CHARACTERIZATION OF GENES INVOLVED IN NITROGENASE SYNTHESIS AND REGULATION LOCATED ON BOTH CHROMOSOME AND PLASMID OF *Bradyrhizobium* sp. DOA9

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การศึกษาคุณสมบัติของยีนที่เกี่ยวข้องกับการสังเคราะห์ และการควบคุมการทำงานของเอนไซม์ในโตรจีเนสที่อยู่บนโครโมโซม และพลาสมิดของเชื้อแบรดี้ไรโซเบียมสายพันธุ์ DOA9

นางสาวเจนจิรา วงษ์ดี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

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

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เจนจิรา วงษ์ดี : การศึกษาคุณสมบัติของยีนที่เกี่ยวข้องกับการสังเคราะห์ และการควบคุม การทำงานของเอนไซม์ไนโตรจีเนสที่อยู่บนโครโมโซม และพลาสมิดของเชื้อแบรดี้ไรโซเบียมสายพันธุ์ DOA9 (CHARACTERIZATION OF GENES INVOLVED IN NITROGENASE SYNTHESIS AND REGULATION LOCATED ON BOTH CHROMOSOME AND PLASMID OF *Bradyrhizobium* sp. DOA9)
อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร. พรรณลดา ติตตะบุตร, 141 หน้า.

เชื้อแบรดี้ไรโซเบียมสายพันธุ์ DOA9 ประกอบด้วยดีเอ็นเอจำนวน 2 ชุด คือ โครโมโซม และเมกะพลาสมิดอย่างละ 1 ชุด การวิเคราะห์ลำดับเบสแสดงให้เห็นว่า เชื้อชนิดนี้มียีนที่ใช้ในการสังเคราะห์โครงสร้างหลักของเอนไซม์ไนโตรจีเนสอยู่ทั้งบนโครโมโซม (*nifDKc*) และบนเมกะพลาสมิด (*nifDKp*) อย่างละ 1 ชุด และพบยีนที่ใช้ในการควบคุมการแสดงออกของยีนอื่น ๆ ที่เกี่ยวข้องกับการทำงานของเอนไซม์ไนโตรจีเนสอยู่บนโครโมโซม (*nifAc*) และบนเมกะพลาสมิด (*nifAp*) อย่างละ 1 ชุด เช่นกัน นอกจากนี้ยังพบยีน *nifV* ที่ใช้ในการสังเคราะห์เอนไซม์ homocitrate synthase จึงสันนิษฐานได้ว่าเชื้อ DOA9 สามารถตรึงไนโตรเจนเมื่อเจริญในสภาวะแบบอิสระได้ ดังนั้นวิทยานิพนธ์นี้จึงมีวัตถุประสงค์เพื่อตรวจสอบบทบาทหน้าที่ของยีนต่าง ๆ ดังกล่าวข้างต้น ที่เกี่ยวข้องกับความสามารถในการตรึงไนโตรเจนในเชื้อ DOA9 ภายใต้สภาวะการเจริญแบบพึ่งพาอาศัยกับพืช และแบบอิสระ ทั้งนี้การทดลองเพื่อติดตามการแสดงออกของยีนโดยใช้ *gusA* เป็นยีนเครื่องหมาย พบว่าทั้ง *nifDKc* และ *nifDKp* มีการแสดงออกเมื่อเจริญในสภาวะพึ่งพาอาศัยกับพืช แต่เมื่อเจริญในสภาวะแบบอิสระพบว่ายีน *nifDKc* มีการแสดงออกที่สูงกว่า และเมื่อทำการกลายพันธุ์เชื้อ DOA9 (Δ*nifDKc* หรือ Δ*nifDKp*) แล้วนำไปทดสอบความสามารถในการตรึงไนโตรเจนพบว่า ยีนทั้งสองทำงานร่วมกันเมื่อเจริญภายใต้สภาวะพึ่งพาอาศัยกับพืช *Aeschynomene americana* แต่พบว่ายีน *nifDKc* ทำหน้าที่หลักในการตรึงไนโตรเจนเมื่อเจริญแบบอิสระ อย่างไรก็ตามพบว่าการทำงานของ NifDKp ต้องการเอนไซม์ที่ได้จากการแสดงออกของยีน *nifENX* ซึ่งถูกถอดรหัสแบบต่อเนื่องในชุดยีนเดียวกันกับ *nifDKc* สำหรับบทบาทของยีน *nifA* ที่เกี่ยวข้องกับการควบคุมการแสดงออกของยีนอื่น ๆ ที่เกี่ยวข้องกับการตรึงไนโตรเจน ผลจากการตรวจสอบลำดับกรดอะมิโนของ NifAc และ NifAp พบว่ามีลำดับกรดอะมิโนที่เหมือนกันเพียงร้อยละ 53 ซึ่งเกิดจากการที่โครงสร้างโปรตีนของ NifAp ไม่มีส่วนของ N-terminal domain และเมื่อตรวจสอบระดับการแสดงออกของยีนทั้งสอง พบว่ายีนทั้งสองมีการแสดงออก โดยยีน *nifAp* มีการแสดงออกมากกว่ายีน *nifAc* เมื่อเจริญภายใต้สภาวะพึ่งพาอาศัยกับพืช *A. americana* อย่างไรก็ตาม พบว่าภายใต้สภาวะนี้ ยีน *nifAc* และ *nifAp* สามารถทำงานทดแทนกันได้ แต่กลับ

พบว่ามีเพียง *nifAc* ที่จำเป็นต่อการตรึงไนโตรเจนของเชื้อภายใต้สภาวะการเจริญแบบอิสระ และเมื่อทดสอบเชื้อกลายพันธุ์ของ DOA9 ที่ปราศจากทั้งยีน *nifAc* และ *nifAp* (Ω*nifAc*::Δ*nifAp*) ช่วยยืนยันถึงความสำคัญของ NifA ในการควบคุมยีนอื่น ๆ ที่เกี่ยวข้องกับการตรึงไนโตรเจน อย่างไรก็ตามในสภาวะการเจริญแบบอิสระพบว่า มีเพียงยีน *nifAc* เท่านั้น ที่สามารถทดแทนการทำงานในเชื้อกลายพันธุ์ Δ*nifAc* ได้ ในขณะที่การใช้ยีน *nifAp* และยีน *nifA* hybrid ที่สร้างจากการนำส่วน N-terminal domain ของ *nifAc* เชื่อมต่อกับส่วนกลาง และส่วน C-terminal domain ของ *nifAp* ไม่สามารถช่วยให้เชื้อกลายพันธุ์ Δ*nifAc* กลับมามีกิจกรรมการตรึงไนโตรเจนได้ สำหรับการตรวจสอบบทบาทของ *nifA* ในการควบคุมการแสดงออกของยีนอื่น ๆ โดยใช้วิธี qRT-PCR เพื่อตรวจสอบการแสดงออกของยีนที่สนใจในเชื้อ DOA9 ดั้งเดิม เชื้อกลายพันธุ์ Δ*nifAc* และเชื้อกลายพันธุ์ Δ*nifAp* ในสภาวะการเจริญแบบอิสระพบว่า NifAc ควบคุมการแสดงออกของยีนที่เกี่ยวข้องกับการตรึงไนโตรเจนที่มีตำแหน่งอยู่ทั้งบนโครโมโซม และเมกะพลาสมิด ในขณะที่ทั้ง NifAc และ NifAp สามารถข้ามไปควบคุมการแสดงออกของยีนที่เกี่ยวข้องกับการตรึงไนโตรเจนที่มีตำแหน่งอยู่ทั้งบนโครโมโซม หรือบนเมกะพลาสมิดได้ภายใต้สภาวะพึ่งพาอาศัยกับพืช และเมื่อตรวจสอบการทำงานของยีน *nifV* โดยการสร้างเชื้อกลายพันธุ์ของยีนนี้ คือ Ω*nifV* และ Δ*nifV* แล้วทดสอบความสามารถในการตรึงไนโตรเจนภายใต้สภาวะการเจริญแบบอิสระ พบว่าเชื้อกลายพันธุ์มีประสิทธิภาพการตรึงไนโตรเจนลดลงเมื่อเปรียบเทียบกับเชื้อดั้งเดิม และเนื่องจาก *nifV* มีความสำคัญในการสร้างสารโฮโมซิเทรต จึงได้ทดสอบเติมสารโฮโมซิเทรตลงไปในอาหารที่ใช้ในการเลี้ยงเชื้อกลายพันธุ์นี้ ซึ่งพบว่าสามารถทำให้เชื้อกลายพันธุ์สามารถตรึงไนโตรเจนภายใต้สภาวะการเจริญแบบอิสระได้ดังเดิม และเมื่อทดสอบในสภาวะพึ่งพาอาศัยกับพืช พบว่าเชื้อกลายพันธุ์ไม่มีผลกระทบต่อประสิทธิภาพการตรึงไนโตรเจนในพืชอาศัยที่ใช้ทดสอบส่วนใหญ่ แต่เมื่อทดสอบกับพืช *Indigoferra tinctoria* และ *Stylosanthes hamata* พบว่าปมที่ถูกสร้างโดยเชื้อกลายพันธุ์นี้มีการตายของแบคทีรอยด์มากกว่าเมื่อเทียบกับปมที่ถูกสร้างโดยเชื้อ DOA9 ดั้งเดิมซึ่งพบแบคทีรอยด์ที่ยังมีชีวิต ดังนั้นอาจเป็นไปได้ว่าการขาดยีน *nifV* ส่งผลให้ปมเข้าสู่ระยะเสื่อมสภาพเร็วขึ้นกว่าปกติ ทั้งนี้ความรู้ที่ได้จากงานในวิทยานิพนธ์นี้สามารถนำมาปรับปรุงการใช้ปุ๋ยชีวภาพให้มีประสิทธิภาพการตรึงไนโตรเจนที่เพิ่มขึ้นได้เมื่อนำไปใช้ในการเกษตร

สาขาวิชาเทคโนโลยีชีวภาพ
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JENJIRA WONGDEE : CHARACTERIZATION OF GENES INVOLVED IN NITROGENASE SYNTHESIS AND REGULATION LOCATED ON BOTH CHROMOSOME AND PLASMID OF *BRADYRHIZOBIUM* sp. DOA9. THESIS ADVISOR : ASST. PROF. PANLADA TITTABUTR, Ph.D., 141 PP.

nifDK/*nifA*/*nifV*/ NITROGEN FIXATION/SYMBIOSIS/FREE-LIVING STATE

Bradyrhizobium sp. DOA9 contained 2 replicons of a chromosome and mega-plasmid. Genome analysis of DOA9 indicated that both chromosome (c) and mega-plasmid (p) harbor a set of nitrogenase structural genes named *nifDKc* and *nifDKp*, respectively. Similarly, a *nifA* regulatory gene was also found in one copy on both chromosome (*nifAc*) and mega-plasmid (*nifAp*). DOA9 also contained a *nifV* gene encoded homocitrate synthase suggesting that DOA9 may be able to fix nitrogen under free-living condition. Thus, this thesis aimed at investigating the role of these genes on the nitrogen fixing ability of DOA9 under symbiotic and free-living states. Transcriptional analysis using *gusA* reporter strains showed that both *nifDKc* and *nifDKp* were highly expressed under symbiosis, where *nifDKc* expressed more predominantly under the free-living condition. The gene mutation indicated that both *nifDKc* and *nifDKp* are required for fully function of nitrogenase activity during symbiosis with the host plant *Aeschynomene americana*, while, *nifDKc* was the major contributor for nitrogenase activity during the free-living state. However, the function of NifDKp required the product of *nifENX* genes which are co-transcribed from the *nifDKc* operon. In the case of NifAc and NifAp, both showed only 53% similarity in the amino acid sequence due to the lack of the N-terminal domain in NifAp. Under the symbiosis condition, both *nifAc* and *nifAp* genes expressed where *nifAp* expressed slightly higher than *nifAc*. NifAc and NifAp could replace each other for symbiotic

nitrogen fixing ability. However, only NifAc contributed to a free-living nitrogen fixation. Obviously, the double *nifA* mutation (Δ*nifAp*::Ω*nifAc*) strain could not fix nitrogen neither both symbiotic and free-living conditions. Complementation of the Δ*nifAc* mutant with *nifAc* led to successful nitrogen fixation under free-living condition. However, neither *nifA*p nor the chimeric *nifA* hybrids could complement the nitrogenase activity. The transcriptional profiles under three different backgrounds of wild-type, Δ*nifAc* and Δ*nifAp* strains suggested that NifAc, but not NifAp regulated the expression of other nitrogen fixing genes on both chromosome and mega-plasmid under the free-living condition. Whereas, NifAc and NifAp could cross regulate the expression of nitrogen fixing genes located along the chromosome and mega-plasmid during symbiosis with *A. americana*. To explore the role of *nifV*, two mutants of Ω*nifV* and Δ*nifV* were constructed. The acetylene reduction activity of both mutant strains decreased when compared to wild-type under the free-living condition. Supplementing homocitrate into the culture of Δ*nifV* could restore the nitrogenase activity. Under the symbiotic state, most plant species were not affect when inoculated with mutant strain. However, the high content of dead bacteroids was observed inside the nodule of *Indigofera tinctoria* and *Stylosanthes hamata*. It may be speculated that the lack of *nifV* leads to early senescence of nodules. Knowledge obtained from this study would improve the efficiency of biofertilizer when applied in agriculture.

School of Biotechnology

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CHAPTER I

INTRODUCTION

1.1 Rationale and background

The ability of biological N_2 fixation to convert atmospheric dinitrogen to ammonia is strictly limited to prokaryotes, which can fix nitrogen in free-living or symbiosis with host plant (Hallenbeck, 1987 and Steenhoudt and Vanderleyden, 2000). The importance of symbiotic N_2 fixation as 'green' fertilizers for sustainable agricultural systems cannot be underestimated (Baldani and Döbereiner, 2000). Nitrogenase is the enzyme responsible for biological nitrogen fixation. It catalyzes the conversion of atmospheric nitrogen to ammonia in nitrogen-fixing organisms. However, nitrogenase possesses a high degree of sensitivity towards oxygen thus requiring an oxygen protecting system, which are different in mechanisms by symbiotic and free-living bacteria (Downie, 2000 and Allaway et al., 2001). Therefore, the expression of genes involved in nitrogen fixation and regulation is depended on genetic of each diazotrophic bacteria and the altering of environment. It could be stated that nitrogenase is the main enzyme predicted the potential of biofertilizer.

Nitrogenase is the complex metalloenzyme, which required several genes to encode proteins for structural enzyme synthesis and regulatory function. The nitrogenase genes (*nif* genes) were firstly identified and studied in *Klebsiella pneumoniae*. In rhizobium, *nif* genes are structurally homologous to the *K. pneumoniae nif* genes, and it is inferred that a conserved *nif* gene plays a similar role in rhizobia as in *K. pneumoniae*. Not only *nif* genes, but also *fix* genes are also involved in biological nitrogen fixation

and regulation (Fischer, 1994). The particular "fix genes" are used as essential for nitrogen fixation in symbiotic bacteria. The operon of *nif* genes in rhizobia usually consists of regulatory, structural and cofactor genes. The complex protein of nitrogenase is composed by two protein components, I: FeMo protein or dinitrogenase consists of a complex structure of α (*nifD*) and β subunit (*nifK*) (Beringer, 1984), and II: the *nifH* gene encodes for dinitrogenase reductase which is a homodimeric Fe protein (Roberts et al., 1978). Moreover, nitrogenase requires products of other *nif* genes that are involved in the synthesis of FeMo-cofactor and activator, such as *nifN*, *nifV*, *nifB*, *nifQ*, *nifE*, *nifX*, *nifU*, *nifS* and *nifY* (Hu et al., 2007). Especially, *nifV* has been reported that it is required for nitrogen fixation under free-living state (Ruttimann-Johnson et al., 2001). The expression of these genes is regulated by either the transcriptional activator NifA together with the sigma factor RpoN or transcriptional activator FixK (Batut et al., 1989).

Bradyrhizobium strain DOA9 had isolated from rice by using *A. americana* as a trap legume (Noisangiam et al., 2012). This strain was preliminary found to fix nitrogen under both symbiotic and free-living conditions. DOA9 displays an unusual broad host range extending to peanut, mungbean, and cowpea which are the main legumes crops cultivated in Thailand. Moreover, the DOA9 strain contains a very large symbiotic plasmid of more than 700-kb that harbors the *nod* and *nif* genes. This is the first report showing the presence of a symbiotic plasmid in genus of *Bradyrhizobium*. Interestingly from the genome sequence data, the *fix* and *nif* genes are located on both regions of chromosomal and plasmid DOA9 (pDOA9). Sequence data on chromosome demonstrated the organization of nitrogenase gene contains regulatory genes (*fixL*, *nifA* and *RpoN*), structural genes (*nifHDK*) and activator genes (*nifBENQS* and *V*), while plasmid contains regulator (*fixR*, *nifA* and *RpoN*) and other structural genes (*nifHDK*) (Okazaki et al., 2015).

Although the reiteration of nitrogenase regulatory and structural genes on chromosome and plasmid have been reported in many diazotrophic bacteria, such as in *R. meliloti* carries two extremely large plasmids (mega-plasmids) (Banfalvi et al., 1981) or in *R. leguminosarum* bv. *phaseoli* possesses three identical copies of *nifH* on symbiotic plasmid (pSym), these genes possess different roles and abilities in nitrogen fixation (Girard et al., 1991). Therefore, the activity of nitrogen fixation in diazotrophic bacteria should be suggested more variety and specificity by the responsive process to either their evolution and host plant or environment.

Since, the presence of *nif* reiterated genes in several symbiotic bacteria is remarkable, it could relate the function of these reiterated genes with the ability of nitrogen fixation under different conditions and the unusual of its broad host range. Therefore, the goal of this research is to characterize the nitrogenase functional (*nifD* and *nifV*) and regulatory (*nifA*) elements located on both chromosome and plasmid in *Bradyrhizobium* sp. DOA9 under symbiosis and free-living conditions. The experiment was conducted by using gene comparative analysis, gene disruption, and enzymatic activities techniques to find the distinct function and the regulatory circuitries for symbiosis and nitrogenase activity in DOA9. Knowledge gained from this could be used to improve DOA9 as a board host range biofertilizer that can efficiently survived fix nitrogen under different conditions.

1.2 Objectives

1.2.1 Main objective

To investigate the function and regulatory system of genes involved in nitrogen fixation that located on both chromosome and plasmid of DOA9 under symbiotic and free-living states.

1.2.2 Specific objectives

1.2.2.1 To investigate the function of nitrogenase genes including *nifD* localized on both chromosome and plasmid of DOA9 under both symbiotic and free-living states.

1.2.2.2 To investigate the role of *nifA* located on both chromosome and plasmid of DOA9 involved in regulation of nitrogen fixation under both symbiotic and free-living states.

1.2.2.3 To investigate the function of *nifV* located on the chromosome of DOA9 involved in nitrogen fixation under both symbiotic and free-living states.

CHAPTER II

LITERATURE REVIEWS

2.1 Biological nitrogen fixation

The Biological nitrogen fixation (BNF) had been discovered since 1901 (Beijerinck, 1901) as process finding in the specialized group of prokaryotes. By utilizing the enzyme nitrogenase to catalyze the conversion of atmospheric nitrogen (N_2) to ammonia (NH_3). Plants can readily assimilate NH_3 to produce the nitrogenous biomolecules. These prokaryotes include aquatic organisms, such as cyanobacteria, free-living soil bacteria, such as *Azotobacter*, bacteria that form associative relationships with plants, such as *Azospirillum*, and most importantly, bacteria, such as *Rhizobium* and *Bradyrhizobium*, which form symbioses with legumes and other plants (Postgate, 1982). The reduction of atmospheric nitrogen is a complex process that requires a large input of energy to proceed (Postgate, 1982). The nitrogen molecule is composed of two nitrogen atoms joined by a triple covalent bond, thus forming the molecule highly inert and nonreactive. Nitrogenase catalyzes the breaking of this bond and the addition of three hydrogen atoms to each nitrogen atom. Microorganisms that fix nitrogen require 16 moles of adenosine triphosphate (ATP) to reduce each mole of nitrogen (Hubbell and Kidder, 2009). These organisms obtain this energy by oxidizing organic molecules. Non-photosynthetic free-living microorganisms must obtain these molecules from other organisms, while photosynthetic microorganisms, such as cyanobacteria, use sugars produced by photosynthesis. Associative and symbiotic nitrogen-fixing microorganisms obtain these compounds from their host plants' rhizospheres (Hubbell and Kidder, 2009).

2.2 Nitrogen-fixing symbiotic association: Nodule formation

Rhizobia are taxonomically diverse members of the α-subdivision of the proteobacteria and can exist in two states: as a free-living saprophyte in the soil and in a symbiotic relationship with leguminous plants. The latter interaction begins with a specific molecular signals exchange between the legume and rhizobia. Plant roots secrete many different organic compounds into the soil, some of which allow microorganisms to grow in the rhizosphere and include carbohydrates, amino acids, organic acids, vitamins and phenolic derivatives. In terms of fundamental symbiosis, flavonoids are the most important compounds, as they trigger the induction of bacterial nodulation (*nod*) genes (Redmond et al., 1986). At first step of symbiosis, plant released phenolic root compounds lead to the expression of many rhizobial genes (*nod*) required for nodulation. The products of nod genes are enzymes for the biosynthesis of bacterial nodulation (Nod) factors called lipo-chitooligosaccharides. Nod factor stimulates a series of physiology and development of host plants and lead to nodule formation and rhizobial hosts adaptation (Debelle et al., 2001 and Denarie et al., 1996). On production of Nod factors, the bacteria then surround and attach to the root, causing the root to start to curl. Rhizobia trapped in a curled hair, or between a hair and another cell, proliferate and begin to infect the outer plant cells, which in turn stimulates plant cells to produce infection threads (Martínez-Romero, 2009). Bacteria released from infection threads into the cytoplasm of plant cells are surrounded by the plant plasma membrane and then briefly replicate their DNA and divide before stopping both processes (Asamizu et al., 2008). Finally, the endosymbiotic forms of the bacteria (referred as bacteroids) make up a new organ of the plant on the root (called the root nodule) and begin to fix nitrogen by the action of the enzyme nitrogenase (Kawaguchi and Minamisawa, 2010). Other rhizobia that are adapted to aquatic or semi-aquatic tropical legumes can penetrate their way into emerging nodules at the loose cellular junctions of emerging lateral roots,

a process known as crack-entry. Rhizobia move through the epidermal root cracks and then immediately synthesized the signal compounds, which result to generate the plant-derived plasma membrane surrounded structure of symbiosomes. As mentioned, the *Rhizobium*-legume symbiosis is very specific between both the species of rhizobia and the species of legume (Ide Franzini et al., 2010) and certain bacterial genes will only activate under symbiotic conditions. Nodules formed on different plants by different bacteria nonetheless display striking developmental similarities (Mandel et al., 2009).

2.3 Symbiotic formation by the broad host range rhizobia

Rhizobia establish nitrogen-fixing symbioses by interact with the specific plant root to form nodules. Some rhizobia have a narrow host range, which means to form nodule with a few legume species, while some rhizobia can nodulate with many species of legume, which are called broad host range strains. A narrow host range has been described for *Sinorhizobium meliloti* which induces nitrogen-fixing nodules only on few species of the genera of *Medicago*, *Melilotus*, and *Trigonella* (Schwartz, 2009). In contrast, several *Bradyrhizobium* species have a broader host range as they could nodulate legumes from different families such as Papilionoideae, Mimosoideae, and Phaseoleae (Pinton et al., 2007). The modifications of chemical and molecular forms at the Nod factor structure are further important affect to host range (Roche et al., 1996).

Induction of nod genes is controlled by the NodD protein consist of the LysR family of transcription regulators (Schwartz, 2009). Some proportion of NodD represented specific transcription factors, which may be involved in controlling host range. In *B. japonicum*, an isoflavonoid stimulating two-component regulatory NodVW is important process for nodulation of mung bean (*Vigna radiata*), cowpea (*Vigna unguiculata*) and siratro (*Macroptilium atropurpureum*), but this signaling is slightly contributed to activate soybean symbiosis (Bogino et al., 2015 and Loh et al., 1997).

In the process of nodule development have been reported that bacterial lipopolysaccharides and exopolysaccharides implicate as the early bacterial signals (Gourion et al., 2015). For example, *R. meliloti* invade root nodules via a tube of plant host called the infection thread that depend on the production of exopolysaccharides (EPSs). EPSs are composed of capsular polysaccharides (CPS) and extracellular polysaccharides (EPS) that different in their numbers of polymerization (Kaci et al., 2005). EPS-deficient (exo) mutants of several *Rhizobium* sp. do not form normal nitrogen-fixing nodules instead they induce abnormal pseudo-nodules in legume roots which are lack of bacteroids (Niehaus et al., 1993; Staehelin et al., 2006). Exopolysaccharides may function as signal molecules that induce host initiation of infection thread formation and allow the rhizobia to enter the plant (van Workum et al., 1998). They may act as host specificity factors which enable the bacteria to bind to specific plant lectins (Staehelin et al., 2006).

Although, Rhizobia utilize several processes to form functional nodules, molecular elements play the important role about this process considered to secretion systems including specialized type III (T3SS), type IV (T4SS) and possibly type VI (T6SS) secretion systems (Hubber et al., 2004 and Soto et al., 2006). It was found that several S*inorhizobium*, *Mesorhizobium* and *Bradyrhizobium* strains use both T3SS and T4SS to mediate their interactions with legume hosts. In these rhizobia, the activation of symbiotic T3SS or T4SS was found to be coordinated with the expression of nodulation genes; NodD/ NodVW or NodD-VirA-VirG regulatory cascades (Hubber et al., 2004 and Krause et al., 2002). In the broad host range, *Sinorhizobium* sp. strain NGR234 performed the important of T3SS on nodulation with the host: it is globally positive for interaction with the *Tephrosia vogelii* host, neutral for *Vigna unguiculata* and negative for *Pachyrhizus tuberosus* (Deakin and Broughton, 2009, Marie et al., 2004 and Schmeisser et al., 2009). From the current data, it has been expected that secreted effectors T3SS and T4SS modulate plant by the presence of infecting rhizobia (Deakin

and Broughton, 2009). All together it becomes clear that, Nod factors, surface polysaccharides and secreted effectors, the combined effects of nodulation-specific secreted proteins contribute to define the host range of rhizobia.

2. 4 Nitrogen Fixation

2.4.1 Nitrogen Fixation under free-living state

Many species of the family Rhizobiaceae possess the ability to fix atmospheric nitrogen, a mechanism that is exclusive to prokaryotes (Reed et al., 2011). The bacterial genes for nitrogen fixation fall into two broad categories. The gene sets that have homologies among organisms (e.g. *Klebsiella* spp.) that can fix nitrogen in the free living state are known as *nif* (Dixon and Kahn, 2004a). Whilst those that are unique to symbiotic nitrogen fixation are known as *fix* (Dixon and Kahn, 2004a). In all diazotrophic proteobacteria have been reported that the transcriptional activator NifA is required for expression of the nitrogen fixation (*nif*) genes (Steenhoudt and Vanderleyden, 2000). Expression and activity of *nifA* is regulated in response to the environmental factors and nitrogen (Dixon and Kahn, 2004a). Most of the free-living diazothrophs, NifA activity is not directly sensitive to oxygen (Ausubel and Ow, 2012), but some is regulated in response to the signals of molecular oxygen and combined nitrogen through a second regulator NifL, which the *nifL* and *nifA* genes together form an operon. The expression of the *nifLA* operon is regulated by the nitrogen status of the cell by the NtrB/NtrC two component regulatory system (de Bruijn et al., 2012).

2.4.2 Nitrogen Fixation under symbiosis state

Symbiotic nitrogenase genes in generally can be involved to *nod*, *nif*, and *fix* genes. The *nif* and *fix* genes are organized in distinct clusters that perform species specific, such as in *R. meliloti*, *B. japonicum*, and *A. caulinodans*. Although the particular

"*fix* gene" is used as essential for nitrogen fixation in symbiotic bacteria but do not have a homologous copy in *K. pneumoniae*. Expression of *Rhizobium* sp. *nif* genes consists of regulatory, structural and cofactor genes, which are regulated by either the transcriptional activator NifA together with the sigma factor RpoN or transcriptional activator FixK. These are induced under microaerobic conditions by the transcriptional activator FixJ that phosphorylated by FixL. It is the activated FixJ protein that induces the transcription of *nifA* and *fixK* (Batut et al., 1989). The role of FixK in the regulatory system varies in different symbiotic diazotrophs for example in *A. caulinodans*, FixK rather than FixJ directly activates NifA expression. Furthermore, *nif* genes expression in *Bradyrhizobium* sp. is regulated by both the oxygen-responsive FixL–FixJ and the redox-responsive RegS–RegR systems. In which the homologues of redox-sensitive RegS and RegR controlled *nifA* expression during the symbiosis condition. In *K. pneumoniae*, the level of gene cascade involves regulation of NifA activity by the redox-responsive anti-activator protein NifL in response to oxygen then to fixed nitrogen through interaction with the PII-like protein GlnK (Nitrogen regulatory protein P-II) (Jack et al., 1999). Mutations within these genes resulted in *Rhizobium* was still able to undergo nodulation with their legume hosts, but was unable to fix nitrogen (Nod^+ Fix^- phenotype). While *Rhizobium* fix nitrogen within the nodules produced by their host, a few have been shown to exhibit this property when growing in pure culture; *Azorhizobium* and some *Bradyrhizobium* strains exhibit low levels of nitrogen fixation activity in older cultures (Franche et al., 2009).

2.4.3 Biochemical and molecular biology of nitrogenase

Nitrogenase is the two-component enzyme complex responsible for the process of nitrogen fixation and is structurally highly conserved throughout nitrogen-fixing bacteria (Figure 1.). The α and β subunits of component I (the dinitrogenase or MoFe protein) are encoded from *nifD* and *nifK*, respectively; component II (the

dinitrogenase reductase or Fe protein) is encoded from *nifH*. Component I requires a co-factor, encoded by *nifB*, *nifE* and *nifN* genes, which is believed to be the site of substrate binding and reduction (Kennedy, 2012). The genes *nifH*, *nifM*, *nifQ* and *nifV* are also required for synthesis and maturation of the active enzyme complex. The enzyme is slow in its action, large in size and can account for up to 30% of the protein present in bacteroids (Fani et al., 2000).

Oxygen concentration is the trigger factor for nitrogen fixation process, and the oxygen sensing *fixLJK* regulate this process in many rhizobia. Nitrogenase made within these bacteroids converts N_2 into NH_3 by reduction of dinitrogen gas and protons as indicated in the following equation of $8H^+ + N_2 + 8^{e-} \rightarrow 2NH_3 + H_2$. This is a very energy intensive process (Hernando et al., 1998). Since nitrogen is highly inert at normal atmospheric temperature and pressure, this process requires a minimum of 16 ATP molecules per molecule of nitrogen reduced. It has been estimated that the energy requirements under certain circumstances may be as high as 42 ATP molecules per molecule of nitrogen fixed (Kennedy, 2012). Therefore, bacteroids need to respire at a high rate to generate the ATP required for nitrogen fixation, but it is believed that part of their metabolism is shut down on entering symbiosis with the plant (Madsen et al., 2010). It has always been believed that the plant provided carbon to the rhizobia (in the form of dicarboxylates) for respiration, in return for fixed nitrogen (in the form of ammonium); this was later revised to fixed nitrogen (ammonium) and alanine (Allaway et al., 2001).

2.4.4 Interaction of symbiotic nitrogen fixing rhizobium and its host plant during symbiosis

The plant provides the bacteria with the environment conditions controlled amounts of oxygen, dicarboxylates (taken in by rhizobia via the dicarboxylate transport (DCT) system) and glutamate (or glutamine), which is then used for respiration in the

rhizobia via the tricarboxylic acid (TCA), generating the ATP required for nitrogen fixation. In return, the bacteria supply the plant with ammonia, aspartate and alanine. Aspartate is converted into asparagine that is used by the plant, whilst the ammonia serves to replace the glutamate that was donated to the bacteroid (Lodwig et al., 2003).The amino acid cycling between rhizobia and legume has significant consequence on their symbiosis.

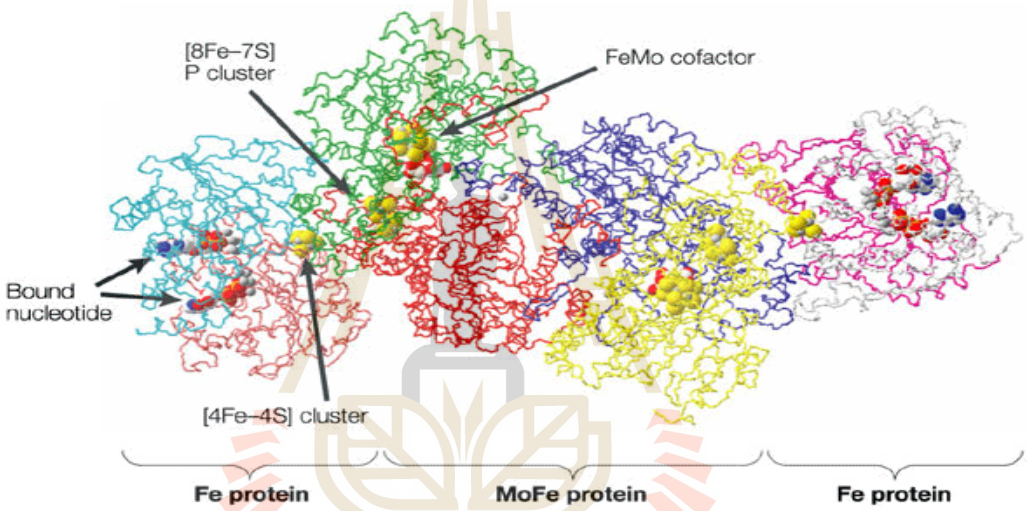

Figure 2.1 The model of nitrogenase proteins Complex (Fe and MoFe protein) of *Azotobacter vinelandii*. The subunits of the two Fe protein dimers are colored in blue, brown, purple and grey. The alpha-subunits of the MoFe protein are coloured in green and yellow, with beta-subunits in red and Blue. The oxygen-sensitive metalloclusters and bound nucleotides are shown in space fill (Dixon and Kahn, 2004b).

