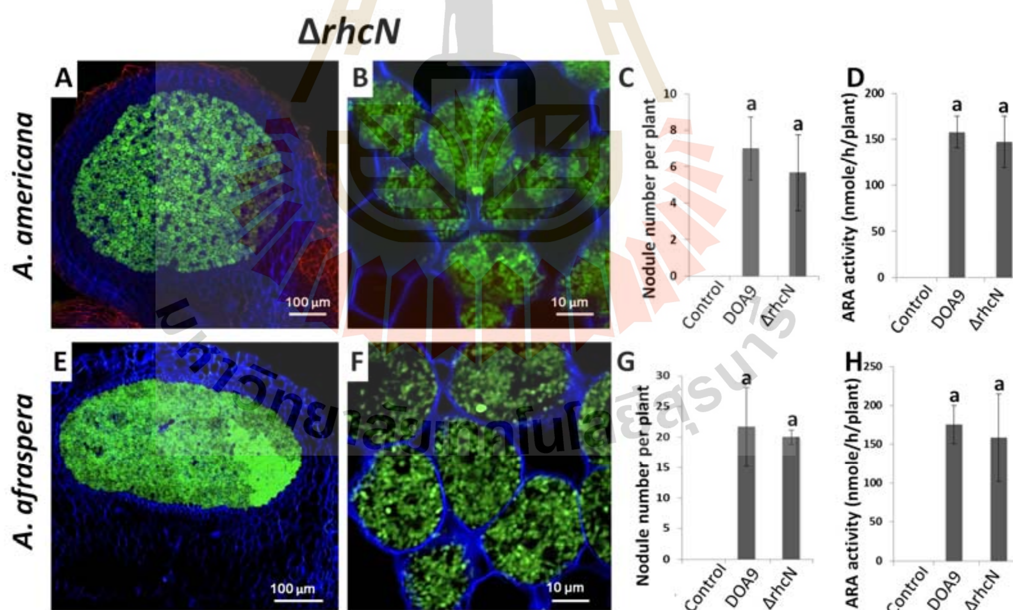


Figure 4.11 Symbiotic interaction of T3SS-no effect group. Nodule phenotype, nodule numbers and ARA activity of *A. americana* (A-D)*, A afraspera* (E-H), *I. tinctoria* (I-L), *D. tortuosum* (M-P) and *M. atropurpureum* (Q-T) after inoculated with Δ*rhcN*.

