SYMBIOTIC MEGAPLASMID OF *BRADYRHIZOBIUM* SP. DOA9 AFFECTING LEGUME-RHIZOBIA SPECIFICITY IN NON SYMBIOTIC *BRADYRHIZOBIUM COSMICUM* S23321

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สภาวะพึ่งพาอาศัยของแบรดี้ไรโซเบียมที่ไม่สร้างปม *BRADYRHIZOBIUM COSMICUM* S23321 หลังจากการส่งถ่ายเมกะพลาสมิด PDOA9

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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พลาสมิดขนาดใหญ่ pDOA9/*Bradyrhizobium* sp. DOA9/*Bradyrhizobium cosmicum* S23321

พลาสมิดขนาดใหญ่ DOA9 (pDOA9) ที่มียีน *nod, nif* และ Type three secretion system (T3SS) ถูกพบในเชื้อแบคทีเรีย *Bradyrhizobium* sp. สายพันธุ์ DOA9 เป็นครั้งแรก ในการศึกษานี้มีวัตถุประสงค์เพื่อส่งถ่ายพลาสมิด pDOA9 ไปสู่เชื้อแบคทีเรีย *Bradyrhizobium* โดยใช้เชื้อสายพันธุ์ ORS278 ที่มีพลาสมิด pDOA9 เป็นเชื้อส่งถ่าย pDOA9 ไปสู่เชื้อ *B. cosmicum* S23321 ซึ่งเป็นเชื้อที่ไม่มียีน *nod,* และ T3SS/T4SS จากผลการทดลองพบว่า สมบัติการสร้างปมในพืชจากเชื้อสายพันธุ์ ORS278 และ S23321 ที่มี pDOA9 มีสมบัติการสร้างปมที่แตกต่างจากเชื้อ DOA9 ถึงแม้ว่าเชื้อทั้ง 3 สายพันธุ์จะมีพลาสมิด pDOA9 เหมือนกัน โดยพบว่า pDOA9 เป็นสาเหตุการยับยั้งการสร้างปมโดยเชื้อ ORS278 แต่ เชื้อ S23321 ที่มี pDOA9 สามารถเกิดปฏิสัมพันธ์แบบพึ่งพาอาศัยกันกับพืชตระกูลถั่ว การศึกษาแสดงให้เห็นว่าเชื้อ S23321 ที่ได้รับพลาสมิด pDOA9 (S23321:pDOA9) สามารถสร้างปฏิสัมพันธ์แบบพึ่งพาอาศัยกันกับพืชตระกูลถั่วได้ 2 ลักษณะคือ ยีน *nod* บน pDOA9 ทำให้ลดความจำเพาะต่อเกิดปฏิสัมพันธ์แบบพึ่งพาอาศัยกันกับพืชตระกูลถั่ว และสามารถสร้างปมกับถั่วลิสง (*Arachis hypogea*) (Dalbergoid)) ถั่วเขียว (*Vigna radiata* cv. SUT4 (Millitoid)) ได้อย่างมีประสิทธิภาพ ในขณะที่เชื้อ DOA9 สร้างปมที่ตาย (necrotic nodule) กับถั่วเขียว ลักษณะที่สองคือ ระบบ T3SS มีผลต่อประสิทธิภาพการเกิดปฏิสัมพันธ์แบบพึ่งพาอาศัยกัน โดยผลของการสร้างปมของเชื้อ S23321 ที่มี pDOA9 (S23321:pDOA9ΩrhcN) มีความสอดคล้องกับเชื้อ DOA9 คือ เมื่อทำลายการทำงานของระบบ T3SS ทำให้ลักษณะของการสร้าปมกับ *Crotalaria juncea* ดีขึ้น นอกจากนี้ การทำลายการทำงานของยีนที่เกี่ยวกับการสร้างปม (nodulation genes) (S23321:pDOA9Ω*nodB*) ส่งผลให้ยับยั้งการสร้างปมกับพืชตระกูลถั่วทุกสายพันธุ์อย่างสมบูรณ์ ผลดังกล่าวแสดงให้เห็นว่า พลาสมิด pDOA9 ได้รับการปรับเปลี่ยนให้ขยายความจำเพาะเจาะจงกับพืชอาศัยให้กว้างขึ้น โดยอาจเกิดไปพร้อมกับวิวัฒนาการของพืชตระกูลถั่ว และการเข้าสร้างปม ซึ่งขึ้นอยู่กับชุดโครโมโซมของผู้รับพลาสมิด รวมถึงข้อจำกัดของพืชตระกูลถั่ว

นอกจากนี้อีกหนึ่งวัตถุประสงค์ ต้องการทราบบทบาทที่แน่ชัดของกลุ่ม *nod* ยีนบนเชื้อ *Bradyrhizobium* sp. DOA9 ประกอบด้วยยีน *nodA* 2 ชุด คือ *nodA1* และ *nodA2* และยังมียีน

nodD 2 ชุด คือ *nodD1* และ *nodD2* โดยบทบาทของชุดยีนดังกล่าวยังไม่มีการศึกษามาก่อน ผลการทดลองพบว่า การทำลายการทำงานของยีน *nodD1* หรือ *nodD2* เพียงยีนเดียว ไม่มีผลต่อลักษณะของพืชเมื่อเปรียบเทียบกับพืชที่ปลูกด้วยเชื้อ DOA9 โดยเชื้อที่ทำลาย *nodD1* (**Ω***nodD1*) และ *nodD2* (**Ω***nodD2*) ไม่ทำให้ระดับการสร้างปม น้ำหนักแห้งพืช และระดับการตรึงไนโตรเจนลดลง ผลของเชื้อที่ทำลายการทำงานของยีน *nodA1* (**Δ***nodA1*) และ *nodA2* (**Δ***nodA2*) พบว่ายีน *nodA1* ไม่ใช่ตัวกำหนดสมบัติการสร้างปมของเชื้อ DOA9 ผลการทดสอบพืชพบว่า **Δ***nodA1* ไม่มีผลต่อการลดจำนวนปม น้ำหนักแห้ง และระดับการตรึงไนโตรเจน ในขณะที่ ยีน *nodA2* มีบทบาทสำคัญต่อการเข้าสร้างปมของเชื้อ DOA9 เชื้อ **Δ***nodA2* ไม่สามารถสร้างปมกับพืชตระกูลถั่วทุกสายพันธุ์ที่ทดสอบ การวิเคราะห์โครงสร้าง Nod-Factors (NFs) ของเชื้อ DOA9 พบว่ามีโครงสร้างคล้ายกับ NFs ที่พบในเชื้อ *Rhizobium* sp. NGR234 ในขณะเชื้อ **Δ***nodA2* ไม่พบการสร้าง NFs สำหรับเชื้อ **Δ***nodA1* พบว่า NFs ที่สร้างมีเพียงโมเลกุลหลักที่คล้ายกับ NFs ที่พบใน DOA9 แต่ไม่พบ NFs ที่มี C18:1 acyl group จากผลดังกล่าวสรุปได้ว่า DOA9 ใช้ชุดยีน *nodD1* และ *nodD2* เพื่อกระตุ้นการการลอกรหัสพันธุกรรมชุดยีนที่เกี่ยวข้องกับกระบวนการสร้างปม ยีนทั้ง 2 มีการทำงานที่ซ้ำซ้อนกัน สมบัติการเข้าอาศัยร่วมกับพืชที่หลากหลายของเชื้อ DOA9 นี้ เป็นไปได้ว่ามาจากการที่จำนวนชุดยีน *nod* หลายชุด ซึ่งมีผลต่อของโครงสร้าง NFs

สาขาวิชาเทคโนโลยีชีวภาพ
ปีการศึกษา 2564

ลายมือชื่อนักศึกษา_____
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DYAH WULANDARI : SYMBIOTIC MEGAPLASMID OF *BRADYRHIZOBIUM* SP. DOA9 AFFECTING LEGUME-RHIZOBIA SPECIFICITY IN NON SYMBIOTIC *BRADYRHIZOBIUM COSMICUM* S23321. THESIS ADVISOR : PROF. NEUNG TEAUMROONG, Dr. rer. nat., 182 PP.

SYMBIOTIC MEGAPLASMID pDOA9/*Bradyrhizobium* sp. DOA9/*Bradyrhizobium cosmicum* S23321

The megaplasmid DOA9 (pDOA9) carrying *nod, nif, fix* and T3SS genes was first found in *Bradyrhizobium* sp. DOA9. This study aimed to transfer the pDOA9 from DOA9 into different background chromosomes than DOA9 and *Bradyrhizobium* sp. ORS278. The chimeric ORS278 carrying pDOA9 was used as a donor for transferring pDOA9 to the *B. cosmicum* S23321 which is free-living with the absence of *nod,* T3SS and T4SS genes. The result of plant nodulation was varied among DOA9, chimeric ORS278 and S23321 strains even though it carried the same symbiotic plasmid pDOA9. The pDOA9 brings incompatible factors on legume symbiosis in chimeric ORS278, but the result in chimeric S23321 is able to contribute the symbiosis compatibility on legumes. This study indicated that S23321 was able to gain the symbiotic function of pDOA9 in two manners. Firstly, the *nod* genes broaden the host range in all legumes tested and successfully formed the active nodule with *Arachis hypogea* (Dalbergoid) and *Vigna radiata* cv. SUT4 (Millitoid), while the DOA9 wild type only performs necrotic nodule in *V. radiata* SUT4. Secondly, the Type three secretion system (T3SS) affects the degree of effectivity in symbiosis. The chimeric mutant T3SS (S23321:pDOA9Ω*rhcN*) congruence with DOA9, mutation in T3SS gave better nodulation in *Crotalaria juncea*. Besides, the mutation of nodulation gene (S23321:pDOA9Ω*nodB*) completely abolished the nodulation in all tested legumes. These outcomes implied that pDOA9 has been decorated for broadening host specificity along with legumes evolution time and the compatibility of nodulation depending on the background of the chromosome of the recipient as well as the legumes host restriction.

Bradyrhizobium sp. DOA9 is the broad host range with divergent *nod* containg strain. DOA9 carry two copies of *nodA* genes, *nodA1* and *nodA2* as well as two copies of *nodD* genes, *nodD1* and *nodD2*. The roles of both genes remained to be elucidated. The result showed that the mutation of a single copy of *nodD1* (**Ω***nodD1*) or *nodD2* (**Ω***nodD2*) resulting in a plant phenotype similar to DOA9 WT. **Ω***nodD1* or **Ω***nodD2* were not significantly reduced in nodule number, plant dry weight and nitrogen fixation. **Ω***nodD1* and **Ω***nodD2* showed functionally redundant. The result of **Δ***nodA1* and **Δ***nodA2* showed that the *nodA1* was not the symbiotic determinant in DOA9 nodulation. The plant test showed that **Δ***nodA1* was not significantly reduced in nodule number, plant dry weight, and nitrogen fixation but the *nodA2* played an important role in the general nodulation of DOA9. **Δ***nodA2* showed aborted nodulation in all plant tests. Analysis of Nod-Factor (NF) of DOA9 shares high similarity to the major NF of the *Rhizobium* sp. NGR234. Analysis of the **Δ***nodA2* revealed that no NFs were detected either in the conditions with or without flavonoid induction in the mass spectrometry (MS) positive mode. In **Δ***nodA1*, NFs were found but the main identified molecule was not similar as in DOA9 WT and the NF bearing the C18:1 acyl group was not detected. In conclusion, DOA9 required both copies of *nodD1* and *nodD2* for activation of *nod* genes transcription, both genes showed functionally redundant. The broad host range properties of DOA9 possibly derived from the decoration of the core *nod* genes that affect the NF structure functions.

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LIST OF ABBREVIATIONS

°C	=	degree Celsius
%	=	percentage sign
µg	=	microgram
µl	=	microlitre
µm	=	micrometer
ATP	=	Adenosine triphosphate
ANOVA	=	Analysis of variance
dai	=	days after inoculation
DNA	=	deoxyribonucleic acid
DOA	=	Department of Agriculture
et al.	=	Et alia (and other)
h	=	hour
L	=	litre
M	=	molarity
mg	=	milligram
min	=	minute
ml	=	milliliter
mm	=	millimeter
N_2	=	Nitrogen
OD	=	Optical Density
ARA	=	Acethylene Reduction Assay
PBS	=	Phosphate-Buffered Saline
PCR	=	Polymerase Chain Reaction
rpm	=	Rounds Per Minute
SD	=	Standard Deviation
SUT	=	Suranaree University of Technology
USA	=	United States of America
w/v	=	weight per volume

LIST OF ABBREVIATIONS (Continued)

YEM	=	Yeast Extract Mannitol
YMB	=	Yeast Extract Mannitol Broth
AG	=	Arabinose-Gluconate
BNF	=	Biological Nitrogen Fixation
bp	=	base pair
CFU	=	Colony-forming unit
DAI	=	Days after inoculation
v/v	=	volume per volume
dNTP	=	deoxynucleotide 5’ triphosphate
DOA	=	Department of Agricultural
EPS	=	extracellular polysaccharides
FAO	=	Food and Agriculture Organization
mM	=	Millimolar
NCBI	=	National Center for Biotechnology Information
NF	=	Nod factors
ng	=	nanogram
nm	=	nanometer
RNA	=	ribonucleic acid
RNS	=	Root Nodule Symbiosis
RT-PCR	=	Reverse Transcription Polymerase Chain Reaction
USDA	=	United States Department of Agriculture

CHAPTER I
INTRODUCTION

1.1 Significances of study

Plasmids are extrachromosomal, self-replicative, and important DNA vehicles that can be transmitted vertically or horizontally (James et al., 2016). Genetic information carried on plasmid corresponds usually to accessory genes that have certain function such as antibiotic resistance, detoxification, virulence, catabolism of uncommon metabolites, or capacity to invade certain tissues (Bedhomme et al., 2017). The notion of the symbiotic genes (*nod, nif,* and *fix*) that are required for rhizobia to permit them to develop a symbiosis with legumes and this symbiotic toolkit are generally found on a symbiotic plasmid or on genomic island that can transfer between strain by Horizontal Gene Transfer (HGT). The Bradyrhizobia strains generally harbor their symbiotic genes on genomic island, but it exists few exceptions such as the DOA9 strain that harbor a large symbiotic plasmid containing *nod, nif* and T3SS genes. Insterestingly, this strain also contains *nif* genes on chromosome (Okazaki et al., 2015). The transconjugation of pDOA9 into photosynthetic *Bradyrhizobium* sp. ORS278 was successfully constructed in previous study. Interestingly, symbiotic pDOA9 in chimeric ORS278 : pDOA9 showed confound the symbiotic relation in the original host (*Aeschynomene indica* and *A. evinia*), but do not provide the symbiotic relationship with NodFactor-dependent host as found in DOA9 (Songwattana et al., 2019). This finding suggested that, (i) symbiotic tools required for classical nodulation process could render incompatible the symbiotic interaction with NF-independent manner. (ii) The pDOA9 acquirement of ORS278 could not provide the symbiotic properties to ORS278 dual some incompatible factors regarding to the phylogenetically distant.

To completely demonstrate the function of symbiotic tools on pDOA9. In this thesis determined transferring of pDOA9 into *B. cosmicum* S23321 which is phylogenetic close to *B. diazoefficiens* USDA110 but absence the symbiotic island on

its genome. The nodulation properties of new chimeric S23321:pDOA9 was investigated with all legumes which are originally nodulated by DOA9. In this study, we also examine the symbiotic properties depending on the factors from T3SS on the pDOA9 plasmid. Thus, T3SS lacking pDOA9 (pDOA9Ω*rhcN*) was also contributed into S23321 (S23321:pDOA9Ω*rhcN*) and observed the nodulation on legume tests.

1.2 Research objective

1.2.1 Main objective

1.2.1.1 The main objective of this study was to determine the function of genes involved in symbiotic megaplasmid pDOA9 of *Bradyrhizobium* sp. DOA9 on the symbiotic properties of non-symbiotic *B. cosmicum* S23321 including broad host range features.

1.2.1.2 To demonstrate the function of *nod* genes of *Bradyrhizobium* sp. DOA9 determining the nodulation ability of host specificity on different legumes.

1.2.2 Specific objectives

1.2.2 1 To transfer the pDOA9 from *Bradyrhizobium* sp. DOA9 into *B. cosmicum* S23321 using bacterial conjugation system.

1.2.2.2 To determine the nodulation capacity of transconjugant S23321:pDOA9 in various legumes which belongs to 3 tribes includes Genistoid, Dalbergoid and Milletoid

1.2.2.3 To determine the function of genes involved in symbiotic on pDOA9 including *nod*, *nif*, and T3SS which is involve in the symbiotic of *B. cosmicum* S23321

1.2.2.4 To determine the function of genes involved in symbiotic on chromosome S23321 including Glycosyltransferase-39 (*Gly*), O-antigen, mutltidrug transporter A (*mdtA)* and mutltidrug transporter B (*mdtB)*

1.2.2.5 To analyze the function of two copies of nod genes, *nodA1* and *nodA2*, also *nodD1* and *nodD2*

1.2.2.6 To demonstrate the nodulation phenotypes of DOA9 with different legumes plant hosts by inoculation with mutant *nod* strains

1.2.2.7 To determine the Nod-Factor structure of *Bradyrhizobium* sp. DOA9

1.3 Hypotheses

1.3.1 The symbiotic properties of pDOA9 could contribute to the mode of life of S23321.

1.3.2 The *nod* cluster located on pDOA9 could be a main factor for symbiotic interaction with legumes.

1.3.3 T3SS on pDOA9 could be the one of symbiotic determinant for nodulation with legumes.

1.3.4 Either *nif* gene located on pDOA9 or chromosome of S23321 could encourage the N_2 fixation efficiency under free living and in symbiotic conditions.

1.3.5. The genes from background chromosome S23321 are possibly one of the symbiotic determinant in the nodulation efficiency of chimeric.

1.4 Scope of limitations

1.4.1 The contribution of symbiotic plasmid *Bradyrhizobium* sp. DOA9 into the non-symbiotic *Bradyrhizobium* sp. S23321 was on the focus of this study. This study aims to obtain the transconjugant using bacterial conjugation system, then to test the stability of the plasmid in transconjugant under correspondence antibiotic in three generations.

1.4.2 The verification of all transconjugants were performed by molecular technique such as PCR amplification with the specific primers.

1.4.3 The nodulation test and host specificity were performed in various leguminous plants by inoculation of wild type and transconjugant under symbiotic state experiment representing 3 legume tribes: Genistoid, Dalbergoid and Milletoid.

1.4.4 To determine the function of symbiotic gene from pDOA9 in S23321, derivative mutant pDOA9*,* Ω*nodB* and ΩT3SS were transferred into S23321 and observed the nodulation capacity in 3 legume tribes compared with wild type strain and DOA9 mutant derivatives.

1.4.5 To determine the function of symbiotic gene in background chromosome S23321. The gene expression of symbiotic gene from chromosome S23321 was evaluated using qRT-PCR.

1.4.6 To determine the function of each copy of *nod* genes in pDOA9 including *nodA1, nodA2*, *nodD1* and *nodD2* by deletion mutant in *nodA1* and *nodA2* and insertion mutant in *nodD1* and *nodD2.*

1.4.7 To assess the symbiotic ability of each mutant *nodA1, nodA2*, *nodD1* and *nodD2* in 3 legume tribes: Genistoid, Dalbergoid and Milletoid.

1.4.8 To describe the NodFactor (NF) structure of *Bradyrhizobium* sp. DOA9 and their derivatives mutants by LCMS-MS.

CHAPTER II

LITERATURE REVIEW

2. 1 Biological Nitrogen Fixation (BNF)

Nitrogen is a key macro element required by all living organisms (Burris and Roberts, 1993). To stay productive in sustainable farming systems, it is required to supplant the reserves of nutrients lost from the soil. The inputs of Nitrogen (N) resources into agricultural systems in the form of N-fertilizer, or the derivatives from atmospheric N_2 thru Biological Nitrogen Fixation (BNF) (Peoples et al., 1995). Biological N_2 fixation is a symbiotic process (Lindström and Mousavi, 2020), during plants provide shelter and carbon as an energy source to the symbiotic microorganisms in exchange for bacteria converts the atmospheric nitrogen (N_2) into ammonia (N_3) that can be readily assimilated by the host plant (Skorupska et al., 2010; Lazali et al., 2013; Lindström and Mousavi, 2020). BNF is highly distributed amongst the Bacteria and Archaea but is not found in eukaryotes.

Nitrogen-fixing bacteria are found in a wide variety of habitats including both soil and marine environments, in free living and in symbiotic associations with termites, ferns, woody plants and legumes (crop, pasture, green manures and trees), *Casuarina*, and *Azolla* (Dixon and Kahn, 2004). The different N_2-fixing organisms and symbioses found in agricultural and terrestrial natural ecosystems are shown in Figure 2.1 In 1888 Hellriegel and Wilfarth published their findings on N_2 fixation by leguminous plants in symbiotic association with rhizobia, it was recognized that inoculation of seed with rhizobia would help ensure that the leguminous roots would nodulate and fix N_2 (Wilson, 1940).

Nitrogenases are complex metalloenzymes which catalyse the biological reduction of dinitrogen to ammonia. The process of fixing atmospheric N_2 is energy intensive, requiring 16 molecules of adenosine triphosphate (ATP) to fix 1 mol of N_2, as depicted in Figure 2.2.

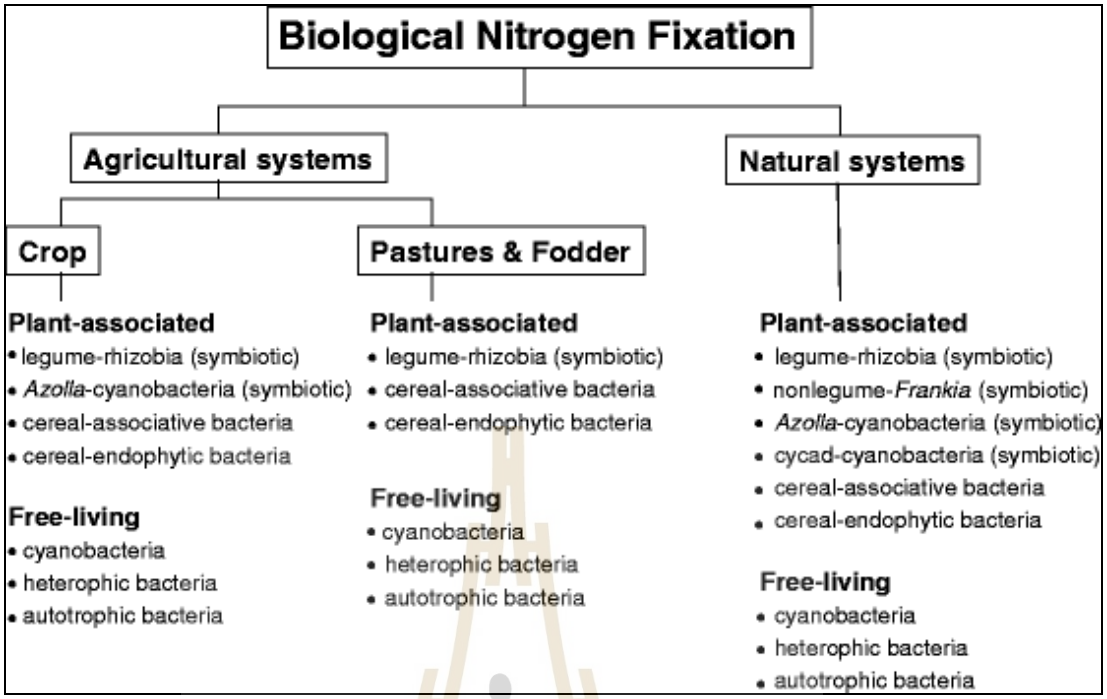

Figure 2.1 Biological N_2-fixing agents in agricultural and terrestrial natural systems (Herridge et al., 2008)

$$N_2 + 8\ e^- + 8\ H^+ + 16\ MgATP \longrightarrow 2\ NH_3 + H_2 + 16\ MgADP + 16P$$

Figure 2.2 Stoichiometry of dinitrogen reduction under optimal conditions (Dixon and Kahn, 2004).

According to the metal composition, Nitrogenase contains two components which is MoFe protein (catalytic unit) and Fe protein (reductase unit) with a size range of 220-250 and 50-60 kD depending on organism (Mortenson and Thorneley, 1979; Halbleib and Ludden, 2000). Both of the nitrogenase component proteins are oxygen sensitive with swift oxidation of the catalytic site (MoFe protein) and reductase component (Fe protein) (Marchal et al., 2000). The oxygen sensitivity of the Fe protein is conferred by a surface-exposed [4Fe-4S] cluster that bridges the two subunits of the dimer. The MoFe protein contains two types of metal centres: the P cluster (an [8Fe-7S] cluster), and the FeMo cofactor ($MoFe_7\ S_9$ homocitrate), which is the site of substrate reduction as depicted in Figure 2.3 from *Azotobacter vinelandii* nitrogenase structure (Dixon and Kahn, 2004).

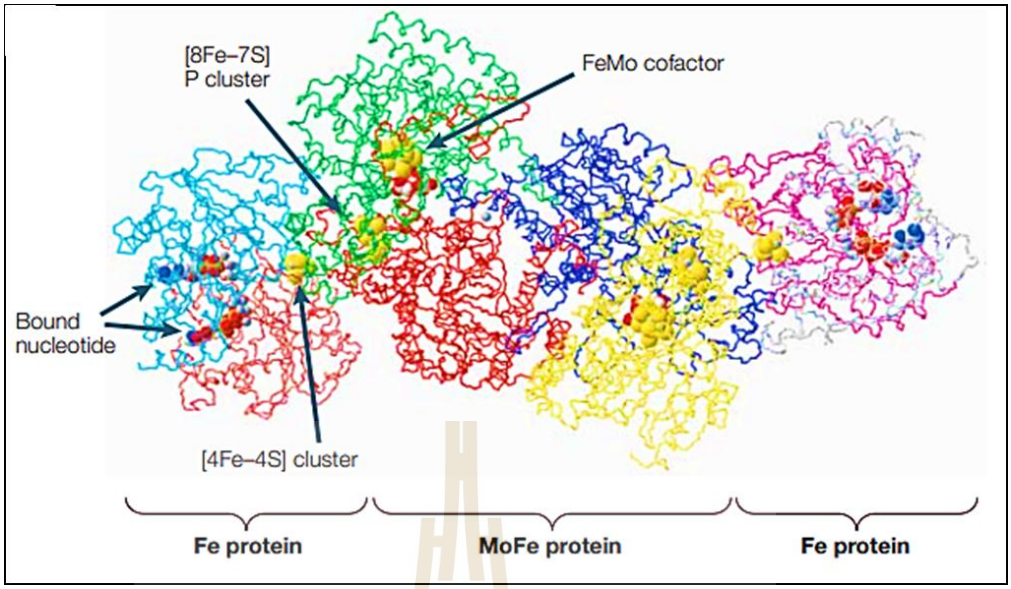

Figure 2.3 Nitrogenase structure (Dixon and Kahn, 2004).

In legume root nodules, the nitrogenase secured from oxygen by structural walls and through binding of free oxygen by leghemoglobin. Free-living bacteria are an indirect source of N_2 without host specificity which hires different tactics for shielding the nitrogenase from oxygen and consecutively facing high demand of respiration (Oelze, 2000; Ott et al., 2005). Therefore, in cereals the improvement of biological nitrogen fixation through genetic engineering or synthetic biology of free-living diazotroph, associative symbionts, and rhizobial symbionts are being focused (Goyal et al., 2021).

Nodulation and nitrogen fixation process in rhizobia is mostly regulated by *nod, nif* and *fix* genes (Table 1). Nod and Nif proteins are encoded by auxiliary genes of bacteria that are located in mobile genetic elements like plasmids, symbiotic islands and chromids. Thus, these sets can be transferred horizontally in high frequencies within the species of a bacterial genus and infrequently between genera (Remigi et al., 2016).

Table 2.1 The list of the most common rhizobial *nod, nif* and *fix* genes (Laranjo et al., 2014; Lindström and Mousavi, 2020).

Genes	Function of gene product
Nodulation genes	
nodA	Acyltransferase
nodB	Chitooligosaccharide deacetylase
nodC	N-acetylglucosaminyltransferase
nodD	Transcriptional regulator of common nod genes
nodIJ	Nod factor transport
nodPQ	Synthesis of Nod factor substituents
nodX	Synthesis of Nod factor substituents
nodEF	Synthesis of Nod factor substituents
other *nod* genes	Several functions in synthesis of Nod factors
nol genes	Several functions in synthesis of Nod factors substituents and secretion
noe genes	Synthesis of Nod factos substituents
Nitrogen fixation genes	
nifH	Dinitrogenase reductase (Fe protein)
nifD	α subunits of dinitrogenase (MoFe protein)
nifK	β subunits of dinitrogenase (MoFe protein)
nifA	Transcriptional regulator of the other *nif* genes
nifBEN	Biosynthesis of the Fe-Mo cofactor
fixABCX	Electron transport chain to nitrogenase
fixNOPQ	Cytochrome oxidase
fixLJ	Transcriptional regulators
fixK	Transcriptional regulator
fixGHIS	Copper uptake and metabolism
fdxN	Ferredoxin

The minimal set of genes vital for nitrogen fixation are *nifHDK* (nitrogenase enzyme complex) and *nifENB* (Fe-Moco biosynthesis) and present in all rhizobia. Other *nif* genes including *nifTXQWZSUV* required in FeMoco synthesis, the Fe-S

cluster synthesis and electron transport. The distribution of *nif* genes may vary depend on *rhizobium* genomes. Few of free-living diazotroph absent of *nif* genes but it can be substituted with other housekeeping genes such as *iscA/nifU* and *iscS/nifS* or can be supplemented by the FEN1 homocitrate synthase gene *(nifV)* of host plants (Hakoyama et al., 2009; Nouwen et al., 2017). The transcription regulator NifA regulated the expression of *nif* genes in free-living diazotrophs under microoxic environment. The *ccoNOQP* (formerly *fixNOQP*) and *ccoGHIS* (formerly *fixGHIS*) genes regulate the cytochrome *cbb3* oxidase with high affinity for oxygen to cope the microoxic condition. In several rhizobia for example, *Azorhizobium caulinodans, Paraburkholderia phymatum,* and *P. tuberum* able to fix nitrogen in free-living and under symbiotic condition (De Meyer et al., 2016).

The dual regulation of NifA by oxygen may prevent the synthesis of nitrogenase in conditions where this enzyme would be inactive. In symbiotic stage, rhizobia have to handle with the contradictive situation where the nitrogenase is sensitive to O_2 while bacteria are strictly aerobic (Sciotti et al., 2003; Bobik et al., 2006). The next is *fix* genes (*fixABCX*) regulates the electrons transfer to nitrogenase (Ledbetter et al., 2017) are well conserved in most rhizobia (Black et al., 2012).

Mutation related *nif* genes affects the effectiveness of nitrogenase. The *nif* genes controlled by *nifA* which has role in association with sigma factor *RpoN* (σ^{54}) of RNA polymerase (Dixon and Kahn, 2004). Mutation in the region of *nifL* in *Azotobacter* sp. positively affects the regulatory gene *nifA* and increase the N_2 fixation ability and lessen the dependence on N fertilizer under field conditions of wheat cultivation (Bageshwar et al., 2017). Moreover, mutation on *nifL* in *A. vinelandii* and glutamine synthetase promoter resulting greater NH_4 concentration (Bali et al., 1992) and robust propagation of microalgae and promoted growth in cucumber plants without additional N_2 (Ambrosio et al., 2017). The BNF organisms impressively affect the crops management practices and boosted the yield from 60% (Orr et al., 2011) up to 80% (Skorupska et al., 2010; Santachiara et al., 2017), compared with unfertilized soils, consequently decrease the amount of N_2 needed especially in low inputs farming systems (Hardarson & Atkins, 2003).

2.2 Nodulation Mechanisms

Nodule developmental stage (root infection and organogenesis) in most rhizobia thru activation of the common symbiosis signaling pathway (CSSP) via the synthesis and secretion of Nod Factors (NFs) (D'Haeze & Holsters, 2002). It is initiated via an exchange of plant flavonoid from legumes with rhizobial molecules and is accompanied by a series of signal transductions inside. Nitrogen fixation in symbiotic nodules involves an enzyme complex called nitrogenase, which is composed of two enzymes, nitrogenase (Mo-Fe protein) and nitrogenase reductase (Fe protein). The nodules begin to fix nitrogen when leghemoglobin (Lb) is expressed (branching stage) (Yuan et al., 2017).

2.2.1 Nod dependent and Independent Strategies

Nodulation mechanism can be divided into two pathways. The first is *nod*-dependent strategy, *rhizobia* sense compounds such as flavonoids and betaines secreted by the host root and respond by inducing the expression of *nod* genes. The *nod* genes encode approximately 25 proteins required for the bacterial synthesis and exportation of NFs. NFs is a lipooligosaccharide signal consisting of a chitin backbone, four to five N-acetylglucosamine units in length, with a lipid attached to the nonreducing end and host-specific modifications on the backbone. 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 (*nodABC*) and specific modifications of NFs common to bradyrhizobia. NFs are exported via a type I secretion system encoded by the *nodIJ* genes, and recognized by a cognate host receptor complex, comprising *MtNFP* and *MtLYK3* in *Medicago truncatula* (Mt) and *LjNFR5* and *LjNFR1* in *Lotus japonicus* (Lj). The perception of NFs initiates the phosphorylation of these receptors which induces the activation of the CSSP (Kouchi et al., 2010; Oldroyd, 2013). Then the CSSP activates the specific transcription factors *NSP1, NSP2, NIN, ERN1* and *ERN2* coordinating the nodule development and rhizobial infection in the different root tissues (Cerri et al., 2016, 2017; Vernié et al., 2015). The developmental changes could be seen in the host plant early in the nodulation process, including root hair deformation, membrane depolarization, intracellular calcium oscillations, and the initiation of cell division in the root cortex, which

establish a meristem and nodule primordium (Madsen et al., 2003; Radutoiu et al., 2003; Gage, 2004). Along with the infection process, rhizobia also synthesize a panel of Surface polysaccharides, Exopolysaccharides, Lipopolysaccharides and Cyclic β Glucans (EPS, LPS, CGs) to avoid plant defense responses. In particular rhizobia, the symbiotic formation is supported by T3SS and T4SS. Released bacteria from infection threads remained enclosed by a peribacteroid membrane of the plant later on forming symbiosomes in the plant cytosol. In order to survive in acidic environment in peribacteroid space, rhizobia require the surface polysaccharides LPS. In symbiosomes, bacteria differentiate into bacteroids able to fix atmospheric N_2 into NH_3. Bacteroid terminal differentiation activated by Nodule Cysteine Rich (NCR) peptides present in plants from IRLC and Dalbergioid clades and important the bacterial peptide transporter (*BacA* or *BclA*). Bacteroids used cytochrome *cbb3* oxidase for microaerobic respiration in the microoxic atmosphere in symbiosomes and allowed the nitrogen fixation. Carbon sources are supllied by the plant as C4 dicarboxylic acids (Fig. 2.4) (Haag et al., 2011; Guefrachi et al., 2015; Tang and Capela, 2020).

Another mechanism is *nod*-independent pathway. In the nod-independent rhizobia, the mechanism inducing the CSSP is unknown (Tang and Capela, 2020). A NF-independent strategy was uncovered a decade ago in photosynthetic *Bradyrhizobium* (PB) lacking the *nodABC* genes and nodulating certain *Aeschynomene* CI group III in example *A. indica* nodulated by PB including *Bradyrhizobium* sp. strains BTAi1, ORS278 and ORS285 (Bonaldi *et al.,* 2010; Bonaldi *et al.,* 2011, Chaintreuil et al., 2018; Giraud et al., 2007; Okazaki et al., 2016). Mutation analyses of the *nod* genes in the strain ORS285 did not affect nodulation of *A. indica* and *A. sensitiva* (CI group III) but blocked nodulation of the original host plant *A. afraspera* (*nod*-dependent plant).

Crack entry is initiated at the emergence sites of lateral roots. From these primary infection pockets, some tropical rhizobia form infection threads, whereas other rhizobia do not. In most cases, rhizobia then become internalized in plant cells

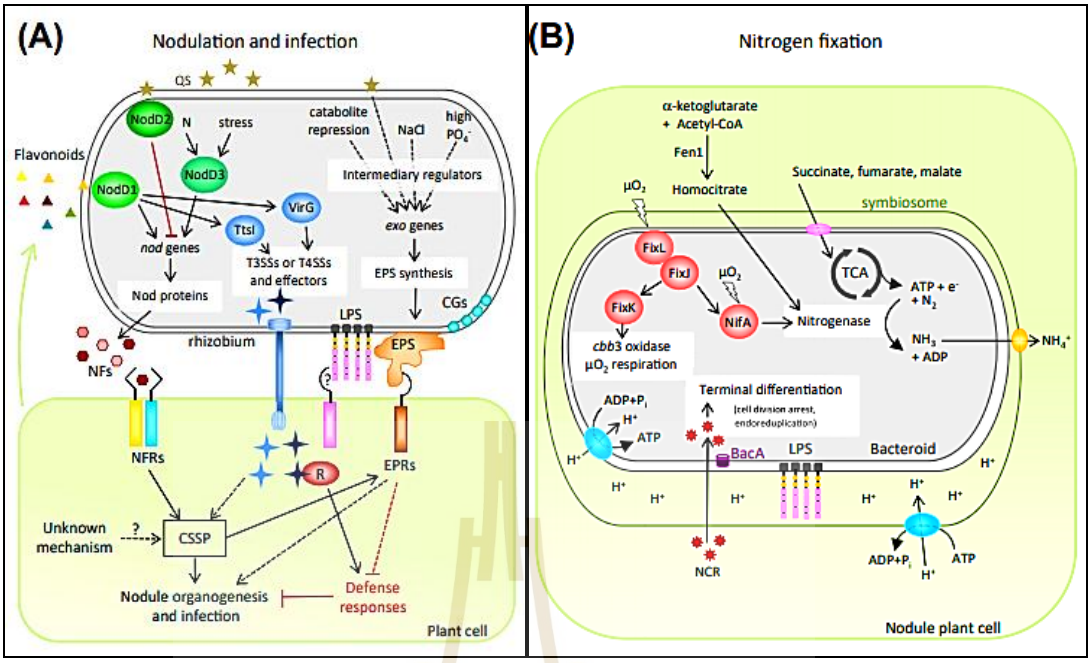

Figure 2.4 The genetic nodulation, infection (A) and nitrogen fixation process (B) in rhizobia (Tang and Capela, 2020).

via an endocytosis-like process that remains largely unknown. The need for one initial plant signal remains to be demonstrated. The bacteria enter in the plant via cracks in the epidermis which result from the emergence of lateral roots. Accumulation in these infection zones of cytokinin-like compounds synthesized by the bacteria might directly bypass the early nod factor signaling pathway and inducing nodule organogenesis (Masson-Boivin et al., 2009). Interestingly, *nod* deletion mutant of strain ORS285 retains the ability to nodulate *A. sensitiva* and *A. indica* (CI group III) but not the other species (Giraud et al., 2007), a hypothetical scheme is proposed for the *nod*-independent process is shown in figure 2.5. This result suggested that a group of rhizobia, currently limited to PB uses a *nod*-independent strategy to enter into symbiosis with some *Aeschynomene* species. In the *nod*-independent pathway other bacterial functions can also improve or impede the infection process and thus modulating host range: examples are T3SS and T4SS that translocate effectors in plant cells and that trigger or suppress plant defense reaction (Hubber et al., 2004; Bartsev et al., 2004) and the HrrP peptidase which

cleaves host- encoded signaling NCR peptides (Price et al., 2015). Only for *B. elkanii* USDA61 processes T3SS as an alternative way for nodulation with rj4 soybean.

The bacterial factors involved in NF-independent strategy have not been completely elucidated so far. Nevertheless, two key proteins of the *CSSP, SymRK* and *CCaMK,* as well as the *LHK1* cytokinin receptor critical for the nod-dependent nodulation process were shown to be also essential for the nod-independent process (Fabre et al., 2015). Currently, a transcriptomic analysis of the nod-independent *A. evenia-Bradyrhizobium* ORS278 symbiotic interaction identified transcripts of an *NFP/NFR5* ortholog and orthologs of most of the genes belonging to the CSSP *(MtDMI2/LjSYMRK, LjCastor-MtDMI1/LjPollux-LjNENALjNup85-LjNup133, MtIPD3/ LjCYCLOPS* and *MtDMI3/LjCCaMK),* underpinning the involvement of the same plant signaling pathway in both nod-dependent and nod-independent symbioses (Gully et al., 2018). To this point it is indistinct whether this NF-independent strategy restricted merely in Aeschynomene-Bradyrhizobia, is either a mark of an ancient story.

