ROLE OF TYPE IV SECRETION SYSTEM (T4SS) IN *Bradyrhizobium*-LEGUME SYMBIOSIS

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บทบาทของระบบ Type IV secretion system (T4SS) ต่อการอาศัยอยู่ร่วมกันของแบรดี้ไรโซเบียมกับพืชตระกูลถั่ว

นางสาวปราณีต วังไธสง

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คำสำคัญ : *Bradyrhizobium* sp. SUTN9-2/การอาศัยอยู่ร่วมกัน/Type IV secretion system

Bradyrhizobium sp. SUTN9-2 เป็นเชื้อที่มีพืชอาศัยที่สามารถเข้าสร้างปมกับพืชตระกูลถั่วได้หลายชนิด ดังนั้นเชื้อสายพันธุ์นี้จึงถูกเลือกโดยมีวัตถุประสงค์เพื่อศึกษาบทบาทของระบบ type IV secretion system (T4SS) ที่มีต่อความสัมพันธ์แบบการอยู่ร่วมกันระหว่าง *Bradyrhizobium* และพืชตระกูลถั่ว เนื่องจาก *Bradyrhizobium* sp. SUTN9-2 มี 2 ชุดยีนของ T4SS อยู่บนโครโมโซมที่จัดอยู่ในกลุ่ม *tra/trb* operon และมีการจัดเรียงตัวของกลุ่มยีน *copG*, *traG* และ *virD2* ที่เป็นเอกลักษณ์ภายในทั้งสอง operon จากการวิเคราะห์ความสัมพันธ์ทางพันธุกรรม (phylogenetic tree) ของยีน *traG* แสดงให้เห็นหลักฐานการถ่ายโอนยีนแบบแนวนอน (horizontal gene transfer) ของยีน *traG* ที่เป็นยีนในระบบ T4SS ระหว่างสกุล *Bradyrhizobium* และ *Mesorhizobium* อย่างไรก็ตามไม่พบยีน *virD2* ในกลุ่ม *Mesorhizobium* ยกเว้นใน *M. oppotunistum* WSM2075 แสดงให้เห็นถึงการจัดเรียงของกลุ่มยีน *copG*, *traG* และ *virD2* ที่มีลักษณะเฉพาะในสกุล *Bradyrhizobium* จากการวิเคราะห์สายวิวัฒนาการของกลุ่มยีน *copG*, *traG* และ *virD2* ของ *Bradyrhizobium* sp. SUTN9-2 แสดงให้เห็นว่าชุดยีนที่ 1 และชุดยีนที่ 2 ถูกจัดกลุ่มอยู่ในกลุ่มที่แตกต่างกันภายในสกุล *Bradyrhizobium* โดยT4SS copy 1 ($T4SS_1$) ของ *Bradyrhizobium* sp. SUTN9-2 มีความคล้ายคลึงกันอย่างมีนัยสำคัญกับสายพันธุ์ *Bradyrhizobium* ที่สำคัญสองสายพันธุ์ ได้แก่ *B. diazoefficiens* USDA110 ซึ่งเป็นหัวเชื้อสำหรับถั่วเหลืองที่นำมาใช้ในทางการค้า และ *B. yuanmingense* ซึ่งเป็นสายพันธุ์ไรโซเบียมหลักที่เกี่ยวข้องกับถั่วฝักยาว (*Vigna unguiculata)* และถั่วเขียว (*V. radiata*) ในพื้นที่กึ่งเขตร้อน ดังนั้นจึงได้ศึกษาบทบาทของ $T4SS_1$ ในการปฏิสัมพันธ์ทางชีวภาพระหว่าง *Bradyrhizobium* sp. SUTN9-2 และพืชตระกูลถั่ว เพื่อให้บรรลุเป้าหมายนี้จึงได้ทำลายชิ้นยีน $T4SS_1$ ประกอบด้วยยีน *copG*, *traG* และ *virD2* รวมทั้งนำยีน $T4SS_1$ ดังกล่าวกลับเข้ามายังเชื้อกลายพันธุ์อีกครั้งหรือที่เรียกว่า complementation ($T4SS_{1compl}$) และทดสอบกับพืชตระกูลถั่วที่เป็นตัวแทนจาก Dalbergioids, Millettiods และ Genistoids ผลลัพธ์แสดงให้เห็นผลในเชิงบวกของ $T4SS_1$ ต่อการเข้าสร้างปม เนื่องจากการทำลายยีน $T4SS_1$ ออกไปนั้นส่งผลให้ทั้งจำนวนปมลดลง และประสิทธิภาพของเอนไซม์ไนโตรจีเนสก็ลดลงเช่นกัน ในทางตรงกันข้ามประสิทธิภาพในการเข้าสร้างปมจะได้รับการฟื้นฟูอย่างสมบูรณ์โดยเชื้อ $T4SS_{1compl}$ ในระดับที่มีความใกล้เคียงกับเชื้อ

ดั้งเดิม จึงเป็นการเน้นย้ำถึงบทบาทที่สำคัญของ T4SS ที่มีปฏิสัมพันธ์ทางชีวภาพระหว่าง *Bradyrhizobium* sp. SUTN9-2 และพืชตระกูลถั่ว