The plant provides amino acids to the bacteroids, allowing them to shut down ammonium assimilation; likewise the bacteroids must export ammonia to the plant in order to obtain amino acids (Franche et al., 2009). The plant cannot dominate the relationship by restricting amino acid availability since the bacteroids act as plant

organelles and are responsible for the aspargine synthesis of the plant. This demonstrates a special phenotype of mutualism between the symbiotic bacteria and host plant, rather than dominance on either side (Emerich et al., 2009). This process continues until the plant dies hence releasing its nitrogen into the biomass and contributing to the nitrogen cycle. *Rhizobium*-legume symbiosis is the primary source of fixed nitrogen in land-based systems, providing well over half of the biological source (González and Gonzalez-López, 2013).

2.5 Organization and function of nitrogen fixing genes clusters in symbiotic bacteria

Rhizobial *nif* genes are structurally homologous to the *K. pneumoniae nif* genes (Arnold et al., 1988) and it is implied that a conserved *nif* gene performs a similar role in rhizobia as in *K. pneumoniae*. While *fix* genes are used as essential gene for nitrogen fixation but do not have a homologous analogues to *K. pneumoniae*. The *nif* and *fix* genes of several diazotrophic bacteria (for example *Rhizobium* sp., *Bradyrhizobium* sp., and *Sinorhizobium* sp.) are organized in different clusters. It could be indicated that both gene structure and genomic location of these genes are species specific. Obviously, the distribution of nitrogen fixation genes in *K. pneumonia* is not as close as in between those bacteria, which 20 *nif* genes are organized in 8 operons within 24 kb of total DNA fragment (Gage, 2004, Madsen et al., 2010 and Schmitz et al., 2002). *R. meliloti* obtains two extremely large plasmids (me-gaplasmids) of 1,400 kb and 1,700 kb of respective pSym-a (me-gaplasmid 1) and pSym-b (mega-plasmid 2) (Banfalvi et al., 1981, Burkardt et al., 1987, Hynes et al., 1986 and Sobral et al., 1991). This strain obtains both cluster I (*nifHDKE*, *nifN*, *fixABCX*, *nifA*, *nifB*, *frdX*) and cluster II (*fixLJ*, *fixK*, *fixNOQP*, *fixGHIS*) on megaplasmid 1 (Batut et al., 1985). DNA sequence of cluster II genes at downstream of the *nifHDKE* operon and are transcribed in opposite orientation.

Furthermore, a functional duplication of the region spanning *fixK* and *fixNOQP* is presented at upstream of *niffHDKE*. The gene cluster of common *nodABC* is located in between regions of *nifE* and *nifN* (David et al., 1988). And additional genes required for an effective symbiosis are located on mega-plasmid 2 and on the chromosome (Hynes et al., 1986 and Watson et al., 1988). Generally, *B. japonicum* does not have plasmid. Therefore, the symbiotic island containing *nif*, *fix* and *nod* genes of this strain are located on the chromosome. Cluster I of *B. japonicum*, which contains the nitrogenase structural genes, is located at a distance of 240 kb away from cluster II, which includes the *fixRnifA* operon, *fixA*, and 11 nod genes. Cluster III, carrying *fixLJ*, *fixK2*, and the *fixNOQP* operon, maps at ca. 770 kb away from cluster II (Bradburne et al., 1994 and Kündig et al., 1993). Moreover, additional gene regions that are involved in nodulation (*nodVW*) or expression is induced under symbiotic conditions (*rpoN1*, *groESL3*, and *ndp*) are present close to or within this part of the *B. japonicum* chromosome (Kündig et al., 1993).

In *Bradyrhizobium* sp. DOA9 genome consists chromosome and one mega-plasmid of 7,114,514 bp and 736,163 bp, respectively. This strain contains a complete cluster of nitrogen-fixing genes (*nif-fix*) on chromosome and incomplete cluster of *nif-fix* genes on mega-plasmid (Okazaki et al., 2015). DNA sequence of regulatory *fixLJKRnifA* located on chromosome at downstream of the structural *nifDK* and accessory *nifENXBQVW* operons, which are transcribed in opposite orientation. Whereas, the plasmid DNA sequence of regulatory *nifAfixR* obtains at upstream of the structural *nifDK* by lack of any accessory genes, and this plasmid *nif-fix* genes transcribed in the same orientation. Furthermore, the genes required for an effective symbiosis are located on mega-plasmid, which the gene cluster of common *nodABC* is located in upstream regions of regulatory *nifAfixR*. However, the function of these repetitive genes involved in nitrogen fixation that located on both chromosome and plasmid of DOA9 has never been investigated.

2.6 Regulatory cascades of *nif* and *fix* genes expression in symbiotic bacteria

In vivo nitrogen fixation required high energy up to 400 mol of ATP for reduction 1 mol of dinitrogen to ammonia. However, the nitrogenase is oxygen sensitive response, these can suggest that all diazotrophs have developed regulatory mechanisms that permit a specific control of nitrogenase synthesis. The gene expression for nitrogen fixation is processed by cascades of hierarchically organized regulatory genes. Their cooperative action allow the bacteria to response optimal environmental conditions and to transfer this indication to the level of gene expression required for nitrogen fixation (Gordon and Wheeler, 2012).

2.6.1 Nitrogen fixation regulatory control in free-living bacteria

Nitrogen and oxygen are critical signals from environmental conditions regulate the *nif* gene expression in *K. pneumoniae*. This physiological control is mediated through the general nitrogen regulatory system (*ntr*) and the *nif*-specific regulatory genes, *nifLA* (de Bruijn et al., 2012). While, expression of nitrogen fixation genes in symbiotic diazotrophs is regulated predominantly in response to the cellular oxygen conditions. The lack of nitrogen control mechanism particularly relates to the physiological conditions under which symbiotic nitrogen fixation. Bacteroids are normally demanded with combined nitrogen by host plant during fixing nitrogen. Nevertheless, elements of an *ntr*-like system are also found in rhizobia; however, their regulatory role in nitrogen fixation is different from that in *K. pneumoniae*.

2.6.2 Nitrogen fixation regulatory control in symbiotic bacteria

The model of regulatory gene involved in regulation of symbiotic nitrogen fixation of representative strain *R. meliloti*, *B. japonicum*, and *S. meliloti* is presented in Figure 2. It is clear that all these organisms use largely identical regulatory elements (FixL, FixJ, FixK, NifA, RpoN) but only *B. japonicum* use combined regulatory element

FixR (Dixon and Kahn, 2004b). In *S. meliloti*, FixK expression is negatively regulated by FixT, which inhibits the activity of FixL. However, the regulation is different depend on species-specific networks. The NifA controls expression of the nitrogenase structural genes and genes encoding essential functions by communicates of their RpoN-dependent promoters. Hence, this process of regulation is similar to the situation in *K. pneumonia* (Barnett et al., 2001 and Finan et al., 2001). Alternatively, the FixLJ and FixK proteins *of R. meliloti, B. japonicum,* and *S. meliloti* constitute a different regulatory cascade which may be specific for symbiotic diazotrophs when no functionally proteins have been found in free-living diazotrophs. A function of this cascade involves the regulation of the *fixNOQP* genes, whereas it is significant to participate in *nifA* regulation. In *R. meliloti* and *S. meliloti,* the functional FixJ and FixK perform as direct positive regulators of *nifA* gene. In contrast, *nifA* expression in *B. japonicum* is mainly independent of the FixLJ-FixK interaction (Dixon and Kahn, 2004b and Fischer, 1994). Similarly, the regulation of nitrogen fixation varies among *R. meliloti*, *B. japonicum*, and *S. meliloti* by nitrogen response regulator NtrC. In *B. japonicum,* it has been reported that no such role to attribute the regulation of nitrogen fixation by NtrC, whereas NtrC of *R. meliloti* contributes to the regulation of *nifA* at different levels of biological significance when compare with the oxygen responsive system (Batut et al., 1993 and Schulze, 2004).

NifV or Homocitrate sysnthase is essential for nitrogen fixation, example in the genome sequence of *Cupriavidus taiwanensis* has one annotated copy of *nifV*. This microsymbiont, strain that has been reported to be able to fix-nitrogen in the free-living state (Amadou et al., 2008). The assay of acethylene reduction showed strains can be divided into two groups which are able to fix high levels of nitrogen under *ex planta* and do not fix. Absent of *nifV* is from many rhizobium strains that can fix-nitrogen only

in symbiotic states (Oldroyd et al., 2011). It cause of the activation of *fen1* expression in determinate nodule tissue to produce this homocitrate synthase during symbiotic nitrogen fixation (Hakoyama, Niimi, Watanabe, Tabata, Matsubara, Sato, Nakamura, Tabata, Jichun and Matsumoto, 2009). While, it is presents in strains that also fix nitrogen in *ex planta* states such as *A. caulinodans*, *Bradyrhizobium* spp. BTAi1 and ORS278 (Terpolilli et al., 2012). In Hoover (1988), strains that reduced amounts of acethylene when grown on medium with 2-oxoglutatrate as a carbon source. Several strains which have highest acethylene reduction such as *B. elkanii*, *B. japonicum* strain UADA20, these species are likely to have homostrate synthase activity (NifV). Thus, possess of NifV activity could be involved to diversity among rhizobia (Hoover, T. R. et al., 1988).

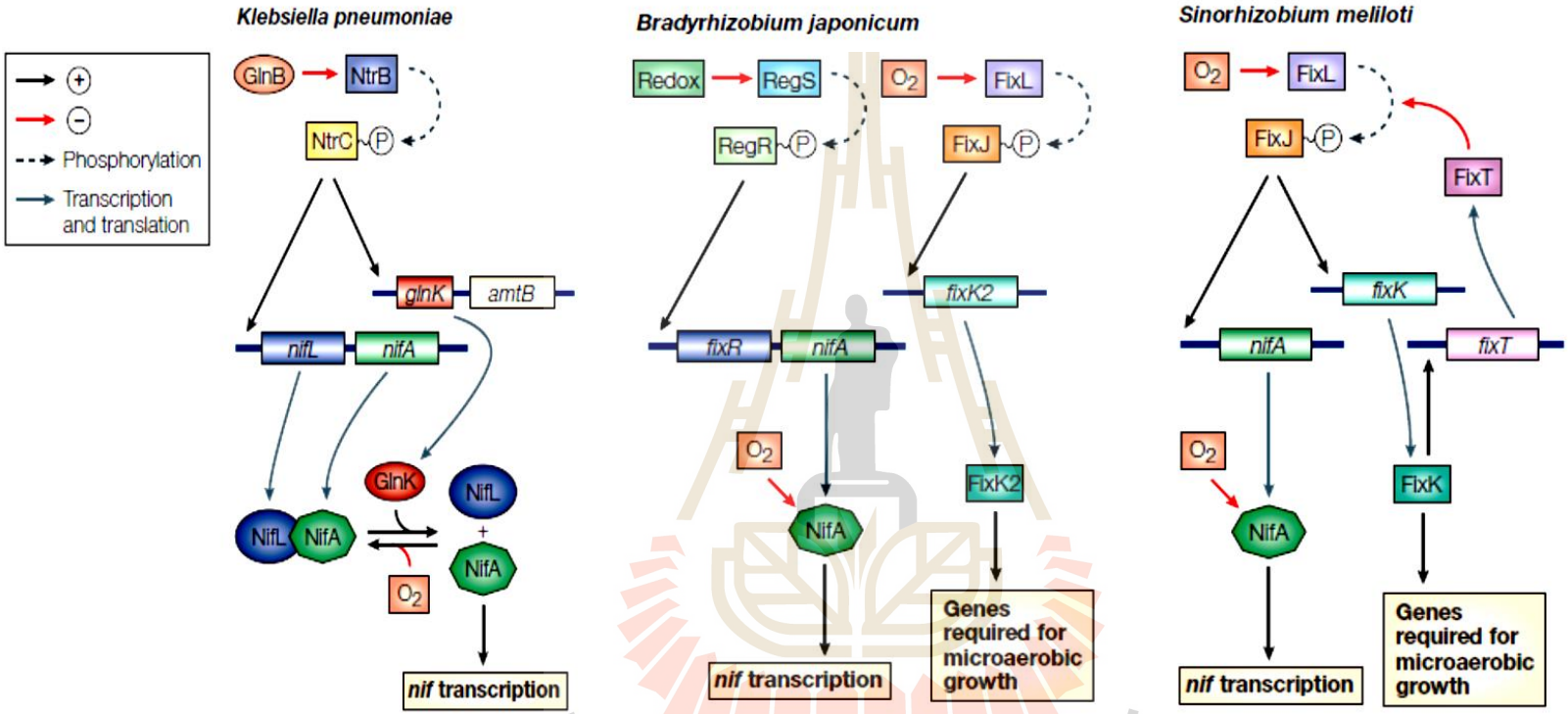

Figure 2.2 Comparison of regulatory cascades controlling *nif* transcription in the free-living diazotroph *K. pneumoniae* and in the symbiotic diazotrophs *S. meliloti* and *B. japonicum* (Dixon and Kahn, 2004b).

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CHAPTER III

Two *nifDK* clusters located on both the chromosome and megaplasmid of *Bradyrhizobium* sp. DOA9 contribute differently to the nitrogenase activity during symbiosis and free-living growth

3.1 Introduction

The agronomical and ecological importance of legumes can largely be attributed to their ability to form a nitrogen fixing symbiosis with specific bacteria known as rhizobia. These bacteria housed in nodules, usually found on the root system, are able to reduce atmospheric nitrogen in ammonia to the plant's benefit. Nitrogen fixation involves an enzyme complex called nitrogenase, which is composed of two enzymes, nitrogenase (Mo-Fe protein) and nitrogenase reductase (Fe protein). The Mo-Fe protein is composed of two pairs of different subunits encodes by the *nifD* and *nifK* genes while the Fe protein is composed of two identical subunits encoded by the *nifH* gene. In *Sinorhizobium meliloti* and *Azorhizobium caulinodans* the *nifHDK* genes are organized in an operon whereas in *Bradyrhizobium japonicum* USDA110 the *nifH* gene is located at several kb of the *nifDK* operon (Fischer 1994). Besides these 3 structural *nif* genes, other accessory *nif* genes are required for: i) FeMO-co biosynthesis (*nifBENXQSUVY*), ii) nitrogenase maturation (*nifZM*) and iii) electron donnation (*nifFJ*) (Masson-Boivin et al. 2009). All these *nif* genes are under the control of the activator NifA protein which acts in association with the RNA polymerase sigma factor RpoN (σ^{54}) (Dixon and Khan, 2004).

The way *nifA* transcription is regulated, differs markedly among rhizobia (Fischer, 1994). Similarly, the accessory *nif* genes content fluctuates between the rhizobium species, some species such as *Rhizobium leguminosarum* contain only five additional accessory genes while photosynthetic *Bradyrhizobium* strains as well as *A. caulinodans* contain up to 10 additional accessory genes (Masson-Boivin et al. 2009). In particular, these last strains have the *nifV* gene that permits the synthesis of homocitrate which is incorporated into the FeMo cofactor of dinitrogen reductase (Hoover et al. 1988). Thank to the occurrence of the *nifV*, these bacteria are able to fix nitrogen under free-living conditions unlike most rhizobia who lack this gene. In contrast, most rhizobia are able to fix nitrogen during symbiosis because the plant provides homocitrate to the bacteria which permits to insure the formation of a functional nitrogenase (Hakoyama et al. 2009).

All the *nif* genes are generally found on the same DNA region that also encompassed other symbiotic genes such as the *nod* genes required for the synthesis of the Nod Factors. In *Rhizobium* and *Sinorhizobium* species, this symbiotic region is found on a plasmid whereas in *Mesorhizobium* and *Bradyhizobium* strains this symbiotic region is integrated on the chromosome (MacLean et al. 2007). However, it was described for the first time of a *Bradyrhizobium* strain DOA9, that does contain a symbiotic plasmid of 700kb harboring the *nif* and *nod* genes (Okazaki et al., 2015). This non-photosynthetic *Bradyrhizobium* strain isolated from rice field soil using *Aeschynomene americana* as a trap legume (Noisangiam et al., 2012), was found able to induce nitrogen-fixing nodules on a broad range of legume hosts (Teamtisong et al. 2014).

Interestingly, sequence genome analysis of DOA9 strain revealed that both the chromosome and the plasmid harbor a full set of *nifHDK* genes split in both cases on two clusters, *nifH* on one side and *nifDK* on the other side (Figure. 3.1) (Okazaki et al. 2015). Similarly, the *nifA* regulatory gene is also found in two copies present on both the chromosome and the plasmid. In contrast, a survey of the accessory *nif* genes revealed

that all of them are on the chromosome (Table 3.1). Another unusual aspect of the *nif* genes content in this bacteria is also the presence of a *nifV* gene suggesting that the DOA9 strain could be able to fix nitrogen under free-living conditions similarly as observed for the photosynthetic *Bradyrhizobium* strains.

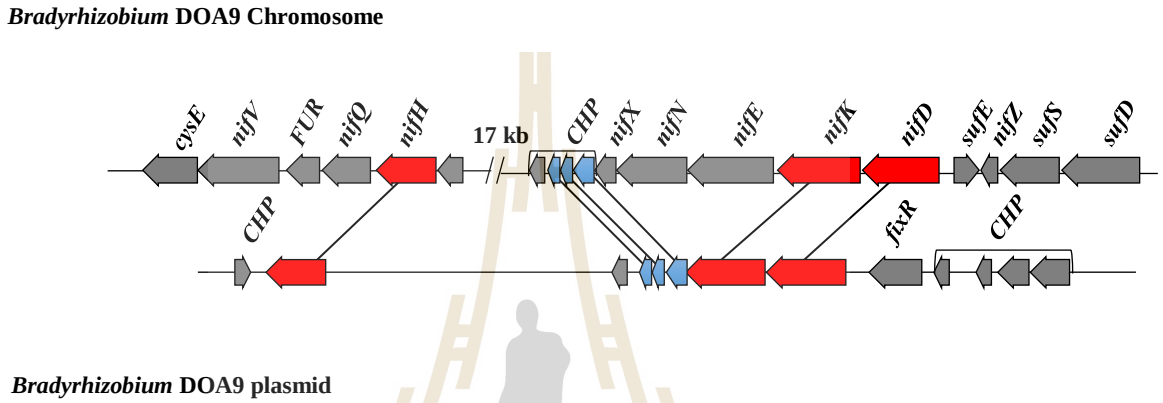

Figure 3.1 Genetic organisation of the *nifH, nifD* and *nifK* genes (in red) found in both the chromosome and the plasmid of the DOA9 strain. Note in the chromosome the presence of the *nifV* gene indicative of the ability of the strain to fix under free-living conditions. In blue, the genes that are found in synteny in both replicon and in grey, the genes specific to each replicon. CHP; Conserved Hypothetical Protein.

Table 3.1 Distribution of *nif* genes between the chromosome and plasmid replicons identified in *Bradyrhizobium* sp. DOA9.

Gene	DOA9 chromosome	DOA9 plasmid
nifH	BRADOA9_v1_51512*	BRADOA9_v1_p0648
nifD	BRADOA9_v1_51562	BRADOA9_v1_p0592
nifK	BRADOA9_v1_51561	BRADOA9_v1_p0593
nifB	BRADOA9_v1_51543	-
nifE	BRADOA9_v1_51560	-
nifN	BRADOA9_v1_51559	-
nifX	BRADOA9_v1_51558	-
nifQ	BRADOA9_v1_51511	-
nifS	BRADOA9_v1_42985	-
nifV	BRADOA9_v1_51508	-
nifZ	BRADOA9_v1_51536	-
nifW	BRADOA9_v1_51506	BRADOA9_v1_p0650
nifA	BRADOA9_v1_51570	BRADOA9_v1_p0526
rpoN	BRADOA9_v1_21339	BRADOA9_v1_p0436

* = The gene code based on the annotation by MicroScope version 3.9, France.

– = not gene found

Altogether these genome data suggest that the DOA9 strain possesses two functional nitrogenase complexes that are differently regulated and adapted to its different style of life, which could represent a unique case among the rhizobia. In this study, to explore this possibility, first we checked the ability of this strain to fix nitrogen under free-living state and second, we examined through the construction of reporter and mutant strains the expression of both nitrogenase complexes and their respective contribution in nitrogen fixation in both free-living and symbiotic conditions.

3.2 Objective

To investigate the structural *nifDK* genes involved in nitrogen fixation that located on both chromosome and plasmid of DOA9 under symbiotic and free-living states.

3.2.1 Specific objectives

3.2.1 To verify the nitrogen fixation of *Bradyrhizobium* sp. DOA9 under free-living condition

3.2.2 To determine the expression of *nifDKc* and *nifDKc* under both symbiotic and free-living states

3.2.3 To determine the function of *nifDKc* and *nifDKc* under both symbiotic and free-living states

3.3 Materials and methods

3.3.1 Bacterial strains, plasmids and culture media.

Bradyrhizobium sp. DOA9 strain was obtained from School of Biotechnology, Suranaree University of Technology, Thailand. This bacteria and its derivatives as well as the ORS278 and *B. japonicum* USDA110 were cultured at 28°C in YM medium (Vincent, 1970) or in a BNM-B minimal medium. The BNM-B medium

is a synthetic plant growth medium (Ehrhardt et al. 1992) that has been supplemented with a carbon (succinate) and a nitrogen (glutamate) sources and various oligoelements (Renier et al. 2011). *Escherichia coli* strains were grown in Luria-Bertani medium (LB) at 37°C. When required, the media were supplemented with the appropriate antibiotics. Antibiotics were used at the following concentrations: kanamycin, 50 or 100 µg/ml; streptomycin 200 µg/ml; nalidixic acid, 25 µg/ml and cefotaxime, 20 µg/ml.

3.3.2 Construction of the reporter and mutant strains

Standard molecular biology techniques were used for all cloning work. All primers used for cloning of DNA fragments are listed in Table 3.2. For the construction of the reporter strains DOA9-P*nifDKc* and DOA9-P*nifDKp*, the 500-bp upstream region of each *nifDK* operon was amplified by PCR and cloned into the plasmid pVO155-npt2-cefo-npt2-gfp (Table 3.2). This plasmid which is non-replicative in *Bradyrhizobium* strains is a derivative of the plasmid pVO155 (Oke and Long, 1999) that carries the promoterless *gusA* gene and constitutively expressed *gfp*, kanamycin and cefotaxime genes (Okazaki et al. 2015). For the construction of the two *nifDK* mutants, 300 to 400 base pairs (bp) internal fragments of each *nifD* gene were amplified by PCR and cloned into the plasmid pVO155-Sm-npt2-gfp harboring a streptomycin/spectinomycin-resistance gene. The resulting plasmids were introduced into *E. coli* S17-1 using electroporation (15 kv/cm, 100Ω, and 25µF) and were transferred into *Bradyrhizobium* DOA9 by biparental mating using protocol described in Giraud et al. (2010). Transconjugants were selected on YM plates supplemented with 20 µg/ml nalidixic acid, 20 µg/ml cefotaxime and 200 µg/ml kanamycin or 20 µg/ml nalidixic acid and 200 µg/ml streptomycin.

3.3.3 Complementation of the DOA9Δ*nifDKc* mutant

For complementation of the DOA9Δ*nifDKc* mutant with *nifENX* genes, it was first introduced the amplified promoter region of the *nifDKc* operon into the plasmid

of pMG103-npt2-cefo harboring a constitutive cefotaxime resistance gene leading to the pMG103-npt2-cefo-*pm-nifDKc* plasmid (Table 3.2). Then, the amplified *nifENX* genes were cloned downstream the introduced promoter region (Table 3.2). For complementation of the DOA9Δ*nifDKc* mutant with *nifDKc* genes, the *nifDKc* genes including the 500-bp upstream promoter region was amplified and cloned directly into pMG103-npt2-cefo. These constructed vectors were introduced into the DOA9Δ*nifDKc* mutant by electroporation (17.5 kv/cm, 100Ω, and 25μF). The complemented strains were selected on YM plates supplemented with 20 μg/ml cefotaxime and 100 μg/ml spectinomycin.

3.3.4 Plant cultivation, symbiotic analysis

The symbiosis efficiency of *Bradyrhizobium* DOA9 strain and derivatives were tested with *Aeschynomene americana* No. 281 collected from LSTM's greenhouse. The seeds were surface sterilized by immersion in sulphuric acid under shaking during 45 minutes. Seeds were abundantly washed with sterile distilled water and incubated overnight in sterile water. Seeds were then transferred for one day at 37°C in the darkness on 0.8% agar plates for germination. Plantlets were transferred on the top of test tubes covered by aluminum paper for hydroponic culture in buffered nodulation medium (BNM) (Ehrhardt et al. 1992). Plants were grown in a 28°C growth chamber with a 16-h light and 8-h dark regime and 70% humidity. Seven days after transfer, each seedling was inoculated with one milliliter of cell suspension resulting from a 5 day-old bacterial culture washed in BNM and adjusted to reach an optical density of one at 600 nm. For nodulation and nitrogen fixation assay, 10 to 20 plants per condition were taken at 20 days post inoculation (dpi) and analyzed for the number of nodules and nitrogenase activity as previously described (Bonaldi et al. 2010). The experiments were carried out in duplicate.

Table 3.2 Primers used in this study.

Primers	Sequences (5'-3')	Relevant characteristics
Pm.nifDKp.f/ Pm.nifDKp.r	GAACGT**CTCGAG**CTCGCAGCG CCCTCGACAATAG/CGCTT**TCT** **AGA**CTTCTCCGGATAGACCTTC AAG	Cloning of the 500-bp upstream region of the *nifDKp* operon in pVO155-npt2-cefo-npt2-gfp after digestion of the PCR product by XhoI/XbaI and the plasmid by SalI/XbaI. Plasmid constructed to obtain the reporter strain DOA9-P*nifDKp*.
Pm.nifDKc.f/ Pm.nifDKc.r	GCCTCG**CTCGAG**GGGTCGGAT GCATCAAGCAAG/GACGCT**CTC** **TAGA**GTTGCTAGGCTCATACG AATATC	Cloning of the 500-bp upstream region of the *nifDKc* operon in pVO155-npt2-cefo-npt2-gfp after digestion of the PCR product by XhoI/XbaI and the plasmid by SalI/XbaI. Plasmid constructed to obtain the reporter strain DOA9-P*nifDKc*. The same PCR product was cloned in pMG103-npt2-cefo to obtain the plasmid pMG103-npt2-cefo-pm-*nifDKc* used for complementation.

Table 3.2 Continued.

Primers	Sequences (5'-3')	Relevant characteristics
nifDKp.in.f/nifDKp.in.r	CAAGCA**GTCGAC**CGACGAGGTCTTGAAGGTCTATC/ATGTCCT**T** **CTAGA**GGAAGTCGGAGGTGAACTGCATC	Cloning of internal *nifDp* fragment in pVO155-Sm-npt2-gfp after digestion of the PCR product by SalI/XbaI and the plasmid by SalI/XbaI. Plasmid constructed to obtain the mutant strain DOA9Ω*nifDKp*.
nifDKc.in.f/nifDKc.in.r	GACCG**GTCGAC**GTCGCGCCAAGCACCTCAATG/CTGGAT**TCTA** **GA**GATGATCTTGGCTAGCTTCTTG	Cloning of internal *nifDc* fragment in pVO155-Sm-npt2-gfp after digestion of the PCR product by SalI/XbaI and the plasmid by SalI/XbaI. Plasmid constructed to obtain the mutant strain DOA9Ω*nifDKc*.

Table 3.2 Continued.

Primers	Sequences (5'-3')	Relevant characteristics
nifENX.in.f/nifENX.in.r	GGGGC**TCTAGA**CTCGCGCAGCGGGTCAGGGAATAG/CGTCGG**GAATTC**GGTACCCAGCCGTCTCTGACATGATGTTTC	Cloning of the *nifENX* genes in pMG103-npt2-cefo-pm-*nifDKc* after digestion of the PCR product by XbaI/EcoRI and the plasmid by XbaI/EcoRI. Plasmid constructed to complement the mutant strain DOA9Δ*nifDKc*.
nifDKc.f/ nifDKc.r	GCCTCG**TCTAGA**GGGTCGGATGCATCAAGCAAG/CCCCG**GAATTC**GGCGGCAACTCTCATCGACATCAG	Cloning of the *nifDKc* genes including the 500-bp upstream region in pMG103-npt2-cefo after digestion of the PCR product by XbaI/EcoRI and the plasmid by XbaI/EcoRI. Plasmid constructed to complement the mutant strain DOA9Δ*nifDKc*.

3.3.5 Cytological analysis

To follow the β-glucuronidase activity in the nodules elicited by the reporter strains, 30-to 40-µm-thick sections from fresh nodule samples made using a vibratome (VT1000S; Leica, Nanterre, France) were incubated at 37°C in the darkness in a GUS assay buffer for 1 hour as described in Bonaldi et al. (2010). After staining, the sections were mounted and observed under bright-field illumination with a microscope (Nikon AZ100; Champigny-sur-Marne, France).

3.3.6 Determination of nitrogenase activity under free-living conditions

To determine the nitrogenase enzyme activity under free- living conditions, *Bradyrhizobium* sp. strain DOA9 and derivatives, as well as *Bradyrhizobium japonicum* USDA110 and *Bradyrhizobium* sp. strain ORS278 were grown in 10-ml test tubes (BD Vacutainer, Franklin Lakes, NJ, U.S.A.) containing 2 ml of BNM-B medium (without glutamate) soft agar (0.8%), as described by Alazard (1990). Acetylene was injected to give a final concentration of 10%. The cultures were incubated at 28°C without shaking and gas samples were analyzed at 7 dai for ethylene production by gas chromatography (GC), as described (Renier et al. 2011).

3.3.7 Determination of the β-glucuronidase (Gus) activity under free-living conditions

The two DOA9 reporter strains were grown 4 days in YM medium, collected, and washed with BNM-B medium (without glutamate) as described above. Bacterial cells were inoculated into flasks containing BNM-B medium to obtain an initial OD600 at 0.05. Aliquots 55 ml of culture were injected into 150-ml bottles sealed with rubber stoppers and incubated at 28°C for 11 days without shaking. Replicas of the cultures were simultaneously incubated in cotton-stoppered flasks to evaluate aerobic conditions. At different time points, samples were taken and the β-Glucuronidase activity was measured using thesubstrate p-nitrophenyl glucuronide (PNPG) as described

by Jefferson (1987). β-Glucuronidase units were calculated according to Miller (1972).

3.4 Results

3.4.1 The non-photosynthetic *Bradyrhizobium* strain DOA9 is able to fix nitrogen under free-living conditions

The presence of a *nifV* gene on the DOA9 chromosome indicated the ability of this strain could fix nitrogen under free-living conditions. To test thishypothesis, analysis the nitrogenase activity of this bacteria was tested by culturing on soft-agar in a minimal medium free of nitrogen and under low oxygen tension, i.e. conditions for which *A. caulinodans* and photosynthetic bradyrhizobia are able to fix nitrogen (Alazard, 1990). As shown in Figure 3.2, the strain DOA9 displayed a nitrogenase activity (estimated by acetylene reduction) at a similar level than ORS278 strain. In contrast, no activity could be detected for *B. japonicum* USDA110 used here as negative control. These report was the first finding that a non-photosynthetic *Bradyrhizobium* strain was able to fix nitrogen under free-living conditions.

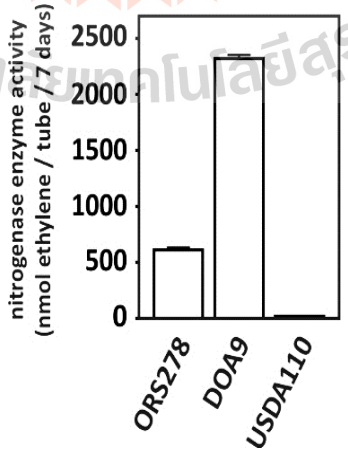

Figure 3.2 Occurrence of a nitrogenase activity determined by the acetylene reduction assay in various *Bradyrhizobium* cultivated in free-living condition.

3.4.2 Both *nifDK* operons were highly expressed under symbiosis but differently regulated under free-living conditions

To determine whether the two *nifDK* operons found on the chromosome (*nifDKc)* and the plasmid *(nifDKp)*, are expressed under free-living and symbiotic conditions, the two reporter strains annotated DOA9-P*nifDKc* and DOA9-P*nifDKp* were constructed by integrating respectively the pVO155-npt2-cefo-npt2-gfp plasmid just downstream the corresponding *nifDK* operon. This plasmid harbors the promoter less *gusA* gene used to follow the *nifDK* expression and a constitutive *gfp* and cefotaxime cartridge used to select the single crossing over event. To test the symbiotic expression of the *nifDK* operons, was selected as host plant *A. americana* from which the DOA9 strain has been isolated (Noisangiam et al., 2012). The observations done at 14 days of post-indoculation (dpi) showed that the two reporter strains were able to nodulate *A. americana* and to fix nitrogen similar to the WT-strain indicating that the integration of the pVO155 plasmid did not disturb their symbiotic performance (Figure 3.3A-F). Cytological analysis revealed that the nodules elicited by the two reporters strains displayed a high ß-glucuronidase activity, in contrast to the WT-nodules for which no activity could be detected (Figure 3.3G-I). It was too highly that no difference could be detected by eyes in the X-Gluc staining of the nodules elicited by the two reporters strains indicating that the two *nifDK* operons were expressed at a similar level during symbiosis with *A. americana*.

