กลไกการอยู่อาศัยแบบพึ่งพาอาศัยซึ่งกันและกันของแบรดีไรโซเบียม ในข้าว และศักยภาพในการประยุกต์ใช้ในระดับแปลงปลูก

นายพงษ์เดช ภิรมย์อยู่

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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SYMBIOTIC MECHANISMS OF CULTIVATED RICE ENDOPHYTIC BRADYRHIZOBIA AND THEIR POTENTIAL FOR FIELD APPLICATION

Pongdet Piromyou

A Thesis Submitted in Partial Fulfillment of the Requirements for the

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SYMBIOTIC MECHANISMS OF CULTIVATED RICE ENDOPHYTIC BRADYRHIZOBIA AND THEIR POTENTIAL FOR FIELD APPLICATION

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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พงษ์เดช ภิรมย์อยู่ : กลไกการอยู่อาศัยแบบพึ่งพาอาศัยซึ่งกันและกันของแบรดีไรโซเบียมในข้าวและศักยภาพในการประยุกต์ใช้ในระดับแปลงปลูก (SYMBIOTIC MECHANISMS OF CULTIVATED RICE ENDOPHYTIC BRADYRHIZOBIA AND THEIR POTENTIAL FOR FIELD APPLICATION) อาจารย์ที่ปรึกษา : ศาสตราจารย์ ดร. หนึ่ง เตียอำรุง 109 หน้า.

การเข้าอยู่อาศัยของแบคทีเรียในสกุลแบรดีไรโซเบียม ไม่เพียงพบในพืชตระกูลถั่ว แต่ยังพบในพืชชนิดอื่น เช่น ข้าว เพื่อที่จะเข้าใจวิวัฒนาการของการอยู่อาศัยแบบพึ่งพาอาศัยซึ่งกันและกันของแบคทีเรียในสกุลแบรดีไรโซเบียมในระบบนิเวศการปลูกข้าวมีผลต่อปฏิสัมพันธ์ระหว่างจุลินทรีย์กับพืชจึงเป็นปัจจัยสำคัญปัจจัยหนึ่งที่ต้องพิจารณาทำการเก็บตัวอย่างข้าว (*Oryza sativa*) จากเนื้อเยื่อส่วนต่างๆ ในระบบการปลูกข้าวแบบหมุนเวียน เพื่อแยกเชื้อแบคทีเรียในสกุลแบรดีไรโซเบียม เชื้อที่แยกมาได้สามารถแบ่งออกเป็น กลุ่มแบรดีไรโซเบียมที่สังเคราะห์ด้วยแสง และแบรดีไรโซเบียมที่ไม่สังเคราะห์ด้วยแสง นอกจากนี้ยังพบว่าแบรดีไรโซเบียมสายพันธุ์ที่แยกได้จากในประเทศไทยสามารถส่งเสริมการเจริญของข้าวสายพันธุ์ไทยได้ดีกว่าข้าวสายพันธุ์ญี่ปุ่น ทำให้สันนิษฐานได้ว่าสายพันธุ์ข้าวเป็นปัจจัยที่ส่งผลต่อปฏิสัมพันธ์ของข้าวและจุลินทรีย์ เป็นที่น่าสนใจว่าในทุกสายพันธุ์ของแบรดีไรโซเบียมมีความคล้ายคลึงของลำดับเบสยีน *nodABC* ในระดับต่ำเมื่อเทียบกับสายพันธุ์ *Bradyrhizobium diazoefficiens* USDA110 และ *Bradyrhizobium* sp. ORS285 จากผลการทดลองนี้จึงทำการตรวจสอบวิวัฒนาการของการอยู่ร่วมกันของพืชและแบรดีไรโซเบียม รวมไปถึงการเข้าอาศัยกับพืชที่ไม่ใช่พืชตระกูลถั่ว โดยให้ความสำคัญกับประเด็นการมีสมบัติสังเคราะห์ด้วยแสง และการเข้าอาศัยอยู่กับพืชโดยไม่ใช้กลุ่มยีน *nod* ต่อไป

กลไกที่เกี่ยวข้องกับเมทาบอลิซึม การปรับตัว และคุณสมบัติอื่นๆที่เป็นประโยชน์ของแบคทีเรียสกุลไรโซเบียมในพืชตระกูลถั่วเป็นที่เข้าใจค่อนข้างมาก แต่กลไกของแบรดีไรโซเบียมที่อาศัยในต้นข้าวนั้นยังไม่เป็นที่เข้าใจ ดังนั้นจึงได้ศึกษาถึงกลไกการเข้าอาศัยและระบบของยีนของแบรดีไรโซเบียมในเนื้อเยื่อข้าวโดยเลือกเชื้อสายพันธุ์SUTN9-2 เพื่อศึกษาการเข้าสู่เนื้อเยื่อข้าว จากการศึกษา การแสดงออกของยีนในแบรดีไรโซเบียมสายพันธุ์ SUTN9-2 ระหว่างที่มีปฏิสัมพันธ์กับข้าว โดยวิธี RT-PCR พบว่า การแสดงออกของยีน *rhcJ, virD4* และ *peces* แสดงออกเมื่อมีการกระตุ้นด้วยสารคัดหลั่งจากรากข้าว เมื่อทดสอบระบบ T3SS ซึ่งเกี่ยวข้องกับการเข้าบุกรุกเนื้อเยื่อข้าวของเชื้อแบรดีไรโซเบียม จากการปลูกเชื้อ SUTN9-2 สายพันธุ์เดิม และสายพันธุ์ที่ถูกทำลายระบบ T3SS ได้แก่สายพันธุ์Δ*rhcJ*-3B, Δ*rhcJ*-18A และ Δ*rhcJ*-27A ของ SUTN9-2 สายพันธุ์เดิมในพืชอาศัยดั้งเดิม(โสนขน: *Aeschynomene americana*) และข้าวสายพันธุ์ปทุมธานี 1 (*O. sativa*

cultivar Pathum Thani 1) พบว่า สายพันธุ์ที่ถูกทำลายระบบ T3SS มีความสามารถในการเข้าบุกรุกสู่เนื้อเยื่อข้าวลดลง เมื่อเปรียบเทียบกับเชื้อสายพันธุ์ดิม แต่ไม่ส่งผลต่อการเข้าปมของพืชตระกูลถั่ว *A. americana* จากผลดังกล่าวแสดงให้เห็นว่า T3SS เป็นปัจจัยหนึ่งในการควบคุมการอยู่อาศัยแบบพึ่งพาอาศัยกันและกันของแบรดีไรโซเบียมกับข้าว

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2557 ลายมือชื่ออาจารย์ที่ปรึกษา_____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

PONGDET PIROMYOU : SYMBIOTIC MECHANISMS OF CULTIVATED RICE ENDOPHYTIC BRADYRHIZOBIA AND THEIR POTENTIAL FOR FIELD APPLICATION. THESIS ADVISOR : PROF. NEUNG TEAUMROONG, Dr.rer.nat., 109 PP.

RICE/ENDOPHYTIC *Bradyrhizobium*/RICE GROWTH/PROMOTION/ ENDOPHYTIC MECHANISM

Bradyrhizobia colonization in plant is found not only in leguminous plants but also in non-leguminous species such as rice. To understand the evolution of endophytic symbiosis of bradyrhizobia, its association with ecosystems of rice plantations was investigated. Samples were collected from various rice (*Oryza sativa*) tissues in crop rotation systems to obtain bradyrhizobial strains. The isolates were separated into photosynthetic bradyrhizobia (PB) and non-photosynthetic bradyrhizobia (Non-PB). Thai bradyrhizobial strains promoted rice growth of Thai rice cultivars better than the Japanese strains. This implies that the rice cultivars have the factors governing rice-bacteria associations. Interestingly, all of these bradyrhizobial strains seemed to exhibit low similarity of *nodABC* genes to those of *Bradyrhizobium diazoefficiens* USDA110 and *Bradyrhizobium* sp. ORS285. From these results, the evolution of plant-bradyrhizobia association including non-legume in terms of photosynthetic lifestyle and *nod*-independent interactions were further investigated.

The metabolic processes, adaptations and beneficial characteristics of rhizobia with leguminous plants are clearly understood whilst the mechanisms of endophytic

bradyrhizobia in rice have not been elucidated so far. Therefore, the determination of the infection process and genetic system of bradyrhizobia in rice tissues was further emphasized. The SUTN9-2 was selected for further determination of rice-*Bradyrhizobium* interactions. The expression of SUTN9-2 genes was examined during the association with rice plants by RT-PCR. The *rhcJ*, *virD4* and *peces* genes of the bacterium were only up-regulated when rice roots exudate was added in to the culture. To examine whether T3SS is involved in bradyrhizobial infection to rice plants, wild-type SUTN9-2 and three T3SS mutant strains (Δ*rhcJ*-3B, Δ*rhcJ*-18A and Δ*rhcJ*-27A of SUTN9-2) were inoculated to the original host plant (*Aeschynomene americana*) and rice plant (*O. sativa* cultivar Pathum Thani 1). The T3SS mutants reduced their ability to invade rice tissues as compared with the wild-type strain, although the phenotypes in *A. americana* was not changed by T3SS mutations. These results suggest that T3SS-dependent effectors are a factor that controls the compatibility of rice-bradyrhizobial association.

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LIST OF ABBREVIATIONS

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

LIST OF ABBREVIATIONS (Continued)

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

CHAPTER I

INTRODUCTION

1.1 Significances of this study

The plant infection and colonization by bradyrhizobia are not only found in leguminous plants but also in non-leguminous species such as rice (Chaintreuil et al., 2000; Okubo et al., 2013). However, the symbiosis mechanisms between *Bradyrhizobium* and rice have been absolutely unclear, although the evolution of bradyrhizobium-rice association may have occurred earlier. In addition, rice is the most important food crop in Asia. The high-yielding rice production requires huge amounts of nitrogen fertilizers. The biological nitrogen fixation (BNF) from rice root-associated bacteria has a great potential to improve the sustainable rice production. Moreover, the rice-legumes rotational cropping systems are useful for rice production since legumes can be planted after rice and nitrogen can be provided from legumes to rice. Bradyrhizobia are well recognized for their ability to fix atmospheric dinitrogen into ammonia in the nodules of legumes, thus providing ammonia to host plants. The bradyrhizobia-legumes symbiosis has been reported to increase the legumes productivity for agricultural farms (Abbasi et al., 2008; Antoun et al., 1998; Stephens and Rask, 2000). However, *Bradyrhizobium* has been well defined as the oligotrophic bacteria, which can survive under the nutrients deprived conditions (Crist et al., 1984; Saito et al., 1998). Besides, a renewed interest in endophytic diazotrophs in graminous plants has also arisen because of their occurrence mainly within plant tissues. The

endophytic diazotrophs actually perform plant growth promotion (Elbeltagy et al., 2001). However, the relationships of endophytic *Bradyrhizobium* with rice cultivar and/or habitats have never been elucidated so far. Thus, the host preference for endophytic bradyrhizobia and rice growth promotion are the critical aspects to be determined before they are used in the fields.

Aeschynomene americana is the leguminous weed grown in rice filed only a few months a year (from July to December) and the *A. americana* can also grow well in next following year as same period of time. Furthermore, *Aeschynomene* establishes a symbiotic relationship with bacteria belonging to the genus *Bradyrhizobim*, and the nitrogen-fixing nodules are formed on root and/or stems (Noisangiam et al., 2012). However, the potential of applying endophytic bacteria is still underexplored (Hurek et al., 2002; Mano and Morisaki, 2008). The previous reports showed that the photosynthetic *Bradyrhizobium* strains form nodules on stems/root of aquatic legumes of the genus *Aeschynomene*, and it can establish the endophytic association with the wild rice species *Oryza breviligulata* (Chaintreuil et al., 2000). Moreover, the bradyrhizobia were isolated from root/stem nodules of *A. americana* (strain DOA9) can establish the symbiosis with rice (Teamtisong et al., 2014). Despite a widespread occurrence of this natural endophytic bradyrhizibium-rice association, much remains unknown about its infection and colonization processes. In addition, the metabolic processes, adaptations, and beneficial characteristics of rhizobia with leguminous plants are clearly understood, but the mechanisms of endophytic bradyrhizobia in rice have not been elucidated so far. Therefore, to obtain the natural endophytic bradyrhizobia from rice, bradyrhizobial strains were isolated on the basis of the oligotrophic property. The siratro (*Macroptilium atropurpureum*) has been used as a

promiscous host plant to screen the *nod*-containing *Bradyrhzobium* from homogenated rice tissues and soil samples (Lima et al., 2009). A selective medium (BJSM) (Tong and Sadowsky, 1994) was also applied for *Bradyrhizobium* isolation. Moreover, to understand the mechanism of plant-bradyrhizobia association, rice endophytic bradyrhizobia were tested with various types of legumes, which are normally found in rice field. The appropriate rice endophytic bradyrhizobia as strain SUTN9-2 was selected. The draft genome of SUTN9-2 was already accomplished. This might facilitate the functional genes analyses regarding the early stages of rice-association. The profile of SUTN9-2 gene expression in early stage of rice (*O. sativa* L. ssp. *indica* cv. Pathum Thani 1) infection and some genes deletion was investigated. In addition, the role of those deleted genes in legume and rice association were compared and discussed.

1.2 Hypothesis

A. The agricultural pracices systems (monoculture-,commercial legumes and/or weed legumes) may be one of factors to maintain putative endophytic bradyrhizobial strains in the ecosystems.

B. The factors that control the compatibility of rice-bradyrhizobial association is governed by rice cultivars.

C. The T3SS was important for legumes-bradyrhizobia interaction. Therfore, the rice endophytic bradyrhizobia may use T3SS system for entering the rice.

1.3 Objectives

1.3.1 General objectives

The general objectives of this research were to understand the relationships of the persistence and the evolution of rice endophytic-*Bradyrizobium* strains from different agricultural systems and rice cultivars. In addition, the mechanisms of rice endophytic were partially explored to determined the rice-*Bradyrhizobium* interaction.

1.3.2 Specific objectives

1. To investigate the endophytic bradyrhizobia from rice tissues grown under different agricultural practice systems.

2. To determine the endophytic bradybacteria localization and the population in rice tissue from various cultivars.

3. To determine the genes involved in infection process of endophytic bradyrhizobia in rice tissues as well as in legumes.

CHAPTER II

LITERATURE REVIEW

2.1 Rice

Rice (*Oryza sativa*) is the most important staple food in Asia. Nearly 90% of the rice fields in the world are located in Asia, where 60% of the world's population lives (Food and Agriculture Organization of the United Nations. 2002). Asian rice feeds more than one-half of the global population and has been become a key model system for plant biology. China and India are the largest for both of producers and consumers of rice (Araújo et al., 2013). However, the world's rice exporting countries are Thailand, India and Vietnam. Rice is the monocotyledonous plant. They belong to the Gramineas (Poaceae) family of the tribe *Oryzeae* and of genus *Oryza*. The local Thai variety, *Oryza sativa* (L.) originated in Asia. Asian rice, is one of world's oldest and most important crop species, having been domesticated beginning some 8,000-9,000 year ago (Fuller et al., 2010; Higham and Lu, 1998; Liu et al., 2007). Genetic analysis has established that rice consists of several genetically differentiated varitety groups, with the two main groups known as *indica* and *japonica* (Garris et al., 2005; Molina et al., 2011).

2.2 Rice endophytic and diazotrophic endophytic bacteria

Endophytic bacteria can be defined as those bacteria that have inhabited for one period of their life cycle into the internal tissue of the plant without making any pathogenic sympthoms or negative effect on their host (Reinhold-Hurek and Hurek, 2011). Later, the definition was extended to the bacteria that colonise the interior and promote benefits to the plant (Santi et al., 2013).

Endophytic bacteria in a single plant host are not restricted to a single species but comprise several genera and species. No one knows if communities inside plants interact, and it has been speculated that beneficial effects are the combined effect of their activities. It was proposed that the bacteria best adapted for living inside plants are naturally selected. Germida et al., (Germida et al., 1998) found that the endophytic population was less diverse than the root-surface population and the endophytes appeared to originate from the latter. (Mavingui et al., 1992) found that there are different populations of *Bacillus polymyxa* in soil, rhizosphere, and rhizoplane and that wheat roots select specific populations. *Rhizobium etli* is found as a natural endophyte of maize plants in traditional agricultural fields in which maize and bean are grown in association (Gutierrez-Zamora and Martınez-Romero, 2001). *In planta* and *ex planta* populations of *Pseudomonas* species could be differentiated by biochemical characteristics (van Peer et al., 1990). Inaddition, various types of rhizobia including *Sinorhizobium meliloti*, *Sinorhizobium* sp. strain NGR234, and *Rhizobium* etli performed sweet potato endophytic properties. Other detected bacteria were *Klebsiella* spp. and *Paenibacillus odorifer* (Reiter et al., 2003).

2.2.1 Diazotrophic endophytic bacteria

The facultative endophytic diazotrophs have a stage in their life cycle in which they exist outside host plant and these are able to colonies both the surface and root interior and to survive well in soil. The group of diazotrophs, composed mostly of *Azospirillum* species, can be considered the starting point most ongoing biological nitrogen fixation programs with non-legume plants worldwild. *Azospirillum* spp. have been found associated with several cereals and forage grasses grown in temperate and tropical climates.

The obligate endophytic diazotrophs are strictly dependent on the plant for their growth and survival and transmission to other plants occurs vertically or via vectors (Hardoim et al., 2008). This group includes *Acetobacter diazotrophicus* and *Azoarcus* spp., *Herbaspirillum seropedicae* and a partially identified *Burkholderia* sp.; these are clustered in the beta subclass of the Proteobacteria (Baldani et al., 1997).

Several diazotrophic species of *Azoarcus* spp. occur as endophytes in the pioneer plant Kallar grass. The purpose of this study was to screen Asian wild rice and cultivated *Oryza sativa* varieties for natural association with these endophytes. Populations of culturable diazotrophs in surface-sterilized roots were characterized by 16S rDNA sequence analysis, and *Azoarcus* species were identified by genomic fingerprints. *A. indigens* and *Azoarcus* sp. group C were detected only rarely, whereas *Azoarcus* sp. group D occurred frequently in samples of flooded plants: in 75% of wild rice, 80% of land races of *O. sativa* from Nepal and 33% of modern cultivars from Nepal and Italy. The putatively endophytic populations of diazotrophs differed with the rice genotype. The diversity of cultured diazotrophs was significantly lower in wild rice species than in modern cultivars. In *Oryza officinalis* (from Nepal) and *O.*

minuta (from the Philippines), *Azoarcus* sp. group D were the predominant diazotrophic putative endophytes in roots. In contrast, their number was significantly lower in modern cultivars of *O. sativa*, whereas numbers and diversity of other diazotrophs, such as *Azospirillum* spp., *Klebsiella* sp., *Sphingomonas paucimobilis*, *Burkholderia* sp. and *Azorhizobium caulinodans*, were increased. In land races of *O. sativa*, the diazotrophic diversity was equally high; however, *Azoarcus* sp. was found in high apparent numbers. Similar differences in populations were also observed in a culture-independent approach comparing a wild rice (*O. officinalis*) and a modern-type *O. sativa* plant: in clone libraries of root-associated nitrogenase (*nifH*) gene fragments, the diazotrophic diversity was lower in the wild rice species. However, the rice endophytic bacteria were displayed in Table 2.1.

Table 2.1 Examples of reported bacterial endophytes and rice harboring them

Endophytes	**references**
Azorhizobium caulinodans, *Sphingomonas paucimobilis*	(Engelhard et al., 2000)
Bradyrhizobium sp. BTAi1	(Chaintreuil et al., 2000)
Rhizobium leguminosarum	(Yanni et al., 1997)
Chromobacterium violaceum, *Sphingobacterium* sp.	(Phillips et al., 2000)
Serratia sp.	(Sandhiya et al., 2005)
Serratia marcescens	(Gyaneshwar et al., 2001)

One of the most case of rice endophytic bacteria studied world wide is Herbaspirillum-rice endophytic association. *Herbaspirillum seropedicae* Z67 was firstly elucidated in term of application as biofertilizer. It was tested with rice seedling cvs. IR42 and IR72. Internal populations peaked at over 10^6 log CFU per gram of fresh weight by 5 to 7 days after inoculation (dai) but declined to 10^3 to 10^4 log CFU per

gram of fresh weight by 28 dai. GUS staining was most intense on coleoptiles, lateral roots, and at the junctions of some of the main and lateral roots. Bacteria entered the roots via cracks at the points of lateral root emergence, with cv. IR72 appearing to be more aggressively infected than cv. IR42. *H. seropedicae* subsequently colonized the root intercellular spaces, aerenchyma, and cortical cells, with a few penetrating the stele to enter the vascular tissue. Xylem vessels in leaves and stems were extensively colonized at 2 dai but, in later harvests (7 and 13 dai), a host defense reaction was often observed. Dense colonies of *H. seropedicae* with some bacteria expressing nitrogenase Fe-protein were seen within leaf and stem epidermal cells, intercellular spaces, and substomatal cavities up until 28 dai. Epiphytic bacteria were also seen. Both varieties showed nitrogenase activity but only with added C, and the dry weights of the inoculated plants were significantly increased. Only cv. IR42 showed a significant (approximately 30%) increase in N content above that of the uninoculated controls, and it also incorporated a significant amount of $^{15}N_2$ (James et al., 2002).

For the bradyrhizobia-rices association, the original study was focused on the photosynthetic bradyrhizobia nodulating the legumes in genus *Aeschenomone*. These bradyrhizobia also presented within the roots of the wetland wild rice *Oryza breviligulata*, which is the ancestor of the African cultivated rice *Oryza glaberrima*. This primitive rice species grows in the same wetland sites as *Aeschynomene sensitiva*, an aquatic stem-nodulated legume associated with photosynthetic strains of *Bradyrhizobium*. Twenty endophytic and aquatic isolates were obtained at three different sites in West Africa (Senegal and Guinea) from nodal roots of *O. breviligulata* and surrounding water by using *A. sensitiva* as a trap legume. Most endophytic and aquatic isolates were photosynthetic and belonged to the same

phylogenetic *Bradyrhizobium/Blastobacter* subgroup as the typical photosynthetic *Bradyrhizobium* strains previously isolated from *Aeschynomene* stem nodules. Nitrogen-fixing activity, measured by acetylene reduction, was detected in rice plants inoculated with endophytic isolates. A 20% increase in the shoot growth and grain yield of *O. breviligulata* grown in a greenhouse was also observed upon inoculation with one endophytic strain and one *Aeschynomene* photosynthetic strain. The photosynthetic *Bradyrhizobium* sp. strain ORS278 extensively colonized the root surface, followed by intercellular, and rarely intracellular, bacterial invasion of the rice roots, which was determined with a *lacZ*-tagged mutant of ORS278. The discovery that photosynthetic *Bradyrhizobium* strains, which are usually known to induce nitrogen-fixing nodules on stems of the legume *Aeschynomene*, are also natural true endophytes of the primitive rice *O. breviligulata*could significantly enhance cultivated rice production(Chaintreuil et al., 2000). These testimonies were concluded that the *Bradyrhizobium* also performed rice endophytic properties.

2.3 *Bradyrhizobium*

Bradyrhizobium species are Gram-negative bacilli (rod shaped) with a single subpolar or polar flagellum. They are a common soil dwelling bacteria that can form symbiotic relationships with leguminous plant species where they fix nitrogen in exchange for carbohydrates from the plant. Like other rhizobia, they capable of fixing atmospheric nitrogen into forms readily available for other organisms to use. They are known as slow grower in contrast to *Rhizobium* species, which are recognized as fast grower. In a liquid media, bradyrhizobia take 3–5 days to develop a moderate turbidity

and 6–8 hours to double in population size. The most preferable carbon source for growth is pentoses (Somasegaran and Hoben, 1994a).