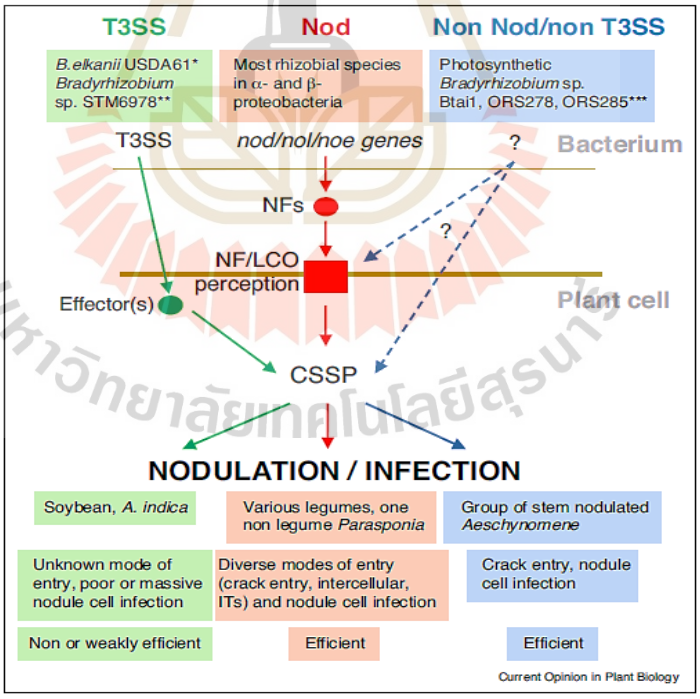

Figure 2.5 Nodulation strategies in rhizobia. Rhizobia induce the formation of nodules on legumes using either a nod-independent (T3SS) or a nod-dependent and the Nod and non-Nod/non-T3SS strategies process (Masson-Boivin and Joel, 2018).

2.2.2 The importance of Surface Polysaccharides in Nodulation Process

Over and above the NFs, the synthesis of surface polysaccharides is necessary along with the development of the symbiotic interaction in both early and late infection stages in all rhizobium-legume symbiotic interactions (Benezech et. al., 2020). The symbiotic functions of these molecules frequently depend on the rhizobium-plant host genotype. There are four main types of rhizobial surface polysaccharides that have been shown to play a role in symbiosis: secreted EPS, KPS and LPS, both anchored in the outer membrane, and cyclic β-glucans mostly located in the periplasm (Downie, 2010; Fraysse et al., 2003; Lopez-Baena et al., 2016). K-antigen polysaccharide (KPS), neutral polysaccharide (NP), gel-forming-polysaccharide (GPS), and cellulose fibrils in determining host range primarily comes from bacterial genetics (Kawaharada et al. 2015; Ghosh and Maiti, 2016).

There are many functions of surface polysaccharides supporting the progression of infection process in legume-rhizobia symbiosis, including deal with variety of biotic and abiotic stresses under symbiotic conditions (osmotic stress, acidification, antimicrobial peptides, or reactive oxygen species) (Arnold, et al., 2018; D'Haeze and Holsters, 2004; Davies and Walker, 2007; Geddes et al., 2014; Ingram-Smith and Miller, 1998; Lehman and Long, 2013; Miller-Williams and Oresnik, 2006). Eventhough the exact function of surface polysaccharides have not been definited yet. Those molecules to be expected warrant the protection of rhizobia either by forming a physical diffusion barrier surrounding the bacteria or by actively inducing pathways that prevent plant defense responses, a essential for nitrogen fixation (D'Antuono et al., 2008; D'Haeze and Holsters, 2004; Jaszeket al., 2014; Jones et al., 2008). The active signaling function of EPS was recently provided by the identification of an EPS receptor in *L. japonicus* (Kawaharada et al., 2015, 2017). Upcoming investigations should be addressed whether other plant receptors not only to EPS but also to the other rhizobial polysaccharides, such as KPS and LPS, also contribute to the symbiosis signaling pathway (Benezech et. al., 2020).

2.2.2.1 Exopolysaccharides (EPS)

EPS functions are the signaling molecule for initial primary attachment via recognition of root-hair lectins by the surface carbohydrates of rhizobial cell (Dazzo et al. 1984; Smit et al., 1992). EPS may improve the chance of

adhesion of bacteria to the tip of growing root hairs and to overcome the plant defense reaction (Becker and Pühler, 1998; Fraysse et al., 2003). These functions required for successful colonization and infection of host plant roots. Though EPS lacking mutants are not significantly affected in adhesion to the roots of several species such as clover (Rolfe et al., 1996), vetch (Van Workum et al., 1998) or alfalfa (Cheng and Walker, 1998). EPS in rhizobia were characterized by biofilm formation that enhanced bacterial cells to better adaptation inside the host roots (Fujishige et al., 2006).

EPS regulatory network was described clearly in *Sinorhizobium meliloti*. It consists of EPS I (succinoglycan) and EPS II (galactoglucan). EPS I plays the dominant function in the invasion of Medicago nodules while EPS II has less effect (Battisti et al., 1992; Cheng and Walker, 1998; Janczarek., 2011).

Several regulatory genes of EPS I and EPS II synthesis *S. meliloti* have been identified either on the chromosome (*mucR, exoR, exoS, exoD, expR, syrM and phoB) or on the megaplasmid pSymB (exsB, exoX and wggR*). Most of the proteins encoded by these genes have been designated as repressors. These include the *exoR, exoS, exoX* and *exsB* genes, which negatively affect EPS I synthesis, and the *mucR,* which negatively regulates EPS II synthesis (Becker et al., 1995; Keller et al., 1995; Reed et al., 1991; Mendrygal and González, 2000; Yao et al., 2004). Contrariwise, the *SyrM* and *PhoB* proteins are positive regulators of EPS I and EPS II production. Among the identified regulators, *MucR* seems to be a global regulatory protein playing a key role in both positive regulation of EPS I synthesis and negative regulation of EPS II synthesis, thus coupling these two biosynthetic pathways (Figure 2.6) (Rüberg et al., 1999; Bahlawane et al., 2008). EPS I synthesis is higher under ammonium or sulfur starvation, high phosphate concentration, hyperosmotic stress as well as high-cell density and repressed by succinate-mediated catabolite repression, while EPS-II production is promoted under phosphate starvation and high-cell density. Many rhizobial EPS regulatory systems are species-specific (Bonomi et al., 2012; Liu et al., 2018; Nishihata et al., 2018).

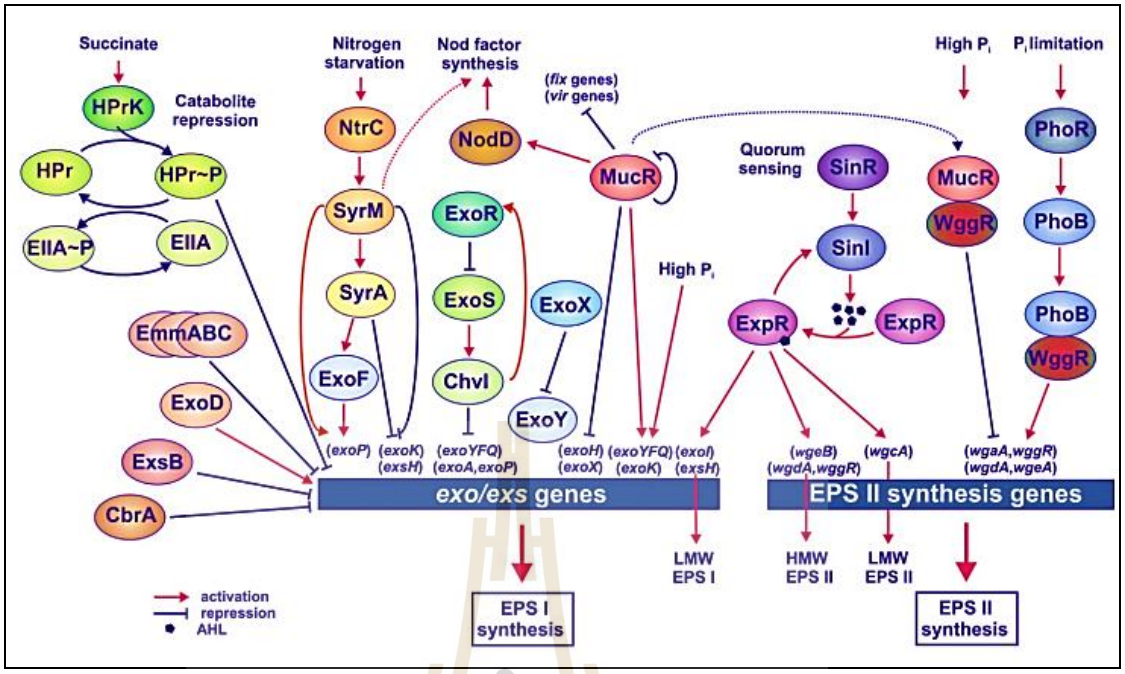

Figure 2.6 The regulation of *S. meliloti* exopolysaccharide (EPS) synthesis, quorum sensing and catabolite repression (Janczarek., 2011).

The EPS structures comprise of repeating unit of heteropolysaccharides which differs in the composition of sugars, their linkage within the unit, the degree of polymerization and the existence of non-carbohydrate substituents such as, acetate pyruvate, and succinate (van Workum et al., 1998; Skorupska et al., 2006; Wang et al., 2018). EPS functions are host specific in a rhizobial-legume interactions, in example: an *exo*U mutant in *M. loti* R7A is incapable to induce infected nodules on *L. japonicus* (Gifu ecotype), but only reduced the infection efficiency on other ecotypes of *L. japonicus* (MG-20 ecotype) (Kelly et al., 2013).

Congruence with that, complementation of EPS deficient strains by natural heterologous EPS produced by other rhizobia does not always lead to a successful infection. For examples, EPS from *R. tropici* or *R. etli* restore the infection ability of *R. leguminosarum* bv. viciae *pss*D mutants but EPS from *S. meliloti, S. fredii* NGR234 or *B. diazoefficiens* cannot (van Workum et al., 1998). Similarly, EPS from *R. leguminosarum* bv. trifolii and *S. fredii* NGR234 cannot restore the defective infection phenotype of *S. meliloti exo* mutants on alfalfa (Battisti et al., 1992).

Specific EPS receptor: *EPR3*, mediates the progression of infection thread (Its) through the root cortex and nodule primordium ensured the role of EPS as vital signaling molecules in *L. japonicus* (Kawaharada et al., 2015, 2017; Muszynski et al., 2016). This receptor is part of the symbiotic signal transduction pathway since its expression is initiated downstream from the CSSP (Kawaharada et al., 2017). Remarkably, *EPR3* orthologs were also present in other legumes: *M. truncatula*, soybean and *A. hypogaea* whose infection via crack entry but not thru ITs (Kawaharada et al., 2015; Karmakar et al., 2019). Most proteins regulatory EPS synthesis encode global regulatory systems that not only control EPS synthesis but also affect multiple bacterial behaviors. The three component system *ExoR/ExoS/ChvI (RSI)* is one vital regulatory system that shifts bacteria from motile free-living cells to non-motile host invading cells by up and down-regulating the expression of hundreds of genes, including EPS I and flagellum genes (Bélanger et al, 2009). Quorum sensing (QS) systems also affect EPS biosynthesis along with a wide-ranging physiological traits in rhizobi, including biofilm formation, swarming motility, T3SS, HGT, cell division, metabolism and transport (Hoang et al, 2004; Krysciak et al., 2014; Calatrava et al., 2018). Their effect on symbiosis can be either no-effect (Krysciak et al., 2014), positive (Zheng et al., 2006; Gurich and Gonzalez, 2009; Yang et al., 2009) or negative (Rosemeyer et al., 1998; Rodelas et al., 1999).

2.2.2.2 Lipopolysaccharides (LPS)

LPS are main constituents of the outer membrane in gram-negative bacteria. LPS, composed of lipid A, a core oligosaccharide and an O-antigen polysaccharide, are anchored in the outer membrane (Janczarek., 2011; Serrato, 2014). Rhizobial lack in LPS biosynthesis have an impact on early stage of symbiosis which abort the formation of ITs and in later stage of symbiosis able to induce a rapid lysis of bacteria after released in the cytoplasm of nodule cells, leads to the formation of non-fixing nodules (Broughton et al., 2006; Campbell et al., 2002; Noel et al., 2000). The O-antigen is the main immunogenic feature in gram-negative bacteria and the most varied part of LPS. Mutants altering the conformation of O-antigen in *R. etli* and *S. fredii* NGR234 are diminished IT development and N_2 fixation (Reuhs et al., 2005). Remarkably, the O-chain modifications in these two rhizobia

happened under symbiotic conditions (Kobayashi et al., 2004; Noel et al., 2004; Broughton et al., 2006; Ojeda et al., 2010).

2.2.2.3 Capsular polysaccharides (CPS)

CPS are tightly associated with the bacterial surface and are neutral or acidic polysaccharides, it consist of of dimeric repeating unit containing one hexose linked with 3-deoxy-D-manno-oct-2-ulosonic acid. The number of CPS gene clusters is variable among rhizobium genomes even within the same rhizobium species (Kiss et al., 2001; Sugawara et al., 2013). CPS structure in most species very similar or even identical to EPS. In contrast to CPS, EPS are weakly associated with the bacterial surface and are released in large amounts into the environment (Fraysse et al., 2003; Skorupska, et al., 2006). The role of rhizobial CPS in legume symbiosis is diverse, for example CPS in *S. meliloti* Rm1021 showed insignificant effect (Pellock et al., 2000) while in *S. fredii* HH103 showed significant effect in nodule organogenesis during symbiosis (Parada et al., 2006). The number of *cps* gene clusters is also diverse amongst rhizobium genomes even within the same rhizobium species (Kiss et al., 2001; Sugawara et al., 2013).

2.2.2.4 Cyclic β-glucan (CG)

The order Rhizobiales are able to synthesize cyclic β-glucans (CGs). Cyclic β-glucans are consist of neutral cyclic homopolymers of β-linked glucose residues frequently substituted by phosphoglycerol, phosphocholin or succinyl groups. The linkage between the glucose residues, either β(1,2) or β(1,3) or β(1,6), is varies among symbionts legume. Though, there is no certainly link between the nature of the linkage and their role on host plants (Breedveld & Miller, 1994). The variability of β-glucans (CGs) showed in soybean symbionts, the different symbionts produces different β-glucans (Crespo-Rivas et al., 2009; Rolin et al., 1992). β-glucans (CGs) function are important for the invasion of nodules. The rhizobia lacks of β-glucans (CGs) in the synthesis or export in the periplasm could abort the IT formation and produce pseudonodule which failed to fix N_2 (Nagpal et.al., 1990; Bhagwat et al., 1992, 1996).

2.2.3 Determinate and Indeterminate nodule

Legume nodules can be categorized into two major groups, based on the perseverance of their apical meristem (Figure 2.7). 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. In order to rhizobial infection requires the production of surface polysaccharides that are recognized by plant receptors. In legumes producing nodule specific cysteine-rich (NCR) peptides peptide transporters (*BacA* or *BclA*) are required for the differentiation of internalized bacteria into functional bacteroids metabolically adapted to N_2-fixation (Guefrachi et al., 2015; Haag et al., 2011).

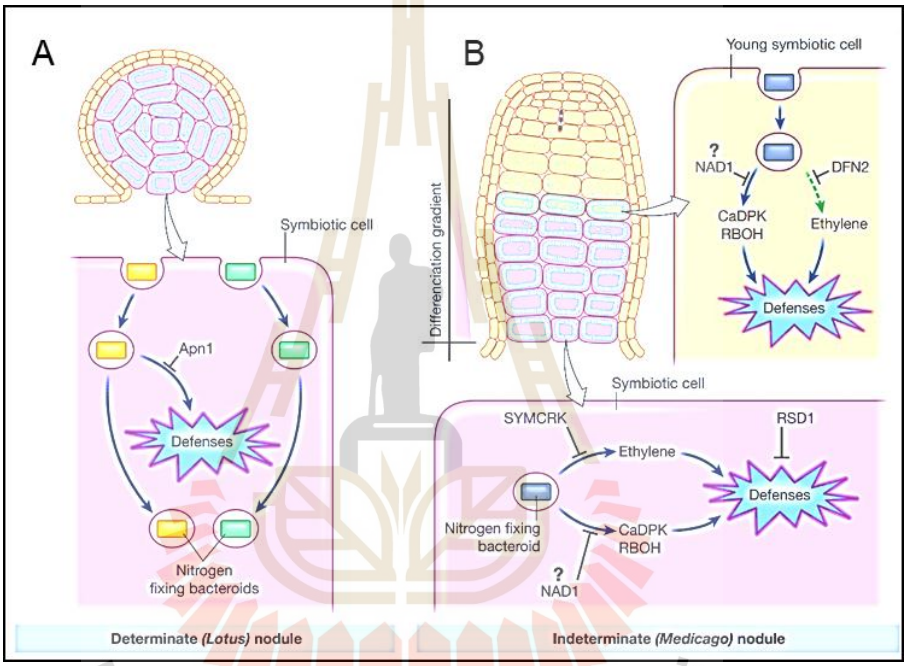

Figure 2.7 Rhizobia internalization in the plant cells of (a) determinate nodules of *Lotus japonicus* and (b) indeterminate nodules of *Medicago truncatula* (Benezech et. al., 2020).

After rhizobial internalization in the nodule, the control of defense is crucial for bacteroid persistence (Benezech et. al., 2020). In the early stage of symbiosis, the defence reaction shown to influence innate immunity at a later stage of the symbiotic process, after nodule formation and after bacteria have been released within the symbiotic cells (Kelly et al., 2018). In *Lotus* nodule (Figure 2.7a), the gene aspartic peptidase nodule-induced 1 avoids the development of plant defence reactions in nodules in a strain-specific way (Gourion and Alunni, 2018; Yamaya-Ito et

al., 2018). In *M. truncatula,* the gene *DNF_2* inhibits the induction of defence reactions in nodules (Figure 2.2b) (Berrabah et al., 2014; Bourcy et al., 2013). Therefore, the rhizobia genotype has a strong effect on the interaction amongst innate immunity and symbiosis. Moreover, aspartic peptidase nodule-induced 1 (*APN1*) and *DNF2* other plant genes, such as regulator of symbiosome differentiation 1 *(RSD1)*, symbiotic cysteine-rich receptor kinase *(SymCRK),* and nodule with activated defenses 1 (nad1), constrain defence reactions after rhizobia internalisation in the symbiotic cells (Berrabah et al., 2014; Domonkos et al., 2017; Sinharoy et al., 2013; Wang et al., 2016). The DNF2 act first then followed by *SymCRK* and *RSD1*, thus activated the ethylene pathway. Ethylene functions in the plant defence responses particularly by directly stimulating the expression of PRRs (Boutrot et al., 2010; Mersmann et. al., 2010; Berrabah et al., 2015).

2.3 Bradyrhizobium

Rhizobia are varied bacteria and recognized in distant phylogenetic branches, interspersed with non-rhizobial bacterium species (Remigi, et al., 2016). To date, equal to 18 different rhizobia genera and hundreds of species have been described among α and β-proteobacteria, hence today denoted as α and β-rhizobia (Tang and Capela, 2020). *Bradyrhizobium* species are Gram-negative bacilli (rod-shaped) with a single subpolar or polar flagellum. They are a communal soil-dwelling microorganism that can be associated with leguminous plants to fix atmospheric nitrogen into forms readily available for other organisms. *Bradyrhizobium* is slow-growing bacteria with 3-5 days to create moderate turbidity and the doubling time 5-7 in liquid media contrasting to *Rhizobium* species, which are considered as fast-growing rhizobia. The best carbon source for growing *Bradyrhizobium* is a pentose (Somasegaran and Hoben, 1994).

2.3.1 *Bradyrhizobium* sp. DOA9

The *Bradyrhizobium* sp. DOA9 was isolated from paddy field soil using *Aeschynomene americana* as trap legume (Noisangniam, et al., 2012), displayed the broad host range nodulation in many legumes including dalbergioid, milletoid and robinoid tribes and also found to be endophyte in rice (Teamtisong *et.al.,* 2014).

Moreover, based on 16S rRNA gene sequences, the DOA9 was grouped into the non-photosynthetic bradyrhizobia which comprised a single chromosome of 7.1 Mbp and plasmid 0.7 Mbp encoding the gene for nodulation (*nod*), nitrogen fixation (*nif*), type III and type IV protein secretion system (T33S and T4SS). T3SS in pDOA9 contains *tts* boxes wich encode for nodulation outer protein, structural components machinery, cupin and some hypotethical protein (Okazaki et al., 2015) (figure 2.8).

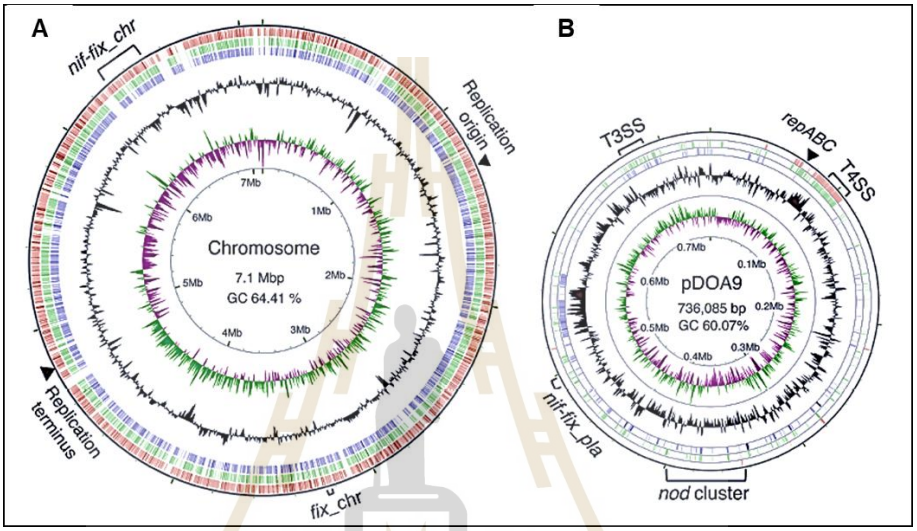

Figure 2.8 The genome structure of *Bradyrhizobium* sp. DOA9. (A) Circular representation of the chromosome of *Bradyrhizobium* sp. DOA9. (B) Circular representation of the plasmid (pDOA9) of DOA9 (Okazaki et al., 2015).

The T3SS cluster of pDOA9 displayed similarity with the plasmid of *Rhizobium*, *Agrobacterium*, and *Bradyrhizobium* sp. BTAi1. The pDOA9 has the same GC content lower in plasmid than in chromosome with the pBTAi1. Based on phylogenetic tree data and gene arrangements, the T3SSs of the bradyrhizobial strains SUTN9-2, DOA1, and DOA9 and the Senegalese strain ORS3257 may share the same origin as depicted in the Figure 2.9 (Piromyou et al., 2019).

The DOA9 contain *nif* genes for nitrogen fixation. The *nif* genes controlled by *nifA* which has role in association with sigma factor RpoN (σ^{54}) of RNA polymerase (Dixon and Kahn, 2004). The DOA9 has capability to fix the nitrogen in free living and in symbiotic condition, also contains two copies of the *nifD* and *nifK* genes with the function encoded the Mo-Fe protein for nitrogenase. The *nifDK* genes, one located

on the chromosome which highly express under free living and one on the symbiotic plasmid which highly express during symbiosis (Wongdee et al., 2016). The chromosome of DOA9 equipped with *nifV* gene with the function in biosynthesis of homocitrate then incorporate into the Fe-Mo cofactor of dinitrogen reductase which makes the DOA9 able to fix the nitrogen under free living condition (Hoover *et al,* 1989). The degree of similarity in term of genes content the genome of DOA9 is more similar to the genome *B. japonicum* USDA110 (Okazaki et al., 2015). The characteristics of *Bradyrhizobium* sp. DOA9 nodulation are described in the table 2.1.

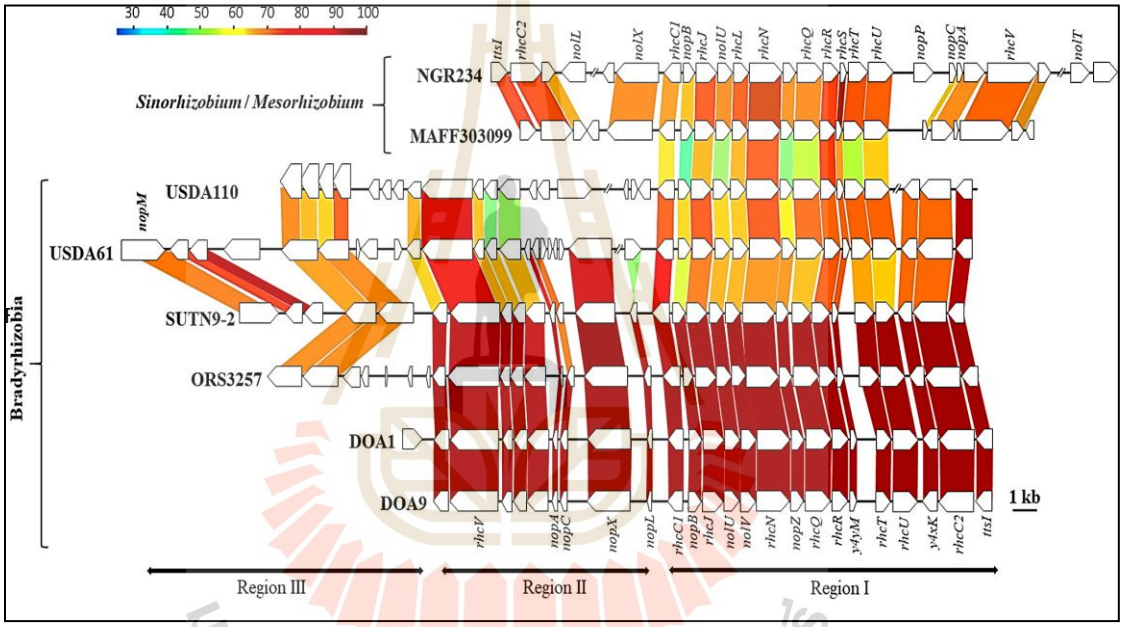

Figure 2.9 Comparison of the gene organizations of the type III secretion system (T3SS) in the bacterial strains *Sinorhizobium* sp. NGR234, *Mesorhizobium loti* MAFF303099, *Bradyrhizobium diazoefficiens* USDA110, *B. elkanii* USDA61, *Bradyrhizobium* sp. SUTN9-2, *Bradyrhizobium* sp. ORS3257, *Bradyrhizobium* sp. DOA1, and *Bradyrhizobium* sp. DOA9 ((Piromyou et al., 2019).

Table 2.2 The characteristics of *Bradyrhizobium* sp. DOA9 wild type (Teamtisong et.al., 2014), and mutant (Songwattana et. al., 2017) in the broad host legumes.

Tribes	Host plant	Wild type	Mutant	
			ΔnodABC genes	ΔT3SS
Genistoid	*Crotalaria juncea*	nod $^{-}$ fix $^{-}$	nod $^{-}$	nod $^{+}$ fix $^{-}$
Dalbergoid	*Aeschynomene americana*	nod $^{+}$ fix $^{+}$	nod $^{-}$	nod $^{+}$ fix $^{+}$
	A. afraspera	nod $^{+}$ fix $^{+}$	nod $^{-}$	nod $^{+}$ fix $^{+}$
	Arachis hypogea	nod $^{+}$ fix $^{+}$	nod $^{-}$	nod $^{+}$ fix $^{+}$
	Stylosanthes hamata	nod $^{+}$ fix $^{+}$	nod $^{-}$	nod $^{+}$ fix $^{+}$
Millitoid	*Vigna radiata* cv (SUT4)	nod $^{+}$ fix $^{-}$	nod $^{-}$	nod $^{+}$ fix $^{-}$
	Marcoptilium artopurpureum	nod $^{+}$ fix $^{-}$	nod $^{-}$	nod $^{+}$ fix $^{-}$
	Indigofera tinctoria	nod $^{+}$ fix $^{-}$	nod $^{-}$	nod $^{+}$ fix $^{-}$

2.3.2 *Bradyrhizobium cosmicum* S23321

Bradyrhizobium cosmicum S23321 is an oligotrophic bacterium isolated from paddy field soil. 16S rRNA revealed that the S23321 close to *B. japonicum* USDA110, a legume symbiont. The ITS region S23321 clustered within a group of Nod factor dependent nodulating strains. For the *nifH* phylogenetic tree, S23321 closer to *Bradyrhizobium* sp. BTAi1 and ORS278 than to the *nif* genes on the symbiosis islands of *B. japonicum* USDA110 (Okubo et. al., 2012). The evolution of *nif* genes and *nod* genes are different. It is generally hypothesized that rhizobial genes related to nodulation (*nod*) and symbiotic nitrogen fixation (*nif*) were acquired by lateral gene transfer in *Bradyrhizobium* because these genes are clustered in a large genomic island known as a symbiosis island, which is characterized by a lower GC content than the rest of the genome. The *nif* genes are present in the chromosome of bradyrhizobia were vertically transferred from their ancestors. From the phylogenetic analysis revealed that *nifH* genes located in the non-symbiotic loci S23321 clustered together with *nifH* in the plasmid and chromosome of strain DOA9. These results indicate that the evolutionary histories of *nif* genes on symbiosis islands and plasmids are distinct (Okubo et al., 2016)

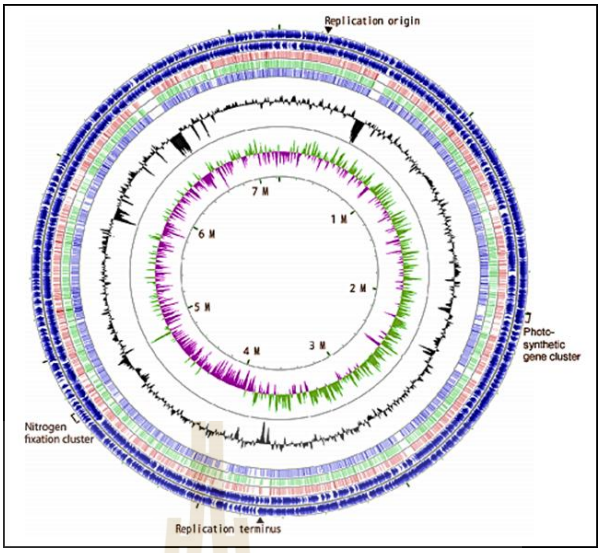

Figure 2.10 Circular representation of the chromosome of *Bradyrhizobium cosmicum* S23321 (Okubo et al., 2012).

The genome of S23321 is a single circular chromosome, 7,231,841 bp in length, with an average GC content of 64.3% (Figure 2.10). The genome contains 6,898 potential protein-encoding genes, one set of rRNA genes, and 45 tRNA genes. Comparison of the genome structure between S23321 and USDA110 showed strong colinearity. However, the symbiosis islands present in USDA110 were absent in S23321, whose genome lacked a chaperonin gene cluster (*groELS3*) for symbiosis regulation found in USDA110. A comparison of sequences around the tRNA-Val gene strongly suggested that S23321 contains an ancestral-type genome that precedes the acquisition of a symbiosis island by HGT. Although S23321 contains a *nif* (nitrogen fixation) gene cluster, the organization, homology, and phylogeny of the genes in this cluster were more similar to those of photosynthetic *Bradyrhizobium* sp. ORS278 and BTAi1 than to those on the symbiosis island of USDA110 (Okubo et. al., 2012).

Furthermore, S23321 possess genes encoding a complete photosynthetic system, many ABC transporters for amino acids and oligopeptides, two types (polar and lateral) of flagella. The presence of *fix*NOPQ genes and six different respiratory chains in the S23321 genome suggested that the terminal oxidase (a cytochrome bc1 complex that functions under low oxygen stress) is not part of symbiotic nitrogen fixation, but instead provides broad adaptation to a wide range of oxygen stress in

the environment. These features suggest that S23321 is able to adapt to a wide range of environments, probably including low-nutrient conditions, with multiple survival strategies in soil and rhizosphere (Okubo et. al., 2012).

Bradyrhizobium cells often show chemoautotrophic growth. The genome survey suggested that S23321 should be able to grow chemoautotrophically using CO and CO_2 as an electron donor and a carbon source, respectively, because of the presence of *cox* and *cbb* genes. Although the S23321 genome possesses many genes for carbon metabolic pathways such as glycolysis, gluconeogenesis, the citrate cycle, the pentose phosphate pathway, and the glyoxylate cycle. It apparently lacks several genes for nitrogen metabolism such as those for N_2O reductase and glutamate dehydrogenase (Okubo et. al., 2012; Patricia et al., 2016).

The S23321 which lack of symbiotic plasmid, could be a model microorganism for non-nodulating soil bradyrhizobia and can be used as a recipient in symbiosis island transfer to study the symbiotic evolution of bradyrhizobia. The interesting features of pDOA9 bring the curiosity how if the pDOA9 transfer to the free living rhizobia and the pDOA9 will or will not stably maintain in the new host. The novelties of leguminous plants-rhizobial interaction may explain by gene loss or lateral transfer of nodulation genes. In order to increase the plasmid stability Harrison & Brockhurst (2012) revealed that under selection pressures favoring the maintenance of plasmid genes and occur the co-evolutionary processes, in example by selection under variance antibiotic concentration. Thus, this research aimed to transfer the plasmid of *Bradyrhizobium* sp. DOA9 (pDOA9) to the free living *Bradyrhizobium* sp. S23321, then demonstrate the infection process, nodulation and evolution mechanisms of the transconjugant in three different tribes of legumes (Dalbergioid, Genistoid and Milletoid).

2.4 Photosynthesis *Bradyrhizobium*-legumes symbiosis

Legumes are the third largest flowering plant family with a global distribution in total approximately 20,000 species and over 700 genera, (Lewis et al., 2005). Legumes play crucial roles in food safety and agro-ecosystems sustainability by providing some benefits at the food and production system levels (Stagnari et al., 2017). *Bradyrhizobium* is **α**-proteobacteria familiy Rhizobiaceae that are common in

soil and induce nodules on plants from the Leguminosae family. Legumes work together with specific soil rhizobia to improve symbiotic interactions that lead to the formation of root nodules. These nitrogen-fixing nodules supporting the plants to grow without the addition of nitrogen fertilizers in farming systems and are of noteworthy in agronomic and ecological field (Atienza and Rubiales, 2017; Vanlauwe et al., 2019).

Rhizobia carry most of the genes specifically required for nodulation either on large (500-kbp to 1.5-Mbp) plasmids or on symbiosis islands. The host range of this strain such as *Medicago sativa* (alfalfa), *M. truncatula, Pisum sativum* (pea), *Vicia* species (vetches), and *Trifolium* species (clovers), *Glycine max* (soybean), *Vicia faba* (bean), and *Lotus japonicus* (Gage, 2004). Some *Bradyrhizobium* strain can grow as heterotrophs, autrothrops, phototrophs and grow symbiotically with stem of the plant genus *Aeschynomene* (Giraud et al., 2007).

Alazard (1985) and Molouba (1999) discovered that the host specificity of *Bradyrhizobium* within different *Aeschynomene* species, have been identified into three different cross-inoculation (CI) groups. *Aeschynomene* species belonging to CI-group 1 (e.g. *A. elaphroxylon* or *A. americana*) are nodulated on their roots by non-photosynthetic. Stem nodulation is restricted to CI-groups 2 (e.g. *A. afraspera*) and 3 (e.g. *A. indica*) by photosynthetic *Bradyrhizobium*. For example, photosynthetic *Bradyrhizobium* strain ORS278 and BTAi1 are able to nodulate *Aeschynomene* belonging to the CI-group 3 but impairs the nodulation ability to plants from CI-groups 1 and 2. Whereas, photosynthetic bradyrhizobia (ORS285) have a rather wide host range and nodulate stems of *Aeschynomene* belonging to CI-groups 2 and 3. The mutation of common *nod* genes in ORS285 did not effected the CI-group 3 nodulation (*A. indica*) but became impaired in its original host (*A. afraspera*). 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 (Giraud et al., 2007). Meanwhile, bacteria in CI-group 3 is photosynthetic rhizobia which can be distinguish into two: First group, contain the canonical *nodABC* genes found among other rhizobia and have a broad host range that extends to all stem-nodulated *Aeschynomene* species, i.e. ORS285. Second group, lack *nodABC* genes and are able to nodulate only a few species of *Aeschynomene*, including *A. sensitiva* and *A.*

indica, i.e. ORS278 and BTAi1 (Giraud et al., 2007, Bonaldi et al., 2011; Giraud et al., 2013).

2.4.1 *Bradyrhizobium* sp. ORS278

Bradyrhizobium sp. ORS278 is a photoheterotrophic photosynthetic strain with strictly aerobic and isolated from stem nodule of *A. sensitiva in* Senegal, Africa. The ORS278 genome consists of a single, circular, chromosome of 7,456,587 base pairs (bp) with GC content 65.5% and 6752 predicted coding sequences (CDSs). The genomes of ORS278 contain 21 putative horizontally acquired genomic islands (HAIs) including a ribulose 1,5-bisphosphate carboxylase (RuBisCo), enzymes involved in nitrogen metabolism, including urease, lipopolysaccharide (LPS) modification enzymes, a type II secretion system, a chemotaxis operon, and a multidrug efflux pump exhibiting hallmarks of recent gene transfer events. No symbiotic genes are found, *nif* and *fix* genes are clustered in a 45-kb region but there is no evidence that this region was acquired by HGT (Giraud et al., 2007).

ORS278 and *Rhodospeudomonas palustris* derive from a common photosynthetic ancestor. Photosynthetic gene cluster (PGC) found in the 45-kb region which consist of *bch* and *crt* genes for bacteriochlorophyll and carotenoid synthesis and the *puf* operon coding proteins of the light harvesting complex (*pufB* and *pufA*) and the proteins of the reaction center complex (*pufL and pufM*). PB enter *Aeschynomene* spp. via cracks in the epidermis that result from lateral root bump (Goormachtig et al. 2004). The photosynthetic *puf* genes found to be important in the stem symbiosis between *Bradyrhizobium* sp. ORS278 and its host plant *A. sensitiva* (Giraud *et al.,* 2000). Moreover, a purine derivative possibly cytokinin may play a key role as the signal in triggering nodule formation. Remarkably, ORS278 naturally associate with a wild rice species, *Oryza breviligulata* and increase the production up to 20% that co-inhabits some tropical marshes of Africa with Aeschynomenes. Furthermore, ORS278 produces canthaxanthine, which has pharmaceutical, agro-alimentary, and cosmetic applications for its coloring and photo-protective properties (Giraud et al., 2007).

The genes required for an efficient symbiosis with *Aeschynomene* sp. in ORS278 including TCA cycle, gluconeogenesis, PHB, and glycogen degradation found to be important in bacteroid metabolism. Novel regulatory and signaling proteins

involved in bacteroid functioning a large sensory histidine kinase act as global regulator for N_2 fixation. ORS278 presents two *kaiBC* operons for central component of the circadian clock system of the *nif* genes would be particularly beneficial to optimize the symbiotic interaction in the stem nodules. ORS278 also encodes a putative transcriptional regulatory protein related to the *MarR* family for oxidoreductase. The *che* operons important for chemotaxis and motility, though, after the infection has been started, the motility is not necessary and *che* operons are implicated in controlling cellular functions other than motility, including biofilm formation, initiation of developmental programs, EPS biosynthesis. D-alanyl-D-alanine carboxypeptidase involved in peptidoglycan biosynthesis and an apolipoprotein *N*-acyltransferase that transfers a fatty acyl group on membrane lipoproteins possibly required for membrane integrity, which is crucial during the early stages of bacterial differentiation within plant cells. (Hickman et al. 2005; Kirby and Zusman 2003; Bible et al. 2008; Bonaldi et al., 2010). Furthermore, ORS278 has C_{26}:25OH very long-chain fatty acid (VLCFA) linked to the lipid A which found to have dual function in this strain. Firstly, playing the critical function in rigidification of the outer membrane, thus assisting ORS278 for successful symbiosis with Aechynomene legume. Secondly, essential for the ORS278 strain to deal with abiotic stresses in free-living conditions (Busset et al., 2017). ORS278 possesses a *luxI-luxR* type Quorum Sensing (QS) system and produces cinnamoyl-HSL as a QS signal important for promotes growth of rice (*O. sativa*) and this finding was vital role of aryl-HSLs in endophytic plant-bacteria interactions (Cai et al., 2020).

2.5 Symbiosis of *Bradyrhizobium* sp. DOA9 with Legumes

Bradyrhizobium sp. DOA9 was able to nodulate a broad range of leguminous hosts by using classical NF dependent pathway and was not form nodules on NF-independent groups of Aeschynomene. The legumes which could nodulate by DOA9 including Dalbergioid tribe (*A. americana, A. afraspera, A. hypogaea,* and *S. hamata*), Millettioid tribe (*M. artopurpureum, I. tinctoria, V. radiata cv*. SUT4, *Lespedeza* sp. and *D. turtuosum*), Genistioid tribe (*Lupinus polyphyllus* and *C. juncea*), and Robinioid tribe (*L. japonicus*), IRLC (Inverted Repeat Lacking Clade) tribe (*Medicago*

sativa), Mimosoidae tribe (*Leucaena leucocephala* and *Samanea saman)*. Moreover, DOA9 was also found to be an endophyte in rice (Teatimsong et al., 2014).

DOA9 was unable to nodulate Soybean even their chromosomes share a high degree of similarity and phylogenetically close to *B. diazoefficiens* USDA110. The reason because of DOA9 absent of *nodY* which was reported to be *Bradyrhizobium* specific and to be induced by soybean seed extract and selected isoflavones, primarily genistein and daidzein, but not by flavones (Banfalvi et al., 1988; Okazaki et al., 2015). DOA9 possess few copies of *nodVW* which is a member of the classical two-component regulatory family and is essential for the nodulation of cowpea, siratro, and mungbean (Sanjuan et al., 1994; Okazaki et al., 2015).