To test the *in vitro* expression of the two *nifDK* operons, different culture conditions of the reporter strains were tested: i) with and without a nitrogen source and ii) under oxygenic and hypoxic conditions. Whatever the conditions used, the growth of the two reporter strains was comparable with a clear positive effect of the oxygen and the nitrogen source (Figure 3.4). However, only a significant β-gucuronidase activity could be detected for the reporter strain DOA9-P*nifDKc* cultivated under low oxygen

tension and without nitrogen, i.e. the conditions previously used to detect a nitrogenase activity. It was worth noting that in such conditions the reporter strain DOA9-P*nifDKp* displayed a very low ß-glucuronidase activity that represent less than 1% the one detected for DOA9-P*nifDKc*. Altogether these data suggest that the two *nifDK* operons ws differently regulated, both was strongly expressed under symbiosis while only the *nifDKc* was expressed at a significant level under free-living state.

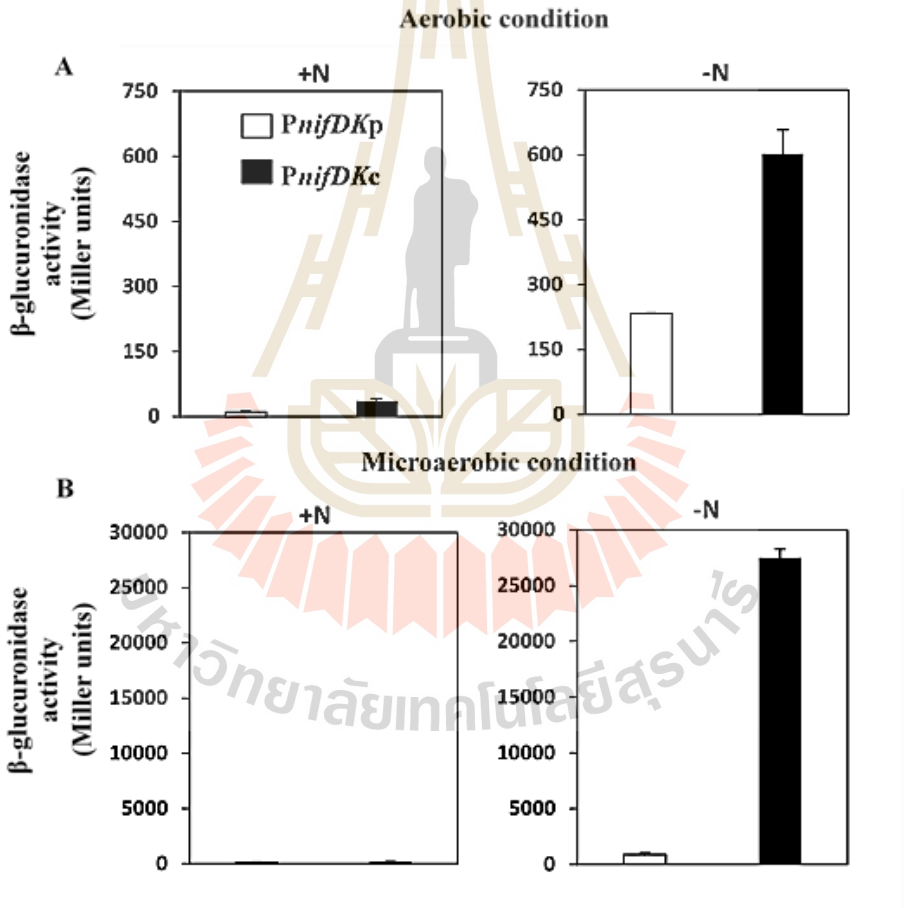

Figure 3.3 Expression of chromosome and plasmid *nifDK* operon of,*Bradyrhizobium* sp. DOA9 in free-living conditions. ß-galactosidase activity of DOA9*PnifDK*p (white bar) and DOA9-P*nifDKc* (black bar) in BNM medium addition of a nitrogen source (+N) and without a nitrogen source (-N), aerobic **(A)** and microaerobic **(B)** conditions.

3.4.3 Both *nifDK*c and *nifDK*p are required for fully function of nitrogenase activity in symbiosis with *A. americana*

To determine the respective contribution of the two *nifDK* operons in the nitrogenase activity of the DOA9 strain during symbiosis, two insertional mutants (DOA9Δ*nifDKc* and DOA9Δ*nifDKp*) were constructed in which, this time, a pVO155 derivative plasmid was integrated in the 5' region of the corresponding *nifD* gene. Observations done at 21-dpi using similarly *A. americana* as host plant showed drastic differences between the two *nifDK* mutations.

The plants inoculated with the DOA9Δ*nifDKc* mutant displayed typical nitrogen starvation symptoms, including foliage chlorosis and reduced plant growth (Figure 3.5A and B). This was correlated with the total absence of a nitrogenase activity (Figure 5I). This mutant induced a higher number of nodules per plant but all of them were hollow from inside and displayed a white color indicating the absence of leghemoglobin (Figure 3.5C and F). All these data indicated a strict fix minus phenotype for this mutant. In contrast, the effect of the *nifDK*p mutation was less drastic but remained significant in comparison to the WT strain. Indeed, the plants inoculated with the DOA9Δ*nifDKp* mutant fixed nitrogen slightly less efficiently (around less 35%) than those inoculated with the WT strain (Figure 3.4I). This lower nitrogenase activity had a direct impact the plant growth (Figure 3.5A and B). The DOA9Δ*nifDKp* mutant also induced a higher number of nodules per plant but, in contrast to the DOA9Δ*nifDKc* mutant, these nodules looked normal with no sign of degeneracy (Figure 3.5C and E).

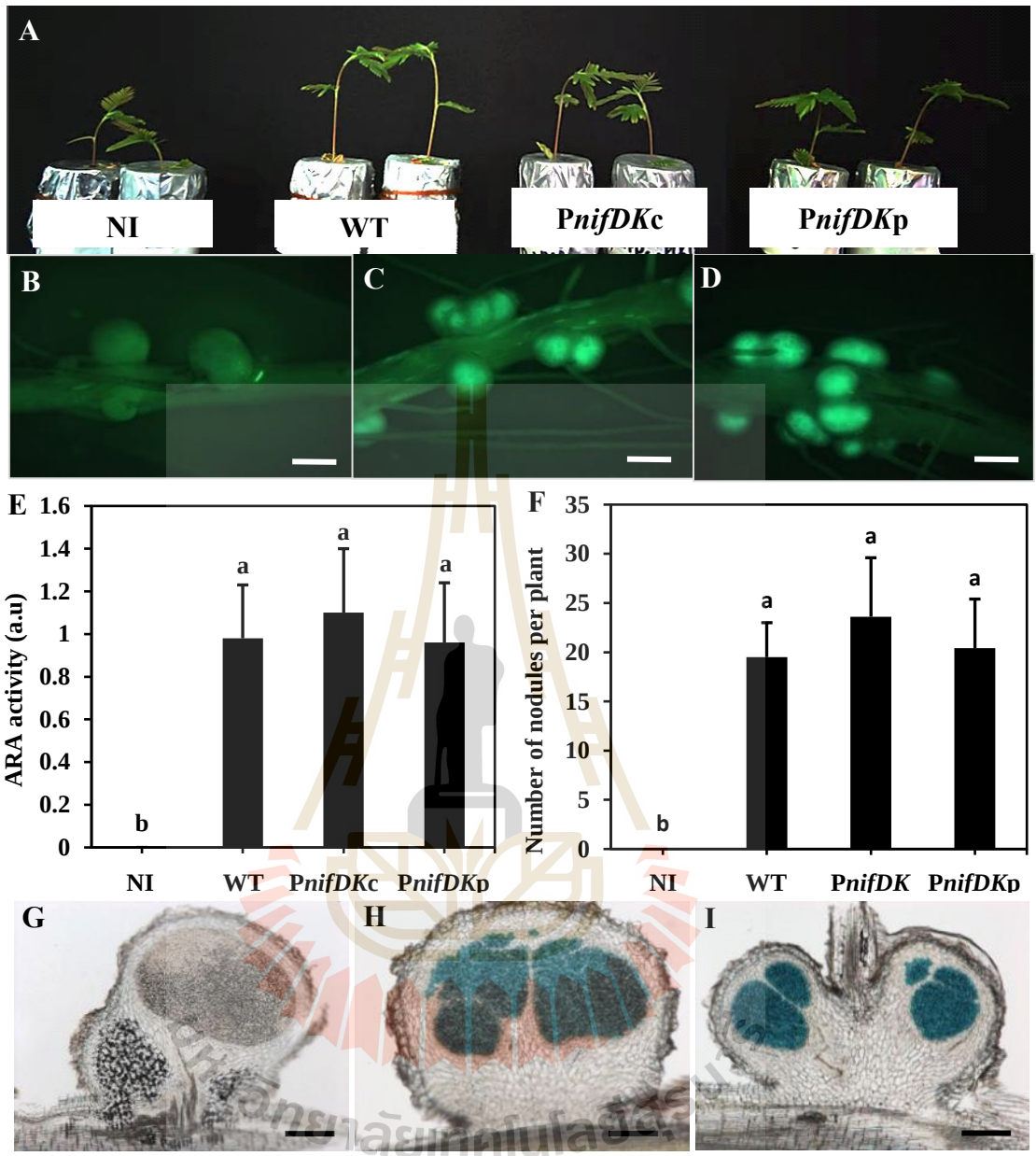

Figure 3.4 The chromosomic and plasmidic *nifDK* operon of *Bradyrhizobium* sp. DOA9 strain are expressed during symbiosis with *A. americana*. (**A)** Comparison of the growth of the plants (aerial part) non-inoculated (NI) or inoculated with WT and the reporter strains DOA9-P*nifDKc* and DOA9-P*nifDKp* (at 14 dpi). (**B-D**) Root nodules observed with a fluorescent stereo microscope equipped with a green fluorescent protein (GFP) filter. (**E**) The occurrence of acetylene reducing activity (ARA) in *A. americana* plants inoculated with WT and the reporter strains DOA9-P*nifDKc* or DOA9-P*nifDKp*. (**F**) Number

of nodules per plant elicited by WT and DOA9-P*nifDKc* and DOA9-P*nifDKp*. (**G-I**) Expression of the *gusA* reporter gene revealed on 40 µm nodule sections stained with X-Gluc. **(G**) nodules elicited by WT; (**H**) nodules elicited by DOA9-P*nifDKc*; (**I**) nodules elicited by DOA9-P*nifDKc*. Scale bars are 1 mm for (**B-D)** and 250 µm **(G-I**). In panels (**E**, **F**) Error bars represent s.e. *(n=10).* Different letters above the error bars indicate significant differences at P<0.05 (Tukey's HSD test).

To explain the differences observed between the two *nifDK* mutants, we considered the possibility of a polar effect of the *nifDKc* mutation on the downstream accessory genes (*nifENX*) which could be co-transcribed with the *nifDKc* genes and which were found in single copy on the genome (Figure 3.1). To test this hypothesis, the pMG103 plasmid harboring either the *nifDKc* genes or the *nifENX* genes were introduced into DOA9Δ*nifDKc* mutant, both constructs put under the control of the *nifDKc* promoter. The pMG103 plasmid was selected because it has been previously found stable in some *Bradyrhizobium* strains (Bonaldi et al. 2011). As shown in Figure 3.5A, G, H, a clear complementation of the nitrogenase activity of the DOA9Δ*nifDKc* mutant could be observed after introduction of the *nifENX* genes but not with the *nifDKc* genes confirming the previous hypothesis. However, it was to highlight that only a partial restoration of the nitrogenase activity was observed after introduction of the *nifENX* genes (Figure 3.5I). This difference observed with the WT strain (around 35%) could be attributed to the fact that in this complemented strain the *nifDKc* genes remained mutated. Altogether, these data indicated the two *nifDK* operons were functional during symbiosis and both of them contribute significantly to the global nitrogenase activity of the DOA9 strain.

3.4.4 The *nifDKc* operon was the major contributor of the nitrogenase activity of the DOA9 strain under free-living conditions

Thank the two previous obtained mutants, the contribution of each *nifDK* operon in the nitrogenase activity of the DOA9 strain cultivated *in vitro*. As expected, the DOA9Δ*nifDKc* mutant was also found to not fix nitrogen in this condition (Figure 3.5J). In contrast, the DOA9Δ*nifDKp* mutant displayed a nitrogenase activity similar than the WT strain indicating that the *nifDKp* operon did not play a significant role in this culture condition. We also analyzed the DOA9Δ*nifDKc* mutant complemented with the *nifENX* genes. In this last case, contrary to the symbiotic conditions, only a very partial restoration of the nitrogenase activity could be detected (Figure 3.5J). This last result was in line with the very low level of expression *nifDKp* operon found in this condition (Figure 3.4).

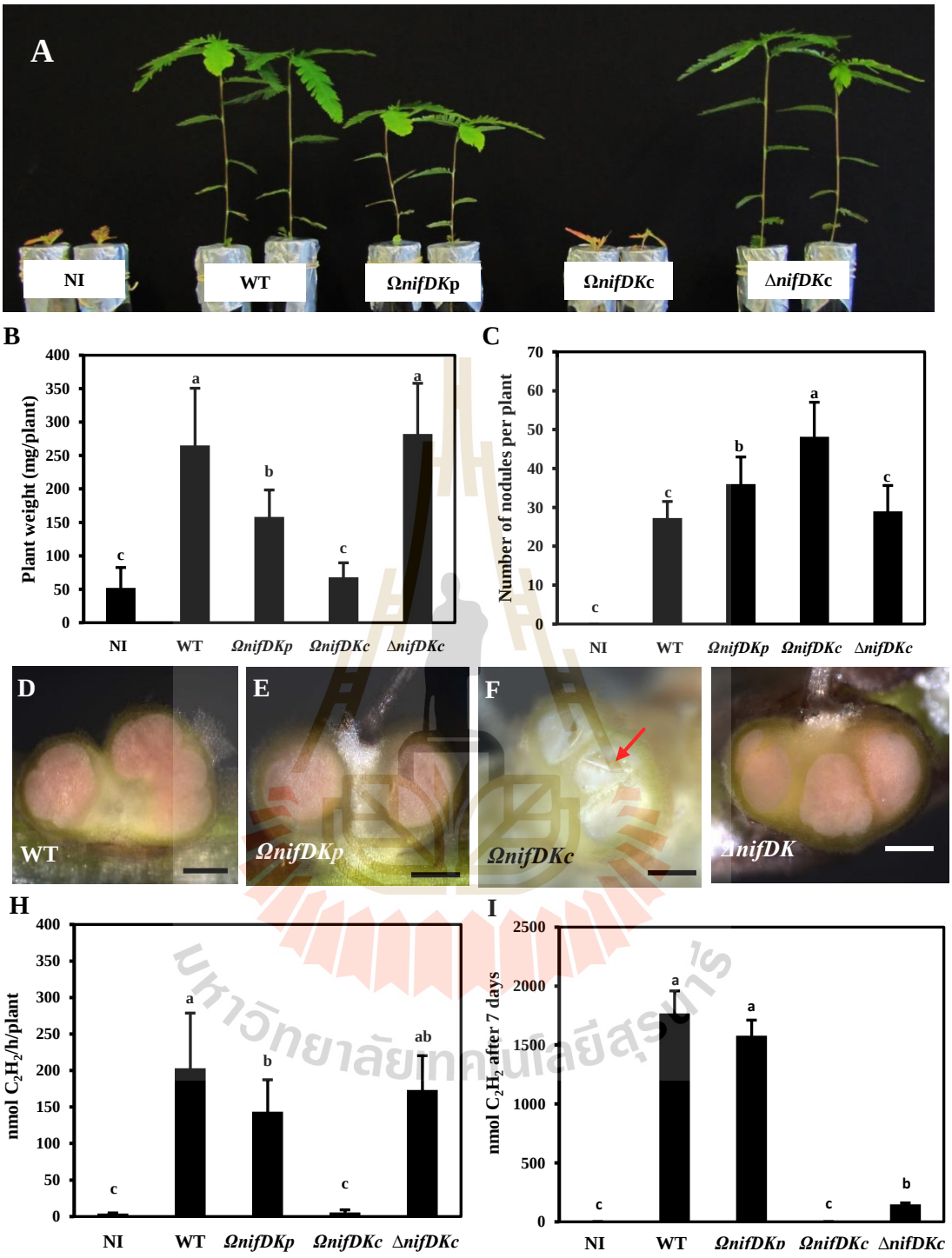

Figure 3.5 Role of plasmid and chromosome *nifDK* operon of *Bradyrhizobium* sp. DOA9 during the symbiotic and free-living states. **(A)** Comparison of the growth of the plants (aerial part) non-inoculated (NI) or inoculated with WT and the mutants DOA9*ΩnifDKp,* DOA9*ΩnifDKc,* DOA9*ΩnifDKc* and DOA9Δ*nifDKc* (at 21 dpi). (**B**) Fresh mass of the plants inoculated with WT and the mutants

DOA9*ΩnifDKp,* DOA9*ΩnifDKc* and DOA9Δ*nifDKc* (at 21 dpi). (**C**) Number of nodules per plant elicited by the WT and the mutants DOA9*ΩnifDKp,* DOA9*ΩnifDKc* and DOA9Δ*nifDKc* (at 21 dpi). (**D-G**) Cross section of nodule elicited by the WT (**D**) and the mutants DOA9*ΩnifDKp* (**E**) and DOA9*ΩnifDKc* **(F)** and the mutant DOA9Δ*nifDKc* (**G**). The red arrow in panels F indicates the digestion of the nodule central tissue. Scale bars are 250 µm for (**D-G)**. (**H**) The occurrence of acetylene-reducing activity (ARA) in *A. americana* plants inoculated with WT and the mutants DOA9*ΩnifDKp* and DOA9*ΩnifDKc* and the DOA9Δ*nifDKc.* (**I**) Occurrence of a nitrogenase activity in WT and the mutants DOA9*ΩnifDKp* and DOA9*ΩnifDKc* and the Δ*nifDKc* cultivated in free-living conditions. In panel (**B**, **C**, **H**, **I**) Error bars represent s.e. *(n=20).* Different letters above the error bars indicate significant differences at $P<0.05$ (Tukey's HSD test).

3.5 Discussion

They were several reports indicating the reiteration of some structural nitrogenase genes in some rhizobium strains. For example, *Azorhizobium caulinodans* or several photosynthetic *Bradyrhizobium* strains (BTAI1, ORS278, S58) contain two *nifH* genes (Norel and Elmerich, 1987; Giraud et al. 2007). More complexly, the *Rhizobium etli* CFN42 strain, initially named *R. phaseoli,* contained two complete *nifHDK* operons (copies a and b) and a truncated *nifHD* operon (copy c) (Quinto et al. 1982; Quinto et al. 1985). In all these different cases, the reiterated genes were found on the same replicon either on the chromosome for the *Bradyrhizobium* strains or on the symbiotic plasmid for the *Rhizobium* species and they were 100% identical or only differ by one or two aa mismatch indicating that these multiples copies most probably result to recent duplication events. The *Bradyrhizobium* sp. DOA9 strain constitutes an unique case

because the reiterated *nifHDK* genes are found on both the plasmid and the chromosome and they were not perfectly conserved (97% of aa identity between the two NifH or NifK proteins and 98.5% between the two NifD). This would suggest a different origin of these structural nitrogenase genes, with an acquisition of the *nifHDKc* genes by a vertical transmission and the one of the *nifHDKp* genes by a horizontal transfer.

However, a phylogenetic analysis conducted on NifH, NifD or NifK sequences from *Bradyrhizobium* strains shows in the 3 cases that the chromosomic and plasmidic copies identified in DOA9 strain were the closest neighbors, which did not support this hypothesis (Figure 3.6). It could be therefore possible that a more ancestral duplication event(s) occurred followed by the transposition of one copy on the plasmid, each gene copy evolving then independently by accumulating different mutations. The absence of accessory *nif* genes on the plasmid would be more in agreement with this last scenario.

The data clearly indicated that the two *nifDK* genes found on the chromosome and the plasmid encode for a functional nitrogenase and both contribute significantly to the nitrogenase activity measured during symbiosis. In contrast, functional differences were observed in free-living conditions, the nitrogenase activity detected resulting mainly to the NifDKc. This is surely related to the fact that in these last conditions, the transcription level of *nifDKp* was found to be very low. These data are reminiscent of those reported on *A. caulinodans*, for which the two *nifH* genes appears to be functionally redundant during symbiosis while *nifH*1 gene was found to be the principal contributor of the nitrogenase activity in free living conditions (Iki et al. 2007). In contrast to the NifHDKc and p, which were well conserved between them, the two NifA and RpoN copies strongly differ with respectively 52% and 55% of amino acid identity. It could be therefore possible that these regulatory proteins behave differently in response to various environmental factors and have distinct regulons by recognizing DNA motifs slightly different. Second, the upstream regions of *nifDKc* and *nifDKp* are

highly divergent and only the *nifDKc* promoter displays a canonical NifA and RpoN binding sites (Figure 3.7). Similar observations could be done with the *nifHc* and *nifHp* promoters region (Figure 3.8). From these observations, a simple regulatory model could be proposed in which the NifAc and RpoNc specifically control the *nifDKc* and *nifHc* genes and vice versa the NifAp and RpoNp specifically control the *nifDKp* and *nifHp* genes. However, a crosstalk between these two regulatory circuits could be also expected considering the fact that the NifDKp required the expression of the accessory *nif* genes to be functional, in particular the *nifENX* which are co-transcribed from the *nifDKc* promoter.

Furthermore, it could be found a perfect *nifA* and *rpoN* binding site in the promoter region of *nifAp* itself suggesting that *nifAp* is under the direct control of NifAc which is in line with the previous remark. It appears therefore that the *Bradyrhizobium* DOA9 strain with its unusual *nif* genes content and its unusual ability to fix nitrogen on both free-living and symbiotic states was an excellent model to progress in the understanding of the function and regulation of rhizobial nitrogenases. In this respect, experiments were presently conducted to clarify the specific regulon of each NifA and RpoN proteins in this bacteria.

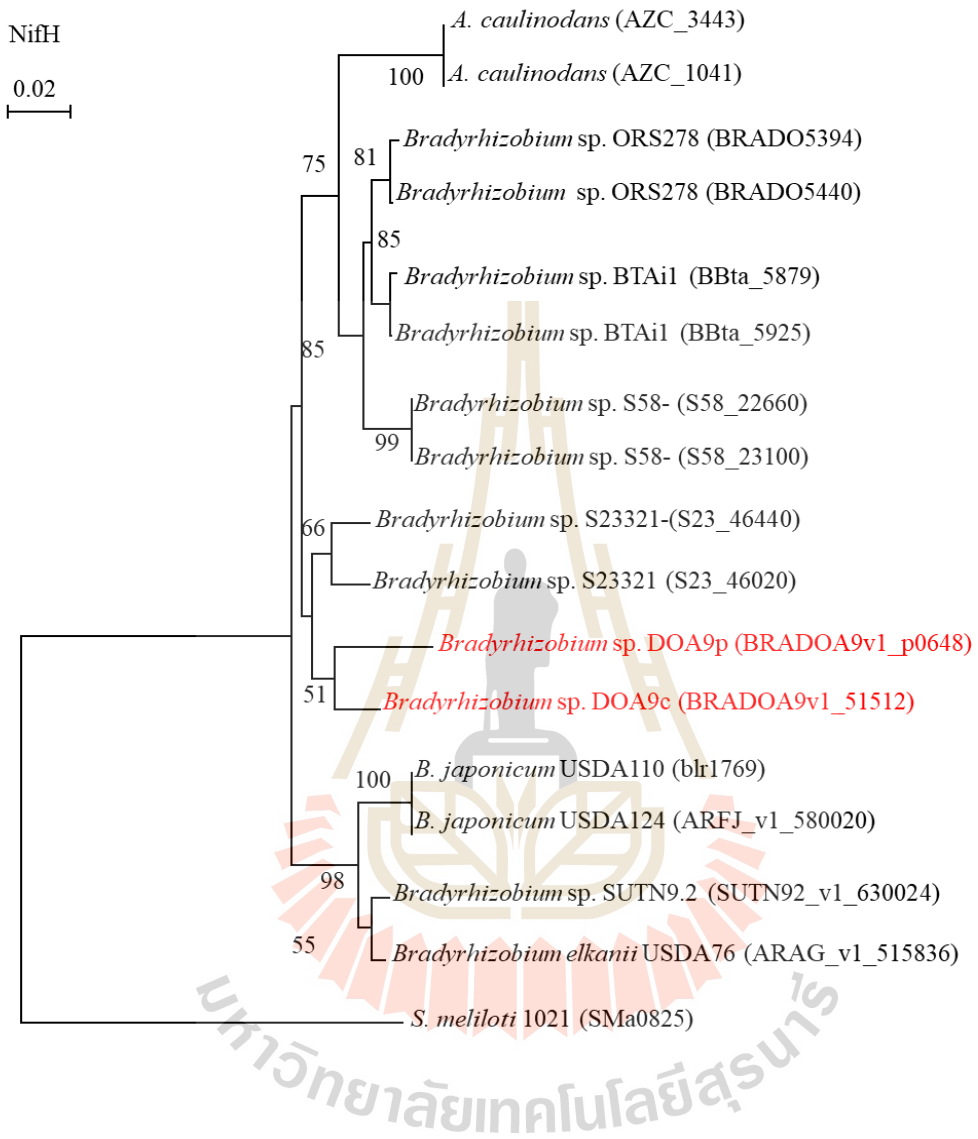

Figure 3.6 Neighbor joining phylogenetic trees of NifH sequence. Scale bars indicate the number of substitutions per site. Bootstrap values (% from 1000 replications replications) are indicated at nodes when higher than 50%.

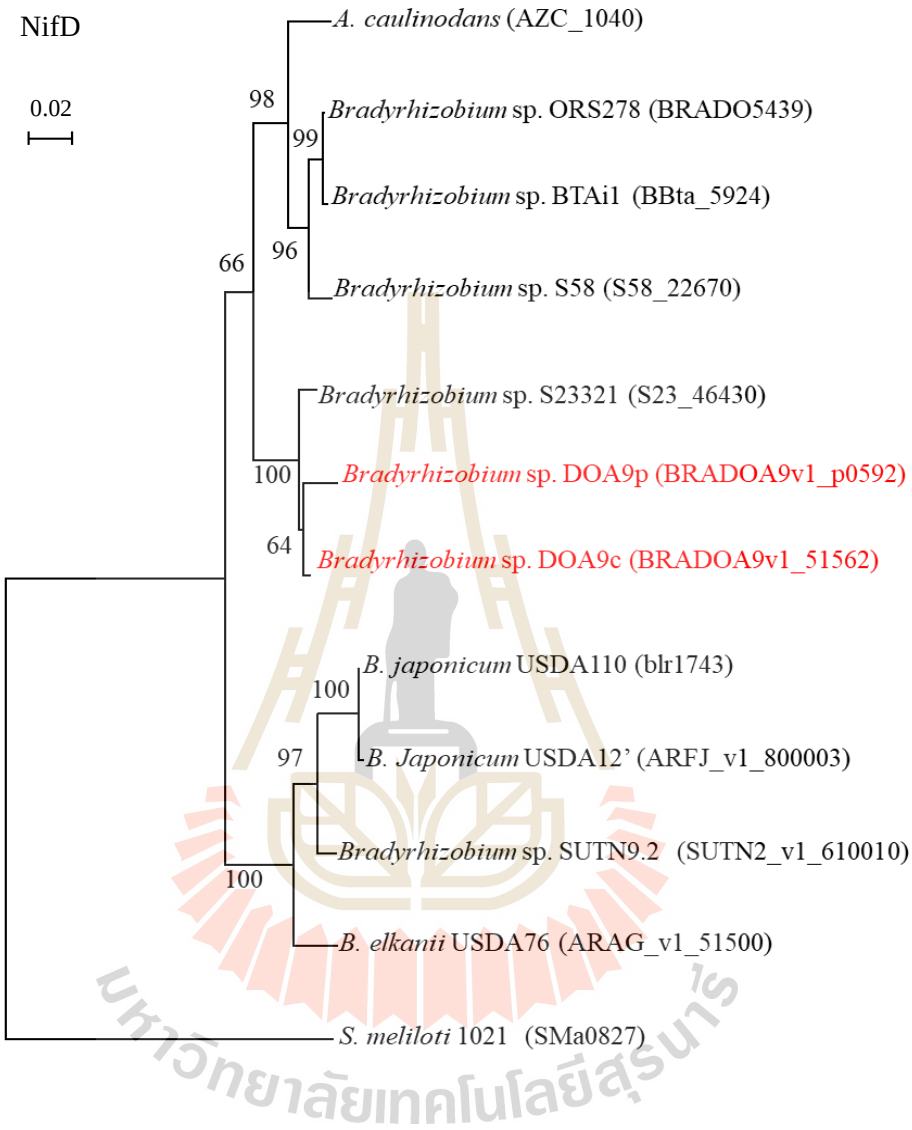

Figure 3.7 Neighbor joining phylogenetic trees of NifD sequence. Scale bars indicate the number of substitutions per site. Bootstrap values (% from 1000 replications replications) are indicated at nodes when higher than 50%.

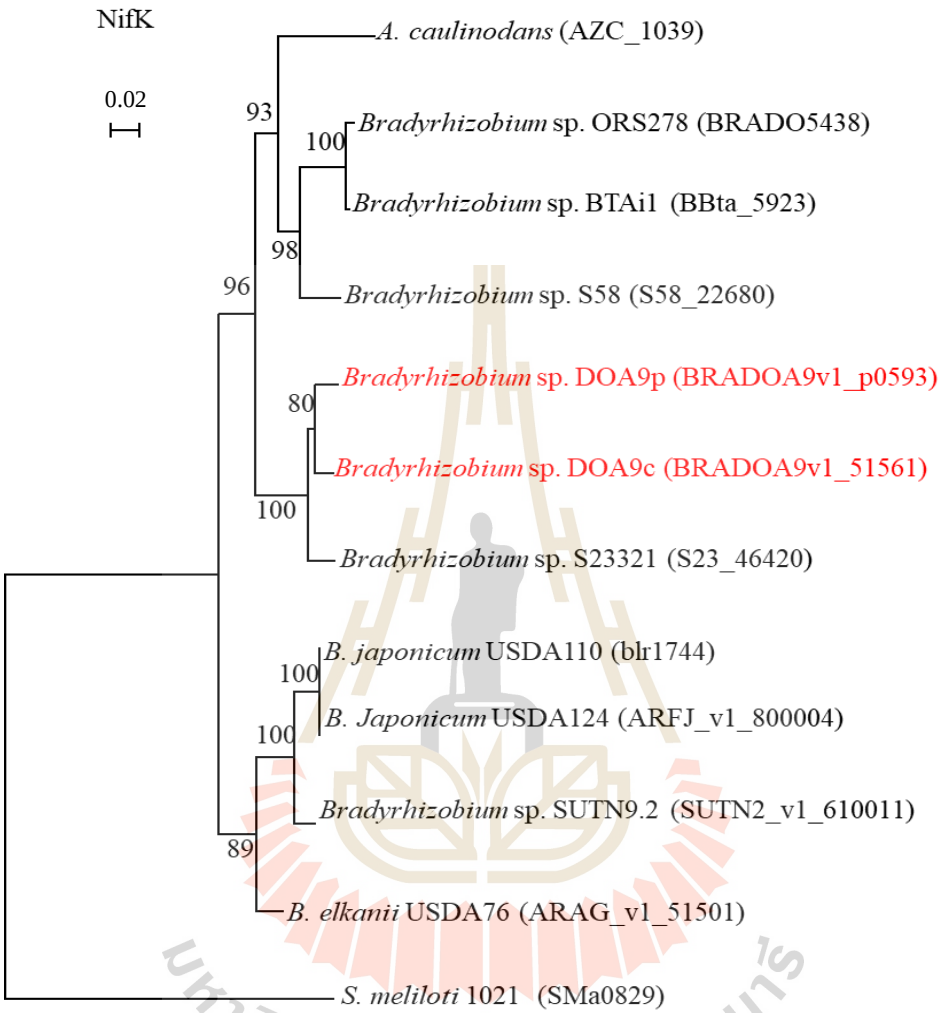

Figure 3.8 Neighbor joining phylogenetic trees of NifK sequence. Scale bars indicate the number of substitutions per site. Bootstrap values (% from 1000 replications replications) are indicated at nodes when higher than 50%.