Bradyrhizobium can form either specific or general symbioses (Somasegaran and Hoben, 1994a). This means that one species of *Bradyrhizobium* may only be able to nodulate one legume species, whereas other *Bradyrhizobium* species may able to nodulate several legume species. The sequences ribosomal RNA are highly conserved among the group, making *Bradyrhizobium* extremely difficult to use as an indicator of species diversity. DNA-DNA hybridizations have been used instead and show more diversity (Rivas et al., 2009). For example, *Bradyrhizobium* species as *B. betae* was isolated from tumor like root deformations on sugar beets, *B. elkanii, B. liaonigense* establish symbiosis with soybeans, *B. japonicum* nodulates soybeans, cowpeas, mung beans, and siratro, *B. yuanmingense* nodulates *Lespedeza*, *B. canariense* nodulates Genistoid legumes, Lupin and Serradella nodule. Recently, strains belonging to *B. japonicum* have been split into two species, *B. japonicum* and *B. diazoefficiens*. The strain USDA110 now belongs to *B. diazoefficiens*, and the type strain for this new species is USDA 110 (Delamuta et al., 2013). In culture-dependent studies, it seems that fast growing γ-Proteobacteria out-grow slower-growing α-Proteobacteria such as rhizobia. It is interesting that, in this case and perhaps in relation to the methodology used, a dominance of rhizobia was observed, accounting for around 50% of the sequences obtained. From previous report display that the photosynthetic-*bradyrhizobia* were isolated from African wild rice and they can promote rice growth (Chaintreuil et al., 2000). Whereas non-photosynthetic bradyrhizobia have never been found inside rice tissues therefore, non-photosynthetic bradyrhizobia in Thailand still unexplored.

2.4 Molecular basis of rhizobium-legume symbiosis

2.4.1 Rhizobial colonization

The rhizobia-legume symbiosis is the most wildely known and popular as the model of plant-microbe interaction. The establishment of symbiosis between legumes and rhizobia involves the activation of genes in both the host and the symbiont and an elaborated exchange of signals (Geurts and Bisseling, 2002). The formation of a root nodule, the specialized organ from a plant host that contains the symbiotic nitrogen-fixing rhizobia, involves two simultaneous processes : infection and nodule organogenesis. The initiation of a root nodule is shown in Figure 2.1.

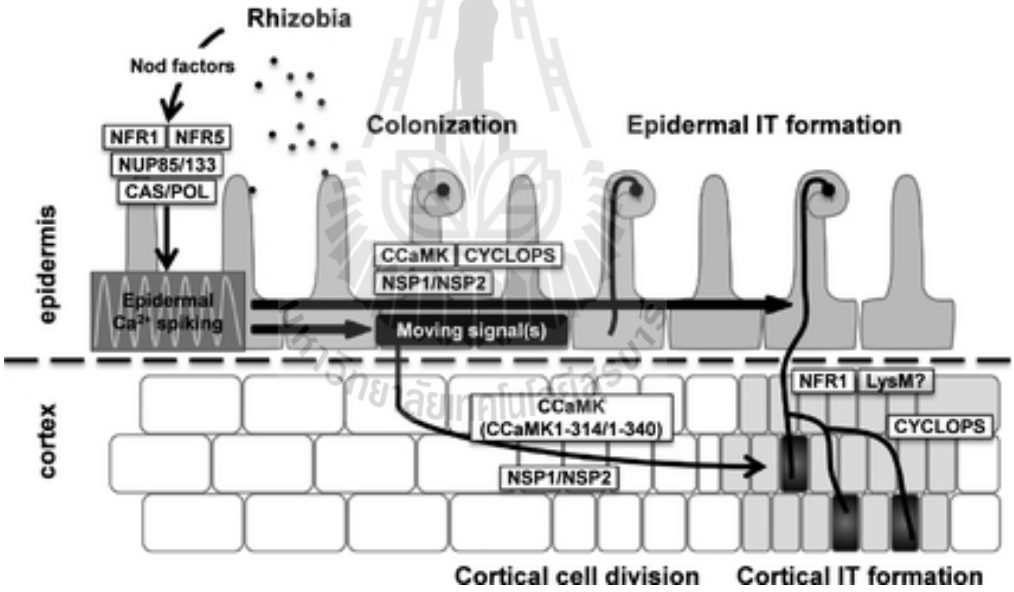

Figure 2.1 Rhizobial colonization (Adapted from (Hayashi et al., 2014)

Rhizobial colonization starts with the releasing of flavonoids by the plant root signal to rhizobia in the rhizosphere, which in turn produce nodulation factors (Nod factors) that are recognized by the plant. Nod factor perception activates the symbiosis signaling pathway, leading to calcium oscillations, initially in epidermal cells but later also in cortical cells preceding their colonization. Rhizobia gain entry into the plant

root by root hair cells that grow around the bacteria attached at the root surface, trapping the bacteria inside a root hair curl.

Infection threads are invasive invaginations of the plant cell that are initiated at the site of root hair curls and allow invasion of the rhizobia into the root tissue. The nucleus relocates to the site of infection, and an alignment of endoplasmic recticulum (ER) and cytoskeleton, known as the pre-infection thread, predicts the path of the infection thread. Nodules initiate below the site of bacterial infection and form by de novo initiation of a nodule meristem in the root cortex. The infection threads grow towards the emergent nodules and ramify within the nodule tissue. In some cases, the rhizobia remain inside the infection threads, but more often, the bacteria are released into membrane-bound compartments inside the cells of the nodule, where the bacteria can differentiate into a nitrogen-fixing state.

2.4.2 Symbiotic signaling

Arbuscular mycorrhiza (AM) fungi produce lipo-chitooligosaccharides (LCOs) structurally closely related to rhizobial Nod factors that induce lateral root formation in plants. It is thus conceivable that production of LCOs by rhizobia was a key step during the evolution of the root nodule symbiosis. This infection is preceded by signal transduction through a so-called ‘common symbiosis pathway’ (Kistner and Parniske, 2002) shared by Root Nodule Symbiosis (RNS) and AM (Figure 2.2).

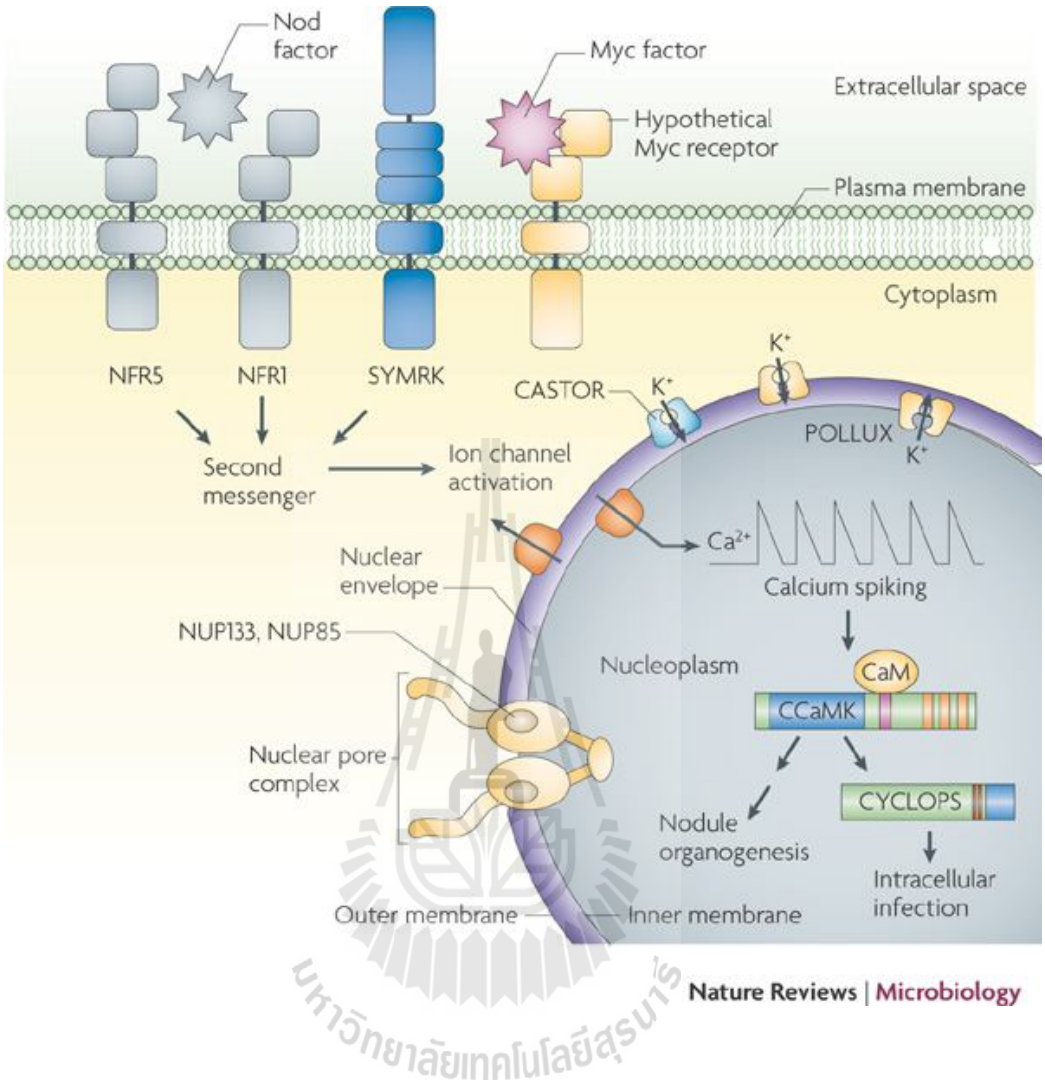

Figure 2.2 Symbiotic signal transduction in plant root cells (Parniske, 2008)

Symbiotic signal transduction starts with the percepting of rhizobial Nod factors (NFs) at the plasma membrane (PM) is mediated by LysM-receptor-like kinases (LYKs) including *L. japonicus* Nod factor receptor 1 (NFR1) and NFR5 and in *Medicago truncatula* LYK3 and NFP (Antolín-Llovera et al., 2012; Haney and Long, 2010; Madsen et al., 2003). An NFR5-like receptor may mediate perception of an AM fungus-derived 'Myc factor' (MF) (Parniske, 2008). PUB1 : plant U-box

protein 1 of *M. truncatula*, is an E3 ubiquitin ligase interacting with the kinase domain of LYK3, and exerts a negative regulatory role on nodulation signaling (Mbengue et al., 2010). SINA : the SEVEN IN ABSENTIA homolog SINA4 interacts with the kinase domain of SYMRK and mediates its relocalization and degradation (Den Herder et al., 2012). The symbiotic receptors at the PM interact with SYMREM1, a remorin protein specifically upregulated during nodulation and required for infection thread (IT) formation (Lefebvre et al., 2010; Tóth et al., 2012). Within minutes, LCO perception at the PM leads to a sustained nuclear Ca^{2+}-spiking response, the generation, decoding and transduction of which is mediated by components common to both types of symbioses (Oldroyd, 2013). These are genetically positioned upstream (SYMRK/DMI2, CASTOR/POLLUX/DMI1, NUP85, NUP133, NENA) or downstream (CCaMK/DMI3, CYCLOPS/IPD3) of the Ca^{2+}-spiking response. Several transcription factors including NSP1/2, NIN and others have been implicated in symbiosis-related gene expression (Kouchi et al., 2010). The observation that autoactive CCaMK does not restore epidermal IT formation in *nfr* mutants suggests the existence of a common *SYM* gene-independent pathway (Hayashi et al., 2010).

2.5 Molecular basis of endophytic interaction

Establishment of endophytes in the host was demonstrated in Figure 2.3. The plant-polymer-degrading enzymes (pectin esterase and cellulase) have considered as another putatively important feature (Sessitsch et al., 2012). These enzyme were normally presented in the bacterial genome and may contribute to endophyte entry and spreading inside rice roots tissues (Sessitsch et al., 2012).

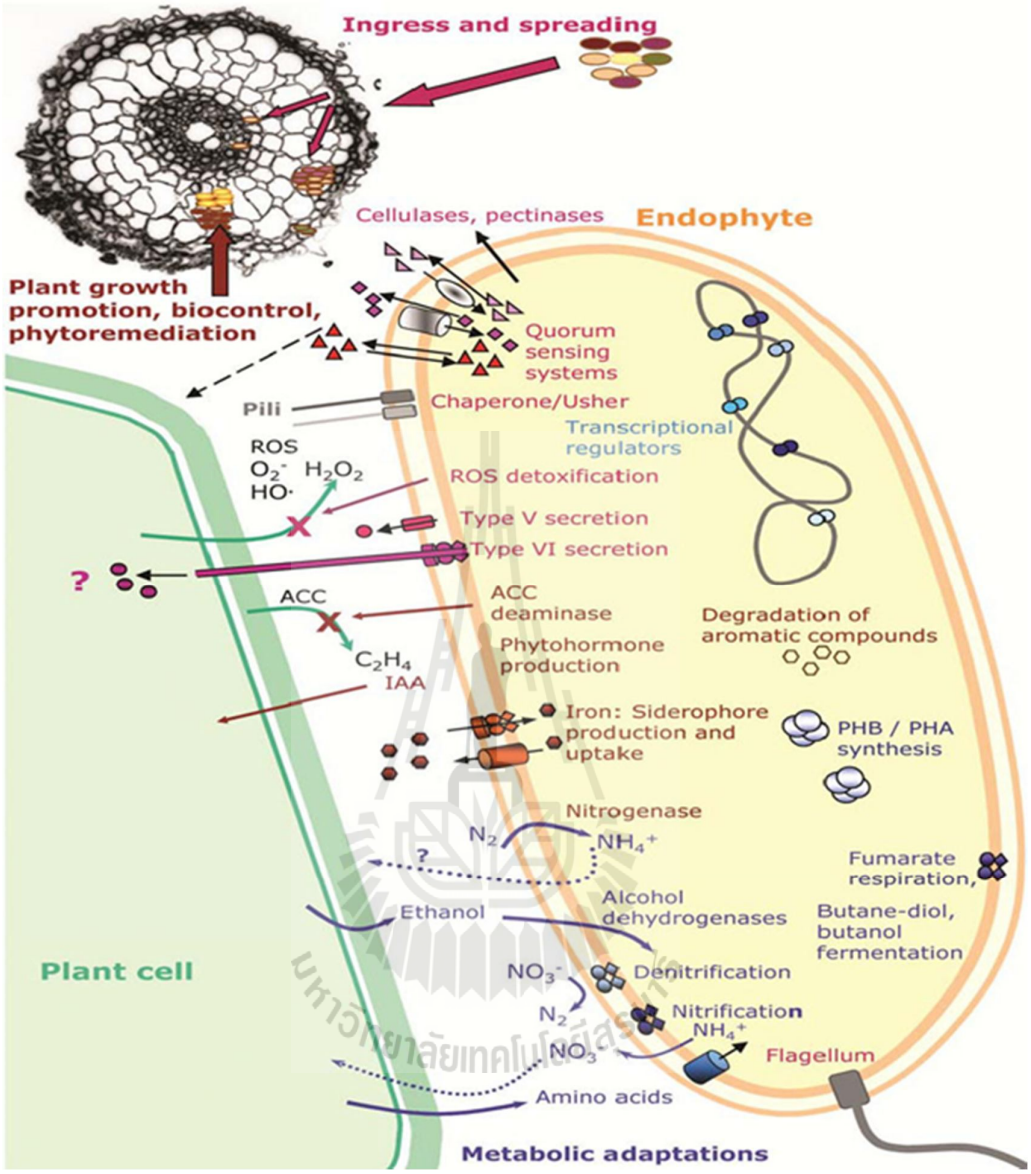

Figure 2.3 Establishment of endophytes in the host (Sessitsch et al., 2012)

In the endophyte mechanisms, all known protein secretion systems for translocation across the cytoplasmic and outer membranes are presented (Sessitsch et al., 2012). An exception is the type III secretion system (T3SS) commonly used by symbiotic (Downie, 2010) and pathogenic bacteria to inject effector proteins directly into the host cytoplasm and, thereby, to modulate the host response. For the T3SS, not all essential

elements could be found in the among all the rice endophytes. This correlates with the suggestion that endophytes, though plant associated, considerably differ from pathogens; genomes of cultivated endophytes do often not contain a T3SS (Reinhold-Hurek and Hurek, 2011), although the *Herbaspirillum seropedicae* genome, for example, contains one (Pedrosa et al., 2011). New targets for functional studies are proteins whose role in interactions has not yet been studied in cultivated endophytes. A high representation of genes encoding components of type VI secretion systems, suspected to deliver effector proteins into cells of eukaryotic hosts (Pukatzki et al., 2009), suggests their importance in beneficial plant–microbe interactions. In rhizobia, the type VI secretion system may contribute to host specificity (Bladergroen et al., 2003). The almost every endophyte genome would contain an *hcp* gene, which encodes a key protein forming the secretion channel. Interestingly, this distribution is also reflected in genomes of cultivated endophytic bacteria: among 12 published genomes, seven harbor at least two putative type VI secretion gene clusters (Reinhold-Hurek and Hurek, 2011).

Hydrolytic, plant-polymer-degrading enzymes are the putatively important feature. High gene abundance (domains represented in cellulases, xylanases, pectinase, and pectin-esterase) suggested more than one set per endophyte genome. Pectinases are especially prominent in the genome and may contribute to endophyte entry into and spreading inside roots by degrading middle lamella (Sessitsch et al., 2012). The role of hydrolytic enzymes has been shown (e.g., for the endoglucanase EglA in the endophyte *Azoarcus* sp. strain BH72 involved in rice root invasion) (Reinhold-Hurek et al., 2006).

Although flagellins are known to elicit an innate immune response in *Arabidopsis* (Zipfel et al., 2004). The motility or flagella mediated adhesion may be required for

establishment in the rice endosphere (Sessitsch et al., 2012). In addition, the genes encoding enzymes potentially involved in the detoxification of reactive oxygen species (ROS), as well as glutathione synthases and also glutathione-S-transferases (GST). Genes encoding glutathione synthetases are generally involved in detoxification of ROS and products of oxidative stress. Because plants are produce a range of ROS in response to colonizing microorganisms (Fouts et al., 2008; Taghavi et al., 2010).

2.6 Architecture of endophyte genomes

The genome sizes and genes content varies strongly in the nine studied endophytes (Figure 2.4), ranging from 3.9 Mbp and 3633 genes in *Gluconacetobacter diazotrophicus* PAl5 and 4.4 Mbp and 3992 genes in *Azoarcus* sp. BH72, to 8.2 Mbp and 7487 genes in *Burkholderia phytofirmans* PsJN (Mitter et al., 2013). It is generally assumed that the genome size correlates with the number of possible lifestyles of a strain; a strain with higher gene content might be able to better deal with diverse environmental conditions. Toft and Andersson (Toft and Andersson, 2010) unveiled in their review that the genome size of bacteria drastically decreases during the evolutional transition from a free-living bacterium to an obligate intracellular symbiont. *B. phytofirmans* PsJN with its large genome is able to colonize various genetically unrelated plants such as potato, tomato, grapevine, maize, switchgrass, sugarbeet, and barley, both endophytically and in the rhizosphere (Mitter et al., 2013). *B. phytofirmans* was first isolated from surface-sterilized onion roots and, in the description of the species *B. phytofirmans* (Compant et al., 2005) was reported to occur in agricultural soils. In contrast, *Azoarcus* sp. BH72, characterized by a rather small

genome size, has only been found in grasses and only reported as endophyte, not in soil (Reinhold-Hurek and Hurek, 1998).

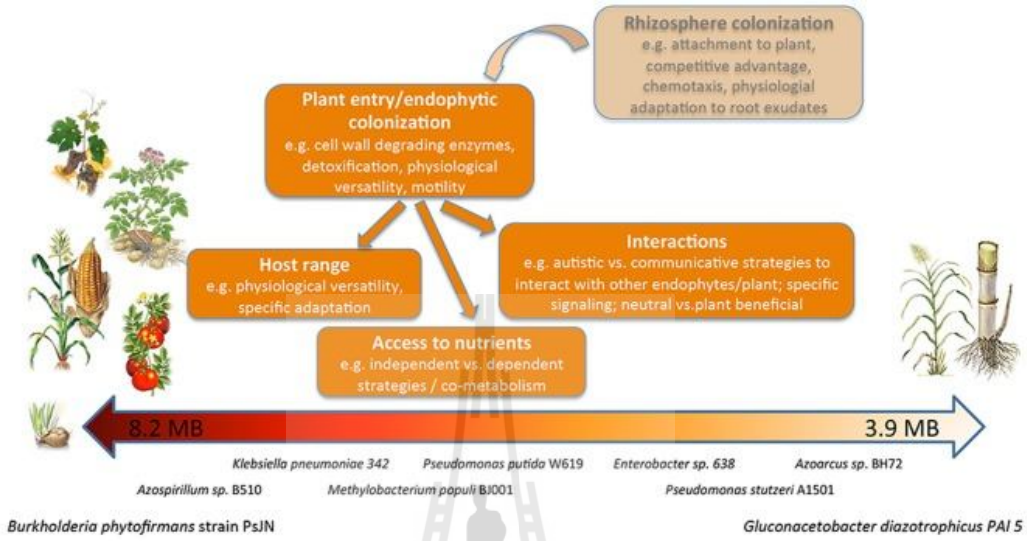

Figure 2.4 The spectrum of endophytes analyzed in this study. Drawing illustrating the differences in genome size and reported host spectrum of the endophytic bacteria analyzed in this study and summarizing the features of the endophytic lifestyle (Mitter et al., 2013)

The genomic comparison revealed a high genetic diversity indicative of the phylogenetic groups the analyzed strains belong to. The characteristics such as motility, chemotaxis, and degradation of plant polymers and organic compounds have been proposed to be necessary for colonization and endophytic life (Reinhold-Hurek et al., 2006). However, some studies suggested that not all these traits are absolutely necessary. One of the surveyed endophytes, *Klebsiella pneumoniae* 342, does not have genes involved in biosynthesis of flagella, the feature that might suggest adaption to the symbiotic life via establishing dense populations inside the host (Fouts et al., 2008). The traits generally encountered in endophyte genomes include detoxification of ROS,

a strategy to deal with plant defense responses similar to plant pathogenic bacteria, as well as the production of plant polymer-degrading enzymes. Similarly, quorum-sensing, an important trait in pathogens' invasion of plants is also prominent in the endophyte genomes.

The application trends of rice endophytes are still intriguing therefore, the application of these organisms for a more sustainable agricultural practice is also reconsidered. In addition, the bradyrhizobia were isolated from root/stem nodules of *A. americana* (strain DOA9) can establish the symbiosis with rice (Teamtisong et al., 2014). Despite a widespread occurrence of this natural endophytic bradyrhizibium-rice association, much remains unknown about its infection and colonization processes.

Far aim: To pave the way for making monocot-plants fix N_2 per se.

To engineer the commercial bradyrhizobium which can multiply and function in plant cells. Therefore, the understanding the initial process of rice infection by bradyrhizobia is needed to explore.

CHAPTER III

MATERIALS AND METHODS

3.1 Endophytic bradyrhizobial isolation from rice tissues grown under different agricultural practice systems

3.1.1 Soil sampling and endophytic bradyrhizobial isolation

Rice (*Oryza sativa* L.) cultivars and rice rhizospheric soil samples used in this study are listed in Table 3.1.

Table 3.1 Rice cultivars used for bacterial isolation, agricultural systems and relevant characteristics

Rice cultivars	Agricultural system	Relevant characteristics
O. sativa* L. ssp. *Indica		
Khao Dowk Mali 105	Rice/Legume crop rotation	Photosensitivity, lowland rice
Leuang Yai 148	Rice/Legume crop rotation	Photosensitivity, lowland rice
Lu Ni	Rice/Legume crop rotation	Photosensitivity, floating rice
Pathum Thani 1	Monoculture system	Non-photosensitivity, lowland rice
	Rice/Legume crop rotation	
Chai Nat 1	Monoculture system	Non-photosensitivity, lowland rice
Suphan Buri 1	Monoculture system	Non-photosensitivity, lowland rice
Patthalung	Monoculture system	Non-photosensitivity, lowland rice

Rice roots and paddy soils were collected and immediately transferred to the laboratory in polyethylene boxes at 4ºC to obtain endophytic bradyrhizobia. The roots and stems were chemically sterilized (3% sodium hypochlorite for 5 min followed by soaking in 70% ethanol for 5 min). The rice roots were thoroughly rinsed with sterilized distilled water at least five times and cut into 4 to 5-cm-long sections. As a control to check superficial contamination for each individual plant, 200 µl of water from the final rinsed were spread on plate count agar (PCA) medium. No individual root samples containing in contamination on PCA plates were retained for isolation of endophytic bradyrhizobia (Baldwin et al., 1996). Sections were aseptically crushed by sterilized mortar and pestle containing 1 ml of sterilized water.

To obtain the endophytic bradyrhizobia from rice tissues, the bradyrhizobial strains were isolated from three different protocols (Figure 4.1). Firstly, to obtain *nod* contained *Bradyrhizobium* strains, the 1 ml of roots homogenate solutions (as mentioned previously) was directly inoculated into germinated siratro (*M. atropurpureum*) seeds. Then, after 1 month, each nodule was crushed and streaked on arabinose-gluconate (AG) agar plates (Sadowsky et al., 1987). Secondly, the oligotrophic-based isolation was considered to reduce growth of other contaminants. The 1 ml of the roots homogenate was added into 50 ml of sterilized water for 1 month, then 0.1 ml of solution was spread on BJSM plates (Tong and Sadowsky, 1994). Finally, the 0.1 ml of roots homogenates was directly spread on BJSM plates (Figure 4.1). In order to select different strains of bacteria, BOX-PCR was carried out for screening the redundant strains (Versalovic et al., 1994). The bacterial strains showing the different BOX-PCR patterns were selected for bacterial identification by 16S rDNA sequencing (Table 3.2).