DOA9 acquired divergent nodulation (*nod*) genes and other symbiosis-related genes such as T3SS and T4SS for broadening their symbiosis. Mutation of T3SS mutant (**Ω***rhcN*) in DOA9 induce the functional nodule in *V. radiata* SUT4 and *C. juncea*, also increase in the nodule number and a dramatic improvement in nodule development and infection of *A. hypogea* and *M. atropurpureum* (Songwattana, 2017). Type Three Effector (T3E) of symbiotic plasmid DOA9 also has role in symbiosis efficiency in this strain, for example the T3E-SkP48 in pDOA9 which contains a shikimate kinase and a SUMO protease (C48 cysteine peptidase) domain is a key factor suppressing nodulation and nodule organogenesis in *Vigna* spp., *C. juncea* and *A. hypogea* by activation of effector-triggered immunity (ETI) via salicylic acid biosynthesis (Piromyou et al., 2021).

2.6 Legume Nodulation and Evolution

Legumes are the third largest family of flowering plants and are extremely varies from tiny herbs to giant trees. There are over 19,000 species in the legume family separated into six subfamilies and then further distributed into narrower groups based on their evolutionary interactions. There are 765 genera the groups one level above species. The phylogenetic tree was built based on sequences from the transcriptomes data, which is expressed as most genes from 463 species and a small number of short sequenced whole genomes from across legume diversity. Over 17,000 legumes species and include all of the species with the ability to fix N_2. Both nodulation and nitrogen fixation invented a single time early in the history of

legumes and other related nitrogen-fixing plants and the whole-genome duplication event at the origin of legumes might have been crucial for the evolution. The multiple gene losses in plants induce incapability to nodulate also occurs during gene duplication/whole-genome triplication events across Fabaceae, and evolutionary changes in genes contributed to their role in nodulation (Zhao et al., 2021).

Papilionoideae and Caesalpinioideae are the largest subfamilies of N_2- fixing Fabaceae species with rhizobial nodules that have been identified (Doyle, 2016). The ancestor of Caesalpinioideae indicated to be non-nodulating species, as reinforced by the non-nodulation state of three successive comrade, *Umtiza* and *Pterogyne* of the lasting Caesalpinioideae. The ancestor of the *Swartzieae* is shown to be nodulating, as is *Vatairea*. After the divergence of the *vataireoid* clade, the common ancestor of the remaining Papilionoideae is also indicated to be nodulating. Furthermore, losses of nodulation were found in *Nissolia, Bowringia,* and *Chesneya* (Zhao et al., 2021). The four order including Fabales, Fagales, Rosales, and Cucurbitales found from single origin N_2-fixing nodulation and followed by massive, multiple losses, given the complexity of the nodulation process that needs many gene functions. The well-supported single clade comprises all N_2 fixing taxa, and the expression results of multiple genes in both rhizobial and actinorhizal-nodulating species. This hypothesis is shown by molecular analysis of multiple gene families related to rhizobial nodulation for N_2 fixation (van Velzen et al., 2017, 2018, 2019; Battenberg et al., 2018; Griesmann et al., 2018; Zhao et al., 2021).

The huge amount of multiple losses of nodulation found in the regulatory of nodulation-related gene expression homologs of *LjNIN* (*Lj = Lotus japonicus*), in multiple non-nodulating species of the nitrogen-fixing clade (Fabales, Fagales, Rosales, and Cucurbitales) (Sprent, 2009; Doyle, 2011; Suzuki et al., 2013; and Griesmann et al., 2018). Legumes form rhizobial nodules that are dissimilar from the actinorhizal nodules in most of the non-legume nitrogen-fixing species distributed in three orders. N_2-fixing actinorhizal nodules possibly ancestral type, for example for Parasponia. Possibly, there are one or many genes shifts to changes actinorhizal nodules to be the rhizobial nodules (van Velzen et al., 2017, 2018, 2019).

The multifaceted legumes expand their preference to the microsymbiont partners. Nowadays, there are 18 α and β proteobacterial genera including *Allorhizobium, Ensifer, Neorhizobium, Pararhizobium, Rhizobium,* and *Shinella* in *Rhizobiaceae; Aminobacter, Mesorhizobium* and *Phyllobacterium* in *Phyllobacteriaceae; Ochrobactrum* in *Brucellaceae; Methylobacterium* and *Microvirga* in *Methylobacteriaceae; Bradyrhizobium* in *Nitrobacteraceae, Azorhizobium* in *Xanthobacteraceae; Devosia* in *Hyphomicrobiaceae;* and the β*-proteobacterial Paraburkholderia, Cupriavidus* and *Trinickia* in *Burkholderiaceae*) consist of rhizobial species, with over 250 legitimate names and represent species. The numbers rapidly increase over the years (de Lajudie et al., 2019).

Bradyrhizobium nodulates with the broadest host range of legume genera and has the largest genomic diversity and *nod* gene diversity of the rhizobia, congruence with the theory that it is the ancestral microsymbiont of legumes (Ormeño-Orrillo & Martínez-Romero, 2019; Parker, 2015). Papilionoideae, Dalbergieae, Genistoid, Mirbelioid and the Phaseoleae seem common associations with the species of *Bradyrhizobium.* However, the number of genetic and molecular mechanisms that govern symbiotic specificity in legume-rhizobia interactions rapidly modify, for example symbiont shift from *Bradyrhizobium* to *Rhizobium* in *P. vulgaris* related species (Servín Garcidueñas et al., 2014). Commonly, it appears that legume species diversification and radiation are often but not always associated with distinct changes in microsymbiont preference. Legumes-rhizobia are varied in the specificity of their symbiotic associations: legumes may be promiscuous or highly specific and rhizobia may have broad or narrow host ranges (Wang et al., 2018). The features of legume-rhizobia symbiosis shown in Table 2.3.

Table 2.3 The hallmark of legume-rhizobia symbiosis.

Feature	**Legume-Rhizobia Symbiosis**
Plant host Phylogenetic occurrence of nodulation Order; Family, Subfamily	Fabales, Leguminosae, Caesalpinioideae (including the mimosoid clade), Papilionoideae
Number of symbiotic species	ca.17.300 - 19.300
Biogeographic distribution	Universally dispersed and extent all dominant terrestrial biomes; abound in the tropics, where they can be foremost components in late successional forests (Yahara et al., 2013)
Habit	Woody trees and shrubs, lianas, herbaceous perennials and annuals
Microsymbiont	Rhizobia: phylogenetically diverse, Gram-negative α and β proteobacteria; facultatively symbiotic
Number of symbiotic species	18 α and β proteobacterial genera; over 250 authentically named and labelled species
N_2-fixation	Most are incapable to fix N_2 ex planta; many lack the *nifV* gene necessary for biosynthesis of the indispensable nitrogenase cofactor homocitrate, which must be provided by the plant (Hakoyama et al., 2009)
Symbiotic signaling	Obligatory
molecules : Plant flavonoids	
Microsymbiont signalling molecules	Lipochitooligosaccharide Nod factors; the transcriptional regulator NodD bind with legume flavonoids, initiating transcription of the canonical nod genes that encode biosynthesis and export of host-specific Nod factors (Fisher & Long, 1992)

Table 2.3 The hallmark of legume-rhizobia symbiosis (Continue).

Feature	Legume-Rhizobia Symbiosis
Symbiotic genes	Rhizobial symbiotic genes (nod, nif and fix genes) are clustered in potentially transferable elements such as symbiotic plasmids or genomic islands (Freiberg et al., 1997; Kaneko et al., 2002); HGT of these sym genes is main key in the evolution of rhizobial strains that have acquired the ability to nodulate with a given legume host (Sullivan & Ronson, 1998; Barcellos et al., 2007 Legume flavonoid-induced effector proteins secreted by T3SS are crucial symbiotic determinants in some rhizobial strains (Songwattana et al., 2017; Staehelin & Krishnan, 2015; Viprey et al., 1998); effector proteins are required for nodulation in Nod Factor independent Aeschynomene-Bradyrhizobium symbioses (Teulet et al., 2019)
Nodule structure	Stem-like organs with a peripheral vascular system and infected cells in the central tissue (Hirsch & Larue, 1997)
Endoreduplication	Infected nodule cells undergo endoreduplication (Suzaki et al., 2014; Vinardell et al., 2003)
Infection threads/ symbiosomes	Low chance the nodulating legumes retain rhizobia within infection threads. In most species, bacteria within the nodule cell are budded off and surrounded by the host plasma membrane only; this new bacteria-plus-membrane compartment is called the symbiosome (Sprent, 2009)
Host control of the microsymbiont	Bacteroid metabolism is significantly reduced and the plant assumes control of more biochemical functions; bacteroids become ammonia plasts (Udvardi and Poole, 2013)

Table 2.3 The hallmark of legume-rhizobia symbiosis (Continue).

Feature	Legume-Rhizobia Symbiosis
Defensin-like peptides	Nodule-specific cysteine-rich (NCR) peptides present in Inverted Repeat Lacking Clade (IRLC) legumes and equivalent peptides found in Aeschynomene impose terminal differentiation on their bacteroids (Alunni and Gourion, 2016; Czernic et al., 2015): bacteroids become enlarged, endoreduplicated, occasionally pleomorphic, and the bacterial membrane turn into more permeable (Mergaert et al., 2006) Rhizobia that are microsymbionts of IRLC legumes that express NCR peptides require the correct BacA protein to warrant the survival of bacteroids within the nodule cells (diCenzo et al., 2017; Karunakaran et al., 2010) Terminally differentiated bacteroids are more efficient for N2 fixation (more N2 fixed per unit of CO2 respired; Kazmierczak et al., 2017; Lamouche et al., 2019)
Autoregulation of nodulation and nitrogen regulation of nodulation	Legumes appear to have a greater capacity for optimising N2 fixation by autoregulation of nodulation and downregulation of rhizobial N2 fixation when levels of soil N are high

Table 2.3 The hallmark of legume-rhizobia symbiosis (Continue).

Feature	Legume-Rhizobia Symbiosis
	In the systemic autoregulation of nodulation (AON) pathway, CLE peptides produced in the root in response to N2 fixation are deliver via the xylem to the shoot, where they are perceived by a receptor complex containing a leucine rich-repeat receptor-like kinase (LRR-RLK). The upcoming signal transduction downregulates production of shoot derived microRNA miR2111, which itself downregulates the root-specific protein TOO MUCH LOVE that prevents nodule organogenesis (Ferguson et al., 2019). In nitrogen regulation of nodulation, CLE peptides encoded by nitrate-induced genes are perceived by root-localized LRR-RLKs, triggering signal transduction and production of a root factor that results in local inhibition of nodule organogenesis (Ferguson et al., 2019)

2.6.1 *Crotalaria juncea*

Crotalaria ("rattlepods"), which includes herbs or shrubs belonging to the Fabaceae, with about 600 species mainly occurring in the tropics, particularly in Africa, establishes symbiotic associations with nitrogen-fixing bacteria belonging to the two genera *Bradyrhizobium* and *Methylobacterium* (Doignon-Bourcier et al., 2000; Sy et al., 2001). *C. juncea* are common green-manure legumes grown in rice cropping systems of the tropics. *C. juncea* has been recommended for warm and temperate regions. Some of the main attributes of this species is due to rapid and high productivity of biomass (8 Mg ha^{-1}) and its high content of foliar nitrogen, obtained by biological nitrogen fixation (BNF) at an average of 150 kg N ha^{-1}. In addition, a characteristic of these species is that they have the ability to establish a promiscuous

and functional symbiosis with the native rhizobia of the soil. The biomass production of *C. juncea* is positively correlated with the recycling of nutrients, the entry of carbon (C) into the soil, and a decrease in the rate of erosion (Li et al., 2012; Poeplau and Don, 2015). Furthermore, high concentrations of foliar N derived from BNF determine a low C/N ratio, which favors the rapid decomposition of plant remains The ease of degradation of this material also facilitates net N mineralization, which can be used by subsequent crops (Berriel, et al., 2020).

2.6.2 *Aeschynomene* legumes

The Aeschynomene genus belongs to the dalbergioid group of the Papilionoideae subfamily. The dalbergioid is a diverse group that includes *A. hypogea* (Qin et al., 2011) and other herbaceous genera, and also woody plants such as the Brazilian rosewood (Doyle and Luckow, 2003). The Aeschynomene genus consists of approximately 150 species, of which 22 species were reported to be nodulated on roots and stems by *Bradyrhizobium* strains (Arrighi et al., 2012). Nodule-forming Aeschynomene species are classified into three different cross-inoculation (CI) groups according to their symbiotic characteristics. Aeschynomene species belonging to CI group 1 (e.g. *A. americana*) are only nodulated on their roots, while CI groups 2 (e.g. *A. afraspera*) and 3 (e.g. *A. indica*) are nodulated on roots and stems (Miche et al. 2010). The most distinctive difference between CI group 2 and 3 species is the nodule initiation mechanism. Symbionts of CI group 2 possess *nodABC* genes and initiate nodulation through NFs, whereas CI group 3 symbioints do not possess *nodABC* genes and initiate nodulation without NFs for example *Bradyrhizobium* strains (BTAi1 and ORS278), which form nodules on roots and stems (Giraud et al. 2007). NF-independent symbiotic process is an ancestral symbiotic system (Madsen et al. 2010, Bonaldi et al. 2011). There are no essential genes (in example: *nod*-like genes) which are required to form a symbiotic relationship with Aeschynomene (CI group 3) (Okubo et al., 2012).

2.6.3 Mung bean *(Vigna radiata)*

The role of Mung bean *(V. radiata)* in biological nitrogen fixation (BNF) over association with native rhizobia, which declines the demand for the N- fertilizer (Herridge et al., 2005). It is extensively cultivated crop in Asian countries. It has well compliance in the degree of salinity and high temperature tolerance, short cycle,

water regime and the low cost for plantation and its grain has high nutritional value in their proteins and peptides (Du et al., 2018; Yi-Shen et al., 2018). Mung bean contribute to its development and grain yield performance thus upsurge its acceptance by farmers (Hanumantharao et al., 2016; Sharma et al., 2016).

V. radiata could nodulated with several group of Rhizobia (Yang et al., 2008; Zhang et al., 2008) including Bradyrhizobia: *B. japonicum* and *B. elkanii* (Zhang et al., 2008; Appunu et al., 2009; Risal et al., 2012; Favero et al., 2021), Sinorhizobia (Hakim et al., 2018; 2020), and Mesorhizobia (Lu et al., 2009). However, it showed low symbiotic specificity with native rhizobia in native soil (Yang et al., 2008; Zhang et al., 2008). The diversity of the native soils microbiome also have significant role in *V. radiata*-bradyrhizobia symbiosis (Favero et al., 2021).

2.6.4 *Arachis hypogaea*

Some rhizobia of the genus *Bradyrhizobium* efficiently nodulate peanuts, the second most important legume crop in the world after soybeans (Urtz and Elkan, 1996). The peanut-rhizobium symbiosis is essential and supplies 55% of nitrogen needs for optimal peanut growth (Hardarson, 1993). Rhizobial infection commonly follows by a “root hair entry” pathway, which involves penetrating via intracellular infection threads and its dominant in most of legumes species, such as soybean (*Glycine max*), *M. truncatula*, and *L. japonicus*, (Bonaldi et al., 2011; Oldroyd et al., 2011). On the other hand, intracellular infection threads are not found in the roots of legumes in *A. hypogaea* L. where the infection occurs intercellularly via “crack entry.” In peanut, the rhizobia enter the root, where root hairs emerge and occupy the space between the root hair wall and adjoining epidermal and cortical cells. They colonize the intercellular spaces and invade sub-epidermal cortical cells via an endocytosis-like process (Okubo et al., 2012a). The invaded cells divide and are incorporated in the nodule tissue. Adjacent cells separate at the middle lamellae, and this space is filled with bacteria, in consequence allocating the bacteria intercellularly (Chandler, 1978). This intercellular infection is considered an evolutionarily prehistoric invasion mechanism (Groth et al., 2010; Madsen et al., 2010), which might be a key to facilitate transforming non-legumes for biological nitrogen fixation via symbiosis (Charpentier and Oldroyd, 2010).

2.6.5 Siratro *(Marcoptilium artopurpureum)*

M. atropurpureum is a perennial herb widely cultivated across tropical and subtropical regions of the world as a forage crop, a soil and pasture improver and for ground cover. It is native to tropical America and has been introduced across Africa, Asia, the Caribbean, Australia and many islands in Oceania. Similarly to other Fabaceae species, *M. atropurpureum* grows in association with *Rhizobium* bacteria, which enables it to fix nitrogen (FAO, 2017). *M. atropurpureum* grows in moist habitats in temperatures ranging from 22°C to 30°C, but can tolerate lower temperatures of 13/15°C. It is extremely drought tolerant due to its deep rooting habit, but it is not tolerant of flooding or waterlogging. It is well adapted to growing under moderately saline conditions gravels with pH ranging from (4.5-) 6.5 to 8.0 (-8.5) (Cook, 2005; FAO, 2017).

As promiscuous legume, *M. atropurpureum* could be infected by several different types of rhizobia and forming root nodule symbiosis (RNS). In addition, this leguminous species was able to associate with *R. japonicum* 143 (Rj-143), *B. japonicum* and *B. elkanii* which were isolated from soybean plants. The nodule type in siratro is determinate nodules with spherical, possess a transient meristem, differentiate in a more synchronous manner and eventually contain populations of bacteroids often considered as developmentally homogenous across a nodule section. By the time, some determinate nodules likewise develop secondary clusters of dividing cells that form new nodule meristems. The developmental and infection processes leading to the formation of proficient nodules where bacteroids can fix nitrogen are complex, and sets of molecular cues exchanged by plants and rhizobia coordinate both of these processes. In such a molecular discourse, signals emitted by one of two partners and the corresponding cognate receptors of the second symbiont were proposed to function together as key-lock systems that define the specificity of rhizobia-legume associations (Unay and Perret, 2020).

2.7 Host Specifity

The reciprocal recognition of a series of molecular signals between the symbiotic partners allows to the successful mutualism, thus admissible or impairing bacterial proliferation at each stage (nodulation, infection, intracellular survival) and determining host specificity. Under nitrogen restricting circumstances, legume roots secrete a mixture of flavonoid compounds into the rhizosphere, and they assist to activate the expression of a group of bacterial nodulation (*nod*) genes, leading to the synthesis of the Nod factor, a lipochitooligosaccharide signal that is essential for initiating symbiotic development in most legumes (Oldroyd et al., 2011). Induction of *nod* gene expression is mediated by the flavonoid activated NodD proteins, which are LysR-type transcription regulators (Long, 1996). NodDs activate *nod* gene expression through binding to the conserved DNA motifs (*nod* boxes) upstream of the *nod* operons (Rostas et al., 1986 and Fisher et al., 1988). NodD proteins from different rhizobia are adapted to recognize different flavonoids secreted by different legumes, and this recognition specificity stipulates an early checkpoint of the symbiosis (figure 4.3). Even though lack of evidence for physical interaction between the two molecules, flavonoids have been shown to be able to stimulate the binding of NodD to *nod* gene promoters in *S. meliloti* (Peck et al., 2006). It is well recorded that crossed strain exchange of *nodD* genes can change the response of the recipient strain to a different set of flavonoid inducers and consequently to the host range (Horváth et al., 1987 and Perret et al., 2000). In example, the transfer of *nodD1* from the broad host range symbiont *Rhizobium* sp. NGR234 to the narrow host range strain *R. leguminosarum* bv. *trifolii* ANU843 enabled the recipient strain to nodulate the non-legume *Parasponia*, because the broad host range NodD1 protein was capable of recognizing a broader spectrum of flavonoid inducers (Bender et al., 1988).

Nod factors contain three to six N-acetylglucosamine residues, and uridyl diphosphate (UDP)-N-acetylglucosamine (GlcNAc) is a common precursor for the biosynthesis of peptidoglycan as well as Nod factor is backbone in different rhizobial species. Nod factors from different rhizobia share the same chitin-like N-acetyl glucosamine oligosaccharide backbone with a fatty acyl chain at the non-reducing end, but differ in their length of the backbone, the size and saturation of the fatty

acyl chain, as well as additional modifications at either end, such as acetyl, methyl, carbamoyl, sulfuryl or glycosyl groups (Long, 1996).

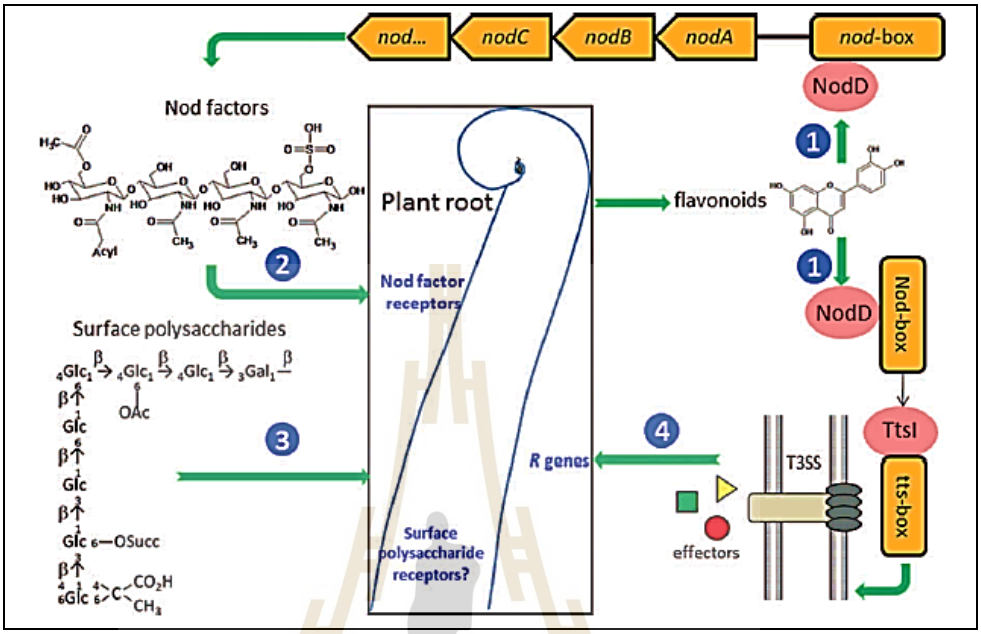

Figure 2.11 Molecular determinants of host specificity during nitrogen-fixing symbiosis (Wang et al., 2012).

Structural variations in Nod factors are a key determinant of host range, because these Nod factors have to be recognized by the host in order to initiate infection and nodulation (Perret et al., 2000 and D'Haeze and Holsters, 2004). The core structure of Nod factor is synthesized by the products of *nod*ABC genes. Mutations in these genes completely abolish symbiosis. Additional genes determine the specific decorations on the Nod factor core, and alterations in these genes often change specificity. For example, abolishing the *nod*E gene in *R. leguminosarum* bv. *Trifolii* changes the identity of the fatty acyl chain attached to the Nod factor (Spaink et al., 1991), and this change severely affects symbiosis with *Trifolium* species while enhancing symbiosis with *P. sativum* and *V. sativa* (Djordjevic et al., 1985 and Spaink et al., 1989). On the contrary, introducing the *nod*EFGHPQ gene cluster from *S. meliloti* into *R. leguminosarum* altered the modifications on Nod factor into the *S.*

meliloti type, and the engineered *rhizobium* became specific to the *S. meliloti* host *M. sativa* (Debelle et al., 1988 and Faucher et al., 1989).

Perception of the Nod-factor signal in legumes is mediated by Nod factor receptors (NFRs). NFRs are plasma membrane-localized serine/threonine receptor kinases that contain LysM motifs in their extracellular domains (e.g. NFR1 and NFR5 in *L. japonicus*; NFP and LYK3 in *M. truncatula*) (Limpens et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003; and Arrighi et al., 2006). These LysM domains are found in a variety of peptidoglycan and chitin binding proteins, suggesting their probable role in direct binding of Nod factors. Direct binding of Nod factors to the extracellular LysM domains of the receptor complex leads to activation of the downstream nodulation signaling pathways (Broghammer et al., 2012). Specificity in Nod-factor binding is widely thought to be critical for recognition between the prospective symbiotic partners. The role of NFRs in determining host specificity was observed by transferring the *Lj*-NFR1 and *Lj*-NFR5 to *M. truncatula* enables nodulation of the transformants by the *L. japonicus* symbiont *M. loti* (Radutoiu et al., 2007). Hereinafter, the specificity for different rhizobial symbionts of two different *Lotus* species is a function of a single amino acid residue within one of the LysM domains of Lj-NFR5 (Radutoiu et al., 2007).

These plant genetic programs can also be activated by bacterial T3SS effectors. Microbe-associated molecular patterns (MAMPs) or other bacterial T3/4SS effectors can induce plant immunity mediated by intracellular or plasma membrane localized receptors. The loss of the ability to express the T3SS was strictly necessary for the early transition towards symbiosis in the experiment (Marchetti et al., 2010), and that subsequent adaptation favored the reuse of regulatory modules leading to massive metabolic and transcriptomic changes (Capela et al., 2017). EPS recognized by plant (EPR3) facilitate infection (Kawaharada et al., 2015). NCRs, which are extremely abundant defensin-like peptides in the nodules of some legumes (Inverted Repeat-Lacking Clade and *Aeschynomene* legumes), can have a positive or negative impact on the intracellular stage. Some NCRs control the differentiation of intracellular bacteria into enlarged polyploid bacteroids (Czernic et al., 2015 and Van de velde et al., 2010) a step required for N_2-fixation but which can be impaired by bacterial peptidases (*HrrPs*) (Price et al., 2015). The bactericidal activity of other

peptides (NFS1 NFS2) affects the intracellular survival of specific bacterial genotypes (Wang et al., 2017 and Yang et al., 2017). The bacterial *BacA/BclA* transporter promotes the import of NCR peptides (Marlow et al., 2009).

Host cannot distinguish between the beneficial and pathogenic symbionts during initial colonization in legume-rhizobia symbiosis (Keller et al., 2018). NF has been the most successful strategy as it is used by most of the rhizobia inspected so far. The symbiotic genes including *nod* and *nif* genes are tightly linked on symbiotic plasmid (*psym*) or genomic island (Lee et al., 2008). Transferring of mobile genetic element is the first step for genetic rewiring of the recipient genome (Ling et al., 2016). However, most of the genes involved in nodulation effectiveness in the later stages, including surface polysaccharides crucial for infection (Pobigaylo et al., 2008), hormones regulation, secondary metabolite production, nitrogen and carbon metabolism and transport (Keller et al., 2018) do not belong to the symbiotic mobile genetic element and must be recruited from the genomic background.

The evolution after acquisition of the symbiotic plasmid followed by genomic remodeling under plant selection pressure, is reinforced by the presence of vivid phenotypic shifts driven by adaptive mutations in experimental evolution studies (Capela et al., 2017 and Clerissi et al., 2018). Post horizontal gene transfer evolution of emerging rhizobia may have been accelerated by error-prone DNA polymerases to create the hypermutagenesis that are present on half of the rhizobial symbiotic plasmids (Remigi et al., 2014 and Clerissi et al., 2018) and facilitated by IS transposition (Zhao et al., 2017).

Rhizobia possess highly complex genomic architectures, consisting of a chromosome and multiple extrachromosomal replicons (plasmids). The chromosome is always the largest replicon in the genome and contains the majority of the core/essential genes (diCenzo and Finan, 2017 and Harrison et al., 2010). Rhizobial plasmids have specific characters, such as miscellaneous genes, independent replication system, self-transmissibility, and instability. These plasmids regulate several cellular metabolic functions and enable the host rhizobia to survive in diverse habitats and even under stress conditions (Zahran, 2017). The symbiotic genes with high sequence similarity can be found in different rhizobia species, conferring nodulation ability with the same legume species; conversely, divergent

genes conferring distinct host ranges can be found in different strains of the same rhizobia species (Remigi et al., 2016).

The advantage of multiple replicons in the genome has been proposed to increase the adaptive potential of a species (Gonza´lez et al., 2006), which, assigned that the presence of multipartite genomes in many rhizobial species reflecting the complex lifestyle adopted by these plant-associated bacteria. The presence of additional replicons in the genome of the **α**-proteobacteria may enhance the bacterium's metabolic or symbiotic capabilities, thus ensuring stable maintenance of the replicon within the population then boardening the host specificity.

The *Bradyrhizobium* sp. DOA9 possesses 4191 replicon in chromosome and 324 replicon in the megaplasmid (Wang et al., 2018). The megaplasmid of DOA9 containing the symbiotic genes *nod* and *nif* genes which have several of copy number, example: *nodA* genes. The *nodA1* gene is located upstream of the *nodBC* genes and the second copy *nodA2* is found in the upstream of *nodIJ* genes. Mutation of the *nodA* gene affected the ability to form nodules and to fix the nitrogen (Songwattana et al., 2017). DOA9 has at least two copies of *nifH* one on the megaplasmid and one on the chromosome, which encode dinitrogenase reductase or Fe protein (Teatimsong et al., 2014). The *nifDK* in the plasmid of DOA9 has important role of nitrogenase structural genes under symbiotic condition. Deletion of the *nifDK* in megaplasmid DOA9 will abort the nitrogen fixation capability (Wongdee et al., 2016). The deletion secretion system (T3SS) also could boarden the host specificity (Songwattana et al., 2017). Thus, the acquisition of divergent *nod*, *nif* genes and secretion system (T3SS) may contribute to the broad host range of DOA9. Some of genus in rhizobia also carry the nonsymbiotic plasmids (Table 2.4). These plasmid encode a diverse assembly of genes, enabling them to collaborate with symbiotic plasmid (pSyms), and offering their host cells substantial abilities to compete in natural communities, catalyzing vast metabolic activities, and sustaining the symbiotic process (Barreto et al., 2012). However, the *Bradyrhizobium* sp. DOA9 is the first genus of bradyrhizobia which carries the symbiotic plasmid and shows broad host range capability. It will be interesting if we can transfer the megaplasmid of DOA9 to the non-nodulating bradyrhizobia, as it might convey the novel trait.

Table 2.4 The architecture of symbiotic nitrogen fixing Rhizobia

Genera	Species	Genomic Architecture	Number	Size (Mb)	G+C%	Host	Reference
Bradyrhizobium	DOA9	Chromosome	1	7.10	64.8	*Aeschynomene americana*	Okazaki et al. (2015)
		Symbiotic plasmid	1	0.74	60	*Vigna radiate (SUT4), Marcoptilium artopurpureum, Indigofera tinctoria, Aeschynomene afraspera, Arachis hypogea and Stylosanthes hamata*	Teatimsong et al. (2014)
	sp. BTAi1	Chromosome	1	8.26	64.92	*Aeschynomenes indica*	Giraud et al. (2007)
		Nonsymbiotic plasmid	1	0.22	60.69		
Mesorhizobium	*huakuii* 7653R	Chromosome	1	6.36	63.3	*Astragalus sinicus*	Wang et al. (2014)
		Nonsymbiotic plasmid	1	0.19	59.8		
		Symbiotic plasmid	1	0.32	59.6		

Table 2.4 The architecture of symbiotic nitrogen fixing Rhizobia (Continue).

Genera	Species	Genomic Architecture	Number	Size (Mb)	G+C%	Host	Reference
Sinorhizobium	meliloti E1021	Chromosome Symbiotic plasmid	1 2	3.65 1.68, 1.35	62.7 62.4, 60.4	Medicago, Melilotus, Trigonella	Capela et al. (2001); Stiens et al. (2008)
	meliloti E2011	Chromosome Symbiotic plasmid	1 2	3.66 1.68, 1.35	62.7 62.4, 60.4	Medicago truncantula	Sallet et al (2013)
	meliloti BL225C	Chromosome Symbiotic plasmid	1 2	3.67 1.69, 1.62	62.8 62.2, 60.1	Medicago, Melilotus, Trigonella	Galardini et al. (2011); Stiens et al. (2008)
	meliloti Rm41	Chromosome Nonsymbiotic plasmid Symbiotic plasmid	1 1 2	3.68 0.25 1.66, 1.56	62.7 59.3 62.3, 60.6	Medicago sativa	Liu et al. (2014)
	meliloti GR4	Chromosome Nonsymbiotic plasmid Symbiotic plasmid	1 2 2	3.62 0.18, 0.23 1.70, 1.42	62.8 60, 58.6 62.4, 60.4	Medicago sativa	Martínez-Abarca et al. (2013)

Table 2.4 The architecture of symbiotic nitrogen fixing Rhizobia (Continue).

Genera	Species	Genomic Architecture	Number	Size (Mb)	G+C%	Host	Reference
	meliloti GR4	Chromosome	1	3.62	62.8	Medicago sativa	Martínez-Abarca et al. (2013)
		Nonsymbiotic plasmid	2	0.18, 0.23	60, 58.6		
		Symbiotic plasmid	2	1.70, 1.42	62.4, 60.4		
	meliloti RMO17	Chromosome	1	3.65	62.8	Medicago orbicularis	Toro et al. (2014)
		Symbiotic plasmid	2	1.62, 1.62	62.6, 62.6		
	meliloti SM11	Chromosome	1	3.91	62.7	Medicago, Melilotus, Trigonella	Schneiker-Bekel et al. (2011); Stiens et al. (2008)
		Nonsymbiotic plasmid	2	0.14, 0.18	59.54, 58.67		
		Symbiotic plasmid	2	1.63, 1.63	62.4, 60.2		
	americanum CCGM7	Chromosome	1	3.65	63	Phaseolus vulgaris	Peralta et al. (2016)
		Nonsymbiotic plasmid	2	0.41, 2.25	60, 62		
		Symbiotic plasmid	1	0.55	58.8		
	americanum CFNEI 73	Chromosome	1	3.76	63	Acacia farnesiana	Peralta et al. (2016)
		Nonsymbiotic plasmid	2	0.22, 2.18	59.7, 62.2		
		Symbiotic plasmid	1	0.59	59		

Table 2.4 The architecture of symbiotic nitrogen fixing Rhizobia (Continue).

Genera	Species	Genomic Architecture	Number	Size (Mb)	G+C%	Host	Reference
	fredii NGR234	Chromosome	1	3.93	63	112 genera legume	Streit et al. (2004)
		Symbiotic plasmid	2	2.43, 0.54	62.3, 58.5		
	medicae WSM419	Chromosome	1	3.78	61.5	Medicago spp.	Garau et al. (2005), Terpolilli et al. (2008)
		Nonsymbiotic plasmid	1	0.22	60		
		Symbiotic plasmid	2	1.57, 1.25	61.5, 59.9		
Rhizobium	leguminosarum 1325	Chromosome	1	4.77	61.1	Trifolium spp.	Howieson et al. (2005), Reeve et al. (2010a)
		Nonsymbiotic plasmid	3	0.52, 0.35, 0.29	58.8, 60.6, 60.4		
		Symbiotic plasmid	2	0.66, 0.83	60.8, 60.4		
	leguminosarum 1689	Chromosome	1	4.85	61.1	Trifolium uniflorum	Terpolilli et al. (2008)
		Nonsymbiotic plasmid	4	0.67, 0.52, 0.26, 0.26	60.9, 61.2, 61, 61		
		Symbiotic plasmid	1	0.34	58.2		

Table 2.4 The architecture of symbiotic nitrogen fixing Rhizobia (Continue).

Genera	Species	Genomic Architecture	Number	Size (Mb)	G+C%	Host	Reference
	leguminosarum WSM2304	Chromosome	1	4.54	61.5	Trifolium polymorphum	Howieson et al. (2005); Reeve et al. (2010b)
		Nonsymbiotic plasmid	2	0.31, 0.26	57.9, 61.4		
		Symbiotic plasmid	2	0.50, 1.27	62, 60.4		
	leguminosarum 3841	Chromosome	1	5.06	61.1	Pisum sativa	Silva et al. (2005), Young et al. (2006)
		Nonsymbiotic plasmid	4	0.15, 0.15, 0.35, 0.87	57.6, 58.7, 61, 61		
		Symbiotic plasmid	2	0.68, 0.49	61, 59.6		
	leguminosarum CB782	Chromosome	1	4.38	61.4	Trifolium semipilosum	Carson et al. (2000)
		Nonsymbiotic plasmid	2	1.56, 0.51	60.3, 61.7		
		Symbiotic plasmid	1	0.25	57.3		
	leguminosarum Vaf10	Chromosome	1	5.04	60.9	Vavilovia formosa	Safronova et al. (2015)
		Nonsymbiotic plasmid	6	1.25, 0.62, 0.31, 0.27, 0.27, 0.25	60, 60.5, 60.2, 61.4, 61.3, 58.9		
		Symbiotic plasmid	1	0.56	58.5		

Table 2.4 The architecture of symbiotic nitrogen fixing Rhizobia (Continue).

Genera	Species	Genomic Architecture	Number	Size (Mb)	G+C%	Host	Reference
	gallicum IE4872	Chromosome	1	4.28	60.1	Phaseolus vulgaris	Silva et al. (2003)
		Nonsymbiotic plasmid	2	0.13, 0.18	58.1, 59		
		Symbiotic plasmid	2	2.39, 0.51	59.2, 58.3		
	gallicum R602	Chromosome	1	4.13	60	Phaseolus vulgaris	Soria-Díaz et al. (2006)
		Nonsymbiotic plasmid	1	0.20	58.6		
		Symbiotic plasmid	2	2.47, 0.51	59.4, 58.2		
	etli 8C-3	Chromosome	1	4.19	59.9	Phaseolus vulgaris	López-Guerrero et al. (2012b)
		Symbiotic plasmid	3	0.43, 2.25, 0.44	58.6, 59.2, 57.9		
	etli IE4771	Chromosome	1	4.48	61.5	Phaseolus vulgaris	Santamaría et al. (2017)
		Nonsymbiotic plasmid	4	0.24, 0.47, 0.73, 0.74	58.8, 61.9, 61.2, 60.7		
		Symbiotic plasmid	1	0.39	57.5		
	etli mim1	Chromosome	1	4.28	61.3	Phaseolus vulgaris	Rogel et al. (2014)
		Nonsymbiotic plasmid	5	0.18, 0.25, 0.27, 0.51, 1.08	61.8, 61.5, 58.4, 61.6, 60.5		
		Symbiotic plasmid	1	0.62	57.9		

Table 2.4 The architecture of symbiotic nitrogen fixing Rhizobia (Continue).

Genera	Species	Genomic Architecture	Number	Size (Mb)	G+C%	Host	Reference
	etli CFN 42	Chromosome	1	4.38	61.3	Phaseolus vulgaris	González et al. (2003); Silva et al. (2005)
		Nonsymbiotic plasmid	4	0.19, 0.18, 0.25, 0.64	58, 61.8, 61.5, 61.2		
		Symbiotic plasmid	2	0.51, 0.37	61.7, 57,8		
	etli TAL 182	Chromosome	1	4.49	61.4	Phaseolus vulgaris	Santamaría et al. (2017)
		Nonsymbiotic plasmid	4	0.17, 0.28, 0.28, 0.28	61.4, 61.2, 61.2, 61.2		
		Symbiotic plasmid	1	0.44	57.9		
	etli Brasil 5	Chromosome	1	4.43	61.7	Phaseolus vulgaris	Santamaría et al. (2017)
		Nonsymbiotic plasmid	3	0.26, 0.44,	62, 62.2, 60.9		
		Symbiotic plasmid	1	1.14	58.1		
	etli CIAT 652	Chromosome	1	4.51	61.7	Phaseolus vulgaris	Ramirez-Romero et al. (1997); Uribe (1993)
		Nonsymbiotic plasmid	1	1.09	60.9		
		Symbiotic plasmid	2	0.41, 0.43	62.2, 57.8		

Table 2.4 The architecture of symbiotic nitrogen fixing Rhizobia (Continue).

sp. NXC14	Chromosome	1	4.49	61.1	Phaseolus vulgaris	Santamaría et al. (2017)
	Nonsymbiotic plasmid	2	0.77, 0.89	61.6, 60.7		
	Symbiotic plasmid	1	0.53	58.2		
sp. CIAT 894	Chromosome	1	4.43	61.5	Phaseolus vulgaris	Santamaría et al. (2017)
	Nonsymbiotic plasmid	4	0.22, 0.27, 0.56, 0.72	61.3, 58.3, 62, 60.9		
	Symbiotic plasmid	1	0.46	58.2		
sp. IRBG74	Chromosome	2	2.84, 2.04	59.3, 59.3	*Sesbania* sp.	Crook et al. (2013), Cummings et al. (2009)
	Symbiotic plasmid	1	0.58	57.3		
tropici CIAT 899	Chromosome	1	3.84	59.9	Phaseolus vulgaris	Hernandez-Lucas et al. (1995), Hungria et al. (2000)
	Nonsymbiotic plasmid	2	0.22, 2.08	58.6, 59.4		
	Symbiotic plasmid	1	0.55	57.6		

2.8 Horizontal Gene Transfer (HGT)

2.8.1 Horizontal Gene Transfer

Conjugation is the most general studied, which is a process demanding direct cell to cell contact via cell surface pili or adhesins (Norman et al., 2009; Guglielmini et al., 2013). The DNA is transferred from the donor to the recipient cell. It is assisted by the conjugative machinery which is encoded either by genes on independently replicating plasmids or by integrative conjugative elements (ICE) in the chromosome (Smillie et al., 2010; Wozniak and Waldor, 2010; Von Wintersdorff et al., 2016). Furthermore, this conjugative machinery may allow the mobilization of plasmids that are non-conjugative, for example in the broad host range *IncQ* plasmids (Meyer, 2009). The slight transfer rates of ICEs and symbiotic plasmids under experimental condition, representing that these transfers are strictly regulated (Fig. 2.12A) (Banuelos-Vazquez et al., 2017).