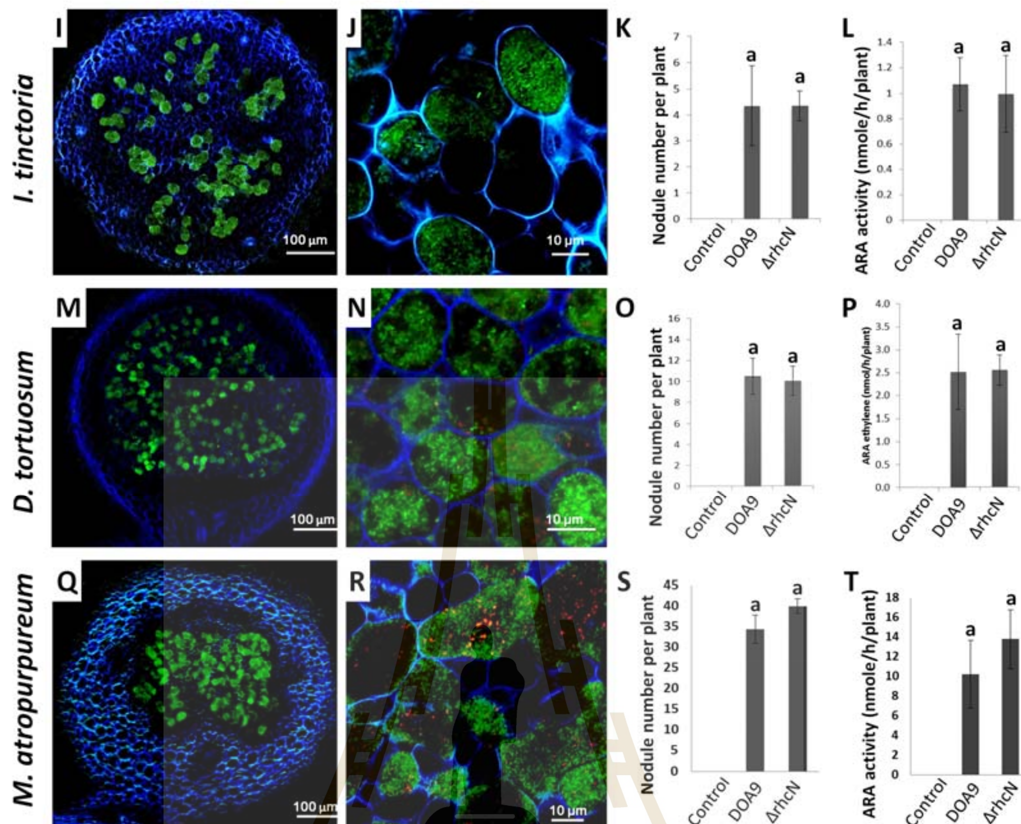


Figure 4.11 (Continued).

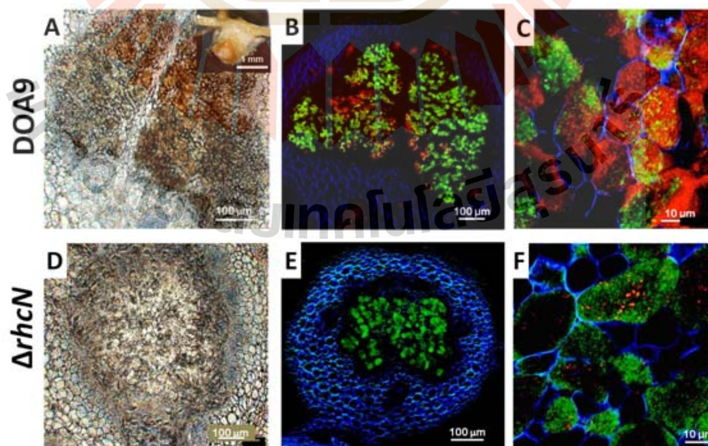


Figure 4.12 The necrotic nodules of siratro after nodulation for 21 days after inoculation. Dead cells were stained with popidium iodide (PI) which indicated in red cells. Green cell are viable cells in nodules.

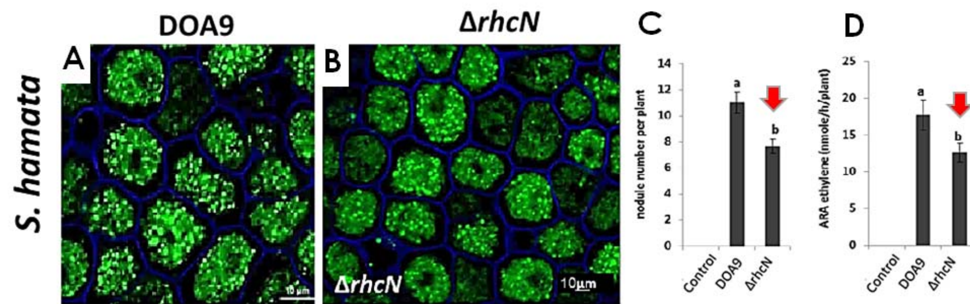
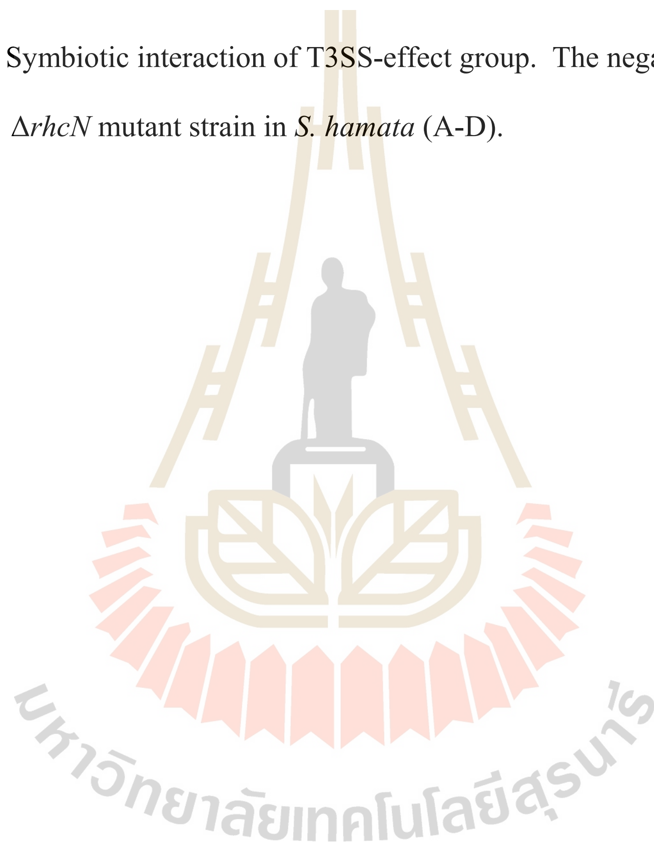


Figure 4.13 Symbiotic interaction of T3SS-effect group. The negative effect from Δ*rhcN* mutant strain in *S. hamata* (A-D).