Table 3.2 Strains and plasmids used in this study, sampling sites and relevant characteristics

Strain or Plasmid	Geographical origin	Relevant characteristics and source of isolation
Strains		
Bradyrhizobium sp.		
SUT-R3	Chiang Mai (19° 13' 11.3"N, 98° 50'51.7"E)	Rice root isolate (rice with crop rotation)
SUT-PR9	Uttaradit (17° 39' 1.9"N, 100° 8' 34.2"E)	Rice root isolate/paddy soil isolate (rice with crop rotation)
SUT-PR48	Surin (14°39'41.3"N, 103°17'09.5"E)	Rice root isolate (rice with crop rotation)
SUT-R55	Lampang (18° 49' 42.5"N, 99° 56' 37.2"E)	Rice root isolate (rice with crop rotation)
SUT-PR64	Khonkaen (16° 14' 13.5"N, 102° 31' 31.1"E)	Rice root isolate (rice with crop rotation)
SUT-R74	Chiangrai (19° 22' 31.7"N, 99° 30' 5.9"E)	Rice root isolate/paddy soil isolate (rice with crop rotation)
DOA1	Lopburi	*A. americana* nodule isolate (paddy crop)
DOA9	Lopburi	*A. americana* nodule isolate (paddy crop)
SUTN9-2	Lampang	*A. americana* nodule isolate (paddy crop)
WD16 [a]	Miyagi prefecture, Japan	Rice root isolate (rice monoculture)
RP5 [a]	Miyagi prefecture, Japan	Rice root isolate (rice monoculture)
RP7 [a]	Miyagi prefecture, Japan	Rice root isolate (rice monoculture)
Escherichia coli S17-1		*pro recA* RP4-2(Tc^s::Mu) (Km^s::Tn*7*); Mob$^+$
Plasmids		
pRK404		Broad-host-range vector
pCAM120		mTn*5*SS*gusA20* in pUT/mini-Tn*5*
pBjGroEL4::dsRed2		mTn*5*dsRed2 in pUT/mini-Tn*5*
pRK2013		helper plasmid

[a] Department of International Environmental and Agricultural Science, Graduate School of Agriculture, Tokyo University of Agriculture and Technology, Tokyo, Japan

3.1.2 Phenotypic characteristics and nitrogen fixation assay

The color and morphology of bradyrhizobial colonies were observed during cultivation on HM agar medium (Cole and Elkan, 1973). In order to detect the photosynthetic pigments from the bradyrhizobial strains, they were grown aerobically at 30°C for 7 days under a 12-h-light/12-h-dark cycle. To determine bacterial pigment production, cell pellets were extracted in the dark with cold acetone-methanol (7:2 [vol/vol]) for 30 min (Lorquin et al., 1997). Absorbance of the supernatant was observed at a wavelength range from 350 to 850 nm. Another methodology to determine the bacterial pigment production as described by Chaintreuil et al. (Chaintreuil et al., 2000) was also conducted. The bradyrhizobia were grown at 30°C on YEM modified agar medium (g/l; K_2HPO_4 0.66, sodium glutamate 2.5, yeast extract 2, NaCl 0.05, $MgSO_4.7H_2O$ 0.1, $CaCl_2.2H_2O$ 0.04, $FeCl_3$ 0.004, $MnSO_4$ 0.01) for 7 days under aerobic conditions on a cycle of 15-h-light/9-h-darkness. A lawn of bacterial colony was analyzed using a Beckman DU40 spectrophotometer (Cary WinUV scan). Absorption spectra were obtained by scanning over a wavelength range from 350 to 900 nm with medium speed. Photosynthetic pigment peaks were detected at 800 and 870 nm.

Acetylene reduction assay (ARA) was carried out to examine the nitrogen-fixing activity in symbiosis form of bradyrhizobial strains. Symbiotic abilities of putative bradyrhizobial strains were determined in Leonard's jars containing sterilized vermiculite and inoculated with 1 ml of bacterial culture, equivalent to 10^7 cells. The nitrogen-fixing activity of the bacterial cultures was examined by ARA as described previously (Prakamhang et al., 2009).

3.1.3 The 16S rRNA, housekeeping genes and phylogenetic analyses

The genomic DNA of bradyrhizobial strains was prepared from the purified bacterial strains grown in HM broth (Piromyou et al., 2011). The 16S rRNA gene was amplified using the primer pair fD1 and rP2 (Allardet-Servent et al., 1993). The DNA primers for housekeeping genes and other DNA primers used are listed in Table 3.3. Gene fragments were amplified using the Go Taq Flexi DNA polymerase kit (Promega, Mannheim, Germany). The PCR products were purified using the Wizard SV gel and PCR clean-up system (Promega, Germany) and sequenced using the same primers as those for the PCR. DNA sequencing was carried out by Macrogen, Inc. (Seoul, South Korea). The DNA sequences were generated and the most closely related sequences were obtained from the NCBI database. The nucleotide sequences were aligned using the CLUSTAL W program, and the phylogenetic trees of the 16S rRNA gene and housekeeping genes sequences were constructed by the maximum likelihood method using PhyML (Guindon and Gascuel, 2003). The confidence levels were estimated for 1,000 replicates. In comparison, phylogenetic trees were also reconstructed by the distance neighbor-joining method (Saitou and Nei, 1987) using the MEGA 4.1 package (Kumar et al., 2008). DNA sequences of each gene from relative strains in the family *Bradyrhizobiaceae*, of other rhizobia, and of outgroups were obtained from the NCBI database.

Table 3.3 Primers used in this study

Target gene	Primer name or primer sequences	Accession no. or description of design	Annealing temperature (ºC)
16S rRNA	fD1	Universal primer for 16S rRNA gene from bacteria	55
	rP2		
dnaK	TSdnaK4	FJ347237	53
	TSdnaK2	FJ347237	
glnB	TSglnBF	FJ347346	53
	TSglnBR	FJ347346	
recA	recA41F	BAC51020	53
	recA640R	BAC51020	
BOX-A1R	BOX-A1R	Repetitive Sequence-based PCR (rep-PCR) for bacteria	63
nodA	nodAF28	NC_004463	45
	nodAR627	NC_004463	
	nodAF25-ORS285	AF284858	46
	nodAR584-ORS285	AF284858	
nodB	nodBF26	NC_ 004463	47
	nodBR625	NC_ 004463	
	nodBF73-ORS285	AF284858	53
	nodBR622-ORS285	AF284858	
nodC	nodCF195	NC_ 004463	45
	nodCR1394	NC_ 004463	
	nodCF197-ORS285	AF284858	53
	nodCR1196-ORS285	AF284858	
pufM	pufM-F(5'CGAGATCATCGGCCTCAACATGTTGG-3')	Designed from *pufM* of *Bradyrhizobium* sp. BTAi1(CP000494) and *Rhodopseudomonas palutris* (AB015977)	62
	pufM-R(5'GAAGCCCATCGTCCAGCGCCAGAAC-3'		
bchL	bchL-F (5'-AAGCAYGACTCSACCTTCAC-3')	Designed from *bchL* of *Bradyrhizobium* sp. BTAi1(CP000494) and *Rhodopseudomonas palutris* BisB5 (CP000283)	53
	bchL-R (5'-CGTTGAACTTGTCGATCTGG-3')		

3.2 Determination of endophytic bradyrhizobial isolation and the population in rice tissue from various cultivars

3.2.1 GUS and DsRed-tagging of endophytic bradyrhizobia and monitoring of root colonization

Bradyrhizobial strains were cultured in HEPES-MES (HM) medium at 30ºC (Cole and Elkan, 1973). The *Escherichia coli* strains were cultured at 37°C in Luria-Bertani (LB) medium (Sambrook et al., 1989). The media were supplemented with the following antibiotics when appropriate: for *E. coli*, 30 µg/ml gentamicin; and 200 µg/ml streptomycin for *Bradyrhizobium*. Bradyrhizobial strains were tagged with mTn5SS*gusA20* (pCAM120) (Table 3.2) and pBjGroEL4::DsRed2 (Okubo et al., 2013) by triparental mating on HM agar plates and using pRK2013 as a helper plasmid (Tamura et al., 2011). The cell slurry was mixed with HM medium and then 100 µl of cell suspension was plated on HM agar plates containing 100 µg/ml streptomycin, 100 µg/ml spectinomycin, and 50 µg/ml polymycin B. The forming colonies were selected as transconjugants.

Seed coats of cultivated rice (Table 3.1) were removed and seeds were surface sterilized with 3% Na_2HPO_4 for 5 min and shaken in 1% hydrogen peroxide solution for 60 min. Seeds were then washed five times with sterilized distilled water with shaking (15 min each). Surface sterilized seeds were gnotobiotically germinated on water agar plate. After 3 days, rice seedlings were transferred into the 80 ml tubes containing 10 ml nitrogen-free plant nutrient solution (Elbeltagy et al., 2001). Three

days after transferring of seedling into the growth medium, GUS-tagged bacteria were inoculated into the growth medium to a density of 10^7- 10^8 cells/ml. Roots of 3 days-old seedling were examined for bacterial colonization by GUS staining. For the detection of β-glucuronidase (GUS) expression of endophyte in plant tissue, GUS staining was performed (Manassila et al., 2007). The roots were embedded in 5% agarose gel, and the 90-μm-thick sections were prepared using vibratome (Microm HM 650V, Thermo Scientific, UK). The plant samples were then directly observed under the light microscope. The DsRed-labeled strains were also inoculated into rice roots. Then, bradyrhizobial localizations were observed under confocal microscope (Nikon Confocal Microscope).

3.2.2 Plant experiments

Leonard's jar assembly was filled with sterilized vermiculite, and nutrient solutions were applied through a wick to provide nutrients to plants (Mae and Ohira, 1981). The whole apparatus was autoclaved (121°C for 25 min) prior to the transplantation of seedlings. Surface-disinfected rice seeds were germinated on sterilized filter sheets in Petri dish. Uniformly germinated seeds were transplanted into the Leonard's jar under aseptic conditions (triple plants per Leonard's jar). One milliliter of 5-day-old inoculum (approx 10^7 CFU/ml) was inoculated to the seedling at 2 days after transplanting. This was conducted as three replicates per single bacterial isolate. Plants were grown under controlled environmental condition of 28 ± 2°C on 12-h-light/12-h-dark cycle at light intensities 400 μE/m^2S and 50% humidity. The plant dry weights were measured after 28 days of planting.

3.2.3 Enumeration of endophytic bradyrhizobia

To enumerate the endophytic bradyrhizobia, the rice roots were surface-sterilized as described above. The rice cultivars used in this study were displayed in Table 3.4.

Table 3.4 Rice cultivars used in this study, geographical origin and relevant characteristics.

Rice cultivars	Geographical origin	Relevant characteristics	Reference
O. sativa* L. ssp. *Indica			
Khao Dowk Mali 105	Chonburi/Thailand	Photosensitivity, lowland rice	Sommai Lertna [a]
Leuang Yai 148	Chiangmai/Thailand	Photosensitivity, lowland rice	Sommai Lertna [a]
Lu Ni	Nakhonratchasima/Thailand	Photosensitivity, floating rice	Sommai Lertna [a]
Pathum Thani 1	Pathumthani/Thailand	Non-photosensitivity, lowland rice	Sommai Lertna [a]
Sang Yod Phatthalung	Phatthalung/Thailand	Photosensitivity, lowland rice	Sommai Lertna [a]
Kasalash	Japan	Non-photosensitivity, lowland rice	Tadashi Sato [b]
O. sativa* L. ssp. *Japonica			
Nipponbare	Japan	Non-photosensitivity, lowland rice	Tadashi Sato [b]

[a] Nakornratchasima Rice Research Center, Phimai, Nakhonratchasima, 30110, Thailand

[b] Graduate School of Life Sciences, Tohoku University, 2-1-1 Katahira, Aoba-ku, Sendai 980-8577, Japan

The surface-sterilized roots were macerated with a sterilized mortar and pestle, diluted in 0.85% of saline solution prior to being spread on YM plates containing streptomycin (300 µg/ml) and X-gluc (10 µg/ml). After 7 days of incubation at 28ºC, the number of the blue colonies was counted to display the bacterial population densities (CFU) in rice root tissues.

3.2.4 Plant nodulation tests

Bradyrhizobial strains were grown for 5 days in HM broth. All plants were grown in a growth chamber under controlled environmental condition of 28 ± 2°C on 12-h-light/12-h-dark cycle at light intensities 400 μE/m^2S and 50% humidity. Mungbean (*V. radiata*), soybean (*Glycine max* cv.SJ5) and siratro (*M. atropurpureum*) seeds were sterilized (Somasegaran and Hoben, 1994b). The seeds of *A. americana* (a local Thai variety), *A. indica* (African ecotype) and *A. evenia* (provided by Eric Giraud) were sterilized as described in Noisangiam et al. (Noisangiam et al., 2012). The nodulation test was performed in sterilized plastic pouches for five replicates (Somasegaran and Hoben, 1994b). The root nodules from the plants tested were enumerated at 28 days after inoculation (dai).

3.3 Determination the genes involved in infection process of endophytic bradyrhizobia in rice tissues as well as in legumes

3.3.1 Plant root exudates preparation

Rice seeds were surface sterilized as described above, then the germinated seeds were transferred into the 80-ml tubes (5 seeds/tube) containing 5 ml nitrogen-free medium (Elbeltagy et al., 2001). Seven days after transferring of seedling into growth medium, the medium solutions containing root exudates were transferred into new tubes. The root exudate solutions were repeatedly sterilized through 0.2 μm filter membranes. The root exudate solutions were kept at -20°C before used.

3.3.2 RNA isolation and reverse transcription PCR amplification of candidate genes

The total RNA was extracted from bacterial cells (3 ml of rice root exudate was added into 6 ml of bacterial cell cultures) at 3 hours and directly isolated from rice samples (at 6 h, 12h, 1, 3, and 7 dai) using RNeasy Plant Mini Kit (QIAGEN, USA) according to the manufacturers protocol. RNAs were treated with DNase to prevent contamination of genomic DNA and finally resuspended in diethylpyrocarbonate-treated wate. Reverse-transcription polymerase chain reaction (RT-PCR) was performed on RNA samples, using primers designed to target genes (Table 3.5).

Table 3.5 Primer for Reverse-transcription polymerase chain reaction (RT-PCR)

Target gene	Primer name	Primer sequence (5'→ 3')	Reference or description of design	Annealing temperature used (°C)
16S rRNA	fD1	AGAGTTTGATCCTGGCTCAG	Universal primer for 16S rRNA gene from bacteria	55
	rP2	ACGGCTACCTTGTTACGACTT		
exoB	exoBF	GAATGAGGTGGGTTTCGTTG	Designed from *exoB* of *Bradyrhizobium* sp. SUTN9-2	55
	exoBR	GCCCGTCGTTTATGACAATC		
fliP	fliPF	GATCAGGAACGGCAGGAATA	Designed from *fliP* of *Bradyrhizobium* sp. SUTN9-2	55
	fliPR	GGACATCAGCATCAATCTCG		
rhcJ	rhcJF	CCCGCTACGTCTATGCTCT	Designed from *rhcJ* of *Bradyrhizobium* sp. SUTN9-2	56
	rhcJR	TATTTCGGATCGGAGGACAG		
peces	pecesF	TATCACTCCGTGCAACAAGC	Designed from *peces* of *Bradyrhizobium* sp. SUTN9-2	48
	pecesR	CGGCACTTAGAGTGCAATGA		
virD4	virD4F	GAGACCGAGACC GAGCTCTA	Designed from *virD4* of *Bradyrhizobium* sp. SUTN9-2	55
	virD4R	CTTGATGATGAAGGGCTGCT		
gst	GST_f	CTGGAAGGCCAAGACCAAC	Designed from *gst* of *Bradyrhizobium* sp. SUTN9-2	56
	GST_r	ACCAGATCTTGACCGAGG		
Δ*rhcJ*	Δ*rhcJ*-f	CGTCCCAAATTGGAAGCTAA	Designed from *rhcJ* of *Bradyrhizobium* sp. SUTN9-2 for gene deletion	55
	Δ*rhcJ-r*	CATACTTCTGCGCTGCCATA		

The total RNA was treated with 1 U of RNase free DNase (Promega, USA) and incubated at 37ºC for 30 min. One microliter of stop solution (20 mM DGTA [pH8.0] at 25ºC) was added and incubated at 70ºC for 15 min. The combined RNA was preheated at 70ºC for 5 min and kept on ice until the reverse transcription reaction was added. Reverse transcription reaction mixture containing 4 µl of ImProm-II^{TM} 5X reaction buffer, 3 mM $MgCl_2$, 0.67 mM dNTP mix, 20 U of ribonuclease inhibitor, 1 µl of ImProm-II^{TM} reverse transcriptase (Promega, USA) was adjusted with nuclease-free water to 15 µl. Five microliters of RNA and primer mix were added into reverse transcription reaction mixture, giving a final reaction volume of 20 µl. The solution

was annealed at 25ºC for 5 min, extended at 42ºC for 60 min and inactivated reverse transcriptase at 70ºC for 15 min. Two microliters of the cDNA was amplified by PCR in same condition as DNA amplification. The 16s rRNA gene was used as a standard to calibrate the amount of RNA. All RT-PCR was performed in Thermal cycler BIO-RAD T100^{TM} Thermal Cycle and products were visualized using 1% agarose gel electrophoresis and stained with 0.5 μg/ml of ethidium bromide, then documented on Gel documentation and analysis (Gel Doc XR+ system, BIO-RAD). The relative genes expressions were calculated by using the 16s rRNA gene for normalization.

3.3.3 Genome information and comparison

The draft genome sequences of *Bradyrhizobium* sp. SUTN9-2 and its genes annotation files were determined by a whole-genome shotgun strategy using Sanger sequencing and 454 pyrosequencing. For Sanger sequencing using a 3730*xl* DNA analyzer (Applied Biosystems, Foster City, CA, USA), about 20 μg DNA was sheared using a HYDROSHEAR (Gene Machine, San Carlos, CA, USA) for a shot-insert genomic library, and another 80 μg was sheared for construction of a long-insert library. DNA fragments of 3 kb (for the short-insert library) and 10 kb (for the long-insert library) were subcloned into plasmid vector pTS1 (Nippon Gene, Tokyo, Japan) to construct shotgun libraries (Okubo et al., 2011). Gene for structural RNAs identified by similarity searches againt an in-house structural RNA database that has been constructed from data available in GenBank. rRNAs were predicted on the basis of similarity searches against those of *B. diazoefficiens* USDA110 using BLASTN program. The tRNA, tmRNAs and non-coding RNAs were predicted using the non-

coding RNA sequence database fRNAdb version 3.4 (http://www.ncrna.org/frnadb/) and Rfam version 10.0 (http://rfam.sanger.ac.uk) (Gardner et al., 2009). Protein-encoding regions were predicted using the MetaGeneAnnotator with default parameters (Noguchi et al., 2008).

Some endophytic-related genes were selected for genetic comparisons by using USDA110 genome as non-endophytic strain (Table 3.5) (Sessitsch et al., 2012). The genome sequences of *Bradyrhizobium* sp. SUTN9-2 were compared using the program GenomeMatcher (Ohtsubo et al., 2008) at the nucleotide level. The annotated genome sequences of USDA110 were obtained from Genome Assembly/Annotation Projects (NCBI database).

3.3.4 Protein extraction and MALDI-TOF-MS/MS analysis

Bradyrhizobim sp. SUTN9-2 were grown at 28ºC for 48 h in 1,000 ml AG medium (Sadowsky et al., 1987) in the absence or in the presence of rice and *A. americana* root exudates (10% volume/volume). The extracellular proteins were prepared as described previously (Okazaki et al., 2009). The protein samples were subjected to SDS-PAGE analysis (5-20% gradient gel in Tris-glycine buffer). Small pieces of the Coomassie-stained gel corresponding to bands of interest were digested with trypsin. Peptides were analyzed by using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS/MS) (TOF/TOF 5800 system; AB Sciex, Foster City, CA). Proteins were identified by the MS-Fit software

program (http://prospector.ucsf.edu/prospector/mshome.htm) with the USDA110 database as a reference (Kaneko et al., 2002).

3.3.5 Construction of *Bradyrhizobium* sp. SUTN9-2 mutants

To construct *Bradyrhizobium* sp. SUTN9-2 (*rhcJ*) deletion mutants, DNA fragments corresponding to the upstream and downstream regions of *rhcJ* gene were individually amplified by PCR (Table 3.5). Bacterial strains and plasmids used in these experiments were listed in Table 3.6 The PCR products as 1,065-bp KpnI-BamHI and 1,059-bp BamHI-XbaI fragments into the pGEM®-T Easy vector (Promega), yielding the plasmids pGEM®-TUP and pGEM®-TDOWN, respectively. The 1,065-bp KpnI-BamHI fragment from pGEM®-TUP was then cloned into pGEM®-TDOWN, yielding the plasmid pGEM®-TUP/DOWN. Finally, a 2.124-kb KpnI-XbaI from pGEM®-TUP/DOWN was cloned into the Km^r-suicide vector pK18mobsacB (Schäfer et al., 1994) to yield the plasmid pK18mobsacB-UP/DOWN. The antibiotic genes (spectinomycin/streptomycin) cassette were inserted into pK18mobsacB-UP/DOWN (between upstream and downstream fragments) to yield the plasmid pK18mobsacB-UP/DOWN/Spc^r/Sm^r. Plasmid was transferred by conjugation from *E. coli* DH5α to *Brdyrhizobium* sp. SUTN9-2 for markerless mutant construction. Triparental matings were conducted using pRK2013 as a helper plasmid (Tamura et al., 2011). Km^r transconjugants were selected and grown in the presence of 10% sucrose to force loss of the vector-encoded *sacB* gene. The resulting 3 colonies (named as Δ*rhcJ*-3B, Δ*rhcJ*-18A and Δ*rhcJ*-27A) were checked for Km sensitivity. The desired mutations in *rhcJ* were confirmed by sequence analysis and PCR.

Table 3.6 Strains and plasmids used in this study

Strain or Plasmid	Relevant characteristics
Strains	
Bradyrhizobium sp. SUTN9-2Δ*rhcJ*	SUTN9-2 derivative, *rhcJ*::Ω cassette; Sp^r, Sm^r
Escherichia coli S17-1	*pro recA* RP4-2(Tc^s::Mu) (Km^s::Tn*7*); Mob^+
E. coli DH5α	*recA*; cloning strain
Plasmids	
pRK404	Broad-host-range vector
pCAM120	mTn*5*SS*gusA20* in pUT/mini-Tn*5*
pHP45Ω	Plasmid carrying a 2.0-kb Ω cassette; Sp^r, Sm^r, Ap^r
pK18mob-sacB	Cloning vector; pMB1ori oriT, oriV, sacB; Km^r
pRK2013	ColE1 replicon carrying RK2 transfer genes; Km^r
pK18mobsacB-UP/DOWN/Spc^r/Sm^r	pK18mobsacB carrying 2.123-kb *rhcJ* deletion fragment; Km^r, Spc^r, Sm^r

3.3.6 Statistical analyses

The experimental data were statistically analyzed according to Steel and Torrie (Steel and Torrie, 1980), and means were compared by Duncan's Multiple Range Test (Duncan, 1955).

CHAPTER IV

RESULTS

4.1 Bacterial isolation

The putative rice endophytic bacteria including bradyrhizobia were isolated from two different agricultural practice systems including rice monoculture and rice/legumes crop rotational systems along with the procedures depicted in Figure 4.1. Sixty-two bacterial colonies were obtained from the samples of monoculture system from the central part of Thailand, while 91 colonies were isolated from the samples of crop rotation system from every regions of Thailand (Table 3.2 and Table 4.1).