Transformation is the uptake, integration, and functional expression of naked fragments of extracellular DNA. Specialized or generalized transduction, bacteriophages may transfer bacterial DNA from an earlier infected donor to the recipient cell. The first transformation investigations direct genetic exchange between different strains of *Streptococcus pneumonia* (Griffith, 1928). There are several requirement for transformation occurred. The DNA should be present in the extracellular environment, the recipient should be in competence stage and the translocated DNA should be stabilized, either by integration into the recipient genome, or by re-circularization (in the case of plasmid DNA) (Fig. 2.12B) (Thomas and Nielsen, 2005).

Bacteriophages play significant role in shaping the bacterial microbiome in any situation. Through specialized or generalized transduction, bacteriophages can transfer genes that are beneficial to their microbial hosts, in turn promoting their own survival and dissemination. Along generalized transduction, bacterial DNA possible accidentally loaded into the phage head (revealed as a phage with a red DNA strand). Thru specialized transduction, genomic DNA neighboring the prophage DNA is co-excised and loaded into a new phage (Fig. 2.12C) (Modi et al., 2013; Von Wintersdorff et al., 2016).

The first discovery of Gene transfer agents (GTA) was from *Rhodobacter capsulatus* (RcGTA) in 1974. GTAs are bacteriophage-like particles that bring random pieces of the producing cell's genome. The amount of DNA packaged by the GTAs is not enough to encode all of their protein components, making them unable to self-propagate (Lang and Beatty, 2007). GTA particles possibly liberated over cell lysis and scattered to the recipient cell, it use the combination of transduction and transformation for cell entry (Fig. 2.12D) (Brimacombe et al., 2015; Von Wintersdorff et al., 2016).

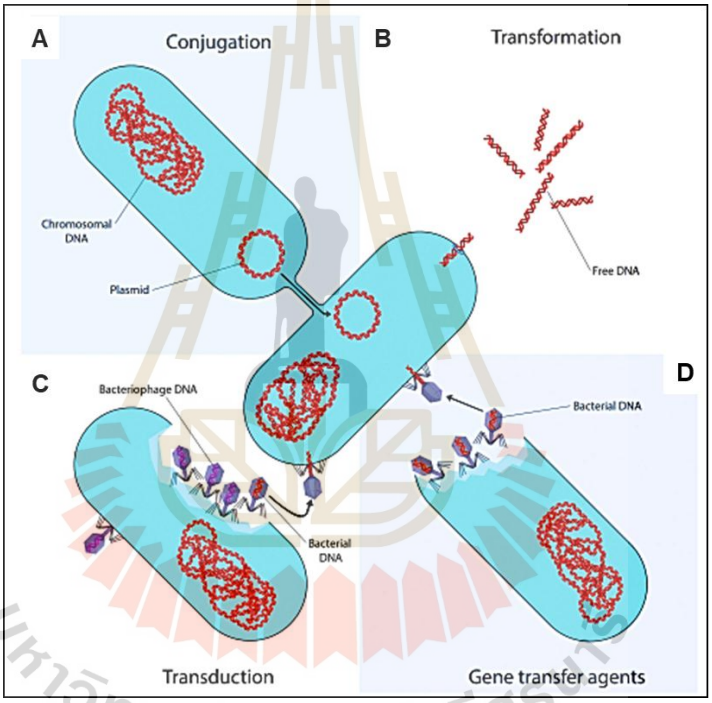

Figure 2.12 Horizontal Gene Transfer (HGT) Mechanism. Each region denote different method of gene transfer. Conjugation (A), Transformation (B), Transduction (C), Gene Transfer Agents (GTAs) (Von Wintersdorff et al., 2016).

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 (HGT). The symbiotic bacterium of genus *Rhizobium* carries

high molecular weight plasmids (90-350 x 10^6) (Banu & Prasad, 2017). 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. According to Barnett et al. (2001) megaplasmid pSymA of *S. meliloti* contains gene clusters for nitrogen fixation. The symbiotic plasmids are possible transfer by the horizontal gene transfer. Horizontal gene transfer (HGT) of genomic islands is a driving force of bacterial evolution. The intertwined interaction between plasmid and the host should incur significant fitness of the host or even has negatively impact host fitness (Dietel et al., 2018).

The symbiotic genes involved in the nodulation (*nod*) and nitrogen fixation (*nif*) and T3SS genes of *R. leguminosarum*, *S. meliloti*, *S. fredii* and *M. loti* were located on large plasmids varying in size from 100-300 Mb, called megaplasmid (Gonzalez et al., 2006; Galibert et al., 2001; Kaneko et al., 2000). 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 (Cytryn et al., 2008).

There are two central regulatory mechanisms of conjugative transfer were investigated in rhizobial *sym* plasmids in *Rhizobium* and *Sinorhizobium* genera. The first mechanism via quorum sensing (QS) system with the genes the *traI/traR/traM* genes. In certain amount with high level cell density, QS molecules produced by the autoinducer synthase *traI* binds to the regulator *tra*R which trigger the activation of *tra/trb/virB* (controlled functions important for the DNA transfer including the conjugative T4SS) and *rep* (controlled genes necessary for segregation/partition and replication of the plasmid) genes as well as *traM,* a gene encoding an anti-activator of *traR.* Additional levels of the regulation acting either in parallel or upstream the *traI/traR/traM* system were investigated in several rhizobia. For examples, in *R. leguminosarum*, the *bisR* response regulator and the *cinI/cinR* QS system order the expression of *traR* (Danino et al.,2003) whereas in *R. etli*, the *LuxR*-type regulator *CinR* positively regulate the expression of *traI* (Tun-Garrido, et al. 2003).

The second mechanism of regulation is QS-independent programs, designated in *R. etli* CFN42, implicate the *rct*A repressor of the *tra* and *virB* genes (Pérez-Mendoza et al., 2005) and another regulator *rct*B that oppressive the

repressive effect of *RctA*. Additionally, in *S. meliloti*, the expression of *rctB* is repressed by the regulator *RctR* and activated by the response regulator *rctC* in response to a yet unidentified signal (Nogales et al, 2013). Homologs of *rctR* and *rctC* are present in *S. fredii* NGR234 but are absent in *R. etli,* presenting the existence of different signaling cascades and regulation mechanisms of plasmid transfer in rhizobia. The strict and complex regulations of DNA transfers opposite with the transfer frequencies detected in natural populations of legume symbionts. It indicated that either environmental factors in the plant rhizosphere encourage the transfers of symbiosis genes or infrequent transfers are swiftly fixed in a population when it offers a strong adaptive benefit, such as the capacity to infect and proliferate within legume plants. Root exudates were also displayed to augment conjugal plasmid transfers amongst bacteria in the rhizosphere, showing that HGTs can be favoured in the rhizospheric environment (Mølbak, et al., 2007).

2.8.2 Post Horizontal Gene Transfer Regulations

HGT of symbiosis genes is not straightway sufficient all the time to change the recipient bacterium into an efficient legume symbiont and multiple genomic alterations may be necessary to gain succeed symbiosis. In agreement with this, HGT observed in nature often results in the evolution of novel rhizobia that are suboptimal for N_2 fixation. In example, the introduction of *Biserrula pelecinus* legume in Western part of Australia with its compatible symbiont *M. ciceri* bv. *biserrulae* led to the rapid evolution of novel *Mesorhizobium* strains that have acquired the symbiosis genomic island from *M. ciceri* inoculant but ineffective to fix N_2. This suggests that the symbiotic potential of transconjugant must be optimized first in these strains in order to gain efficient rhizobia (Nandasena et al., 2007).

In addition, the experimental transfers of *sym* plasmid to distant non rhizobial bacteria in the laboratory were usually unsuccessful leading either to non-nodulating strains or to strains able to induce ineffective pseudonodules (Hirsch et al., 1984; Marchetti et al., 2010). The optimization begin as integration of symbiosis genes into the endogenous regulatory circuit in example recruiting the diversity of regulatory systems controlling nitrogen fixation genes in rhizobia (Dixon and Kahn, 2004), genome remodelling allelic variations, gene inactivations, gene recruitments, gene duplications, regulatory rewirings (Masson-Boivin and Sachs, 2018). In point of

fact, the integration of laterally acquired genes into the existing regulatory network was shown to be a laborious process with never ending years (Lercher and Pal, 2008). In HGT of *sym* plasmid, rhizobia largely recruit resident genes for symbiosis. The absent of *nod* and *nif* genes can be employed from housekeeping genes either directly (*glmS* substitute *nodM, iscA/iscS* substitute *nifU/nifS)* or through the gene duplication and integration into the symbiotic region (*glmS/nodM* or *cysDNC/nodPQ*) (Galibert et al., 2001). Aforementioned section, protein secretion systems were regularly employed in rhizobia to contribute NFs to activate the CSSP and/or reduce plant defense responses. The merging of secretion systems in the nodulation regulatory circuit controlled their expression with *nod* genes confirms their vital role in the symbiotic adaptation (Chun and Sadowsky, 2017).

Moreover, the functions necessary for N_2 fixation, like heme biosynthesis or central metabolism functions, are encoded by native genes from background chromosome that are used to fulfill symbiosis. The phylogenetic analysis of approximately 500 genes identified to be involved in symbiosis collected from 41 rhizobial strains discovered expansive recruitment of lineage-specific genes (Tian et al., 2012). Prominent examples of exploitation of native genes from background chromosome of recipient for symbiosis are the *exo, exs, lps,* or *pss* genes, essential for infection and whose phylogeny is consistent with the phylogeny of species (Black et al., 2012; Wang et al., 2018). Few rhizobia have also exploited their intrinsic metabolic properties, such as methylotrophy in *Methylobacterium nodulans* or photosynthesis in *Bradyrhizobium* sp. ORS278, to gain better symbiosis (Giraud et al., 2000; Jourand et al., 2005). Ultimate post-transfer adaptive evolution happened over various processes such as allelic variation, gene inactivation or deletion, or additional horizontal gene acquisition. The degree of genomic modifications requisited for adaptation into legume symbiosis is unrevealed yet and perhaps depends on the recipient genome and the host genotype. The HGT between close and pre-adapted genomes possibly instantly get efficient symbiosis whereas transfers amongst distant genera would demand extra adaptation steps to enable symbiosis. Longstanding co-evolution of rhizobia-legume hosts should also intensify the number of genome adaptation to improve the mutualistic interactions. The success story of HGT between rhizobia-legume roots depicted in Fig. 2.13 (Masson-Boivin, and Sachs, 2018).

The transfer of symbiotic plasmid (*psym*) conveying the crucial symbiotic genes *(nod/nif/fix)* via HGT into the soil from rhizobia to native bacteria in soil including non-rhizobium strains leads to a proto-rhizobium which requires further genome remodeling under the plant selection pressure to turn into an effective N_2 fixing rhizobium (Masson-Boivin and Sachs, 2018).

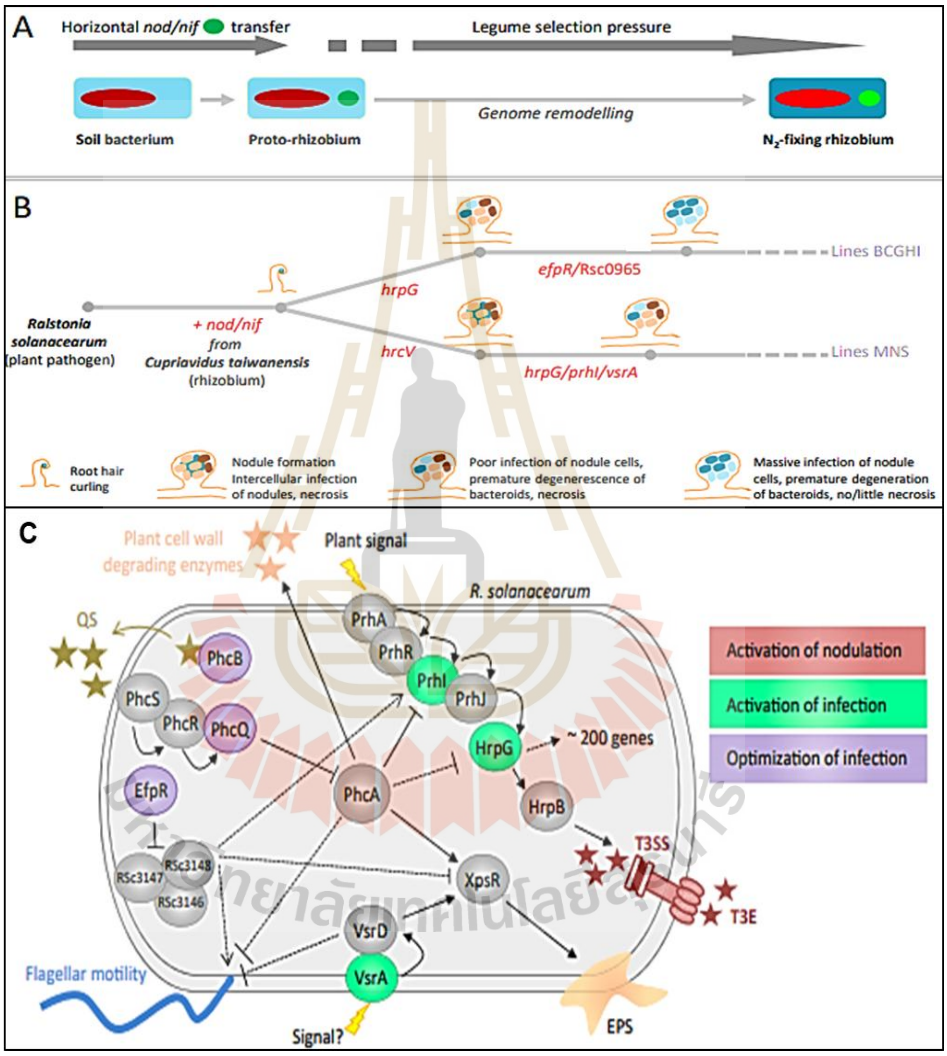

Figure 2.13 Experimental evolution of legume symbionts thru the horizontal gene transfer (HGT). The evolutionary model for the emergence of NF-dependent rhizobia via HGT (A) (Masson-Boivin & Sachs, 2018). The Experimental evolution of the plant pathogen *R. solanacearum* into legume symbionts (B) (Masson-Boivin and Sachs, 2018). Genetic rewiring of the *R. solanacearum* virulence regulatory network leading to symbiotic adaptation (Tang and Capela, 2020; Tang et al., 2020).

As soon as the *nod/nif* genes from *Cupriavidus taiwanensis,* the symbiont of *Mimosa pudica,* were hosted into the plant pathogen *Ralstonia solanacearum*. The subsequent chimeric strain was able to produce Nod Factors and induce root hair curling but unable to form nodules on *M. pudica* (Masson-Boivin and Sachs, 2018). The capacity to achieve the two first steps of symbiosis, nodulation and intracellular infection, was continuing attained in parallel lineages evolved by repeated inoculation to *M. pudica*). Mutations allowing the main symbiotic changes were identified (Capela et al., 2017; Guan et al., 2013; Marchetti et al., 2010). Nodulation was attained via inactivation by stop mutation of the T3SS in *hrcV/hrp*G genes. The first level of intracellular infection was achieved via a *hrp*G stop mutation or a combination of a *hrc*V mutation and other virulence regulators such as *hrp*G, *prh*I *or vsr*A. Further mutations affecting global virulence regulatory pathways such as *efp*R or the *phc* QS pathway necessary for optimization of intracellular infection (Masson-Boivin and Sachs, 2018).

The virulence chief regulatory *hrp*G not only regulates the T3SS via *hrp*B but also approximately 200 other genes some of them interfere with intracellular infection of *M. pudica* nodules. The *phc* QS system regulates the activity of *phc*A, which in turn controlled the expression of hundreds genes including *hrp*G regulatory cascade component, EPS forming, cell wall degrading enzymes, and motility. Another two virulence global regulators are *vsr*A and *efp*R present much the same functions as *phc*A. Genes mutated in the evolution experiment are colored according to their associated symbiotic phenotypes relate with QS, EPS, T3SS, and T3E. Dashed and straight lines indicate indirect and direct controls, respectively (Figure 2.13) (Tang and Capela, 2020; Tang et al., 2020).

2.9 References

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CHAPTER III

SYMBIOTIC CONTRIBUTION OF NON-NODULATING *BRADYRHIZOBIUM COSMICUM* S23321 AFTER TRANSFERRING OF THE SYMBIOTIC PLASMID PDOA9

3.1 Abstract

The megaplasmid DOA9 (pDOA9) carrying *nod, nif, fix* and T3SS genes was first found in *Bradyrhizobium* sp. DOA9. This study aimed to transfer the pDOA9 from DOA9 into different background chromosomes than DOA9 and *Bradyrhizobium* sp. ORS278. The chimeric ORS278 carrying pDOA9 was used as a donor for transferring pDOA9 to the *B. cosmicum* S23321 which is free-living with the absence of *nod* and T3SS/T4SS genes. The result of plant nodulation was varied among DOA9, chimeric ORS278 and S23321 strains even though it carried the same symbiotic plasmid pDOA9. The pDOA9 brings incompatible factors on legume symbiosis in chimeric ORS278, but the result in chimeric S23321 is able to contribute the symbiosis compatibility on legumes. This study indicated that S23321 was able to gain the symbiotic function of pDOA9 in two manners. Firstly, the *nod* genes broaden the host range in all legumes tested and successfully formed the active nodule with *Arachis hypogaea* (Dalbergoid) and *Vigna radiata* cv. SUT4 (Millitoid), while the DOA9 wild type only performs necrotic nodule in *V. radiata* cv. SUT4. Secondly, the type three secretion system (T3SS) affects the degree of effectivity in symbiosis. The chimeric mutant T3SS (S23321:pDOA9Ω*rhcN*) congruence with DOA9, mutation in T3SS gave better nodulation in *Crotalaria juncea*. Besides, the mutation of nodulation gene (S23321:pDOA9Ω*nodB*) completely abolished the nodulation in all tested legumes. These outcomes implied that pDOA9 has been decorated for broadening host specificity along with legumes evolution time and the compatibility of nodulation depending on the background of the chromosome of the recipient as well as the legumes host restriction.

3.2 Introduction

Plasmids are extrachromosomal, self-replicative, and important DNA vehicles that can be transmitted vertically or horizontally. Genetic information carried on plasmid corresponds usually to accessory genes that have certain function such as antibiotic resistance, detoxification, virulence, catabolism of uncommon metabolites, or capacity to invade certain tissues (James et al., 2016). The notion of the symbiotic nodulation *(nod)* and nitrogen fixation *(nif/fix*) genes that are required for rhizobia to permit them to develop a symbiosis with legumes and this symbiotic toolkit are generally found on a symbiotic plasmid or on genomic island that can transfer between strain by Horizontal Gene Transfer (HGT) (Bedhomme et al., 2017). Bradyrhizobia generally harbor their symbiotic genes on genomic island, but it exists few exceptions such as the *Bradyrhizobium* sp. DOA9 harbor a large symbiotic plasmid containing *nod, nif* and T3SS genes. Insterestingly, this strain also contains *nif* genes on chromosome (Okazaki et al., 2015). The transconjugation of pDOA9 into photosynthetic *Bradyrhizobium* sp. ORS278 was successfully constructed in previous study. Interestingly, symbiotic pDOA9 in chimeric ORS278:pDOA9 showed confound the symbiotic relation in the original host (*Aeschynomene indica* and *A. evinia*), but do not provide the symbiotic relationship with nod factor (NF)-dependent host as found in DOA9 (Songwattana et al., 2019). This finding suggested that, (i) symbiotic tools required for classical nodulation process could render incompatible the symbiotic interaction with NF-independent manner. (ii) The pDOA9 acquirement of ORS278 could not provide the symbiotic properties to ORS278 due to some incompatible factors regarding to the phylogenetically distant.

The beginning of this study was fulfill the curiosty from the result of Songwattana *et. al.,* (2019). Why the pDOA9 showed the strong negative effect on the original host of *Bradyrhizobium* sp. ORS278 which was *A. indica* and *A. evenia* after transferring the pDOA9. Symbiotic tools in pDOA9 in *Bradyrhizobium* sp. ORS278 are sufficient to form the pseudo/white nodules in *C. juncea* and *M. artropurpureum*, but no any nodulation in other legumes which were originally nodulated by DOA9. Since the background of ORS278 was Photosynthetic *Bradyrhizobium* (PB)-strain and NF-independent symbiotic strain.

To scrutinize the evolutionary relationships, S23321 was the sister taxa of *B. cosmicum* 58S1 which is the *Glycine max* cv. AC Orford symbiont. From the internal node of *B. cosmicum* S23321 and 58S1 found in the same branch with *B. lupini* USDA-3051 and *B. canariense* SEMIA-928 which is *Lupinus agustifolius* and *Lupinus* sp. symbiont, meaning that they shared the most common ancestor. However, the branch length showed that the *Lupinus* symbiont is older than S23321 (Wasai-Hara et al., 2020). The close phylogenetic background of chromosome S23321 with many symbiotic bradyrhizobia is possible to make this strain become symbiotic strain after the transfer of symbiotic plasmid.

To completely demonstrate the function of symbiotic tools on pDOA9, this study determined transferring of pDOA9 into *B. cosmicum* S23321 which is phylogenetic close to *B. diazoefficiens* USDA110 but absence the symbiotic island on its genome. The nodulation properties of new chimeric S23321:pDOA9 was investigated with all legumes which are originally nodulated by DOA9. In this study, we also examine the symbiotic properties depending on the factors from *nod* and T3SS on the pDOA9 plasmid. Thus, T3SS lacking pDOA9 (pDOA9Ω*rhcN*) was also contributed into S23321 (S23321:pDOA9Ω*rhcN*) and observed the nodulation on legume tests.

3.3 Research objective

3.3.1 Main objective

The main objective of this study was to determine the function of genes involved in symbiotic megaplasmid pDOA9 of *Bradyrhizobium* sp. DOA9 on the symbiotic properties of non-symbiotic *B. cosmicum* S23321 including broad host range features.

3.3.2 Specific objectives

1. To transfer the pDOA9 from *Bradyrhizobium* sp. DOA9 into *B. cosmicum* S23321 using bacterial conjugation system

2. To determine the nodulation capacity of transconjugant S23321:pDOA9 in various legumes which belongs to 3 tribes includes Genistoid, Dalbergoid and Milletoid

3. To determine the function of genes involved in symbiotic on pDOA9 including *nod*, *nif*, and T3SS which is involve in the symbiotic of *B. cosmicum* S23321

4. To determine the expression of genes involved in symbiotic on chromosome S23321 including glycosyltransferase-39 (Gly), O-antigen, mutltidrug transporter A (mdtA) and mutltidrug transporter B (mdtB)

3.4 Materials and Methods

3.4.1 Bacterial strains and culture media

Bradyrhizobium strains used in this study (Table 3.1) were cultured in Arabinose-Gluconate (AG) medium (Sadowsky et al., 1987) at 28°C on a rotary shaker at 180 rpm for 5 days. *Escherichia coli* strain was cultured in Luria Bertani (LB) (Bertani, 1951) at 37°C on a rotary shaker at 180 rpm for 18 h. The culture media for derivative mutants or recombinant plasmid carrying strains were supplemented with an appropriate antibiotics at the following concentrations: 20 µg/ml cefotaxime (cefo), 50 µg/ml nalidixic acid (nal) and 200 µg/ml spectinomycin (spec).

3.4.2 Construction of chimeric strains

The chimeric *B. cosmicum* S23321 carrying pDOA9 (S23321:pDOA9) and its derivative chimeric *nodB* mutant (S23321:pDOA9Ω*nodB*) and *rhcN* mutant (S23321:pDOA9Ω*rhcN*) strains were obtained by triparental mating of *Bradyrhizobium* sp. ORS278:pDOA9, ORS278:pDOA9Ω*nodB* or ORS278:pDOA9Ω*rhcN* (donor strain), *B. cosmicum* S23321 (recipient strain) and *E. coli* PRK2013 (helper strain) (Figurski and Helinski, 1979; Okazaki et al., 2004) (Fig.3.1). Briefly, 5 days culture of chimeric *Bradyrhizobium* sp. ORS278 or their drivatives, *B. cosmicum* S23321, and also 18 h culture of helper *E. coli* PRK2013 were washed with AG broth for *Bradyrhizobium* and LB for *E. coli* PRK2013 without addition of antibiotics. The mixed culture with ratio of recipient:donor:helper (5:3:1) was dropped onto the AG medium containing 10 mM $MgCl_2$ to facilitate a better mating process and incubated at 30°C for 3 days. The colony grown on the plate was resuspend with AG and spreaded on the AG supplemented with mixture of three antibiotics (spec, nal, and cefo). The incubation was placed under the light for enhance the accumulation of canthaxanthin production (Lorquin et al., 1993). This step to distinguish the orange colony

morphology of ORS278 with chimeric S23321 (Fig.S1). The colourless colony of chimeric S23321 was repeatedly re-streaked on the AG plate containing spec, nal, and cefo. The colony morphology of the transconjugant was also observed and compared with the S23321 and DOA9 WT.

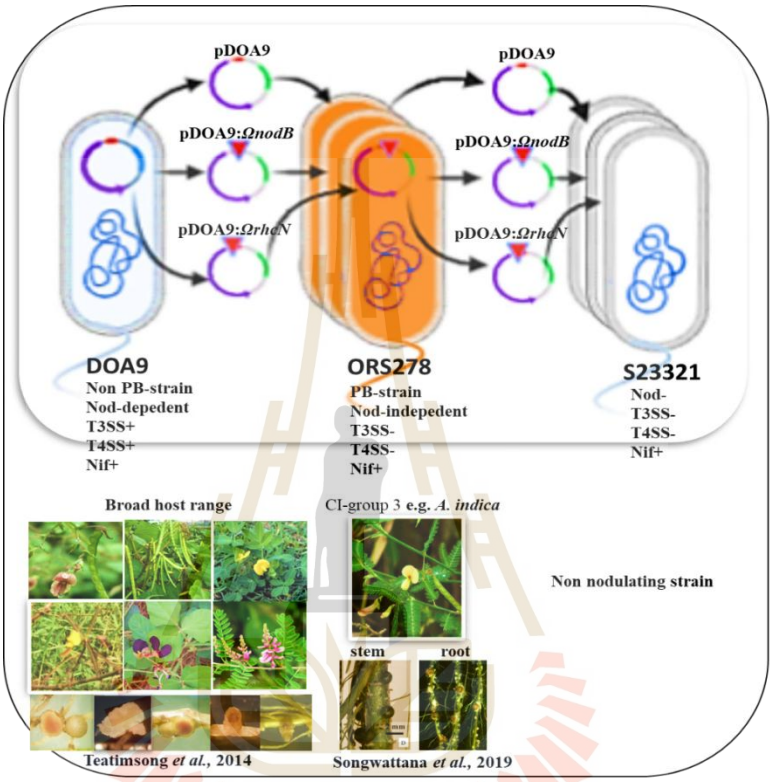


Figure 3.1 The schematic transfer of symbiotic megaplasmid (pDOA9) from *Bradyrhizobium* sp. DOA9 to the free living strain *B. cosmicum* S23321. The board host range properties of *Bradyrhizobium* sp. DOA9 found in Teatimsong et al., 2014. The previous transfer of symbiotic megaplasmid (pDOA9) to the photosynthetic *Bradyrhizobium* sp. ORS278 found in Songwattana et. al., 2019.

The transconjugants were confirmed by PCR amplification using 2 pairs of specific primer for gene located on the S23321 chromosome (cS23321) (*nif*A and *bchL*) genes, 2 pairs of specific primer for gene located on the ORS278 chromosome (cORS278) (hypothetical protein and *lysE* transporter) genes. The pDOA9 was confirmed using the specific primers for genes located on the pDOA9, including

moeB, *repA*, *trbG*, *nodA*, *nodB*, *nifD*, *hupK*, *nodD2*, *rhcN*, and GAJ3851 (Table 3.2). The cycling conditions for PCR program were 5 min at 95°C for activation, followed by 35 cycles of 95°C for 30 s for denaturation, 55°C for 30 s for annealing, 72°C for 30 s for elongation and a final cycle 72°C for 10 min for the final elongation. The PCR amplification was illuminated by 1% agarose gel with 1×TAE buffer, and then subjected to electrophoresis at 100 V for 30 min.

Table 3.1 The bacterial strains used in this study.

Strain	Characterictics	Reference
Bradyrhizobium sp. DOA9	Non-photosynthetic strain, isolated from paddy field using *A. americana* as trap legume,	Noisangiam et al., 2012
Bradyrhizobium sp. DOA9Ω*nodB*	Nod-dependent strain	Songwattana et al., 2017
Bradyrhizobium sp. DOA9Ω*rhcN*	*nodB* mutant of DOA9 strain obtained by integration of pVO155-npt2-cefo^r- npt2-gfp; cefo^r km^r	Songwattana et al., 2017
Bradyrhizobium sp. ORS278:pDOA9	*rhcN* mutant of DOA9 strain obtained by integration of pVO155-npt2-cefo^r- npt2-gfp; cefo^r km^r	Songwattana et al., 2019
Bradyrhizobium sp. ORS278:pDOA9 Ω*nodB*	*Bradyrhizobium* sp. ORS278 (sp^r) containing pDOA9 plasmid (pDOA9-pK18mob-sacB-cefo^r), sp^r km^r cefo^r	Songwattana et al., 2019
Bradyrhizobium sp.ORS278:pDOA9 Ω*rhcN*	*Bradyrhizobium* sp. ORS278 (sp^r) containing pDOA9Ω*nodB* plasmid, sp^r km^r cefo^r	Songwattana et al., 2019
Bradyrhizobium cosmicum sp. S23321	*Bradyrhizobium* sp. ORS278 (sp^r) containing pDOA9Ω*rhcN* plasmid, sp^r km^r cefo^r	Okubo et. al., 2012
Bradyrhizobium sp. S2:pDOA9	Non-photosynthetic strain, isolated from paddy soil Non-nodulating strain	This study
Bradyrhizobium sp. S2:pDOA9Ω*nodB*	*Bradyrhizobium cosmicum* S23321 (sp^r) containing pDOA9 plasmid (pDOA9-pK18mob-sacB-cefo^r), sp^r km^r cefo^r	This study
Bradyrhizobium sp. S2:pDOA9Ω*rhcN*	*Bradyrhizobium cosmicum* S23321 (sp^r) containing pDOA9Ω*nodB* plasmid, sp^r km^r cefo^r	This study
Eschericia coli PRK2013	Helper plasmid; mob+, tra+, km^r	Figurski and Helinski, 1979

Table 3.2 The primers used in this study.

Strain	Sequence (5'-3')	References
Primer		
Primers for confirmation the chromosome S23321		
bchL		
bchL-S23321.f	5'-CAAGCATGACTCGACCTTCA-3'	This study
bchL-S23321.r	5'-GCGAAGATCGAGTCGAAATCA-3'	
nifA		
nifA-S23321.f	5'-CATTGAGCGCACCGGTAAAC-3'	This study
nifA-S23321.r	5'-CATTGCGAGGATTGCTGGAGA-3'	
Primers for confirmation pDOA9		
moeB		
moeB-252.f	5'- GAGCCGTCCTTCAGCAATTA-3'	Songwattana et al.,
moeB-253.r	5'-GCGACTTCTCAGACCATCATAC-3'	2019
repA		
repA-250.f	5'-AAGAGAACGGCACCTTGTATG-3'	Songwattana et al.,
repA-251.r	5'-TCCGAGGTCATCAGGAGAAA-3'	2019
trbG		
trbG-254.f	5'-CGTGTGTCACTTGAGACCAA-3'	Songwattana et al.,
trbG-255.r	5'-GAAGGCGCGATAATTGAGGA-3'	2019
nodA2		
nodA2-196.f	5'-GGCGTTCAATGCAAGACC-3'	Songwattana et al.,
nodA2-197.r	5'-GCGTACAATCCGAGTTCAG-3'	2019
nodB		
nodB-202.f	5'-GGTATGCGACAGAACATCCAC-3'	Songwattana et al.,
nodB-203.r	5'-CGCACGTATATGTCTCAGCC-3'	2019
nifDp		
nifDp-4218.f	5'-GAGCCGAACGACCAACCA-3'	Songwattana et al.,
nifDp-4219.r	5'-GCGTCGCCGCCGATATTGTAG-3'	2019
hupK		
hupK-256.f	5'- CGCAACGAGATCGACATCA-3'	Songwattana et al.,
hupK-257.r	5'-GGTAGTCGCCTGCATCATT-3'	2019
nodD2		
nodD2-130.f	5'-GTCTTGCGAGCCCTTTACAC-3'	Songwattana et al.,
		2019

Table 3.2 The primers used in this study (Continue).

Strain	Sequence (5'-3')	References
nodD2-131.r	5'-CGCAAGCTGACATTTGAGAA-3'	Songwattana et al., 2019
rhcN		
rhcN-4083.f	5'-GCAACAATCAGGCCATAATCAAG-3'	Songwattana et al., 2019
rhcN-4084.r	5'-AGGGCATCTCCAATATGACGTTC-3'	
GAJ3851		
GAJ3851.f	5'-GCTCATGGGTGGGTTGAATA-3'	Songwattana et al., 2019
GAJ3851.r	5'-TCCTGATTGGAACGGTTAAGG-3'	
Primers for confirmation the chromosome ORS278		
Hypothetical protein		
Hp.f	5'-GATTGCCCATGGAATGGCAG-3'	This study
Hp.r	5'-CATAGCCTCTCTCTGGCAG-3'	
LysE		
LysE.f	5'-CAAAGGGAAACCCGATTGG-3'	This study
LysE.r	5'-CAATAGCGCGCTGAAGAAC-3'	
Primers for qRT-PCR		
16S		
16S.f	5'-ACTCCTACGGGAGGCAGCAG-3'	This study
16S.r	5'- ATTACCGCGGCTGCTGG-3'	
O-antigen		
O-antigen.f	5'-GTCAGCCCTTCAGGAAATTCGG-3'	This study
O-antigen.r	5'-CATGATTTCCGCGGGCTTC-3'	
Glycosyltransferase		
Gly.f	5'-GTTCCTGTTCGTCGTGTTGATC-3'	This study
Gly.r	5'-CATAACCATGGGTGTTGCGATAG-3'	
MdtA		
MdtA.f	5'-CATTCAGACCTTCGGAGACCAC-3'	This study
MdtA.r	5'-GTCTGTTCGCCTATGTGATCGG-3'	
MdtB		
MdtB.f	5'-GATGCCGATCAGCAAGACGAG-3'	This study
MdtB.r	5'-CTATGAGAGCTACATTCATCCGC-3'	

3.4.3 Plasmid stabilitiy test

The plasmid stability test assessment was followed by Songwattana et al., 2019. For this experiment, the chimeric S23321:pDOA9 and its derivative (S23321:pDOA9Ω*nodB,* S23321:pDOA9Ω*rhcN*) were cultured for 1 week in an AG broth in the absence of antibiotic selection and then spread on AG agar. After growth, 100% of the colonies were able to grow when the mixture of antibiotics spectinomycin 200 µg/ml, nalidixic acid 50 µg/ml, and cefotaxime 20 µg/ml were added and amplified a specific gene marker of pDOA9 of the plasmid was obtained.

3.4.4 Determination of nitrogen fixation in chimeric compared with wild-type DOA9 under free-living stage

The efficiency of nitrogenase activity under free-living stage was measured in DOA9, S23321, and the chimeric S23321:pDOA9. They were grown under microaerobic condition in 10 ml test tubes (BD Vacutainer, Franklin Lakes, NJ, U.S.A.) containing 2 ml of buffered nodulation medium-broth (BNM-B) medium with 10 mM succinate as carbon source, supplemented with cocktail of vitamins and also with the presence and the absence of a NH_4NO_3 as nitrogen source to determine nitrogenase enzyme activity under free-living conditions (Alazard, 1990). The cultures were grown and harvested by centrifugation at 4000 rpm for 15 minutes. The cells were washed three times with sterilized Buffered Nodulation Medium (BNM) broth then measured the density using a spectrophotometer with the wavelength 600 nm and adjusted until reach the final total bacterial cell culture equal to 15 (Sun et al., 2001). Then, 2 ml of mixed culture with 1% bacterial cells in BNM medium were transferred into 10 ml tube with rubber cap for three replications. The nitrogenase activity were measured by removing 1 ml air inside and injected subsequently 1.0 ml acethylene into the tube. Acetylene was injected to reach a final concentration of 10% v/v. The cultures were incubated for 7 days at 28°C without shaking and manually mixed every 2 days. Gas chromatography was conducted to measure the peak height of ethylene (C_2H_4) and acetylene (C_2H_2) with 1 ml gas samples from the bottles using PE-alumina packed column with 150°C of injector, 200°C of oven, and 50°C of Flame Ionization Detector (FID) (Renier et al., 2011). Nitrogenase activity units were calculated according to Wongdee et al,. (2016).

3.4.5 Plant cultivation and symbiosis analysis

The symbiosis efficiency and the nodulation ability of transconjugants were tested using the legumes (Table 3.3), including the Genistioid tribe: *Crotalaria juncea,* Dalbergioid tribe: *Aeschynomene americana, A. afraspera, Arachis hypogaea*, and *Stylosanthes hamata,* Milletioid tribe: *Vigna radiata* cv. SUT4, *Marcoptilium artopurpureum*, and *Indigofera tinctoria*. Seeds of *C. juncea, A. hypogaea and V. radiata* cv. SUT4 were surface sterilized in 95% ethanol for 10 sec before added 3% sodium hypochlorite to immerse the seed completely. While the seeds of *A. americana, S. hamata* and *M. artopurpureum* were surface sterilized in 95% ethanol for 10 sec before added 98% H_2SO_4 for the appropriate time (Teatimsong et al., 2014) until the pericarp of seeds were gone. After draining for 5 min, seeds were rinsed ten times with sterilized water. The sterilized seeds were placed on the plate containing sterilized 0.8% (w/v) water agar and kept in the dark place for 1-2 days. Germinated seeds were grown in Leonard's jar filling with sterilizing vermiculite (Somasegaran and Hoben, 1994) for the *A. hypogaea, V. radiata* and *C. juncea*. For the *A. americana, A. afraspera, A. hypogaea*, *S. hamata, M. artopurpureum*, and *I. tinctoria* were grown in falcon tubes containing BNM medium (Ehrhardt et al., 1992) and grown under the controlled environmental condition of 28±2°C on a 16-h-light/8-h-dark cycle at light intensities 300 μE/m^2S and 50 % humidity. Five days after planting, each seedling was inoculated with 1 ml of a cell suspension at approximate density of 1×10^8 cells/ml. Nodulation and nitrogen fixation abilities were measured after 21 days post-inoculation (dpi). Five plants were analyzed for the number of nodules and nitrogenase activity using acetylene reduction assay (ARA) (Bonaldi et al., 2010). Briefly, all nodules were collected from each plant and placed in headspace bottles with 10 % (v/v) of acetylene and incubated at 28°C for 1 h. Gas chromatography was conducted to measure the peak height of ethylene and acetylene with 1 ml gas samples from the bottles using the same condition as mentioned above. The experiments were carried out in five replicate.

Table 3.3 The list of legumes plant for symbiosis test, School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand.

Tribes	Host plant
Genistioid	*Crotalaria juncea*
Dalbergioid	*Stylosanthes hamata*
	Arachis hypogaea
	Aeschynomene americana
	Aeschynomene afraspera
Milletioid	*Vigna radiata* cv SUT4
	Marcoptilium artopurpureum
	Indigofera tinctoria

3.4.6 Cytological analysis

The cytological analysis in the nodules was performed as described by Songwattana et. al., 2019. The fresh nodule was sectioned with the thickness 40-50 µm using a VT1000S vibratome (Leica Nanterre, France). Then, the plant cell wall of the nodule was stained with 0.01% calcoflour (plant staining dye) for 20 min, then followed by incubation in mixing 30 µM propidium iodine (PI) and 5 µM Syto9 (1:1) in 50 mM Tris-HCl pH 7.0 buffer for 25 min. The Syto9 was colored green and used to identify the living cells while the PI was stained red to identify the dead cells (Haag et al., 2011). After staining, the sections were mounted and observed under the confocal laser scanning microscope (Olympus Fluoview FV1000, USA). The calcofluor was excited at 405 nm laser line and detected with a 460-500 nm emission filter. The Syto9 was excited with the 488 nm and detected at 490-522 nm. The PI was excited at 535 nm and detected at 617-636 nm. The overall images were remodeled with NIS elements software (Nikon), and images were adjusted for publication purposes.

3.4.7 Root exudates preparation and Bacterial induction

The sterilized legume seeds were germinated and transferred into tubes containing buffered nodulation (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 using a filter

paper with the diameter 0.45 µm once, followed by diameter 0.20 µm twice equipped with vacuum pump. The contamination in the root exudates was checked by spread 100 µl of the filtrate of root exudate on Yeast extract Mannitol (YM) medium (Somasegaran and Hoben, 1994). Root exudates were stored at -20˚C for future use. The mid-log phase culture bacteria of S23321WT and chimeric S23321:pDOA9 were washed and adjusted the OD 600 of approximately 0.4 with YM supplemented with 1/3 (v/v) of the root exudates or purified flavonoids (20 µM of naringenin in final concentration, Sigma-Aldrich, USA). The sterilized BMN medium was used as negative induction. The induced bacteria were cultured at 28℃ for 24 h. Bacterial cells were collected by centrifugation at 4,000×g for 10 min, at 4℃ and immediately frozen in liquid nitrogen and stored at -80℃ for further total RNA isolation.