นอกจากนี้ยังได้ศึกษาบทบาทของแต่ละยีนภายในชิ้นส่วน $\Delta T4SS_1$ ที่ประกอบด้วย $copG_1$, $traG_1$, และ $virD2_1$ ของ *Bradyrhizobium* sp. SUTN9-2 อีกด้วย โดยการสร้างเชื้อกลายพันธุ์ของแต่ละยีนข้างต้นและทดสอบกับถั่วเขียวสายพันธุ์ มทส.4 พบว่า $\Delta traG_1$ และ $\Delta virD2_1$ มีผลให้ประสิทธิภาพการเข้าสร้างปมลดลงในช่วงเริ่มต้นของการติดเชื้อ แต่สามารถฟื้นคืนความสามารถในการเข้าสร้างปมใหม่เมื่อเวลาผ่านไป ในทางตรงกันข้าม $\Delta copG_1$ แสดงการสูญเสียความสามารถในสร้างปมและการตรึงไนโตรเจนโดยสิ้นเชิง ซึ่งแสดงให้เห็นถึงบทบาทสำคัญของ $copG_1$ ที่มีต่อความสัมพันธ์แบบการอยู่ร่วมกัน *Bradyrhizobium* sp. SUTN9-2 และพืชอาศัย ถึงแม้ว่า $\Delta copG_2$ จะแสดงผลเชิงลบเล็กน้อยต่อการเข้าสร้างปมด้วยจำนวนปมที่เพิ่มขึ้น แต่กิจกรรมของเอนไซม์ไนโตรจีเนสกลับลดลงเมื่อเทียบกับสายพันธุ์ดั้งเดิม นอกจากนี้ใน $\Delta copG_1$ ไม่พบการแสดงออกของยีนที่เกี่ยวข้องกับการสร้างปม ได้แก่ *nodD2*, *nodA* และ *nodC* ในขณะที่มีระดับการแสดงออกของยีนโครงสร้าง $T4SS_1$ ประกอบด้วย $traG_1$ และ $trbE_1$ ในระดับที่สูง ผลลัพธ์การทดลองแสดงให้เห็นว่ายีน $copG_1$ มีบทบาทสำคัญหลายอย่างในการปฏิสัมพันธ์ทางชีวภาพ ซึ่งรวมถึงความมีชีวิตของแบคทีเรีย การเข้าสร้างปม และมีแนวโน้มที่จะทำหน้าที่เป็นตัวยับยั้ง (repressor) ยีนโครงสร้าง T4SS ในขณะที่ยีน $traG_1$ และ $virD2_1$ มีความสำคัญในเริ่มต้นของกระบวนการการอาศัยอยู่ร่วมกันทางชีวภาพระหว่าง *Bradyrhizobium* sp. SUTN9-2 และพืชตระกูลถั่ว จากองค์ความรู้ใหม่นี้สามารถสร้างโอกาสสำหรับการวิจัยในอนาคตเกี่ยวกับบทบาทของ $copG_1$ และปฏิสัมพันธ์กับยีนและกระบวนการอื่น ๆ ที่เกี่ยวข้องที่เกี่ยวข้องกับกระบวนการอาศัยอยู่ร่วมกัน นอกจากนี้งานวิจัยยังเน้นย้ำถึงความสำคัญของ T4SS ต่อการปฏิสัมพันธ์ทางชีวภาพระหว่าง *Bradyrhizobium* sp. SUTN9-2 และพืชตระกูลถั่ว เพิ่มความเข้าใจเกี่ยวกับความสัมพันธ์ระหว่างพืชตระกูลถั่วกับไรโซเบียมอย่างถ่องแท้ ความเข้าใจนี้มีศักยภาพที่จะนำไปใช้ในการเกษตร ปรับปรุงประสิทธิภาพการเพิ่มจำนวนการเข้าสร้างปม และการตรึงไนโตรเจนในพืชตระกูลถั่ว ซึ่งนำไปสู่การเพิ่มผลผลิตทางการเกษตรอย่างยั่งยืน

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PRANEET WANGTHAISONG : ROLE OF TYPE IV SECRETION SYSTEM (T4SS) IN *Bradyrhizobium*-LEGUME SYMBIOSIS. THESIS ADVISOR : PROF. NEUNG TEAUMROONG, Ph.D., 104 PP.

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Bradyrhizobium sp. SUTN9-2 is a broad host range strain capable of nodulating with various legume hosts. This strain was selected with the specific purpose to examine the role of the type IV secretion system (T4SS) in the symbiotic process between *Bradyrhizobium* and legumes. This is due to the chromosome of *Bradyrhizobium* sp. SUTN9-2 which carries 2 copies of T4SS containing the *tra/trb* operon and a unique gene arrangement of the *copG*, *traG*, and *virD2* gene cluster was discovered within both operons. A *traG* gene phylogenetic tree analysis revealed evidence of horizontal gene transfer of the *traG* gene in T4SS between the genera *Bradyrhizobium* and *Mesorhizobium*. However, the *virD2* gene was not found in *Mesorhizobium*, except for a specific case in *M. opportunistum* WSM2075. This finding suggested an arrangement of the *copG*, *traG*, and *virD2* gene cluster that is unique to the *Bradyrhizobium* genus. Phylogenetic analyses of *copG*, *traG*, and *virD2* of *Bradyrhizobium* sp. SUTN9-2 showed that copies 1 and 2 of these genes grouped in different clades within genus *Bradyrhizobium*. Interestingly, T4SS copy 1 ($T4SS_1$) of *Bradyrhizobium* sp. SUTN9-2 exhibited significant similarity to two important *Bradyrhizobium* strains including *B. diazoefficiens* USDA110, a commercial soybean inoculant, and *B. yuanmingense*, the primary rhizobia species associated with *Vigna unguiculata* and *V. radiata* plants in subtropical regions. As a result, this study aimed to investigate the role of T4SS1 in the symbiotic interactions between *Bradyrhizobium* sp. SUTN9-2 and leguminous plants. To achieve this, the construction of T4SS1 ($copG_1$, $traG_1$, and $virD2_1$ genes) deletion and their complementation ($T4SS_{1compl}$) were performed and tested with the representative legumes from the Dalbergioids, Millettiods, and Genistoids. The results demonstrated a positive effect of $T4SS_1$ on nodulation, as in the deletion of $T4SS_1$, both nodule number and nitrogenase activity were reduced. In contrast, nodulation efficiency was successfully restored by $T4SS_{1compl}$ resembling levels observed in the wild type (WT).

These findings emphasize the important role of T4SS in the symbiotic interaction between *Bradyrhizobium* sp. SUTN9-2 and its leguminous hosts.

Furthermore, the role of each *copG*, *traG*, and *virD2* genes within T4SS1 fragment of *Bradyrhizobium* sp. SUTN9-2 was further investigated. Mutants were constructed and tested on *V. radiata* cv. SUT4. It was discovered that Δ$traG_1$ and Δ$virD2_1$ reduced infection efficiency in the early stage of infection, but they regained their invasion abilities over time. As opposed to Δ$copG_1$ which exhibited a complete loss of the ability in nodule organogenesis and nitrogenase activity, highlighting the critical role of the $copG_1$ gene in symbiotic interactions. Although the Δ$copG_2$ showed a slightly negative effect on nodulation by increasing nodule numbers but lower nitrogenase activity when compared to the WT. Moreover, the expression of nodulation genes (*nodD2*, *nodA*, and *nodC*) were not detected in the Δ$copG_1$, while high expression levels of $T4SS_1$ structural genes including $traG_1$ and $trbE_1$ were observed. These results suggested that the $copG_1$ gene played multiple crucial roles in the symbiotic interaction, including contributing to bacterial viability, involvement in nodulation and most likely acting as a repressor for T4SS structural genes. Meanwhile, $traG_1$ and $virD2_1$ were essential in the early stages of symbiotic interaction between *Bradyrhizobium* sp. SUTN9-2 and leguminous plants. This novel approach might create opportunities for future research on the role of $copG_1$ and its interactions with other genes and other pathways involved in symbiosis. Furthermore, the research underscores the importance of T4SS in the symbiotic interaction between *Bradyrhizobium* sp. SUTN9-2 and leguminous hosts, deepening our understanding of successful legume-*Rhizobium* symbiosis. This understanding has the potential to be applied in agriculture, improving nodulation efficiency and nitrogen fixation in legume crops leading to enhanced agricultural productivity and sustainability.