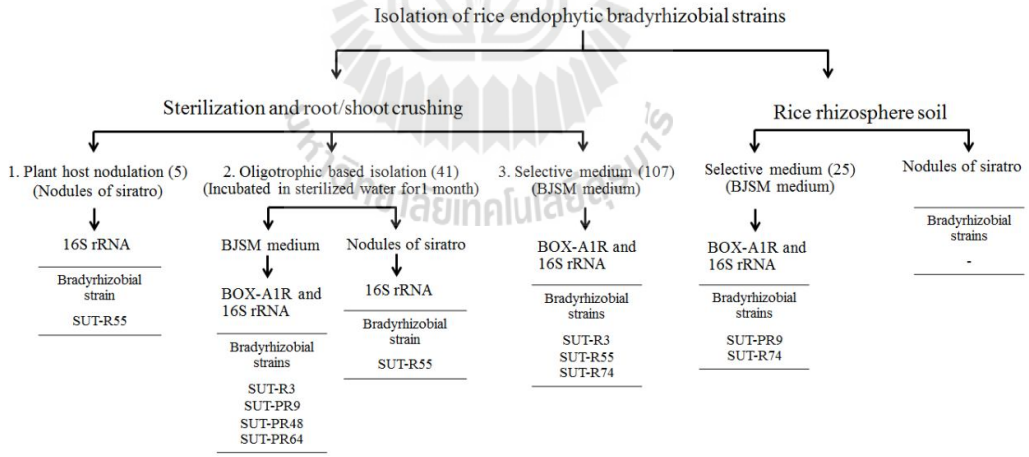

Figure 4.1 Scheme for the isolation of endophytic bradyrhizobial strains: Name of strains with "P" means photosynthetic bradyrhizobial strain while without "P" indicating the non-photosynthetic bradyrhizobial strain. The numbers inside parentheses represent the numbers of bacterial isolated strains from each isolation procedure.

Table 4.1. Putative rice endophytic strains and geographical origin

Monoculture system		
Isolates	**Genus (along with code of isolate, respectively)**	**Geographical origin: province in Thailand (Latitude and Longitude)**
Central part		
SUT-R11-15	*Methylobacterium* sp.(1), *Acidovorax* sp.(1), *Agrobacterium tumefaciens*(1), *Serratia marcescens*(1), *Chryseobacterium* sp.(1)	Suphan Buri 14°13'60.0"N 100°03'05.2"E
SUT-S11-13	*Methylobacterium* sp.(1), *Agrobacterium tumefaciens*(1), *Ralstonia* sp.(1)	
SUT-R16-19	*Sphingomonas* sp.(1), *Chryseobacterium* sp.(2), *Agrobacterium tumefaciens*.(2), *Agrobacterium tumefaciens*(3)	Ang Thong 14°36'06.5"N 100°22'46.4"E
SUT-S14-15	*Chryseobacterium* sp.(2), *Ralstonia* sp.(2)	
SUT-R20-26	*Chryseobacterium* sp.(3), *Agrobacterium tumefaciens*(3), *Ralstonia* sp.(3), *Agrobacterium* sp.(4), *Agrobacterium tumefaciens*(5), *Acidovorax* sp.(2), *Ralstonia* sp.(4)	Sing Buri 14°55'14.1"N 100°24'07.1"E
SUT-S16	*Acidovorax* sp.(3)	
SUT-R27-31	*Ralstonia* sp.(5), *Acidovorax* sp.(4), *Ralstonia* sp.(6), *Sphingomonas* sp.(1), *Agrobacterium tumefaciens*(6)	Chai Nat 15°15'46.8"N 100°02'54.9"E
SUT-S17	*Acidovorax* sp.(4)	
SUT-R32-35	*Rhodopseudomonas* sp.(1), *Burkholderia* sp.(1), *Ralstonia* sp.(7), *Burkholderia gladioli*(2)	Phra Nakhon Si Ayutthaya 14°19'55.9"N 100°35'14.0"E
SUT-S18	*Burkholderia gladioli*(2)	
SUT-R36-40	*Burkholderia* sp.(3), *Chryseobacterium* sp.(4), *Agrobacterium* sp.(7), *Ralstonia* sp.(8), *Burkholderia gladioli*(4)	Uthai Thani 15°19'28.9"N 100°04'40.2"E
SUT-S19	*Burkholderia gladioli*(4)	
SUT-R41-43	*Ralstonia* sp.(9), *Ensifer adhaerens*(1), *Serratia marcescens*(1)	Nakhon Sawan 15°29'10.5"N 100°07'54.9"E
SUT-S20-23	*Ralstonia* sp.(9), *Burkholderia gladioli*(5), *Acidovorax* sp.(5), *Agrobacterium tumefaciens*(8)	
SUT-R44-45	*Burkholderia gladioli*(6), *Serratia marcescens*(1)	Lopburi 14°46'08.0"N 100°38'01.0"E
SUT-S24-26	*Burkholderia gladioli*(6), *Acidovorax* sp.(6), *Burkholderia* sp.(7)	
SUT-R46-47	*Agrobacterium* sp.(9), *Agrobacterium tumefaciens*(10)	Pathum Thani 14°01'27.5"N 100°27'48.5"E
SUT-S27-28	*Burkholderia* sp.(8), *Ralstonia* sp.(10)	
SUT-R49-52	*Rhodopseudomonas* sp.(2), *Ralstonia* sp.(11), *Burkholderia gladioli*(9), *Paenibacillus* sp.(1)	Nonthaburi 13°55'13.1"N 100°17'09.6"E
SUT-S29-30	*Burkholderia gladioli*(9), *Burkholderia* sp.(10)	

Table 4.1. Putative rice endophytic strains and geographical origin (continue)

Rice with crop-rotation system		
Isolate	**Genus (along with code of isolate, respectively)**	**Geographical origin: province in Thailand (Latitude and Longitude)**
Northern part		
SUT-R1-2 and R55	*Ralstonia* sp.[12], *Rhodopseudomonas* sp.[3], *Bradyrhizobium* sp. SUT-R55[1]*	Lampang 18°49'42.5"N 99°56'37.2"E
SUT-S1	*Ralstonia* sp.[12]	
SUT-R3	*Bradyrhizobium* sp. SUT-R3[2]*	Chiang Mai 19°13'11.3"N 98°50'51.7"E
SUT-S2-4	*Ralstonia* sp.[13], *Burkholderia* sp.[11]	
SUT-R4-6 and R74	*Burkholderia cepacia*[12], *Acidovorax* sp.[7], *Chryseobacterium* sp.[5], *Bradyrhizobium* sp. SUT-R74[3]*	Chiangrai 19°22'31.7"N 99°30'5.9"E
SUT-S5-6	*Chryseobacterium* sp.[5], *Burkholderia gladioli*[13]	
SUT-R7-9	*Methylobacterium* sp.[2], *Burkholderia cepacia*[14], *Methylobacterium* sp.[3]	Nan 18°38'44.2"N 100°45'30.8"E
SUT-S7-8	*Agrobacterium* sp.[11], *Ralstonia* sp.[14]	
SUT-R10	*Bradyrhizobium* sp. SUT-PR9[4]*	Uttaradit 17°39'1.9"N 100°08'34.2"E
SUT-S10	*Burkholderia gladioli*[15]	
Northeastern part		
SUT-R53-54	*Agrobacterium* sp.[12], *Methylobacterium* sp.[4]	Si Sa Ket 15°06'05.3"N 104°07'25.2"E
SUT-S31	*Methylobacterium* sp.[4]	
SUT-R56-57	*Burkholderia gladioli*[15], *Chryseobacterium* sp.[6]	Udon Thani 17°20'08.3"N 102°49'34.3"E
SUT-S32	*Herbaspirillum seropedicae*[1]	
SUT-R58-61	*Herbaspirillum seropedicae*[1], *Stenotrophomonas maltophilia*[1], *Chryseobacterium* sp.[7], *Acinetobacter calcoaceticus*[1]	Yasothon 15°54'33.4"N 104°18'02.1"E
SUT-S33	*Stenotrophomonas maltophilia*[1]	
SUT-R62-63	*Serratia marcescens*[1], *Chryseobacterium* sp.[7],	Nong Bua Lam Phu 17°14'45.1"N 102°23'02.2"E
SUT-S34	*Serratia marcescens*[1]	
SUT-R64	*Bradyrhizobium* sp. SUT-PR64[5]*	KhonKaen 16°14'13.5"N 102°31'31.1"E
SUT-S35-36	*Herbaspirillum seropedicae*[1], *Methylobacterium* sp.[5]	

Table 4.1. Putative rice endophytic strains and geographical origin (continue)

Rice with crop-rotation system		
Isolate	**Genus (along with code of isolate, respectively)**	**Geographical origin: province in Thailand (Latitude and Longitude)**
Northeastern part (continue)		
SUT-R65-68	Uncultured bacterium, *Flavobacterium* sp.(1), *Serratia marcescens*(1), *Herbaspirillum seropedicae*(1)	Mukdahan 16°35'55.5"N 104°30'23.4"E
SUT-S37-38	*Herbaspirillum seropedicae*(1), *Burkholderia gladioli*(16)	
SUT-R69-72	*Stenotrophomonas maltophilia*(1), Uncultured bacterium, *Chryseobacterium* sp.(8), *Acinetobacter calcoaceticus*(1)	Ubon Ratchathani 14°56'51.6"N 104°52'25.1"E
SUT-S39-40	*Stenotrophomonas maltophilia*(1)	
SUT-R73	*Chryseobacterium* sp.(8)	Chaiyaphum 15°41'30.5"N 101°57'02.1"E
SUT-S41-43	*Stenotrophomonas maltophilia*(1), Uncultured bacterium, *Ralstonia* sp.(15)	
SUT-R75-78	*Methylobacterium radiotelerans*(6), *Acidovorax sp.*(8), *Agrobacterium* sp.(12), *Stenotrophomonas maltophilia*(1)	Kalasin 16°42'13.9"N 103°25'14.8"E
SUT-S44-48	*Acidovorax* sp.(8), *Stenotrophomonas maltophilia*(1), *Chryseobacterium* sp.(9), *Ralstonia* sp.(16), *Serratia marcescens*(1)	
SUT-R48	*Bradyrhizobium* sp. SUT-PR48(6)*	Surin 14°39'41.3"N 103°17'09.5"E
SUT-S49-53	*Agrobacterium* sp.(13), *Methylobacterium* sp.(7), *Ralstonia* sp.(17), *Chryseobacterium* sp.(10), *Burkholderia gladioli*(16)	
Southern part		
SUT-R79-82	*Rhodopseudomonas* sp.(4), Uncultured bacterium, *Burkholderia gladioli*(17), Uncultured bacterium,	Phetchaburi 13°11'00.2"N 99°50'39.6"E
SUT-S54-56	*Rhodopseudomonas* sp.(4), *Agrobacterium* sp.(14), *Stenotrophomonas maltophilia*(1)	
SUT-R83-86 and R48	*Stenotrophomonas maltophilia*(1), *Agrobacterium* sp.(15), *Burkholderia* sp. (18), *Acidovorax* sp.(9), *Bradyrhizobium* sp. SUT-PR48(6)*	Prachuap Khiri Khan 12°36'43.4"N 99°51'40.5"E
SUT-S57-58	*Acidovorax* sp.(10), *Rhodopseudomonas* sp.(5)	
SUT-R87-90	*Agrobacterium* sp.(16), *Methylobacterium radiotelerans.*(6), *Rhodopseudomonas* sp.(6), *Burkholderia gladioli*(19)	Surat Thani 9°10'32.5"N 99°11'21.1"E
SUT-S59	*Acidovorax* sp.(11),	
SUT-R91	*Rhodopseudomonas* sp.(6)	Nakhon Si Thammarat 8°39'30.1"N 99°55'22.0"E
SUT-S60-62	*Acidovorax* sp.(12), *Agrobacterium* sp.(16), *Ralstonia* sp.(18)	

The numbers inside parentheses represent the different BOX-A1R PCR pattern in same genus. (*): putative rice endophytic bradyrhizobial strains

Five isolates were obtained from root nodules of siratro while 41 isolates were obtained from oligotrophic-based isolation. In addition, 107 isolates were obtained directly from BJSM medium. A total of 153 bacterial colonies were screened to remove the redundant strains using BOX-A1R PCR. The remaining 98 strains showing different BOX-A1R fingerprints (data not shown) were chosen for 16S rRNA identification. Only 6 strains of putative rice endophytic bradyrhizobia from each of the isolation strategies were obtained (Figure 4.1), while the other isolates were classified into four groups including alpha-proteobacteria (*Agrobacterium* sp., *Methylobacterium* sp., and *Rhodopseudomonas* sp.), firmicutes, flavobacteria, and beta-proteobacteria (*Ralstonia* sp., *Burkholderia* sp. and *Acidovorex* sp.) (Figure 4.2).

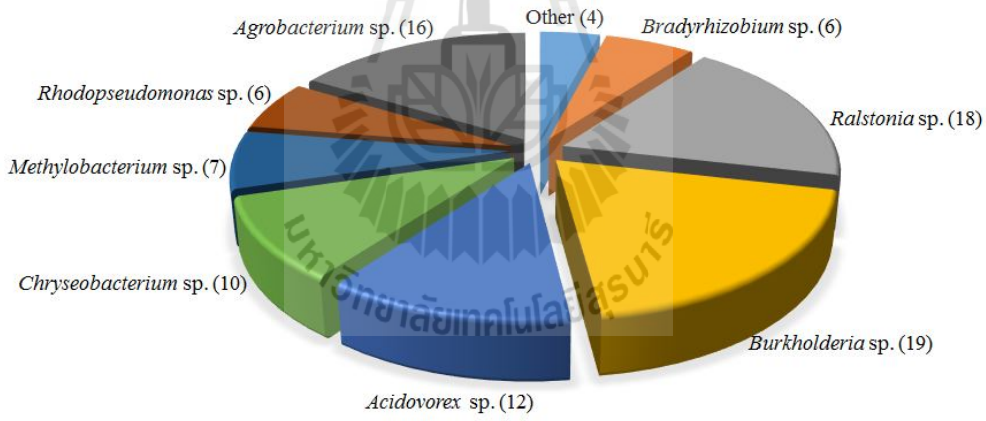

Figure 4.2 Rice endophytic bacteria: numbers inside parentheses represent different BOX-A1R-PCR pattern as same genus.

In addition, rice endophytic bradyrhizobial strains (DOA1, DOA9 and SUTN9-2) isolated from *A. americana* L. (weeds grown in rice fields) (Noisangiam et al., 2012) were also used in this study, and three strains, RP5, RP7 and WD16, from *O. sativa* L.

ssp. *japonica* cv. Nipponbare in Japan were also used as putative Japanese rice endophytes representatives (Shinoda *et al.* unpublished).

To enhance the opportunity to acquire rice endophytic bradyrhizobia, the sterilized rice roots and shoots homogenate solutions were applied into three procedures. Firstly, to obtain the *nod*-dependent bradyrhizobia, the nodulation test with siratro was conducted. Only isolate SUT-R55 was obtained from root nodules of siratro. Secondly, in order to avoid growth of other contaminants, the oligotrophic isolation approach (10 % [vol/vol] of homogenate solution was incubated into sterilized water for 1 month) was applied with rice tissue prior to testing on BJSM medium and siratro nodulation. Most of the 6 bradyrhizobial strains, SUT-R3, SUT-PR9, SUT-PR48, SUT-R55 and SUT-PR64, can be isolated from this procedure. Finally, three strains, SUT-R3, SUT-R55 and SUT-R74, were also directly obtained from homogenate and grown on bradyrhizobial selective (BJSM) agar plates. The rice rhizospheric soils were also used for the bradyrhizobia isolation. The strains obtained from these soil samples were SUT-PR9 and SUT-R74, when cultivated in BJSM medium but not found from siratro nodulation test (Figure 4.1).

In addition, most of putative rice endophytic bradyrhizobia from Thailand were found exclusively from the crop rotation system, whereas no bradyrhizobial isolates were obtained from monoculture system (Table 3.2 and Table 4.1).

4.2 Phenotypic characteristics and phylogenetic analysis of 16S rRNA and housekeeping genes

To determine the relationships of bradyrhizobial strains among their members of the *Bradyrhizobiaceae*, the nucleotide sequences from various reference *Bradyrhizobium* members and *Rhodopseudomonas* species were used to construct the phylogenetic tree. *Mesorhizobium loti* ATCC33669 and *Bosea thiooxidans* DSM9653 were chosen as outgroup strains to the root of phylogenetic tree (Figure 4.3).

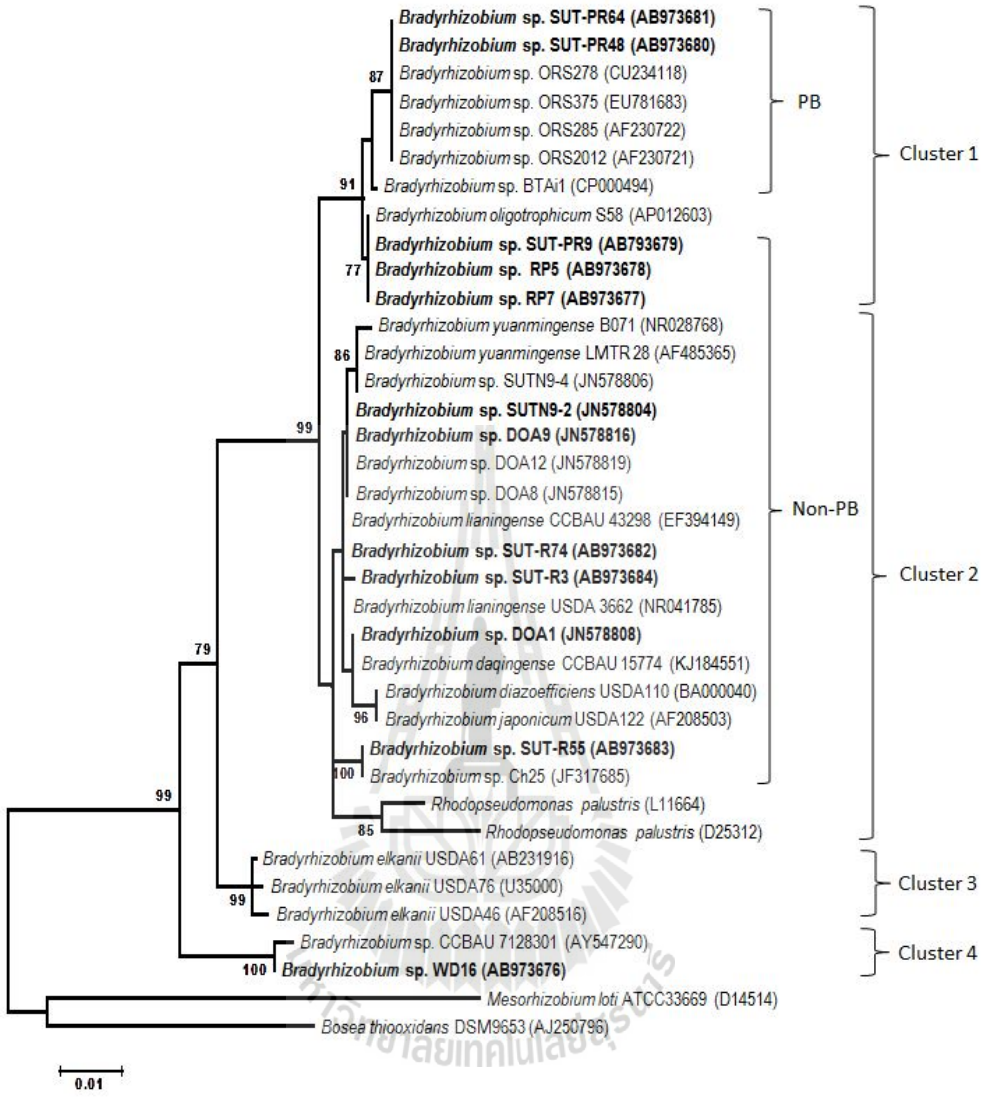

Figure 4.3 Neighbor-joining trees based on sequences of 16S rRNA genes showing classification of the photosynthetic bradyrhizobia (PB) and non-photosynthetic bradyrhizobia (Non-PB) strains isolated from rice tissues. Bootstrap values are expressed as percentages of 1,000 replications. The bar represents 1 estimated substitution per 100 nucleotide positions. The evolutionary distances were computed using Kimura two-parameter method and are shown in the units representing the number of base substitutions per site.

On the basis of 16S rRNA gene sequences similarity, the phylogenetic tree could be divided into 4 major clusters. The cluster 1 comprises bradyrhizobial strains SUT-PR64, SUT-PR48, SUT-PR9, RP5 and RP7. Only strains SUT-PR9, RP5 and RP7 separated slightly from strains in the PB group. Strains SUT-PR48 and SUT-PR64 were closely related with *Bradyrhizobium* sp. ORS278, *Bradyrhizobium* sp. ORS285 and *Bradyrhizobium* sp. ORS375. The bradyrhizobial members in the cluster 2 belonged to various groups of *Bradyrhizobium* species (*Bradyrhizobium* sp., *B. yuanmingense*, *B. japonicum*, *B. lianingense*, and *B. diazoefficiens*), *Bradyrhizobium* sp. S23321 (non-symbiotic strain), and *Rhodopseudomonas* members. Three stains from rice tissues including SUT-R3, SUT-R55, SUT-R74 and *A. americana* isolated strains (SUTN9-2, DOA1 and DOA9) belonged to this cluster, with strong bootstrap support (99%) separated from the cluster 1. In the cluster 3 of phylogenetic tree, only *B. elkanii* species were located. Finally, strain WD16 was located in the cluster 4 of phylogenetic tree. The phylogenetic tree based on sequences of *dnaK*, *recA*, and *glnB* was also constructed. The taxonomic positions of the bradyrhizobial strains in the combination tree (Figure 4.4) were almost concordant with their taxonomic positions in the 16S rRNA gene tree (Figure 4.3). In the combination tree, the strains were clearly separated into two clusters. In order to construct the correlation between phenotypic characteristics and phylogenetic positions, the photosynthetic pigment productions in bradyrhizobial strains were considered.

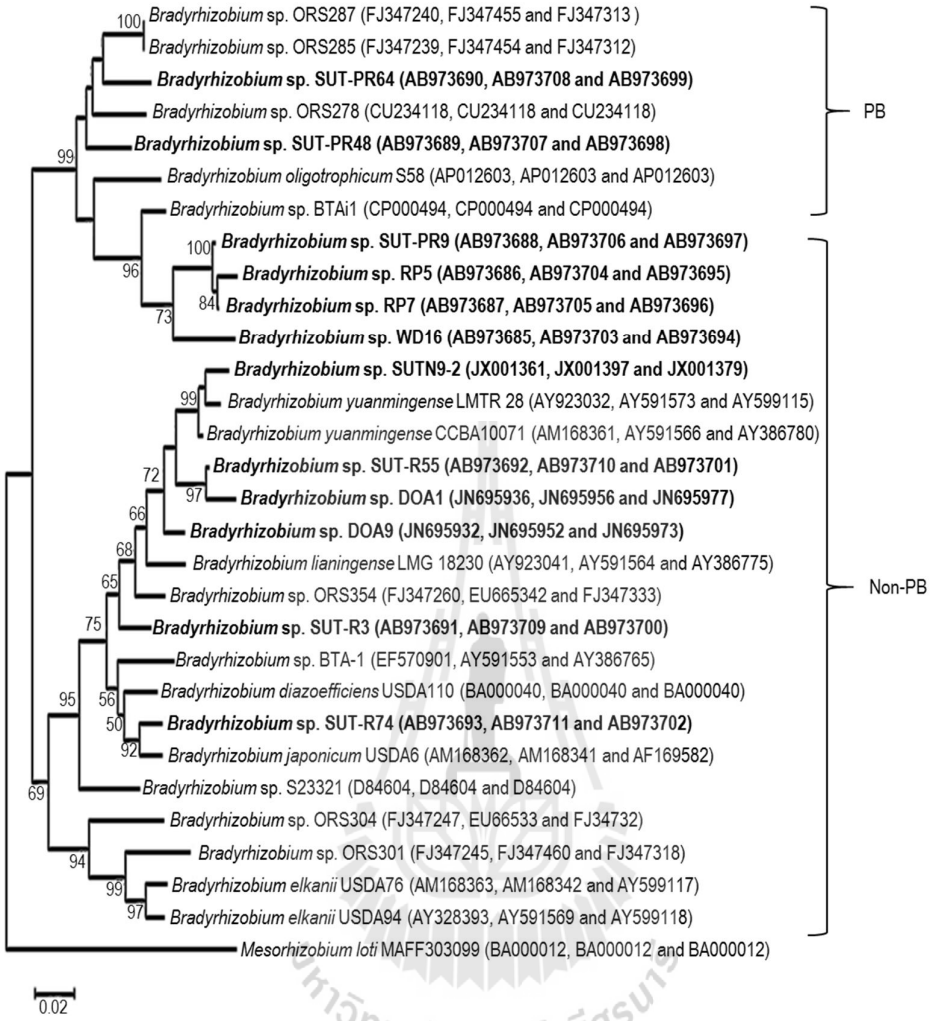

Figure 4.4 Maximum likelihood tree based on combined sequences of housekeeping genes (*dnaK*, *recA* and *glnB*), showing classification of photosynthetic bradyrhizobia (PB) and non-photosynthetic bradyrhizobia (Non-PB) strains isolated from rice tissues. Bootstrap values are expressed as percentages of 1,000 replications. The bar represents 1 estimated substitution per 100 nucleotide positions.