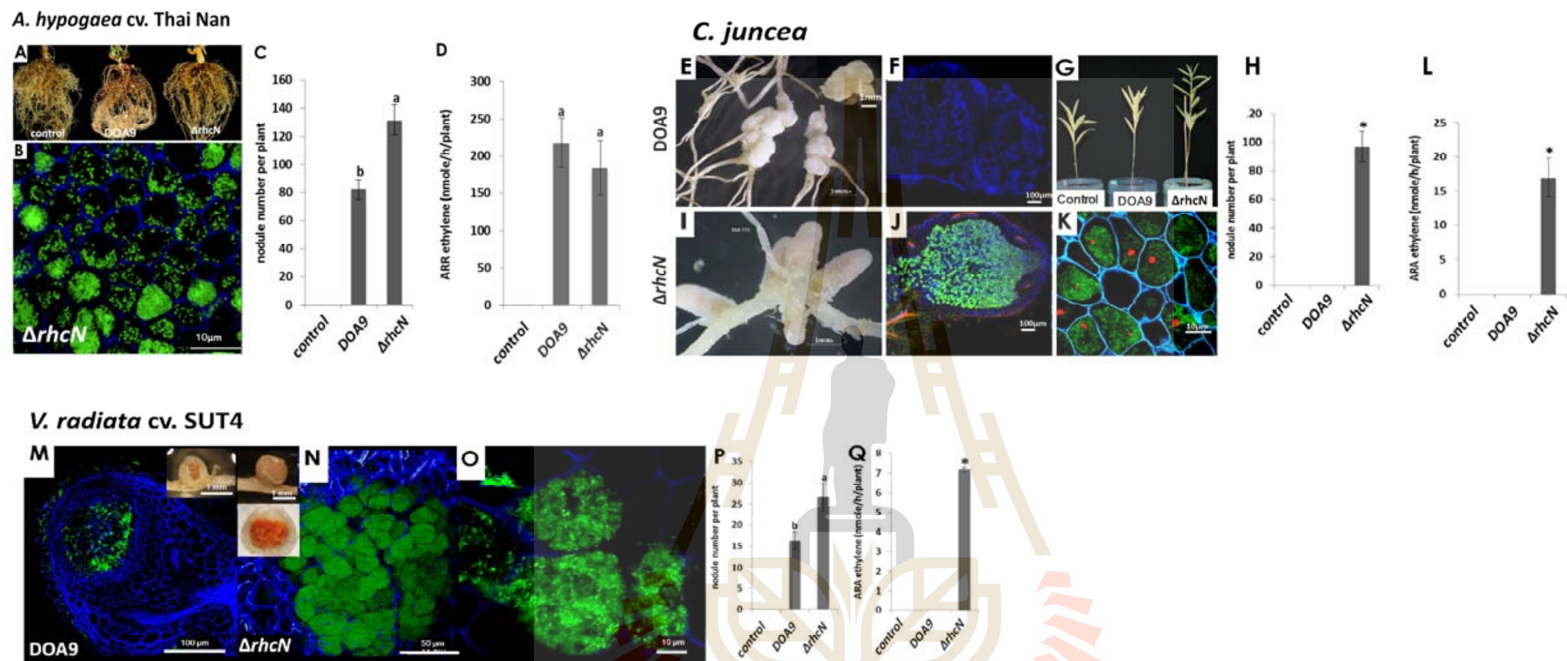


Figure 4.14 Symbiotic interaction of T3SS-effect group. The positive effect from Δ*rhcN* mutant strain included *A. hypogaea* cv. Thai Nan (A-D), *C. juncea* (E-K) and *V. radiata* cv. SUT4 (M-Q).

Table 4.1 Total plant dry weight (mg) of all plants tested after inoculated with *Bradyrhizobium* sp. DOA9, mutant and complement strains such as $\Delta nodA_1$, $\Delta nodA_2$, $\Delta nodB$, $\Delta rhcN$ and pMG103-nodA1.