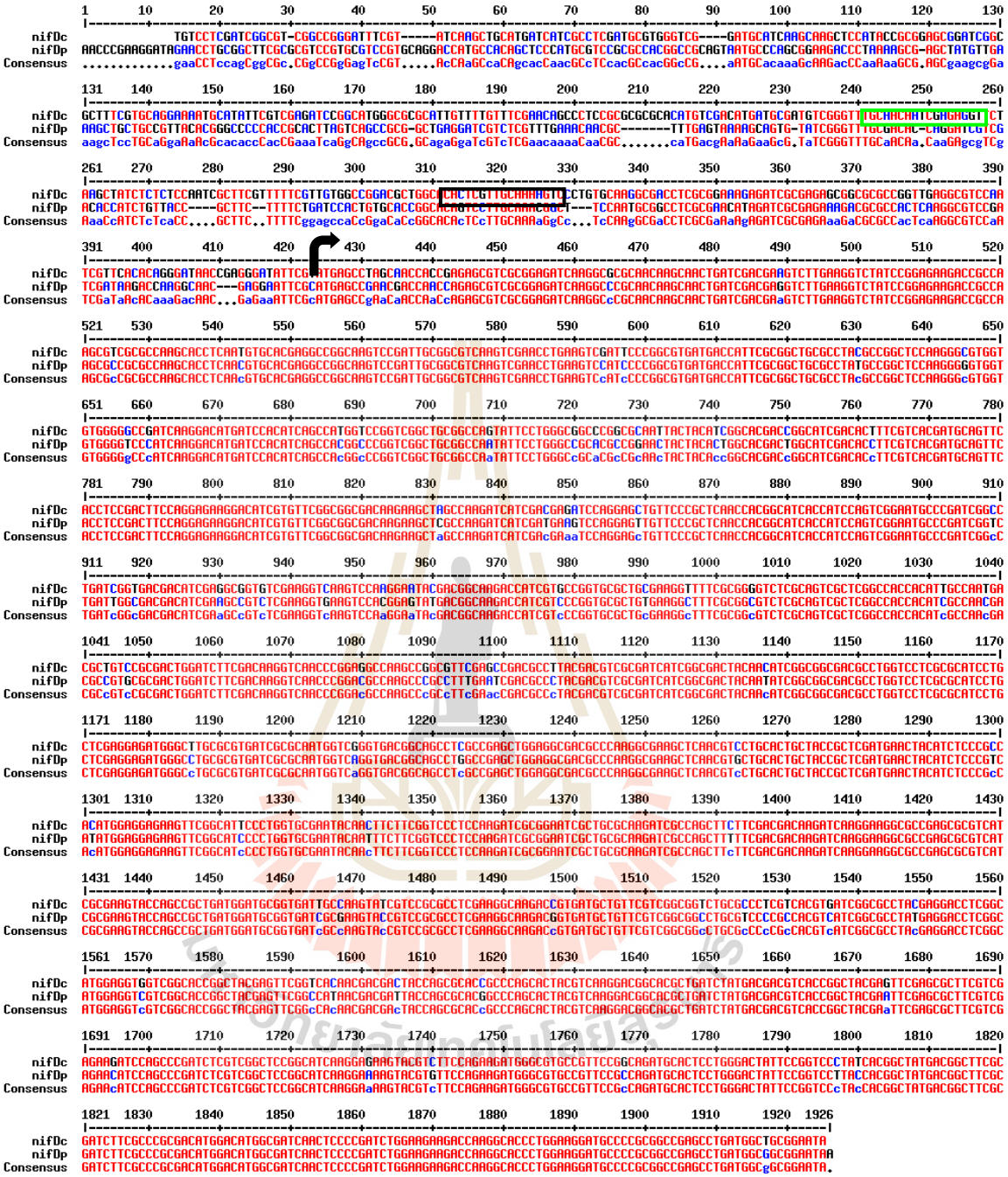

Figure 3.9 Alignment of *nifDc* and *nifDp* including the 400-bp upstream region. In green, it is indicated a putative NifA box and in black, a RpoN box. The arrow indicates the starting codon.

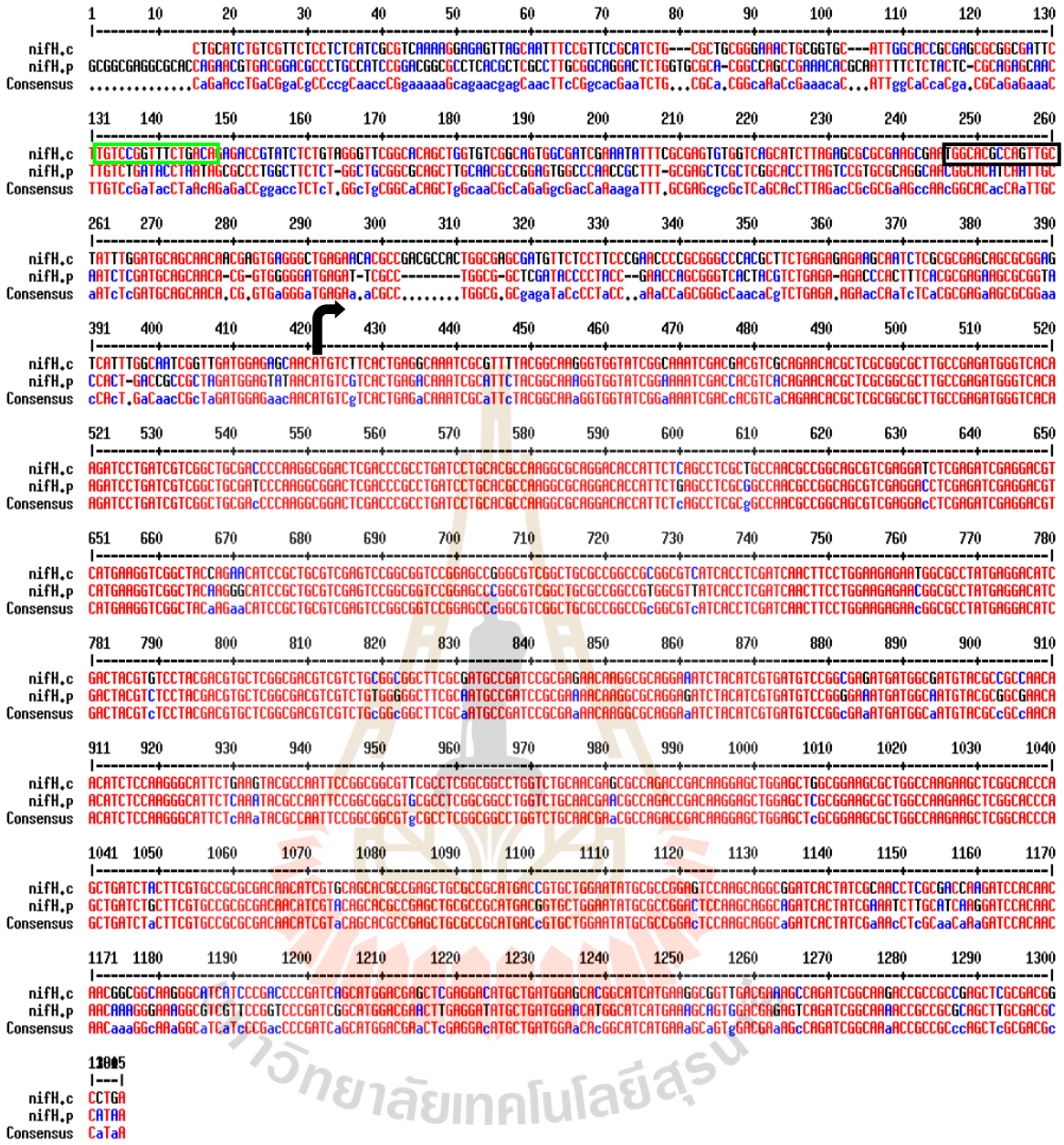

Figure 3.10 Alignment of *nifHc* and *nifHp* including the 400-bp upstream region. In green, it is indicated a putative NifA box and in black, a RpoN box. The arrow indicates the starting codon.

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CHAPTER IV

Bradyrhizobium sp. strain DOA9 displayed the two NifA regulatory proteins that replaced their functions for nitrogen fixation under symbiosis but not in free-living condition

4.1 Introduction

The number species of Rhizobiaceae possess of the nitrogenase enzyme activity to convert atmospheric nitrogen to ammonia, a mechanism that is exclusive in prokaryotes. The gene expression for nitrogen fixation is processed by cascades of hierarchically organized regulatory genes (Ratet et al., 1989). Their cooperative action allows the bacteria to response an optimal environmental conditions before transferring this signal cascade to the level of gene expression required to accomplish the nitrogen fixation (Gordon and Wheeler, 2012). Nitrogen and oxygen are the critical signals that regulate the *nif* genes expression in most diazotrophic bacteria. This physiological control is mediated through the general nitrogen regulatory system (*ntr*) and the *nif*-specific regulatory genes (*nifAL*) (de Bruijn et al., 2012). However, the nitrogen fixation genes in symbiotic diazotrophs are regulated predominantly in response to the oxygen conditions.

The models of nitrogen fixing regulatory circuit in symbiotic strains of *Rhizobium* sp., *Bradyrhizobium japonicum*, and *Sinorhizobium meliloti* have been documented. In *Rhizobium* sp. and *S. meliloti* use mostly identical regulatory elements including FixL, FixJ, FixK, NifA or FixRNifA, and RpoN in the oxygen responsive FixLJ-FixK cascade performing as direct positive regulators on *nifA* operon (Thöny et al. 1987 and 1989). In contrast, *B. japonicum* uses the oxygen responsive RegSR-NifA cascade

to induce expression of the *nifA* operon which is preceded by appropriate promoters (Dixon and Kahn, 2004b and Fischer, 1994). The induction of genes controlled by both FixLJ-FixK and RegSR-NifA cascades requires very low oxygen condition because of the oxygen sensitivity of NifA. Responding to low oxygen or anoxic conditions, the functional NifA and RpoN proteins are required for regulation of other *nif* and *fix* genes expression (Torres et. al., 2014). The phosphorylation-responsive NifA protein in cooperate with RNA polymerase containing RpoN interact on the upstream activator sequence (UAS) and form a DNA loop induced by the integration host factor (IHF) to initiate the transcription of other *nif* genes involved in nitrogen fixation (Dixon and Kahn, 2004a). Therefore, NifA is one of the key proteins to control the nitrogen fixing ability of bacteria under different environmental conditions.

The number of *nifA* genes in the chromosome could be varied from one to two copies dependent on each rhizobial strain. For example, *Mesorhizobium loti* contains two copies of the *nifA* gene, *nifA1* and *nifA2*, both genes are located on ICEMlSym^{R7A}. The *nifA1* gene is similar and located in the same genomic context as *nifA* from *R. etli*, *R. leguminosarum*, *Rhizobium* sp. strain NGR234, and *S. meliloti* (Santero et al., 1989). In contrast, *nifA2* is highly similar to *nifA* from *B. japonicum* and does not located adjacent to the known nitrogen fixation genes (Sullivan et al., 2001). The two genes are not functionally redundant since the *M. loti nifA2* mutant formed Fix minus nodules, whereas *nifA1* mutant did not show symbiotically impaired (Nukui et. al., 2006). In *Bradyrhizobium* sp. DOA9, two copies of *nifA* were also found to locate one on the chromosome and another one on the mega-plasmid (Okazaki et al., 2015). Strain DOA9 was isolated from rice field soil using *Aeschynomene americana* as a trap legume (Noisangiam et al., 2012). This strain displayed an unusual broad host range as indicated previously (Teamtisong et al., 2014). Interestingly, DOA9 also has ability to fix the nitrogen under the free-living condition (Wongdee et al., 2016). Scenario of genetic

organization suggests that *nifA* on chromosome should belong to the *fixRnifA* operon, whereas *nifA* on the plasmid was stand alone. Nevertheless, the structural *nifH*, *nifDK* and regulatory *rpoN* genes were found on both replicons. Data from previous research indicated that the structural NifDK on both replicons of DOA9 are required to full fill the functional of nitrogenase activity when symbiosis with *A. americana*, while only the structural NifDK genes on the chromosome are mainly required during free-living state (Wongdee et al., 2016). Therefore, it is interesting to understand whether NifA on both replicons regulate the expression of other nitrogen fixing genes differently under different life-styles of strain DOA9.

Thus, in this present work we aimed at investigation and analysis of two regulatory *nifA* genes in *Bradyrhizobium* sp. DOA9 under both symbiotic and free-living conditions. After DNA and amino acid sequence analyses, the Gus-fusions of *nifAc* and *nifAp* were constructed to determine the expression of genes under both symbiotic and free-living conditions. Then, the single and double *nifA* mutants as well as the hybrid proteins of *nifAc-NifAp* were constructed and tested their function involving in nitrogen fixing ability of DOA9. Finally, the expression of some *nif* genes under three different backgrounds, including DOA9 wile-type, Δ*nifAc*, and Δ*nifAp* mutant strains were determined to elucidate the genes that could be regulated or cross-regulated by NifAc or NifAp. From these experiments, the function of both *nifA* genes and their regulatory pathways were proposed.

4.2 Objectives

4.2.1 To analyze the genetic similarity and organization of genes involved in nitrogen fixation in DOA9 compared with other nitrogen-fixing bacteria

4.2.2 To verify the expression of two *nifA* genes under both symbiotic and free-living conditions

4.2.3 To investigate the role of *nifA* located on both chromosome and plasmid of DOA9 involved in regulation of nitrogen fixation under both symbiotic and free-living conditions

4.3 Materials and methods

4.3.1 Bacterial strains and culture media

Bradyrhizorium sp. DOA9 wild-type was obtained from School of Biotechnology, Suranaree University of Technology, Thailand, while all mutants were constructed in Laboratoire des Symbioses Tropicales et Méditerranéennes (LSTM), France. These bacterial strains were grown at 28°C for 4 days in YM medium (Vincent, 1970) or in a BNM-B minimal medium. (Ehrhardt et al. 1992 and Renier et al. 2011). All *Escherichia coli* strains were grown in LB medium at 37°C for 1 day. Each medium was supplemented with the following antibiotics when appropriate, for *E. coli*, 20 µg/ml Cefotaxime and for *Bradyrhizobium* mutant, 20 µg/ml Cefotaxime and 50 µg/ml Kanamycin.

4.3.2 DNA manipulations, PCR amplification and sequencing

The bacterial DNA and plasmids were extracted by Genomic DNA and Plasmid purification kits (Promeca, Germany). DNA fragments were purified from agarose gels using the illustra GFX PCR DNA and Gel Band Purification Kits (GE Healthcare, USA). DNA fragments were eluted and kept into freezing at -20°C until used. PCR assays were performed by Go Taq Flexi DNA polymerase kits (Promega, Germany) following the manufacturer's instructions. The primers used are listed in Table 4.1. The cleaned PCR products were sequenced using the same primers as those for the PCR. DNA sequencing was carried out by Genoscreen (Lille, France).

4.3.3 Construction of the reporter and mutant strains

All DNA fragments were amplified using the primers that are listed in Table 4.1. To construct of the reporter strains DOA9-P*nifAc* and DOA9-P*nifAp*, the 500-bp and upstream region of each respective *nifAc* and *nifAp* operon was amplified by PCR and cloned into the plasmid pVO155-npt2-cefo-npt2-gfp. This plasmid is a derivative of the plasmid pVO155 and it could not eplicated in *Bradyrhizobium* strains (Oke and Long 1999).This plasmid carries the promoterless *gusA* gene and constitutively expressed *gfp*, kanamycin and cefotaxime genes (Okazaki et al. 2015). To construct the two DOA9Ω*nifA* (insertion) mutants, 300 to 400 base pairs (bp) internal sequence of each *nifA* gene were amplified by PCR and cloned into the plasmid pVO155-Ce-npt2-gfp harboring a cefotexime resistance gene. To construct the Δ*nifA* in *Bradyrhizobium* sp. strain DOA9, the 1,700 bp upstream and downstream fragments of *nifA* gene were amplified using the overlap PCR technique. Then, the fragment was cloned into the plasmid pK18-Ce-mob-sacB harboring a cefotexime resistance gene. To transfer each constructed plasmid into DOA9 to create mutants, each constructed plasmid was introduced into *E. coli* S17-1 using electroporation (15 kv/cm, 100Ω, and 25μF) and then transferred into *Bradyrhizobium* sp. strain DOA9 by bi-parental mating using protocol described in Giraud et al. 2010. To create the double *nifA* genes mutation, the *E. coli* S17-1 containing constructed plasmid using for create the DOA9Ω*nifAp* mutant were transferred into the DOA9Δ*nifAc* strain by bi-parental mating. Transconjugants were selected on YM plates supplemented with 20 μg/ml Nalidixic acid, 20 μg/ml Cefotaxime and 200 μg/ml Kanamycin. Whilst, the two mutant strains of Δ*nifA* (deletion mutant) were subjected to select the transconjugants showing the sensitive growth on the YM containing 10%sucrose plates to obtain the mutant without *nifA* and plasmid harboring. These deletion mutants were maintained in YM without antibiotic.

4.3.4 Complementation of the DOA9Δ*nifAc* and Δ*nifAp* mutant

For complementation of the DOA9Δ*nifAc* and Δ*nifAp* mutants with respective *nifAc* and *nifAp* genes, it was first introduced the amplified promoter region of the *nifDKc* operon into the pMG103-npt2-cefo that succeeded in the previous experiment (Chapter III). Then, the amplified *nifAc* and *nifAp* genes were cloned downstream of the introduced promoter region (Figure 4.1B). The complementation of the DOA9Δ*nifAc* with respective *nifAc* and *nifAp* genes, each *nifA* genes was amplified and cloned directly into pMG103-npt2-cefo including the 500-bp upstream promoter region. These constructed vectors were introduced into DOA9Δ*nifAc* mutant by electroporation (17.5 kv/cm, 100Ω, and 25μF) for complementation.

Concerning the protein sequence of NifA, the NifAp of DOA9 lacked of N-terminal part. Thus, the two different fragments of chimeric NifA hybrid under constitutive expression of *nptII* promoter in pMG103:npt2:Pmnpt2-MCSR were constructed (Figure 4.1). Based on animo acid sequence analysis of both NifAc and NifAp, the first chimeric NifA hybrid was established by joining two fragments of NifAc-N-terminal (aa position 1-80) with the NifAp-C-terminal (aa position 81-579), whereas second chimeric NifA hybrid was also established by joining two fragments of NifAc-N-terminal (aa position 1-220) with the NifAp-C-terminal (aa position 221-579). The constructed plasmids were introduced into *E. coli* S17-1 before transferring each hybrid NifA into the DOA9Δ*nifAc* mutant by bi-parental mating. The complemented strains were selected on YM plates supplemented with 20 μg/ml cefotaxime and 200 μg/ml kanamycin (Figure 4.1).

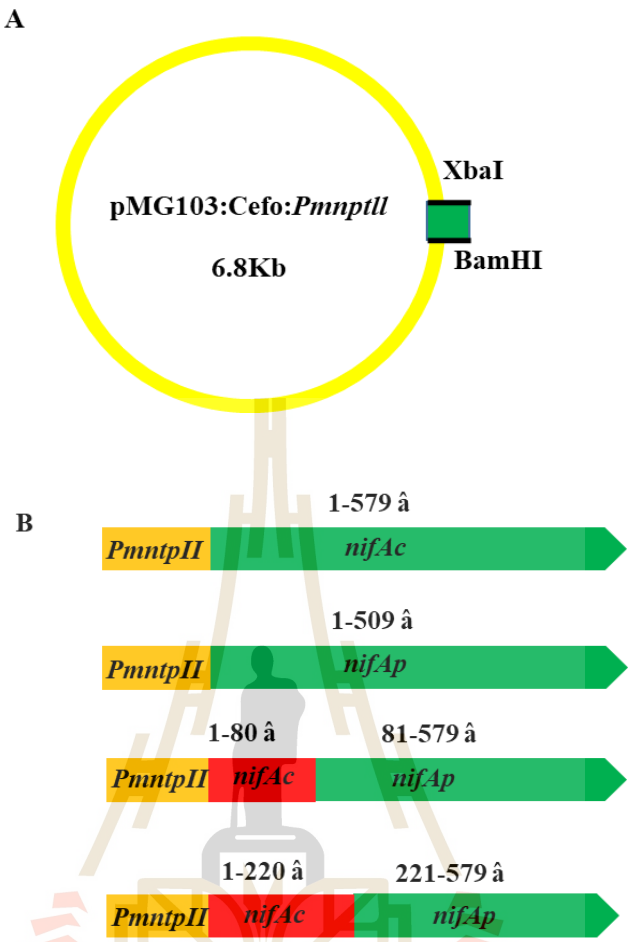

Figure 4.1 The construction for complementation of DOA9Δ*nifAc* mutant using pMG103:Cefo:Pmntp:MCSR (A) containing four different fragments of *nifAc*, *nifAp* and two hybrid fragments of *nifAc*:*nifAp* under the control of nptllI promoter (B).

Table 4.1 Primers used in this study.

Primers	**Sequences (5'-3')**	**Relevant characteristics**
PmNifA-DOA9c.f/ PmNifA-DOA9c.r	CGCACC**GTCGAC**GATCAAGA CCGACATCCTGTC/ AACCGA**TCTAGA**AGCATTCA GCATGACGGAATC	Cloning of the 466 bp upstream region of the *fixRnifAc* operon in pVO155-npt2-cefo-npt2-gfp after digestion of the PCR product by SalI/XbaI and the plasmid by SalI/XbaI. Plasmid constructed to obtain the reporter strain DOA9-P*nifAc*.
PmNifA-DOA9p.f/ PmNifA-DOA9p.r	GTACCG**GTCGAC**GAGATCGC GCTGACCGGTATCTTC/ CAGTCG**TCTAGA**AGGCGGGA TGGGTCGCAATATTTTC	Cloning of the 451 bp upstream region of the *nifAp* operon in pVO155-npt2-cefo-npt2-gfp after digestion of the PCR product by SalI/XbaI and the plasmid by SalI/XbaI. Plasmid constructed to obtain the reporter strain DOA9-P*nifDKc*.
NifA.DOA9c.in.f/ NifA.DOA9c.in.r	GTACCG**GTCGAC**GAGATCGC GCTGACCGGTATCTTC/ CAGTCG**TCTAGA**AGGCGGGA TGGGTCGCAATATTTTC	Cloning of the 313 bp of intenal *nifAc* fragment in pVO155-Cefo-npt2-gfp after digestion of the PCR product by SalI/XbaI and the plasmid by SalI/XbaI. Plasmid constructed to obtain the mutant strain DOA9Ω*nifAc*.

Table 4.1 Continued.

Primers	**Sequences (5'-3')**	**Relevant characteristics**
NifA.DOA9p.in.f/ NifA.DOA9p.in.r	AGCAA**GTCGAC**GACGTTCAT CGCCGTTCCGATC/ GCAGC**TCTAGA**CTGCGACCG ACAACGCCAGGTAC	Cloning of the 285 bp of internal *nifDc* fragment in pVO155-Cefo-npt2-gfp after digestion of the PCR product by SalI/XbaI and the plasmid by SalI/XbaI. Plasmid constructed to obtain the mutant strain DOA9Ω*nifAp*.
up.nifA.DOA9c.f/ up.nifA.DOA9c.r	GCGCC**GGATCC**GAGATCAA GGAGCGGTTAGACG/ ACAGTCGTCG**AAGCTT**GGAA TCTCCGTTGCCCGTTAC	792 bp upstream fragment of the *nifAc* gene obtain in pGemT-Am. The PCR product use to amplify the Overlap fragment with 759 bp downstream fragment of the *nifAc* gene by using Pfu polymerase. HindIII use to cut between up-dw fragments to insert with appropriate antibiotic resistant gene.
dw.nifA.DOA9c.f/ dw.nifA.DOA9c.r	CGGAGATTCC**AAGCTT**CGAC GACTGTCGCAGATGCGAC/ GCAAG**TCTAGA**CCGAGCTTC TCGTAGGTCTTGAGCAACTCT CATCGACATCAG	759 bp downstream fragment of the *nifAc* gene obtain in pGemT-Am. After PCR amplification, the Overlap fragment of *nifAc* gene clone into pK18-mobB-Cefo after digestion of the PCR product by BamHI/XbaI and the plasmid by BamHI/XbaI. Plasmid constructed to obtain the mutant strain DOA9Δ*nifAc*.

Table 4.1 Continued.

Primers	Sequences (5'-3')	Relevant characteristics
up.nifA.DOA9p.f/ up.nifA.DOA9p.r	GGCGG**GGATCC**GTTTGCACCAT GTCCGACGAATC/ CTCCGGTCAA**AAGCTT**CAAGAC TTGGACACACCACGGAATG	781 bp upstream fragment of the *nifAp* gene obtain in pGemT-Am. The PCR product use to amplify the Overlap fragment with 813 bp downstream fragment of the *nifAp* gene by using Pfu polymerase. HindIII use to cut between up-dw fragments to insert with appropriate antibiotic resistant gene.
dw.nifA.DOA9p.f/ dw.nifA.DOA9p.r	CGGAGATTCC**AAGCTT**CGACGA CTGTCGCAGATGCGAC/ GCAAG**TCTAGA**CCGAGCTTCTC GTAGGTCTTGAGCAACTCTCATC GACATCAG	759 bp downstream fragment of the *nifAp* gene obtain in pGemT-Am. After PCR amplification, the Overlap fragment of *nifAp* gene clone into pK18-mobB-Cefo after digestion of the PCR product by BamHI/XbaI and the plasmid by BamHI/XbaI. Plasmid constructed to obtain the mutant strain DOA9Δ*nifAp*.
NifA.comp.DOA9c.f/ NifA.comp.DOA9c.r	TCGGC**ACTAGT**GATGTAACGGG CAACGGAGATTC/ GTTGCAGGAATTC**GGATCC**CAA GTACGATCATAGCTTCTTTAG	Cloning of the *nifAc* gene in pMG103-npt2-Cefo- npt2-MCS2 (use a constitutive promoter) after digestion of the PCR product by SpeI/BamHI and the plasmid by SpeI/BamHI. Plasmid constructed to complement the mutant strain DOA9Δ*nifAc*.

Table 4.1 Continued.

Primers	Sequences (5'-3')	Relevant characteristics
NifA.comp.DOA9p.f/ NifA.comp.DOA9p.r	CATTC**ACTAGT**GTGTCCAAGTC TTGCCGAAAATG/ GTTGCAGGAATTC**GGATCC**CA AGTACGATCATAGCTTCTTTAG	Cloning of the *nifAp* gene in pMG103-npt2-Cefo- npt2-MCS2 (use a constitutive promoter) after digestion of the PCR product by SpeI/BamHI and the plasmid by SpeI/BamHI. Plasmid constructed to complement the mutant strain DOA9Δ*nifAc*.
NifA1.hybrid.DOA9c.r NifA1.hybrid.DOA9p.f	TCAGTCCAGC**AAGCTT**GGCCC CGACCGTGAGATCCGGAATG/ GGTCGGGGCC**AAGCTT**GCTGG ACTGAAGGAACCGATCAGAG	Cloning of the *nifA*-hybrid 1 gene in pMG103-npt2-Cefo- npt2-MCS2 (use a constitutive promoter) after digestion of the PCR product by HindIII and the plasmid by HindIII. Plasmid constructed to complement the mutant strain DOA9Δ*nifAc*.
NifA2.hybrid.DOA9c.r NifA2.hybrid.DOA9p.f	GTACCGTTAT**AAGCTT**CCTGCG GCGGTCGCGTGCCGGCT/ CCGCCGCAGG**AAGCTT**ATAAC GGTACCTGGCGTTGTCGG	Cloning of the *nifA*-hybrid 2 gene in pMG103-npt2-Cefo- npt2-MCS2 (use a constitutive promoter) after digestion of the PCR product by HindIII and the plasmid by HindIII. Plasmid constructed to complement the mutant strain DOA9Δ*nifAc*.

4.3.5 Plant cultivation and analysis under symbiotic condition

The symbiosis efficiency of *Bradyrhizobium* DOA9 strain and derivatives were tested with *Aeschynomene americana* No. 281 collected from LSTM's greenhouse. The seeds were surface sterilized by immersion in sulphuric acid under shaking during 45 minutes. Seeds were abundantly washed with sterile distilled water and incubated overnight in sterile water. Seeds were then transferred for one day at 37°C in the darkness on 0.8% agar plates for germination. Plantlets were transferred on the top of test tubes covered by aluminum paper for hydroponic culture in buffered nodulation medium (BNM) (Ehrhardt et al. 1992). Plants were grown in a 28°C growth chamber with a 16-h light and 8-h dark regime and 70% humidity. Seven days after transfer, each seedling was inoculated with one milliliter of cell suspension resulting from a 5 day-old bacterial culture washed in BNM and adjusted to reach an optical density of one at 600 nm. For nodulation and nitrogen fixation assay, 10 to 20 plants per condition were taken at 20 days post inoculation (dpi) and analyzed for the number of nodules and nitrogenase activity as previously described (Bonaldi et al. 2010). The experiments were carried out in duplicate.

4.3.6 Cytological analysis

To follow the β-glucuronidase activity in the nodules elicited by the reporter strains, 30-to 40-µm-thick sections from fresh nodule samples made using a vibratome (VT1000S; Leica, Nanterre, France) were incubated at 37°C in the darkness in a GUS assay buffer for 1 hour as described in Bonaldi et al. 2010. After staining, the sections were mounted and observed under bright-field illumination with a macroscope (Nikon AZ100; Champigny-sur-Marne, France).

4.3.7 Determination of nitrogenase activity under free-living conditions

To determine the nitrogenase enzyme activity under free- living conditions, *Bradyrhizobium* sp. strain DOA9 and derivatives, were grown in 10 ml test tubes (BD, Vacutainer, Franklin Lakes, NJ, U.S.A.) containing 2 ml of BNM-B medium (without glutamate) soft agar (0.8%), as described by Alazard (1990). Acetylene was injected to give a final concentration of 10%. The cultures were incubated at 28°C and gas samples were analyzed 7 day of cultivation for ethylene production by gas chromatography, as described (Renier et al. 2011).

4.3.8 Determination of the β-glucuronidase (Gus) activity under free-living conditions

The two DOA9 reporter strains were grown 4 days in YM medium, collected, and washed with BNM-B medium (without glutamate) as described above. Bacterial cells were inoculated into flasks containing BNM-B medium to obtain an initial OD600 at 0.05. Aliquots 55 ml of culture were injected into 150-ml bottles sealed with rubber stoppers and incubated at 28°C for 11 days without shaking. At 7 days of cultivation, samples were taken and the β-Glucuronidase activity was measured using the substrate p-nitrophenyl glucuronide (PNPG) as described by Jefferson (1987). β-Glucuronidase units were calculated according to Miller (1972).

4.3.9 RNA purification, cDNA synthesis and qRT-PCR

The expression of genes involved in nitrogen fixation of strain DOA9 was determined from cell grown under free-living condition and from the bacteroid obtained from nodule of *A. americana* under symbiotic condition. For free-living condition, the bacterial cells were grown for 7 days without shaking in liquid Bnm medium supplementing with 1mM succinate and mixed Vitamins. For harvesting, cultures were added with 1:10 volume of “stop solution” (10% Tris-HCl-buffered phenol [pH8] in ethanol) and removed from the liquid medium by centrifugation for 10 min (10,000 rpm,

4°C). The cells were frozen in liquid nitrogen and stored at –80°C. Analysis under symbiotic condition, RNA isolation from bacteriods were processed from approximately 1 g of frozen nodules by homogenize with a tungsten carbide bead (3mm; Qiagen, Germany) in a 2-ml microcentrifuge tube. The total RNA was isolated from the free-living bacterial cells and disrupted nodule with the hot (65°C) phenol-extraction procedure described previously (Babst et al. 1996). RNA was purified and treated with DNase using mini-prep kits (Qiagen, Valencia, CA, and U.S.A.). Then, the cDNA was synthesized by iScript TM Reverse transcription Supermix for RT-qPCR (Bio-Rad, Germany). The 10-50 ng of each cDNA sample were added to PowerUP^{Tm} SYBRTM Green master mixed buffer (Applied Biosystems, US & Canada) and appropriate amount of specific primers (listed in Table 4.2) were used in the qRT-PCR analyses using annealing temperature at 55°C for all reactions. The expression of target genes was relatively compared with the expression of housekeeping gene, dnaK using the Applied Biosystems, QuantStudio Design & Analysis Software.

Table 4.2 Primers used in qRT-PCR experiment.

Gene name	Gene description	Forward/ Reverse (5'-3')	Annotated file
nifAc	Nitrogen fixing regulator gene located on DOA9 chromosome	GCACCCCGGCGACACCGGCTTTG/ GGTGTCGAACACGTCGCTATTG	BRADOA9_v1_51570
nifAp	Nitrogen fixing regulator gene located on DOA9 plasmid	CGGTGCTCTTACGAGGCGAGAC/ CCAGTAGCGGAATGACGCTCAGG	BRADOA9_v1_p0526
rpoNc	Nitrogen fixing regulator gene located on DOA9 chromosome	CCACCTATACCGAATGGGGC/ CCACCTATACCGAATGGGGCG	BRADOA9_v1_21339
rpoNp	Nitrogen fixing regulator gene located on DOA9 plasmid	CGACGATCTTCCCTCTCAGC/ GGGATGTTAGAGCGAGTCCG	BRADOA9_v1_p0436
nifDKc	Operon of nitrogenase structural genes on DOA9 chromosome encoded for Dinitrogenase	GGGTCGGATGCATCAAGCAAG/ GTTGCTAGGCTCATACGAATATC	BRADOA9_v1_51562

Table 4.2 Continued.