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LIST OF ABBREVIATIONS

ADP	=	Adenosine diphosphate
AG	=	Arabinose-Gluconate
ANOVA	=	One-way analysis of variance
ARA	=	Acetylene reduction assay
ATP	=	Adenosine triphosphate
att	=	Conserved attachment site
avh	=	Avirulence
BNF	=	Biological control nitrogen fixation
C_2H_2	=	Acetylene
C_2H_4	=	Ethylene
cDNA	=	Complementary deoxyribonucleic acid
cefo	=	Cefotaxime
CI	=	Cross-inoculation
CM	=	Cytoplasmic membrane
$copG_1$	=	*copG* copy 1
$copG_2$	=	*copG* copy 2
Ct	=	Cycle threshold
DNA	=	Deoxyribonucleic acid
dpi	=	Day post inoculation
e^-	=	Electron
et al.	=	et alia (and others)
Fe	=	Iron
FID	=	Flame ionization detection
GC	=	Gas Chromatography
h	=	Hour
H^+	=	Proton
H_2	=	Hydrogen gas
ICE	=	Integrative and conjugative element

LIST OF ABBREVIATIONS (Continued)

ICE	=	Inner membrane
kg	=	Kilograms
km	=	Kanamycin
LB	=	Left border
LB	=	Luria-Bertani
LCO	=	Lipochito-oligosaccharide
ml	=	Milliliter
mm	=	Millimeter
n	=	Number
N	=	Nitrogen
N_2	=	Nitrogen gas
nal	=	Nalidixic acid
NF	=	Nod factor
NFR	=	Nod factor receptor
NH3	=	Ammonia
nm	=	Milliliter
nmol	=	Nanomoles
nod	=	Nodulation gene
NodD	=	Nodulation protein
Nop	=	Nodulation outer protein
ns	=	Not statistically significant
OM	=	Outer membrane
p-value	=	Probability value
PB	=	Photosynthetic bradyrhizobia
PBS	=	Phosphate buffered saline
PCR	=	Polymerase chain reaction
PI	=	Propidium iodide
Pi	=	Inorganic phosphate
pSym	=	Symbiotic plasmids

LIST OF ABBREVIATIONS (Continued)

qRT–PCR	=	Quantitative reverse transcription polymerase chain reaction
RB	=	Right border
rep	=	Replication gene
repA	=	*Replication A* gene
rhc	=	*Rhizobium conserved* gene
RNA	=	Ribonucleic acid
rRNA	=	Ribosomal ribonucleic acid
sm	=	Streptomycin
SYTO9	=	Green-fluorescent nucleic acid stains
T-DNA	=	Transferred DNA
T1SS	=	Type I secretion system
T3Es	=	T3SS effectors
T3SS	=	Type III secretion system
T4SS	=	Type IV secretion system
T6SS	=	Type VI secretion system
$T4SS_1$	=	T4SS copy 1
$T4SS_{1compl}$	=	T4SS1 complementation
$T4SS_2$	=	T4SS copy 2
T6SS	=	Type VI secretion system
tag	=	Type six accessory gene
Ti	=	Tumor inducing
$traG_1$	=	*traG* copy 1
$trbE_1$	=	*trbE* copy 1
Tss	=	Type six secretion
TtsI	=	Transcriptional activator of T3SS
vir	=	*Virulence* gene
$virD2_1$	=	*virD2* copy 1
v/v	=	Volume per volume

LIST OF ABBREVIATIONS (Continued)

µl	=	Microliter
µg	=	Microgram
µM	=	Micrometer
Ω	=	Omega
%	=	Percentage sign
℃	=	Degree Celsius

CHAPTER I
INTRODUCTION

1.1 Significances of study

Bradyrhizobium is a type of Gram-negative soil bacteria that belongs to the alpha-proteobacteria group. It forms a symbiotic relationship with plants which involves the exchange of signaling molecules. This symbiosis leads to the development of specialized structures called nodules. Inside these nodules, there are bacteroids that have the ability to convert atmospheric dinitrogen (N_2) into ammonia (NH_3) through nitrogen fixation. This conversion allows nitrogen to be incorporated into organic molecules making it accessible for plant growth (Stevenson, 1965). Typically, *Bradyrhizobium* requires nod factors (NFs) to establish symbiosis with various leguminous plant hosts using a mechanism known as nod factor (NF)-dependent symbiosis. The symbiotic process is initiated by plants through the secretion of flavonoids or phenolic compounds which diffuse across the rhizobial membrane and bind to a specific protein called NodD. This binding activates NodD, thereby triggering the expression of nodulation (*nod*) genes including the common *nodABC* genes in rhizobia. The *nodABC* genes are responsible for synthesizing NFs which are lipochitooligosaccharides (LCOs) serving as crucial signaling molecules. These NFs play a crucial role in initiating the symbiotic relationship and facilitating the organization of nodules (Rhijn & Vanderleyden, 1995).

Although NFs are commonly involved in the symbiotic relationship between rhizobia and legumes. There are exceptions in *Aeschynomene*-bradyrhizobium symbiosis, especially in photosynthetic bradyrhizobia (PB) including *Bradyrhizobium* sp. BTAi1 and *Bradyrhizobium* sp. ORS278 which capable of nodulating *Aeschynomene* cross-inoculation (CI) group 3 without the presence of *nodABC* genes, a phenomenon referred to as NF-independent which limited to a very few host plants. These PB strains may use an alternative mechanism for invasion into host plant, brarhizobia enter the roots through crack entry rather than via the traditional

pathway (Giraud et al., 2007; Noisangiam et al., 2012). Despite this different entry pathway, the nodulation process still occurs using similar downstream signals that are present in NF-dependent nodulation (Bonaldi et al., 2011). An intriguing observation is that *Bradyrhizobium* sp. ORS285 harbors plasmid-borne nod genes and T3SS on its chromosome. Interestingly, the *nodB* derivative of *Bradyrhizobium* sp. ORS285 loses the ability to nodulate in CI group 2 but retains nodulation ability in CI group 3. These findings suggest that *Bradyrhizobium* sp. ORS285 can establish a nitrogen-fixing symbiosis with *Aeschynomene* plants through both NF-dependent and NF-independent mechanisms depending on the *Aeschynomene* species (Giraud et al., 2007).