Tested plants / strains		Total plant dry weight (mg)						
		Control[1]	**DOA9**	***ΔnodA1***	***ΔnodA2***	***ΔnodB***	***ΔrhcN***	**pMG103-nodA1**
Genistioids	*C. juncea*	169±20 ab[2]	152±4.9 a	144±3.7 a	166±20 ab	163±21 ab	196±38.3 b	- [3]
Dalbergioids	*A. americana*	13.1±2.2 b	67.4±4.9 a	62.3±5 a	22.8±3.7 c	18.6±3.1 cd	53.6±9.9 b	-
	A. afraspera	54.4±7.9 b	78.2±6.9 a	71.0±8.3 a	63.0±6 b	57.2±9.3 b	79.2±2.8 a	-
	A. hypogaea cv. Thai Nan	1200±219 b	2112±330 a	1017±24 b	1005±62 b	830±99.3 b	1779±248 a	1119±64.1 b
	S. hamata	5.0±0.6 c	7.8±1 a	5.9±0.8 abc	5.7±0.9 bc	5.4±0.6 c	6.4±1.1 ab	7.1±0.54 a
Millitioid	*M. atropurpureum*	31.2±5.0 a	27.6±5.8 a	30.2±4.2 a	30.3±5.4 a	25.9±4.6 a	27.1±5.1 a	25.6±4.2 a
	I. tinctoria	5.1±0.6 a	5.1±0.7 a	5.0±0.6 a	5.0±0.7 a	5.0±0.8 a	5.0±0.8 a	-
	V. radiata cv. SUT4	45.6±9.0 a	47.2±9.3 a	46.2±7.1 a	42.4±9.5 a	42.5±13.5 a	45.6±13.4 a	-
	D. tortuosum	6.5±1.2 a	6.3±1.1 a	7.0±1.1 a	5.6±1.2 a	6.0±1.4 a	7.3±1.1 a	-

Values are shown as Mean±SD of at least triplicates, [1] : uninoculation control, [2] a-d means in a column followed by the same letter are not significantly different by Dancan's multiple range test at the 5% ($p \leq 0.5$), [3] means not observed in this study.

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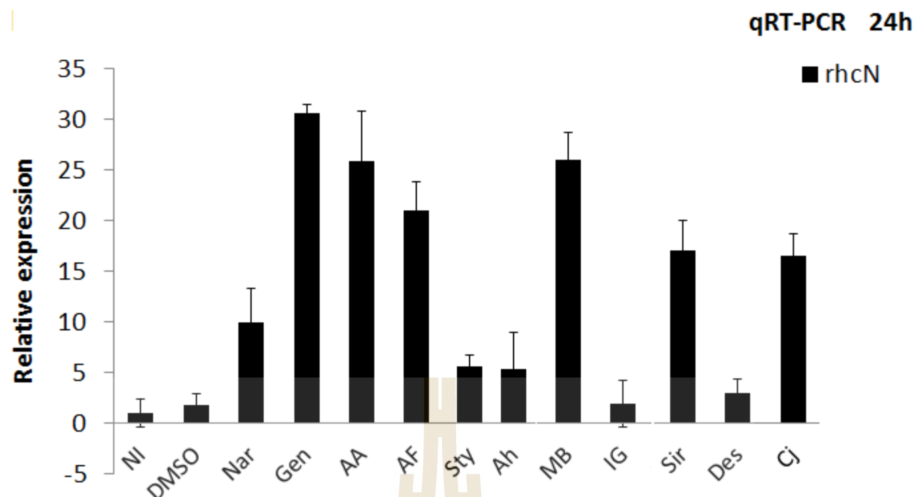

Figure 4.15 The relative expression of *rhcN* of *Bradyrhizobium* sp. DOA9 (wild-type) after induced with Nar = Naringenin, Gen= Genistein. DMSO and non-induced samples were used as negative control. The root exudates from legumes were indicated as AA = *A. amercana*, AF = *A. afraspera*, Sty = *S. hamata*, Ah = *A. hypogaea* cv. Thai Nan, MB = *V. radiate* cv. SUT4, Ig = *I. tinctoria*, Sir = *M. atropurpureum* (siratro), Des = *D. tortuosum* and Cj = *C. juncea.* The expressions under induced with legumes root exudates or flavonoids for 24h were observed by using RT-PCR (A) and qRT-PCR (B).