Gene name	Gene description	Forward/ Reverse (5'-3')	Annotated file
nifDKp	Operon of nitrogenase structural genes on DOA9 plasmid encoded for Dinitrogenase	CTCGCAGCGCCCTCGACAATAG/ CTTCTCCGGATAGACCTTCAAG	BRADOA9_v1_p0592
nifHc	Nitrogenase structural gene on DOA9 chromosome encoded for nitrogenase reductase	CGACCACGTCACAGAACAC/ CCTTGTAGCCGACCTTCATG	BRADOA9_v1_51512
nifHp	Nitrogenase structural gene on DOA9 plasmid encoded for nitrogenase nitrogenase reductase	AAGGGTGGTATCGGCAAATC/ GCAGCGGATGTTCTGGTAG	BRADOA9_v1_p0648
dnaK	Housekeeping gene use as internal gene in this experiment	GAAGTGCTGCGCATCATCAA/ TCCTTCTGGAATTCGTCGGC	BRADOA9_v1_21393
nifDKp	Operon of nitrogenase structural genes on DOA9 plasmid encoded for Dinitrogenase	CTCGCAGCGCCCTCGACAATAG/ CTTCTCCGGATAGACCTTCAAG	BRADOA9_v1_p0592

4.4 Results

4.4.1 *nifA* located on both chromosome and plasmid of *Bradyrhizobium* sp. strain DOA9

Based on genome analysis (Okazaki et al., 2015), *nifA* genes were located one copy on chromosome (*nifAc*) and another one on the mega-plasmid (*nifAp*) of DOA9. On chromosome, *nifAc* was located downstream of *fixR* in the operon as *fixRnifAc*, which was located in upstream region of the *fer* gene (the iron-sulfur protein that mediate electron transfer in a range of metabolic reactions) and on the upstream of the operon *sufBCD* (the SufBCD complex, proteins contribute to the assembly or repairing of the oxygen-labile iron-sulfur clusters under oxidative stress). The *nifAc* was also located 6,441 bp upstream of the operon of *nifDKENX*. On the plasmid, *nifAp* was located in between the unknown proteins and around 5,589 bp away from the *nodA* (Figure 4.2). The amino acid sequence alignments of NifAc (579 aa) and NifAp (503 aa) showed 53% of homology scores (Figure 4.3). Similarly, phylogenetic tree based on amino acid sequence indicates NifAp was not-closely related and clearly separated from NifA of other *Bradyrhizobium* strains (Figure 4.4). NifAc of *Bradyrhizobium* sp. strain DOA9 was closely related to *Bradyrhizobium* sp. strain S23321, which was either saprophytic or non-symbiotic *Bradyrhizobium* sp. strain. Based on NifAc amino acid sequence comparison, NifAp lacked of 70 amino acids on N-terminal site and also a part of amino acid sequence (at position 131 to 220) was altered (Figure 4.3). The promoter analysis using the Protein Pattern Search online program and manual suggests that both *nifAc* and *nifAp* preceded with the NifA and RpoN-binding sites. The consensus sequence of NifA and RpoN-binding sites were located at position -109 and -56 bp, respectively from the *nifAc* start codon on chromosome, while the NifA binding site was found at position -107 bp from the *nifAp* start codon on the mega-plasmid.

However, the RpoN-binding site could be also manually predicted at position 351 bp away from *nifAp* start codon. The binding site of RpoN (TGG-N_{10}-TGT) in front of *nifAp* was slightly different from the consensus sequence (TGG-N_9-TGC) (Table 4.3). Therefore, there is a possibility that both *nifAc* and *nifAp* could be expressed in DOA9.

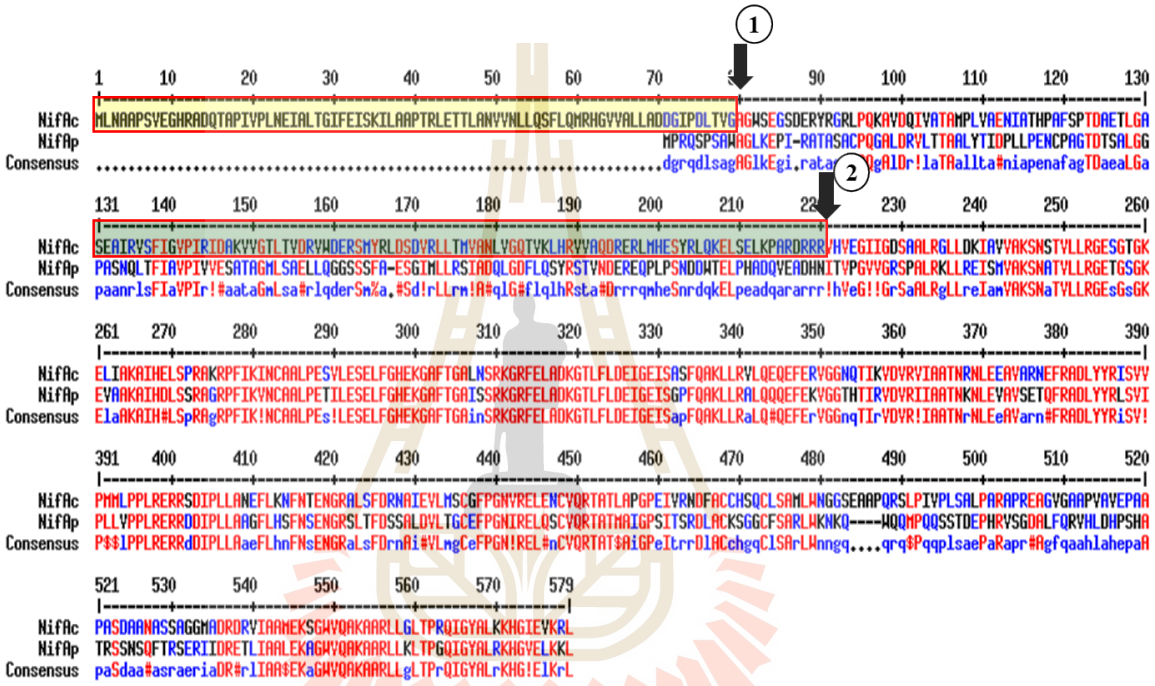

Figure 4.2 The comparison of Amino acid alignment between NifAc and NifAp fragments. This alignment indicated two differences at N-terminal region when compared to NifAc, region 1 (aa position 1 to 80) and region 2, (aa position 131 to 220 amino acids.

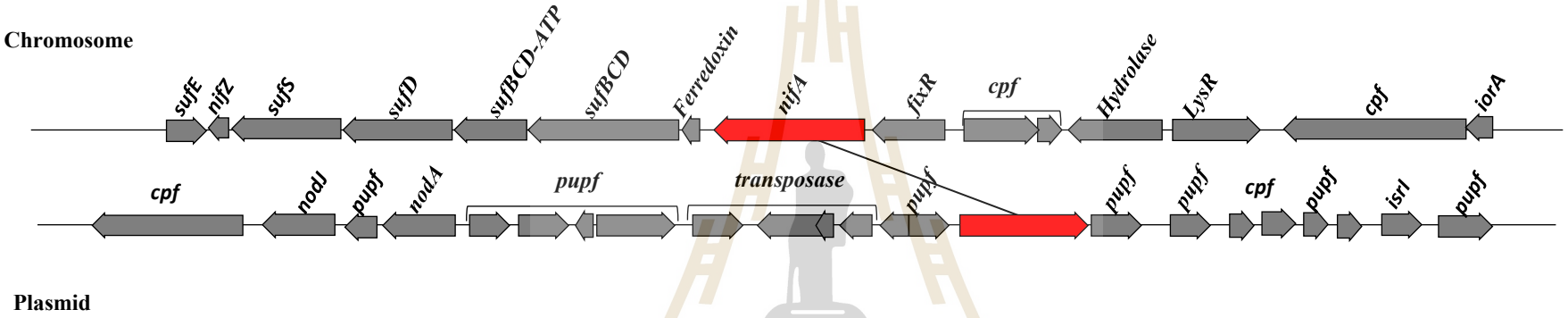

Figure 4.3 Gene organization of the two *nifA* genes (in red) located on both the chromosome and the plasmid of the DOA9strain. From the gene annotation indicates that the *nifA* gene on chromosome (*nifAc*) probably used upstream promoter of *fixR* gene. In grey, the genes specific to each replicon. *cpf*; conserve of protein function, *pupf*; Putative of unknown protein function.

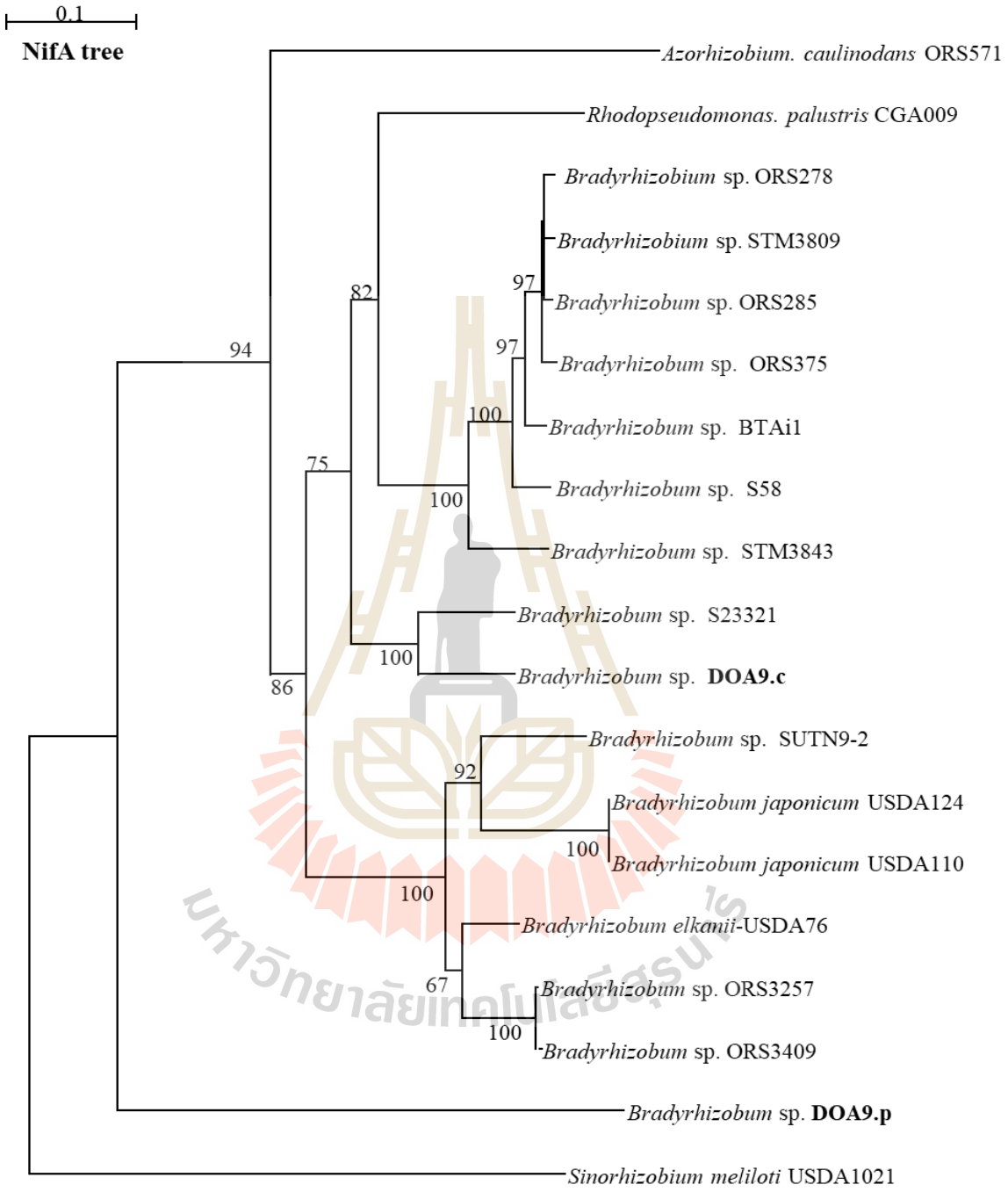

Figure 4.4 The NifA phylogenetic tree of the DOA9 (on chromosome and plasmid) comparing to the other *Bradyrhizobium* strains.

Table 4.3 Analysis of RpoN-NIfA binding site sequences located upstream of nitrogen fixing genes on both chromosome and plasmid of *Bradyrhizobium* sp. strain DOA9 using the Protein Pattern Search online program and manual analysis.

***nif* genes**	**RpoN-binding box**	**NifA-binding box**
nifAc	TGGCCCAATTCCTGC	TGTCGCGAACTCGACA
nifAp	TGGGGACCGGATTGT	TGTCGATCGCAGAACA
rpoNc	TGGGAACGGTTCTTGC, (N_{10})	TGTACATCGGGCTCG
rpoNp	TGGTTAGTCGAAAGCA, (N_{10})	CGTCTGCGTGCCTAACA, (N_{11})
nifDKc	TGGCACACTCGTTGC	TGTTTTGTTTCGAACA
nifDKp	CGGCACAGTCCTTGC	TGTATCGGGTTTGCGACA, (N_{12})
nifHc	TGGCACGCCAGTTGC	TGTCCGGTTTCTGACA
nifHp	TGGCTTCTCTGGCTGC, (N_{10})	TGTCTGATACCTAATA

4.4.2 The β-glucuronidase activity of *PmnifA-gus* fusion DOA9 reporter strains indicates *nifAp* expressed higher than *nifAc* under both symbiosis and free-living states

The two reporter strains (*PmnifAc-gus* and *PmnifAp-gus*) were constructed and inoculated on to germinated seed of *A. americana* as a host plant. Plants at 14 days after inoculation (dai) showed that both reporter strains were able to nodulate and even fix nitrogen similar to the wild-type (WT)-strain indicating that these *gus*-fusion did not affect to the symbiotic performance (Figure. 4.5A-G). Cytological analysis revealed that the root nodules were occupied by the two reporter strains and contained ß-glucuronidase activity, in contrast to the WT-nodules for which no activity could be detected (Fig. 4.5E). The X-Gluc staining of the nodules elicited by the two reporter strains indicates that the two *nifA* operons were differentially expressed during symbiosis with *A. americana*. The nodules elicited with *PmnifAp-gus* fusion strain showed the dark-blue color indicating of higher Gus activity when compared to the nodule of *PmnifAc-gus* fusion strain (Figure. 4.5F-G).

Moreover, both *PmnifA-gus* fusion strains were tested under free-living condition. The culture at 7 dai was observed for the ß-glucuronidase activity. Surprisingly, the amount of ß-glucuronidase activity was much lower than that of levels shown in the plant nodule (with the Miller units equal to 22.23 and 30.51 for *PmnifAc-* and *PmnifAp-gus* fusion strains, respectively under free-living stage) (Figure 4.5H). These results indicated that both *nifAc* and *nifAp* were expressed in DOA9 during symbiotic and free-living conditions by which *nifAp* expressed in higher level than that of *nifAc*. Thus, the function of these two genes involving in nitrogen fixation of DOA9 was further examined.

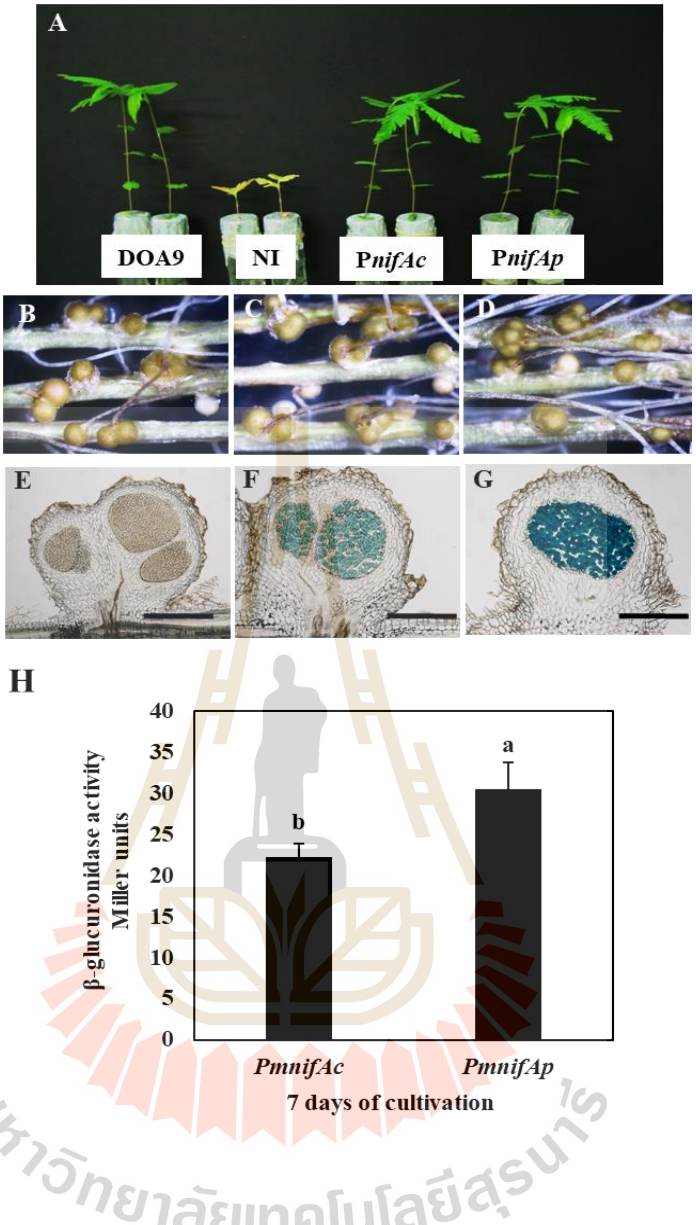

Figure 4.5 Two *nifA* genes of *Bradyrhizobium* sp. DOA9 strain are expressed during symbiosis with *A. americana*.

A. Comparison of the growth of the plants (aerial part) non-inoculated (NI) or inoculated with WT and the reporter strains DOA9-P*nifAc* and DOA9-P*nifAp* (at 20 dpi). (**B-D**) Root nodules observed with a stereomicroscope equipped. (**B**) Nodules elicited by WT; (**C**) Nodules elicited by DOA9-P*niAc*; (**D**) Nodules elicited by DOA9-P*nifAp*. (**E-G**) Expression of the *gusA* reporter gene revealed on 40 µm nodule sections stained with X-Gluc. **(E**) Nodules elicited by WT; (**F**) nodules elicited by DOA9-P*nifAc*; (**G**)

nodules elicited by DOA9-P*nifAp*. Scale bars are 1 mm for (**B-D)** and 250 µm **(E-G**). And (H), the β-glucuronidase activity of two *PmniA*-gus fusion strains indicating the expression of *nifA* chromosome and plasmid operons of *Bradyrhizobium* sp. DOA9 in free-living condition.

4.4.3 *nifAc* and *nifAp* genes in *Bradyrhizobium* sp. DOA9 strain could function stead of each other for regulation of the nitrogen fixation when symbiosis with *A. americana*

Here, the three different mutants of DOA9 by insertion (DOA9Ω*nifAc* and DOA9Ω*nifAp*), deletion (DOA9Δ*nifAc* and DOA9Δ*nifAp*) and double mutations (DOA9Δ*nifAp::*Ω*nifAc*) were constructed. These mutant strains were inoculated on *A. americana* and observed the result at 20 dai. Plants inoculated with each mutant displayed the same phenotypes, which was same as plants inoculated by DOA9 wild-type. All inoculated plans showed the similar results for the plant phenotypes, nitrogenase activity and root nodule numbers. Clearly, the presence of a single *nifAc* or *nifAp* gene in DOA9 could compensate for the nitrogenase activity and plant growth when symbiosis with *A. americana* (Figure 4.6A-C and 4.7A-). In contrast, the plants inoculated with *nifA* double mutation showed the drastic effect on plant with the nitrogen starvation symptoms, by producing the foliage chlorosis and reducing plant growth as similar to the phenotype of non-inoculated plants (Figure 4.6A and 4.7A). The absence of total nitrogenase activity was detected in both non-inoculated plants and inoculated plants with *nifA* double mutation strain (Figure 4.6B). This mutant induced smaller nodules and slightly higher number of nodules (Figure 4.6C), which were appeared senescence of white color nodule from inside (Figure 4.6G). Missing of both *nifAc* and *nifAp* genes in DOA9 (Δ*nifAp::*Ω*nifAc*) indicates a particular Fix^- phenotype in inoculated plants but not from a single *nifA* mutation. Therefore, the symbiotically

nitrogen fixation in this DOA9 could probably regulate by either NifAc or NifAp, which required for the regulation of other nitrogen fixing genes to transcribe.

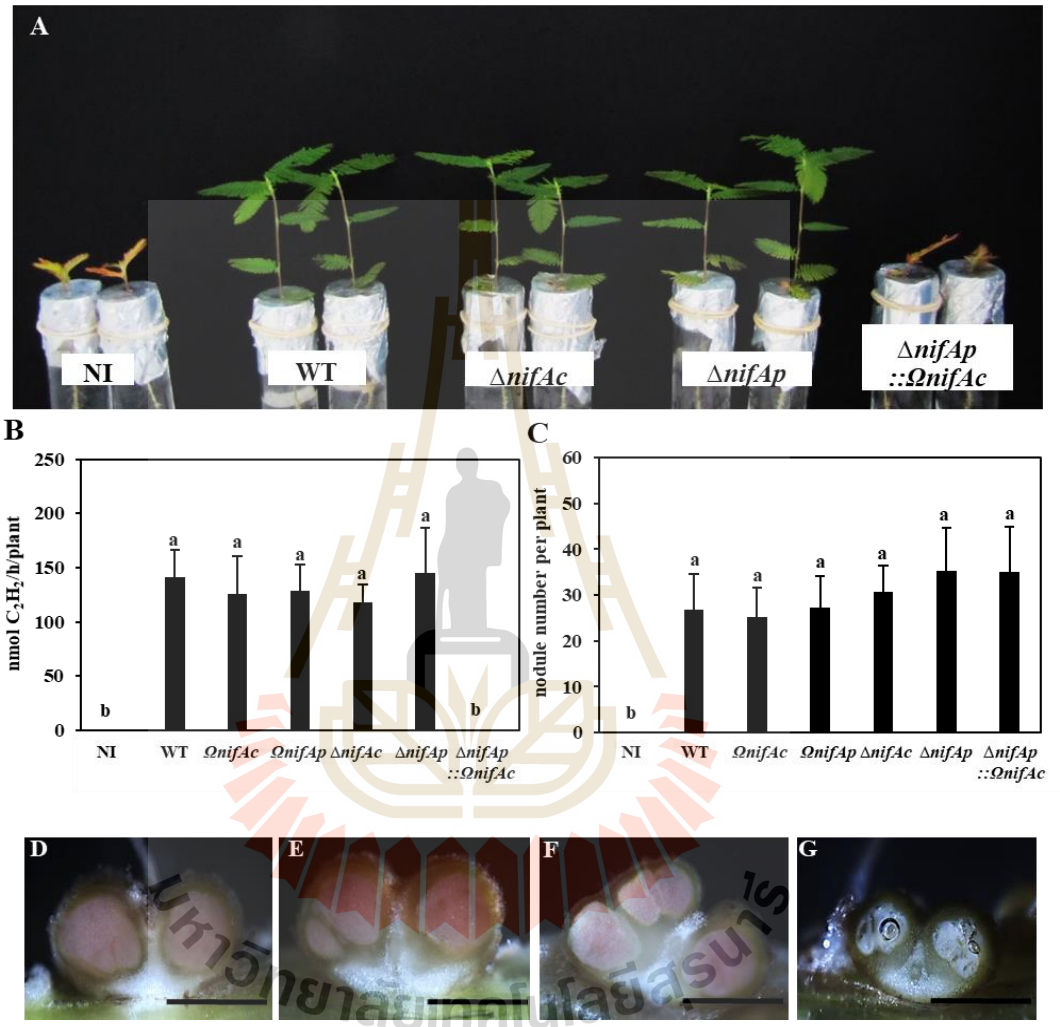

Figure 4.6 The presence of *nifA* genes in *Bradyrhizobium* sp. DOA9 strain, both are responsible to control the symbiotic nitrogen fixation with *A. americana*. **(A).** Comparison of the plants phenotypes (aerial part) non-inoculated (NI) or inoculated with WT and the mutant strains Δ*nifAc*DOA9, Δ*nifAp*DOA9 and DOA9Δ*nifAp*::Ω*nifAc*DOA9 (at 20 dpi). Because, result indicated no significant differences between the plant phenotypes inoculated with insertion mutant strains DOA9Ω*nifAc*, DOA9Ω*nifAp* (**Figure 4.7**) and clean mutant strains DOA9Δ*nifAc*, DOA9Δ*nifAp*. **(B)** The amount of acetylene reducing

activity (ARA) in *A. americana* plants inoculated with WT and the mutant strains. (**C**) Number of nodules per plant nodulated by WT and with insertion mutant strains and clean mutant strains. (**D**) Nodules nodulated by WT; (**E**) Nodules nodulated by DOA9Δ*nifAc*; (**D**) Nodules nodulated by DOA9Δ*nifAp;* **(G**) nodules elicited by DOA9Δ*nifAp*::Ω*nifAc*. Scale bars are 1 mm for (D-**G)**. In panels (**B**, **C**) Error bars represent s.e. *(n=10).* Different letters above the error bars indicate significant differences at P<0.05 (Tukey's HSD test).

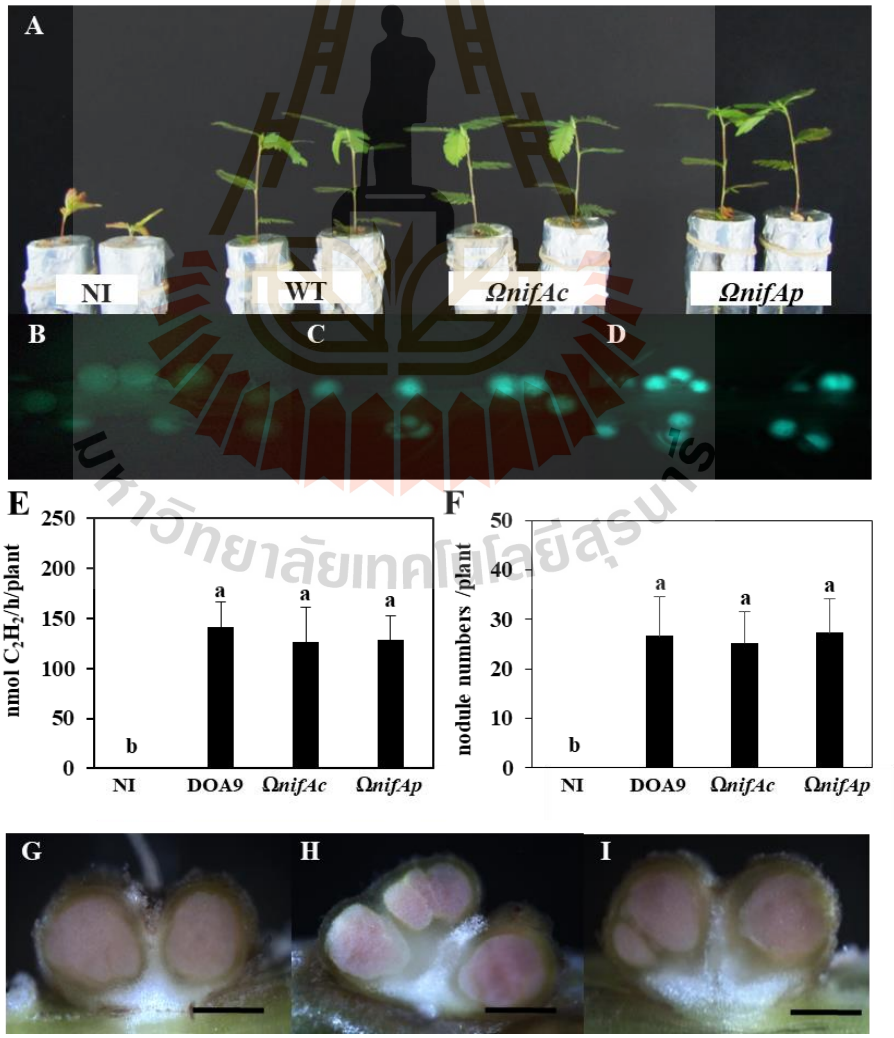

Figure 4.7 The insertion mutation of *nifA* indicated the presence of chromosome and mega-plasmid *nifA* genes *in Bradyrhizobium* sp. DOA9 strain and both are

responsible for controlling the symbiotic nitrogen fixation with *A. americana* (A). Comparison of the plants phenotypes (aerial part) non-inoculated (NI) or inoculated with WT and the mutant strains DOA9Ω*nifAc* and DOA9Ω*nifAp* (at 20 dpi). (B-D) Root nodules observed with a fluorescent stereomicroscope equipped with a green fluorescent protein (GFP) filter. (B) Nodules elicited by WT; (C) Nodules elicited by DOA9*ΩnifAc*; (D) Nodules elicited by DOA9*ΩnifAp*. (E) The occurrence of acetylene-reducing activity (ARA) in *A. americana* plants inoculated with WT and the insertion mutant strains *DOA9ΩnifAc* and *ΩDOA9nifAp*. (F) Number of nodules per plant elicited by WT and DOA9*ΩnifAc* and DOA9*ΩnifAp*. (G-I) Cross section of nodule elicited by the WT (G) and the mutants DOA9*ΩnifAc* (H) and DOA9*ΩnifAp* (I). Scale bars are 250 µm for (D-G). In panels (E, F) Error bars represent s.e. *(n=10)*. Different letters above the error bars indicate significant differences at $P<0.05$ (Tukey's HSD test).

4.4.4 The *nifAc* was mainly required for regulation of nitrogen fixation activity in DOA9 strain under free-living conditions.

All *nifA* mutants were subjected to examine the nitrogen fixing activity of bacterial cells grown in semi-solid BNM medium after 7 days. Obviously, the *nifAc* mutants including DOA9Ω*nifAc* and DOA9Δ*nifAc* were not able to fix nitrogen during free-living life (Table 4.4). In contrast, DOA9Ω*nifAp* and DOA9Δ*nifAp* mutants similarly performed the nitrogenase activity compared to the WT strain suggesting that the NifAp did not display a significant control of nitrogen fixation in free-living condition. It was found that the single *nifAc* and double *nifA* mutations had the same result of no nitrogenase activity indicating that the NifAc not NifAp regulate nitrogen fixation process during free-living life (Table 4.4).

Since the information of genetic analysis predicted the lacking of the N-terminal part in NifAp was mainly found, it could be hypothesized that this lacking of N-terminal part may cause a non-functional of NifAp under free-living condition. Construction of chimeric NifA hybrid protein using different lengths of NifAc (N-terminal part) connected with NifAp (C-terminal part) was considered for complementation of nitrogen fixing ability in DOA9Δ*nifAc* mutant (Figure 4.1).

Based on this hypothesis, DOA9Δ*nifAc* mutant was introduced with different fragments of *nifAc*, *nifAp*, *nifA-hybrid 1* and *nifA-hybrid 2* genes and then tested under free-living condition. DOA9Δ*nifAc* was successfully complemented by only *nifAc* gene fragment that improved nitrogenase activity similarly to the WT and DOA9Δ*nifAp* strains (Table 4.4). However, *nifA-hybrid 1* and *nifA-hybrid 2* were not successfully complemented in DOA9Δ*nifAc*. We proposed that the structure of functional NifA protein could be more complicated to synthesize via the chimeric hybrid fragments. Nevertheless, the regulatory *nifAp* gene was expressed, although it did not function in free-living condition of strain DOA9. Therefore, the questions remained whether NifAc regulated other *nif* genes located on the chromosome or on the mega-plasmid under free-living condition, and whether NifAc and NifAp had the cross-regulation among genes located on both replicons during symbiosis state.

Table 4.4 Acetylene reduction by respective cultures of WT and the mutant strains in liquid cultures of nitrogen-free (Bnm) medium under free-living concentration.

Treatments*	**Acetylene reduction [1] (nmol /h/culture)**	**SD**
Non-inoculation	ND	ND
DOA9WT	3,643.55	±18.01
Ω*nifAc*	ND	ND
Δ*nifAc*	ND	ND
Ω*nifAp*	3,588.72	±74.99
Δ*nifAp*	3,547.54	±35.54
Ω*nifAp::*Δ*nifAp*	ND	ND
Δ*nifAc::pMG103:nifAc*	3,535.37	±137.48
Δ*nifAc::pMG103:nifAp*	ND	ND
Δ*nifAc::pMG103:nifA hybrid1*	ND	ND
Δ*nifAc::pMG103:nifA hybrid2*	ND	ND

* = Nitrogen-starved cells were inoculated into glass vials containing 50 mL of medium and 150 mL of gas headspace and incubated at 30 °C for 7 days.

[1] = Each value represents a mean

SD = Standard deviation determined from three replicates

ND = non detected data

4.4.5 Both NifAc and NifAp performed the cross-regulation on the expression of nitrogen fixation involving genes during symbiosis with *A. americana*, but only NifAc regulated the expression of nitrogen fixing genes located on both replicons during free-living condition

The quantitative reverse transcriptase-PCR (qRT-PCR) were used to analyze the expression of other genes relevant to nitrogen fixation in the background of Δ*nifAc* and Δ*nifAp* mutants. The transcriptional profiles were compared to the wild-type strain when symbiosis with *A. americana* (Figure 4.8A). *In planta* experiment, all tested genes were differentially expressed in the wild-type, while no expression of *nifAc* and *nifAp* was observed in each particular mutant. The lower expression level of *nifAc* than that of *nifAp* was also observed by qRT-PCR similar to the previous Gus staining detection on nodule. However, lower expression of *nifAc* did not affect the symbiotic performances. In addition, the *rpoN* which was encoded for the sigma factor (σ^{54}) which responsible for regulation of nitrogen fixation process was also expressed, also found that the expression of *rpoNp* was expressed higher than *rpoNc* in all the DOA9 wild-type, Δ*nifAc*, and Δ*nifAp* backgrounds. This could be suggested that *rpoNp* is mainly expressed during symbiosis with *A. americana*. Moreover, both *nifHc* and *nifHp* were expressed at very high level, which confirmed the presence of nitrogenase activity under symbiosis. Interestingly, genes of *rpoNc*, *rpoNp*, *nifDKc*, *nifDkp*, *nifHc* and *nifHp* were expressed either in the background of Δ*nifAc* or Δ*nifAp* mutants or the level of expression was similar to that of wild-type. This result indicated the cross regulation between NifAc and NifAp on genes located on both the mega-plasmid and on the chromosome under symbiosis condition.