All of the strains formed typical slow-growing *Bradyrhizobium* colonies on HM agar plate (4-7 days). From a total of 9 strains, the strains SUT-PR48 and SUT-PR64

could synthesize the pink/orange pigments when cultured on yeast-mannitol agar and broth, whereas the pigments formation could not be detected from strains DOA1, DOA9, SUTN9-2, SUT-R3, SUT-PR9, SUT-R55, SUT-R74 and Japanese bradyrhizobial strains (Figure 4.5).

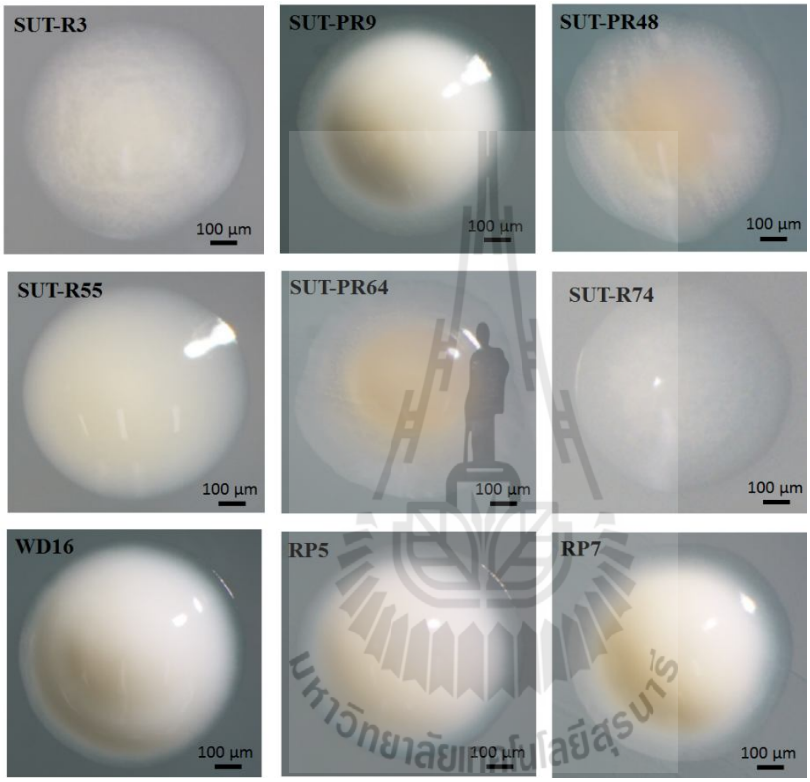

Figure 4.5 Phenotypic colonies of all bradyrhizobial isolated strains

In order to confirm photosynthetic characteristics on the basis of photosynthetic pigment production, the extraction of pigment using acetone-methanol method was carried out. The results showed also the same trend as the results obtained from colony color formations. (Figure 4.6).

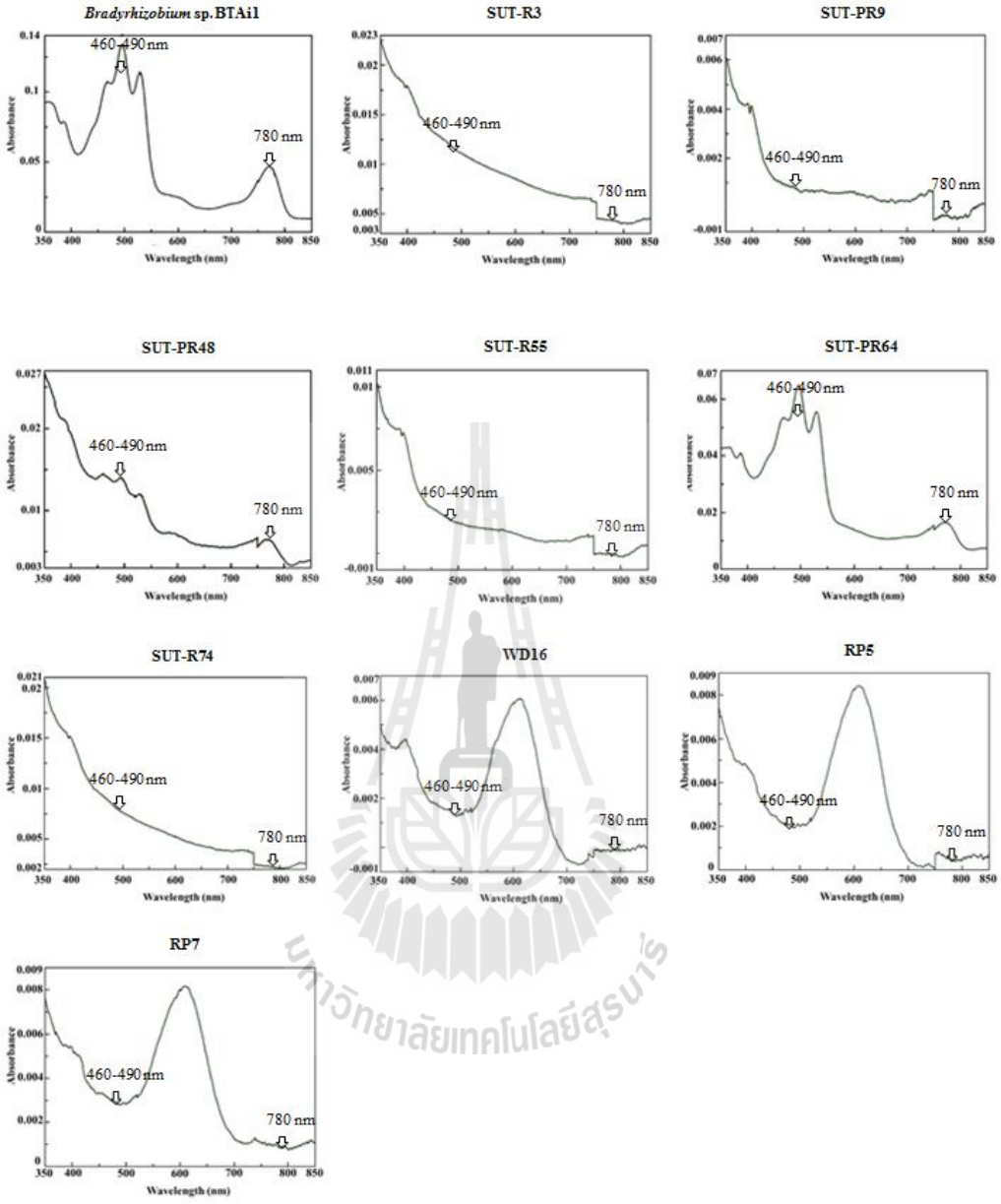

Figure 4.6 Absorption spectra of acetone-methanol (7:2, vol/vol) extracts of *Bradyrhizobium* sp. BTAi1, and all rice isolates in this study

In addition, the light-harvesting complex I (*puf*) and bacteriochlorophyll biosynthesis (*bch*) genes were also used to determine the photosynthetic related genes from every

isolates. Degenerated primers were designed from both of the photosynthetic bacteria, *Bradyrhizobium* sp. and *Rhodopseudomonas palustris*. The *pufM* was perfectly conserved in *Bradyrhizobium* strains ORS285, BTAi1, SUT-PR48 and SUT-PR64. However, the *pufM* bands were still detected from non-photosynthetic *Bradyrhizobium* strains S23321. In contrast, the specific band of *pufM* gene could not be detected in the strains USDA110, SUT-R3, SUT-PR9, SUT-R55, SUT-R74, WD16, RP5 and RP7. The *bchL* gene was amplified from *Bradyrhizobium* strains BTAi1, ORS285, SUT-PR48 and SUT-PR64, whereas the specific band of *bchL* gene could not be detected from *Bradyrhizobium* strains USDA110, S23321, SUT-R3, SUT-PR9, SUT-R55, SUT-R74, WD16, RP5 and RP7 (Figure 4.7).

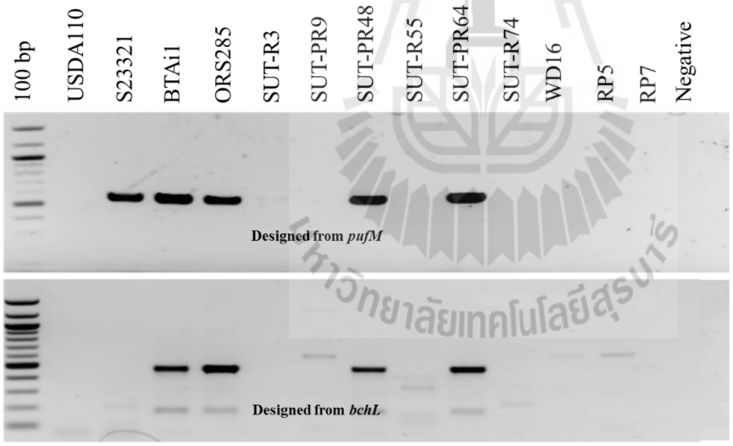

Figure 4.7 Photosynthetic related genes determination: the PCR amplification of *pufM* and *bchL* was carried out. Degenerate primers were designed from both the photosynthetic *Bradyrhizobium* sp. and their closest photosynthetic relative *Rhodopseudomonas palustris*.

The results from phylogenetic tree analyses were mainly congruent with pigment production characteristics as described before. On the basis of phylogenetic tree and phenotypic properties, endophytic bradyrhizobial strains might be classified into two main groups: PB and Non-PB groups. Nevertheless, the strains SUT-PR9, RP5, RP7 and WD16 separated slightly from PB cluster. To confirm again the photosynthetic property of this group, strain SUT-PR9 was chosen as the representative of strain RP5 and RP7 to determine the pigment production by another method described by Chaintreuil et al. (Chaintreuil et al., 2000). The photosynthetic pigment at 800 and 870 nm was not detected from SUT-PR9 (Figure 4.8).

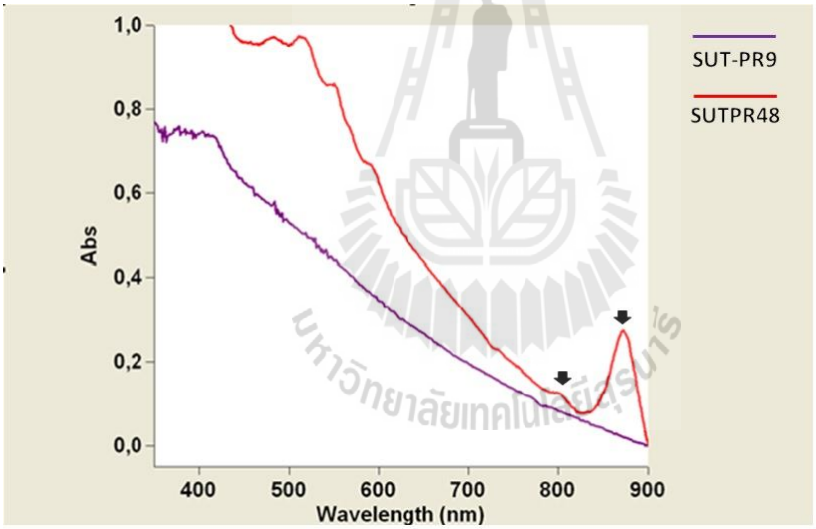

Figure 4.8 Photosynthetic pigment determination of strains SUT-PR9 and SUT-PR48; Absorption spectra were obtained by scanning over a wavelength range from 350 to 900 nm with medium speed. Photosynthetic pigment peaks present at 800 and 870 nm.

Therefore, strains SUT-PR9, RP5, RP7 and WD16 were classified as Non-PB strain based on photosynthetic pigment production (Figure 4.8).

4.3 Rice growth promotion

To examine whether the isolated strains are rice endophytes, they were tagged with GUS reporter gene, and observed under light microscopy (Figure 4.9).

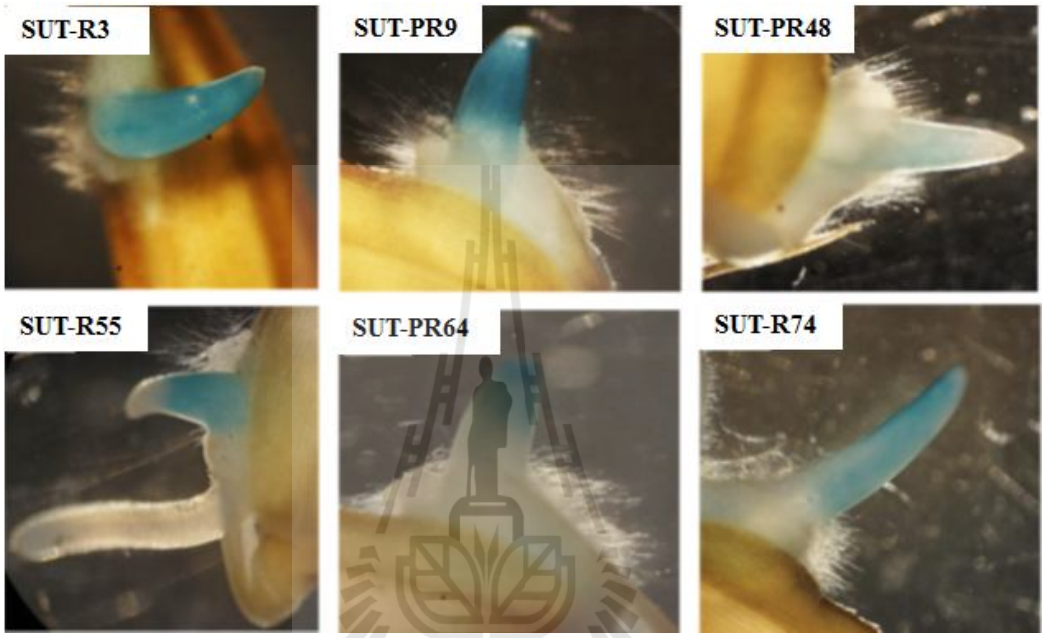

Figure 4.9 The colonization of putative endophytic bradyrhizobial strains

The effects of bradyrhizobial strains on rice biomass in Leonard's jar were compared among three rice cultivars. In this experiment, nitrogen source for plant at 0, 0.1, and 1 mM NH_4NO_3 was applied into N-free medium. Among three concentrations of nitrogen source, only the 0.1 mM NH_4NO_3 showed significant rice growth promotion between bradyrhizobial inoculation treatments and un-inoculation control (data not shown). Therefore, the 0.1 mM NH_4NO_3 was further used to determine the rice growth promotion (Figure 4.10).

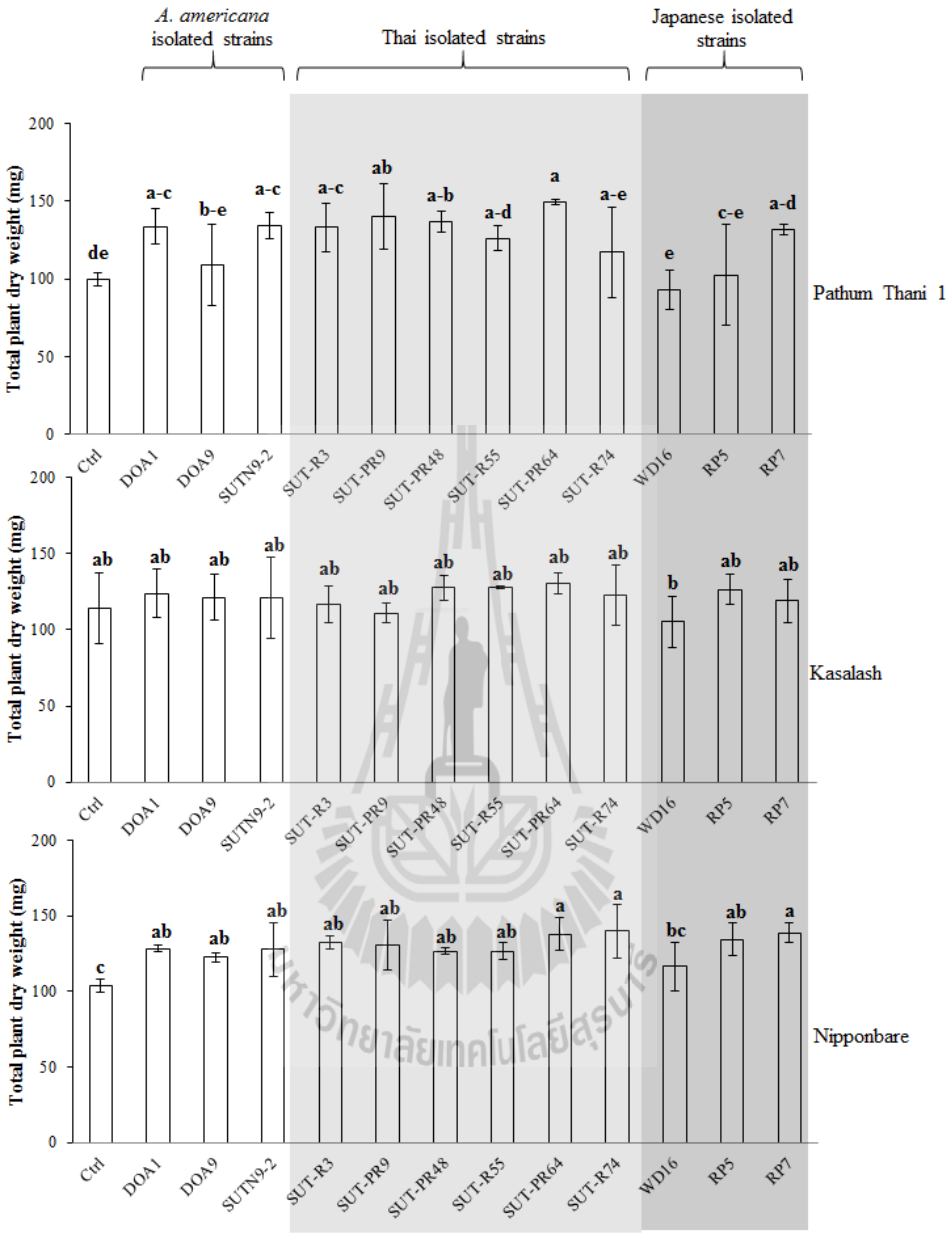

Figure 4.10 The effects of putative rice endophytic bradyrhizobium on rice biomass in Leonard's jar were compared among three rice cultivars (*O. sativa* L. ssp. *indica* cv. Pathum Thani 1, *O. sativa* L. ssp. *indica* cv. Kasalash and *O. sativa* L. ssp. *japonica* cv. Nipponbare).

The rice growth promotion given by Thai bradyrhizobial strains was significantly higher than that of un-inoculated control, except strains SUT-R55 and SUT-R74, which were not able to promote rice growth when compared with un-inoculated control. The tendency of strains SUT-PR9 and SUT-PR64 could promote higher rice biomass than other strains. In addition, the strains SUT-PR9 and SUT-PR64 could enhance rice biomass 40% and 50% higher than un-inoculated control treatment, respectively. Among the Japanese bradyrhizobial strains, only strain RP7 was able to promote higher rice biomass (cultivar Pathum Thani 1) than un-inoculated control but without a statistical difference (32% higher than un-inoculated treatment). Generally, Thai bradyrhizobial strains could promote rice growth better than those of Japanese bradyrhizobial strains especially when inoculated with Thai rice cultivars, whereas the rice growth promotion of cultivar Kasalash could not clearly be observed from all bradyrhizobial strains. Nevertheless, almost all of the bradyrhizobial strains (except WD16) could promote rice cultivar *O. sativa* Nipponbare significantly higher than un-inoculated control.

Because bradyrhizobial isolates from *A. americana* nodules (DOA1, DOA9 and SUTN9-2) were phylogenetically intermixed in rice bradyrhizobia (Figure 4.3), the inoculations of rice cultivar *O. sativa* Pathum Thani 1 with strains DOA1 and SUTN9-2 were also tested and could significantly promote higher rice biomass than that of un-inoculated control as well. In contrast, the DOA9 did not show the effectiveness in rice growth promotion.

A bacterial strain performing the highest rice growth promotion in each rice cultivar was selected. The strain SUTN9-2 was chosen to represent as *A. americana*

isolated strain, while the SUT-R3 and SUT-PR64 were deputized for Thai rice isolated strains from Non PB and PB, respectively. Besides, the Japanese strain RP7 was also selected to determine rice growth promotion in 4 more Thai rice cultivars (Figure 4.11).

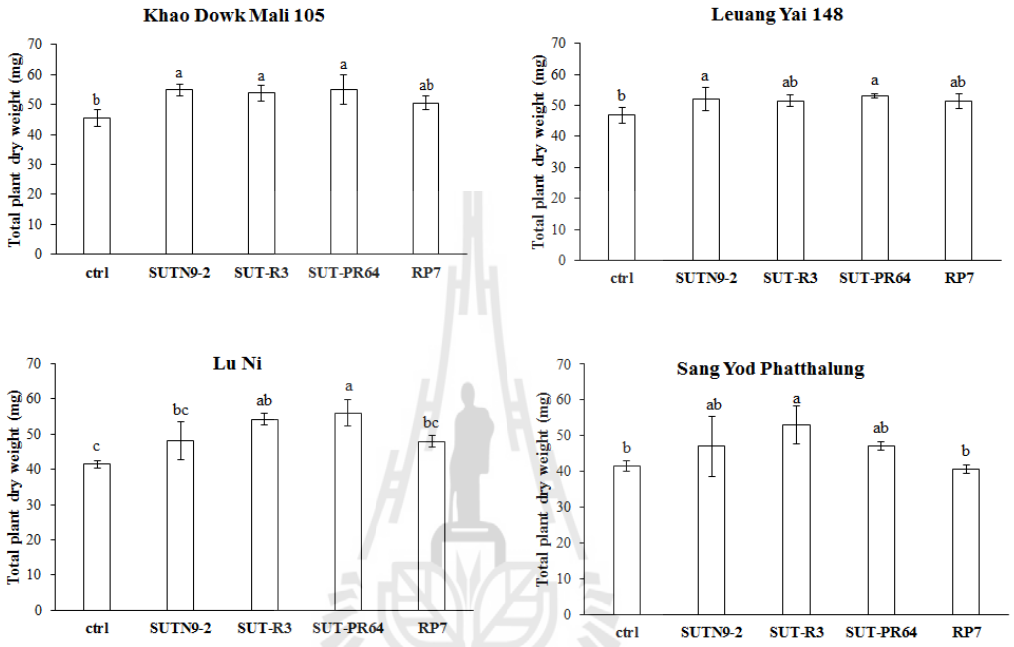

Figure 4.11 The effects of putative rice endophytic bradyrhizobium on rice biomass in Leonard's jar were compared among four rice cultivars (*O. sativa* L. ssp. *indica* cv. Khao Dowk Mali 105, *O. sativa* L. ssp. *indica* cv. Leuang Yai 148, *O. sativa* L. ssp. *indica* cv. Lu Ni and *O. sativa* L. ssp. *indica* cv. Sang Yod Phatthalung.

For rice cultivar *O. sativa* Khao Dowk Mali 105, strains SUTN9-2, SUT-R3 and SUT-PR64 could promote rice growth significantly better than those of un-inoculated control and Japanese strain. Likewise, with rice cultivar *O. sativa* Leuang Yai 148, both strains of SUTN9-2 and SUT-PR64 yielded a positive effect on rice growth promotion. In case of rice cultivar *O. sativa* Lu Ni, strains SUT-R3 and SUT-PR64 could promote rice

growth significantly higher than that of un-inoculated control. As the same trend of rice cultivar *O. sativa* Sang Yod Phatthalung, the strain SUT-R3 could promote significantly higher rice biomass than that of un-inoculated control. In contrast, the rice growth promotion derived from RP7 inoculation did not affect all of Thai rice cultivars (Figure 4.10 and Figure 4.11).

4.4 Endophytic bradyrhizobium population from different rice cultivars

The putative Thai rice endophytic bradyrhizobial populations in rice root were around 2.5 $\log_{10}$ CFU/g root fresh weight in rice cultivar *O. sativa* Pathum Thani 1 at 7 dai (Figure 4.12A). The tendency of bacterial population in tissue of rice cultivar Pathum Thani 1 was significantly higher than those of cultivars *O. sativa* Kasalash and Nipponbare while bradyrhizobial populations of *O. sativa* Kasalash and Nipponbare were around 1.0 $\log_{10}$ CFU/g root fresh weight at 7 dai.