In addition to NFs, the signal exchange between bradyrhizobia and legumes during symbiosis also involves secretion systems. Various rhizobia have been found to possess three types of secretion systems similar to those used by pathogenic bacteria including type III, IV and IV secretion systems (T3SS, T4SS and T6SS). These systems are involved in symbiotic interactions and play a crucial role in delivering virulence factors, effector protein or macromolecules directly into the cells of the host organism (Sugawara et al., 2013). There are many reports about T3SS and T6SS in symbiosis process of rhizobia. T3SS has been identified in *Rhizobium, Sinorhizobium, Mesorhizobium*, and *Bradyrhizobium*, and its effects on symbiosis with the host can vary either positive, negative, or neutral (Hubber et al., 2004; Fauvart & Michiels, 2008; López-Baena et al., 2008; Okazaki et al., 2009; Kimbrel et al., 2013; Okazaki et al., 2013). While, The T6SS has been identified and studied in several species of rhizobia, including *R. leguminosarum*, *B. japonicum*, *M. loti*, *S. saheli*, and *S. fredii* (Nelson & Sadowsky, 2015; Sousa et al., 2021). Additionally, the impact of symbiotic interactions has been observed in *R. etli* Mim1 and *Bradyrhizobium* sp. LmicA16 depending on the host plant. The T6SS is also crucial for competitiveness in *Azorhizobium caulinodans* ORS571 (Salinero-Lanzarote et al., 2019). Similar to T3SS and T6SS, the T4SS in some species has been found to impact symbiotic interactions. However, there has been relatively less research conducted on the effects of T4SS in the bradyrhizobia genus (Hubber et al., 2004; Hubber et al., 2007; Jones et al., 2007). Interestingly, PB strains lack a T3SS except *Bradyrhizobium* sp. ORS285. ORS285 T3SS mutant revealed that the T3SS can have both positive and

negative effects on the interaction with NF-dependent but not necessary for the interaction with any NF-independent *Aeschynomene* species tested. These findings suggest that legumes can establish two types of NF-independent symbiotic processes with rhizobia, one involving the T3SS and another utilizing an unknown mechanism (Okazaki et al., 2016).

Bradyrhizobium sp. SUTN9-2 is a Gram-negative soil bacteria isolated from *A. americana* in Thailand, which demonstrates an impressive ability to nodulate a wide range of legume species and acts as a rice endophyte (Piromyou et al., 2015). *Bradyrhizobium* sp. SUTN9-2 also serves as a valuable model for studying the symbiotic relationship between *Vigna radiata* and bradyrhizobia, as this particular strain has demonstrated the ability to form symbiotic nodules with a wide range of tested *V. radiata* cultivars (Piromyou et al., 2019). While the nodulation process of *Bradyrhizobium* sp. SUTN9-2 generally relies on NF-dependent pathway for nodule organogenesis in legume plants, its interaction with *Aeschynomene* symbiosis is particularly intriguing (Noisangiam et al., 2012; Hashimoto et al., 2019). This is because *Bradyrhizobium* sp. SUTN9-2 can nodulate even *Aeschynomene* CI group 3, which typically only interacts with PB through an NF-independent process (Noisangiam et al., 2012). Within the chromosome of *Bradyrhizobium* SUTN9-2 harbors two secretion systems namely, T3SS and T4SS which actively participate in symbiotic interactions (Piromyou et al., 2015; Hashimoto et al., 2020). The impact of the T3SS on symbiotic interactions in *Bradyrhizobium* SUTN9-2 is influenced by the specific plant species involved *A. americana* exhibits a neutral response to the T3SS, while *V. radiata*, *Macroptilium atropurpureum*, and *Lotus* spp. experience a strong negative effect. Notably, the T3SS has been found to have a positive effect exclusively *in V. radiata* CN72 (Piromyou et al., 2019; Jiménez-Guerrero et al., 2022). On the other hand, there is no reported evidence of T4SS in *Bradyrhizobium* species during symbiotic interactions with legumes.

In the genome of *Bradyrhizobium* sp. SUTN9-2, two distinct clusters of T4SS have been identified. These clusters are situated in different regions on the chromosome and belong to the *tra/trb* operon, which shares homology with the T4SS found in *Agrobacterium*. T4SS cluster 1 exhibits similarity with the T4SS of *B. diazoefficiens* USDA110, a non-PB that acts as an endosymbiont in *Glycine max*

(soybean). On the other hand, T4SS cluster 2 displays similarity with the T4SS of *Bradyrhizobium* sp.BTAi1, which is a PB that nodulates with CI group 3 and acts as an endophyte in rice (Piromyou et al., 2015). Moreover, there is no reports regarding the role of T4SS of bradyrhizobia symbiosis. To elucidate the role and function of the T4SS in *Bradyrhizobium* sp. SUTN9-2 is of particular interest due to it have ability to nodulate with a wide range of legume species.

1.2 Research objectives

To investigate the function and impact of T4SS in the symbiotic relationship between *Bradyrhizobium* sp. SUTN9-2 and legume.

CHAPTER II

LITERATURE REVIEWS

2.1 Biological nitrogen fixation (BNF)

The demand for nitrogen (N) in crop production has significantly increased since the mid-twentieth century. This increase is due to advancements in the productivity of major food crops like wheat, rice, and maize which are important for a substantial portion of the global population's staple diet (Ladha et al., 2022). Nitrogen plays a crucial role in various essential biochemical compounds within living cells. These compounds include nucleotide phosphates, amino acids, proteins, and nucleic acids, which are necessary for cellular processes and functions (Rascio & Rocca, 2013).