3.4.8 Quantification of gene expression by qRT-PCR

Total bacterial RNA was extracted from induced cells using the RNeasy® Mini Kit (QIAGEN, USA) in accordance with the manufacturer's protocol. Total RNA was treated with RNase-free DNase I (NEB, USA) for 30 min at 37℃. Complementary DNA (cDNA) was synthesized from 500 ng total RNA using *iScript reverse transcription supermix* (Biorad) according to the manufacturers' protocols. Fifty ng of cDNA was subjected to real time PCR using specific primers of O-antigen, glycosyltransferase family 39 (Gly), multidrug transporter A (mdtA), and multidrug transporter B (mdtB) in chromosome S23321. All primer sets used in the expression analysis were listed in Table S1.

For qRT-PCR, briefly cDNA of each sample was mixed with Luna® Universal qPCR Master Mix (NEB) following the manufacturer's protocol and performed the cycling condition by QuantStudio™ 3 Real-Time PCR System (ThermoFisher, USA). The qPCR amplification was performed under cycling condition following; hold stage at 95℃ for 2 min; PCR stage: 40 cycles at 95℃ for 15 s, 60℃ for 30 s. Melt curve stage: 95℃ for 15 s, 60℃ for 1 min, and a final at 95℃ for 15 s. 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 three replicates PCR amplifications were performed for each sample.

3.4.9 Phylogenetic tree constructions

The multiple sequence alignment of the 34 retrieved sequences is performed with the ClustalW software. Using the multiple sequence alignment of the ClustalW, the phylogenetic trees are constructed by the Neighbor joining method in the Molecular Evolutionary Genetics Analysis (MEGA) software. It showing the relationships between the S23321 species and the recognized species of the genus *Bradyrhizobium.* Bootstrap values after maximum-likelihood analysis are indicated in the nodes and branches, respectively.

3.4.10 Statistical analysis

Data in all experiments were analyzed using SPSS software (SPSS versions 17.0 Windows; SPSS Inc., Chicago, IL) to confirm the statistically significant value in the F-test ($p \leq 0.05$) analysis of variance (ANOVA). The post hoc test was used Tukey's HSD test at $p \leq 0.05$) (Tukey, 1949).

3.5 Result

The beginning of this study was fulfill the curiosty from the result of Songwattana et. al., 2019. Why the pDOA9 showed the strong negative effect on the original host of *Bradyrhizobium* sp. ORS278 which was *A. indica* and *A. evenia* after transferring the pDOA9. Symbiotic tools in pDOA9 in *Bradyrhizobium* sp. ORS278 are sufficient to form the pseudo/white nodules in *C. juncea* and *M. artropurpureum*, but no any nodulation in other legumes which were originally nodulated by DOA9. Since the background of ORS278 was PB-strain and NF-independent symbiotic strain.

To scrutinize the evolutionary relationships S23321 was the sister taxa of *B. cosmicum* 58S1 which is the *Glycine max* cv. AC Orford symbiont (Wasai-Hara et al*.,* 2020). From the internal node of *B. cosmicum* S23321 and *B. cosmicum* 58S1 found in the same branch with *B. lupini* USDA-3051 and *B. canariense* SEMIA-928 which is *Lupinus* sp. and *L. agustifolius*. symbiont, meaning that they shared the most common ancestor. However, the branch length showed that the *Lupinus* symbiont is older than S23321 (Fig 3.2). The close phylogenetic background of chromosome S23321 with many symbiotic bradyrhizobia is possible to make this strain become symbiotic strain after the transfer of pDOA9.

3.5.1 Conjugative transfer of pDOA9 into non-nodulating Bradyrhizobium cosmicum S23321

The pDOA9 as well as a series of derivatives (pDOA9Ω*nodB* and pDOA9Ω*rhcN*) were introduced into the PB strain ORS278. The use of an appropriate cocktail of antibiotics combined with a color criteria of the colonies permit to isolated white S23321 transconjugant harboring the pDOA9 (Fig. 3.3A). Moreover, the screening of chimeric S23321 strain was conducted on the medium containing antibiotics (spectinomycin 200 µg/ml, cefotaxime 20 µg/ml, and nalidixic acid 50 µg/ml). The success of all chimeric strains was confirmed by PCR using couple of primers specific of the gene located on the chromosome of S23321, ORS278, and the pDOA9 (Fig. 3.4). The chimeric and their derivatives were able to detect the genes on cS23321 and pDOA9 but the genes located on cDOA9 and cORS278 were not detected. Moreover, based on colony morphology of all chimeric strains was similar to the S23321 (Fig. 3.3B). The stability of the plasmid showed 100% in chimeric and mutant chimeric strains S23321:pDOA9; S23321:pDOA9Ω*nodB* and S23321: pDOA9Ω*rhcN* by following the method from Songwattana et.al.,(2019).

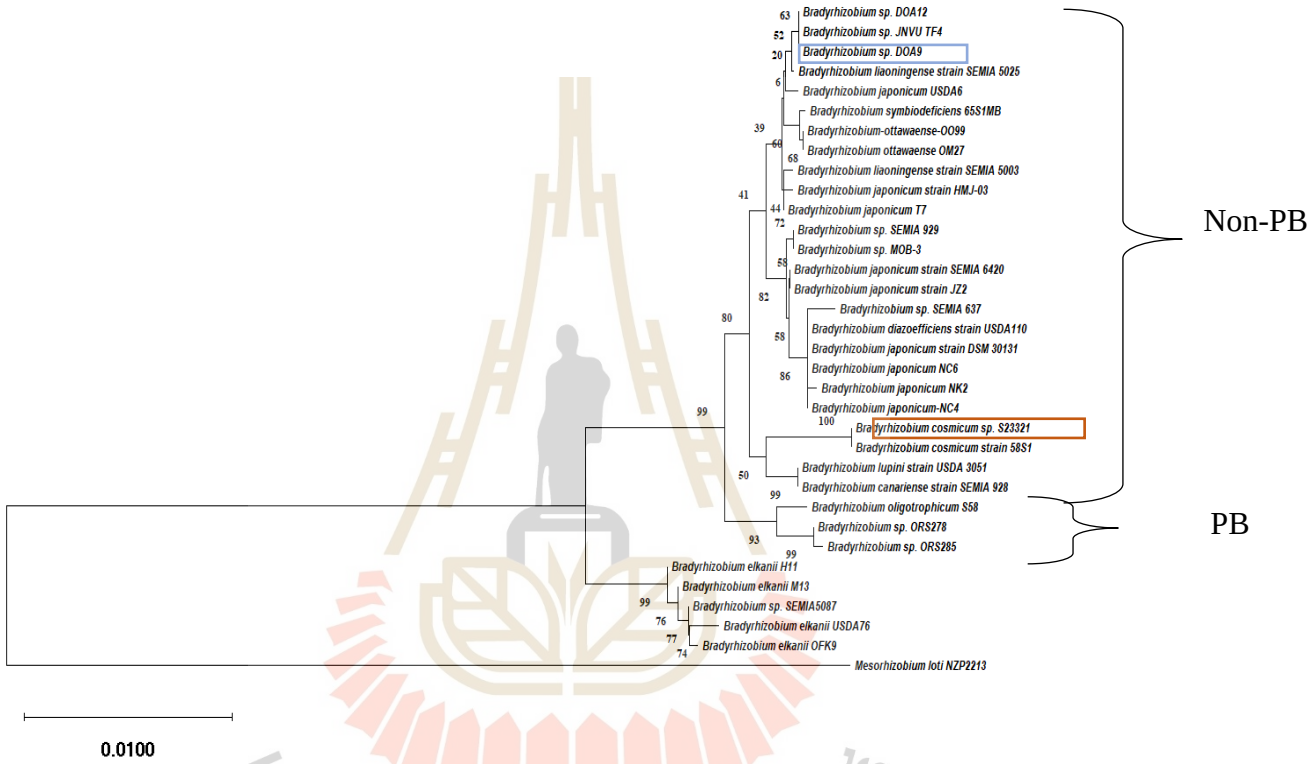


Figure 3.2 Neighbor-joining tree based on 16S rRNA gene sequences of *Bradyrhizobium cosmicum* S23321, showing relationships among *Bradyrhizobium* spp. Scale bar: 0.01 nucleotides substitution per site. Numbers on branches indicate confidence limits estimated from bootstrap analysis of 1,000 replicates.

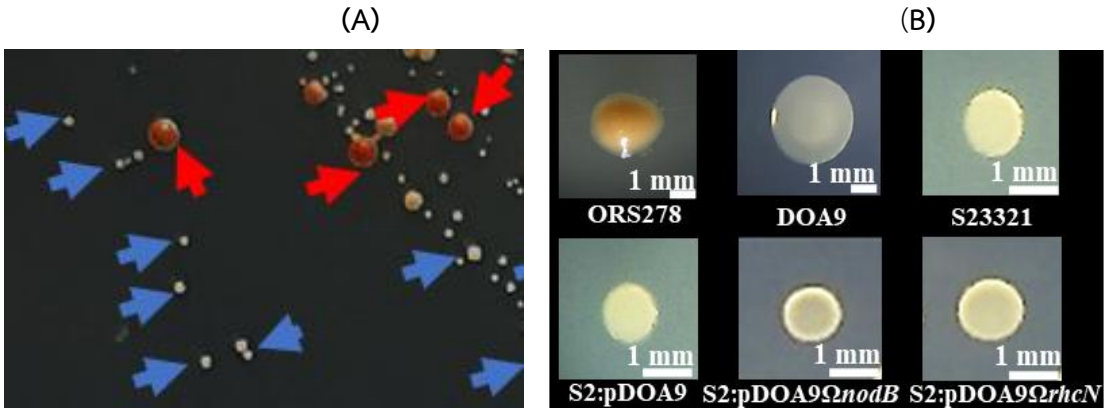


Figure 3.3 Screening process to choose the transconjugant based on colony morphology using AG medium with antibiotic (spectinomycin 200 µg/ml, cefotaxime 20 µg/ml, nalidixic acid 50 µg/ml). Red arrow =ORS278:pDOA9, Blue arrow = S2:pDOA9. Different colony color of chimeric strain (A) and colony morphology of *Bradyrhizobium* used in this study (B). The wild type grow in AG medium while the chimeric and mutant chimeric grow on AG medium with antibiotic (spectinomycin 200 µg/ml, cefotaxime 20 µg/ml, nalidixic acid 50 µg/ml). S2 = S23321.

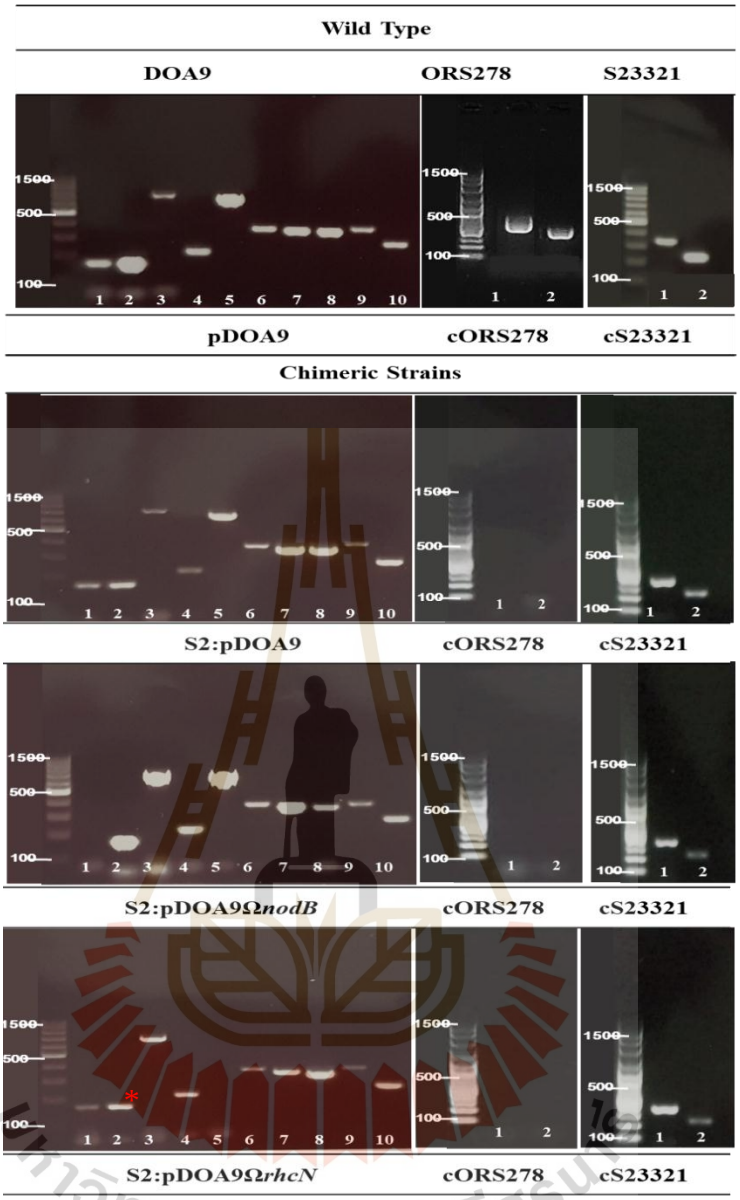


Figure 3.4 PCR verification of chimeric by PCR method using specific primers for plasmid DOA9 (pDOA9), chromosome ORS278 (cORS278), chromosome S23321 (cS23321). The plasmid of DOA9 in chimeric strains confirmed by various primers: 1 = *nodB*, 2 = *nodA2*, 3 = *nifDp*, 4 = *nodD2*, 5 = *rhcN*, 6 = *repA*, 7 = *moeB*, 8 = *trbG*, 9 = *HupK* and 10 = C-terminal C48 (SUMO) peptidase domain (GAJ38051). The chromosome of ORS278 in chimeric strains confirmed by 1=Hypothetical protein and 2 = *lysE* transporter specific in ORS278. The chromosome of S23321 in chimeric strains confirmed by 1 = *Bchl* and 2 = *nif*A specific in S23321. S2 = S23321. * = mutation.

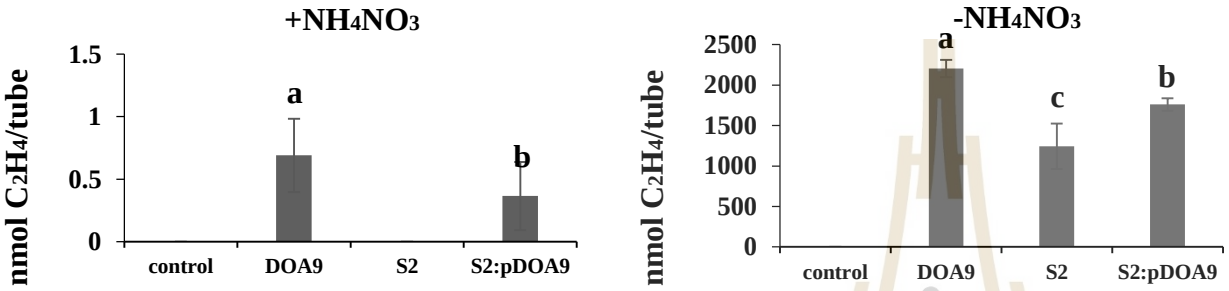
3.5.2 The transfer of pDOA9 increase the ability of chimeric strain to fix N_2 higher under free living stage

The free-living stage of S23321, DOA9 and S23321:pDOA9 in microaerobic condition and in absence of the N source affected the nitrogenase activity displaying higher in all strains when it compared with the presence of N source (NH_4NO_3) (Fig.3.5A). The presence of N source hindered the ability of nitrogenase activity in all treatments including S23321 which was not detected and if compared with DOA9 and S23321:pDOA9, it showed very low activity less than 1 nmol ethylene/culture. The result without N source indicated that DOA9 significantly showed the highest nitrogenase activity under free-living condition 2,205.2 nmol ethylene/culture compared with S23321WT which significantly lower 1,243.1 nmol ethylene/culture, S23321:pDOA9 displayed significantly higher 1,761.0 nmol ethylene/culture when compared with S23321 WT. In addition, the *nif* cluster of cS23321 was found to be highly conserved with the chromosome DOA9 than *B. diazoefficiens* USDA110. The *nif* genes analysis of S23321 showed that in the chromosome of S23321 possesses 2 copies of *nifD, nifS, nifZ* and *nifH* (Fig. 3.5B).

3.5.3 Restoration of S23321 properties by symbiotic tools acquirement

The plant test result showed that S23321:pDOA9 failed to nodulate the DOA9 original host *A. americana.* and *A. afraspera*. The S23321:pDOA9 was success to nodulate *A. hypogaea*, plant phenotype showed that only DOA9 WT performed the compatible symbiosis. However, the plant phenotype inoculated with S23321:pDOA9 also started healthy after 35 dpi. The plant dry weight showed not significantly different when compared DOA9 WT with S23321:pDOA9 (Fig. 3.6a). The nodule number derived from S23321:pDOA9 (190 nodules/plant) was higher when compared with the DOA9 WT (35 nodules/plant) (Fig. 3.6b). The late nodulation affected the nitrogen fixation ability that would not reach the optimum, only 110.7 nmol ethylene/h/plant lower than DOA9 WT 2,717.3 ethylene/h/plant (Fig. 3.6b). The nodule phenotype derived from S23321:pDOA9 showed the pink and active nodule (Fig. 3.6g). From the confocal observation, the bacteroid cells of S23321:pDOA9 were fully residing all symbiosome. Both DOA9 WT and S23321:pDOA9 displayed the green color of bacteroid indicated that the living bacteroid inside the nodule (Fig. 3.6i-l).

A


+NH4NO3
nmol C2H4/tube
1.5
1
0.5
0
a
b
control
DOA9
S2
S2:pDOA9
-NH4NO3
nmol C2H4/tube
2500
2000
1500
1000
500
0
a
c
b
control
DOA9
S2
S2:pDOA9

B

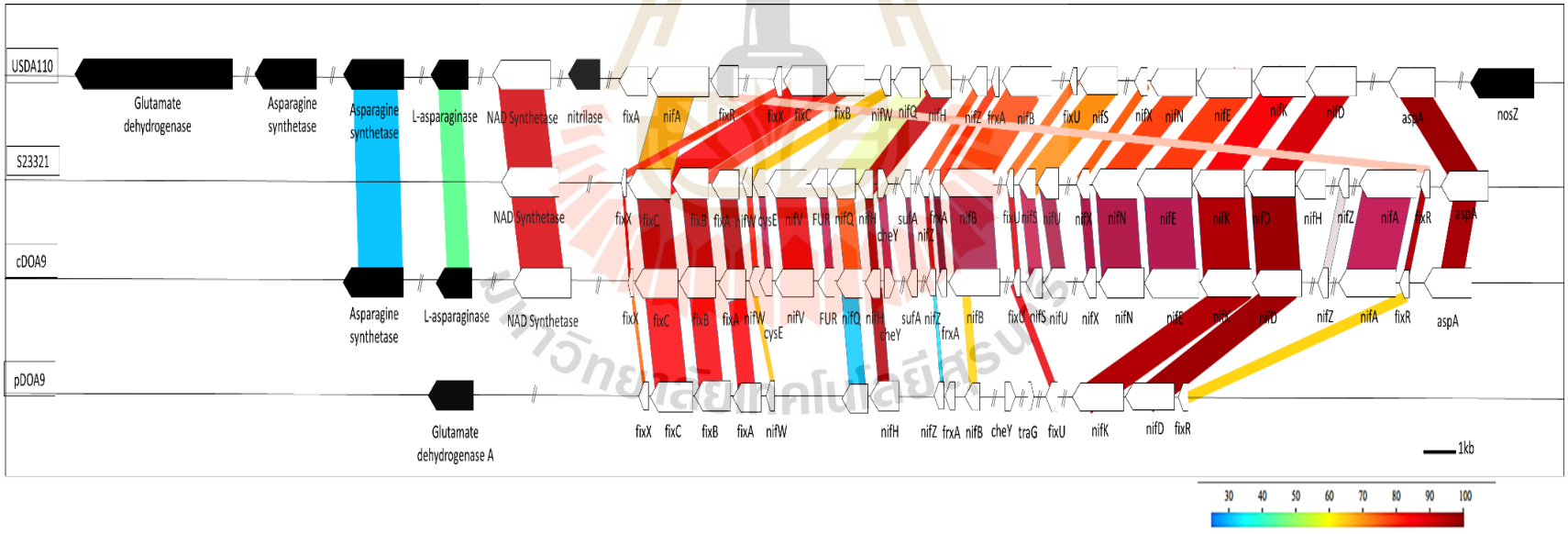

USDA110
S23321
cDOA9
pDOA9
Glutamate dehydrogenase
Asparagine synthetase
Asparagine synthetase
L-asparaginase
NAD Synthetase
nitrilase
fixA
nifA
fixR
fixX
fixC
fixB
nifW
nifQ
nifH
nifZ
frxA
nifB
fixU
nifS
nifX
nifN
nifE
nifK
nifD
aspA
nosZ
NAD Synthetase
Asparagine synthetase
L-asparaginase
NAD Synthetase
cysE
nifV
FUR
cheY
sufA
nifH
nifB
nifU
nifA
fixR
aspA
Glutamate dehydrogenase A
traG
1kb
30
40
50
60
70
80
90
100

Figure 3.5 A manifestation of nitrogenase activity determined by acetylene reduction assay (ARA) in *Bradyrhizobium* sp. DOA9 WT (DOA9), S23321 WT (S2), and chimeric strain S23321:pDOA9 (S2:pDOA9) cultivated under the free-living condition with and without the present of nitrogen source (NH_4NO_3) (A). The *nif/fix* gene organization of S23321 compared with plasmid (pDOA9) and chromosome of DOA9 (cDOA9) and also USDA 110 using GenomeMatcher (B). Black color bar indicated genes that S23321 was absent. Double slash marks represent DNA regions that were not shown. Colored strips represent the conserved gene regions between the compared strains, and the color indicates the percentage similarity. Error bars represent standard deviation (SD) (n=5). Different letters above the error bars indicate significant differences at $P<0.05$ (Tukey's HSD test).

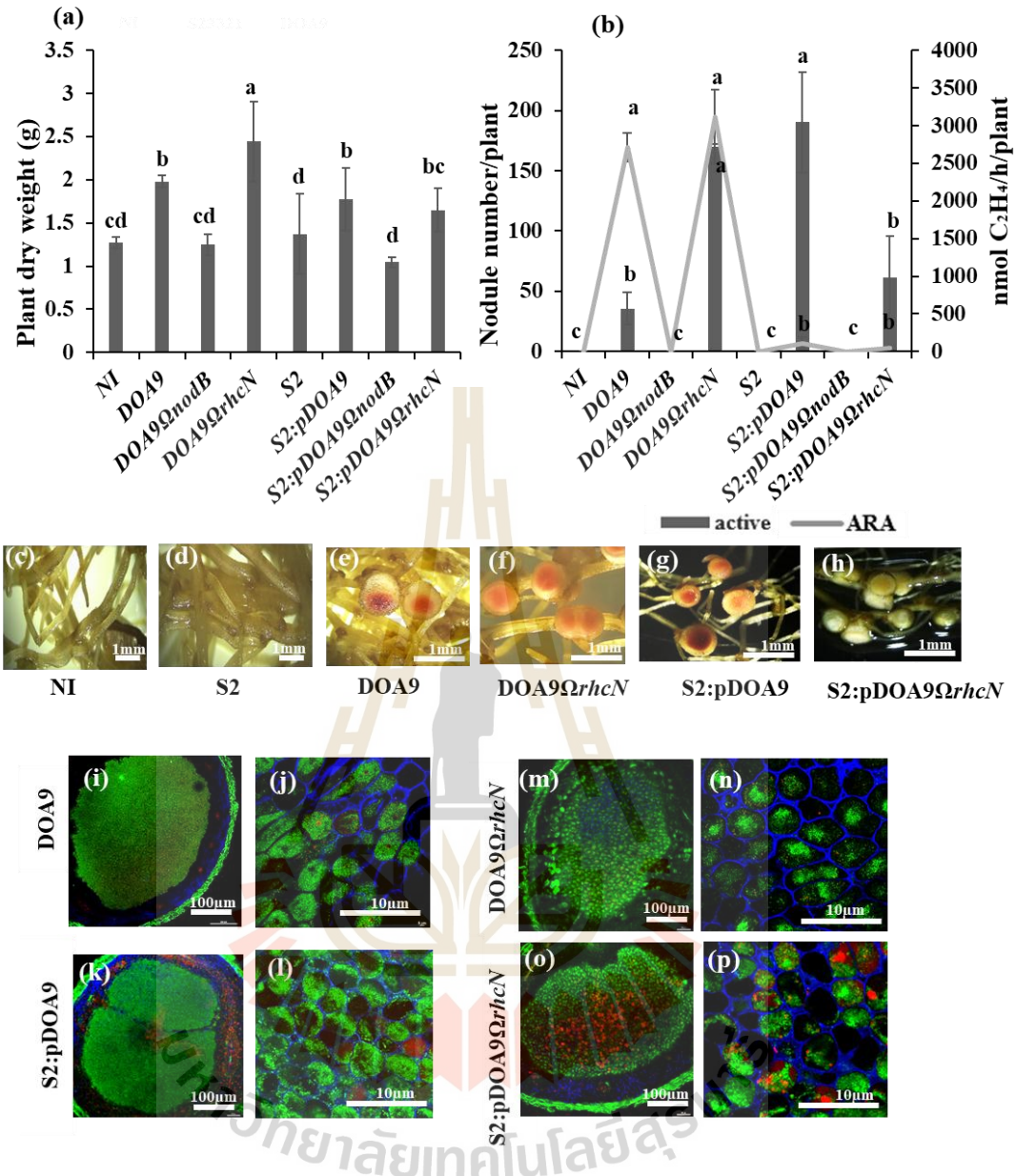


Figure 3.6 Nodulation in *A. hypogaea* (a-p). The plant dry weight (a). The nodule number/plant and nitrogenase activity (nmol C_2H_4/h/plant) (b). The comparison of the nodule morphology (c-h). The root nodules observed with a stereomicroscope and the bacteroid inside of the nodule were observed by confocal laser scanning microscope tools after stained with Calcofluor M2R, Syto9 and Propidium Iodine (PI) (i-n). Active nodule number indicated in bar and ARA indicated in line. Error bars represent standard deviation (SD) (n=5). Different letters above the error bars indicate significant differences at $P<0.05$ (Tukey's HSD test). S2 = S23321.

In *Vigna radiata* cv. SUT4 (Milletioid) displayed positives responses from the chimeric strains (Fig. 3.7). DOA9 WT induced the defective nodule in *V. radiata* with low nitrogenase activity (Fig. 3.7b, and e). Notwithstanding, the chimeric strain S23321:pDOA9 had significantly induced the active nodule with higher nitrogenase activity than DOA9 WT. According to the ARA in *V. radiata*, the nitrogenase activity of chimeric strain had significantly higher with 64.0 nmol ethylene/h/plant when compared with DOA9 WT 14.9 nmol ethylene/h/plant (Fig. 3.7b). The plant dry weight showed significantly different when compared DOA9 WT with S23321:pDOA9 (Fig. 3.7a). It seemed that the background of S23321 is more compatible with *V. radiata* when compared with DOA9 WT inoculation. The plant phenotype derived from chimeric strain was not different in *V. radiata*. The nodule formed by DOA9 WT only had necrotic nodules (Fig. 3.7e) whilst the chimeric performed both active and inactive nodules (Fig. 3.7g). The bacteroid inside of the nodule DOA9 stained red means almost of the cells were dead (Fig. 3.7i, and j). The bacteroid of S23321:pDOA9 stained green means the cells were lived and active (Fig. 3.7k, and l).

In *C. juncea*, the nodulation of S23321:pDOA9 in this plant similar to DOA9 WT which perform only pseudonodules (Fig. 3.8e,g). The plant dry weight showed not significantly different when compared DOA9 WT with S23321:pDOA9 (Fig. 3.8a). ARA also showed low in both DOA9 WT with S23321:pDOA9 (Fig. 3.8b). The bacteroid inside of the nodule was empty (Fig. 3.8 (b, i, and j). Mutation in the *nodB* aborts the nodulation in all chimeric plant tests. *nodB* is important for NF production, and it seemed that the *nod* genes in pDOA9 was ultimately pivotal for chimeric nodulation in all legumes.

Mutation in the *nodB* aborts the nodulation in all chimeric plant tests (Fig 3.6b; 3.7b; 3.8b; and 3.9b). *nodB* is important for NF production, and it seemed that the *nod* genes in pDOA9 was ultimately pivotal for chimeric nodulation in all legumes. Moreover, the DOA9**Δ**pDOA9 displayed lost ability of nodulation in all plants which means that the nodulation of DOA9 only depend on their symbiotic megaplasmid pDOA9 (Fig.3.9).

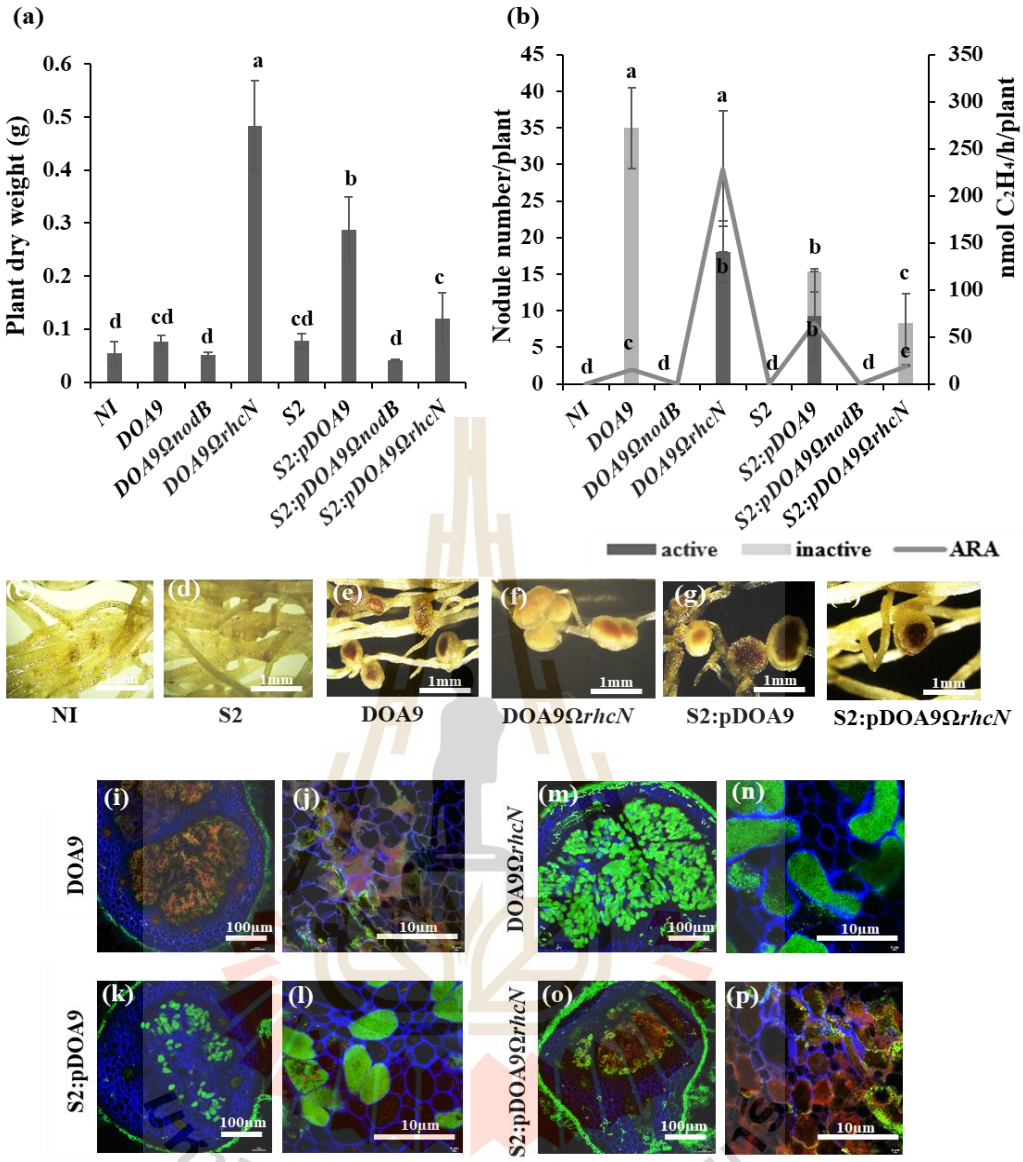


Figure 3.7 Nodulation in *V. radiata* (a-p). The plant dry weight (a). The nodule number/plant and nitrogenase activity (nmol C_2H_4 /h/plant) (b). The comparison of the nodule morphology (c-h). The root nodules observed with a stereomicroscope and the bacteroid inside of the nodule were observed by confocal laser scanning microscope tools after stained with Calcofluor M2R, Syto9 and Propidium Iodine (PI) (i-n). dark grey bar = number of active nodule, light grey bar = number of inactive nodule. ARA indicated in line. Error bars represent standard deviation (SD) (n=5). Different letters above the error bars indicate significant differences at $P<0.05$ (Tukey's HSD test). S2 = S23321.

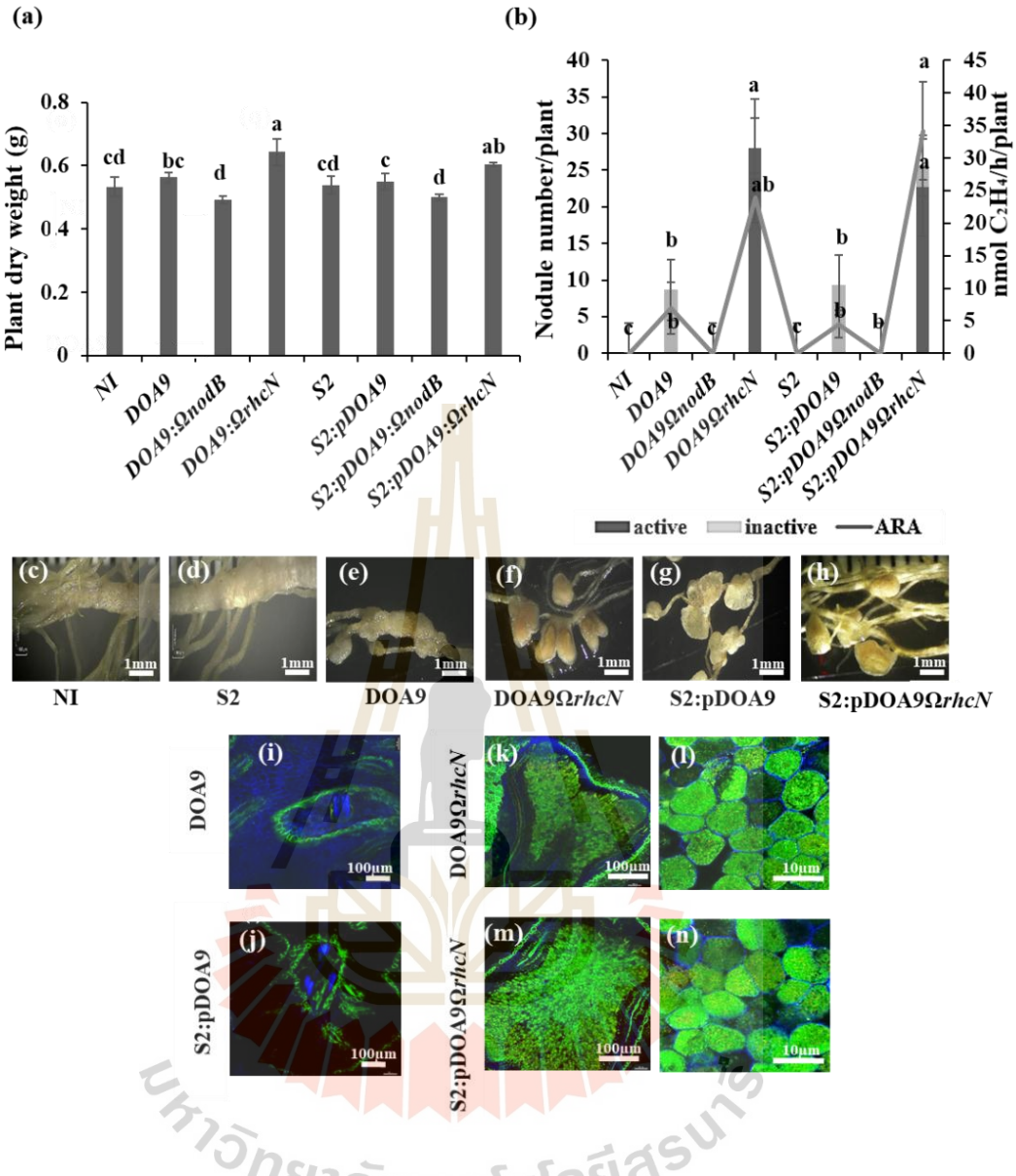


Figure 3.8 Nodulation in *C. juncea* (a-p). The plant dry weight (a). The nodule number/plant and nitrogenase activity (nmol C_2H_4 /h/plant) (b). The comparison of the nodule morphology (c-h). The root nodules observed with a stereomicroscope and the bacteroid inside of the nodule were observed by confocal laser scanning microscope tools after stained with Calcofluor M2R, Syto9 and Propidium Iodine (PI) (i-n). dark grey bar = number of active nodule, light grey bar = number of inactive nodule. ARA indicated in line. Error bars represent standard deviation (SD) (n=5). Different letters above the error bars indicate significant differences at $P<0.05$ (Tukey's HSD test). S2 = S23321.

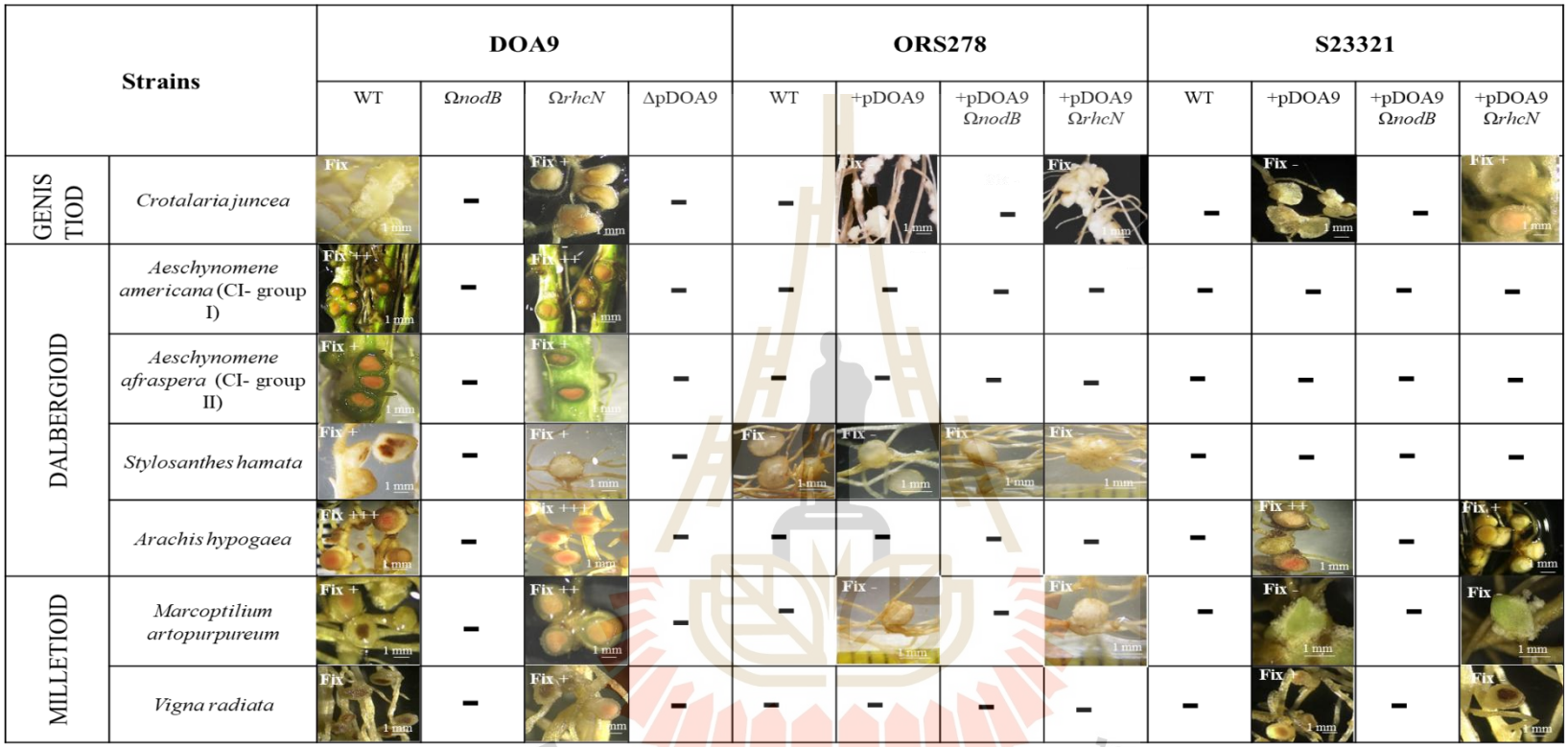


Figure 3.9 The overall plant test result among *Bradyrhizobium* sp. including DOA9 wild type, DOA9**Ω***nodB*, DOA9**Ω***rhcN,* and DOA9**Δ**pDOA9 (curing plasmid). ORS278, including ORS278 wild type, chimeric ORS278:pDOA9, ORS278:pDOA9**Ω***nodB* and ORS278: pDOA9**Ω***rhcN*. S23321, including: S2:pDOA9, S2:pDOA9**Ω***nodB* and S2:pDOA9**Ω***rhcN*. The legume test from four tribes including: Genistoid: *C. juncea*, Dalbergoid: *A. americana*, *A. afraspera, S. hamata,* and *A. hypogaea*. Milletioid: *M. artopurpureum* and *V. radiata*.