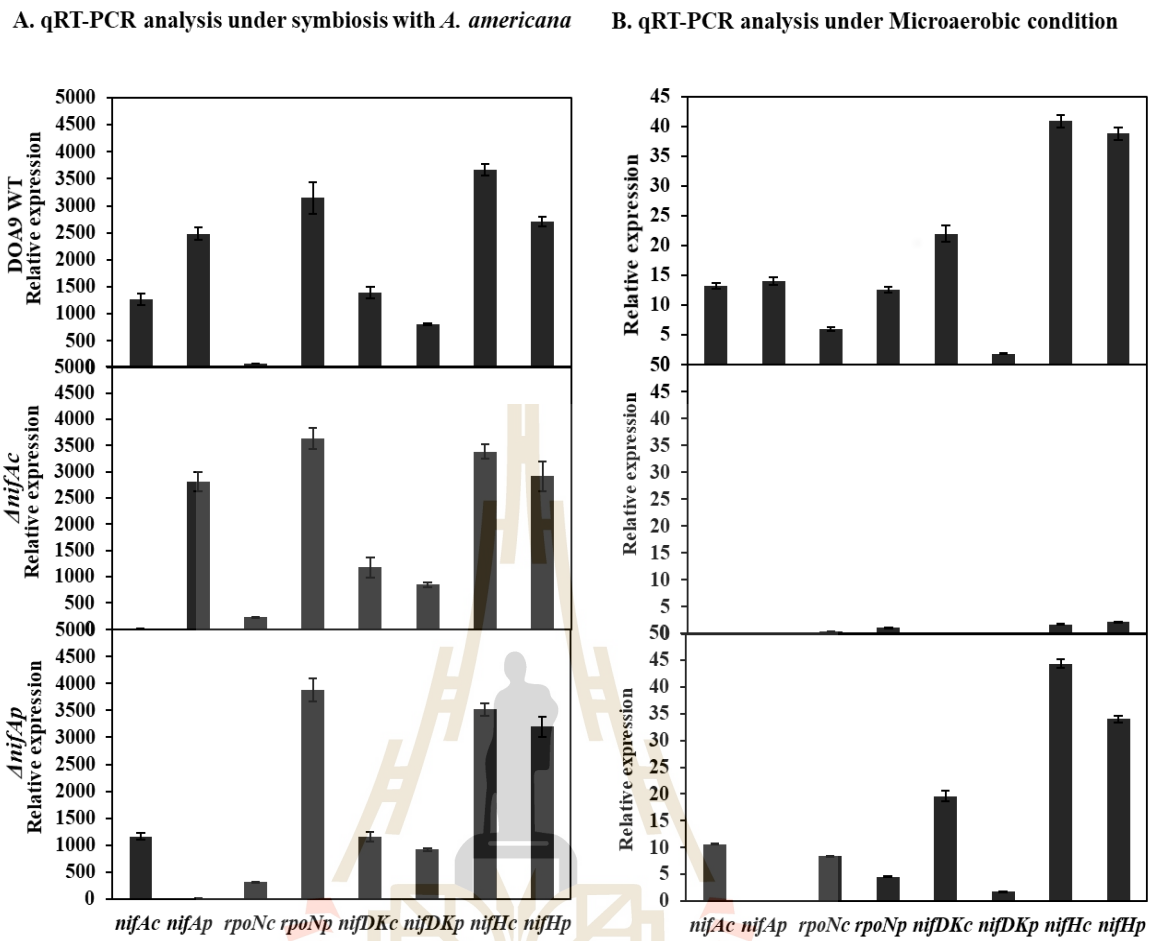

Figure 4.8 Expression of nitrogen fixing genes; *nifAc*, *nifAp*, *rpoNc*, *rpoNp*, *nifDKc*, *nifDKp*, *nifHc* and *nifHp* in the background of DOA9WT, DOA9*ΔnifAc*, and DOA9*ΔnifAp*.

The bacterial strains were cultured *in planta* as a symbiosis with *A. americana* for 20 days (A) and under *in vitro* as a free-living growth in Bnm medium (B). Total RNA was extracted, transcribed and subjected to qualitative reverse transcription-PCR with the internal standard of *dnaK*. All data were from one representative experiment that was repeated three times. Error bars indicate SD. P values were calculated using one-tailed tests. P=0.001.

The experiment was also performed under free-living condition. The results showed that both *nifAc* and *nifAp* responded under micro-aerobic condition in the wild-type background (Figure 4.8B). Both *rpoNc* and *rpoNp* expressions were also detected in the wild-type strain with the low expression level of *rpoNc* gene. Similarly, the two *nifDc* and *nifDp* expressions were detected in DOA9WT. It was found that *nifDc* was expressed higher than *nifDp*, this result was consistent with the β-glucuronidase activity of DOA9 growing under free-living condition as previously shown by Wongdee et al. (2016).

Genes of *rpoNc*, *rpoNp*, *nifDKc*, *nifDkp*, *nifHc* and *nifHp* were expressed in Δ*nifAp* background similar to those of wild-type. However, an obvious low level of gene expression was observed in the Δ*nifAc* background, only the genes of *nifHc*, *nifHp* and *rpoNp* genes were slightly expressed. These results indicated that only *nifAc* was required for transcription of nitrogen fixing genes located on both chromosome and mega-plasmid under free-living condition (Figure 4.8B). Therefore, the Fix$^-$ phenotype was correlated with the low level of gene expression in the Δ*nifAc* mutant under free-living condition. Taking together, it would be concluded that NifAc not NifAp was the main regulator for the transcription of nitrogen-fixing genes, and NifAc could cross regulate those genes located on the mega-plasmid of DOA9 under the free-living life.

4.5 Discussion

Bradyrhizobium sp. DOA9 contained two *nifA* genes by which *nifAc* and *nifAp* working redundancy under symbiotic condition, while only *nifAc* was required for nitrogen fixation under free-living condition. However, double mutation of *nifAc* and *nifAp* (DOA9Δ*nifAp::*Ω*nifAc*) indicated that NifA was an essential regulator to initiate the transcription of other genes involving in nitrogen fixation. The Fix$^-$ phenotype was observed in plants inoculated with the double *nifA* mutant strain, although high amounts of nodule number was observed when compared to the single *nifA* mutant strains and

WT. Increase of nodule number was similar to several reports in nitrogenase Fix$^-$ mutants, and this finding could be involved in the host plant control that try to ensure the amount of fixed nitrogen by increase nodule formation (Masson-Boivin and Sachs 2018). The mutation of *nifA* genes in *R. meliloti*, *B. japonicum*, and *A. caulinodans* were also completely abolished symbiotic nitrogen fixation (Fix$^-$ phenotype) (Alvarez-Morales and Hennecke 1985). Similarly, no free-living nitrogenase activity was measurable in *nifA* mutants of *B. japonicum* and *A. caulinodans* (Sanjuan and Olivares 1991). These works refered the common function of the NifA proteins as transcriptional activator for nitrogenase encoding genes and genes encoding accessory functions.

However, *nifA* is specific in each group of nitrogen fixing bacteria. It has been reported that *nifA* of *K. pneumoniae* cannot complement the Fix$^+$ activity of *B. japonicum* and *R. meliloti nifA* mutants, although NifA of *K. pneumoniae* is able to activate the promoters of the nitrogenase structural genes of *R. meliloti* and *B. japonicum* when constructed in *E. coli* as a host (Alvarez-Morales and Hennecke 1985; Sanjuan and Olivares 1991). Since NifAp of strain DOA9 was quite different when compared with NifA among a group of *Bradyrhizobium*, this was a surprise situation where NifAp could activate Fix$^+$ phenotype of DOA9 when symbiosis with *A. americana*. More discussions were emphasized below.

Although the presence of multiple copied of *nifA* in nitrogen fixing bacteria has been described elsewhere (Sullivan et al., 2013 and Okubo et al., 2016), the position of the gene organization was relatively different among the bacterial strains. Three clusters of *nifA* gene organization types in the rhizobial genome has previously been proposed. The cluster I type was found in fast-growing rhizobia strains, such as *R. meliloti*, *R. leguminosarum* bv. *viciae* and bv. *trifolii*. The *nifA* gene was located in between the *fixABCX* operon and the *nifB* gene (Quinto et al., 1985). For the cluster II type, *nifA* was located in the same promoter with *fixR* as *fixRnifA* operon, which was located at

upstream of *fixA* gene. This cluster II type was found especially in *B. japonicum*. For the cluster III type, it was found in *A. caulinodans*, the *nifA* gene was organized as similar to cluster I, but the downstream was usually followed with the *nifIHDKE* operon (Halbleib and Ludden 2000 and Dixon and Kahn 2004a). In the case of *Bradyrhizobium* sp. DOA9, two *nifA* genes were found one on the chromosome and another one on mega-plasmid. The genetic organization suggested that *nifA* on chromosome (*nifAc*) was similar to that of cluster II type, which was normally found in *B. japonicum*. Interestingly, the genetic organization of *nifA* gene on mega-plasmid (*nifAp*) could not be classified. It could be possible that the *nifAp* gene was occurred from the gene duplication or through the transposon mutagenesis. However, the *nifAp* gene of DOA9 was clearly expressed and function especially under symbiosis condition even the gene was stand alone and away from other *fix* or *nif* gene clusters.

Higher expression of *nifAp* than *nifAc* was observed in DOA9 under both symbiotic and free-living conditions. It could be explained that the promoter region of *nifAp* was probably stronger than that of *nifAc*. The strong promoter was classified based on the affinity of the RNA polymerase and sigma factor during the transcription. The experiment of the replacing of adenine at position -12 by a cytosine in the mutant promoter (*Pm*) and after transformation of pRJ9309::UAS-*Pm*::*nifAB*j into the *E. coli* cells, had increased the β-Galactosidase activity from 25.4 to 106.0 Miller units under microaerobic condition (Hauser et al., 2007). Moreover, Barrios et al. (1995) and Martı´nez et al. (2004) suggested that the absence of a well-conserved UAS for potential NifA binding could affect the expression of *nifA*. In our experiment, the promoters of *nifAc* and *nifAp* were manually analyzed. It was found that the UAS of nifAc consense with regular RpoN and NifA binding sites, while the RpoN binding site of *nifAp* showed one base pair different (Table 4.1). It might be postulated that the mutation of RpoN binding site on UAS-*nifAp* might increase the affinity of RNA polymerase and sigma

factor and enhanced the transcription under both symbiotic and free-living conditions. Therefore, the promoter and UAS analysis of regulatory *nifAc* and *nifAp* would be interested to explore the complex binding site in the mode of activating nitrogen fixation in the strain DOA9. Although nifAp expressed in high level, the translation of NifAp did not function under free-living condition.

NifA proteins are highly conserved in their central and C-terminal domains. The C-terminal domain of NifA performs the DNA-binding site domain that contains a helix-turn-helix motif particular for binding to upstream activator sequences of 5'-TGT-N_{10}-ACA-3' basically bound to nucleotides upstream position -50 to -200 of the transcriptional point of other *nifA*-regulated genes (Salazar et al., 2010; Sullivan et al., 2013). The central domain, which consists of approximately 500 nucleotides is implicated for binding and hydrolysis of ATP. This central domain is required to recognize the specific sigma factor (Ratet et al., 1989). On the other hand, the rare conserve region of approximately 200 nucleotides at the amino-terminal (N-terminal) domain is predicted for the regulatory function. This catalytic domain interacts to the sigma factor-RNA polymerase holoenzyme to initiate the transcription of downstream genes. Based on amino acid sequence analysis, NifAc of strain DOA9 was similar to other bradyrhizobia, while NifAp showed clearly out of the group. It was found that the N-terminal domain of DOA9 NifAp mainly differ from that of NifAc. Alteration in N-terminal amino acid sequence might cause the change in gene regulation under different conditions. NifAp plays a role in the regulation of nitrogen fixing genes in symbiosis but not under free-living condition. This result was similar to NifA of *R. leguminosarum* biovar *trifolii* which lack of N-terminal domain, its NifA was required for symbiotic nitrogen fixation but not for the free-living state. It indicates that this N-terminal domain is not essential for NifA function under symbiotic condition (Iismaa and Watson 1989; Reed et al., 2011). However, the N-terminal part of NifA may be required to form an

active conformation of protein during free-living condition, which may be essential to response with higher concentration of oxygen that influence the regulation of nitrogen fixation in the cell. In this study, the regular N-terminal part of DOA9 NifAc was used to create a chimeric NifA hybrid protein with the C-terminal part of NifAp. However, the hybrid proteins could not restore the free-living nitrogen fixing ability of DOA9Δ*nifAc* mutant. It was possible that the structural protein of functional NifA could be more complicated to synthesize through the chimeric hybrid fragments even this regulatory NifAp was expressed but no function was observed in free-living condition. On the other hand, FixR protein which is absence in *nifAp* operon, might be specifically required for the functional NifAp under free-living condition. It has been shown that the active NifA protein in *E. coli* could not be observed in the absence of FixR (Kullik et. at., 1989).

Under symbiotic condition, the qRT-PCR analyses confirmed that both *nifAc* and *nifAp* of DOA9 WT were expressed and genes including *rpoN*, *nifDK*, and *nifH* located on both chromosome and mega-plasmid were expressed. Interestingly, the expression pattern of these genes in the background of DOA9Δ*nifAc* and DOA9Δ*nifAp* was similar to that of WT strain. These results indicated that NifAc and NifAp could cross regulate the expression of nitrogen fixing genes located on both chromosome and mega-plasmid. This was the first report of showing the cross regulation between the regulatory NifA on different DNA replicons (chromosome and mega-pasmid). From previous studied, *Mesorhizobium loti* R7A also contained two copies of nifA (*nifA1* and *nifA2*). However, it was found that only NifA2 mainly regulate the expression of *rpoN2* for the nitrogen fixation activity under symbiotic condition and both *nifA2* and *rpoN2* were located on the same replicon in the symbiosis island ICEMlSym^{R7A} (Sullivan et al., 2013). However, the transcription profile in the absence of NifAp could not observed in DOA9 under free-living condition. It confirmed that NifAp did not have a role on regulation of

nitrogen fixing genes under this condition. On the other hand, NifAc was responsible for the regulation of nitrogen fixing genes on both chromosome and mega-plasmid. The transcription profile was similar to that of gene expression in the background of DOA9WT in free-living condition. This could be explained from the NifAc amino acid sequence which was highly match with the NifA proteins found among bradyrhizobia that can fix nitrogen under both symbiotic and free-living conditions (Figure 4.2).

Based on the results obtained in this study, if could be proposed that the nitrogen fixing regulatory model controlled by NifAc and NifAp in *Bradyrhizobium* sp. DOA9 under symbiotic (Figure 4.9) and free-living conditions (Figure 4.10). Under symbiotic condition, the NifAc regulates the expression of *nifAc* itself and might require RpoNc for enhancing the transcription due to the presence of NifA and RpoN binding site in the UAS (Table 4.1). NifAc also regulates other nitrogen fixing genes, including *rpoNc*, *nifDKc* and *nifHc* genes which was involved in the nitrogen fixing ability of DOA9. On the mega-plasmid, NifAp had the same role as NifAc by controlling the expression of *nifAp* itself, *rpoNp*, *nifDKp*, and *nifHp*. The cross regulation between NifAc and NifAp was proposed to be occurred under symbiotic condition. NifAc could cross regulate the expression of nitrogen fixing genes on the mega-plasmid including *rpoNp*, *nifDKp*, and *nifHp*. In the same manner, NifAp could also cross regulate those genes located on the chromosome (Figure 4.9). In free-living life of DOA9, *nifAp* expresses in higher level than *nifAc*, but the translated NifAp protein could not regulate other nitrogen fixing gene under this condition. Nevertheless, NifAc can regulate the nitrogen fixing genes on located on chromosome and also could cross regulate those genes on the mega-plasmid (Figure 4.10). This mode of NifA regulation for nitrogen fixing ability in the strain DOA9 differs from models studied in other bradyrhizobia.

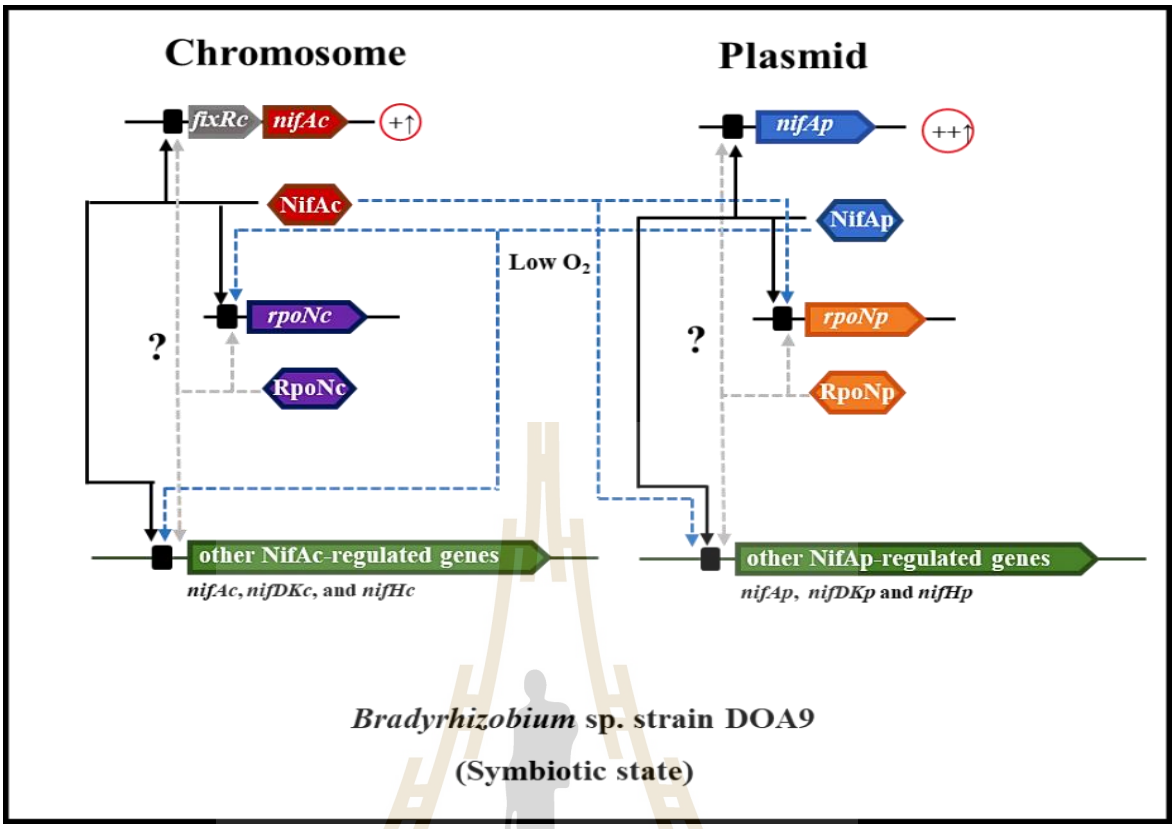

Figure 4.9 Schematic model of the regulation for nitrogen fixation process in *Bradyrhizobium* sp. strain DOA9 during symbiotic state.

In symbiosis with *A. americana*, both *nifA* genes in DOA9 were expressed and functional for regulation of nitrogenase activity. The cross regulation could be possibly occurred among chromosome and plasmid by both regulatory NifAc and NifAp (Dash blue-line). Similar to the free-living condition, activate the expression of other nitrogen fixing genes might cooperate with the conjunction between both NifA and RpoN (Dash grey-line) at the upstream activating site (UAS) that containing both NifA and RpoN binding sequences. While, the information about both *rpoN* genes for regulation of nitrogen fixation in DOA9 was still unknown (?).

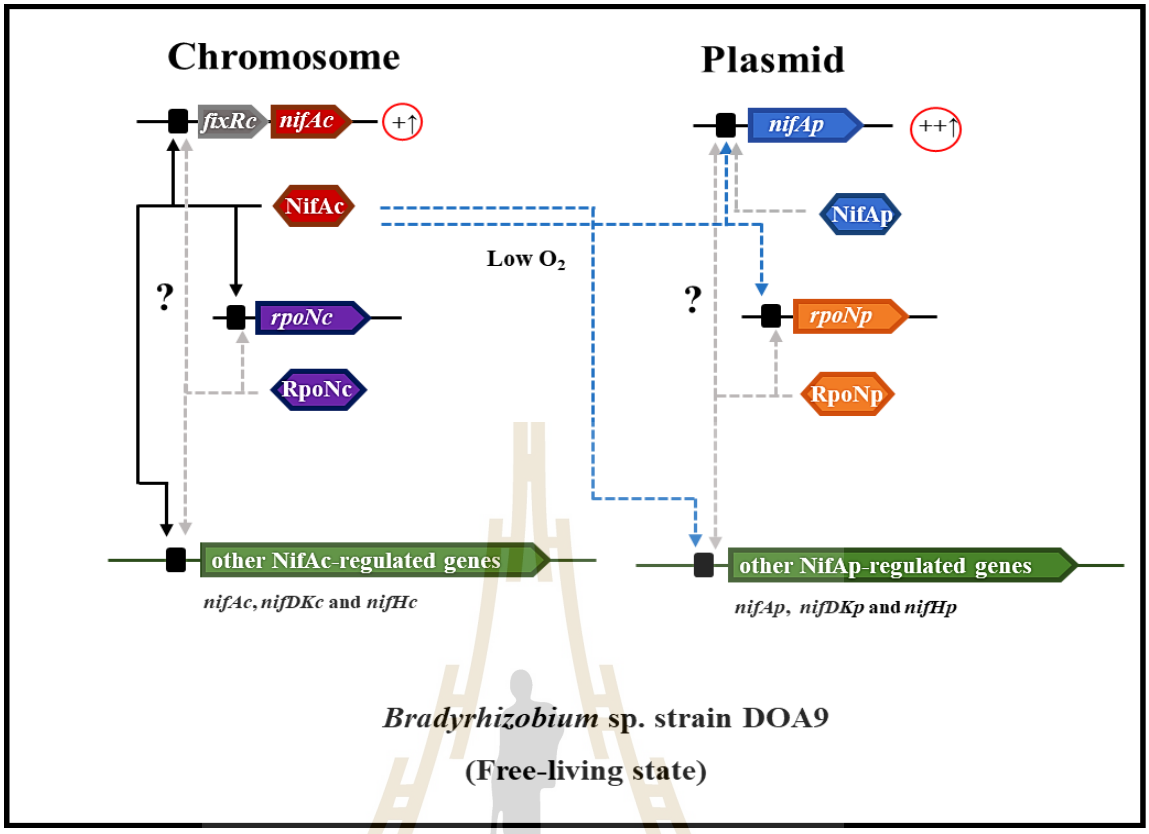

Figure 4.10 Schematic model of the regulation for nitrogen fixation process in *Bradyrhizobium* sp. strain DOA9 during free-living state.

In response to low O_2 condition by phosphorylation and activates expression of two *nifA* genes which were presented in DOA9 that *nifAp* is expressed higher than *nifAc* (++↑). NifAc was mainly regulated the expression of other nitrogen fixing genes (+↑) under free-living condition and may probably cross regulate the other genes on plasmid as well (Dash blue-line). NifAp may form as inactive protein, which could activate some of nitrogen fixing genes with very low expression level (-↓). Activating the expression of nitrogen fixing genes might cooperate with the conjunction between both NifA and RpoN (Dash grey-line) at the upstream activating site (UAS) that containing both NifA and RpoN binding sequences. However, the information about both *rpoN* genes for regulation of nitrogen fixation in DOA9 was still unknown (?).

This study provided the evidences to show the role of two *nifA* genes located on chromosome and mega-plasmid of DOA9. However, it general accepted *nifA* was important for controling the nitrogen fixation, but the *rpoN* has been characterized as a regulator for nitrogen fixation in nitrogen fixing bacteria. In DOA9, two *rpoN* genes were also detected on both replicons. Therefore, the two RpoN of *Bradyrhizobium* sp. DOA9 might also plays a role in regulation of the nitrogen fixation which especially were related to symbiotic condition. For future work on elucidation the NifA-RpoN regulation in this bacterium that may possesses different regulatory genes for nitrogen fixation under different conditions will be explored.

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CHAPTER V

Presence of *nifV*, homocitrate synthase involved in the efficiency of nitrogenase activity in *Bradyrhizobium* sp. strain DOA9 under free-living state rather than under symbiosis with legume plant

5.1 Introduction

Beneficial effects between leguminous plant and rhizobia, is most extensively studied as model of plant-microbe interactions. These symbiosis are involved with several approaches, including infection, nodulation and nitrogen fixation processes (Franche et al., 2009). Successful of initial symbiotic process reached to establish biological nitrogen fixation by specific symbiotic bacteria. Symbiotic bacteria produced nitrogenase, an enzyme to converse the atmospheric nitrogen to become a nitrogen containing compounds in host plant (Bloemberg and Lugtenberg, 2001, Emerich et al., 2009 and Franche et al., 2009).

Nitrogenase is the two-components enzyme complex responsible for the process of nitrogen fixation and is structurally highly conserved throughout nitrogen-fixing bacteria (Fani et al., 2000). The α and β subunits of two components composed of component I (dinitrogenase or MoFe protein) is encoded by *nifD* and *nifK*, and component II (dinitrogenase reductase or Fe protein) is encoded by *nifH*. Component I requires a co-factor that encoded from *nifBENXQSUVY* genes, which is formed the site of substrate binding and reduction (Kennedy, 2012 and Rubio and Ludden, 2008). The

other *nifZMQ* and *nifFJ* genes are also required for nitrogenase maturation and electron donation of the active enzyme complex (Fani et al., 2000). In addition, the catalytic center of dinitrogenase normally binds with the FeMo cofactor which homocitrate is ligated. Homocitrate is synthesized by the *nifV*-encoded enzyme called homocitrate synthase, which catalyzes the condensation of acetyl coenzyme A and 2-oxoglutarate to be homocitrate (Hu et al., 2005 and Masukawa et al., 2007).

The presence of a *nifV* gene in some bacteria could be related to their ability to fix nitrogen under free-living conditions which is an uncommon characteristic of rhizobia (Hu et al., 2005). Under symbiosis, it has been analyzed that the plant, *Lotus japonicus* provided homocitrate to bacteroid that lack of *nifV* to ensure the formation of a functional nitrogenase by using the homocitrate synthase encoded from *FEN1* gene (Hakoyama et al., 2009). This is one of reasons why most rhizobia display a nitrogenase activity only when symbiosis with some host plants.

The genomic data analysis displayed that the strain DOA9 possesses a unique system of two structural *nifDK* and two regulartory *nifA* and *rpoN* genes located on both chromosome and plasmid. The strain DOA9 is able to fix nitrogen under both symbiotic and free-living conditions similar to the photosynthetic *Bradyrhizobium* strains. Thus, the presence of *nifV* in this bacterium which located on the chromosome may be involved in the ability of DOA9 to fix nitrogen under free-living condition. Moreover, the role of *nifV* on nitrogen fixation under symbiosis condition with different host plant species is another aspect to be examined to understand the role of *nifV* in DOA9.

5.2 Objectives

5.2.1 To analyze the genetic similarity and organization of *nifV* involved in nitrogen fixation in DOA9 compared with other nitrogen-fixing bacteria

5.2.2 To investigate the function of nitrogenase genes *nifV* located on chromosome of DOA9 involved in nitrogen fixation under both symbiotic and free-living states

5.3 Materials and methods

5.3.1 Bacterial strains and culture media.

All bacterial strains and plasmids were obtained from School of Biotechnology, Suranaree University of Technology, Thailand, while all mutants were constructed in Laboratoire des Symbioses Tropicales et Méditerranéennes (LSTM), France. These bacterial strains were grown at 28°C for 4 days in YM medium (Vincent, 1970) or in a BNM-B minimal medium (Ehrhardt and Atkinson, 1992 and Renier et al., 2011). All *Escherichia coli* strains were grown in LB medium at 37°C for 1 day. Each medium was supplemented with the following of antibiotics when appropriate, for *E. coli*, 20 µg/ml Cefotaxime, and for *Bradyrhizobium* mutant, 200 µg/ml Cefotaxime and 50 µg/ml Kanamycin.

5.3.2 Construction of NifV phylogenetic tree

Phylogenetic analysis was performed by comparing the NifV sequences of *Bradyrhizobium* sp. DOA9 to other Bradyrhizobia (Fig 5.1). The amino acid sequences were obtained from the online sources including NCBI and GenomeScope.The sequences were aligned using the CLUSTAL W program. Neighbor-joining trees were constructed using MEGA version 6 (Tamura et al., 2013), and 1,000 bootstrap replicates were used to generate a consensus tree.

5.3.3 DNA manipulations, PCR amplification and sequencing

For the bacterial DNA and plasmids were extracted by Genomic DNA and Plasmid purification kits (Promeca, Germany). Whilst, DNA fragments were purified from agarose gels using the illustra GFX PCR DNA and Gel Band Purification Kits (GE Healthcare, USA). These were eluted and kept into freezing at -20°C until used. PCR assay was performed by Go Taq Flexi DNA polymerase kits (Promega, Germany.) by following the manufacturer's instructions. The primers were used for the insertion and clean mutation are including of '5 to 3' primer, nifV.XbaI-D9.f (TGGCGGCTCGAGG CGAGCACCGACTTTGCTTCGT), nifV.SbfI-D9.f GCGAGACTCAGAGGTGCTGTC CGAGCTGAAGCA), up.nifV.D9.f (TGCACCGGATCCCCTTGCACGCTTCTGCAA-T), up.nifV.D9.r (ATTGGAGGACGCAAGCTTGGGTATCATGGCCTGCATCGT), dw.nifV.D-9.f (GCCATGATACCCAAGCTTGCGTCCTCCAATCATTCATTC), dw.-nifV.D9.r (ACGACGTCTAGATGCACGGATTGCAACGATTTC). The cleaned PCR products were sequenced using the same primers as those for the PCR. DNA sequencing was carried out by Genoscreen (Lille, France).

5.3.4 Construction of the mutant strains using insertion and clean mutations

All DNA fragments were amplified by using the primers are listed above. For the construction of the Ω*nifV* mutant, 300 base pairs (bp) internal fragments of *nifV* gene were amplified by PCR and cloned into the plasmid pVO155-Ce-npt2-gus-gfp harboring a cefotexime resistance gene. To construct the Δ*nifV* in *Bradyrhizobium* sp. strain DOA9, approximately 1,600 bp internal fragments of *nifV* gene were amplified using the overlap PCR and these fragments were cloned into the plasmid pNTPS129:*sacB*. The constructed plasmids were introduced into *E. coli* S17-1 using electroporation (15 kv/cm, 100Ω, and 25μF) and were transferred into *Bradyrhizobium* sp. strain DOA9 by bi-parental mating method (Giraud et al., 2010). Transconjugants were selected on YM plates and supplemented with 20 μg/ml nalidixic acid, 20 μg/ml

cefotaxime and 200 µg/ml kanamycin. Whilst, the *nifV* clean deletion mutant was selected twice on YM containing 10% sucrose plates to obtain the mutant without *nifV* and plasmid harboring. These mutants were maintained in YM without any antimicrobial.

5.3.5 Plant cultivation, symbiotic analysis

The symbiosis efficiency of *Bradyrhizobium* DOA9 strain and derivatives were tested with legume plants including *Aeschynomene americana* No. 281 (Mardagusga ecotype) and other interested plants. The seeds were surface sterilized by immersion in sulphuric acid under shaking during 45 minutes. Seeds were abundantly washed with sterile distilled water and incubated overnight in sterile water. Seeds were then transferred for one day at 37°C in the darkness on 0.8% agar plates for germination. Plantlets were transferred on the top of test tubes covered by aluminum paper for hydroponic culture in buffered nodulation medium (BNM) (Ehrhardt and Atkinson, 1992). Plants were grown in the light room at 28°C with a 12-h light and 12-h dark regime. Seven days after transfer, each seedling was inoculated with one milliliter of cell suspension resulting from a 5 day-old bacterial culture washed in BNM and adjusted to reach an optical density of one at 600 nm. At 20 (*A. americana*, *A. afraspera* and *A. hypogea*) or 30 dai (others), the plant growth was compared with non– inoculated control plants and the nodule number, plant fresh weight and mean nitrogenase enzyme activity as analyzed by the ARA assay was determined (Bonaldi et al., 2011). The experiments were carried out in 10 replicates.

5.3.6 Cytological analysis

To follow the β-glucuronidase activity in the nodules elicited by the reporter strains, 30-to 40-µm-thick sections from fresh nodule samples made using a vibratome (VT1000S; Leica, Nanterre, France) were incubated at 37°C in the darkness in a GUS assay buffer for 1 hour as described in Bonaldi et al. (2010). After staining, the sections

were mounted and observed under bright-field illumination with a microscope (Nikon AZ100; Champigny-sur-Marne, France).For the live-dead staining was used with the nodules elicited with the DOA9Δ*nifV* strains, the nodule sections were incubated for 10 minutes in live/dead staining solution (5 µM SYTO 9 and 30 µM propidium iodide (PI) in 50 mM Tris pH 7.0 buffer; Live/Dead BacLight, Invitrogen, Carlsbad, CA, USA). Sections were then removed and incubated an additional 15 min in 10 mM phosphate saline buffer containing calcofluor white M2R (Sigma, Munich, Germany) to a final concentration of 0.01% (wt/vol) to stain the plant cell wall (Okazaki et al., 2016). The stained nodule sections were observed under a confocal laser-scanning microscope (Carl Zeiss LSM 700, Jena, Germany). Setting of excitation wavelengths were used at 405, 488 and 555 nm to detect the emission signal of Calcofluor, SYTO 9 and PI, respectively (Giraud et al., 2013).

5.3.7 Determination of nitrogenase activity under free-living conditions

To determine the nitrogenase enzyme activity under free-living conditions, *Bradyrhizobium* sp. strain DOA9 and derivatives, were grown in 10-ml test tubes (BD Vacutainer, Franklin Lakes, NJ, U.S.A.) containing 2 ml of BNM-B medium (without glutamate) soft agar (0.8%), as described by Alazard (1990). Acetylene was injected to give a final concentration of 10%. The cultures were incubated at 28°C and gas samples were analyzed daily for ethylene production by gas chromatography, as described (Renier et al., 2011).