Since most of the putative Thai rice endophytic bradyrhizobial strains presented the better response to *O. sativa* Pathum Thani 1 than other cultivars, the bacterial colonization of this cultivar was further determined (Figure 4.12B). The bacterial populations among Thai strains were not significantly different at 7 dai, but the population significantly increased at 30 dai, except the densities of SUT-R3 and SUT-R55, which were not significantly different at each dai. The proliferation of all strains showed the highest numbers at 30 dai, then the numbers slightly declined when plant age increased (Figure 4.12B). However, the population densities of the SUT-PR9 and

SUT-PR64 still prolonged their population until 60 dai. In addition, the populations of both SUT-PR9 and SUT-PR64 strains were higher than those of other strains. However, all strains still persisted in rice root tissues at 90 dai while the bacterial cell numbers of strain SUT-PR9 were significantly higher than those of all tested strains.

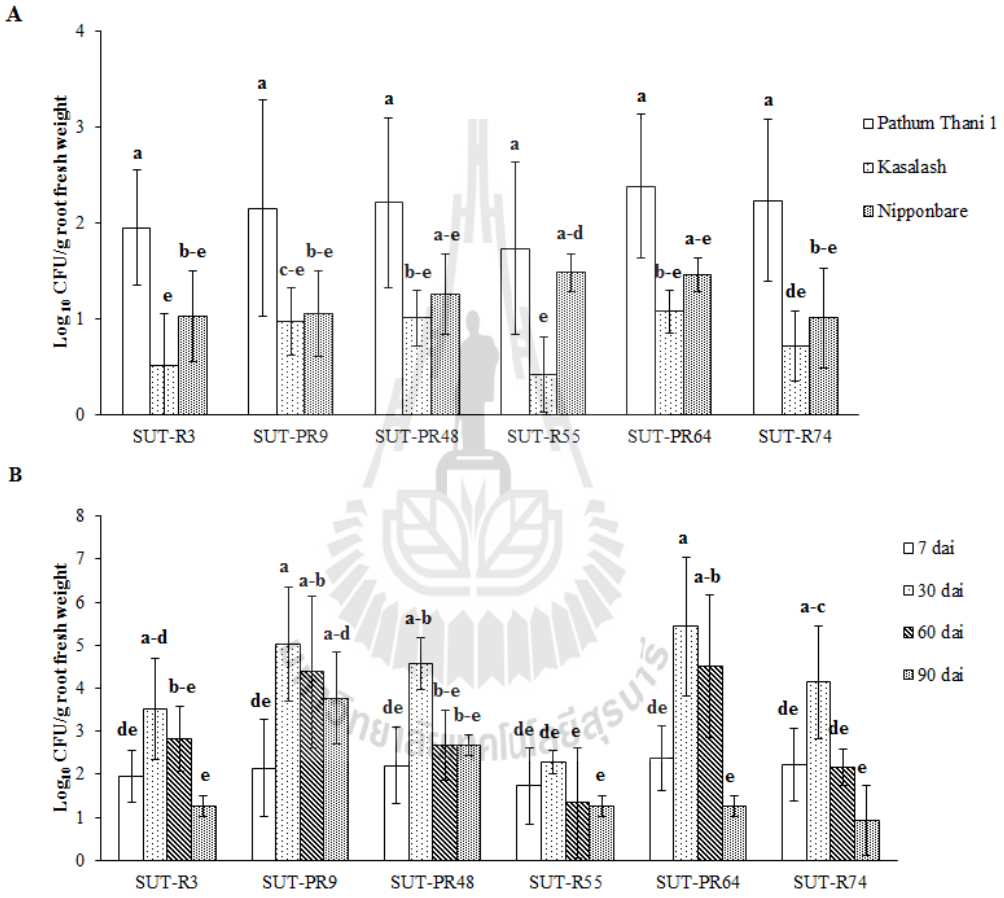

Figure 4.12 Enumuration of bradyrhizobia in rice root tissues: (A) The putative Thai rice endophytic bradyrhizobial populations strains in rice root of three rice cultivars (*O. sativa* L. ssp. *indica* cv. Pathum Thani 1, *O. sativa* L. ssp. *indica* cv. Kasalash and *O. sativa* L. ssp. *japonica* cv. Nipponbare) were determined at 7 dai, (B) The putative Thai rice endophytic bradyrhizobial populations strains in rice root of *O. sativa* L. ssp. *indica* cv. Pathum Thani 1 at each dai.

In addition, 6 more rice cultivars were also tested in terms of bacterial colonization in rice root tissues (same as in Figure 4.11 and including 2 Japanese cultivars). Four bacterial strains performing the highest rice growth promotions, SUTN9-2, SUT-R3, SUT-PR64 and RP7, were also used. For rice cultivar *O. sativa* Khao Dowk Mali 105, the populations of SUTN9-2, SUT-R3 and SUT-PR64 were significantly higher than that of RP7 at 7 dai. The cell numbers of both SUTN9-2 and SUT-PR64 strains could be found higher in rice cultivar *O. sativa* Leuang Yai 148 and Lu Ni than that of RP7. In case of *O. sativa* Sang Yod Phatthalung, all tested strains could increase their cells around 2.5 $\log_{10}$ CFU/g root fresh weight, except Japanese strain RP7 that showed a clearly low amount of bacterial population (Figure 4.13).

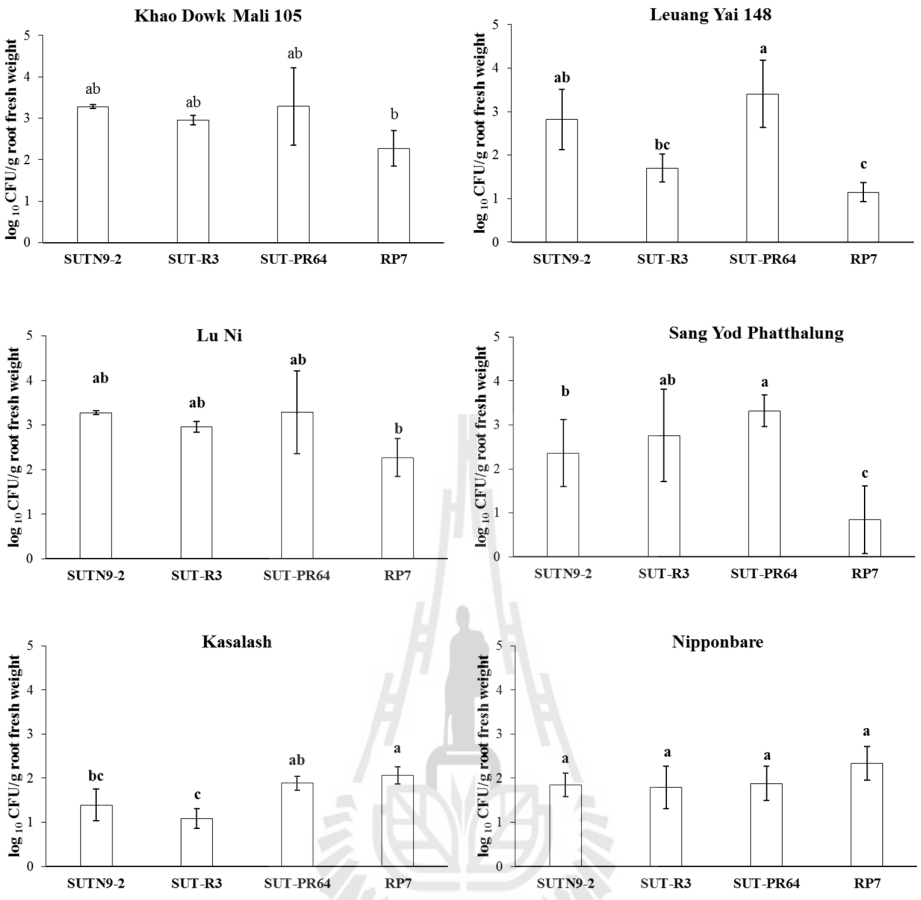

Figure 4.13 Enumuration of bradyrhizobia in rice root tissues: A bacterial strain performing the highest rice growth promotion in each rice cultivar was selected. Strain SUTN9-2 was represented as *A. americana* isolated strain, strains SUT-R3 and SUT-PR64 were deputized for Thai rice isolated strains from Non PB and PB, respectively, the Japanese strain RP7 was also selected to determine rice growth promotion in 6 more Thai rice cultivars (*O. sativa* L. ssp. *indica* cv. Khao Dowk Mali 105, *O. sativa* L. ssp. *indica* cv. Leuang Yai 148, *O. sativa* L. ssp. *indica* cv. Lu Ni and *O. sativa* L. ssp. *indica* cv. Sang Yod Phatthalung, *O. sativa* L. ssp. *indica* cv. Kasalash and *O. sativa* L. ssp. *japonica* cv. Nipponbare.

Since strains SUT-PR9 and SUT-PR64 promoted the highest rice biomass in Leonard's jar experiment, both strains were tagged with GUS/DsRed reporter genes to determine their localization *in planta*. Both strains were capable of the rapid colonization of rice root as in cortical cell layers and xylem occupation of rice roots at 3 dai (Figure 4.14A and Figure 4.14B).

However, the colonization of strain SUT-PR9 was especially dense at the surface and root hair zones of rice root at 7 dai (Figure 4.14C and Figure 4.14D), while the strain SUT-PR64 was able to heavily colonize and invade the rice tissues from epidermis cell to endodermis cell at 7 dai (Figure 4.14E and Figure 4.14F).

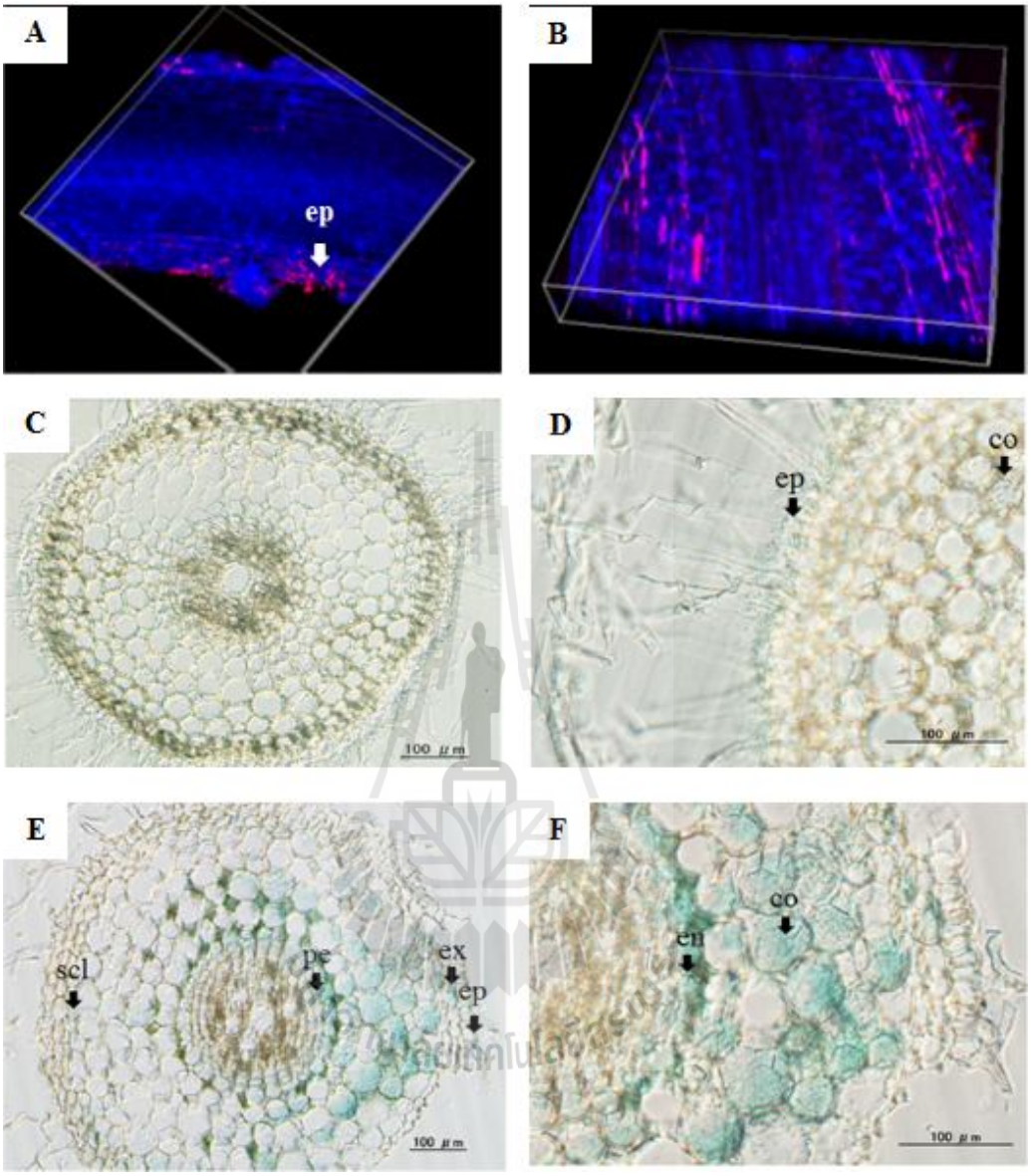

Figure 4.14 The localization of bradyrhizobial strains SUT-PR9 and SUT-PR64 at 3 dai. (A and B) the localization of rice roots by DsRed-tagged SUT-PR9 and SUT-PR64 respectively. (C and D) the localization of rice roots by GUS-tagged SUT-PR9 at 7 dai., (E and F) the localization of rice roots by GUS-tagged SUT-PR64 at 7 dai. The letters showed the bacterial localization inside rice root tissues: (ep); epidermis, (en); endodermis, (ex); exodermis, (co); cortex and (scl); sclerenchyma.

4.5 Nodulation test and *A. indica* growth promotion

The types of leguminous plants were selected on the basis of their frequent presence in rice field environments as genus *Aeschynomene* including *A. americana* and *A. indica*. Besides, the commercial leguminous plants including *V. radiata* and *G. max* normally used for rice-legume crop rotation system in Thailand were also included. The results of nodulation test with the various leguminous plants are summarized in Table 4.2.

Table 4.2 Plant nodulation test in this study

***Bradyrhizobium* strains**	**Nodulation on [a]**					
	A. americana	***A. indica* (Africa)**	***A. evenia***	***M. atropurpureum***	***G. max***	***V. radiata***
PB						
SUT-PR48	-	+	+	-	-	-
SUT-PR64	-	+	+	-	-	-
BTAi1	-	+	+	-	-	-
Non-PB						
SUT-PR9	-	+/-	+/-	-	-	-
WD16	-	+/-	+/-	-	-	-
RP5	-	+/-	+/-	-	-	-
RP7	-	+/-	+/-	-	-	-
SUT-R3	-	-	-	-	-	+
SUT-R55	+	-	-	+	-	+
SUT-R74	-	-	-	-	-	-
USDA110	-	-	-	+	+	+

[a] +: nodules number was higher than 50 nodules/plant with reddish nodules.

+/-: nodules number was lower than 10 nodules/plant with white nodules.

-: no nodules were detected.

The PB strains including SUT-PR48 and SUT-PR64 (Figure 4.3) could produce up to 100 nodules per plant of CI group III, *A. indica* and *A. evenia* at 28 dai. However, only the strain SUT-PR9 (Non-PB) can form nodules in *A. indica* and *A. evenia* but with less number (tentative 5 nodules per plant). In contrast, the nodulations of *A. indica* and *A. evenia* could not be detected when inoculated with Non-PB group strains. Only the strain SUT-R3 formed nodules in *V. radiata*. The strain SUT-R55 could effectively nodulate *A. americana*, *M. atropurpureum* and *V. radiata* while SUT-R74 could not nodulate all leguminous plants tested. *B. diazoefficiens* USDA110 had the ability to form root nodules with *M. atropurpureum*, *G. max* and *V. radiata*, while *Bradyrhizobium* sp. BTAi1 could only form root/stem nodules with *A. indica* and *A. evenia*.

Among the Thai isolated strains, SUT-PR9 was used as a Non-PB representative although it was closely related to PB strains. Therefore, *A. indica* as an appropriate plant for PB groups was used for evaluating the symbiotic efficiency. Total plant dry weights derived from PB strains SUT-PR48 and SUT-PR64 were significantly higher than those of Non-PB strains inoculation (Figure 4.15), while SUT-PR9 showed low efficiency for *A. indica* growth promotion and its nitrogen fixation when compared with PB strains, SUT-PR48 and SUT-PR64 (Figure 4.15).

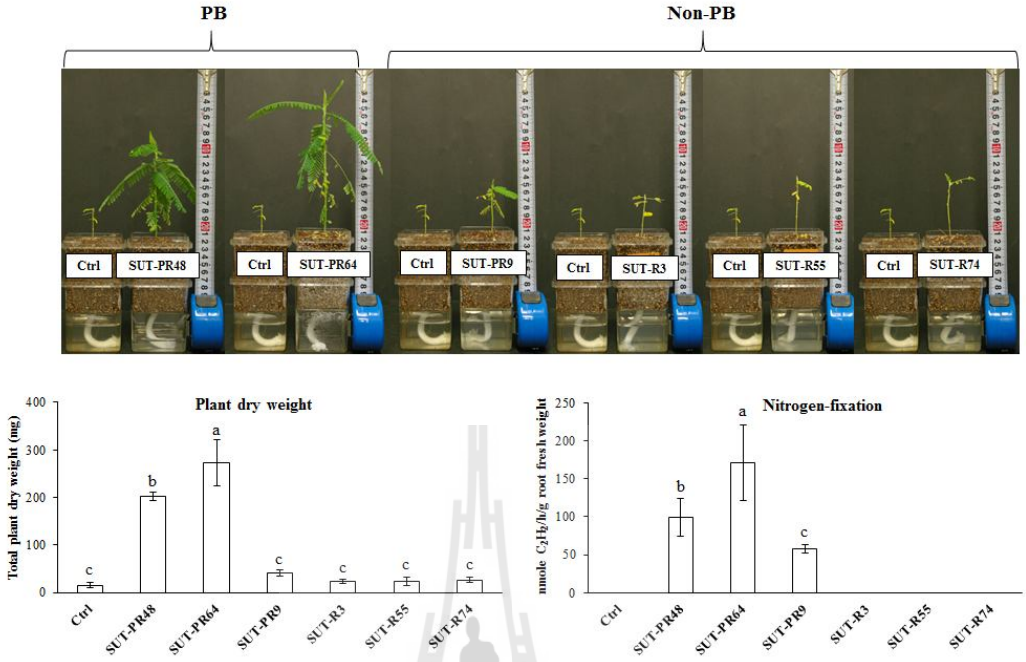

Figure 4.15 *A. indica* growth promotion and nitrogen-fixation : (PB); Photosynthetic *Bradyrhizobium* and (Non-PB); Non-Photosynthetic *Bradyrhizobium*

To detect the nodulation genes among bradyrhizobial isolated strains, the PCR amplification of *nodA*, *nodB* and *nodC* was carried out. The primer sets were designed from *B. diazoefficiens* USDA110 and *Bradyrhizobium* sp. ORS285. None of specific band of *nodA*, *nodB* and *nodC* was obtained from putative endophytic bradyrhizobial strains (Figure 4.16).

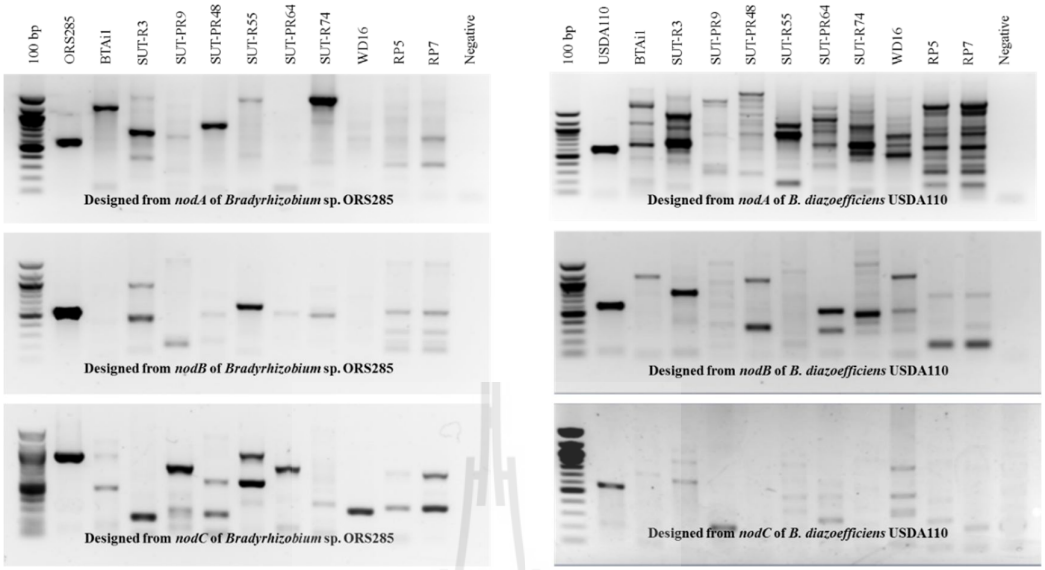

Figure 4.16 Nodulation gene determination: the PCR amplification of *nodA*, *nodB* and *nodC* was carried out. The primers set were designed from *B. diazoefficiens* USDA110 and *Bradyrhizobium* sp. ORS285.

Since the strain SUTN9-2 showed the better rice (*O. sativa* Pathum Thani 1) growth promotion than other *A. americana* nodulating strains, and the bradyrhizobial populations of SUTN9-2 inside rice roots tissues were significantly higher than both strains of DOA1 and DOA9 (data not show). Therefore, the strain SUTN9-2 was selected for further examination on the rice-*Bradyrhizobium* symbiotic mechanism. Prior to determine the symbiotic interaction of strain SUTN9-2, their localization *in planta* was determined by GUS reporter gene. The strain SUTN9-2 was capable of the rapid colonization of rice root as in cortical cell layers of rice roots at 7 dai. The main population of strain SUTN9-2 was especially dense at epidermis and endodermis cells of rice roots tissues (Figure 17A). Furthermore, the scanning electron microscope

clearly displayed the bradyrhizobial localization inside roots tissues (Figure 17B) and may localize intracellularly.

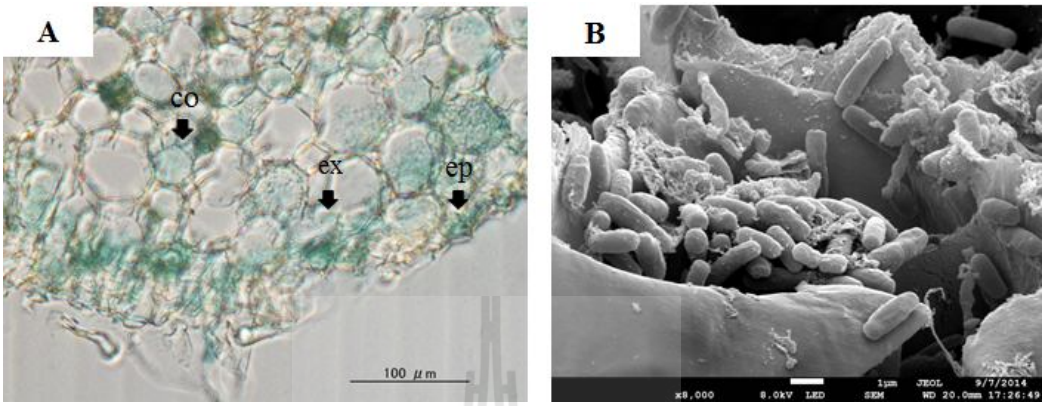

Figure 4.17 The SUTN9-2 localization *in planta* was determined by (A): GUS reporter gene and (B): scanning electron microscope.

4.6 Bradyrhizobial proteins induction with rice root exudate

In or der to determine the secreted proteins from tested bradyrhizobial strains from *A. americana* and rice root exudates, the SDS-PAGE (Figure 4.18A) and MALDI-TOF (Figure 4.18B) techniques were imployed . The strains USDA110 and USDA122 were also used for reference strains by using genistein as flavonoid derivative inducer. For the up-regurated band (red arrow) of strain SUTN9-2 were selected from SDS-PAGE gel and futher analyzed by MALDI-TOF-MS/MS. The proteins were identified by the MS-Fit software program. The result from band C was presented of flagella protein and band D was showed of cellulase.