3.5.4 Effect of T3SS restrict the nodulation effectiveness

In *C. juncea*, the S23321:pDOA9Ω*rhcN* performed active nodules and significantly increase plant dry weight (Fig. 3.8a,f, and h), nodule number (Fig. 3.8b) and nitrogen fixation ability (Fig. 3.8b). The pseudonodule was derived from S23321:pDOA9 inoculation (Fig. 3.8g), while the nodules turned to pinkish nodules after S23321: pDOA9Ω*rhcN* inoculation (Fig. 3.8h). Bacteroid observation revealed that the S23321:pDOA9Ω*rhcN* displayed live bacterial cell (Fig. 3.8m-n). However, the plant phenotype was not healthy. Contrariwise, once the presence of a functional T3SS leads to an increasing in nodule number and nitrogen fixation efficiency, the secreted proteins may suppress plant defense reactions, and increase nodulation efficiency. In this study, the T3SS pro-host properties may act as a positive determinant. Mutation of the *rhcN* gene leads to less nodulation and nitrogen fixation in chimeric S23321:pDOA9Ω*rhcN* with *A. hypogea* (Fig. 3.6b) and *V. radiata* (Fig. 3.7b). This result incongruence with the result from the original strain DOA9Ω*rhcN* in both legumes, while mutation T3SS brings a better effect to the nodulation (Fig. 3.6f and 3.7f).

S23321:pDOA9Ω*rhcN* could nodulate the *A. hypogea*, with low numbers when compared with DOA9Ω*rhcN* (Fig. 3.6b). The nitrogenase activity was found to be the lowest when compared with other treatments (Fig. 3.6b). The nitrogenase activity derived from chimeric and mutant chimeric strains reduce almost half-strength than DOA9. Plant dry weight also showed significantly reduced in S23321:pDOA9Ω*rhcN* when compared with DOA9Ω*rhcN* (Fig. 3.6a). The nodule phenotype of S33212: pDOA9Ω*rhcN* under the light microscope showed the brown and senescence part in the middle of the nodule (Fig. 3.6f, and h).

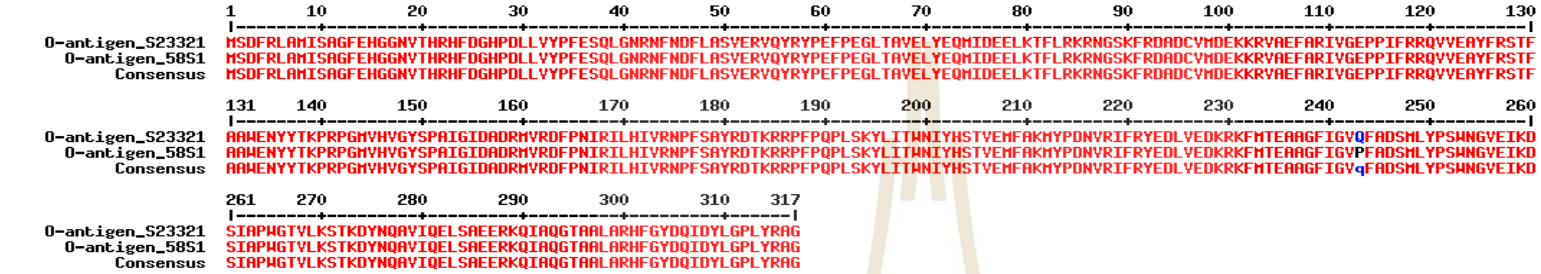
In *V. radiata,* S23321:pDOA9Ω*rhcN* induced the defective nodule (Fig. 3.7h), had low nitrogenase activity (Fig. 3.7b) and showed significantly lower of plant dry weight (Fig. 3.7a). However, when compared with DOA9Ω*rhcN* possesses highest nitrogenase activity (Fig. 3.7b) and had a full green bacteroid (Fig. 3.7m-n), while S23321:pDOA9Ω*rhcN* displayed necrotic nodules (Fig. 3.7g) which showed the red cells color or dead cells after stained with PI (Fig. 3.7o-p). However, all of treatments displayed not healthy plant phenotype.

3.5.5 Preliminary observation of crosstalks of pDOA9 and S23321 chromosome

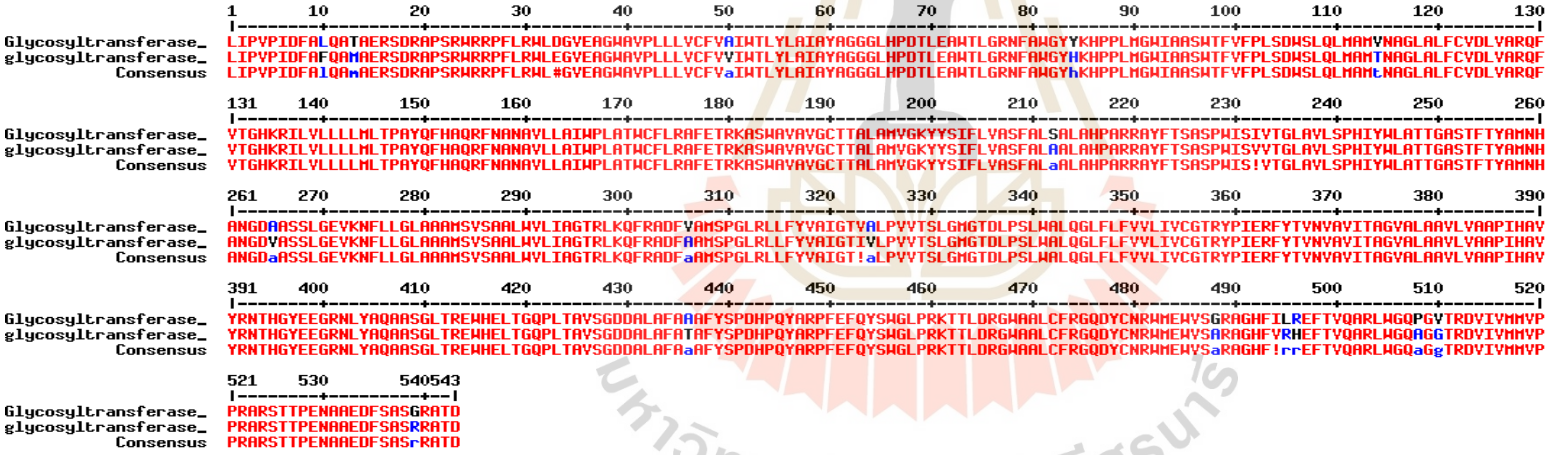
According to Doin de Moura et al., (2020) the genes in the chromosome which have effect to the nodulation in several rhizobia including : O-antigen, mutltidrug transporter A (mdtA) and mutltidrug transporter B (mdtB). All those genes from the cS23321 were further analysed by gene expression using qRT-PCR. Those genes found to have low similarity when compared with cDOA9 and cORS278. Based on multiple alignment of amino acid sequence, all those genes shares high similarity only with *Glycine max* symbiont *B. cosmicum* 58S1 (Fig. 3.10).

The gene expression was investigated in S23321 WT and chimeric strain with the induction of root exudate from few legumes including *V. radiata, C. juncea,* and *A. americana,* respectively. The substrate naringenin 20μM was used as the standard flavonoid induction. The root exudate from plant was chosen based on their nodulation ability. The expression of glycosyltransferase (gly), O-antigen, mutltidrug transporter A and B (mdtA, mdtB) showed increase under the induction of *V. radiata* and *C. juncea* when compared with non-induction and induction with naringenin in the S23321 WT (Fig. 3.11A). The S23321 wild type without any induction was used as the control treatment. The expression of S23321 with naringenin induction was not changed when compared with non-induction. All of the expression with induction of naringenin around 0.9-1.1 fold. The expression of S23321 with *V. radiata* induction was showed up regulated the expression around 3.9-7.1 fold. The expression of S23321 with *C. juncea* induction was showed up regulated the expression around 1.8- 2.3 fold. While, expression of all genes found to be down regulated under induction *A. americana,* around 0.3-0.4 fold. Different result found in the chimeric strain, some regulator on pDOA9 reduce the expression of all genes up to 100 fold in all treatments (Fig. 3.11B). The chimeric expression was not under control of naringenin induction because the expression was lower when compared with the non-induction. Moreover, the expression of genes under induction *V. radiata* and *C. juncea* root exudate were lower than non-induction. The expression of chimeric under induction of *A. americana* root exudate found higher when compared with the induction of *V. radiata* and *C. juncea* root exudate, but not significantly different when compared with the non-induction. However, all those genes were not symbiotic determinant in case of legume-chimeric S23321:pDOA9 symbiosis

O-antigen



Glycosyltransferase



Multidrug transporter A

```
                1       10        20        30        40        50        60        70        80        90       100       110       120       130
                |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
MdtA_S23321]    LTRRPKLGVALFGILAIAAVAFGLWYELIGGVKPVEAAAQSASSQAAAKIPVSSFVVKKADFPVHTYGLGVVSPFKTVTVKSRVDGQITKVFFKQGQMVKEGDPLLEIDQRPFTAALEQAVAKKAQDEAN
mdtA_58S1]      LTRRPKLGVALFGILAIAAVAFGLWYELIGGVKPVEAAAQSASSQAAAKIPVTSFVVKKADFPVHTYGLGVVSPFKTVTVKSRVDGQITKVFFKQGQMVKEGDPLLEIDQRPFTAALEQAVAKKAQDEAN
Consensus       LTRRPKLGVALFGILAIAAVAFGLWYELIGGVKPVEAAAQSASSQAAAKIPVsSFVVKKADFPVHTYGLGVVSPFKTVTVKSRVDGQITKVFFKQGQMVKEGDPLLEIDQRPFTAALEQAVAKKAQDEAN

                131     140       150       160       170       180       190       200       210       220       230       240       250       260
                |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
MdtA_S23321]    LKNDQLNLERFQKLAKQQFETQQNLDAQQALVDQMTAQVKGDQAAIDNAQTNLGYTSIKAPISGRTGFRLVDPGNIVHAADTTGIVTIAQLQPIAVQYTEPEEQLQAIDKAYDSGEVPVEALTSDGTRTL
mdtA_58S1]      LKNDQLNLERFQKLAKQQFETQQNLDAQQALVDQMTAQVKGDQAAIDNAQTNLGYTSIKAPISGRTGFRLVDPGNIVHAADTTGIVTIAQLQPIAVQYTEPEEQLQAIDKAYDSGEVPVEALTSDGTRTL
Consensus       LKNDQLNLERFQKLAKQQFETQQNLDAQQALVDQMTAQVKGDQAAIDNAQTNLGYTSIKAPISGRTGFRLVDPGNIVHAADTTGIVTIAQLQPIAVQYTEPEEQLQAIDKAYDSGEVPVEALTSDGTRTL

                261     270       280       290       300       310       320       330       340       350       360       370       380       390
                |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
MdtA_S23321]    SRGRLAIMDNSVSQATGTISLKARFDNKDNALWPGLSVTTRMLIDTRKDVIVVPQDGVQHALAGLFAYVIGANGKVSAKPIKVSQSGDANAVVSEGLNVGDKIVVAGQSRLFDGALVDDKPQVAAAAPPT
mdtA_58S1]      SRGRLAIMDNSVSQATGTISLKARFDNKDNALWPGLSVTTRMLIDTRKDVIVVPQDGVQHGPAGLFAYVIGANGKVSAKPIKVSQSGDANAVVSEGLNVGDKIVVAGQSRLFDGALVDDKPQVAAAAPPT
Consensus       SRGRLAIMDNSVSQATGTISLKARFDNKDNALWPGLSVTTRMLIDTRKDVIVVPQDGVQHalAGLFAYVIGANGKVSAKPIKVSQSGDANAVVSEGLNVGDKIVVAGQSRLFDGALVDDKPQVAAAAPPT

                391     400403
                |--------+--|
MdtA_S23321]    TTSASQ--IGPAD
mdtA_58S1]      TTSASQAKIGPAD
Consensus       TTSASQ..IGPAD
```

Multidrug transporter B

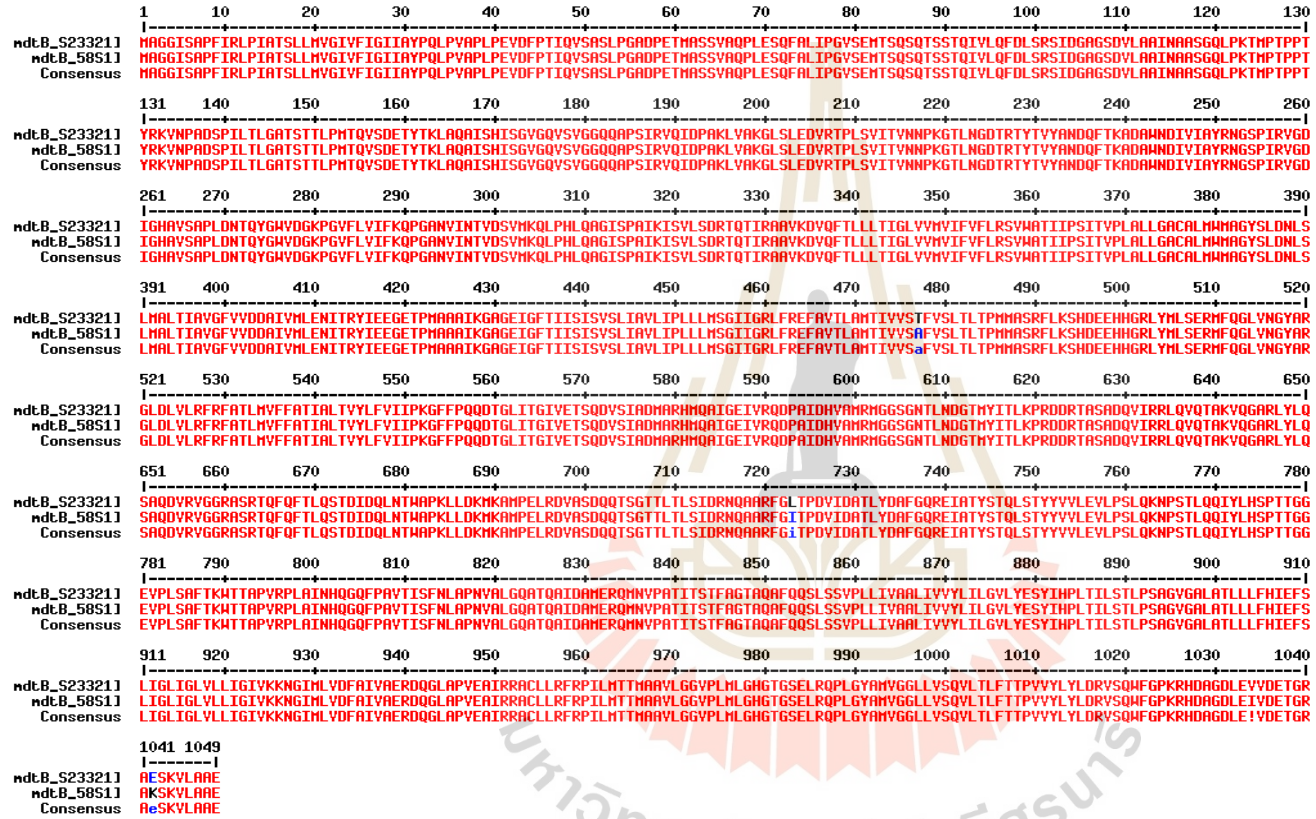
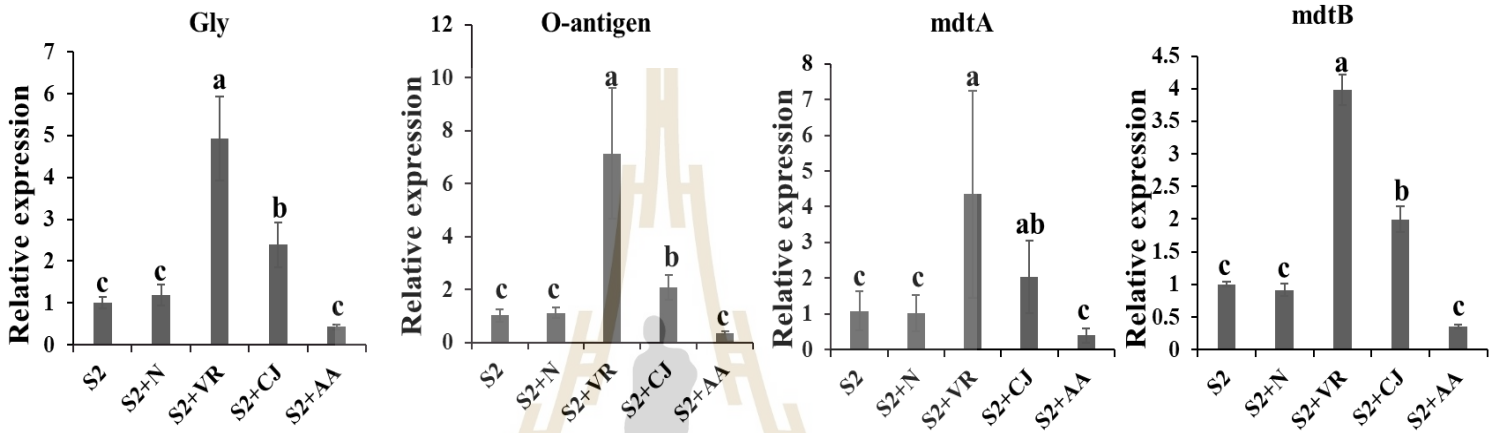


Figure 3.10 Percent similarity of amino acid in O-antigen, glycosyltransferase, multidrug transporter A (mdtA), multidrug transporter B (mdtB), genes relate with successful symbiotic in the background chromosome *B. cosmicum* S23321 compared with *G. max* symbiont *B. cosmicum* 58S1 using Multiple sequence alignment with hierarchical clustering program.

A


Gly
O-antigen
mdtA
mdtB
Relative expression
S2
S2+N
S2+VR
S2+CJ
S2+AA

B

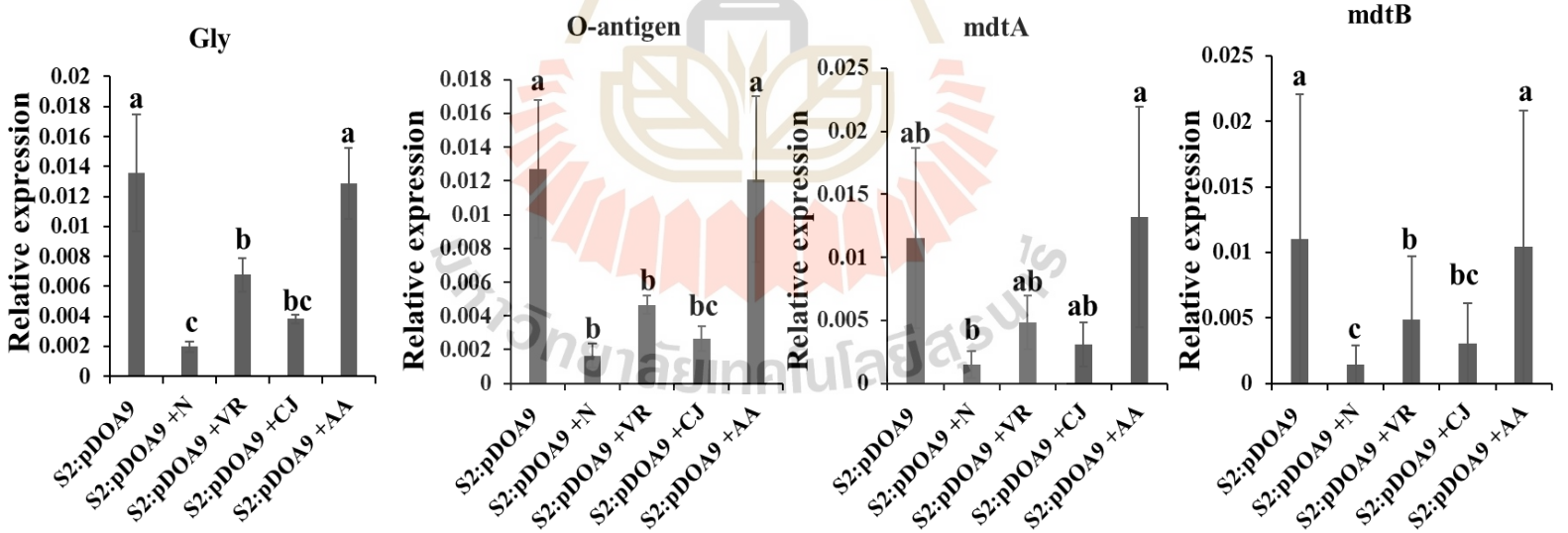

Gly
O-antigen
mdtA
mdtB
Relative expression
S2:pDOA9
S2:pDOA9 +N
S2:pDOA9 +VR
S2:pDOA9 +CJ
S2:pDOA9 +AA

Figure 3.11 Relative gene expression of genes in the membrane cells of S23321 including glycosyltransferase (gly), O-antigen, multidrug transporter A (mdtA), and multidrug transporter B (mdtB) genes relate with successful symbiotic in the background chromosome *B. cosmicum* S23321 (A) compared with chimeric S2:pDOA9 (B). N= naringenin 20 μM, VR= *V. radiata* root exudate, CJ= *C. juncea* root exudate, AA= *A. americana* root exudate. Error bars represent standard deviation (SD) (n=5). Different letters above the error bars indicate significant differences at $P<0.05$ (Tukey's HSD test). S2= S23321.

Statistical Analysis: Data was subjected to analysis of variance (ANOVA) and the significance of mean values were tested at 5% significance level by Duncan's Multiple Range Test.

3.6 Discussion

3.6.1 The nif/fix gene background of S23321

B. cosmicum S23321 is non-nodulating *bradyrhizobium* with a genome size of 7,231,841bp. This strain lacked symbiotic genes (Okubo et al., 2012). The presence of extra-chromosomal replicons of pDOA9 in chimeric strain offers the addition of *nod*, *nif/fix* genes, T3SS, and type four secretion system (T4SS). Moreover, pDOA9 increases the copy number of *nif* and *fix* genes in chimeric strain (Fig. 3.5A). From our result, the transfer of pDOA9 brings additional *nif* gene to the chimeric genome, which could intensify the ability of S23321:pDOA9 to fix the N_2 under the free-living stage. However, the nitrogenase activity was increased only in the absence of N source in the medium. One of the major constraints measuring ARA under the free-living (FL) stage depends on the availability of the N source and O_2 condition. Nitrogenase activity requires a microaerobic condition with the concentration of 1% up to below 21% O_2 mimicking the environmental condition to achieve optimal stability and function of the N-fixing catalyst nitrogenase (Haskett et al., 2021). The lower O_2 content, the better nitrogenase activity. As previous result indicated that the presence of pDOA9 could increase the nitrogenase activity of chimeric strain under free-living condition, possibly *nifA* or other *nif/fix* genes in pDOA9 have not only work under the symbiotic condition but may also increase the nitrogenase activity under free living. However, the expressions in chimeric strain was lower when compared with DOA9 WT. The reason still unknown, though if compared with closely related strain *B. diazoefficiens* USDA110, S23321 lacks genes for several enzymes involved in nitrogen metabolism, such as: nitrilase, L-asparaginase, asparagine synthase, glutamate dehydrogenase, and *nosZ* for final step of denitrification (Okubo et al., 2016) which probably affected lower the N-fixation efficiency when compared with DOA9 WT. The pDOA9 could substitute few genes including glutamate dehydrogenase A (*gdhA*) which present in the megaplasmid, but apparently not all

that genes present in pDOA9. The absence genes in S23321 showed in black color bar (Fig. 3.5B).

The *nif* cluster of cS23321 was found to be more conserved when compared with the pDOA9 and *nif* gene cluster from symbiotic strain (de Matos et al., 2021). Non-symbiotic and photosynthetic supergroup strains often carry *nif*-gene clusters with a GC % content indifferent from the genome average and these clusters were postulated to be ancestral to symbiotic clusters derived from last common ancestor (LCA) (Okubo et al., 2016). The *nif* cluster in nonsymbiotic were characterized by the presence of *sufBCDSE* genes, which involved in FeS cluster synthesis (Takahashi and Tokumoto, 2002), just upstream of the *nifDK* genes. Also, a *nifA* regulatory gene is usually close to nonsymbiotic but more distant in symbiotic (de Matos et al., 2021). The *nif* genes analysis of S23321 showed that in the chromosome of S23321 possesses 2 copies of *nifD, nifS, nifZ* and *nifH* (Fig. 3.5B). The presence of duplicates *nif* genes also found in phylogenetically diverse strain usually with little divergent in nucleotide sequences, and are possibly paralogous in origin (de Matos et al., 2021). The abundance of *nif* genes especially *nifH*, possibly related with lifestyle and specific environment subtype, e.g. in coral metagenome sample rich of *nifH,* while in mangrove metagenome samples was depleted. *nifH* from symbiotic members (Sym) is enriched in "root metagenome" samples, while free living *nifH* exhibits a high proportion in phyllosphere samples (Tao et al., 2021).

The *nifA* in the DOA9 is the regulator to initiate the transcription of other genes involving in nitrogen fixation. *NifAc* and *nifAp* in the DOA9 both functioned under symbiotic conditions, while *nifAc* was indispensable for nitrogen fixation under the free-living condition and the *nifAp* under symbiotic conditions (Wongdee et al., 2018). Interestingly, based on the nucleotide sequence, the *nifAc* in the DOA9 80.42% similarity with the *nifA* S23321 when compared with the *nifAp* DOA9 69,49% (Fig. 3.5B). Based on amino acid sequencing, the N-terminal 70 amino acid of *nifA* S23321 completely different from the *nifAp* DOA9 due to lack of the N-terminal domain *nifAp.* Furthermore, the *nifAc* S23321 compared with *nifAc* DOA9 only different 27 amino acid due to lack of the N-terminal domain. The N-terminal domain expected as the regulatory function and modification of this might cause the change in gene regulation under different conditions (Wongdee et al., 2018). The

function of *nifA* in the S23321 might be similar to the *nifAc* DOA9 but it premature to conclude. The next addressed is the *nifDK* as the nitrogenase Mo-Fe protein subunit alpha and beta. The *nifDK* in cDOA9 is the main contributor for the nitrogenase activity under free living state while *nifDKp* primarily under symbiosis, but both are imperative for full function of nitrogenase activity during symbiosis (Wongdee et al., 2016). Fascinatingly the S23321 has 2 copies of *nifD*, which highly similar with the *nifDc* DOA9. S23321 also has 2 copies of *nifH* (nitrogenase Mo-Fe protein) which is high similarity with the *nifHc* and *nifHp* DOA9 (Fig. 3.5B). The mutation of the *nifA, nifD* and *nifH* in S23321 and pDOA9 in the next study needs to be done to observe the regulatory *nif* gene in the chimeric strain. Other *nif* genes such as *nifK,* and *nifV* among S23321, pDOA9, and cDOA9 are also showed high similarity in the amino acid sequence.

S23321 posses *nifV* gene sequence which 82.49% similarity with *nifV* from cDOA9. The *nifV* gene encodes a homocitrate synthase, responsible for the synthesis of homocitrate, a nitrogenase cofactor essential for nitrogenase activity (Nouwen et al., 2017). In DOA9, *nifV* is important under free-living condition. Mutation of *nifV* was not effect for the symbiosis on *A. americana* in DOA9 but affect in Brdyrhizobial strain SUTN9-2 and ORS285 in few plants (Hashimoto et al., 2019). S23321 must adapt to physiological changes to cope with the tremendously low oxygen accessibility (microoxia) within nodules. The important gene which regulates this process for bacterial respiration inside nodules is *cbb3* oxidase (*Fix*NOQP) genes (Fernández et al., 2019) and S23321 owns it. The *cbb* cycle function is also as an electron sink to equilibrate the red/ox balance or the bacteroids using it as an important carbon supply. Therefore, S23321 has a prompt ability to change the behavior from a free-living state to be the endosymbiont legume.

3.6.2 Effective nodules in *V. radiata* and A. *hypogaea* from chimeric S23321: pDOA9

The actives nodules derived from S23321:pDOA9 was found in *V. radiata* and *A. hypogea.* Contrary to the DOA9, the *V. radiata* cv. SUT4 can be nodulated by S23321:pDOA9 and perform the active nodule while the DOA9 WT only performs necrotic nodule. The T3SS and type three effector (T3E) did not harm the nodulation on the S23321:pDOA9 but the mutation in injectisome T3SS (*rhcN)*

changing the nodule from the active to necrotic nodule. According to Piromyou et al., 2018, T3SSs of *Bradyrhizobium* sp. DOA9 showed negative effects on nodulation in *V. radiata* cv. KPSII and CN72, whereas the T3SS of *Bradyrhizobium* sp. SUTN9-2 showed no effect on symbiosis. In addition, T3SS had the positive effect on *B. diazoefficiens* USDA110 in KPSII and the negative effect in CN72. Therefore, the effect of mutation T3SS is must be cultivar-specific.

The S23321:pDOA9 was unable to nodulate the original host *A. americana* (Fig. 3.9) due to failed in host plant recognition and lack some of key determinants which requires from cDOA9. The *nod* genes in DOA9 not only found in megaplasmid but also in the chromosome. For example: *nodVW* is associate with classical two component regulatory family and it is crucial for the nodulation of cowpea, siratro, and mungbean (Sanjuan et al., 1994). DOA9 possesses seven copies of *nodV* (five on the chromosome and two on the plasmid) and 10 copies of *nodW* (seven on the chromosome and three on the plasmid). *nodVW* in chromosome DOA9 found to be high similarity with *B. diazoefficiens* USDA110 and was considered to recognize plant flavonoids contributing to the nodulation of a broaden host range by increasing NF synthesis in combination with *nodD* (Okazaki et al., 2015). However, the *B. diazoefficiens* USDA110 unable to nodulate *A. americana* (Noisangiam et al., 2012). Although many of *nodVW* high similar with *B. diazoefficiens* USDA110, some of *nodV* (BDOA9_0203630) and *nodW* in the plasmid (BDOA9_0203420) showed low similarity. Moreover, the *nodV* (BDOA9_0120640) in the chromosome of DOA9 from BlastP showed no similarity with *B. diazoefficiens* USDA110 but similar with *B. japonicum* WSM2793, *Methylobacterium* sp. 10, and *Rhizobium* sp. PDO1-076 (Okazaki et al., 2015). All of them are roots-nodule symbiont, for example: *B. japonicum* WSM2793 was isolated from roots of *Rhynchosia totta* in South Africa (Reeve et al., 2015). More than 20 years, *Methylobacterium* spp. strains have been isolated from root nodules of legumes belonging to the genera *Crotalaria* and *Lotononis* (Fabaceae and Crotalariae) (Jaftha et al. 2002; Samba et al. 1999; Sy et al. 2001a and b). *Rhizobium* sp. PDO1-076 was isolated from surface-sterilized fine root of *Polpulus deltoids* on the Oak Ridge Reservation, East Tennessee (Brown et al., 2012) and found to be high similarity with crown gall infected cherry root-stalk from *Agrobacterium tumefaciens* str. Cherry 2E-2-2 (Zhang et al., 2014). More important the chromosome of *B.*

cosmicum S23321 absence of *nodV* (BDOA9_0120640). This gene possibly one of symbiotic determinant for roots-nodule of *A. americana.*

The nodulation in *A. hypogea* occurs via a "crack entry" mechanism (Howieson and Dilworth, 2016). It is believed to be evolutionarily more ancient than infection via root hairs (Sprent, 2007). However, the presence of *nod*ABC genes in pDOA9, which are required for the synthesis of the lipo-chitooligosaccharides backbone, suggests that the S23321:pDOA9 nodulates *A. hypogea* in a NodFcator (NF) -dependent manner. This result confirmed by mutation of the *nodB* gene in pDOA9 abolished the nodulation (Fig. 3.9). According to Morgante et al., 2005, EPS plays an important role in establishing an effective symbiosis through the crack entry in *A. hypogea*. It seems not only EPS but also the T3SS affect positively in *A. hypogea* symbiosis by suppressed the defense system of the plant. Mutation of T3SS lessens the nodule number as well as nitrogen fixation capacity of chimeric strain, the nodule morphology was changed from red to white color after *rhcN* mutation (Fig. 3.6). However, the late nodulation by S23321:pDOA9 inoculation was found.

The analysis of the genes that could be engaged in the nodulation found out the chromosome S23321 was more similar to the DOA9 than in ORS278. It is because the chromosomal DNA of ORS278 phylogenetically distant from DOA9. The successful symbiosis obliges the two partners should be compatible with each other in every stages of symbiotic development (Wang et al., 2018). The host specific-adaptation notion discriminates against environmental necessities for bacteroids in which specific genes are transcribed and translated. This is one of the reasons why the rhizobia could not nodulate all legumes, it depends on host restriction as well. Unlike DOA9, the chimeric and all mutant chimeric strains incapable to nodulate the original host which is the *nod*-dependent legumes of *A. americana* and *A. afraspera* (Fig. 3.9). The chimeric strains (S23321:pDOA9; S23321:pDOA9Ω*nodB;* and S23321:pDOA9Ω*rhcN*) may fail in host plant recognition due to the incomplete key determinant which requires from chromosome DOA9.

3.6.3 Effect of Type Three Secretion System (T3SS) from chimeric S23321: pDOA9ΩrhcN in legume test

The S23321 possesses the genes corresponding T2SS and T3SS for the flagellar assembly system, while T3SS for pathogenicity or nodule formation is absent

in this species (Okubo et al., 2012). Based on our result, the T3SS dependent legume is divided into two mechanisms. First, the secreted proteins negatively strike at the nodulation process, possibly by inducing plant defense reaction and in this manner prevent the chimeric strain to form nodulation or reduce the nitrogen fixation ability. After mutation in the *rhcN* gene, it restores for better nodulation and nitrogen fixation. Mutation in the original strain DOA9Ω*rhcN* leads to better nodulation in *C. juncea, V. radiata*, and *M. artopurpureu* (Songwattana et al., 2017). The chimeric ORS278:pDOA9Ω*rhcN* restored the nodulation in *A. evinia* and *A.indica* which previously performed senescence nodule after transferring of pDOA9. The presence of certainly incompatible determinants on the pDOA9 was obviously in charge of the loss of the symbiotic performance of ORS278:pDOA9. This assumed the incompatible factor came from one of few secreted or effector protein from pDOA9 (Songwattana et al., 2019). In the case of S23321:pDOA9Ω*rhcN* acquired better nodulation in *C. juncea* when compared with chimeric ORS278:pDOA9Ω*rhcN*.

Contrariwise, once the presence of a functional T3SS leads to an increasing in nodule number and nitrogen fixation, the secreted proteins may suppress plant defense reactions, and increase nodulation efficiency (Songwattana et al., 2017). In this case, the T3SS pro-host properties may act as a positive determinant. Mutation of the *rhcN* gene leads to less nodulation and nitrogen fixation in chimeric S23321:pDOA9Ω*rhcN* with *A. hypogea* and *V. radiata* (Fig. 3.6 and Fig. 3.7). This result incongruence with the result from the original strain DOA9:Ω*rhcN* in both legumes, while mutation T3SS brings a better effect to the nodulation. This study propose two hypotheses from this result. First, in the original strain of DOA9 perhaps the chromosome retained the negative factors which lead to the failure of performing the effective nodule in *V. radiata*, also in pDOA9 may contain both of the positive and the negative effectors and affect nodulation. However, the mutation in T3SS may disable the plasmid to secret some of the negative effectors. Second, the transfer of pDOA9 to the S23321 is enough for the chimeric strain to induce the active nodule in *V. radiata* and *A. hypogea*, but the mutation in T3SS reduce the nodulation in both plants meaning there are possibly positive factors from the chromosome of S23321 and the positive regulation of those factors is under the control via T3SS on pDOA9.

3.6.4 Which one is the key determinant: The symbiotic megaplasmid, the chromosome recipient, or the legume host?

The symbiotic variations of rhizobia with distinct host specificity principally depend on the genotypes of their symbiotic plasmids (Ji et al., 2010; Rogel et al., 2014). The diversity of symbiotic plasmids occurs in the Rhizobiaceae pan-genome, and in some phylogenetically closely related species, carry similar genomic backgrounds compatible with the same symbiotic plasmid (Yang et al., 2020). In concordance with the previous study, the result of the legume test of S23321:pDOA9 was found to be more compatible than ORS278:pDOA9 due to the phylogenetic distance of the recipient background.

The HGT of pDOA9 to the S23321 had a significant cost to the gene expression related to surface polysaccharides and the multidrug transporter genes (Fig. 3.11). The S23321 WT under the induction of flavonoid from root exudate resulting the gene expression upregulated, especially *V. radiata* and *C. juncea.* The chimeric S23321:pDOA9 able to perform nodulation in those legumes. However, after the transfer of pDOA9 significant cost in the gene expression was downregulated up to 100 fold. The plasmid-chromosome crosstalk happened after acquiring the pDOA9 to the recipient S23321. The new plasmid carriage due to HGT event affects the bacterial replication, expression machinery and reduces the competitiveness of the chromosomal recipient (Park and Zhang., 2012; Platt et al., 2012; Baltrus et al., 2013; Vial and Hommais, 2020).

The S23321 seems to adapt to the pDOA9 to increase the existence in its chromosome even it has an effect on its normal metabolism. Congruence with this result, the host fitness in *Pseudomonas fluorescense* reduces 25% after acquiring the plasmid pQBR10 (425kb) (Harrison et al., 2015). This phenomenon could be compensated by adaptive evolution (Harrison and Brockhurst, 2012). A previous study from Vial and Hommais, (2020) mentioned that the plasmid-specific regulators control gene expression. The *pSymA* in *S. meliloti* proven to regulate another replicon (chromid or chromosome) in the induction of plant flavonoids during the *nod* genes are expressed (Chen et al., 2000; diCenzo et al., 2018). Moreover, in *Klebsiella pneumonia, E.coli* and *Salmonella enterica* after acquiring of pKpQIL

plasmid, it has changed in their expression in chromosomes involved in carbohydrate metabolism and transport (Buckner et al., 2018) and energy production (Lang and Johnson., 2015). The *nodD3* overexpression in *pSymA, syrM,* and *fixJ* in *S. meliloti* repressed the expression of chromosomal genes such as flagellum and chemotaxis genes (Barnett et al., 2004), also the activation of transcription of *fixJ* as a regulator of nitrogen fixation in the plasmid could regulate several chromosomal genes due to the presence of *fixJ* binding sites in their promoters (Bobik et. al., 2006). The plasmid and chromosome crosstalk via several ways and more than 10 plasmid regulators showed control of the gene expression of chromosome (Vial and Hommais, 2020) for example, NrcR transcriptional factor in *R. tropici,* the pRtcCIAT899c could regulate the extracellular polysaccharide for adherence and motility (Del Cerro et al., 2016). The regulator QfsR (Quorum sensing flagellar sRNA regulator) could interact with several polycistronic mRNA localized on different replicon, from plasmid to chromosome and vice versa (Diel et al., 2019). Also, H-NS (histone-like nucleoid structuring) by plasmid are stealth protein which as one of the key role communication between chromosome and plasmid (Vial and Hommais, 2020) and still many other regulators which need to be elucidated.

Meta-analysis of 18 transcriptome and 1 proteome data sets published in 13 different studies on various symbioses rhizobia (*Rhizobium, Sinorhizobium, Bradyrhizobium, Azorhizobium* and *Mesorhizobium*). During symbiosis, the downregulated traits in the chromosome were analyzed. The data sets that were shared with growth-arrested cells include translation (ribosomal proteins), cell envelope biogenesis (outer membrane proteins, peptidoglycan, and exopolysaccharide synthesis), intracellular trafficking, cell cycle control and DNA replication, signal transduction, motility, and the FoF1 -type ATPase. Moreover, in order to adapt in the microoxic condition the downregulate gene generally found in carbohydrate transport and metabolism, translation, ribosomal structure and biogenesis, chromatin structure and dynamics, cell cycle control, cell division, chromosome partitioning, amino acid transport and metabolism, intracellular trafficking, secretion and vesicular transport, extracellular structures, replication, recombination and repair chromatin structure and dynamics. Meanwhile, the upregulated genes/protein including energy production and conversion, inorganic ion

transport and metabolism, posttranslational modification, protein turnover, chaperones (Ledermann et al., 2021). According to the chimeric S23321:pDOA9 result for the gene expression. It may be possible that the genes related with cell wall/membrane/envelope biogenesis and extracellular structures including O-antigen and glycosyltransferase were downregulated. Moreover, for signal transduction mechanisms, intracellular trafficking, secretion and vesicular transport such as Mutlidrug transporter A (mdtA) and Mutlidrug transporter B (mdtB) were also downregulated.

The same plasmid, with different background chromosomes, and also different host plants concomitantly will give different traits in chimeric-legume symbiosis specificity and compatibility. It refers to results that the S23321:pDOA9 has a wide-ranging effect with each legume test (Fig. 3.9). The foregoing result mentioned that the response of acquisition of the symbiotic plasmid in the recipient on the rhizobial-legume interaction divided into three scenario (Laguerre et al., 2003; Tong et al., 2020). First, those which strictly selected on both chromosomal and symbiosis gene backgrounds, for example in the legume plant of *Medicago sativa* L. Second, those which strictly selected on symbiosis gene background only, such as in the legume plant of common bean, *Aspalathus carnosa* and *Robinia pseudoacacia* L. (Ulrich and Zaspel, 2000; Moulin, et al., 2001). The third, those which are extensively acquired with several different symbiotic genes, for example in *Glycine max* and *Sophora flavescens* nodulations (Zhang et al., 2011; Jiao et al., 2015).