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CHAPTER V

DISCUSSIONS AND CONCLUSIONS

5.1 Discussions

5.1.1 First symbiotic megaplasmid found in *Bradyrhizobium* strain

Broad-host-range symbionts normally harbor multiple replicons of symbiotic genes on their genome. For example, *Sinorhizobium* sp. NGR234, which has an extremely broad host range and contains three replicons: a symbiosis plasmid, a megaplasmid, and a chromosome (Flores et al., 1998); *S. fredii* USDA257 has a chromosome and a plasmid (Schuldes et al., 2012); *S. fredii* HH103 has one chromosome and five plasmids (Weidner et al., 2012); DOA9 has a chromosome (cDOA9) and a megaplasmid (pDOA9) larger than 352 kb. A great many natural isolates of *Rhizobium* spp. carry a variety of large plasmids (MacLean et al., 2007). Genes for a few functions have been found on these large plasmids, particularly including *nod* and *nif* genes of fast-growing rhizobia plasmid. In contrast, the published genomes of *Bradyrhizobium* strains are consisted of a chromosome only, except that *Bradyrhizobium* sp. BTAi1 also harbors a plasmid of 228 kb (Cytryn et al., 2008). To date, there have been no published reports of symbiosis genes localized on plasmids of *Bradyrhizobium* (MacLean et al., 2007). Remarkably, symbiotic islands of *B. japonicum* are located on chromosome, while symbiosis genes of *Sinorhizobium* sp. are located on plasmids (Galibert et al., 2001). These characteristics may provide for contributing to the broad host range of these rhizobia. It will be interesting to find

out that symbiosis genes on plasmid have extent host range. DOA9 had at least two copies of *nifH*, one on each of the megaplasmid and the chromosome. *nifH* is present in two copies also in photosynthetic *Bradyrhizobium* ORS278 and in BTAi1 (Nzoué et al., 2009), and in one copy in *B. diazoefficiens* USDA110 (Kaneko et al., 2002), in all cases on the chromosome. We had already sequenced *nodA* of DOA9 (Accession No. DF 820426.1), which is correspondent fragment of the *nodA*probe sequence that was used in this study, and restriction sites of *EcoR*I, *Hind*III, *Bgl*II, or *Not*I was not identified in *nodA* gene. So that the hybridization data probed by *nodA* suggests that DOA9 had at least two copies of *nodA* on the megaplasmid. An increase copy number of symbiotic region had been indicated to promote plant phenotype. The inoculation of alfalfa with a moderate increase in copy number of symbiotic region of *S. meliloti* resulted in enhancement of plant growth (Castillo et al., 1999).

Therefore, this was the first report of symbiosis genes of *Bradyrhizobium* sp. located on a megaplasmid. Detailed investigation of both replicons of DOA9 would be useful. In addition, the genome sequence on *Sinorhizobium* sp. strain NGR234, *S. fredii* USDA257, and *S. fredii* HH103 share a high degree of synteny (Schmeisser et al., 2009; Schuldes et al., 2012; Weidner et al., 2012), then the host range of rhizobia might be correlated with the number of specialized protein secretion systems they carry (Schmeisser et al., 2009). Thus, it could be interesting to compare and demonstrate the protein involved in secretion systems of DOA9.

Since many of photosynthetic bradyrhizobial (PB) strains were found to be the naturally endophytic with rice (Chaintreuil et al., 2000), the general conclusion was that rice probably evolved approximately 120 million years before

legumes (Allen, 2002 and Lavin et al., 2005). Thus, PB strains might also be the ancestor of non-photosynthetic bradyrhizobia (van Berkum et al., 1995). In the present study, DOA9 shows characteristics of broad host range in legume and rice infection (Teamtisong et al., 2014). Therefore, DOA9 might be the interval of evolution of the NFs-independent symbiotic system but later this strain might loss the photosynthetic activity. Moreover, DOA9 is a divergent *nod*-containing strain rendering incomplete NFs production. This may also support the previous idea that DOA9 would associate with host plants under *nod* independent manner since and during its evolution (Teamtisong et al., 2014). To understand clearly the nodulation mechanisms, symbiotic genes disruption focusing on *nod* and T3SS genes were investigated.