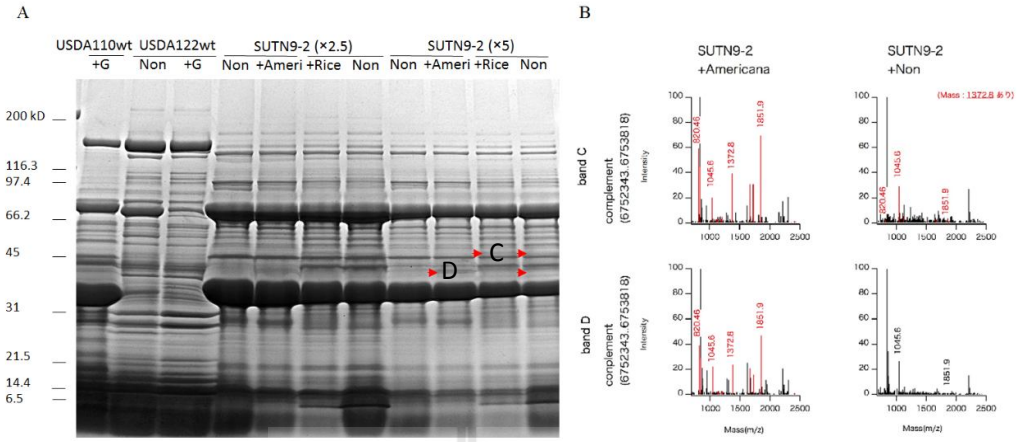

Figure 4.18 The protein analyses of root exudate induction (A)**:** Protein inductions of SUTN9-2 with *A. americana* and rice root exudates under SDS-PAGE analyses and (B): MALDI-TOF-MS/MS analysis of peptides derived from band "C and D" from wild-type *Bradyrhizobium* SUTN9-2. The amino acid sequences of respective mass fragments were identified by MS/MS analysis as follows: a, SSAPYGSFSR.

4.7 General draft genome description

The genome of *Bradyrhizobium* sp. SUTN9-2 is a circular chromosome of 8,060,204 bp with an average GC content of 63.4% (Figure 4.19). The interested nucleotide sequences from draft genome were converted to amino acid sequence then, the total ORFs were predicted using MataGeneAnotator (Noguchi et al., 2008). The function of predicted protein-coding genes were manually annotated through comparisons with

the NCBI nr and Swiss-Prot databases by a jamboree consortium (Okubo et al., 2011)

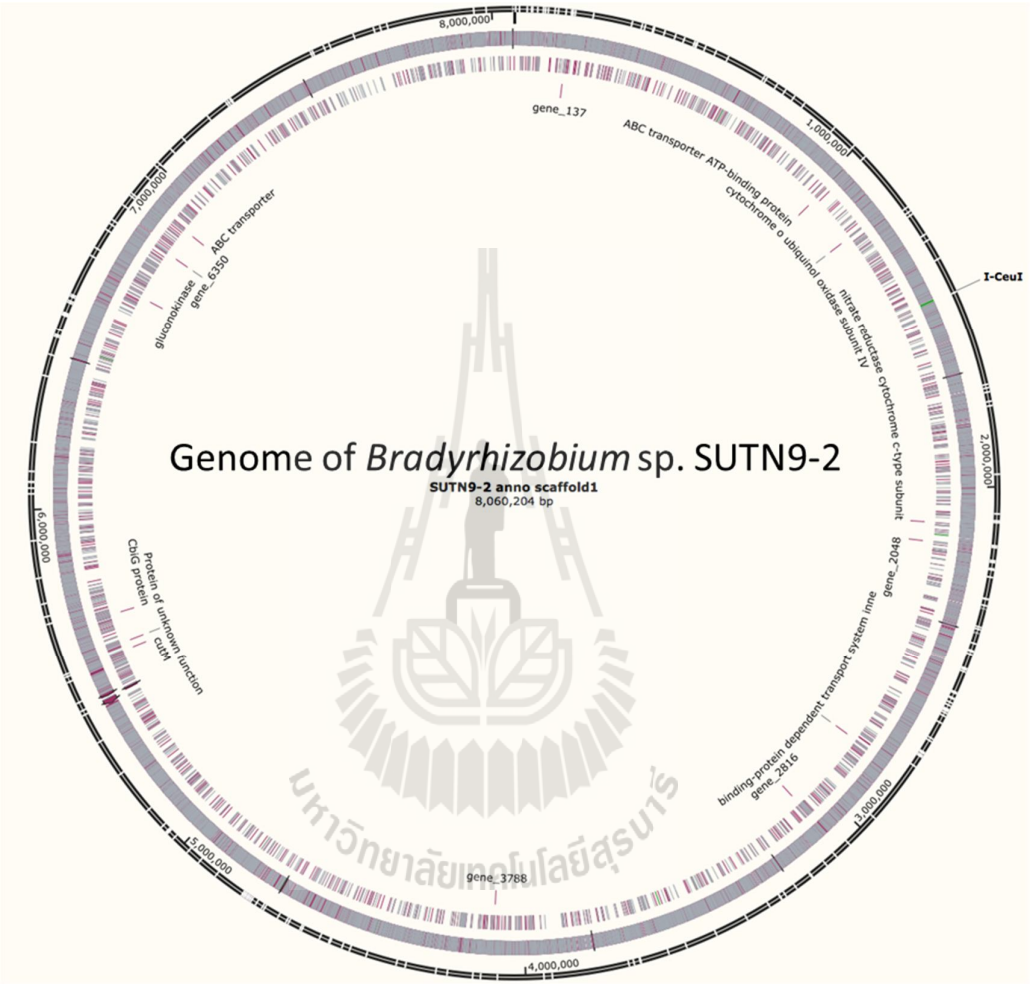

Figure 4.19 Circular representation of the chromosome of *Bradyrhizobium* sp. SUTN9-2

4.8 Genes expression of SUTN9-2 under supplementation of rice root exudate and in planta

In order to detect the genes expression in SUTN9-2 which involve in rice association at the early stage as 3 hours, the root exudate supplementation experiment was carried out. The genes expression were performed on RNA samples, using primers designed to target exoporysacharide production (*exoB*), flagella biosynthetic protein (*fliP*), type 3 secretion component (*rhcJ*), type 4 secretion component (*virD2*), pectiesterase (*peces*) and glutathione-s-transferase (*gst*) (Table 3.4). The relative expression levels of *exoB* gene from rice root exudate induction was significantly up-regulated higher than un-induction treatment (Figure 4.20). Whereas the relative expression levels of *fliP* gene was not significantly different between with and without root exudate inductions. Interestingly, the *rhcJ*, *virD4* and *peces* genes were only up-regulated when rice roots exudate were applied. Whereas *gst* expression was not detected at 3 hours of root exudate induction.

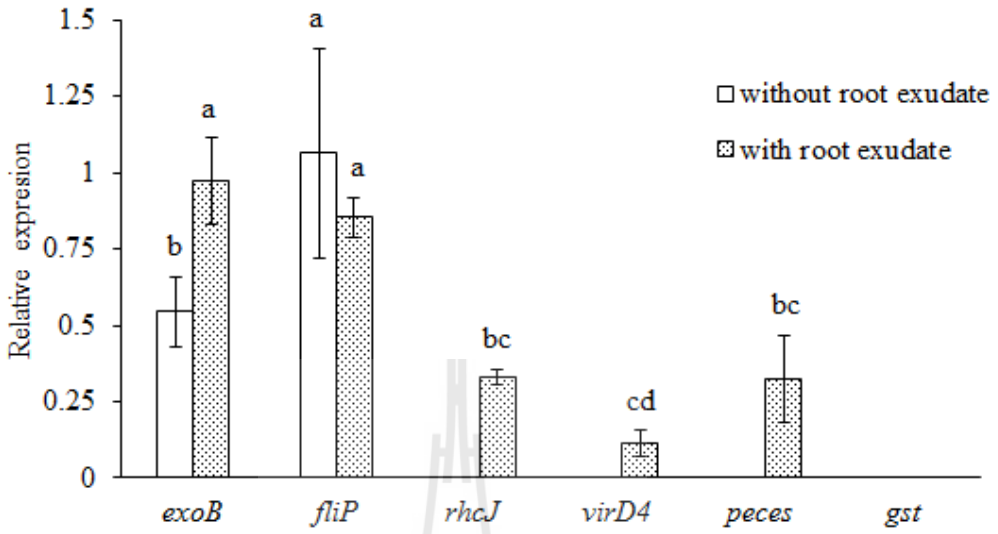

Figure 4.20 Some genes expression (with and without rice roots exudate induction): The letters inside parenthesis showed the target genes (*exoB*); exoporysacharide production, (*fliP*); flagella biosynthetic protein, (*rhcJ*); type 3 secretion component, (*virD4*); type 4 secretion component, (*peces*); pectiesterase and (*gst*); Glutathione-s-transferase.

In planta, several genes such as *exoB*, *rhcJ*, *virD4* and *peces* were highly expressed during 6-24 hours after inoculation (Figure 4.21). In addition, the genes encoding enzymes involved in the detoxification of reactive oxygen species (ROS), glutathione-*S*-transferases (*gst*) were slightly expressed after 12 hours after inoculation. Whereas *fliP* expressions slightly decreased when plant age increased.

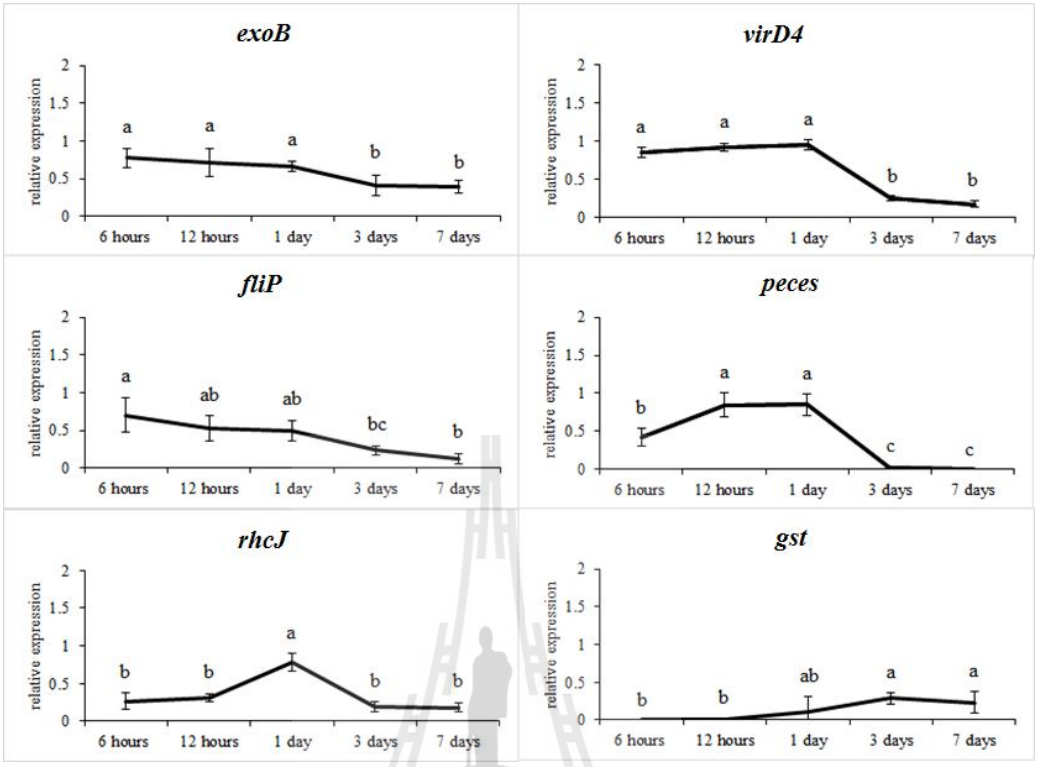

Fig 4.21 Some genes expression *in planta*: The letters inside parenthesis showed the target genes (*exoB*); exoporysacharide production, (*fliP*); flagella biosynthetic protein, (*rhcJ*); type 3 secretion component, (*virD4*); type 4 secretion component, (*peces*); pectiesterase and (*gst*); glutathione-s-transferase.

The copy number of bradyrhizobial genes expressed *in planta* was displayed in figure 4.22. Interestingly the copy number of the detoxification gene (*gst*) in strain SUTN9-2 genome were highest (26 copies) among tested genes.

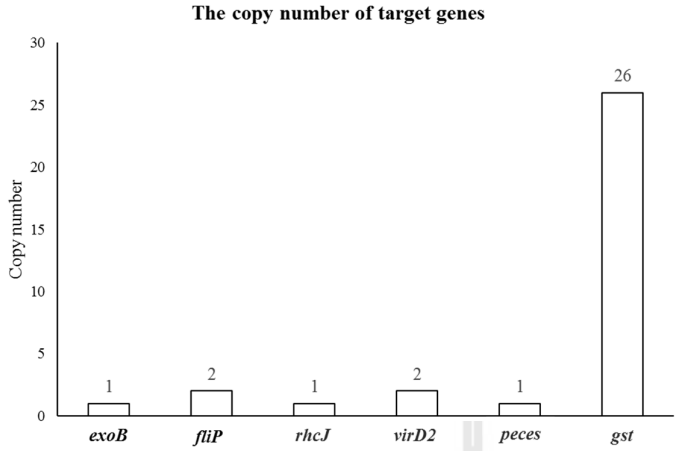

Figure 4.22 The copy number of target genes in draft genome sequence of SUTN9-2

4.9 Some genes organization in SUTN9-2

In order to determine the some genes organization of the *Bradyrhizobium* sp. SUTN9-2, the draft genome sequences were used to reveal the genes arrangement. Amino acid sequences comparison of T3SS clusters among non-endophytic *B. diazoefficiens* USDA110 with rice endophytic strains SUTN9-2 and DOA9 revealed low conserved sequence (figure 4.23). Several ORFs were not detected from rice endophytic strains SUTN9-2 and DOA9, for examples, genes of putative *nopE1* and *nopB*. In addition the amino acid sequences of T3SS between SUTN9-2 and DOA9 highly conserved among their genomes. The putative *nopA* and *nopX* only presented in T3SS cluster of rice endophytic strains (SUTN9-2 and DOA9) (Figure 4.23). Moreover, the putative *nopM* was only detected from T3SS cluster of strain SUTN9-2. These results was intriguing because the T3SS of strain SUTN9-2 was similar to rice

endophytic strain DOA9. Therefore, T3SS was selected to further gene deletion experiment.

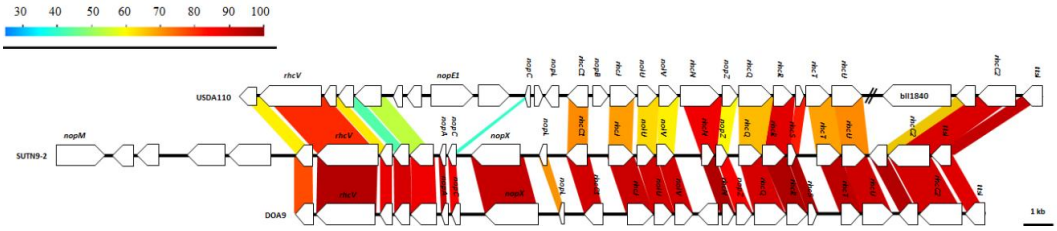

Figure 4.23 The gene comparison of the type three secretion cluster (T3SS) of strains USDA110, SUTN9-2 and DOA9

The type 4 secretion system (T4SS) of strain SUTN9-2 contained two clusters. For the T4SS cluster 1 displayed both in *B. diazoefficiens* USDA110 and SUTN9-2 and showed high similarly of amino acid sequences and genes organization, except that the directions of transcription regulator were different (Figure 4.24A). In contrast, the T4SS cluster arrangement of DOA9 partially differed from strain SUTN9-2 and amino acid sequences of strain DOA9 were absolutely different when compared with SUTN9-2 and USDA110. The genes arrangement in cluster 2 of T4SS of strain SUTN9-2 were highly similar to the T4SS of *Bradyrhizobium* sp. BTAi1 (Figure 4.24B). Therefore, the T4SS was not considered for further knockout experiment because the T4SS organizations and amino acid sequences of strain SUTN9-2 largely differed from rice endophytic strain DOA9.

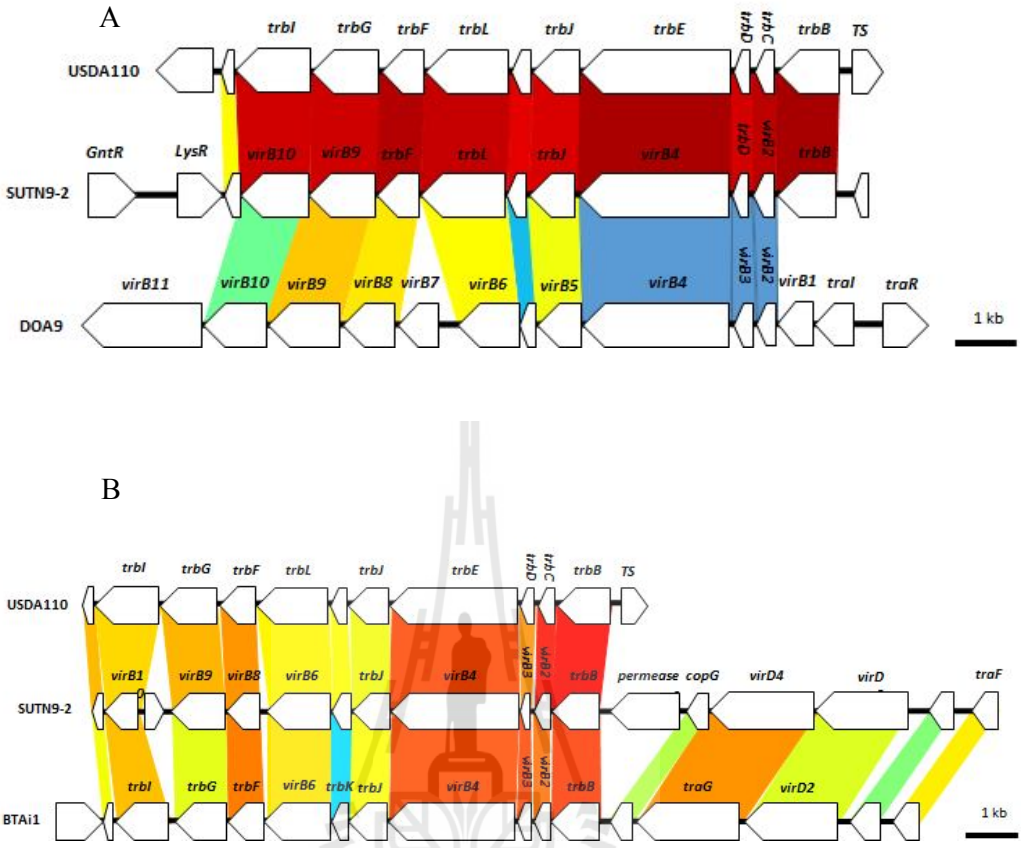

Figure 4.24 The gene comparison of the type four secretion cluster (T4SS) of strains USDA110, SUTN9-2 and DOA9: (A) T4SS cluster1 and (B) T4SS cluster 2

The flagella genes of SUTN9-2 were similar to those of *B. diazoefficience* USDA110 in terms of gene sequences and gene orientation (Figure 4.25). Most of the flagella genes were compactly clustered in two regions including cluster 1 and cluster2 (Figure 4.25A and Figure 4.25B, respectively). Perhaps the bacterial movement was interrupted if they lose the flagella apparatus. Thereby, the flagella genes of SUT9-2 were not considered further disruption experiment.

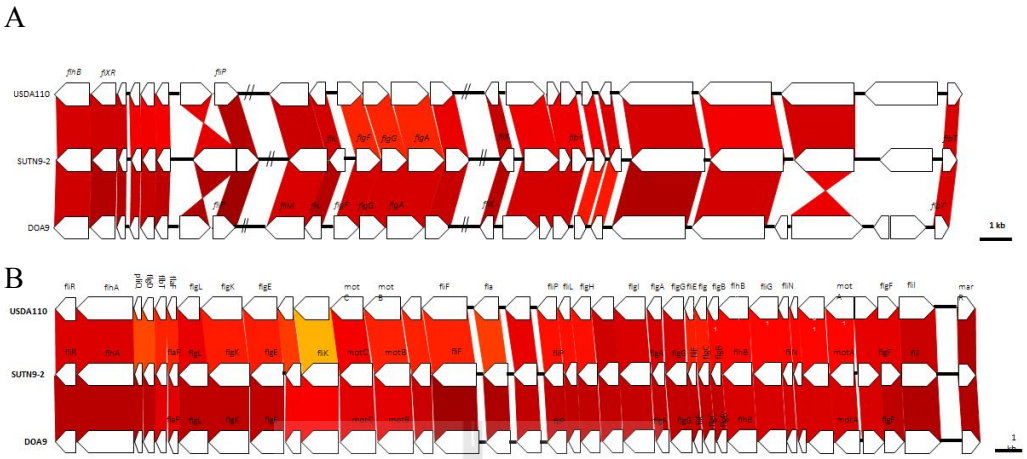

Figure 4.25 The gene comparison of the flagella components of strains USDA110, SUTN9-2 and DOA9: (A) flagella cluster1 and (B) flagella cluster 2

4.10 Symbiotic characteristics of T3SS mutants

The symbiotic characteristics of the SUTN9-2 wild type and the T3SS mutant strains (Δ*rhcJ*-3B, Δ*rhcJ*-18A and Δ*rhcJ*-27A) were tested with host *A. americana* and *O. sativa* cultivar Pathum Thani 1 (Figure 4.26).

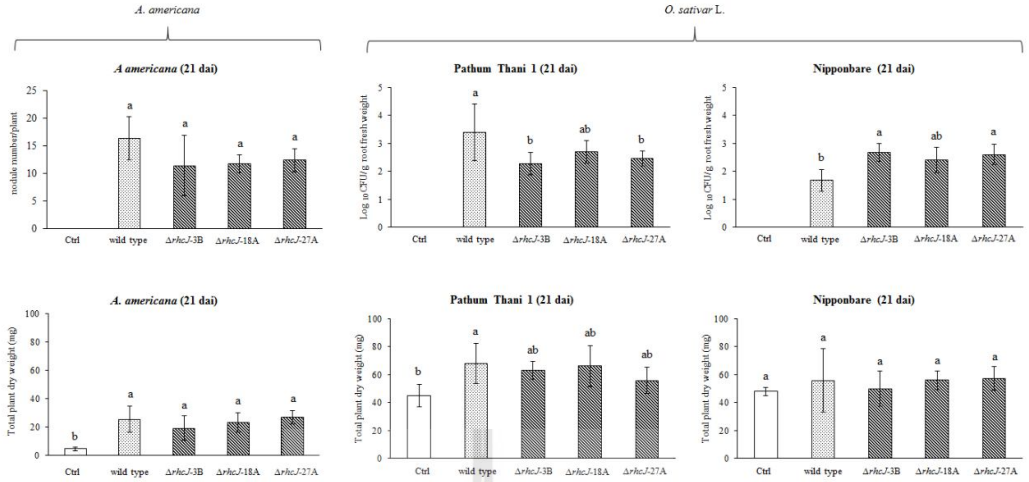

Figure 4.26 The symbiotic characteristics of the SUTN9-2 wild type and the T3SS mutant strains (Δ*rhcJ*-3B, Δ*rhcJ*-18A and Δ*rhcJ*-27A) were tested with host *A. americana*, *O. sativa* cultivar Pathum Thani 1and *O. sativa* cultivar Nipponbare.

On genuine host, the nodule numbers formed by mutant strains were not significantly different when compared with wild type strain, but the tendency of nodules numbers derived from *rhcJ* mutant strains were slightly lower than in wild type strain. The plant growth promotions were not statistically different between wild type and *rhcJ* mutant strains inoculations (Figure 4.26). In case of *O. sativa* cultivar Pathum Thani 1, the population numbers of mutant strains from rice tissues were significantly lower than that of wild type strain (approximately 1 magnitude), except strain Δ*rhcJ*-18A. On the contrary, the populations of mutant strains were statistically higher than that of wild type strain when they were tested with *O. sativa* L. ssp. *japonica* cv. Nipponbare (Figure 4.26). The rice growth promotions of cultivar Pathum Thani 1 were not significantly different between wild type and mutants strains inoculations. However

the wild type strain was able to promote higher rice biomass (cultivar Pathum Thani 1) than un-inoculated control. The *rhcJ* mutant strains could enhanced rice biomass when compared with un-inoculated control but without a statistical difference. In case of rice cultivar Nipponbare, the rice growth promotions were not detected when inoculated with both of wild type and *rhcJ* mutant strains (Figure 4.26).