Recurrent adjustments of chimeric and mutant chimeric have been found during plasmid transfer. The experimental and metabolic modeling scrutiny denote that the alteration to the nodule environment depends on accessory plasmid genes (Ramachandran et al., 2011; Barreto et al., 2012; diCenzo et al., 2016; Palmer et al., 2016; Zahran, 2017; Yang et al., 2020). pDOA9 possesses a unique characteristics compared with other symbiotic plasmids from 24 rhizobia that included four genera and 11 species. The pDOA9 being designated as the out-group located far from other plasmids based on common gene analyses of symbiotic plasmids *nod* (*nod*CIJ) and *fix* (*fix*ABC) (Wang et al., 2018).

This discovery is fascinating and underpinning for some future works. (i) Is that the background chromosome of S23321 more compatible with some legumes

and do not possess any negative factors/effectors if compare with the chromosome of DOA9 ? (ii) Can the negative factors/effectors found in the pDOA9 but the chromosome S23321 could cope with those negative factors/effectors ? It remains a hint. The next notion will be interesting if we study more about factors/effectors which has a positive and negative effects with the nodulation in the original strain DOA9, to know more which factors have been decorated their function for broadening host specificity along with legumes evolution time then after that we can transfer again to the S23321 or other *Bradyrhizobium* strains with powerful and impeccable pDOA9.

3.7 Conclusion

This study is the first fact-finding in non-nodulating and the free-living non photosynthetic *Bradyrhizobium cosmicum* S23321 can be transformed to be the endosymbiotic fixing nodule by simply transfer the megaplasmid of DOA9 (pDOA9). The proximity in phylogenetic distance between the chromosome DOA9 and chromosome S23321 brings the benefit to the nodulation. However, not all legume plants can be nodulated by chimeric S23321:pDOA9 which generally nodulated by DOA9.

3.8 References

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CHAPTER IV

THE *NOD* GENES OF BROAD HOST RANGE *BRADYRHIZOBIUM* SP. DOA9 DETERMINING THE LEGUME NODULATION AND HOST SPECIFICITY

4.1 Abstract

Bradyrhizobium sp. DOA9 is the broad host range with divergent *nod* containing strain. DOA9 carry two copies of *nodA* genes, *nodA1* and *nodA2* as well as two copies of *nodD* genes, *nodD1* and *nodD2*. The roles of both genes remained to be elucidated. The result showed that the mutation of a single copy of *nodD1* (Ω*nodD1*) or *nodD2* (Ω*nodD2*) resulting in a plant phenotype similar to DOA9 WT. Ω*nodD1* or Ω*nodD2* were not significantly reduced in nodule number, plant dry weight and nitrogen fixation. Ω*nodD1* and Ω*nodD2* showed functionally redundant. The result of Δ*nodA1* and Δ*nodA2* showed that the *nodA1* was not the symbiotic determinant in DOA9 nodulation. The plant test showed that Δ*nodA1* was not significantly reduced in nodule number, plant dry weight, and nitrogen fixation but the *nodA2* played an important role in the general nodulation of DOA9. Δ*nodA2* showed aborted nodulation in all plant tests. Analysis of Nod-Factor (NF) of DOA9 shares high similarity to the major NF of the *Rhizobium* sp. NGR234. Analysis of the Δ*nodA2* revealed that no NFs were detected either in the conditions with or without flavonoid induction in the Mass Spectrometry (MS) positive mode. In Δ*nodA1*, NFs were found but the main identified molecule was not similar as in DOA9 WT and the NF bearing of C18:1 acyl group was not detected.

4.2 Introduction

Legume nodulation is facilitated by several genes, both in the host legume and in the bacterium sides. *Bradyrhizobium* sp. DOA9 is a broad host range isolated from the root nodules of the *Aeschynomene americana*. DOA9 is able to induce nitrogen-fixing nodules on legumes belonging to Dalbergioid, Milletioid and Genistioids tribes,

and also could be rice endophytic association (Teamtisong et al., 2014). The symbiotic property of DOA9 is found in megaplasmid DOA9 (pDOA9) with the size 736,085-bp and containing classical nodulation (*nod)* genes required for the early stages of symbiotic nodule formation (Okazaki et al., 2015). Therefore, the nodulation type derived by DOA9 is Nod-Factors (NFs) dependent manner.

The Nod-Factors (NFs)-dependent interaction is usually found in *Bradyrhizobium* nodulating *Aeschynomene* CI group I and II (Bonaldi et al., 2010 and Miche et al., 2010). NFs biosynthesis is commonly facilitated by NodD proteins which belong to the *LysR* family of transcription regulators. The rhizobial regulatory *nodD* gene plays a crucial role of coordinating the transcription of the other nodulation genes. It is constitutively expressed and activated by the common *nodABC* and host-specific nodulation genes in a flavonoids-dependent manner, function as early stages of molecular signaling and host-legume nodulation 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 the rhizobial genomes indicated that one or multiple copies of *nodD* can be found depending on the rhizobial species (Perret et al., 2000; Peck et al., 2006; del Cerro et al., 2015; Acosta-Jurado et al., 2019).

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; Xie et al., 2012). NFs consist of an oligomeric 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 *nod* genes, *nodA* (acyl transferase), *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 *nod* genes of DOA9 are highly divergent in gene organization and homology when compared with many other rhizobia. The *nod* genes of DOA9 found both in chromosome and plasmid. Most of the *nod* genes involved in NF

biosynthesis, including common *nod* genes (*nodABC*) are located on pDOA9, but few are also found on the chromosome e.g. *nolG, nodV, nodW, nodQ, nodN,* and *nodT*. Moreover, additional *nod* genes including *nodQ, nodP,* and *noeE* may have roles in nodulation and host specificity (Okazaki et al., 2015). Based on that background, it was interesting to know the function of *nod* genes by mutation in the *nod* genes regulator *nodD* and in the *nodA* genes which served as acyltransferase in NFs structure. Both of that genes were located in the *nod* cluster with two copies and found to be important genes in nodulation between many others rhizobia with legumes.

Two copies of *nodD* are presented on pDOA9, *nodD1* is upstream of *nodA1BCSUI* operon and *nodD2* located 0.27 Mb away from *nod* cluster (Fig. 4.3.) (Okazaki et al., 2015). In *Rhizobium tropici* CIAT899 *nodD2* also controls other functions that contribute to nodulation, and it also activates the *nod*-gene transcription in the presence of some flavonoids (del Cerro et al., 2015). Thus, *nodD2* in pDOA9 may require optimizing the interaction with each of its legume hosts. pDOA9 comprises two different copies of *nodA* genes (*nodA1* and *nodA2*), a single of *nodB* and *nodC* genes. The *nodA1* is found at the vicinity of the *nodBC* genes, and *nodA2* was found just upstream of the *nodIJ* genes. This study focusing on both *nodD* and *nodA* which located in the *nod* cluster and possibly are important for legumes-host specificity.

The acquisition of divergent *nod* genes may broaden the host range of DOA9 which *nodD* was as regulator and *nodA* was responsible for adding acyl chains in the NFs-structure and may broaden the host range with legumes. The analysis of symbiotic genes involved in infection process of divergent-*nod* (Δ*nodA1,* Δ*nodA2,* Ω*nodD1,* and Ω*nodD2*) in DOA9 will be further investigated. This data provided significant information about the function of divergent-*nod* genes and the structural analysis of NFs in *Bradyrhizobium* sp. DOA9 which never been elucidated so far. These study offered more understanding in infection pathways as well as broad host-range symbiosis characteristics in DOA9 with leguminous plants.

4.3 Research objective

4.3.1 Main objective

To demonstrate the function of *nod* genes of *Bradyrhizobium* sp. DOA9 determining the nodulation ability of host specificity on different legumes

4.3.2 Specific objectives

1. To analyze the function of two copies of *nod* genes, *nodA1* and *nodA2*, also *nodD1* and *nodD2*

2. To demonstrate the nodulation phenotypes of DOA9 with different legumes plant hosts by inoculation with mutant *nod* strains

3. To determine the Nod-Factor structure of DOA9

4.4 Materials and Methods

4.4.1 Bacterial strains and culture conditions

The bacterial strains used in this study were listed in Table 4.1. *Bradyrhizobium* sp. DOA9 (Noisangiam et al., 2012) and its derivatives were grown in modified Yeast-Mannitol (YM) medium (Giraud et al., 2000) at 28°C under aerobic condition for 5 days. *Escherichia coli* strains were cultured at 37°C in Luria Bertani (LB) medium for 18 h (Sambrook et al., 2001). Antibiotics were supplemented in medium at the following concentrations: 200 µg/ml streptomycin (sm), 20 µg/ml cefotaxime (cefo), 20 µg/ml nalidixic acid (nal) and 50 µg/ml kanamycin (km).

4.4.2 Construction of mutant strains

Standard molecular biology techniques were used for all cloning work. The single cross-homologous recombination technique was used for constructing the insertion mutant of nodulation genes. For this purpose, an internal fragment of *nodD1*= 256 bp, *nodD2*= 239 bp and *nodA2*=290 bp were amplified by polymerase chain reaction (PCR) with primers described in Table 4.2. The internal fragment was then digested by *Xba*I and *Sal*I and was cloned into the plasmid pVO155-nptII-GFP-nptII-cefo (Wongdee et al., 2016). The recombinant plasmids were introduced into *E.coli* S17-1 by electroporation (15 kv/cm, 100 Ω, and 25 µF) and was transferred into *Bradyrhizobium* sp. DOA9 by bi-parental mating as described by Giraud et al. (2010). The transconjugants were spread on YM medium supplemented with 20 µg/ml cefotaxime (cefo), 20 µg/ml nalidixic acid (nal) to select for the single

homologous recombination events (annotated as Ω*nodD1*and Ω*nodD2)*. The mutant strains were verified by PCR using external primers paired with specific primers of internal GUS and cefo resistance genes to determine the insertion recombinant. The Ω*nodB* construction as the control of *nod* gene experiment was provided by Songwattana et al., (2019). All the plasmids and primers used for the mutation were described in Table 4.1 and Table 4.2 Moreover, all mutant confirmation was described in the Fig. 4.1.

The method for the double crossed-homologous recombination technique was conducted as follows. The *nodA1* and *nodA2* mutants were obtained after deletion of a 555 bp fragment *nodA1* and a 594 bp fragment *nodA2*, respectively. The upstream and downstream flanking DNA (around 700 to 800-bp) of *nodA1* and *nodA2* genes were amplified and merged by overlap extension PCR. The overlap fragments were cloned into pNPTS129.km^{r}.sacB (Tsai and Alley, 2000) at the *BamHI*/*Xba*I. Afterwards, a cefotaxime resistance cartridge was cloned into the *Hind*III site between the upstream and downstream fragments. The recombinant plasmids were introduced into DOA9 by biparental mating. The screening of the 1st cross recombination was selected on YM+cefo+nal, and consequently selected on YM supplemented with 10% sucrose for screening the double cross recombination event. The mutant strains were verified by PCR using specific primer sets on plasmid and outside of homologous regions. The deletion mutant of these genes later denoted as Δ*nodA1* and Δ*nodA2.* Map of mutant construction and verification are indicated in the Fig. 4.2. All the plasmids and primers were used for the mutation described in Table 4.1 and 4.2.

Table 4.1. Bacterial strains, plasmid and plant tested used in this study.

Strains	Relevant characteristic	Source of reference
Bradyrhizobium sp. DOA9	Non-photosynthetic strain, isolated from paddy field using *A. americana* as trap legume, Nod-dependent strain	Noisangiam et al., 2012
DOA9Δ*nodA1*	Deletion of *nodA1* of pDOA9 by double crossed recombination with up/down *nodA1*:cefo^r.pNPTS129-*sacB*-Km^r	This study
DOA9Δ*nodA2*	Deletion of *nodA2* of pDOA9 by double crossed recombination with up/down *nodABC*:cefo^r.pNPTS129-*sacB*-Km^r	This study
DOA9Ω*nodD1*	Insertional *nodD1* of pDOA9 by integration of pVO155-nptII-GFP-nptII-cefo; cefo^r Km^r at *SalI*/*Xba*I	This study
DOA9Ω*nodD2*	Insertional *nodD2* of pDOA9 by integration of pVO155-nptII-GFP-nptII-cefo; cefo^r Km^r at *SalI*/*Xba*I	This study
DOA9Ω*nodB*	Insertional *nodB* of pDOA9 by integration of pVO155-nptII-GFP-nptII-cefo; cefo^r Km^r at *SalI*/*Xba*I	Songwattana et al., 2019
Eschericia coli S17-1	*recA thi pro hsdR* [res– mod+][RP4::2-Tc::Mu-Km::Tn*7*] λ *pir* phage lysogen, Sm^r/Tp^r	Simon et.al., 1983

Table 4.1 Bacterial strains, plasmid and plant tested used in this study (Continue).

Plasmid	Relevant characteristic	Source of reference
pVO155-nptII-cefo^{r}-gusA-nptII-gfp	Cloning vector for single crossed recombination pUC119-derived sucide vector with GusA gene, GFP and cefo cassette	Wongdee et al., 2016
pVO155-nptII-Sp/Sm^{r}-gusA-nptII-gfp	Cloning vector for single crossed recombination pUC119-derived sucide vector with GusA gene, GFP and Sp/Sm cassette	Songwattana et al.,2017
pNPTS129.km^{r}.sacB	Cloning vector for double crossed recombination with sucrose addition and cefo$^{r.}$	Tsai and Alley, 2000.
Strains	**Relevant characteristic**	**Source of reference**
Bradyrhizobium sp. DOA9	Non-photosynthetic strain, isolated from paddy field using A. americana as trap legume, Nod-dependent strain	Noisangiam et al., 2012
DOA9**Δ**nodA1	Deletion of nodA1 of pDOA9 by double crossed recombination with up/down nodA1:cefor.pNPTS129-sacB-Kmr	This study
DOA9**Δ**nodA2	Deletion of nodA2 of pDOA9 by double crossed recombination with up/down nodABC:cefor.pNPTS129-sacB-Kmr	This study
DOA9**Ω**nodD1	Insertional nodD1 of pDOA9 by integration of pVO155-nptII-GFP-nptII-cefo; cefor Kmr at SalI/XbaI	This study
DOA9**Ω**nodD2	Insertional nodD2 of pDOA9 by integration of pVO155-nptII-GFP-nptII-cefo; cefor Kmr at SalI/XbaI	This study
DOA9**Ω**nodB	Insertional nodB of pDOA9 by integration of pVO155-nptII-GFP-nptII-cefo; cefor Kmr at SalI/XbaI	Songwattana et al., 2019
Eschericia coli S17-1	recA thi pro hsdR [res– mod+][RP4::2-Tc::Mu-Km::Tn7] **λ** pir phage lysogen, Smr/Tpr	Simon et.al., 1983

Table 4.2 List of primers used in this study for mutant construction and verification.

Construction			
Name	**Name of primer**	**Sequence 5'→ 3'**	**Relevant characteristics**
DOA9Ω*nodD1*	nodD1.int.f	TCTGTTGTCGACGAACGGCCGTGAACTAGTCTTG	Amplification of 256-bp internal fragment of the
	nodD1.int.r	CGCATCGTCTAGAGGCACCAGCTCGAATTTAATGC	*nodD1* and cloned into pVO155-nptII-GFP-nptII-cefo at the *sal*I/*Xba*I sites. This construction was used to obtain the DOA9Ω*nodD1* mutant by single crossing over.
DOA9Ω*nodD2*	nodD2.int.f	CATGCGGTCGACGGAGTTGATGCCCACACCACTC	Amplification of 239-bp internal fragment of the
	nodD2.int.r	CCGGCTTCTAGA CGAACGGCAGCAACTCGAAAC	*nodD2* and cloned into pVO155-nptII-GFP-nptII-cefo at the *sal*I/*Xba*I sites. This construction was used to obtain the DOA9Ω*nodD2* mutant by single crossing over.
DOA9Δ*nodA1*	up.nodA1.del.f	ACCGCCGGATCCAGGACTGCAGCTCATAGGCAACAC	Amplification of the upstream and downstream
	up.nodA1.del.r	TTGTCTGTCACGGAAGCTTACATGTCTCCTGCGCG TAGTCCGCGAATG	flanking DNA of the *nodA1*. The PCR fragments were merged by overlap xtension PCR and cloned into
	dw.nodA1.del.f	GGCAACACGTGCCACCGTGACAGACAA ACGACTACACTTCA	pNPTS129 at the *Bam*HI/*Xba*I sites. The cefotaxime resistance cartridge was then cloned in the *Hin*dIII
	dw.nodA1.del.r	CGCTCTTTCTAGACGCTGAGAGGATCGTGT AGGATGAAATC	site between the upstream and downstream fragments. This construction was used to obtain the DOA9Δ*nodA1* mutant by double crossing over.

Table 4.2 List of primers used in this study for mutant construction and verification (Continue).

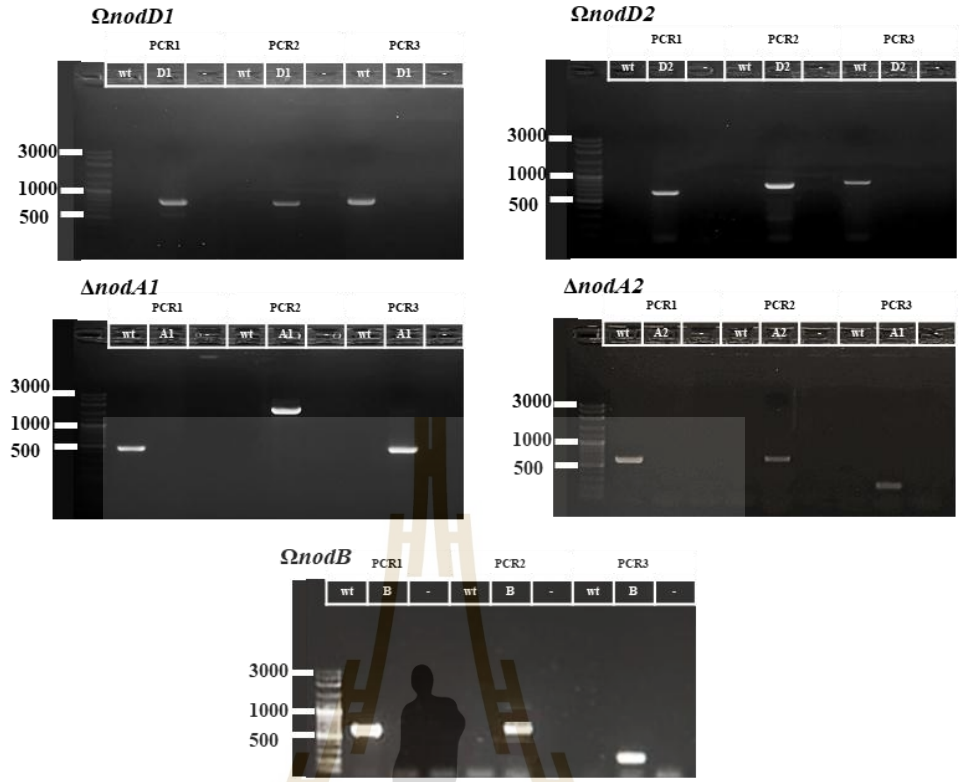
Construction			
Name	**Name of primer**	**Sequence 5'→ 3'**	**Relevant characteristics**
DOA9ΔnodA2	up.nodA2.del.f	TCGCCGGGATCCAGAACGACAGATAGGAACGATTTAC	Amplification of the upstream and downstream flanking DNA of the nodA2. The PCR fragments were merged by overlap xtension PCR and cloned into pNPTS129 at the BamHI/XbaI sites. The cefotaxime resistance cartridge was then cloned in the HindIII site between the upstream and downstream fragments. This construction was used to obtain the DOA9ΔnodA2 mutant by double crossing over.
	up.nodA2.del.r	GACTTCACAGCTCAAGCTTATGGCGCATGAATGCTCTCCTGCTTTC	
	dw.nodA2.del.f	TTCATGCGCCATAAGCTTGAGCTGTGAAGTCGCAAATCGCC	
	dw.nodA2.del.r	GCGACCTCTAGACCCACCAAGTAATCGATATAC	
DOA9ΩnodB	nodB.int.f	TGACTCGTCGACGACGCCGCACCTCTTGGATGTTTTAG	Amplification of 287-bp internal fragment of the nodB and cloned into pVO155-nptII-GFP-nptII-cefo at the sall/XbaI sites. This construction was used to obtain the DOA9ΩnodB mutant by single crossing over.
	nodB.int.r	GTTCCGTCTAGAGCCCATAGGGCGCACGTATATG	

Table 4.2 List of primers used in this study for mutant construction and verification (Continue).

Verification				
Name	**PCR set**	**Name of primer**	**Sequence 5'→ 3'**	**Relevant characteristics**
DOA9Ω*nodD1*	PCR1	Cefo. F	GCTATGGCACCACCAACGATATC	Amplification of pVO155-nptII-GFP-nptII-cefo plasmid insertion in the 256-bp internal fragment of the *nodD1.* The positive amplification of PCR1 and PCR2 was detected on DOA9Ω*nodD1*, while DNA fragment performed by PCR3 amplification was undetected.
		Ext-D1.r	CCATGCTCCTGCATATACCA	
	PCR2	Ext-D1.f	GCTTGCATTTCGTCGTGTTC	
		Gus.r	GCACAGCAATTGCCCGGCTTTCTTG	
	PCR3	Ext-D1.f	GCTTGCATTTCGTCGTGTTC	
		Ext-D1.r	CCATGCTCCTGCATATACCA	
DOA9Ω*nodD2*	PCR1	Cefo. F	CGATGCGTTCTGTTCCAAAC	Amplification of pVO155-nptII-GFP-nptII-cefo plasmid insertion in the 239-bp internal fragment of the *nodD2.* The positive amplification of PCR1 and PCR2 was detected on DOA9Ω*nodD2*, while DNA fragment performed by PCR3 amplification was undetected.
		Ext-D2.r	GCACAGCAATTGCCCGGCTTTCTTG	
	PCR2	Ext-D2.f	CGATGCGTTCTGTTCCAAAC	
		Gus.r	GCACAGCAATTGCCCGGCTTTCTTG	
	PCR3	Ext-D2.f	CGATGCGTTCTGTTCCAAAC	
		Ext-D2.r	GCACAGCAATTGCCCGGCTTTCTTG	
DOA9Δ*nodA1*	PCR1	Ext-A1.f	CGACCATGCGGGGAGAACAAAG	Amplification of the upstream and downstream flanking DNA of the *nodA1*. The PCR fragments were merged by overlap extension PCR and cloned into pNPTS129 at the *Bam*HI/*Xba*I sites. The cefotaxime resistance cartridge was then
		Ext-A1.r	GTGTGGCAGGCAATGTGGGATAG	
	PCR2	Ext-A1.f	CGACCATGCGGGGAGAACAAAG	
		Cefo.r	CCTCCCGACTGCCGCTCTA	
	PCR3	Cefo.f	GCTATGGCACCACCAACGATATC	

Table 4.2 List of primers used in this study for mutant construction and verification (Continue).

Verification				
Name	**PCR set**	**Name of primer**	**Sequence 5'→ 3'**	**Relevant characteristics**
		Ext-A1.r	GTGTGGCAGGCAATGTGGGATAG	cloned in the *Hin*dIII site between the upstream and downstream fragments. This construction was used to obtain the DOA9Δ*nodA1* mutant by double crossing over.
DOA9Δ*nodA2*	PCR1	Ext-A2.f	GGAGGTCTGCAGTCTTGCAT	Amplification of the upstream and downstream flanking DNA of the *nodA2*. The PCR fragments were merged by overlap extension PCR and cloned into pNPTS129 at the *Bam*HI/*Xba*I sites. The cefotaxime resistance cartridge was then cloned in the *Hin*dIII site between the upstream and downstream fragments. This construction was used to obtain the DOA9Δ*nodA2* mutant by double crossing over.
		Ext-A2.r	CCAACCGTCGTGTATTGATG	
	PCR2	Ext-A2.f	GGAGGTCTGCAGTCTTGCAT	
		Cefo.r	CCTCCCGACTGCCGCTCTA	
	PCR3	Cefo.f	GCTATGGCACCACCAACGATATC	
		Ext-A2.r	CCAACCGTCGTGTATTGATG	
DOA9Ω*nodB*	PCR1	Ext-B.f	CGGCTTCAGTGGAGAACGTGCTG	Amplification of pVO155-nptII-GFP-nptII-cefo plasmid insertion in the 287-bp internal fragment of the *nodB*. The positive amplification of PCR1 and PCR2 was detected on DOA9Ω*nodB*, while DNA fragment performed by PCR3 amplification was undetected.
		Gus.r	GCACAGCAATTGCCCGGCTTTCTTG	
	PCR2	Sm/Sp.f	GGTATGCGACAGAACATCCAC	
		Ext-B.r	GCACAGCAATTGCCCGGCTTTCTTG	
		Ext-B.f	GGTATGCGACAGAACATCCAC	
	PCR3	Ext-B.r	CGCACGTATATGTCTCAGCC	



	PCR1	PCR2	PCR3
DOA9Ω*nodD*1	Cefo.F/Ext.D1.R	Ext.D1.F/Gus.R	Ext.D1.F/Ext.D1.R
DOA9Ω*nodD2*	Cefo.F/Ext.D2.R	Ext.D2.F/Gus.R	Ext.D2.F/Ext.D2.R
DOA9Δ*nodA*1:*cefo*	Ext.A1.F/Ext.A1.R	Ext.A1.R/Cefo.R	Ext.A1.F/Cefo.F
DOA9Δ*nodA:2cefo*	Ext.A2.F/Ext.A2.R	Ext.A2.R/Cefo.F	Ext.A2.F/Cefo.R
DOA9Ω*nodB*	Cefo.F/Ext.B.R	Ext.B.F/Gus.R	Ext.B.F/Ext.B.R

Figure 4.1 PCR analysis of representative *nod* gene mutants (Ω*nodD1*, Ω*nodD2*, Ω*nodB*, Δ*nodA1* and Δ*nodA2* in *Bradyrhizobium* sp. DOA9. Genotypes were verified by PCR amplification with respective primers. The PCR product from the wild-type locus (lane 1), the mutant gene (lane2) and negative control (lane3). Ω*nodD1*, Ω*nodD2* and Ω*nodB* have used insertion mutation strategy while Δ*nodA1* and Δ*nodA2* were used deletion mutation strategy. Agarose gel electrophoresis results of PCR verification of mutant strains. Molecular weight marker is VC 100-bp DNA Ladder, Vivantis.

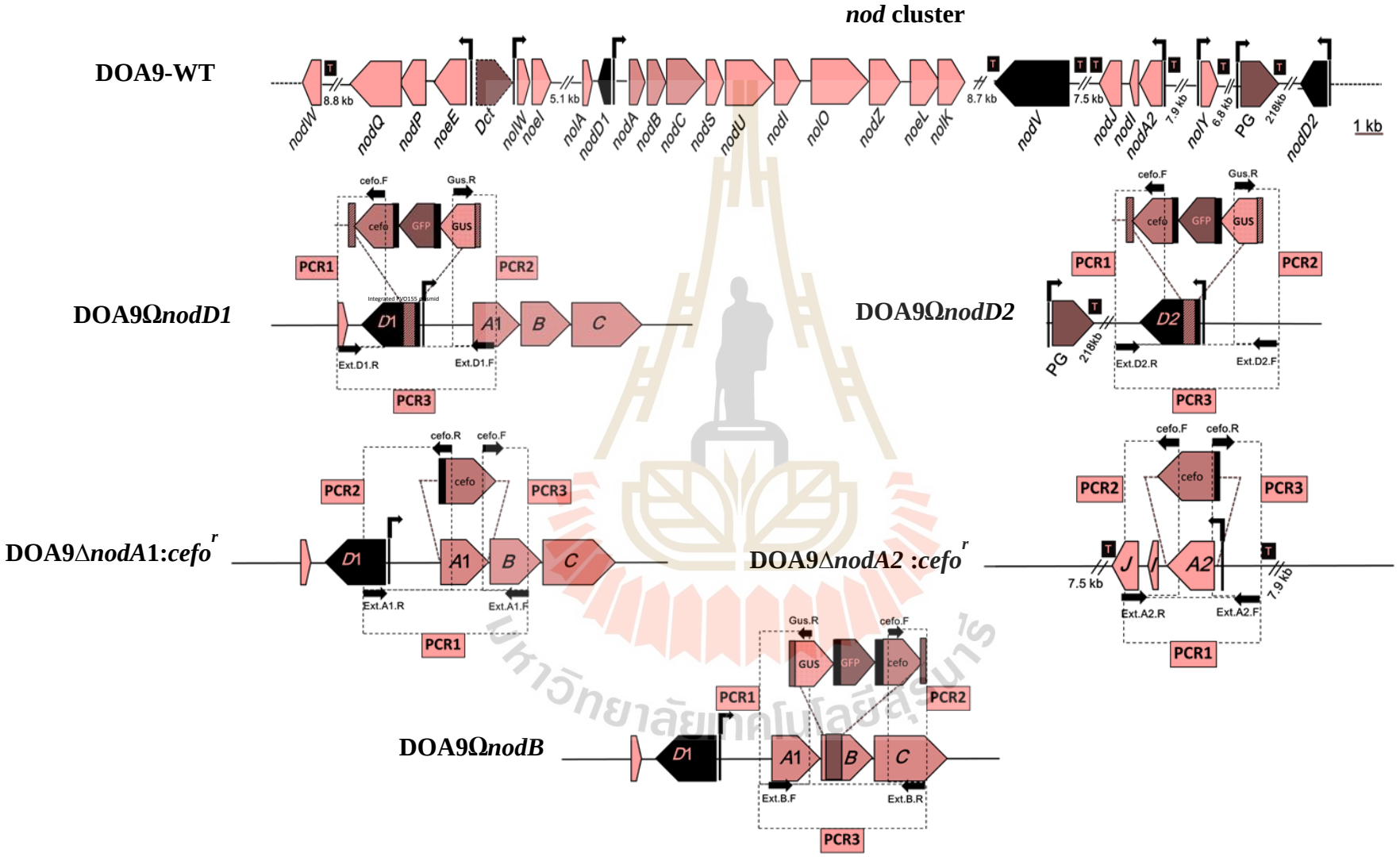

nod cluster
DOA9-WT
nodW
nodQ
nodP
noeE
Dct
nolW
noeI
nolA
nodD1
nodA
nodB
nodC
nodS
nodU
nodI
nolO
nodZ
noeL
nolK
nodV
nodJ
nodI
nodA2
nolY
PG
nodD2
8.8 kb
5.1 kb
8.7 kb
7.5 kb
7.9 kb
6.8 kb
218kb
1 kb
DOA9ΩnodD1
cefo.F
Gus.R
cefo
GFP
GUS
PCR1
PCR2
PCR3
D1
A1
B
C
Ext.D1.R
Ext.D1.F
DOA9ΩnodD2
D2
Ext.D2.R
Ext.D2.F
DOA9ΔnodA1:cefo^r
cefo.R
Ext.A1.R
Ext.A1.F
DOA9ΔnodA2 :cefo^r
J
I
A2
Ext.A2.R
Ext.A2.F
DOA9ΩnodB
Ext.B.F
Ext.B.R

Figure 4.2 Genetic organization of *nod* gene cluster of *Bradyrhizobium* sp. DOA9. The orientations and sizes of the ORFs are indicated by arrows; orange and orange grey present a common *nod* genes, black indicates putative regulatory *nod* genes, and dark gray indicates non-relative *nod* genes including C4-dicarboxylic acid (Dct) and Endo-polygalacturonase (PG). Location and orientation of *nod-box* motifs are shown by black rectangular arrows. Construction map of *nod* mutant strains were explained in the picture by indicating the binding site of specific primers that lists in the table of verification method.

4.4.3 Plant nodulation and symbiosis analysis

The symbiosis efficiency and the nodulation ability of DOA9 and mutants were tested using the legumes, including Dalbergioid tribe: *Aeschynomene americana, A. afraspera, Arachis hypogaea*, and *Stylosanthes hamata,* Milletioid tribe: *Marcoptilium artopurpureum*, and *Indigofera tinctoria* (Table 4.1). Seeds of *A. hypogaea* was surface sterilized in 95% ethanol for 10 sec before added 3% w/v sodium hypochlorite to immerse the seed completely. While the seeds of *A. americana, A. afraspera, S. hamata, I. tinctoria* and *M. artopurpureum* were surface sterilized in 95% ethanol for 10 sec before added 98% Sulfuric acid for the appropriate time (Teatimsong et al., 2014) until the pericarp of seeds were gone. After draining for 5 min, seeds were rinsed ten times with sterilized water and soaked overnight. The sterilized seeds were placed on the plate containing sterilized 0.8% (w/v) water agar and kept in the dark place for 1-2 days. The plants growth condition followed by Songwattana et al., (2017). Five days after planting, each seedling was inoculated with 1 ml of a cell suspension at approximate density of 1×10^8 cells/ml. Nodulation and nitrogen fixation abilities were measured after 21 days post-inoculation (dpi). Five plants were analyzed for the number of nodules and nitrogenase activity using acetylene reduction assay (ARA) as previously described (Bonaldi et al., 2010). Briefly, all nodules were collected from each plant and placed in headspace bottles with 10 % (v/v) of acetylene and incubated at 28 ºC for 1h. Gas chromatography was conducted to measure the peak height of ethylene and acetylene with 1 ml gas samples from the bottles using the same condition as mentioned above.

4.4.4 Extraction and Mass Spectrometry (MS) analysis of Nod factors

A 500 ml bacterial culture (OD=1) was induced by 5 mM genistein in final concentration prior to centrifuged at 8000 g for 45 min. One hundred fifty ml Butanol (ButOH) was added into the supernatant for 1h under stirring. The butanol phase was recovered and kept at 4°C. A second butanol extraction was performed on the water phase. One hundred fifty ml of water was added into the pooled butanol phases. After 1h, the butanol phase was recovered and dried under vacuum at 45°C. The resulting extract was resuspended with 100 ml water and 150 ml ethyl

acetate was added. After 15 min, the water phase was recovered and dried under vacuum at 45°C. The extract was resuspended in 50% Ethanol.

For chromatographic separation and mass spectral analysis, an Acquity H-Class UPLC system (degasser, quaternary gradient pump, autosampler, column thermostat and diode array detector) was used coupled with a Waters Synapt G2-S LC/MS system equipped with ESI ion source (Waters, Milford, USA). UPLC separations were achieved on a ACQUITY UPLC® BEH C18 column 1.7 µm (50 mm x 2.1 mm i.d.; Waters). The mobile phase consisted of solvent A (0.1 %, formic acid v/v, in water) and solvent B (0.1 % formic acid v/v in acetonitrile) with the following gradient: 0-3 min 5-35 % B, 3-6 min 35-100 % B, holding at 100 % B for 2 min, 8-10 min, 100-5% B. The column was maintained at 45°C and the injection volume was 1 µl. The solvent flow rate was 0.4 $ml.min^{-1}$, and column temperature was set at 25°C. Electrospray conditions were as follows: drying gas (N_2) temperature, 450°C; flow rate, 12 $l.min^{-1}$; nebulizer pressure, 6 bar (N_2); cone voltage, 30 V; capillary voltage, 3000 V. Full scan mass spectra were recorded in positive or negative ion mode over an m/z range of 50-1200.

4.4.5 Phylogenetic tree

The multiple sequence alignment of the *nodA* and *nodD* retrieved sequences was performed with the ClustalW software. Using the multiple sequence alignment of the ClustalW the phylogenetic trees were constructed by the Neighbor joining method in the Molecular Evolutionary Genetics Analysis (MEGA7) software (Kumar et al., 2016). It showed the relationships between two copies of *nodD1* and *nodD2* also *nodA1* and *nodA2* genes and the recognized species of the genus *Bradyrhizobium*. Bootstrap values after maximum-likelihood analysis were indicated in the nodes and branches, respectively. The amino acid alignment was performed by MultAlin program (Corpet, 1988).

4.4.6 Statistical analysis

One-way analysis of variance (ANOVA) followed by post hoc tests (Tukey's tests at $P \leq 0.05$) was performed using SPSS software (SPSS version 17.0 windows: SPSS Inc., Chicago, IL) (Bryman et al., 2011). P-values<0.05 were considered statistically significant.

4.5 Results

4.5.1 Gene organization and phylogenetic tree analysis of *nodA1*, *nodA2, nodD1* and *nodD2*

As deliniated in the genome of *Bradyrhizobium* sp. DOA9, pDOA9 comprised two different copies of *nodA* genes (*nodA1* and *nodA2*), a single of *nodB,* and *nodC* genes. The *nodA1* was found at the vicinity of the *nodBC* genes, and *nodA2* was found just upstream of the *nodIJ* genes. Two copies of *nodD* were presented on pDOA9, *nodD1* was upstream of *nodA1BCSUI* operon and *nodD2* located 0.27 Mb away from *nod* cluster (Okazaki et al., 2015) (Fig.4.3).

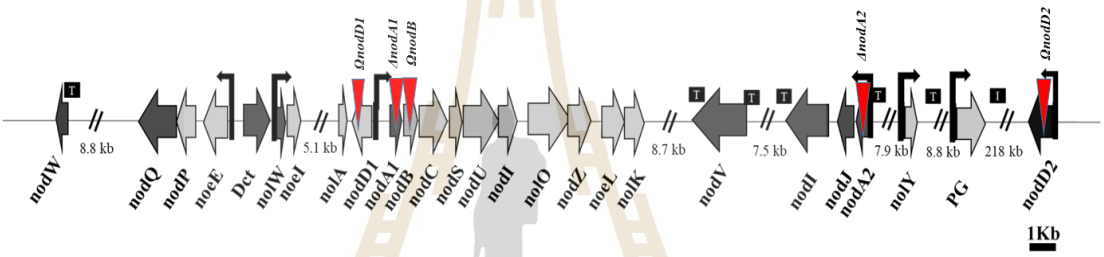


Figure 4.3 Genetic organization of *nod* gene cluster of *Bradyrhizobium* sp. DOA9. The mutation in the nodulation genes in *Bradyrhizobium* sp. DOA9, including Ω*nodB,* Ω*nodD1*, Ω*nodD2,* Δ*nodA1,* and Δ*nodA2.* T indicated as transposase. The ┐ indicated promoter. The red triangles above the genetic map represent the mutant used in this study. The // indicated as the distance of each genes.

The *nodA* (*nodA1* and *nodA2)* and *nodD (nodD1* and *nodD2)* phylogenetic tree was constructed and compared to other bradyrhizobial containing *nod* genes (Fig. 4.4). The *nodA1* is the outgroup of *nodA2.* The *nodA1* shares similarity with copies in other photosyntetic *Bradyrhizobium* strains, such as *Bradyrhizobium* sp. ORS285 which is able to establish *nod* factor-dependent or *nod* factor-independent symbiosis with *Aeschynomene* legumes (Gully, et al., 2017). *Bradyrhizobium* sp. ORS352 which is able to nodulate *A. afraspera*. Also, *Bradyrhizobium* sp. ORS287 which is able to nodulate *A. sensitiva* (Doignon-Bourcier et al., 2000). While *nodA2* shares similarity with other *Bradyrhizobium* such as *B.*

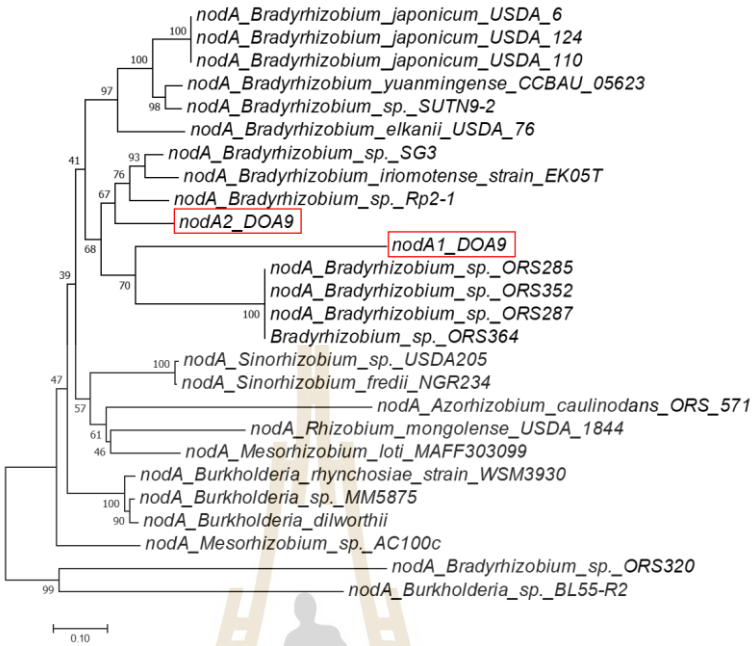
iriomotense EK05T was isolated from a tumor-like root of the legume *Entada koshunensis* from Iriomote Island in Japan (Islam et al., 2008). Phylogenetic analyses of the *nod* genes showed that common *nod* genes of DOA9 were not placed on classified branches of the known *nod* gene-containing rhizobia strains (Fig. 4.4A). The *nodD1* shares similarity with the copies in many other *Bradyrhizobium* strains, while the *nodD2* found in the same clade with *Bradyrhizobium* sp. ORS285 (Fig. 4.4B). The amino acid consensus of *nodD1* and *nodD2* shared high consensus color from MultAlin (Fig. 4.5B) with the percent identity 62.78%. While, the amino acid consensus of *nodA1* and *nodA2* were shared low consensus color each other from MultAlin (Fig. 4.5A) with the percent identity only 37.37%. These results implied that DOA9 maintain a double copies of common regulatory *nodD* and divergenet *nodA* genes on the symbiotic island. To clarify understand the function of each copies of common regulatory *nodD* and divergenet *nodA* genes in the legume nodulation and host specificity, mutation of individual copy of *nodD* and *nodA* was elucidated under symbiotic condition.