Despite being the most abundant element in Earth's atmosphere, constituting around 79% of its composition nitrogen primarily exists in an inaccessible form that cannot be directly utilized by biological systems (Grzyb et al., 2021). As a result, organisms, including plants, rely on soil microorganisms to convert atmospheric nitrogen (N_2) into an accessible form before it can be assimilated (Vitousek & Matson, 2012). In modern times, the primary sources of N supply are biological nitrogen fixation (BNF) and nitrogen fertilizers produced through the Haber-Bosch processes (Ladha et al., 2022). These sources play pivotal roles in meeting the nitrogen requirements across diverse domains including agriculture and industrial applications. The BNF is accomplished by diazotrophs, which utilize the enzyme nitrogenase to catalyze the essential process of biological nitrogen reduction. Nitrogenase is responsible for facilitating the conversion of N_2 into a biologically usable form ammonia (NH_3), thus enabling the incorporation of nitrogen into organic molecules (Stevenson, 1965). BNF has served as the predominant source of reactive nitrogen utilized in agriculture. The reaction for BNF can be represented as follows:

$$N_2 + 8H^+ + 8e^- + 16ATP \longrightarrow 2NH_3 + H_2 + 16ADP + 16Pi$$

In this reaction, N_2 combines with 8 protons (H^+), 8 electrons (e^-), and the energy from 16 molecules of adenosine triphosphate (ATP) to yield 2 molecules of ammonia (NH_3), 1 molecule of hydrogen gas (H_2), and the corresponding products of ATP hydrolysis: 16 molecules of adenosine diphosphate (ADP) and 16 inorganic phosphate (Pi). This process represents the reduction of atmospheric nitrogen to produce ammonia, which can then be utilized by living organisms for various biological processes (*Biology of the Nitrogen Cycle*, 2007). The nitrogenase complex encoded by the *nifHDK* genes is composed of two primary components. The first component is a homodimeric iron metalloprotein (Fe) that binds two molecules of adenosine triphosphate (ATP). The second component is a tetrameric metalloprotein that contains a molybdenum-iron (Mo-Fe) complex. These components work in conjunction within the nitrogenase complex to facilitate the enzymatic process of nitrogen fixation (Rascio & Rocca, 2013). Nitrogenase is carried out by a diverse group of prokaryotes known as nitrogen-fixing or diazotrophic microorganisms. These microorganisms are widely distributed in various ecosystems and can exist as free-living organisms or form symbiotic associations with different plant species (*Biology of the Nitrogen Cycle*, 2007). The symbiotic association between legumes and diazotrophic microorganisms ensures a consistent provision of carbon sources and a regulated supply of oxygen, establishing the necessary conditions for highly efficient N_2 fixation. In contrast, free-living N_2 fixing microorganisms primarily utilize the fixed nitrogen for their own biomass and its availability to plants occurs indirectly through the subsequent mineralization of this biomass (Shin et al., 2016).

2.2 The *Rhizobium*-legume symbiosis plays a crucial role in the process of biological nitrogen fixation

In the realm of agriculture, the symbiotic BNF exhibited by legumes holds significant recognition as the foremost and highly impactful source of biologically fixed N (Kennedy & Islam, 2001). The symbiotic associations between N_2 fixing bacteria, specifically Rhizobium species and leguminous plants play a crucial role in ecological systems. These associations are ecologically significant due to their widespread occurrence and their ability to provide a substantial supply of nitrogen to

ecosystems. Approximately 90% of leguminous plants are capable of forming symbiotic associations with aerobic diazotrophic Gram-negative bacteria known as rhizobia including *Rhizobium*, *Azorhizobium*, *Mesorhizobium*, *Sinorhizobium*, and *Bradyrhizobium*. Different species of rhizobia establish symbiotic relationships with specific legume plant species, contributing to the diversity and specificity of these interactions (Rascio & Rocca, 2013). The BNF has a global impact, annually contributing an estimated 139-170 million tons of N to arable soils. Microorganisms in symbiotic systems are responsible for fixing the majority, around 70-80%, of this N, with non-symbiotic microorganisms fixing the remaining 20-30% (Grzyb et al., 2021). Within the symbiotic association between Rhizobium sp. bacteria and legumes, a substantial amount of N, ranging from 100 to 200 kg per hectare per year, is contributed to the soil. In contrast, free-living bacteria fix N at a significantly lower rate, typically less than 10 kg per hectare per year. This disparity can be attributed to the limited timeframe during which free-living diazotrophs actively engage in N_2 fixation, primarily during their growth phase (Schulze & Mooney, 1993; Babik & Elkner, 2002). Overall, the symbiotic associations between N_2 fixing bacteria and leguminous plants are fundamental for nitrogen supply in ecosystems, highlighting their ecological importance and their role in maintaining soil fertility and ecosystem productivity.

2.3 Nodulation mechanisms

2.3.1 Rhizobial and nodulation process

2.3.1.1 Nod factor-dependent

Rhizobia are a group of Gram-negative bacteria and classified to alpha-proteobacteria which consisting of the genera *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium* play a crucial role in symbiosis (Berkum et al., 1998). The *Rhizobium*-legume symbiosis depends on the exchange of signals between the partners to generate specialized structures called nodules. During the initiation of nodulation, flavonoids and isoflavonoids secreted by legume roots activate the nodulation protein (NodD). NodD proteins belong to the LysR family of transcriptional regulators to stimulate the expression of nod genes by binding to specific DNA motifs known as nod boxes which located upstream of the

nod operons (Fisher et al., 1988; Kamboj et al., 2010). This activation triggers the synthesis of lipochito-oligosaccharide (LCO) compounds called nod factors (NF) (Oldroyd et al., 2011). NF acts as signal molecules that interact with nod factor receptors (NFR) present in the plant. This interaction triggers the activation of a plant symbiotic signaling pathway by initiating cell division in the root cortex forming a specialized structure where the rhizobia bacteria can reside and perform nitrogen fixation. Inside the nodules containing the rhizobia differentiate into bacteroids which can convert atmospheric nitrogen into a form usable by the plant (Bladergroen & Spaink, 1998; Oldroyd et al., 2011).