5.1.2 Functional analyses of *nod* genes and T3SS in legume nodulation

The first signal for the initiation of the symbiotic interactions with legumes is NFs secreted from rhizobium (Geurts et al., 2005). *nodABC* have been collectively termed as a common *nod* genes for NF biosynthesis. NFs are structurally and functionally conserved among leguminous species (Kondorosi et al., 1989). NFs is the number of β-1,4-linked N-acetyl-D-glucosaminyl residues (*nodABC*) and the substitution at the terminal residues (e.g. *nod*, *nol*, *noe*) determined host specificity and a broad host range with plant species. Previous report indicated that acyl transferase *nodA* gene may contribute to the host-specific determinant (D'Haeze and Holsters, 2002). The broad host range and divergent of nodulation (*nod*) genes on symbiotic megaplasmid in *Bradyrhizobium* sp. DOA9 have been elucidated in previous reports (Okazaki et al., 2015 and Teamtisong et al., 2014). To understand the determinants of broad host DOA9, the function of divergent *nod* gene ($\Delta nodA_1$,

Δ*nodA*$_2$, Δ*nodB*) in DOA9 were investigated by insertional mutation and evaluated with varieties of legumes. The results demonstrated that *nodA*$_2$, *nodB* could be a main key for NFs synthesis because they lack the ability of nodulation in all plants tested when inoculated with mutant strains. Whereas, Δ*nodA*$_1$ strain impaired the nodulation capacity when inoculated with *S. hamata*, *A. hypogae* and *M. atropurpureum*. These results implied that *nodA*$_1$ and *nodA*$_2$ could be responsible to the acyl-chain substitutions at the non-reducing terminal residue of NFs backbone and contributed to broad host range ability. The sequence analysis of *nodA*$_2$ showed close homology with the reference strains while *nodA*$_1$ indicated low homologous and orthologous in the database. The copy named *nodA*$_2$ was part of a gene cluster including *nodIJ* (Figure 4.3) which are required for the secretion of NFs (Aoki et al., 2013). The *nodIJ* genes are usually co-transcribed with the *nodABC* genes as reported in *R. galegae, S. meliloti* and *R. leguminosarum*. In case of *nodA*$_1$, it is located upstream of *nodBC* and could process under a same promoter as elucidated in several strains (Egelhoff et al., 1985; Fellay et al., 1998; Loh and Stacey, 2003 and Rossen et al., 1984). Thus, polar affect from insertional mutant could be expected. The *nodA1* mutation could affect the expression of *nodBC* and similarly the *nodA*$_2$ mutation could abolish the expression of *nodIJ*. Interestingly, interrupted *nodA*$_1$ strains retained the nodulation ability in many plants tested. Moreover, *nodB* expression in *nodA*$_1$ mutant strain was retained after induction (Figure 4.8) and displays in different kinetics when compared with the *nodA*$_1$ (Figure 4.7B and C). These findings suggested that *nodB* could be expressed by an independent promoter. Another possibility was that the *nodA*$_1$ mutation could not affect to the open reading frame (ORF) of the downstream

genes (*nodBC)*. These explanations would be reasonable for nodulation capacity of Δ*nodA*$_1$ with plant belonging to *nodA*$_1$-independent were retained (Figure 4.5).

For *nodA*$_1$-dependent plants (*M. atropurpureum* and *S. hamata*), nodulation was impaired but the wild-type nodules numbers were restored in mutant strain of *nodA*$_1$ complementation (pMG103-nodA1) (Figure 4.6H-U). In the case of *A. hypogae*, the restoration is only partial (Figure 4.6A-E). It was possible that the expression of *nodA*$_1$ from the introduced plasmid was not sufficient for a fully function of the corresponding protein. Moreover, *nodA*$_2$ expression was expressed only weakly in presence of *A. hypogae* root exudates (Figure 4.7). Therefore, most of the NFs molecule synthetized might lack the N-acyl chain that could require for triggering the symbiotic programs. Currently, two copies *nodA* paralogs were discovered in *Rhizobium* strains isolated from nodules of Mimosa in French Guiana and New Caledonia (Klonowska et al., 2012 and Mishra et al., 2012). The functions for those of the *nodA* have not been reported so far.

The establishment of the rhizobium-legume symbiotic interaction required a perfect recognition of Nod-factors (NFs) by plant receptors (Bensmihen et al., 2011 and Gourion et al., 2015). NF receptors (NFR) were receptor-like kinase with extracellular domain of N-acetyl-glucosamine-binding lysine motifs (LysM). These LysM domains, originally indicated in bacteria and also represented in plant genomes (Böhm et al., 2014 and Gust et al., 2012). The extracellular region of LysM domains usually have 3 LysM domains (LysM1, LysM2, LysM3) and that each of these LysM domains is quite different in sequence (Wang et al., 2014). Two homologous NFR in *Lotus japonicus*, NFR1 and NFR5 were both important for the early symbiotic interaction with NFs. Nevertheless, the specificity of LjNFR5 in