4.5.2 Effect of *nodD1* and *nodD2* mutation in different legumes

To determine the nodulation properties according to the function of regulatory transcription of *nodD1* and *nodD2* genes, insertion mutant strains (Ω*nodD1* and Ω*nodD2)* were inoculated into the variety of legumes species (Fig. 4.6). In *A. americana* (Fig. 4.6A) and *A. afraspera* (Fig. 4.6B), both mutants were not affect the nodulation and nitrogen fixation, but for the plant dry weight in *A. afraspera* was decreased when compared with the DOA9 WT. In *Aeschynomene* plant, the fuction of *nodD1* and *nodD2* could substitute each other. The insertion mutant of *nodD1* and *nodD2* was not affected to the plant dry weight and nitrogen fixation.

In *A. hypogaea,* the nodule number, plant dry weight and nitrogenase activity were not significantly different when compared between DOA9 with Ω*nodD1* and Ω*nodD2* (Fig. 4.6C). The nitrogenase activity of DOA9 was slightly higher 180 nmol ethylene/h/plant when compared with Ω*nodD1* and Ω*nodD2* approximately 120-170 nmol ethylene/h/plant. It seemed that full symbiosis of both plants required both *nodD1* and *nodD2*, and the regulations of each copy of *nodD* was the same manner.

A. *nodA*

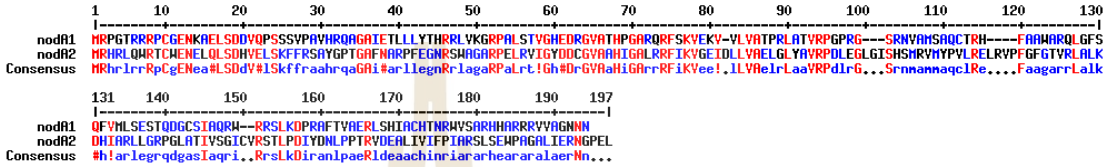


B. *nodD*

Figure 4.4 Neighbor-joining phylogenetic trees obtained from *nodA* (A) and *nodD* (B) protein sequences showing the relationships between 2 copies of *nodD* (*nodD1* and *nodD2*) and *nodA* (*nodA1* and *nodA2*) of *Bradyrhizobium* sp.

DOA9 with the representative *Bradyrhizobium* strains and the related rhizobial species using Kimura two parameter. Percentage at nodes refer to significant bootstrap values of 1000 replicates. The evolutionary distances were computed using the Maximum Composite Likelihood method in MEGAX.

A. *nodA*



B. *nodD*

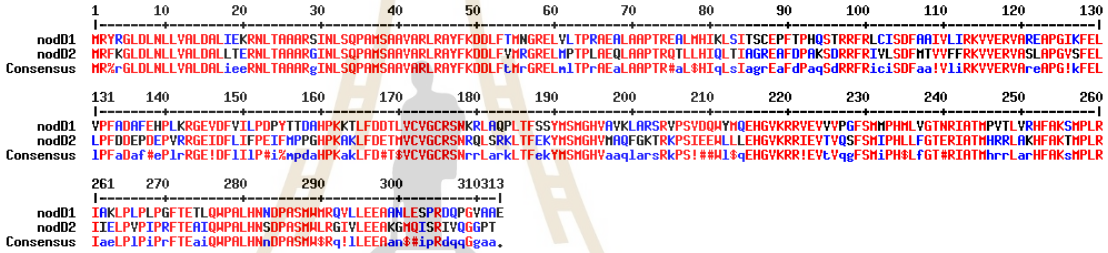


Figure 4.5 Amino acid sequence alignment between 2 copies of *nodA* (*nodA1* and *nodA2*) (A) and *nodD* (*nodD1* and *nodD2*) (B) derived from *Bradyrhizobium* sp. DOA9 was generated by MultAlin program (Corpet, 1988). Amino acid residues are color indicated according to their conservancy. Red represents a high consensus with 90% similarity, blue represents a low consensus with less than 50% similarity, and black means neutral consensus.

In case of *S. hamata* (Fig. 4.6D) and *I. tinctoria* (Fig. 4.6F), the nodule number, plant dry weight and nitrogenase activity were not significantly different when compared between DOA9 with **Ω***nodD1* and **Ω***nodD2*. The plant dry weight and nitrogenase activity reduced only in mutant **Ω***nodD2,* this result designated that the *nodD2* might played a major role as an activator of *nod* gene transcription in these plants. The plant dry weight of DOA9 was higher 9 mg/plant when compared with **Ω***nodD2* 7 mg/plant. Moreover, the nitrogenase activity of DOA9 was higher 0.7

nmol ethylene/h/plant than Ω*nodD2* 0.4 nmol ethylene/h/plant. In *I. tinctoria*, the plant dry weight and nitrogenase activity reduced only in mutant Ω*nodD2.* The plant dry weight of DOA9 was higher 0.58 mg/plant when compared with Ω*nodD2* only 0.45 mg/plant. Moreover, the nitrogenase activity of DOA9 was higher 0.55 nmol ethylene/h/plant than Ω*nodD* only 0.45 nmol ethylene/h/plant.

The results revealed that Ω*nodD1* and Ω*nodD2* mutant strains were not reduce the nodule number but significantly reduced the nitrogenase activity in *M. artopurpureum* (Fig. 4.6E). The nitrogenase activity was lower in Ω*nodD1* and Ω*nodD2* when compared with DOA9 WT, from 2.6 ethylene/h/plant in DOA9 WT reduce to 0.7-0.8 nmol ethylene/h/plant in Ω*nodD1* and Ω*nodD2*.

These results indicated that the *nodD1* and *nodD2* were required for the establishment of the symbiotic interaction of DOA9 with the host plants but mutation in one of *nodD1* or *nodD2* genes were not significantly affect the nodulation meaning that the *nodD1* and *nodD2* were functionally redundant.

4.5.3 Effect of *nodA1* and *nodA2* mutation in different legumes

To determine the nodulation according to the function of *nodA1* and *nodA2*, deletion mutant strains (Δ*nodA1*, Δ*nodA2)* were inoculated to the variety of legumes species compared with insertion *nodB* mutant strain (Ω*nodB*) which is nodulation lacking strain (Songwattana, 2016). The results revealed that Δ*nodA2* mutant strains lost their ability to induce nodules on all the plants tested (Fig. 4.7). These results indicated that *nodA2* was required for the establishment of the symbiotic interaction of DOA9 with the host plants and the *nodA2* was essential for NFs synthesis. To investigate the function of *nodA2*, the single crossed homologous recombination of *nodA2* was performed. The Ω*nodA2* was used in order to manage the selection process using spectinomycin 200 mg/ml antibiotic. For complementation process, the cefo 20 mg/ml was used as the antibiotic screening. The same antibiotic screening cefo 20 mg/ml was found in Δ*nodA2* and in complementation might bring difficulties in screening process. Thus, in this experiment used the Ω*nodA2* for complementation. Confirmation the *nodA2* function by complementation was tested in three plants which representatives from each group as the compatible and incompatible plant in DOA9, were showed that the nodulation in legumes were all fully restored (data not shown, Songwattana, Thesis 2016).

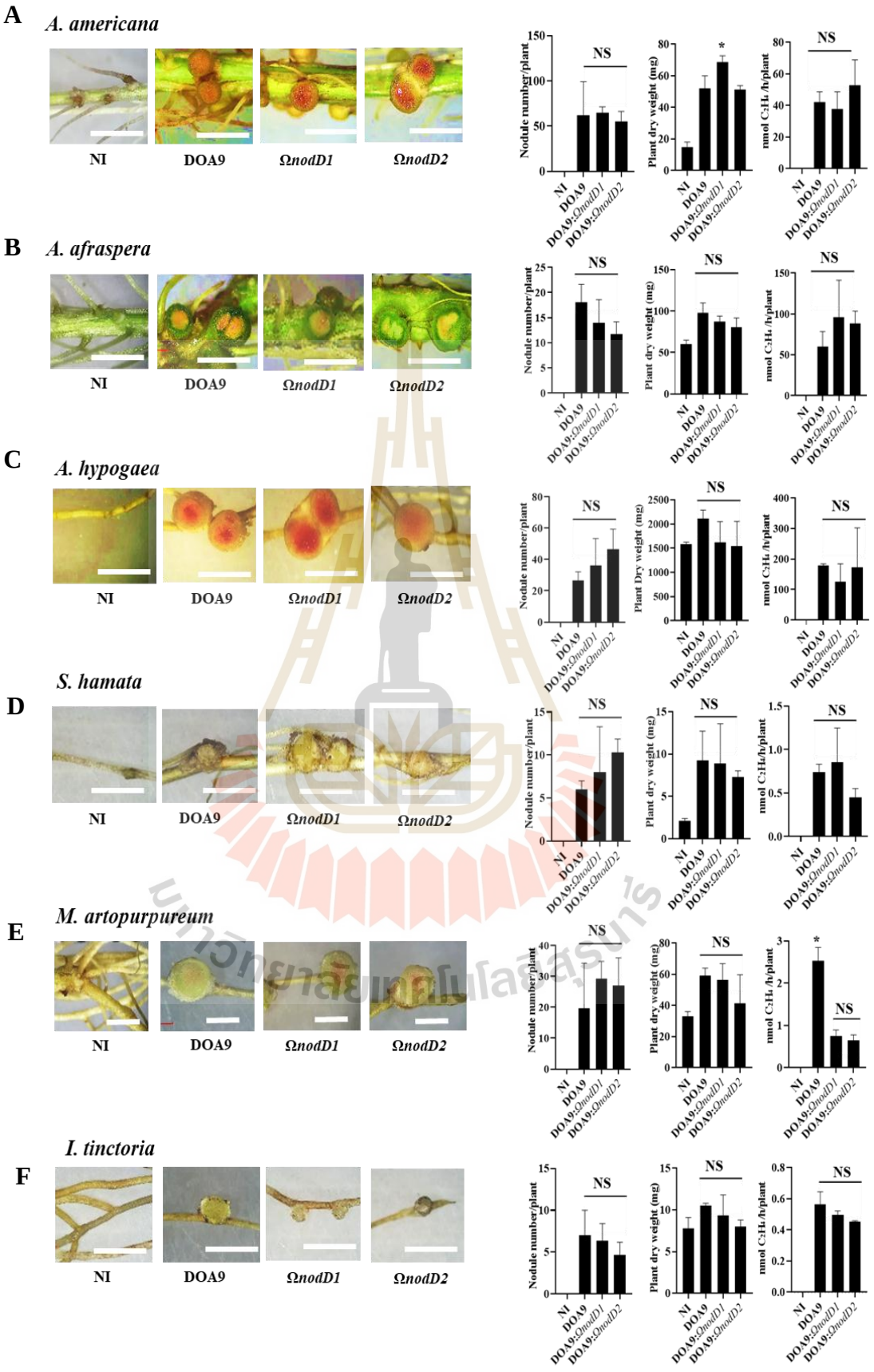

A
A. americana
NI
DOA9
ΩnodD1
ΩnodD2
Nodule number/plant
Plant dry weight (mg)
nmol C2H2 /h/plant
NS
*
B
A. afraspera
C
A. hypogaea
Plant Dry weight (mg)
D
S. hamata
E
M. artopurpureum
F
I. tinctoria
DOA9:ΩnodD1
DOA9:ΩnodD2

Figure 4.6 Function of regulator *nodD1* and *nodD2* in different legumes including: *A. americana* (A), *A. afraspera* (B), *A. hypogaea* (C), *S. hamata* (D), *M. artopurpureum* (E) and *I. tinctoria* (F). Plants were observed in the plant dry weight, nodule number/plant and nitrogenase activity (nmol C_2H_4/h/plant). The comparison of the nodule morphology and root nodules were observed with a stereomicroscope. Error bars represent standard deviation (SD) (n=5). NS above the error bars indicated not significantly difference at P<0.05 (Tukey's HSD test) while * above the error bars indicate significantly difference at P<0.05 (Tukey's HSD test). Scale bar in nodule pictures = 1mm.

Δ*nodA1* displayed a nodulation and a nitrogenase activity similar when compared with the WT. Those plants including *A. americana* (Fig. 4.7A) and *A. afraspera* (Fig. 4.7B) displayed the nodule number with the same as WT around 60 nodules/plant in *A. americana* and around 15 nodules/plant in *A. afraspera*, but slightly decrease in **Δ***nodA1* even not significantly different. For nitrogenase activity, the result was slightly decrease in **Δ***nodA1* when compared with DOA9 WT even not significantly different. *A. hypogaea* (Fig. 4.7C) from Dalbergioids tribe, **Δ***nodA1* displayed the nodule numbers higher than the DOA9 WT and was also exhibited higher nitrogenase activity. **Δ***nodA1* was not significantly reduce the nodule number and N_2 fixation in few plants including *M. atropurpureum* (Fig. 4.7E) from Milletioids tribe, *S. hamata* (Fig. 4.7D) from Dalbergioids tribe, and *I. tinctoria* (Fig. 4.7F) were displayed not significant reduction in nodule numbers and nitrogenase activity after deletion of *nodA1*. In *M. atropurpureum*, the nodule number derived from **Δ***nodA1* (11 nodules/plant) was not significantly lower when compared with the DOA9 WT (19 nodules/plant). The low nodule number of **Δ***nodA1* was also affected the nitrogen fixation ability only 1.8 nmol ethylene/h/plant lower than DOA9 WT 2.6 nmol ethylene/h/plant. Similar result in *S. hamata*, the nodule number derived from **Δ***nodA1* (4 nodules/plant) was lower when compared with the DOA9 WT (6 nodules/plant). The nodule numbers in **Δ***nodA1* were low and affected the nitrogen fixation ability 0.5 nmol ethylene/h/plant lower than DOA9 WT 0.7 nmol ethylene/h/plant (Fig. 4.7D).

To confirm the function of *nodA2*, a fragment of 1153-bp encompassing the complete *nodA2* gene and its upstream promoter region was cloned into replicative pMG103-npt2-cefo, annotate as *nod-box.nodA2*:pMG103-npt2-cefo^{r}. The recombinant plasmid *nod-box.nodA2*:pMG103-npt2-cefo^{r} was introduced into Ω*nodA2* which was conducted by using the pVO155-nptII-Sp/Sm^{r}-gusA-nptII-gfp plasmid for interrupting the internal fragment of the *nodA2,* annotate as Ω*nodA2*::NB-*nodA2*. The nodulation phenotype of Ω*nodA2* was similar to those of Δ*nodA2* by completely abolished the symbiosis with all plants tested. After complementation, Ω*nodA2*::NB-*nodA2* was investigated the nodulation abilities with three representative plants from each group as the compatible and incompatible plant in DOA9, including *A. americana* and *A. hypogaea* as compatible plant, and *M. atropurpureum* as incompatible plant. The result showed that Ω*nodA2*::NB-*nodA2* was completely restored the nodulation properties in these legumes tested (Data not shown, Songwattana Thesis., 2016). This suggested that DOA9 used *nod*-dependent strategy to endosymbiosis with leguminous plants and *nodA2* plays a main role for the establishment of the symbiotic interaction of DOA9 with the host plants. Moreover, *nodA2* could require for the NFs biosynthesis. Thus, NFs structure produced from Δ*nodA1* and Δ*nodA2* were analyzed comparing with NFs derived from DOA9 WT.

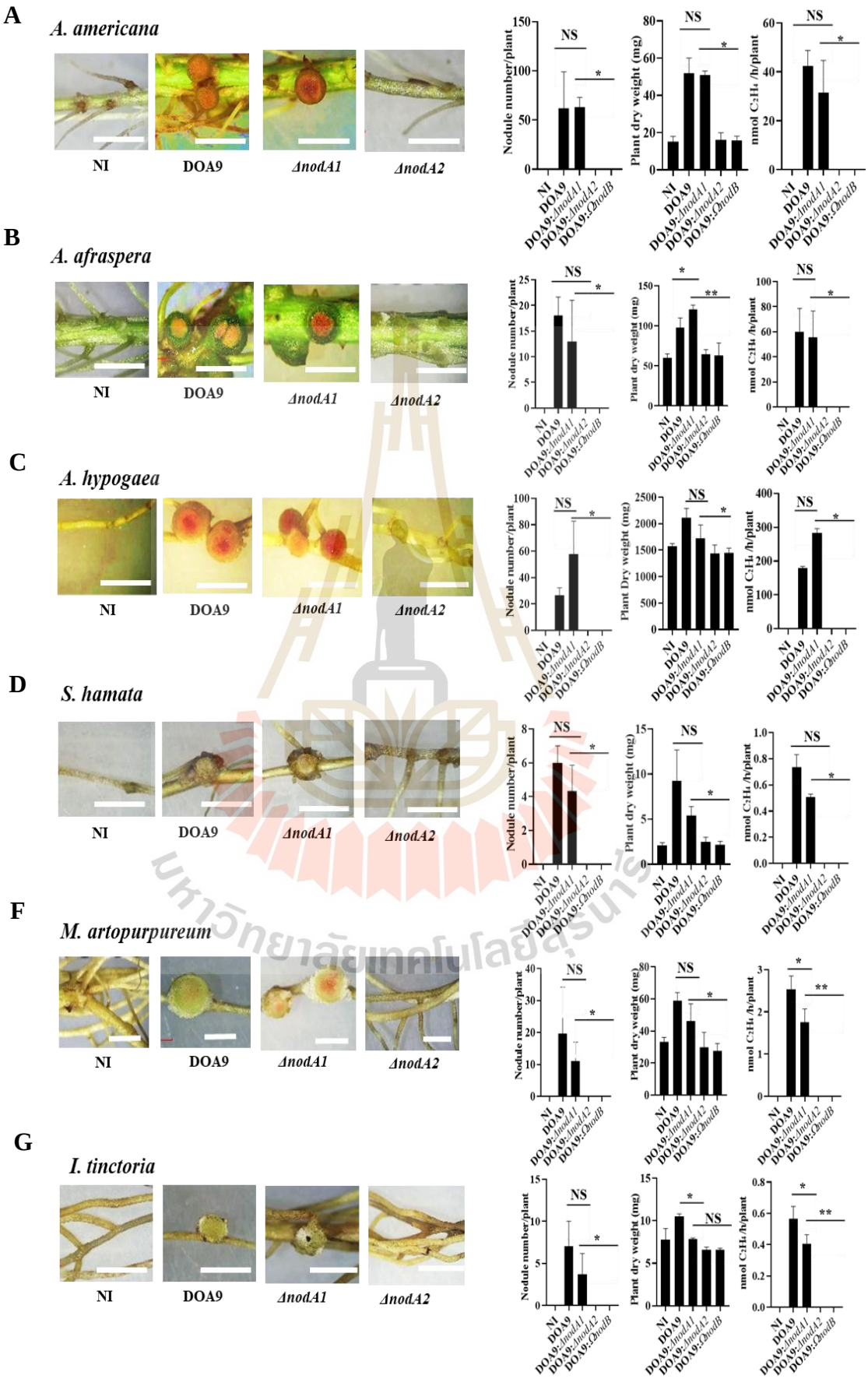

A
A. americana
NI
DOA9
ΔnodA1
ΔnodA2
B
A. afraspera
C
A. hypogaea
D
S. hamata
F
M. artopurpureum
G
I. tinctoria
Nodule number/plant
Plant dry weight (mg)
Plant Dry weight (mg)
nmol C2H4 /h/plant
NS
DOA9:ΔnodB

Figure 4.7 Function of regulator *nodA1* and *nodA2* in different legumes including: *A. americana* (A), *A. afraspera* (B), *A. hypogaea* (C), *S. hamata* (D), *M. artopurpureum* (E) and *I. tinctoria* (F). Plants were observed in the plant dry weight, nodule number/plant and nitrogenase activity (nmol C_2H_4 /h/plant). The comparison of the nodule morphology and root nodules were observed with a stereomicroscope. Error bars represent standard deviation (SD) (n=5). NS above the error bars indicated not significantly difference at P<0.05 (Tukey's HSD test) while * above the error bars indicate significantly difference at P<0.05 (Tukey's HSD test). Scale bar in nodule pictures = 1mm.

4.5.4 Structure of Nod Factors (NFs)

The LC-MS analysis of the DOA9 flavonoid induced extract showed that major peak was at m/z = 1594.7. It has been identified as the NFs build by five N-Acetyl Glucosamine (NAG) residues, bearing a N-methyl group, a vaccenic acid and two carbamoyl groups at the non-reducing end, and a sulphated methylfucose on the reducing end (NodDOA9(V, C18:1, NMe, Cb, Cb, SMe-Fuc), identical to the major NF of the *Rhizobium* sp. NGR234 (Price et al, 1992). The MS/MS analysis of this molecule revealed a fragmentation representative of NFs with sequencial mass of 203 due to the successive loss of NAG (Fig. 4.8, Table 4.3). A number of NF derivatives have been identified at m/z = 1596.7 (the acyl group is C18:0), 1391.7 (four NAG residues instead of five), 1580.7 and 1582.7 (loss of a methyl group on NF with C18:1 or C18:0 acyl chain) and 1551.7 (loss of a carbamoyl group) (Table 4.3). Analysis of the *nodA2* mutant revealed that no NFs were detected neither in the conditions with and without induction of flavonoid. In the Δ*nodA1*, NFs were found but the main molecule identified was at m/z = 1596.7 (NodDOA9(V, C18:0, Cb, Cb, SMe-Fuc) and the NF bearing the C18:1 acyl group was not detected.

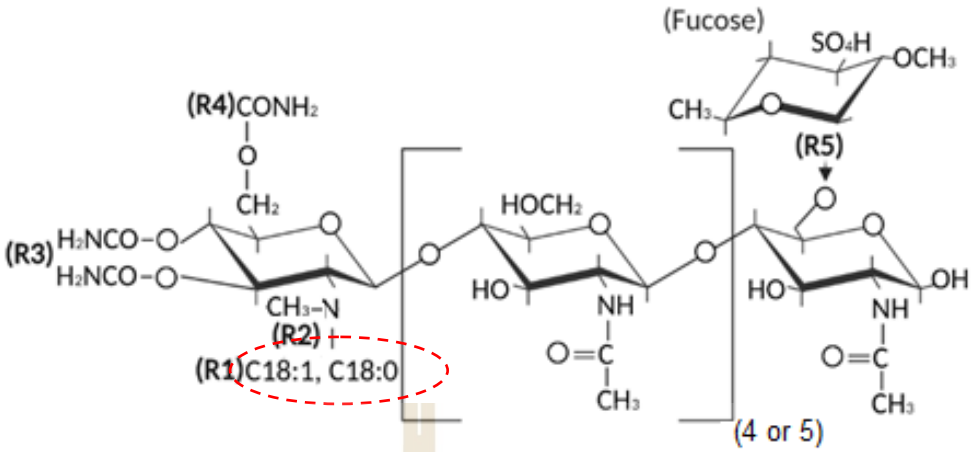


Figure 4.8 Nod Factors structure and derivatives produced by *Bradyrhizobium* sp. DOA9. NF structures of *Bradyrhizobium* sp. DOA9 represented with the number of GlcNAc residues 4 or 5 in the backbone. R1 represents the type of fatty acid, identified as saturated or unsaturated fatty acids (C18:1, C18:0) in the red circle, and chemical substitutions: N-methyl group at glucosamine non-reducing residue (R2), carbamoyl (R3 and R4), methylfucosyl and sulfate (R5).

Table 4.3 The structure of Nod factor and derivatives produced by *Bradyrhizobium* sp. DOA9. NF structures are represented with the number of GlcNAc residues in the backbone. R1 represents the type of fatty acid, identified as saturated or unsaturated fatty acids (C18), and chemical substitutions: N-methyl group at glucosamine non-reducing residue (NMe), carbamoyl (Cb), methylfucosyl (MeFuc) and sulfate (S).

$[M - H]^-$ (*m/z*)	GlcNAc residues	R1	R2	R3	R4	R5
1594.7	5	C18:1	NMe	Cb	Cb	SMeFuc
1596.7	5	C18:0	NMe	Cb	Cb	SMeFuc
1391.7	4	C18:1	NMe	Cb	Cb	SMeFuc
1580.7	5	C18:1	H	Cb	Cb	SMeFuc
1582.7	5	C18:0	H	Cb	Cb	SMeFuc
1551.7	5	C18:1	NMe	H	H	SMeFuc

4.6 Discussion

4.6.1 The divergent *nod* genes of DOA9 affect the degree of specificity in symbiosis

The importance of bradyrhizobial *nodD* genes were produce transcriptional regulators together with appropriate inducer flavonoid compounds, activate the other symbiotic nodulation (*nod*) genes and initiate the nodule formation process. *Bradyrhizobium* sp. DOA9 produce two copies of *nodD1* and *nodD2*, where *nodD1* is upstream of *nodA1BCSUI* operon and *nodD2* is located 0.27 Mb away from *nod* cluster. Some *Bradyrhizobium* and *Rhizobium* produce few copies of *nodD* genes with different function. Subjecting to the species, the copy number of *nodD* genes is varied. In *R. leguminosarum* bv. *trifolii* which has only one copy number of *nodD,* the mutation in this gene resulting in a loss in nodulation (Hungria et al., 1993). The different results showed in *S. meliloti, R. leguminosarum* bv. *phaseoli* and *B. japonicum* which was present more than one copy number of *nodD*. The mutation of one copy of *nodD* from multiple copies did not fully repressed the nodulation and nitrogen fixation (Garcia et al., 1996; Broughton et al., 2000). The *nodD1* of *R. tropici* strain CIAT 899 played a major role in nodulation (van Rhijn et al., 1993). *R. tropici* strain CIAT 899 is broad host range with high tolerance of abiotic stresses and carrying 5 copies of *nodD*. The function of each copy of *nodD* was different. Example: *nodD3* is an activator of *nodD1,* both in the presence of apigenin and salt stress. In contrast, *nodD4* might be an inducer with apigenin and a repressor under saline stress, whereas *nodD5* was an inducer under both conditions (del Cerro et al., 2015), as *nodD1* mutants failed to form nodules with *Lotus burtii*, whilst *nodD2* or *nolR* inactivation led to an extension of host range to *L. japonicus* Gifu (Acosta-Jurado et al., 2019).

PB strain *Bradyrhizobium* sp. ORS285 able to perform nodulation in NF-dependent and NF-independent *Aeschynomene* species. In *A. afraspera* (NF-dependent legume), deletion of both *nodD1* and *nodD2* was affect on the symbiosis, but was not affect the symbiotic interaction between ORS285 and *A. indica* (NF-independent legume) symbiosis. Moreover, when the expression of *nod* genes was artificially induced by adding naringenin to the plant growth medium, the nodulation

of *A. indica* was delayed and resulted in lower nodule numbers. Meaning that the presence of *nod* genes in NF-independent pathway hinders the symbiosis (Nouwen et al., 2016).

In the case of *Bradyrhizobium* sp. DOA9, the result showed that mutation in one copy of *nodD* was not affect with the nodule number, plant dry weight and nitrogen fixation ability except in *M. artopurpureum*. It is possible that their function is alternate and in order to stop the nodulation completely, it needs the double mutation in both copies of *nodD* from DOA9. Besides *nodD* genes, the nodulation in siratro (*M. atropurpureum*) relies on *nodV/W* in the chromosome of DOA9. This gene positively regulate the transcription of one or several unknown genes involved in the nodulation. It has been proposed that *nodV/W* recognizes different host flavonoids and combined with *nodD,* it may increase Nod-signal synthesis (Siqueira et al., 2014). However, in siratro possibly required two copies of *nodD1* and *nodD2* to interact with *nodV/W* for fully function of nodulation and nitrogen fixation.

Furthermore, the *nodD* genes could induce the expression of *ttsI* which trigger type III secretion system (T3SS) expression as an alternative infection processes to T3SS-dependent plant such as *A. hypogaea* and *M.artopurpureum* in DOA9 (Songwattana et al., 2017), **Ω***rhcN* (T3SS) showed repress the plant defense reactions resulting an increasing in the nodule number and efficiency of nitrogen fixation. The *nodD1* and *nodD2* possibly regulated the T3SS in the same manner, thus the mutation in single copy of *nodD1* or *nodD2* were not displayed any different effect in T3SS-dependent plant.

The multiple copies of *nodA* genes were found in pDOA9 as characteristic that may contribute to the broad host range of this strain. The importance for *nodA* as acylated of Nod factor-backbone chitin oligosaccharide structure. It has been designated that the nature of the Nod factor acyl group attached by *nodA* can contribute to the determination of host range (Debelle F et al., 1996). In *R. tropici* CIAT 899 and *Rhizobium* sp. PRF 81 possess multiple copies of *nodA,* for example in symbiotic plasmid *R. tropici* CIAT 899 produce three copies of *nodA* which were not functional redundant copies. The function of *nodA1* and *nodA2* were directed the biosynthesis and incorporation of ***α,β***-unsaturated acyl

chains of NF, while *nodA3* was not provide any indication of its acyl chain specificity. Additional and divergent *nodA* genes likely expand the diversity of NF acyl chains and may contribute to widening the host range in these strains (Ormeño-Orrillo et al., 2012). Transfer of *nod* genes between rhizobial strains may allow the recipient to colonize natural hosts of the donor strain. For example, the transfer of *nod* genes from *S. meliloti* plasmid permits the formation of fix⁻ nodules with *Medicago sativa* in the original host and the deletion of essential *nod* genes in *R. leguminosarum* lead to lost nodulation ability (Debellé et al., 1988; Walker et al., 2020).

The main symbiotic function of *nodA* gene in pDOA9 was not found in the common *nod* gene cluster but found separately in the upstream of the *nodIJ* genes which indicated as *nodA2*. The *nodA2* gene might function as the first signaling molecule (NFs) where mutation of this gene led to a complete absence of nodule formation in all plants tested. From the analysis of NF structure showed that mutation in *nodA2* aborting the biosynthesis of NF in the condition with and without flavonoid inductions. The confirmation of crucial role of *nodA2* in nodulation was proven by complementation (Songwattana., 2016). The result displayed the complementation of *nodA2* in DOA9, the ability of DOA9 to nodulate all legumes plant test was restored (data not shown). Whilst *nodA1* was not function and provide any indication of its acyl chain specificity in all plant tested. From the analysis of NF structure showed that mutation in *nodA1* resulting the NF structure lost of C18:1 acyl group. However, the main NFs molecule was similar between **Δ***nodA1* with WT. We postulate perhaps the mutation causing reshuffling of structural NF. The amino acid consensus of *nodA1* and *nodA2* shared low similarity, the difference in *nodA* proteins display different specificity for the acyl chains (Debellé et al., 1996; Ritsema et al.,1996) and effect with the *nodA* structure which consequently affect the amino acid sequence of *nodA.*

The *nodA1* gene is located with *nodBCSUI* operon and close to *nodD1* which encoded the synthesis the core of NFs upon induction flavonoids. The *nodA1* implied in unsaturated fatty acid in NFs structure. ***Δ****nodA1* under flavonoid induction resulting the NFs structure without unsaturated fatty acid. In DOA9, the changes of unsaturated fatty acid to saturated fatty acid in acyl group which incorporate to NFs. The *nodA2* gene, which is shared same operon with *nodA2IJ* and found distant to

nodD2, implied this *nod* gene is the crucial gene which encoded the NFs synthesis. Δ*nodA2* under flavonoid induction resulting no any NFs structure was synthesis. Moreover, no other symbiotic-related genes have been identified in its vicinity. Based on phylogenetic tree, the distant range between *nodA1* and *nodA2* possibly they recruited in different time by DOA9 during evolution for broadening the host range and increasing specificity in DOA9-legume symbiosis. Since the *nodA1* similar with many *nodA* from PB strain, it is possible the DOA9 acquired this *nod* gene from previous photosynthetic bradyrhizobia, while *nodA2* more similar with non-PB strain (Fig. 4.4A). The *nodA1* gene is more conserved at the nucleotide sequence level between these different species and biovars than the *nodA2*. However, *nodA1* are not functionally equivalent and are important determinants of host specificity in DOA9. After evolution, *nodA* may duplicate themselves but one of them may not function because of frame shift mutation and later on the *nodA2* my recruit from another strain to fulfill the symbiotic function. The broad host range properties from symbiotic pDOA9 may derived from many copies of *nod* genes and also the NF structure.

4.6.2 DOA9 Nod factor Structures (NFs)

Bradyrhizobium sp. DOA9 is a broad host range that effectively nodulates several legumes. The DOA9 NF was found to have a high similarity with NGR234 strain (Price et al., 1992). Nod factors are lipo-chitooligosaccharides (LCOs), which consist of a polymer of three to five N-acetyl glucosamine (GlcNAc) residues (the chitin backbone) with β-(1,4) linkages modified with a long-chain fatty acyl group and various other functional groups (Dénarié, et al, 1996). Although NF share the same basic structure of a chitooligosaccharide (CO) chain connected to a fatty acid, they can be greatly altered by the bacteria once face with the host flavonoid. This has given rise to an exceptional diversity of NFs across rhizobial species (Long, 1996). Most rhizobia rely on LCOs to associate with their legume hosts and legumes can perceive LCOs down to 10-14 mol/L concentrations (Sun, et al. 2015; Kidaj et al., 2020).

The NFs variations usually found in the degree of chitooligosaccharide polymerization, the nature of the fatty acid and modification in both non-reducing and reducing terminal (Mergaert et al., 1997). As result in Δ*nodA1,* substitutions on the fatty acid chain from both saturated and unsaturated fatty acid in DOA9 WT

becoming saturated fatty acid only in Δ*nodA1* was not affect in the host specificity with different legumes in this experiment. However, this result may differ if test with many other tribes of legumes plant, because every plant has their own specificity with their symbiont which defines the *nod* genotype. NF structure analysis from many rhizobia found that there is correlation between the type of NF synthesis and host range. However, the requirement for NFs structure are not always strict for example the case of Δ*nodA1* in DOA9. Another example *Phaseolus* sp. can be nodulated by rhizobia producing NFs with different structures. NFs are not the only determinant for broaden the host range, flavonoid signal induction, rhizobial surface components (LPS, KPS, EPS) (Wang et al., 2018) or secreted protein effectors (T3SS/T4SS) (Songwattana et al., 2017) interacting with their plant receptor also play a role in partner choice.

The proteins encoded by the *nodABC* genes are central in NF synthesis and exist in all nodulating rhizobia. Δ*nodA2* resulting abolished NFs synthesis completely. The NFs perception and signaling pathway induce several responses including ion fluxes, Calcium oscillation, nodulins expression and the reorganization of cytoskeleton. However, the concentration needed to induce the downstream signaling require very low concentration of NFs with compatible receptors legume plants (F Debellé, 2013). From the result of NFs structure, no any signal could be detected, meaning that not even low concentration of NFs could produce in Δ*nodA2* under flavonoid induction. Δ*nodA2* indicated a role as first signaling of NFs structure synthesis and as main structure of NFs backbone. Moreover, the presence of *nodA1* was not equivalent function if compared with *nodA2*. Moreover, the Δ*nodA2* was not affect the expression of downstream gene *nodIJ*, which encode the ATPase and permease of an ABC-type transport system (data not show). The *nodIJ* found in all NF producing rhizobia, and play a role in NF secretion (Downie, 1998).

DOA9 has similar NFs structure with *Rhizobium* sp. NGR234 which possess the broad host range feature. NGR234 synthesis NFs with one or two carbamoyl groups, a palmitoyl or stearoyl fatty acid at the nonreducing end and a 2-O-methylfucosyl branch that is either unsubstituted, sulphated or acetylated at the reducing end which resulting the rich variation in NFs structure (Price et al., 1992). While, the DOA9 possess five N-Acetyl Glucosamine (NAG) residues, bearing a N-

methyl group, a vaccenic acid and two carbamoyl groups at the non-reducing end, and a sulphated methylfucose on the reducing end. Those similar feature of NFs structure between DOA9 and NGR234 may as indicator for broad host range ability. NFs structure of DOA9 core part is controlled by common *nodABC* genes, while the decoration via additional chemical group in the reducing terminus of the oligo-chitin backbone which is a sulphated methylfucose similar with NGR234. In strain NGR234, these decorations encode by *nodH* and *noeE* served as sulfotranferases which modify NF with a sulfate group at the reducing terminus and *nodPQ* as the special donor of sulfate group. Those target for action of the broad group of bacterial enzymes (Tikhonovich and Provorov, 2007). The *noeE* gene in few rhizobia including *R. fredii* USDA257 was important for nodulation in few plants *Calopogonium caeruleum* and mutation in *noeE* abolishes the production of sulphated LCOs thus failed to nodulate *Pachyrizus tuberosus* (Hanin et al., 1997). The DOA9 possibly produce several structure of NFs in response with flavonoid as a signal molecule from legume host in order to broaden the host range. Since DOA9 has additional *nod* genes in the chromosome and plasmid which possibly expected that extra chemical groups affect in the NFs structure synthesis and has it roles as broadening the host range. NFs structures and functions are complex, the broad-host-range NGR234 strain produce approximately 18 different LCOs and all host specific parameters in their chemical structures. *B. japonicum* produce a mixture of tetrameric and pentameric NF which showed different function, the first in charge only for triggering the host receptor and second for autoregulation of the *nod* gene activities (Tikhonovich and Provorov, 2007). It is important to note that in DOA9, the *nodA2* was the one of main gene for synthesis the core structure of NFs, and the host specificity based on the decoration in the core structure with additional chemical groups encode by many other *nod* genes in the chromosome and plasmid.

4.7 Conclusion

The symbiosis compatibility between *Bradyrhizobium* sp. DOA9 with several legumes host is determined by the divergent of *nod* genes in pDOA9. Analysis of *nodD1, nodD2, nodA1* and *nodA2* sequence together with additional NF structure

determination allow us to follow the evolution of NF structures in the course of the legume-*rhizobium* co-evolution. The regulation of *nod* genes expression was controlled by *nodD1* and *nodD2* which are redundant in functions. NFs analysis showing that in DOA9 WT the main structure was (NodDOA9 (V, C18:1, NMe, Cb, Cb, SMe-Fuc), while the *nodA1* is required for the synthesis of the C18 polyunsaturated chains in NFs structure. The *nodA1* was important for the Nod factor N-acyl moiety. However, the changes of fatty acid chain was not affect to the nodulation with legume test. The *nodA2* was important as first signaling of NFs structure synthesis, while **Δ***nodA2* abort all nodulation in legume test and no any NFs structure were synthesis. By studying each function of *nod* genes, it will extend the knowledge that divergent *nodA* genes are likely to expand the diversity of NF acyl chains and the *nodA2* as main regulator for NFs synthesis.

4.8 References

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APPENDIX

Appendix 1 Yeast Extract Mannitol (YEM) medium (Somasegaran and Hoben, 1994)

D-Mannitol	10	g
K2HPO4	0.5	g
MgSO4·7H2O	0.2	g
NaCl	0.1	g
Yeast Extract	0.5	g
Distilled Water	1.0	liter
Adjust pH to 6.8 with 0.1 N NaOH		

Appendix 2 LB medium (Bertani, 1951)

Tryptone	10	g
Yeast extract	5	g
NaCl	10	g
Distilled Water	1	l

Appendix 3 Glucuronidase (GUS) assay solution

20 mg·ml-1 X-Gluc in N, N-Dimethylformamide	40.0	µl
SDS	20.0	mg
Methanol	2.0	ml
1 M Sodium phosphate buffer	0.2	ml
Distilled Water	7.76	ml

Appendix 4 N-free Nutrient Solution (Broughton and Dillworth, 1971)

Stock Solutions	Elements	Chemical Formula	MW	g/liter
1	Ca	$CaCl_2.2H_2O$	147.03	294.1
2	P	KH_2PO_4	136.09	136.1
3	Fe	Fe-citrate	355.04	6.7
	Mg	$MgSO_4.7H_2O$	246.5	123.3
	K	K_2SO_4	174.06	87.0
	Mn	$MnSO_4.H_2O$	169.02	0.338
4	B	H_3BO_3	61.84	0.247
	Zn	$ZnSO_4.7H_2O$	287.56	0.288
	Cu	$CuSO_4.5H_2O$	249.69	0.100
	Co	$CoSO_4.7H_2O$	281.12	0.056
	Mo	$Na_2MoO_2.2H_2O$	241.98	0.048

BIOGRAPHY

Mrs. Dyah Wulandari was born on August 18, 1990 in Pati, Semarang, Indonesia. She graduated with a Bachelor Degree of Biology in Diponegoro University in 2014. Later then she continue internship in SanDiego State University, California in 2014-2015. After return to Indonesia, she continue to work as research assistant in Molecular and Applied Microbiology Laboratory (CORES-DU), Diponegoro University, Indonesia. She received SUT-ASEAN Scholarship Phase-II in 2016 to continue study Master-Doctoral degree combined under Supervised Prof. Dr. Neung Teaumroong. Her topic research was "Symbiotic contribution of non-nodulating *Bradyrhizobium cosmicum* S23321 after transferring of the symbiotic plasmid pDOA9."