2.3.1.2 Nod factor-independent

The presence of NF is often referred to as an NF-dependent mechanism (Figure 2.1). Some rhizobia belonging to the photosynthetic bradyrhizobia (PB) group form a symbiotic relationship with *Aeschynomene* plants and lack the canonical *nodABC* genes which are responsible for encoding LCO signal molecules (Giraud et al., 2007). These PB strains have evolved an alternative infection process known as crack entry or intercellular infection (Figure 2.1) (Bonaldi et al., 2011; Okubo et al., 2012; Guha et al., 2022). Generally, the crack-entry infection process has been extensively studied in *Arachis hypogea* (Ibáñez & Fabra, 2011) and has been observed in the *Aeschynomene*-photosynthetic *bradyrhizobia* symbiosis such as *Bradyrhizobium* sp. ORS285 and *Bradyrhizobium* sp. BTAi1 (Giraud et al., 2007; Okazaki et al., 2016). This mechanism allows them to invade the host plant through gaps between axillary root hairs penetrating the cortical cells by invaginating the host cell wall so, this mechanism called NF-independent (Bonaldi et al., 2011).

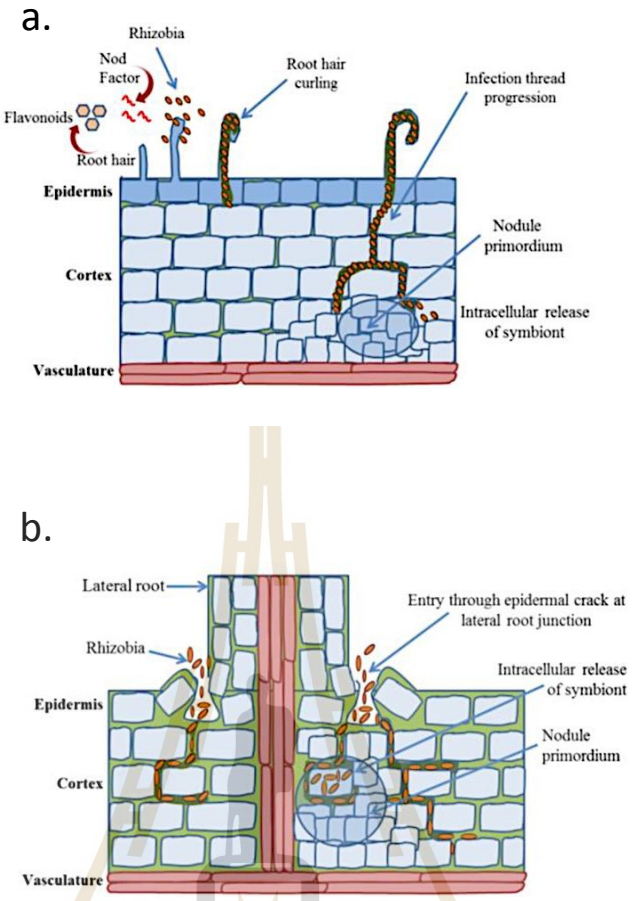

Figure 2.1 Rhizobial invasion can happen through two pathways including a.) root hair invasion via nod-dependent pathway and b.) crack/intercellular invasion via NF-independent pathway (Sharma et al., 2020).

2.4 Comparison of Nod factor-independent Nitrogen Fixation and the Symbiotic Secretion System

2.4.1 Bacterial secretion systems

Bacterial secretion systems are complex nanomachines that facilitate the transport and delivery of a wide range of molecules across bacterial cell membranes. These systems play a crucial role in various biological processes including intercellular communication, nutrient acquisition, pathogenesis and environmental adaptation (Green & Mecsas, 2016).

2.4.1.1 Classification of bacterial secretion systems

Currently, bacterial secretion systems can be classified into various types based on their structure and mechanism of action. The presence of a wide range of secretion systems in bacteria, ranging from type I secretion system (T1SS) to T11SS has been identified in prokaryotic organisms (Figure 2.2) (Pena et al., 2019; Grossman et al., 2021; Filloux, 2022). The secretion system can be classified into several groups based on mechanisms and functions. Firstly, there is the protein translocation group, which includes T1SS, T2SS, T5SS, T9SS, and T10SS (Desvaux et al., 2009; Gawarzewski et al., 2013; Palmer et al., 2021). These systems are primarily involved in transporting proteins and compounds from inside the bacterial cell to the external environment including eukaryotic cells. They play a key role in facilitating the export of molecules outside the cell. Secondly, there are the effector delivery groups which are crucial for bacterial survival, pathogenicity, and symbiotic interactions including T3SS, T4SS and T6SS (Nelson & Sadowsky, 2015). These secretion systems enable bacteria to deliver effector molecules that manipulate host cellular processes, evade immune responses, establish colonization, and promote successful interactions with their hosts. Lastly, T7SS is involved in the secretion of enzymes and factors required for extracellular matrix formation (Kengmo Tchoupa et al., 2020). This enables bacteria to construct biofilms, which are structured communities of bacterial cells embedded in a self-produced matrix. Biofilms provide advantages such as increased resistance to antibiotics, protection from host immune defenses, and enhanced colonization of surfaces (Bowman & Palmer, 2021; Zhou et al., 2022). The latest addition to the secretion systems is the T11SS which has been recently proposed. This system is composed of proteins from the DUF560 family and is responsible for transporting periplasmic cargo across the outer membrane (Grossman et al., 2021, Grossman et al., 2022; Filloux, 2022).

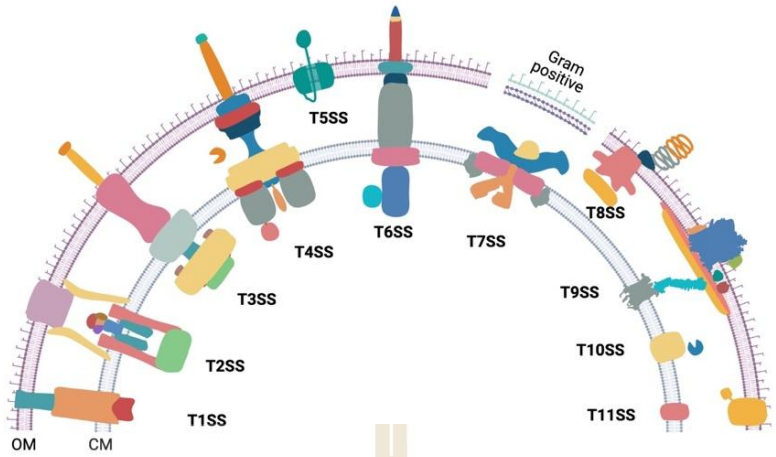

Figure 2.2 The diversity of bacterial protein secretion systems from type 1 to type 11 secretion systems (Trivedi et al., 2022).

2.4.2 The secretion system required for plant-microbe interaction

2.4.2.1 Pathogenicity

Numerous pathogenic bacteria have been observed to employ dedicated secretion systems for the purpose of releasing virulence factors from the bacterial cytosol into host cells or the surrounding host environment (Green & Mecsas, 2016).