5.3.8 Statistical Analysis

Data in all experiments were resolved into elements as mean values and standard deviations with SPSS software (SPSS versions 17.0 Windows; SPSS Inc., Chicago, IL) by Duncan's multiple range test (Duncan, 1955) and Tukey's HSD test (Tukey, 1949).

5.4 Results

5.4.1 Gene organization and NifV phylogenetic tree analysis

The presence of *nifV* on chromosome of strain DOA9 could be involved in the efficient nitrogen fixation activity under free-living condition since most of free-living nitrogen fixing rhizobia also contain this gene. *nifV* of strain DOA9 is located on chromosome at downstream of *nifHQfur* (these genes are encoded for synthesis of nitrogenase iron protein, modular protein and nitrogen fixation protein, respectively) and at the upstream of *cysEnifW* (these genes are encoded for serine acetyltransferase and nitrogenase-stabilizing protein, respectively) (Figure 5.1). The NifV phylogenetic tree was constructed and compared to other bradyrhizobia containing NifV. Analysis of phylogenetic tree suggests that NifV from 16 species was divided into three clades as show in Figure 5.2. Clade I represented the photosynthetic bradyrhizobia, while clade II and III contained non-photosynthetic bradyrhizobia. NifV of strain DOA9 was located in clade II which is located between the photosynthetic and non-photosynthetic bradyrhizobial clades. However, most of bradyrhizobia classified in clade II are able to fix nitrogen under free-living condition. NifV DOA9 showed 97% of amino acid similarity to the non-photosynthetic *Bradyrhizobium* BR10280 which is legume nodulating bacterium isolated from sugarcane roots as endophytic bacterium.

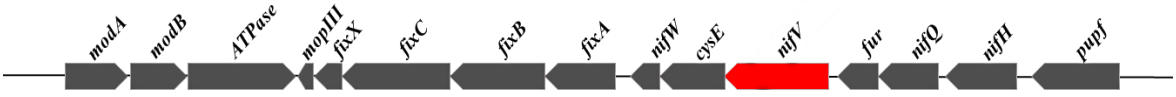

Figure 5.1 Theorganization of *nifV* gene in *Bradyrhizobium* sp. DOA9

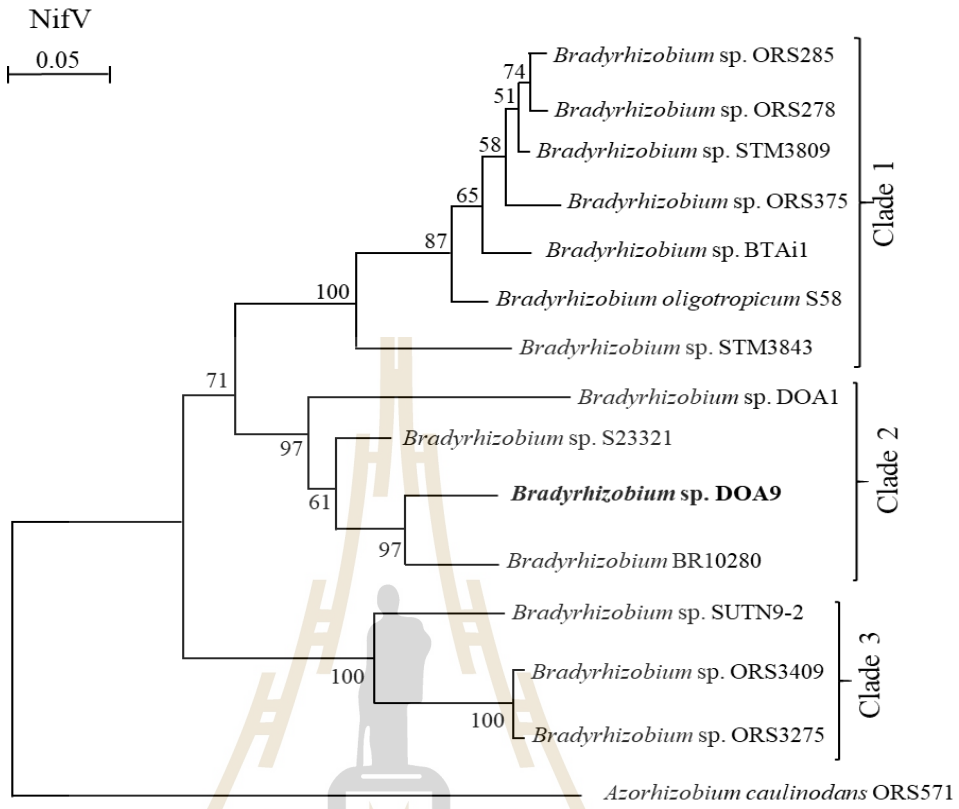

Figure 5.2 The NifV phylogenetic tree of bradyrhizobia.

5.4.2 The nitrogenase efficiency in free-living life of *Bradyrhizobium* sp. DOA9 is involved in the presence of *nifV* gene on chromosome

To investigate the role of *nifV* on nitrogen fixation ability of DOA9 under free-living condition, *nifV* mutant strains were constructed by using the insertion (Ω*nifV*) and deletion (Δ*nifV*) mutation methods. Acethylene Reduction Assay (ARA) was used to determine the nitrogenase activity of DOA9WT in comparison with *nifV* mutants after 7 days of bacterial growth under free-living condition. A similar result was agreed the previously obtained from Wongdee et al. (2016) who found that the nitrogen fixation activity of WT was high under free-living condition with low oxygen and without nitrogen supplement. Interestingly, the nitrogenase activity of DOA9Ω*nifV* and DOA9Δ*nifV* mutants was

significantly decreased when compared to the WT, and the nitrogenase activity of the DOA9Ω*nifV* was lower than DOA9Δ*nifV* (Figure 5.3).

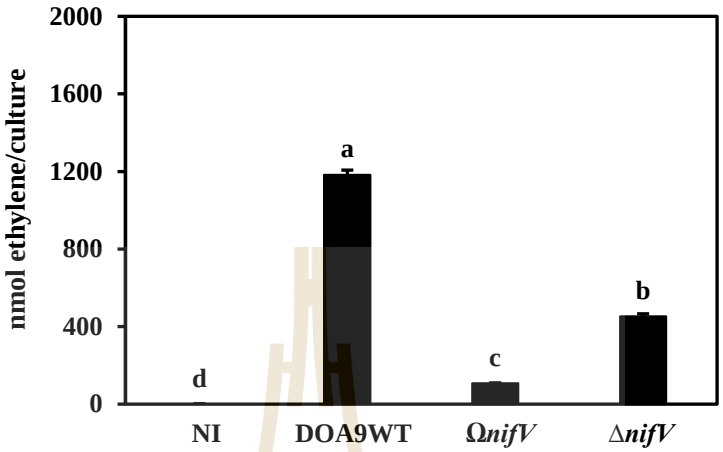

Figure 5.3 Acetylene reduction assay by respective cultures of WT and the mutant strainsDOA9*ΩnifV and* DOA9Δ*nifV*, incubated at 30°C for 7 days under free-living concentration. n= 3.

Since *nifV* is encoded for the homocitrate synthase to synthesize homocitrate which act as the co-factor of dinitrogenase, thus 1 mM homocitrate was supplemented into the culture of *nifV* mutant strains so that these could synthesis the nitrogenase for these mutants under free-living condition. The result showed that supplementation of homocitrate in DOA9Δ*nifV* could restore the nitrogenase activity as similar to the WT, but it could not restore the nitrogenase function of DOA9Ω*nifV* mutant strain (Figure 5.4). It could be explained that the insertion mutation method may cause the polar mutation on the downstream genes that might use the same promoter as *nifV*. These downstream genes might be also important for the nitrogenase activity.

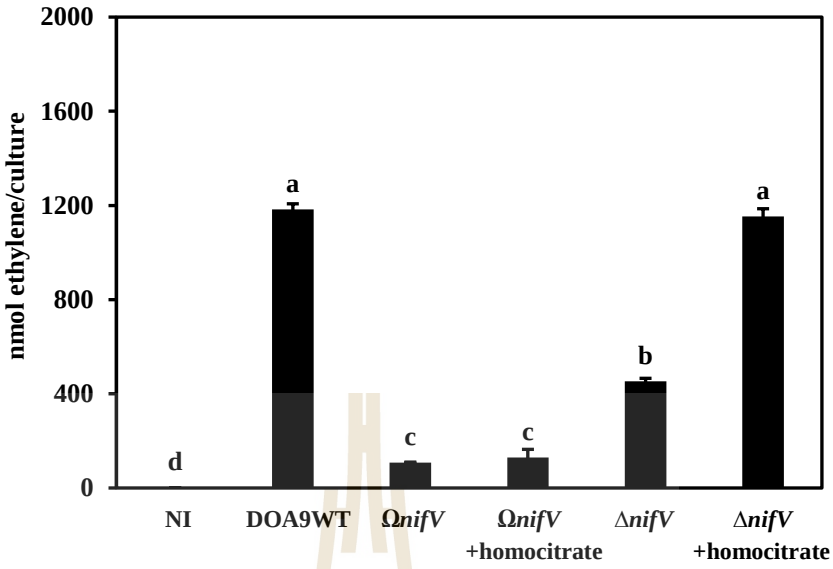

Figure 5.4 The complementation of the mutant strains DOA9Ω*nifV* and DOA9Δ*nifV* by supplementing with 1 mM homocitrate in nitrogen-free (Bnm) medium and grown under free-living concentration. Acetylene reduction was measured at 7 days after inoculation of nitrogen-starved cells into glass vials containing 2 mL of medium and 10 mL of gas headspace and incubated at 30°C. n= 3.

5.4.3 Absence of *nifV* gene in *Bradyrhizobium* sp. DOA9 strain did not affect the nitrogen fixing activity during symbiosis with host plant *A. americana*

The inoculated plants at 20 days after inoculation (dai) showed no significant difference on plant phenotype, ARA activity, numbers of root nodule, and plant fresh weight when compared between plant inoculated with DOA9WT and that of *nifV* mutants (Figure 5.5A-E). The nodule phenotype was observed by staining of *gus*-expression to confirm the infection of DOA9Ω*nifV* and it showed dark blue color. The nodule phenotypes, such as size and area of symbiosome were found similarly in all nodules (Figure 5.5F-H) indicating that both *nifV* mutant strains did not affect the phenotype of symbiotic performance on *A. americana*. However, the presence of *nifV*

gene in DOA9 may affect to the nitrogen fixation activity during symbiosis with other species of legume plants since the strain DOA9 although can nodulate several legumes, the nitrogen fixation efficiency in each plant is different.

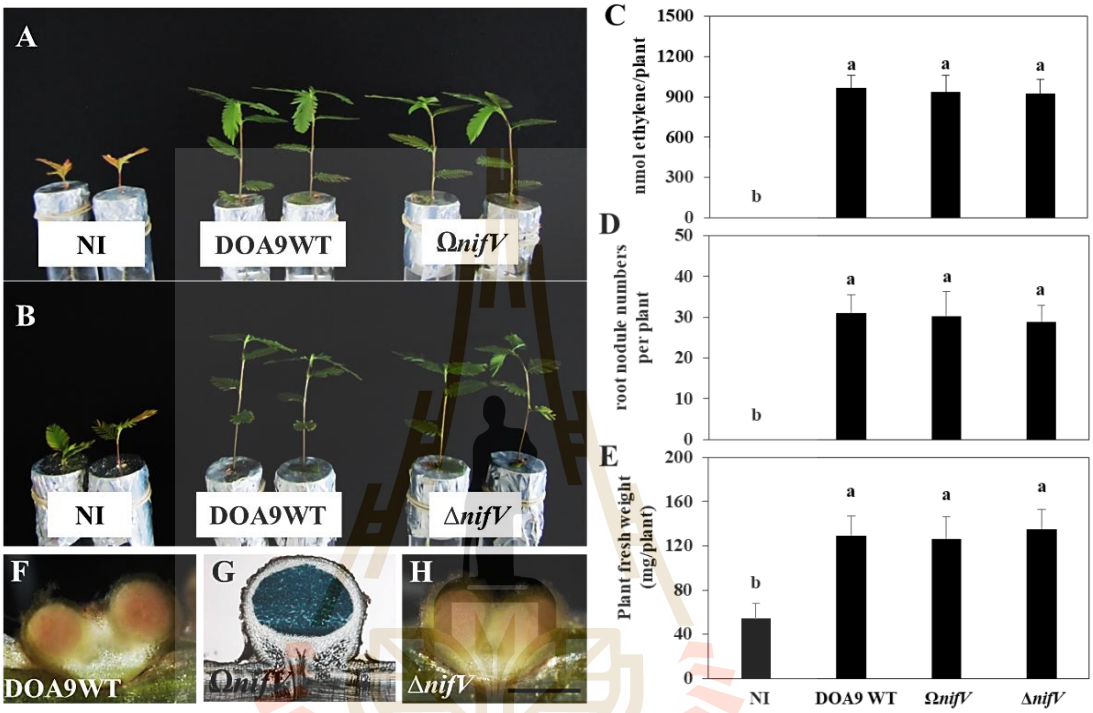

Figure 5.5 Comparison of the plants phenotypes (aerial part) non-inoculated (NI) or inoculated with WT and the mutant strains DOA9*ΩnifV* and *DOA9*Δ*nifV* after 20 days of plant inoculation. The plant phenotype indicated that plant inoculated with the mutant strain DOA9*ΩnifV* and *DOA9*Δ*nifV* were similar to the WT (**A**-B). (**C**) The amount of acetylene-reducing activity (ARA) in *A. americana* plants inoculated with WT and the mutant strains. (**D**) Number of nodules and (**E**) plant fresh weight from plant nodulated by WT and with DOA9Ω*nifV* and *DOA9*Δ*nifV* mutant strains. (**F**) Nodules nodulated by WT; (**G**) Nodules nodulated by DOA9*ΩnifV*; and (**H**) nodules elicited by *DOA9*Δ*nifV*. Scale bars are 1 mm for (**F-H)**. In panels (**C-E**) Error bars represent s.e. *(n=10).* Different letters above the error bars indicate significant differences at P<0.05 (Tukey's HSD test).

5.4.4 NifV is required for symbiotic performances of DOA9 on some leguminous plants

Leguminous plants used in this experiment were selected based on the nodulation ability of strain DOA9 as previously described (Teamtisong et al., 2014). The total of 10 leguminous species were divided into 3 clades, including Genistoids (*Crotalaria juncea*), Dalbergioids (*Aeschynomene americana* (Thai and Madagascar ecotypes), *Aeschynomene afraspera*, *Arachis hypogaea*, *Stylosanthes hamata*) and Millettioids (*Macroptilium atropurpureum*, *Vigna radiata* SUT4, *Desmodium* sp. and *Indigofera tinctoria*) (Table 5.1). The inoculated plants were harvested at different times (at 20 or 30 dai), based on different species of legume. The results showed that 8 species of legume inoculated with DOA9WT and DOA9Δ*nifV* had no significant differences on the nitrogenase activity, numbers of plant nodule, and plant fresh weight (Table 5.1 and Figure 5.6 – 5.8).

Interestingly, only 2 legume plant species, including *I. tinctoria* and *S. hamata* showed significant difference in the nitrogenase activity and plant fresh weight among plants inoculated with DOA9WT and DOA9Δ*nifV* after harvesting at 30 dai (Table 5.1 and Figure 5.9A-B). Plants inoculated with DOA9Δ*nifV* were smaller than that of WT plant, and they appeared of yellow leaves as nitrogen deficiency similar to that of non-inoculated plant (Figure 5.9A). The difference of root nodule numbers between plant inoculated with DOA9WT and DOA9Δ*nifV* could not observe. It was difficult to determine the phenotype differences in *I. tinctoria* inoculated with DOA9WT and DOA9Δ*nifV* at 30 dai by eyes since the plants were very small (Figure 5.9B). However, the nitrogenase activity and plant fresh weight of DOA9Δ*nifV* inoculated plant were significantly decreased from that of DOA9WT inoculated plants (Figure 5.9D and H).

Table 5.1 Characterize of the symbiotic performances on legume plants by inoculating of the WT and Δ*nifV* mutant of *Bradyrhizobium* sp. strain DOA9.

Plant cultivars	**Symbiotic performances after inoculation**	
	DOA9WT	**Δ*nifV***
Genistoids	Nod+Fix-	Nod+Fix-
Crotalaria juncea		
Dalbergioids	Nod+Fix+	Nod+Fix+
Aeschynomene americana (T, M)	Nod+Fix+	Nod+Fix+
Aeschynomene afraspera	Nod+Fix+	Nod+Fix+
Arachis hypogaea	Nod+Fix+	Nod+Fix-
Stylosanthes hamata		
Millettioids	Nod+Fix+	Nod+Fix+
Macroptilium atropurpureum	Nod+Fix-	Nod+Fix-
Vigna radiata (SUT4)	Nod+Fix+	Nod+Fix+
Desmodium sp.	Nod+Fix+	Nod+Fix+/-
Indigofera tinctoria		*

Nod+: the ability of root nodulation by inoculated bacterial strain when compared to the non-inoculated control plants.

Fix+: indicate the nodule phenotype of nitrogen fixation. Fix-: indicate the nodule phenotype of no nitrogen fixation.

*: indicate the plant may have decreased nitrogen fixation.

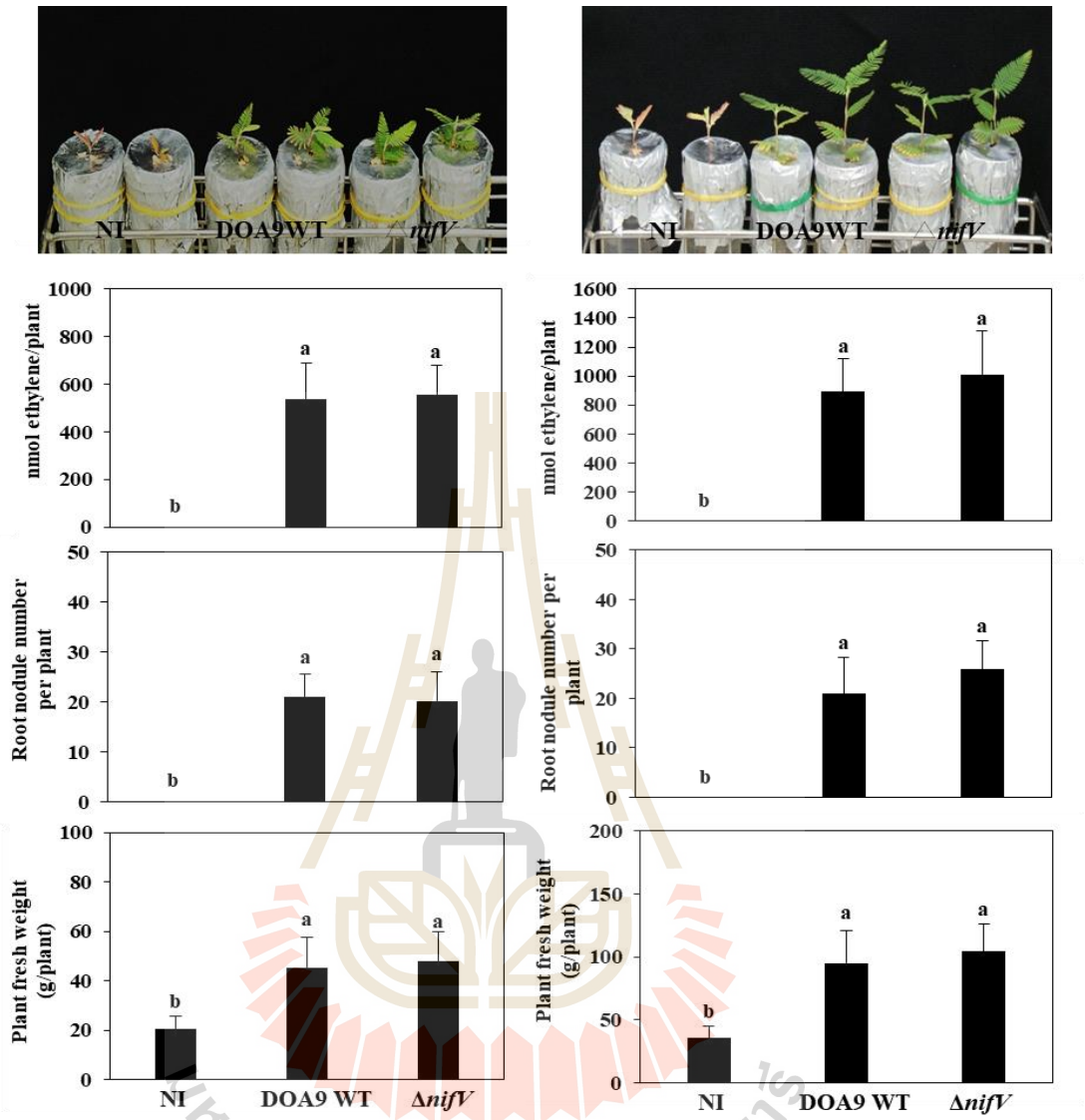

Figure 5.6 Comparison of the plants phenotypes including *Aeschynomene americana* Thai (**A**) and Madagusga (**B**) ecotypes of non-inoculated (NI) or noculated with WT and *DOA9*Δ*nifV* after 30 days of plant inoculation. The amount of acetylene-reducing activity (ARA) in plants inoculated with WT and themutant strains (**C-D**). (**E-F**) Number of nodules and (**G-H**) plant fresh weight from plant nodulated by WT and clean mutant strains. Error bars represent s.e. *(n=10).* Different letters above the error bars indicate significant differences at P<0.05 (Tukey's HSD test).

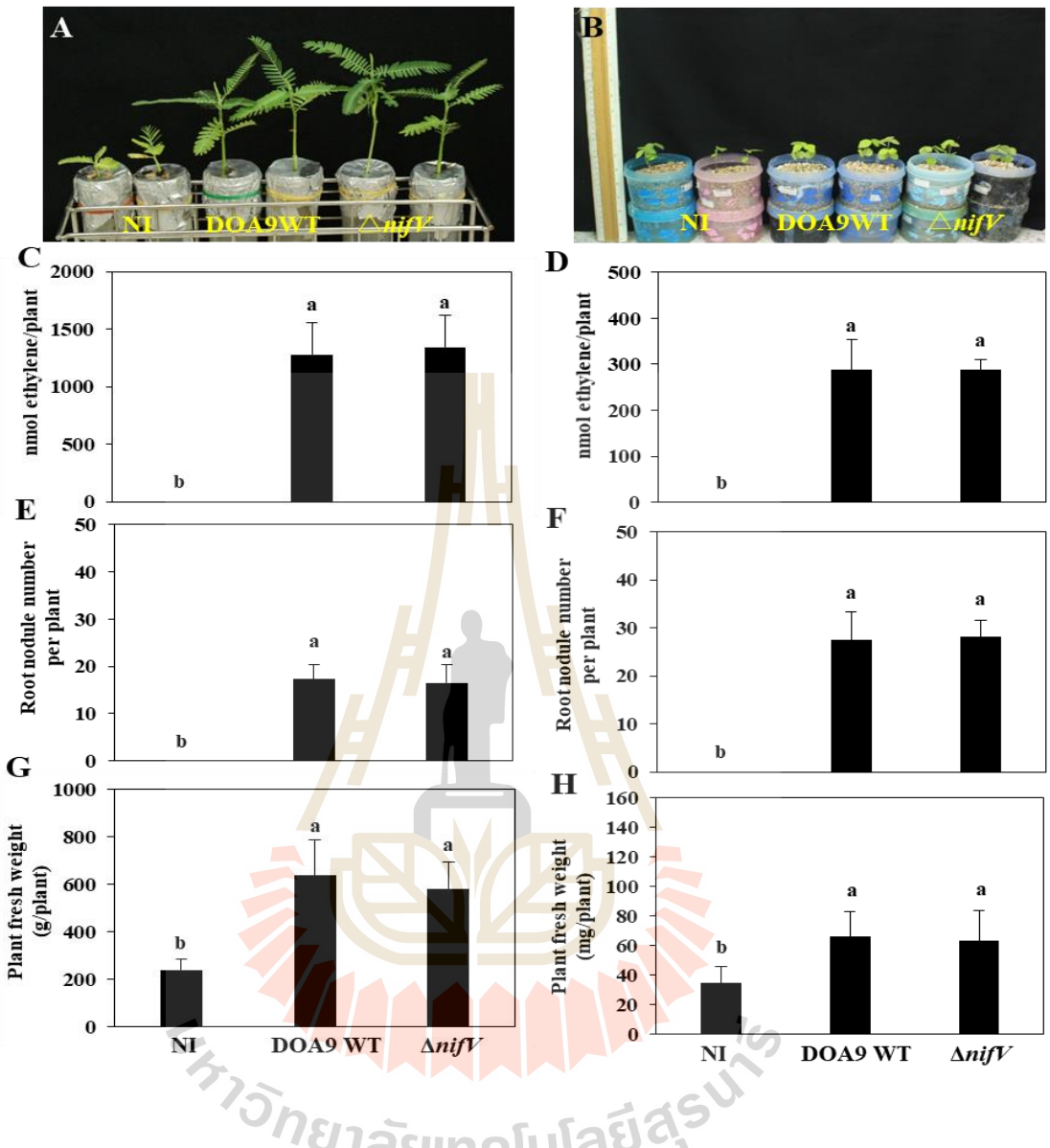

Figure 5.7 Comparison of the plants phenotypes including *A. afaspera* (**A**) and *M. atropurpureum* (**B**) of non-inoculated (NI) or inoculated with WT and *DOA9ΔnifV* after 30 days of plant inoculation. The amount of acetylene reducing activity (ARA) in *A. afaspera* and *M. atropurpureum* plants inoculated with WT and the mutant strains (**C**-**D**). (**E**-**F**) Number of nodules and (**G**-**H**) plant fresh weight from plant nodulated by WT and clean mutantstrains. Error bars represent s.e. *(n=10).* Different letters above the error bars indicate significant differences at P<0.05(Tukey's HSD test).

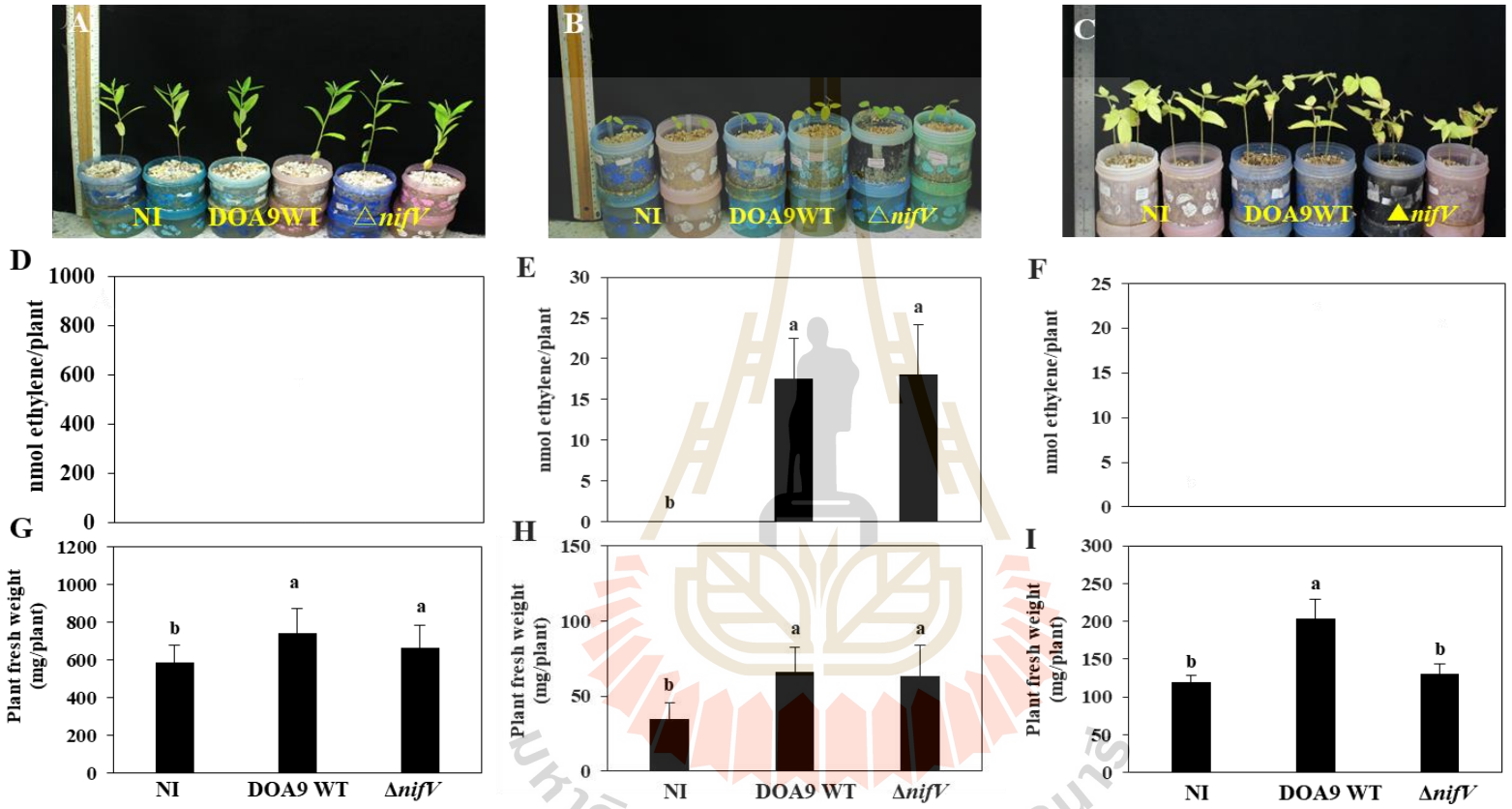

Figure 5.8 Comparison of the plants phenotypes including *C. juncea* (**A**), *Desmodium* sp. (**B**) and *V. radiata* (**C**), of non-inoculated (NI) or inoculated with WT and *DOA9ΔnifV* after 30 days of plant inoculation. Number of nodules (D-F), and plant fresh weight from plant nodulated by WT and clean mutant strains (G-I). Error bars represent s.e. *(n=10).* Different letters above the error bars indicate significant differences at P<0.05 (Tukey's HSD test).

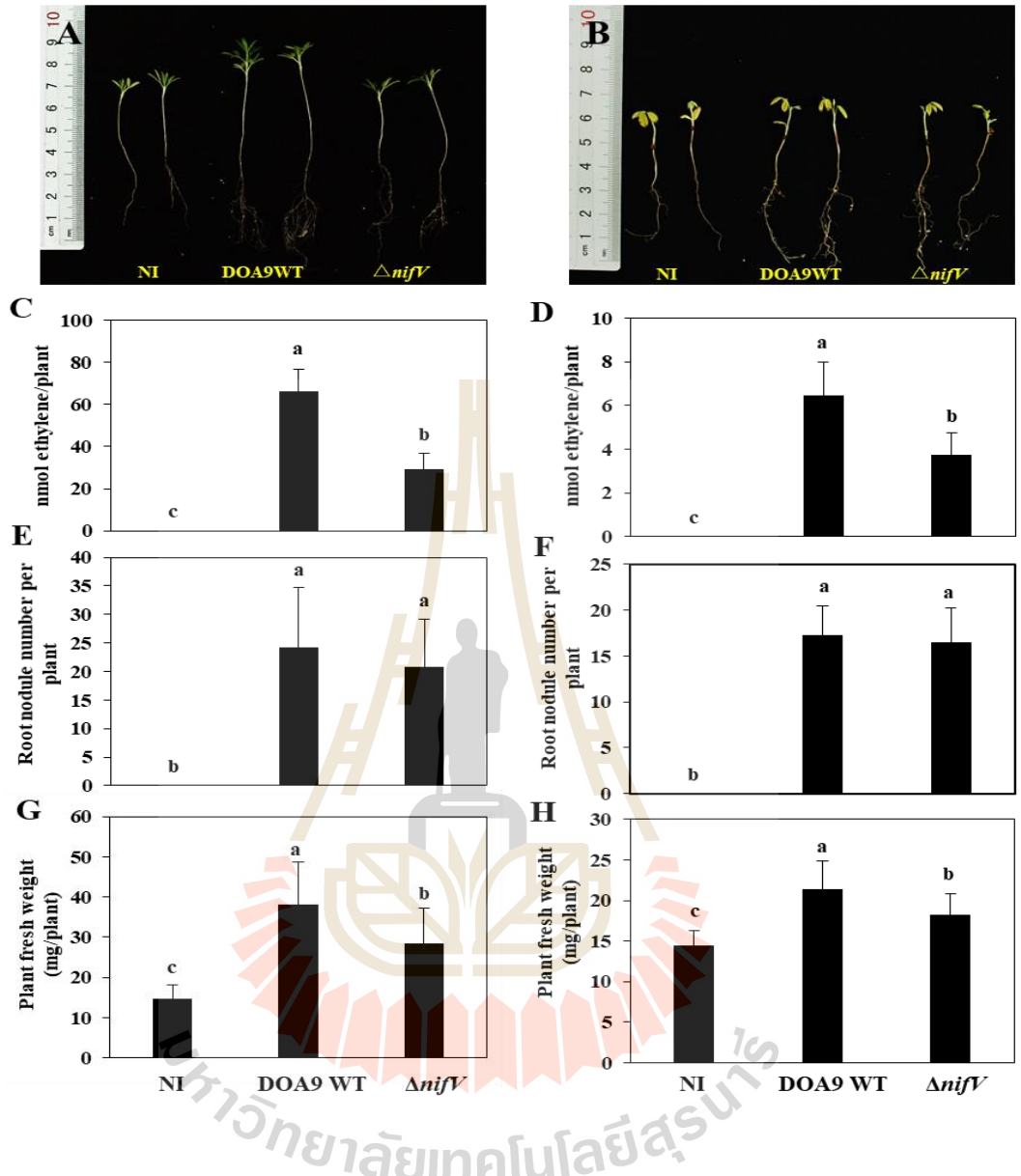

Figure 5.9 Comparison of the plants phenotypes including *S. hamata* (**A**) and *I. tinctoria* (**B**) of non-inoculated (NI) or inoculated with WT and *DOA9*Δ*nifV* after 30 days of plant inoculation. The amount of acetylene educing activity (ARA) (**C**-**D**). Number of nodules (**E**-**F**) and plant fresh weight (**G**-**H**) from plant nodulated by WT and clean mutant strains. Error bars represent s.e. *(n=10).* Different letters above the error bars indicate significant differences at P<0.05 (Tukey's HSD test).