From previous report suggested that the *Bradyrhizobium* sp. SUTN9-2 was isolated from the root nodules of *A. americana* and can nodulate a wide range of leguminous plants including *Macroptilium atropurpureum*, *Arachis hypogaea*, *Vigna radiata* and *A. americana* (Noisangiam et al., 2012). To compare the function of T3SS with some leguminous plants, therefore the symbiotic phenotype of wild type SUTN9-2 and *rhcJ* mutant strains were also tested with two leguminous plant including *V. radiata* and *M. atropurpureum*. The wild type strains could form roots nodules with *V. radiata* L. cv. SUT4 significantly higher than those of *rhcJ* mutant strains. Whereas the wild type strain presented low efficiency for *V. radiata* growth promotion when compared with the mutant strains. But the amount of reddish nodules (approximately 10 to 15 nodules/plant) were detected from mutant strains (data not show).On the *M. atropurpureum* showed similar results with *V. radiata* L. cv. SUT4, which the mutant strains could establish the number of symbiotic roots nodules lower than that of wild type strain (Figure 4.27).

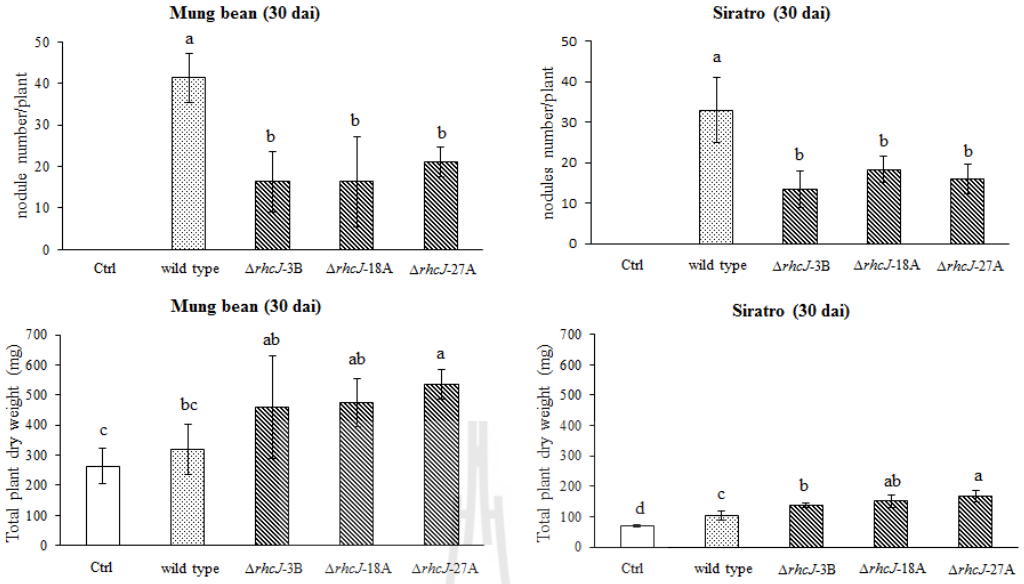

Figure 4.27 Nodulation test and plants growth promotion of wild type SUTN9-2 and Δ*rhcJ* mutant strains were determined with some leguminous plants (Mung bean and Siratro).

CHAPTER V

DISCUSSION

Prior to understand the behavior of bradyrhizobia as endophyte in rice, the advantages of oligotrophic-based isolation were used to reduce the growth of other contaminants, while the bradyrhizobia still survived under nutrients deprived (oligotrophic) conditions. The incubation of homogenates solution as low as 1 ml into large amount of water as 50 ml for 1 month was mimiced the oligotrophic condition. Therefore, almost all of rice oligotrophic endophytic brydyrhizobia in this study were obtained except SUT-R74. Proportion of bradyrhizobial strains and other bacterial endophytes on the basis of culture-dependent approach showed less amount of bradyrhizobia in rice tissues (6 bradyrhizobial strains from 98 bacterial strains). It seemed that the putative endophytic bradyrhozobia may not be dominant species in cultivated rice. This may support the previous report which revealed that proteobacteria are the major endophytic bacteria in rice tissues. Among alpha-proteobacteria (approximately 30% relative abundance of all subdivisions), *Bradyrhizobium* are only found in rice root with 10% relative abundance of total alpha-proteobacteria (Ikeda et al., 2014). The bradyrhizobial isolation was successfully obtained from rice/legume crop rotation system. In contrast, the endophytic bradyrhizobia could not be detected from monoculture system (2-3 crops/year). The data were congruent with Guong et al. (Guong et al., 2012), which revealed that *Bradyrhizobium* sp. and *Herbaspirillum* sp. instantly colonize the interior of rice roots when grown in rotation with a legume crop. Besides, the bacterial community from rice-legumes crop rotation system was

significantly different and higher than those in rice monoculture. Therefore, the appropriate crop rotations provide a feasible practice to maintain the equilibrium in soil microbial environment. These observations raise the hypothesis that the agricultural practice conditions may drive the evolution of bradyrhizobia-host association. This hypothesis may also gain more supportive evidences since *A. americana* bradyrhizobial strains (DOA1, DOA9 and SUTN9-2) perform rice endophytic properties. Furthermore, the bradyrhizobial strains nodulating *A. americana* in this study have never been detected in the paddy soil (Table 3.2), but they can survive during the period without the genuine host, and reform nodules in the following year. They may proliferate their cells in rice tissues even though *A. americana* cannot grow well each year from December to July the following year (tentatively seven months). Actually, *A. americana* is the leguminous weed grown in rice field for only five months a year (from July to December). Therefore, these results may be a part of evidence that confirms the bradyrhizobia-host evolution.

Plant growth promotion properties of rhizobia have been previously reported for various cereals such as wheat, maize and rice (Prévost et al., 2012; Webster et al., 1997; Yanni et al., 1997; Yanni et al., 2001). Most of Thai isolated strains and, especially, SUTN9-2 significantly increase the rice biomass. On the other hand, the growth of Thai rice was not affected by Japanese strains (strains RP5, RP7 and WD16) (Figure 4.10). Moreover, among the three rice cultivars, the *O. sativa* L. ssp. *indica* cv. Pathum Thani 1 responded positively only to putative Thai rice endophytic bradyrhizobia, while this phenomenon was not found in Japanese rice cultivars. These results implied that the rice cultivar is one of the factors that control the compatibility of rice-bacteria association. Besides, this phenomenon displayed the bradyrhizobial host preference

between Thai endophytic bradyrhizobial strains and Thai rice cultivars (Figure 4.11). This scenario is confirmed by a previous report that rice genotypes mainly affected the bacterial association in rice tissues (Sasaki et al., 2013).

The cultivated rice productions are frequently found in an accretion with several aquatic legumes belonging to the genus *Aeschynomene* (*A. americana* and *A. indica*) during the raining season (from August to October). Among these aquatic species, *A. indica* (CI-group III) forms stem and/or root nodules with PB strains (Chaintreuil et al., 2000; Mornico et al., 2011). These data were congruent with our results; the PB strains SUT-PR48 and SUT-PR64 could form nodules with *A. indica* (African ecotype) (Figure 4.15). A previous report showed that photosynthesis plays an important role in the capability of establishing the stem nodules with dalbergioids group (Giraud et al., 2000). Moreover, the dalbergioids group occurred earlier on earth than phaseolids species (tentatively 1.5 million years) (Cannon et al., 2010). Perhaps, the symbiotic evolution of PB group was established on dalbergioids species and the PB still preserve the host specificity. Our results revealed that the PB strains formed symbiosis with both aquatic leguminous plants and non-leguminous species such as rice (Chaintreuil et al., 2000; Okubo et al., 2012). Interestingly, strain SUT-PR9 showed low efficiency in nodulation and nitrogen fixation with *A. indica* (Figure 4.15). Moreover, the photosynthetic pigments of SUT-PR9 were not detected when compared with PB group (Figure 4.8). It seems that the phenotypic properties of SUT-PR9 are still ambiguous. Perhaps, the ability to form nodules without Nod-factor, rather than being driven solely by the plants, has also been dependent on a specific single bacterial evolution/mutation (Chaintreuil et al., 2013). The Calvin-Benson-Bassham (CBB) cycle from *Bradyrhizobium* species plays an important role in chemoautotrophic growth (Masuda

et al., 2009), and it is important for efficient symbiosis with *A. indica* (Bonaldi et al., 2010) via controlling the oxygen tension of early stage of symbiosis (Gourion et al., 2011). In addition, the ancestor of *B. japonicum* and *A. indica* symbionts was photosynthetic free-living bacteria (Mornico et al., 2011). If this scenario is true, it should be hypothesized that the strains SUT-PR48 and SUT-PR64 still preserve some ancestral properties of PB group. In contrast, only SUT-PR9 may partially lose its properties for photosynthetic pigments productions and establish the symbiosis with *A. indica*. Perhaps strain SUT-PR9 is an intermediate between PB and Non-PB as implicated in the branch of evolution among PB strains (Figure 4.3). Furthermore, the photosynthetic related genes were perfectly conserved among PB strains (Fig. 4.7). Even though, the light-harvesting complex (*pufM*) gene still can be detected in non-photosynthetic strains S23321, but the PCR product of bacteriochlorophyll biosynthesis (*bchL*) gene was not detected. However, the *bchL* gene can be found in the strain S23321 genome. This might be because the bchL primer in this experiment was designed based on the sequences of both the photosynthetic *Bradyrhizobium* sp. and its closest photosynthetic relative *Rhodopseudomonas palustris*. Moreover, the *bchL* gene of strain S23321 (BAL75154.1) is not absolutely conserved to those of photosynthetic strains. Thus, *bchL* amplification in S23321 could not be seen because of the incompatibility of the designed primer. The preliminary experiment by Okubo et al. (Okubo et al., 2011) showed that the photosynthetic gene cluster was detected in the genome of non-symbiotic *Bradyrhizobium* strain S23321 that displayed the high similarity to the *A. indica* symbionts, whereas the phylogenetic tree of 16S rRNA gene was closely related to *B. diazoefficiens* USDA110. These testimonies may also support

our hypothesis that the environment condition and/or habitat has been continuously driving the evolution of plant-bradyrhizobia interaction.

In contrast, the root nodules are only found from *A. americana* with Non-PB group (Noisangiam et al., 2012). Similar to this finding, Non-PB strain SUT-R55 can form the effective nodules with *A. americana, M. atropurpureum* and *V. radiata*. Therefore, this is the first report to demonstrate that these Non-PBs are also capable of forming a natural endophytic association with rice. Interestingly, the SUT-R74 only performed rice endophytic property, but it was not able to form the symbiosis with any tested leguminous plants. We predict that SUT-R74 may be one of non-nodulating bradyrhizobial strains. This result was similar to the previous report by Okubo et al. (Okubo et al., 2011) that *Bradyrhizobium* sp. S23321 was not able to form nodules with *M. atropurpureum*. When the PCR amplifications of *nodA*, *nodB* and *nodC* were carried out, none of specific bands of *nodA*, *nodB* and *nodC* were obtained from every rice endophytic bradyrhizobial strain (Figure 4.16). However, strain SUT-R55 could nodulate *A. americana, M. atropurpureum* and *V. radiata*. These data were congruent with a previous report which found that divergent *nod*-containing *Bradyrhizobium* could induce root nodules of *A. americana* and *M. atropurpureum* (Noisangiam et al., 2012). Thus, the rearrangement of nodulation genes sequences may have evolved after the legumes occurred on the planet.

The strain SUTN9-2 significantly increased the rice biomass. In addition, the bacterial population of strain SUTN9-2 was significantly higher than those of strains DOA1 and DOA9. Therefore, the strain SUTN9-2 was further determined the rice endophytic properties. Moreover, the bradyrhizobia-rice symbiosis interactions have

never been elucidated before, thereby the some genes comparisons and genes deletions were further conducted.

The outcome of plant-bacterium interactions is often emphasize by bacterial protein secretion systems (Downie, 2010). From the bacterial genome data, all known protein secretion systems for translocation across the cytoplasmic and outer membranes were presented (data not show). The exceptions particularly in bradyrhizobia, were the type 5 secretion system (T5SS) and type 6 secretion system (T6SS), which their functions were not clearly understood. However the type 3 secretion system (T3SS) commonly used by symbiotic and/or pathogenic bacteria to secrete the effector proteins into the host cells and therefore, to trigger the host cells respond (Downie, 2010). Our results displayed that the T3SS expressions were highly expressed when only roots exudates were applied. In addition, genetic organization and amino acid sequences of T3SS highly conserved in both of rice endophytic strains (SUTN9-2 and DOA9) when compared with non-endophytic strain USDA110. In contrast, the T3SS from rice endophytic strains were partially different from non-rice endophytic strain USDA110 (Figure 4.23). Normally, cluster of genes encoding elements of T3SS were found in the genomes of various rhizobia of non-photosynthetic strain including *B. japonicum* USDA6, *B. diazoefficiens* USDA110 and *B. elkanii* USDA61. Interestingly, the T3SS structure genes of SUTN9-2 highly conserved to non-photosynthetic bradyrhizobia. Whereas the putative effector proteins and hypothetical proteins inside T3SS cluster were highly diverse when compared with references strain USDA110 and USDA61 (data not show). It seems that strain SUTN9-2 still preserved their structural genes but rearranged the genes controlling the putative effector genes. These scenarios implied that strain SUTN9-2 has been rearranging their genes to response to various host plants

along its evolution lines. Therefore, the T3SS may be important for *Bradyrhizobium*-rice symbiosis interactions.

The type 4 secretion system (T4SS) of strain SUTN 9-2 contained two clusters. The genes arrangement of T4SS (cluster 1) was largely different among rice endophytic strains SUTN9-2 and DOA9. Whereas T4SS (cluster 1) organizations and amino acid sequences of strain SUTN9-2 were highly similar to *trp* operon of non-endophytic strain USDA110. Which the *trp* operon was essential for conjugative transfer of Ti-plasmid in *Agrobacterium tumefaciens* (Li et al., 1998). It seems that the T4SS (cluster 1) play a role in the transferring of genetic elements. Interestingly, the cluster 2 of T4SS showed most similar to phosynthetic *Bradyrhizobium* sp. BTAi1 (Figure 4.24B). The cluster 2 of T4SS also contained genes encoding a VirB/D4. The *vir* cluster included genes *virB2* to *virB10*, the products of which form the transmembrane complex and pili required for transferring proteins. Interastingly, the virB/D4 type 4 secretion system have never been detected in bradyrhizobia except *Bradyrhizobium* sp. BTAi1. Moreover, the transcription regulator genes (*virA* and *virG* genes) could not be detected in the SUTN9-2 genome (data not show). Similarly, *virA* and *virG* homologues were absent in *Bradyrhizobium* sp. BTAi1. These finding suggest that the T4SS (cluster 2) of *Bradyrhizobium* spp. SUTN9-2 and BTAi1 share the same origin but differ from other rhizobia. In contrast, these data could also raise another hypothesis that strain SUTN9-2 acquired *vir* operon from photosynthetic-*Bradyrhizobium* sp. BTAi1. If these scenario is true, it should be hypothesized that the strain SUTN9-2 is an intermediate between photosynthetic and non-photosynthetic bradyrhizobial evolutions. However, the amino acid sequences and gene arrangements of strain SUTN9-2 were most similar to *Bradyrhizobium* sp. BTAi1, therefore the T4SS might

important for *Bradyrhizobium*-rice interaction because *Bradyrhizobium* sp. BTAi1 performed also rice endophyte in wild rice (Chaintreuil et al., 2000).

The flagellin is recognized to elicit inert immune response in *Arabidopsis* (Zipfel et al., 2004), but it is still presented in rice endophytic bacteria (Sessitsch et al., 2012). The flagella apparatus (*fliP*) were expressed both with and without rice root exudate inductions (Figure 4.20). *In planta*, the *fliP* expressions declined when plants age increased (Figure 4.21). Perhaps, the flagella mediated the adhesion and may be required to establishment in rice rhizosphere at early stage of bacterial invasions. Then, the *fliP* expressions continuously decreased because it is one of the factor that triggers plants defense systems. These finding was congruent with previous report, which flagella were required for efficient endophytic colonization of rice roots by *Azoarcus* sp. BH72, where flagellins do not appear to act as Pathogen-associated molecular patterns (PAMPS)-eliciting defense responses (Buschart et al., 2012). Moreover, the *fliP* clusters of both DOA9 and SUTN9-2 strains absolutely similar to non-endotpytic strain USDA110. Thus, these genes may not the key factor of rice-*Bradyrhizobium* association.

EPS are high molecular weight sugar polymers secreted by both Gram-negative and positive bacteria with well documented role in bacterial physiology and pathogenicity. The *exoB* expression highly up-regulated when roots exudates were applied. These results were similar to (Jones et al., 2008), which revealed that *Medicago truncatula* roots inoculated with the *Sinorhizobium meliloti* EPS-deficient mutant *exoY* more strongly induced the expression of defense-related genes than roots

inoculated with the wild type strain. It was also suggested that EPS was required for efficient down-regulation of host defense responses during early stages of infection.

The plant-polymer-degrading enzymes (pectin esterase and cellulase) have considered as another putatively important feature (Sessitsch et al., 2012). These enzyme were normally presented in the bacterial genome and may contribute to endophyte entry and spreading inside rice roots tissues (Sessitsch et al., 2012).

Moreover, previous study displayed that the glutathione-*S*-transferases (GST) is generally involved in detoxification of reactive oxygen species (ROS) and products of oxidative stress. Because plants produce a range of ROS in response to abiotic stress or to colonizing microorganisms (Fouts et al., 2008). Interestingly. the various types of detoxification of reactive oxygen species genes in strain SUTN9-2 genome were presented such as GST (26 copies), superoxide dismutases (2 copies), peroxidases (3 copies) therefore, it is not surprising that numerous genes encoding for the detoxification of ROS such as catalases, superoxide dismutase, peroxidase, hydroperoxide reductases and glutathione-S-transferase (GST) in various species of bacterial endophytes (Mitter et al., 2013). An impressively higher number of GST genes was found in the *Bradyrhizobium* sp. SUTN9-2 genome than that of *Burkholderia phytofirmans* PsJN which had 24 copies. The GSTs are enzymes that detoxify endobiotic and xenobiotic compounds by covalent linking of glutathione to hydrophobic substrates (Vuilleumier and Pagni, 2002). In addition, it has been reported that the GSTs related to secondary metabolites degradations (Mitter et al., 2013). The high numbers *gst* genes of strain SUTN9-2 may confer its ability to use of a broad spectrum of plant secondary metabolites. From both functions of GSTs, we might

speculate that the high variety of detoxification enzymes were related with the broad host range of SUTN9-2.

The functional analyses of T3SS with both rice cultivars (*O. sativa* cultivar Pathum Thani 1 and *O. sativa* cultivar Nipponbare) and some leguminous plants. In case of *O. sativa* cultivar Pathum Thani 1, the population numbers of *rhcJ* mutant strains (Δ*rhcJ*) from rice tissues were significantly lower than that of wild type strain (approximately 1 magnitude), but these properties were not detected in japonica rice (*O. sativa* cultivar Nipponbare). It seems that rice cultivars is one of the factor that control the rice-*Bradyrhizobium* associations. These observations confirm hypothesis that the ecosystems have been driving the bradyrhizobia-host interactions. However, the Δ*rhcJ* mutant strains could establish the number of symbiotic roots nodules (*V. radiata* L. cv. SUT4 and *M. atropurpureum*) lower than that of wild type strain. These results might speculate that the T3SS was important for plants-bradyrhizobia interaction. Therfore, the strain SUTN9-2 may use T3SS system for entering the plants.

In conclusion, our findings have provided the testimonies that rice-legume crop rotational system (commercial legumes and/or weed legumes) is one factor to maintain putative endophytic bradyrhizobial strains in the ecosystems. Among Thai rice cultivars, the Thai bradyrhizobial strains could promote rice growth better than Japanese strains. In addition, among the three rice cultivars (Pathum Thani 1, Kasalash and Nipponbare), cultivar Pathum Thani 1 responded positively only to putative Thai rice endophytic bradyrhizobia. In contrast, this phenomenon was not found in Japanese rice cultivar. It seems that rice cultivars are one of the factors that control the compatibility of rice-bradyrhizobial association. In addition, our works provided the

evidence to point out that these Non-PBs are also capable of forming a natural endophytic association with rice. Interestingly, strains SUT-PR9, WD16, RP5 and RP7 showed the Non-PB phenotypes but were genotypically close to PB strains. In addition, all of these endophytic bradyrhizobial strains do not seem to contain typical nodulation genes. The evolution of plant-bradyrhizobia association may have started from photosynthesis lifestyle then they lost their photosynthetic apparatus during the evolution with *nod*-independent manner. The genome comparisons of endophytic-*Bradyrhizobium* strain SUTN9-2 and Non-endophytic *Bradyrhizobium* as *B. diazoefficiens* USDA110 were elucidated in order to understand deeper the mechanism of *Bradyrhizobium*-rice association. The T3SS was important for plants-bradyrhizobia interaction. Therfore, the strain SUTN9-2 may use T3SS system for entering the plants. This would lead to the use of oligotrophic rice endophyte for rice growth promotion in the future.

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APPENDICES

Appendix 1. Yeast Extract Mannitol modified medium (YEM) (Chaintreuil et al., 2000)

D-Mannitol	10.0	g
K_2HPO_4	0.66	g
$MgSO_4 \cdot 7H_2O$	0.1	g
NaCl	0.05	g
$CaCl_2 \cdot 2H_2O$	0.04	g
$FeCl_3$	0.004	g
$MnSO_4$	0.01	g
Sodium glutamate	2.5	g
Yeast Extract	2	g
Distilled Water	1.0	liter

Adjust pH to 6.8 with 0.1 N NaOH

Appendix 2. LB medium (Bertani, G., 1951)

Tryptone	10.0	g
yeast extract	5.0	g
NaCl	10.0	g
Distilled Water	1.0	liter

Appendix 3. HM medium (Cole and Elkan, 1973)

Sodium Glutamate	1.0	g
Na_2HPO_4	0.125	g
$NaSO_4$	0.25	g
NH_4Cl	0.32	g
$MgSO_4 \cdot 7H_2O$	1.8	g
$FeCl_3$	0.004	g
$CaCl_2 \cdot 2H_2O$	0.013	g
HEPES	1.3	g
MES	1.1	g
Yeast extract	1.0	g
L-arabinose	1.0	g
Distilled Water	1.0	liter

Appendix 4. N-free Nutrient Solution (Broughton and Dillworth, 1970)

Stock Solutions	Elements	Form	MW	g/liter
1	Ca	$CaCl_2 \cdot 2H_2O$	147.03	294.1
2	P	KH_2PO_4	136.09	136.1
3	Fe	Fe-citrate	355.04	6.7
	Mg	$MgSo_4 \cdot 7H_2O$	246.5	123.3
	K	K_2SO_4	174.06	87.0
	Mn	$MnSO_4 \cdot H_2O$	169.02	0.338
4	B	H_3BO_3	61.84	0.247
	Zn	$ZnSO_4 \cdot 7H_2O$	287.56	0.288
	Cu	$CuSO_4 \cdot 5H_2O$	249.69	0.100
	Co	$CoSO_4 \cdot 7H_2O$	281.12	0.056
	Mo	$Na_2MoO_2 \cdot 2H_2O$	241.98	0.048

BIOGRAPHY

Mr. Pongdet Piromyou was born on September 2nd, 1983 in Nakhon pathom, Thailand. He graduated with the Bachelor Degree of Public health, Suranaree University of Technology in 2005 and Master Degree enrollment in the School of Biotechnology, Institute of Agricultural Technology, Suranaree University in 2009. During his Ph.D., he presented research work in the The 2nd Asian Conference on Plant-Microbe Symbiosis and Nitrogen Fixation, October 28-31, 2012, Phuket, Thailand (Poster presentation; in "Bradyrhizobia are Natural Endophytes of the Cultivated Rice (*Oryza sativa* ssp. *indica*)"). The 18th International Congress on Nitrogen Fixation, October, 2013, Miyazaki, Japan (Poster presentation; in "The symbiotic mechanism of cultivated rice endophytic bradyrhizobia and their potential for field application"). The 14th International Symposium on Nitrogen Fixation with Non-Legumes, October, 2014, Chengdu, China (Poster presentation; in "The symbiotic mechanism of cultivated rice endophytic bradyrhizobia and their potential for field application").