2.4.2.1.1 *Agrobacterium* harboring the Tumor inducing (Ti) plasmid.

Agrobacterium spp. is a Gram-negative plant pathogenic bacterium which known for inducing crown gall disease through direct transformation of host plant cells (White & Winans, 2007). *A. tumefaciens* infection induces significant physiological alterations in plant tissues. Tumor organogenesis primarily occurs at the site of infection and originates from the cambium layer. Within the cambium undifferentiated cells undergo transformation resulting in the loss of their ability to differentiate into the usual phloem and xylem vessels. As a result, the transport of water and nutrients is adversely affected (Otten et al., 2008). This bacterium carries a significant plasmid called the tumor-inducing plasmid (Ti plasmid), which plays a pivotal role in transferring and integrating DNA into the genome of infected plant cells (Kado & Hooykaas, 1991; Beijersbergen & Hooykaas,

1994). In *Agrobacterium* spp., the Ti plasmid can indeed be classified based on the opine type produced by the bacterium. Opines are small molecules produced by the transformed plant cells infected by *Agrobacterium*. They serve as a source of nutrients for the bacterium and play a role in the establishment of the infection (Cascales & Christie, 2003). There are several types of opines associated with *Agrobacterium* infections including octopine, nopaline, succinamopine, and leucinopine (Moore et al., 1997). The ability to produce specific opines is determined by the presence of the corresponding Ti plasmid in the *Agrobacterium*. In *A. tumefaciens* C58, which serves as a crucial model for investigating the relationship between plants and bacteria, two distinct types of Ti plasmids have been discovered including octopine and nopaline types (Figure 2.3). Normally, the Ti plasmid consists of five key components essential for T-DNA transformation including the T-DNA region, as well as *vir*, *rep*, *tra* and *trb* regions (Engler et al., 1981). The components consist of, i). T-DNA region: the T-DNA region is flanked by specific border sequences, the left border (LB) and right border (RB), which serve as recognition sites for transfer (Kleinboelting et al., 2015). This region carries genes encoding phytohormones (auxins, cytokinins) and opines which act as major carbon and nitrogen sources and play a significant role in the interaction between *Agrobacterium* and plants (Körber et al., 1991 Baek et al., 2003; Flores-Mireles et al., 2012). ii). Virulence genes (*vir*) region: the virulence genes region encodes components of the T4SS responsible for the infection process and transfer of T-DNA from bacteria to the host cytoplasm (Shirasu et al., 1990; Thompson et al., 1988). iii). Replication (*rep*) region: the Ti plasmid encompasses *rep* genes (*repABC*) crucial for vegetative replication and partitioning of the plasmid to ensure maintenance within *Agrobacterium* (Tabata et al., 1989; Pinto et al., 2012). iv). *tra* and *trb* gene regions: the Ti plasmid harbors two gene regions, *tra* and *trb*, which contribute to important functions in *Agrobacterium*. The *tra* genes encode a transcriptional regulator involved in DNA replication and transformation, while the *trb* gene region (*trb* cluster and *traG*) encodes proteins required for Ti plasmid conjugation. This system facilitates the formation of mating pairs and enables the transfer of genetic material between *Agrobacterium* and host cell. v). Opine uptake and catabolism: *A. tumefaciens* C58 has the ability to uptake and catabolize opines which are compounds produced by infected plant cells in

response to Ti plasmids. Specific transporters and enzymes facilitate the uptake and breakdown of opines providing a source of carbon and nitrogen for the bacterium. Opine uptake and catabolism play a significant role in the interaction between *Agrobacterium* and plants contributing to the pathogenicity and survival of the bacterium (Vladimirov et al., 2015). These components collectively contribute to the success of T-DNA transformation and the establishment of the *Agrobacterium*-plant interaction.

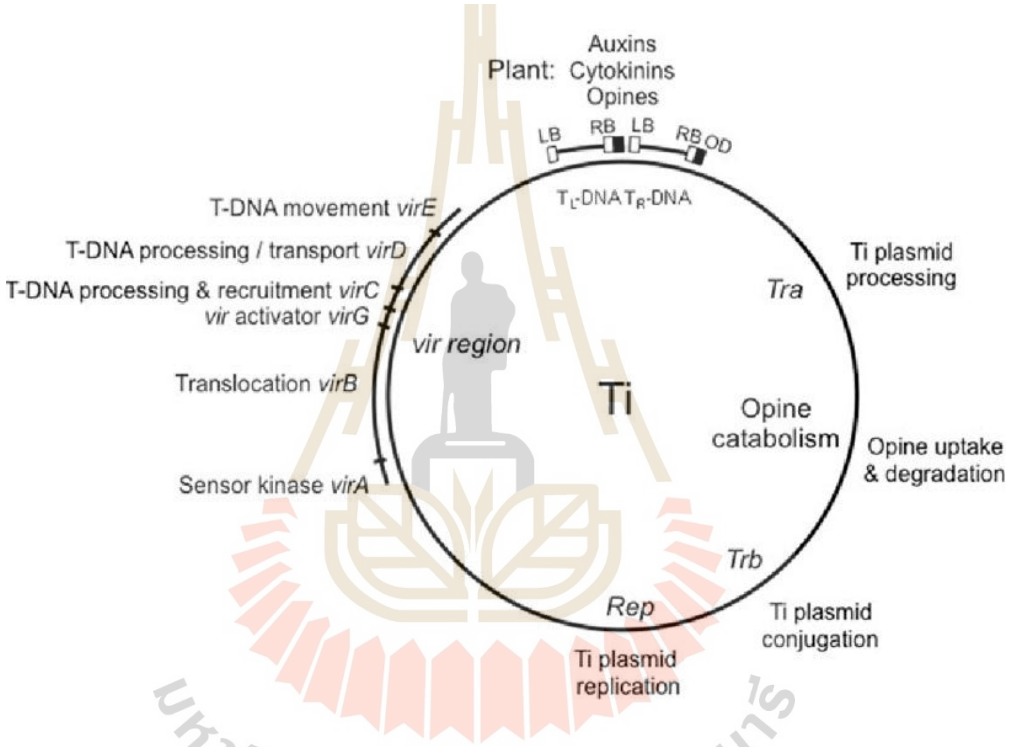

Figure 2.3 The schematic of the octopine-type Ti plasmid illustrates the gene locations responsible for various functions in *Agrobacterium tumefaciens* (Gordon & Christie, 2014).