5.3.5 The root nodules of *I. tinctoria* and *S. hamata* elicited by DOA9Δ*nifV* strain were senescence earlier than nodules elicited by DOA9WT

The nodules elicited by DOA9WT and DOA9Δ*nifV* were observed for the symbiosis features using live-death cells staining. The similar phenotypes of nodule size and symbiosis zone were observed in both WT and Δ*nifV* nodules. However, the dead phenotype of the symbiotic cells with red colour (Figure 5.10) could be observed more in the nodules elicited by DOA9Δ*nifV*. The nodule of DOA9Δ*nifV* in *S. hamata* clearly observed high amount of death cells when compared with the live cell showing green color in the nodules elicited by WT. The earlier senescence of nodule might be suggested that the sanction in nodule elicited by DOA9Δ*nifV* might exist earlier than that of DOA9WT.

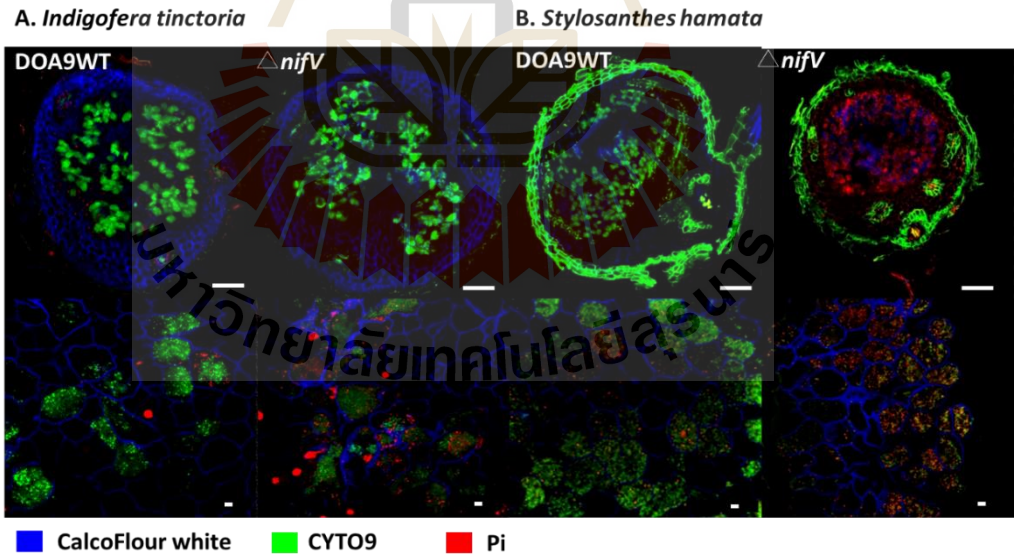

Figure 5.10 Confocal microscopy of bacteroid viability determination by live-dead staining of *I. tinctoria* and *S. hamata* nodules induced by the DOA9 wild type and the *nifV* mutants. Scale bars are indicated 100 and 10 µm for the large and short bars, respectively.

5.4 Discussion

The ability for conversion of atmospheric dinitrogen to ammonia is strictly limited to prokaryotes. This group of organisms, nitrogen fixation is observed in a large number of species belonging to both prokaryotic kingdoms of Archaebacteria and Eubacteria (Beringer et al., 1979 and Martínez-Romero, 2009). The group of symbiotic diazotrophs includes specific species of rhizobia referred the interaction with legume host plant(s) before that contribution of nitrogen fixation. The knowledge of rhizobial strains in free-living state fix no nitrogen but the nitrogen fixing activity is restricted to symbiotic bacteroids indicating the unique form of legume-Rhizobium symbiosis (Beringer et al., 1979 and Zahran, 1999).

The homocitrate synthase encoding *nifV* gene had not been identified in most rhizobium strains, except in the photosynthetic bradyrhizobia and stem nodulating *A. caulinidans* (Alazard, 1990). Many researches proposed that the presence of *nifV* correlated with having nitrogenase activity in their free-living life (Mayer et al., 2002, McLean and Dixon, 1981 and Reed et al., 2011). For example, the nitrogenase activity was detected in the soft-agar and liquid cultures under free-living life of many *Bradyrhizobium* strains, such as *Bradyrhizobium* sp. ORS310 and ORS322 and photosynthetic *Bradyrhizobium* sp. strain ORS285, ORS278 and BTAi1. The *nifV* gene was also detected in *Bradyrhizobium* sp. DOA9 which is an uncommon characteristic of non-photosynthetic bradyrhizobia. The free-living nitrogen fixation ability of this strain was similar to the level has been reported in photosynthetic bradyrhizobia, but it was not equivalent to that of *A. caulinidans* (Wongdee et al., 2016). The phylogenetic tree analysis of bradyrhizobial NifV indicated that NifV of strain DOA9 related to the non-photosynthetic bradyrhizobia that some of them can fix nitrogen under free-living condition (clade II). For example, *Bradyrhizobium* sp. S23321 is a non-photosynthetic and non-symbiotic bradyrhizobium containing *nif* gene clusters, which are similar to

these photosynthetic bradyrhizobia strains ORS278 and BTAi1. Strain S23321 and photosynthetic bradyrhizobia could exhibit the same remarkable feature that they are able to fix nitrogen in the free-living state (Okubo et al., 2012). These evidences might explain the position of DOA9 in phylogenetic tree that located in clade II, which is more closely related to NifV of photosynthetic bradyrhizobia in clade I.

The nitrogenase catalyzes the reduction of nitrogen (N_2) under appropriate condition by the association of two proteins, catalytic dinitrogenase (MoFe protein) which is a catalytic component encoded from *nifDK* and dinitrogenase reductase (Fe protein) which is a reductase component encoded from *nifH* (Hoffman et al., 2014 and Seefeldt et al., 2017). This reaction supplies electrons from reduced ferredoxin or flavodoxin to the catalytic dinitrogenase in an ATP-dependent manner. The catalytic center of dinitrogenase normally requires the FeMo cofactor which homocitrate is ligated to activate the nitrogen fixing activity (Hu et al., 2005, Spatzal et al., 2016 and Varley et al., 2015). Thus, homocitrate is needed to complete the nitrogenase activity.

In *Bradyrhizobium* sp. DOA9, the nitrogenase activity remained around 60 and 10% in the Δ*nifV* and Ω*nifV* mutants, respectively when compared to the WT strain. This result indicates the role of NifV on the efficiency of nitrogenase. The different activities under *in vivo* condition among several *nifV* mutants constructed from various free-living bacteria were studied. In *Klebsiella pneumoniae* and *Azotobacter chroococcum*, their *nifV* mutants contributed about 80 and 0%, respectively of the nitrogenase activity from the wild types, thus the *nifV* mutant of *A. chroococcum* was unable to grow diazotrophically (as the sole source of nitrogen nutrition is N_2) (Evans et al., 1991 and McLean and Dixon, 1981). In contrast, the *nifV* mutants from *A. vinelandii* (Madden et al., 1991) and *Rhodobacter capsulatus* (Evans et al., 1991) remained about 10% of the nitrogenase activities from the respective wild-type strains. However, these mutant strains were still slow growing in diazotrophic condition. According to our result and

the literatures, the question was occurred. As homocitrate is required for nitrogenase activity, why nitrogenase activity still remain in the *nifV* mutant when cultured under free-living condition. The nuclear magnetic resonance and crystallographic analyses had investigated the dinitrogenase purified from a *nifV* mutant of *K. pneumoniae* and it was found that this strain used a modified FeMo cofactor that binds citrate instead of homocitrate to proceed the nitrogenase activity (Liang et al., 1990 and Mayer et al., 2002). In our experiment, supplement of homocitrate in the culture of nifV mutants could restore the nitrogenase activity to the level of WT. This result was similar to the report of several studies, a mutation in the *nifV* gene, was de-repressed for nitrogenase in the presence of homocitrate which restore the activity similar with wild-type properties *in vitro* (Hoover et al., 1988, Liang et al., 1990 and Madden et al., 1991).

However, *in vitro* biochemical studies indicated that the modified citrate-containing dinitrogenase poorly reduces N_2 but the reduction of acetylene and proton under Argon (Ar) proceeds at rates comparable to those for the wild-type enzyme (Hoover et al., 1988). However, homocitrate could complement the nitrogenase activity only in the culture of DOA9Δ*nifV* but not in the DOA9Ω*nifV* mutants. The *nif* gene cluster analysis in DOA9 suggested that insertion mutation Ω*nifV* mutant may disturb the downstream genes by the effect of polar mutation. These downstream *nifV* genes, including *cysE* and *nifW* which were encoded for serine acetyltransferase (a protein is involved in the synthesis of L-cysteine from L-serin) and nitrogenase-stabilizing protein, respectively. From other studies a number of accessory proteins are essential for the maturation and assembly of nitrogenase and it plays a role in the oxygen-protection of the MoFe-protein by direct physical interaction (Lee et al., 1998 and Leish et al., 1993). Thus, supplement of homocitrate could not restore the nitrogenase of DOA9Ω*nifV* mutant.

nifV gene is not present in most of rhizobium species that activity of nitrogen fixation restrict only in symbiosis. This could be explained by the study of Hakoyama et al. (2009) which showed that deletion of the *FEN1* gene encoded for homocitrate synthase in Lotus japonicas provided the ineffective nitrogen-fixing nodules when inoculated with rhizobium strain lack of *nifV*. However, inoculation with specific bacteria carrying either *FEN1* or *nifV* could restored the defect in non-fixing nodules of *FEN1* mutant legume (Hakoyama et al., 2009 and Hoover et al., 1988). Therefore, the function of *FEN1* gene is defined as the root-nodule-specific homocitrate synthase in the host plants, which compensates for the lack of *nifV* gene in most rhizobia by supplementing homocitrate to bacteroids from the plant host cells (Durrant et al., 2006, Hakoyama et al., 2009 and Nouwen et al., 2017). These were used to describe the results found in our plant experiments that most of the plant species showed similar plant phenotype after inoculation with *nifV* mutant compared to the WT. Supplement of plant homocitrate to bacteroids was found in several legume plants, such as *A. americana* and *A. afarspera* (Nouwen et al., 2017). However, some leguminous plants may not be able to provide homocitrate to bacteroids, such as *A. evenia*, *A. sensitiva*, *A. deamii*, and *A. tambacoudensis* during symbiosis with *Bradyrhizobium* sp. ORS285 *nifV* mutant (Nouwen et al., 2017). This result might be used to explain the Fix- phenotype in *S. hamata* and *I. tinctoria* when inoculated with DOA9Δ*nifV*mutant when compared with WT strain. It was possible that *S. hamata* could not provide enough homocitrate to DOA9Δ*nifV* bacteroids resulted in death bacteroids were found earlier than WT nodule.

In the case of *I. tinctoria*, less amount of death bacteroid cells was observed in the nodule elicited by DOA9Δ*nifV* when compared with that of *S. hamata*. It might be due to the difference in plant genotype. It might be possible that *I. tinctoria* produced some amount of homocitrate to bacteroid and lead to slower the nitrogen fixation in bacteroid. Then, the sanction of Fix- bacteroid in nodule could be occurred. In our

experiment, *Crotalaria juncea* and *Vigna radiata* SUT4 showed Fix- phenotype when inoculated with both DOA9WT and DOA9Δ*nifV*, this result suggests the non-compatibility between legumes and rhizobia has more influence on nitrogen fixation efficiency than the presence of *nifV* gene. However, this interaction of non-compatible between DOA9 and some legume plants could be rescued when removed the virulent factor of T3SS. Mutation of *rhcN* gene (a protein involved in the construction of injectisome apparatus) in DOA9 could increase the nitrogenase activity and plant growth when compared to the WT (Songwattana et al., 2017).

Therefore, homocitrate produced from homocitrate synthase in DOA9 was required for efficiency of nitrogen fixation under free-living condition. However, requirement of *nifV* under symbiosis condition might be depended on the host legume species which could or could not produce plant homocitrate. To elucidate the role of *nifV* under symbiosis condition, homocitrate might be used to compensate in planta experiment with *S. hamata* and *I. tinctoria*.

5.5 Reference

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CHAPTER VI

CONCLUSION

From this study, *Bradyrhizobium* sp. DOA9 which contains two replicons of one chromosome and one mega-plasmid was used as a model to investigate the function of some repetitive *nif* genes in correlation with the nitrogen fixation ability under both symbiotic and free-living conditions. The function of structural *nifDK* and the regulatory *nifA* genes which located on both chromosome and mega-plasmid, as well as an accessory *nifV* gene were investigated. The knowledge obtained from this study was summarized as following.

1. The efficiency of nitrogen fixing activity in *Bradyrhizobium* sp. DOA9 under both symbiotic and free-living conditions is depended on several factors.

1.1 Environmental condition: The presence of oxygen and nitrogen mainly affects the nitrogenase activity of DOA9. Low level of oxygen and nitrogen content is required for nitrogen fixation of DOA9 under free-living condition.

1.2 Genetic information: The particular presence of nitrogen fixing genes in bacterial genome is important to predict the ability of nitrogen fixation under various conditions. Therefore, the active nitrogenase found in nitrogen fixing bacteria is involved to presence of the functional protein encoded from regulatory *nifA-rpoN*, structural *nifHDK*, and accessory *nifENXWV* in their genome.

1.3 Biological factor: The compatibility between the host plant and bradyrhizobium does not only affect the nodulation, but also the nitrogen fixation efficiency of bacteroids.

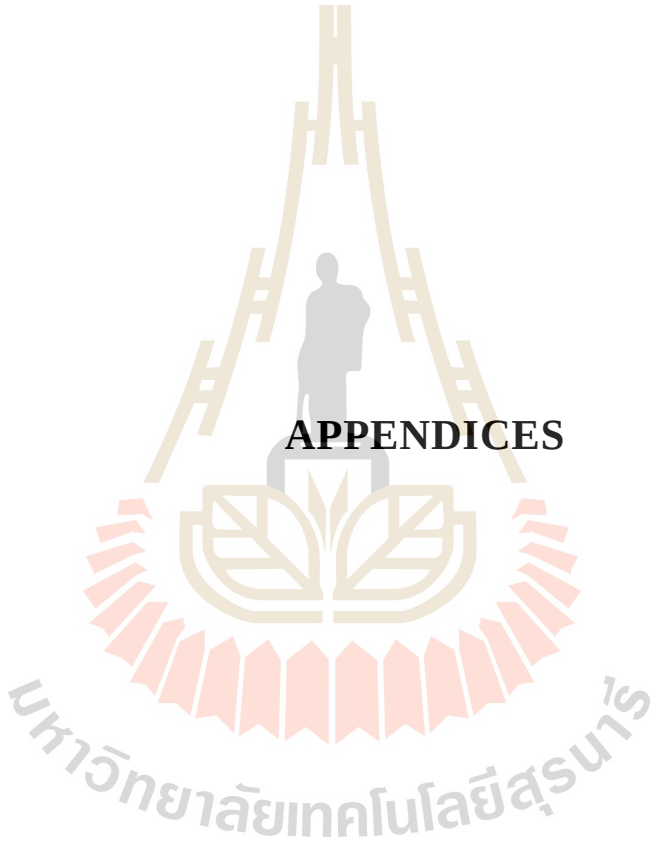
2. The reiteration of nitrogen fixing genes that found in *Bryadyrhizobium* sp. DOA9 strain respond differently for the functional of nitrogenase activity under symbiotic and free-living conditions. Both structural *nifDKc* and *nifDKp* genes are required for symbiosis nitrogen fixation, while only *nifDKc* genes are necessary for free-living nitrogen fixation. On the other hand, *nifAc* and *nifAp* are redundant for symbiotic nitrogen fixation, but only *nifAc* is required for nitrogenase activity under free-living condition.

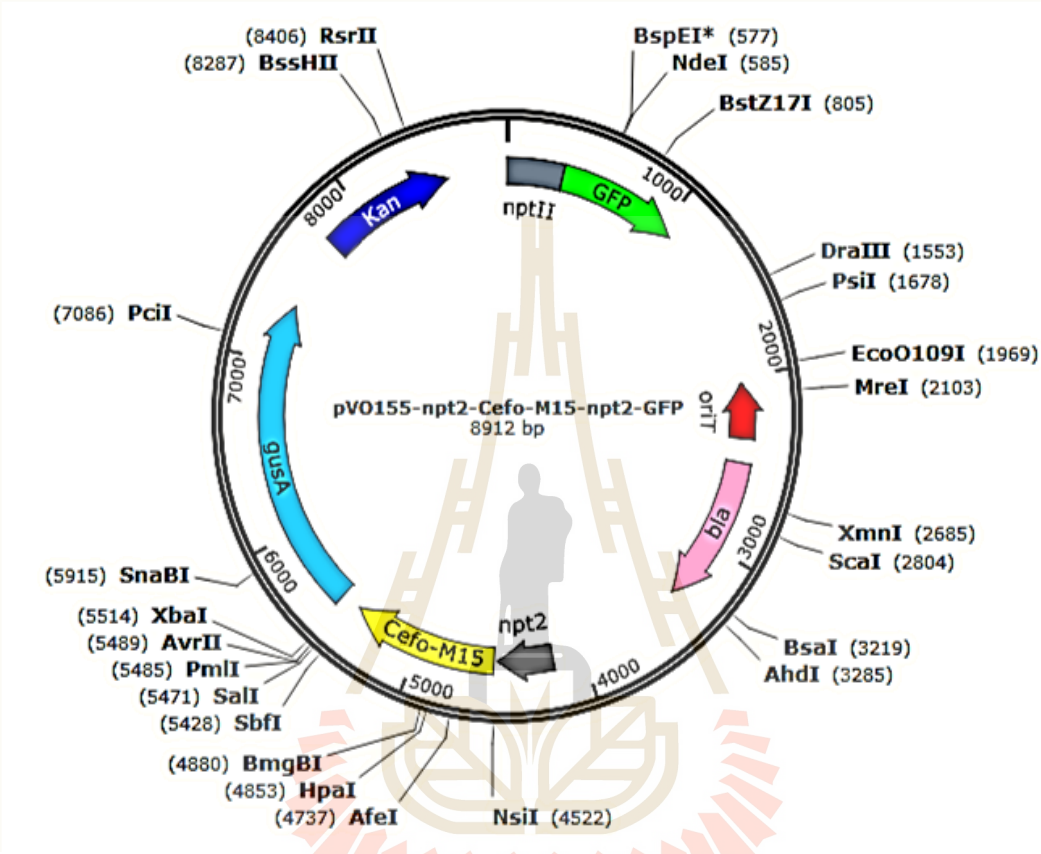
3. The cross regulation of nitrogen fixing genes could be occurred between both chromosome and plasmid by the two regulatory NifA proteins of DOA9. This is emphasized as the first report among bradyrhizobial strains.

4. The presence of *nifV* gene is required for free-living nitrogen fixation of strain DOA9, while *nifV* of strain DOA9 is necessary when symbiosis with some host plants.

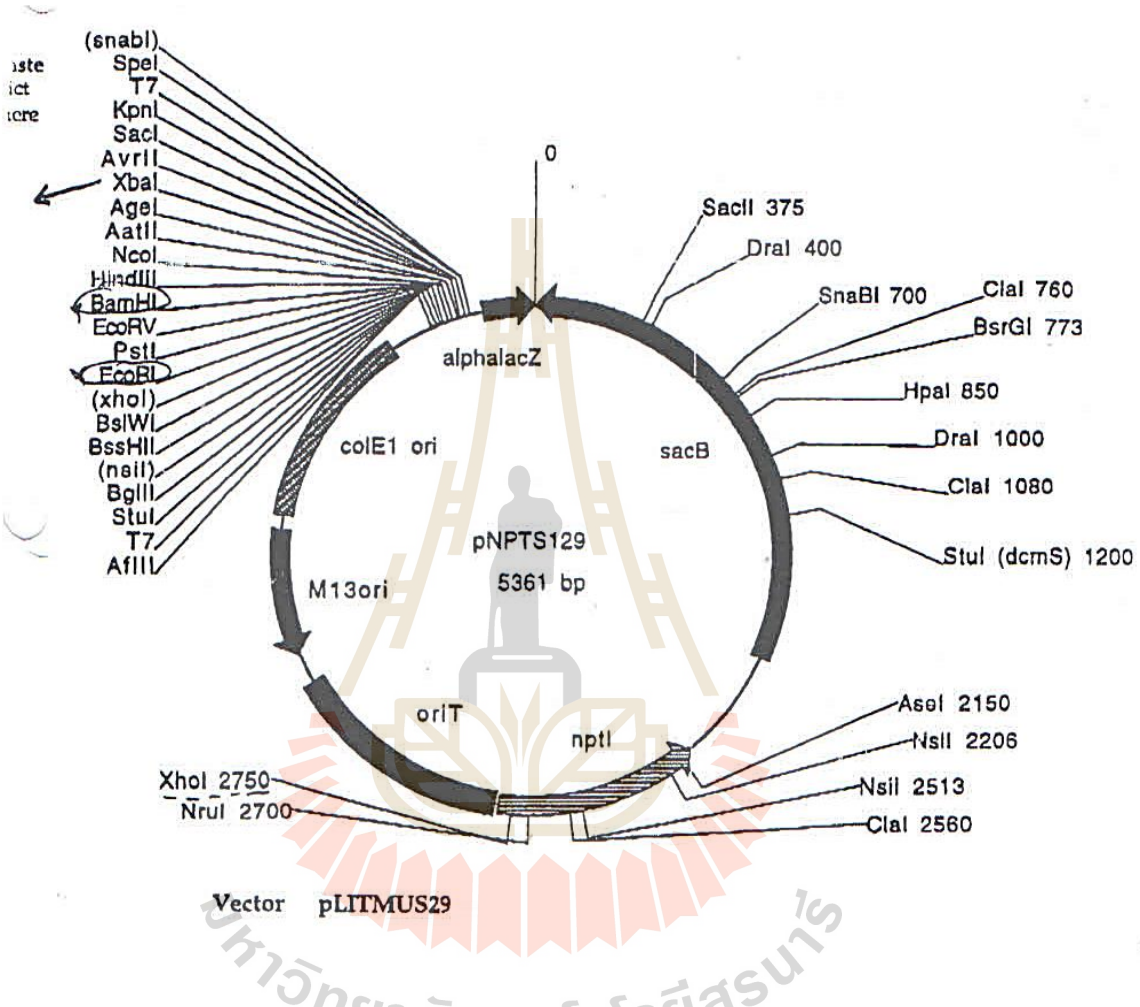
However, further research should be carried out to determine the function of other related genes involving in regulation of nitrogen fixing circuit in DOA9 under different conditions in order to apply the strain DOA9 as a broad host range biofertilizer.

APPENDICES

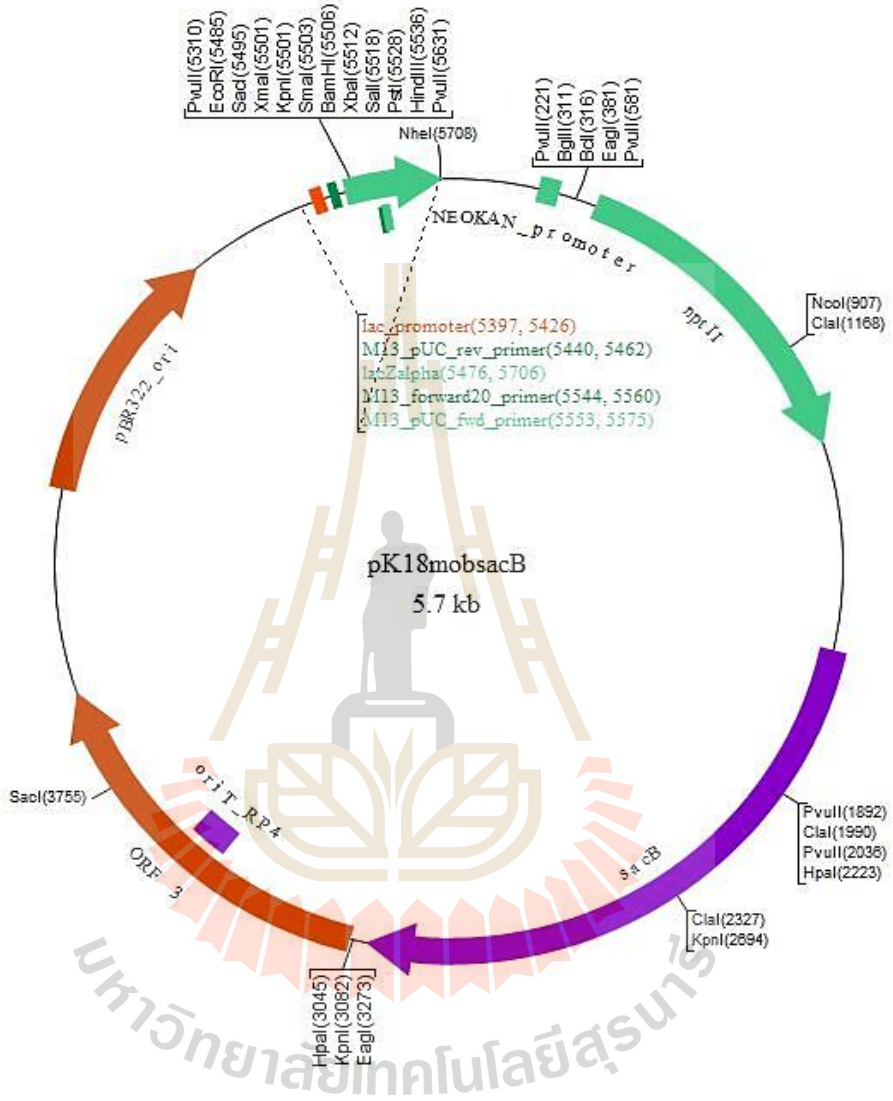




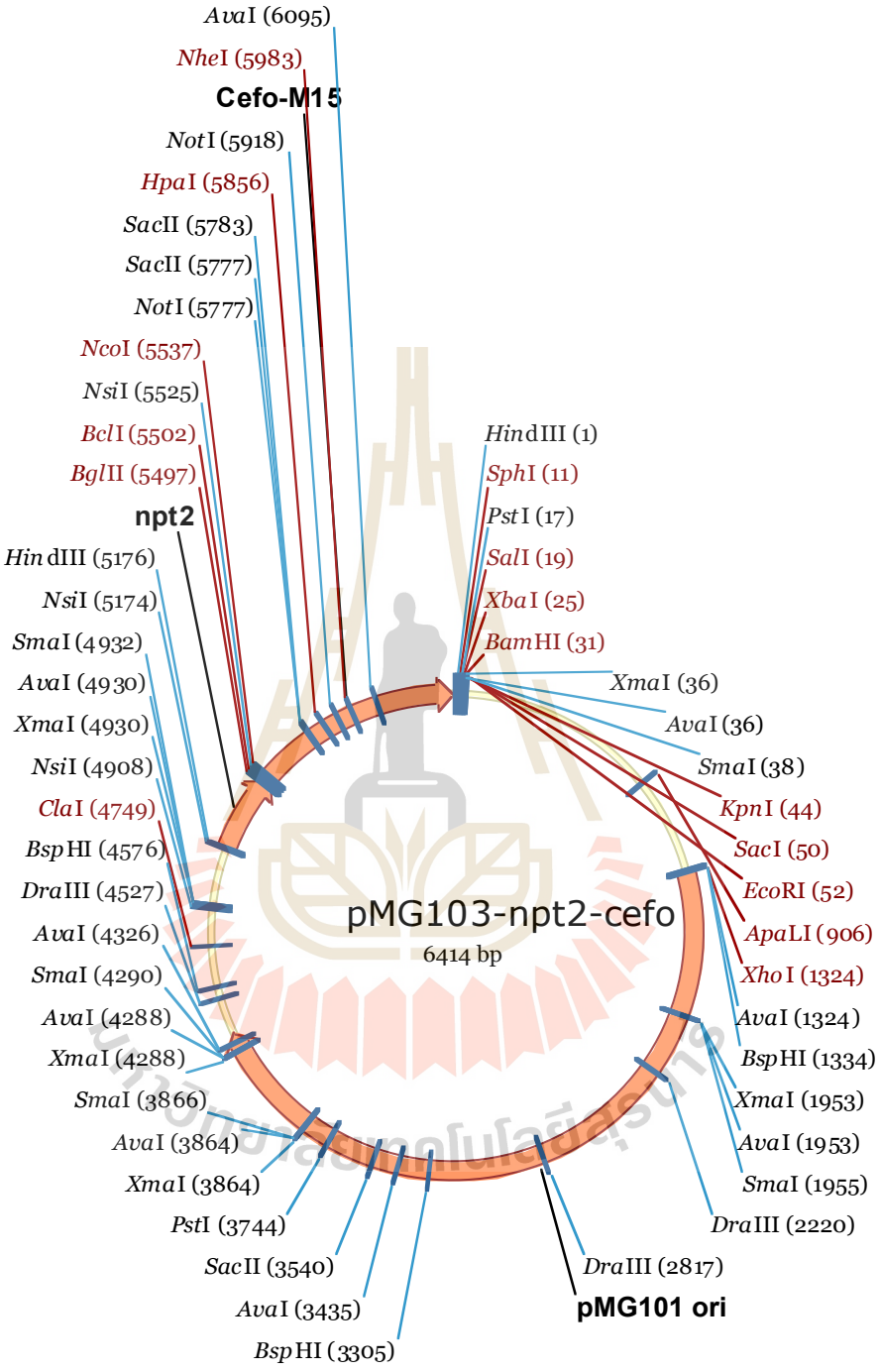
Appendix 1 Plasmid map of pVO155:nptII:Cefo:nptII:GFP



Appendix 2 Plasmid map of pNTPS129-npt2-sacB



Appendix 3 Plasmid map of pK18mab-Cefo-sacB



Appendix 4 Plasmid map of pMG103-npt2-Cefo

Appendix 5. Publications and Conferences

Wongdee, J., Songwattana, P., Noisangiam, R., Nouwen, N., Fardoux, J., Chaintreuil, C., Boonkerd, N., Teaumroong, N., Giraud, E. and Tittabutr, P. Investigation the function of some nitrogenase genes located on chromosome and mega-plasmid of *Bradyrhizobium* sp. DOA9. 12th European Nitrogen Fixation Conference organized in Budapest, Hungary, 25-28 August 2016.

Wongdee, J., Songwattana P., Nouwen N., Noisangiam R., Fardoux J., Chaintreuil C., Teaumroong N., Tittabutr P., and Giraud E. (2016). *nifDK* clusters located on the chromosome and megaplasmid of *Bradyrhizobium* sp. strain DOA9 contribute differently to nitrogenase activity during symbiosis and free-living growth. *Molecular Plant-Microbe Interactions*. 29(10): 767–773.

nifV in DOA9. The 4th Asian Conference on PlantMicrobe Symbiosis and Nitrogen Fixation (APMNF 2016) PENANG, 17 October 2016.

Wongdee, J., Teaumroong, N., Giraud, E. and Tittabutr, P. *Bradyrhizobium* sp. strain DOA9 displays the two NifA regulatory proteins that functional redundancy in symbiosis with *Aeschynomene americana* but not in free-living life. The conference of 20th International Congress on Nitrogen Fixation Granada, Spain, 3-7 September 2017.

BIOGRAPHY

Miss Jenjira Wongdee was born on July 24, 1986 in Nakhonphanom, Thailand. She studied primary school at Ban Nakhae School and in high school at Ban Phang Pittayakhom School. She graduated with the Bachelor's degree of Science of Biology from Mahasarakam University in 200.Then, she obtained the Master's degree of Science in Biotechnology, from Suranaree University of Technology (SUT) in 2008. In 2014, she enrolled at the same school and received a scholarship from the L'Institut de recherche pour le développement (IRD) by enroll to the program of "Allocations de recherche pour une thèse au Sud" (ARTS) in France for conducting her doctoral thesis research. This scholarship gave her opportunity to work with the collaborate team at the Laboratoire des Symbioses Tropicales et Méditerranéennes (LSTM) in France for 6 months in every academic years (starting from the end of 2014 to end of 2016). She had published a part of her works, in the topic of "*nifDK* gene clusters located on the chromosome and megaplasmid of *Bradyrhizobium* sp. strain DOA9 contribute differently to nitrogenase activity during symbiosis and free-living growth"on Molecular Plant-Microbe Interactions in 2016 and performed poster presentation in the topic "Investigation the function of some nitrogenase genes located on chromosome and megaplasmid of *Bradyrhizobium* sp. DOA9" on 12th European Nitrogen Fixation Conference organized in Budapest, Hungary, 25-28 August 2016. Moreover, she had presented the poster in the topic of "*Bradyrhizobiu*m sp. strain DOA9 displays the two NifA regulatory proteins that functional redundancy in symbiosis with *Aeschynomene americana* but not in free-living life" in the conference of 20th International Congress on Nitrogen Fixation at Granada, Spain, 3-7 September 2017.