Lotus species contains to a single Luecine residue within one of the LysM domains (Leu118 and Leu154 within LysM2) that provides strong respond to NF perception and required for NF recognition of *R. leguminosarum* DZL (Madsen et al., 2010; Radutoiu et al., 2007 and Wang et al., 2014). Thus, 510-bp of putative extracellular motifs of NFR5 of all plants tested was investigated and constructed phylogenetic tree (Figure 4.9). Amino acid sequence of NFR5 of all plants tested and other orthologs were grouped following to the tribe clade, except *S. hamata* was closely related to *M. atropurpureum* NFR5. According to the nodulation phenotype responding to *nodA*$_1$ mutation, *nodA*$_1$-independent (green box) and dependent (yellow box) were labeled in tree (Figure 4.9). If considered to the evolution of tribal relationships in the Papilionoideae and symbiotic phenotype, DOA9 most probably start to interact symbiotically with Dalbergioids which estimated 55 million years of evolution (Bertioli et al., 2013). Plant dry weigh of *A. americana*, *A. afraspera, S. hamata* and *A. hypogea* were increased after symbiosis with DOA9 (Table 4.1). While, the symbiotic with plant in Milletioid tribe (more recently) did not induced effective nodules. From these observations implied that during the evolution DOA9 strain has acquired the ability to modify the signal molecules to initiate symbiosis with various legumes but the symbiotic process is still blocked at the N_2 fixation step. The regulation of N_2 fixation depending on plant manner may dominate to the nitrogenase activity after symbiosis. All the results suggested that DOA9 used *nod*-dependent model for early signal interaction. However, the containing of both *nodA*$_1$ and *nodA*$_2$ could be used to restrict symbiosis with specific host. Thus, *nodA* genes likely expand the diversity of Nod factor acyl chains and may contribute to widening the host range in these strains.

Nod factors (NFs) biosynthesis is generally mediated by NodD proteins which belong to the LysR family of transcription regulators. It is constitutively expressed and activates both the common *nodABC* and host-specific nodulation genes in a flavonoids-dependent manner, to initiating the nodulation process (del Cerro et al., 2015; Feng et al., 2003; Kondorosi et al., 1989; Oldroyd, 2013; Wang and Stacey, 1991). Many studies of rhizobial genome indicated that one or multiple copies of *nodD* can be found depending on the rhizobial species. For example, one copy on *R. leguminosarum* pv. phaseoli, preceding the common *nodABC* operon to plays the major role in nodulation (Hungria et al., 1992). The presence of other copies of *nodD* and their regulatory functions of each *nodD* have been reported, such as *nodD2* in *S. fredii* strain NGR 234, *B. japonicum*, and *Bradyrhizobium* (Arachis) showed as a repressor of *nod* gene expression (Fellay et al., 1998; Garcia et al., 1996; Gillette and Elkan, 1996; Loh and Stacey, 2003). In case of DOA9, two copies of *nodD* were presented on pDOA9, *nodD1* is upstream of *nodA1BCSUI* operon and the other (*nodD2* located 0.27 Mb away from nod cluster as indicated in Figure 4.1) (Okazaki et al., 2015). *R. tropici* CIAT899 was reported that *nodD2* also control other functions that contribute to nodulation, and it also activated the nod-gene transcription in the present of some flavonoids (del Cerro et al., 2015). Thus, *nodD2* in DOA9 may require optimizing the interaction with each of its legume hosts. Furthermore, two-component regulator *nodV*/*nodW* are also found on both chromosome and plasmid of DOA9 (Okazaki et al., 2015), which may relate to the ability of nodulation with a variety of legume species. Thus, *nodD* function analysis in DOA9 will be further investigated.

The T3SS rhizobium clusters have been extensively investigated so far. The T3SS clusters required the present of root flavonoids, flavonoid-dependent manner. The transcriptional activator (*ttsI*) is preceded by a nod-box promoter motif and subsequently activates the *ttss* gene cluster and secreted proteins (Krause et al., 2002; S. Okazaki et al., 2009 and Viprey et al., 1998). T3SS may positively or negatively affect symbiosis. The deletion of T3SS in of *Bradyrhizobium* delayed the nodulation on soybean and reduced nodule number on *M. atropurpureum* (Krause et al., 2002). The mutation of T3SS genes in *B. elkanii* USDA61 induced the nodule formation on non-nodulation trait soybean (*Rj2*) (Okazaki et al., 2013, 2009 and Tsukui et al., 2013). In this study, the nodulation of T3SS machinery mutant strain (Δ*rhcN*) showed some defect on plant nodulation, depending on the plant species (Figure 4.11 and 4.13). Nodulation phenotypes responding to Δ*rhcN* were grouped into 2 main groups. First is T3SS-no effect group, Δ*rhcN* mutant strain did not affect to nodules quantity and quality (Figure 4.11). In the order hand, T3SS-effect group showed positive respond with *C. juncae* and *V. radiata* after inoculated with Δ*rhcN* mutant strain (Figure 4.14). T3SS mutant strain improved nodule phenotype better than that of the wild-type. Whereas, *S. hamata* nodules and nitrogenase activity per plant were decreased (Figure 4.13A-D). The plants belonging to T3SS-effect phenomena was able to stimulate the high expression of *rhcN*, especially *C. juncae* and *V. radiate* (Figure 4.14). Although, *M. atropurpureum, A.americana* and *A. afraspera* root exudates showed high expression of r*hcN* but they are less or no affected from *rhcN* mutation (Figure 4.11 and 4.15). As well as in *S. hamata*, *A. hypogaea, I. tinctoria and D. tortuosum* treatments, low expression levels were detected (Figure 4.15).

These data indicated that DOA9 does not process T3SS as the key to initiate the symbiotic interaction with legumes. Nevertheless, T3SS or effectors derived from T3SS might modify the plant immunity in some legume, depending on the plant species. In this study found interested phenotype responding to T3SS lacking strain on *C. juncea* and *V. radiata*. Therefore, effector proteins secreted from T3SS could be a key determinant involved nodulation on *C. juncea* and *V. radiata*. The interruption of gene encoded effector proteins should be explored further.

5.2 Conclusions

This finding has provided that *Bradyrhizobium* sp. DOA9 contains *nod* and *nif* genes on megaplasmid, including a single copy of *nodB* and *nodC,* 2 copies of *nodA* and on plasmid, and *nifH* on plasmid and another on chromosome. Nodulation tests implied that DOA9 processes of *nod*-dependent for endosymbiosis. The divergent *nodA*1 genes are likely expand the diversity of Nod-factor acyl chains, and might broaden the host range of the DOA9. T3SS of DOA9 does not play a crucial role on the nodulation but might process some specific effectors able to modify the plant immunity on plant species. Therefore, the megaplasmid and divergent of *nod* genes in might contribute to the host range nodulation of *Bradyrhizobium* strain DOA9. Both of divergent *nod* gene and T3SS genes in DOA9 could explain the roles of determinant in its broad-host range.

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APPENDIX

Table 1 Yeast Extract Mannitol modified medium (YEM) (Giraud *et al.*, 2000)

Component	gram per liter
D-Mannitol	10
K_2HPO_4	3
$MgSO_4 \cdot 7H_2O$	0.1
NaCl	0.05
$CaCl_2 \cdot 2H_2O$	0.04
$FeCl_3$	0.004
$MnSO_4$	0.01
Sodium glutamate	3
Yeast Extract	2.5

Adjust pH to 6.8 with 0.1 N NaOH

Table 2 LB medium (Bertani, 1951)

Component	gram per liter
Tryptone	10
yeast extract	5
NaCl	5

Adjust pH to 6.8 with 0.1 N NaOH

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Table 3 Modified HM medium (Somasegaran & Hoben, 1994)

Component	gram per liter
Sodium Glutamate	1
Na_2HPO_4	0.125
$NaSO_4$	0.25
NH_4Cl	0.32
$MgSO_4{\cdot}7H_2O$	1.8
$FeCl_3$	0.004
$CaCl_2{\cdot}2H_2O$	0.013
HEPES	1.3
MES	1.1
Yeast extract	0.5
L-arabinose	0.5

Adjust pH to 6.8 with 0.1 N NaOH

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BIOGRAPHY

Ms. Pongpan Songwattana was born on August 15, 1985 in Samut Sakhon, Thailand. She graduated with a bachelor degree of Crop Production of Technology in year 2007 and a master degree in Biotechnology school from Suranaree University of Technology in year 2010. She continue to study a doctoral degree course in school of biotechnology with Prof. Dr. Neung Teaumroung in year 2011. While studying, she received OROG scholarship from SUT to support her tuition and fee. Her research topic was functional analyses of nodulation genes and type III secretion system of Bradyrhizobium sp. DOA9 involved in legumes nodulation. The results from some part of this study have been published in Microbes and Environmental Journal on 2014. She received a scholarship from Franco-Thai for a short study and her research at LSTM-Laboratories, France. She has many an opportunities to learn and exchange her knowledge from other scientific conference and laboratory in Japan.

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