2.4.2.1.2 The T4SS of *Agrobacterium* spp.

The T4SS of *A. tumefaciens* C58 is responsible for delivering the T-DNA into the plant cells and conjugation processes (Hwang et al., 2017). Notably, three distinct types of T4SSs have been identified in *A. tumefaciens* C58, characterized by the presence of *vir*, *avh* and *trb* genes (Sugawara et al., 2013). The *virB* operon plays a crucial role in facilitating the transfer of DNA to the plant

host, while the *trb* system are necessary for the conjugal transfer of the Ti plasmid between *Agrobacterium* and plant host (Bodman et al., 1989). On the other hand, the *avh* cluster of pAtC58 plasmid encode a minimum of 10 proteins, namely AvhB2 through AvhB11. Remarkably, seven of these proteins demonstrate substantial similarity to the corresponding VirB proteins, which are associated with virulence on the Ti plasmid (Chen et al., 2002). While, the Trb system shares amino acid sequence similarities with 6 of the VirB proteins despite having different gene arrangements and this gene cluster required for conjugal transfer (Li et al., 1998).

Based on *A. tumefaciens C58* T4SS, it has been observed that the majority of Gram-negative bacteria with T4SS possess a set of 12 structural proteins which include VirB1-B11 and VirD4. These proteins collaborate to form a multi-protein envelope-spanning transport apparatus serving as a multi-protein transport system. The T4SS apparatus can be categorized into three groups based on their functions including the energy subcomplex, core subcomplex and T pilus subcomplex (Figure 2.4) (Waksman & Orlova, 2014). Energy subcomplex consisting of VirD4, VirB4, and VirB11 proteins that act as an ATPase for T4SS. ATPases are enzymes that hydrolyze adenosine triphosphate (ATP) molecules releasing energy that can be used for various cellular processes. Here, ATPase activity of VirD4, VirB4, and VirB11 proteins provides the energy required for the proper functioning of the secretion apparatus, enabling the transport of substrates across cell membranes (Atmakuri et al., 2004). Beside ATPase function, VirD4 also plays a crucial role in connecting the cytoplasmic ATPases (such as VirB4) with the outer membrane components of the T4SS. It bridges the inner membrane-associated ATPases and the outer membrane channel proteins, facilitating the transfer of substrates across the cell membranes (Redzej et al., 2017).

The core subcomplex consists of several proteins, including VirB6, VirB7, VirB8, VirB9, and VirB10. These proteins play essential roles in the assembly and stabilization of the T4SS apparatus. VirB6 is transmembrane protein involved in the formation of a channel or pore through which substrates are transported during (Jakubowski et al., 2004). VirB7 and VirB9 form a stable complex in the outer membrane of the bacterial cell. While, both VirB8 and VirB10 proteins play crucial roles in the assembly, stability and functioning of the T4SS. They contribute to the formation of a translocation channel and facilitate the movement of substrates

through the secretion apparatus (Das & Xie, 2000; Waksman & Orlova, 2014). The T pilus subcomplex comprises VirB2 and VirB5 proteins which serve as the major and minor components of the T4SS pilus. These proteins function as adhesins, facilitating host-cell targeting by binding to specific receptors on the (Backert et al., 2008).

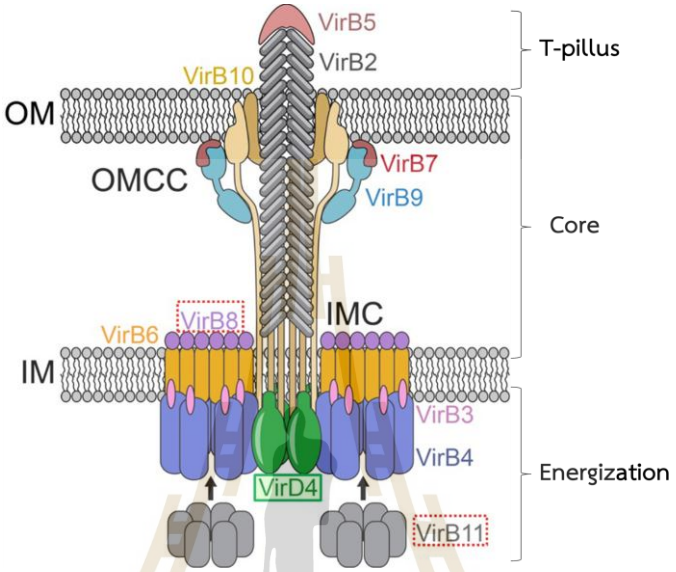

Figure 2.4 The structure of T4SS. This image has been adapted from Álvarez et al., 2020.

2.4.2.1.3 *Agrobacterium* utilizes a T4SS to induce crown gall disease.

The *Agrobacterium*-plant interaction is a intricate process that can be dissected into multiple functional stages to induce crown gall in plants. They required two elements for gall disease development including T-DNA transformation and tumorigenesis (Winans, 1990, 1991; Escobar & Dandekar, 2003; White & Winans, 2007;). The overall process can summarize as following steps (Figure 2.5).

- Recognition of plant wound signals: When plant tissues are wounded, specific chemical signals are released. *A. tumefaciens* detects these signals through the VirA protein, which acts as a sensor kinase located on the bacterial cell membrane.

- Activation of VirA/VirG system: binding of the wound signals, such as acetosyringone, to VirA triggers autophosphorylation of VirA at a histidine residue. The phosphoryl group is then transferred from VirA to the response regulator VirG, activating.
- Transcriptional activation of *vir* genes: activated VirG binds to specific DNA sequences called "*vir*-boxes" in the promoter regions of the *vir* genes. This binding leads to the transcriptional activation of *vir* genes, which encode the Vir proteins required for T-DNA transfer.
- Assembly of T4SS apparatus: the VirB and VirD4 proteins, encoded by the *vir* genes, form the core components of the T4SS apparatus. The VirD4 protein acts as a coupling protein that connects the T-DNA processing complex to the T4SS.
- T-DNA processing: the VirD2 endonuclease nicks the T-DNA at specific border sequences, generating a 5' phosphate and a 3' hydroxyl group at each end. The VirD2 protein remains covalently bound to the 5' ends of the T-DNA, protecting it from degradation.
- Formation of T-pilus and T-complex: the VirB proteins assemble into a translocation channel called the T-pilus, which extends from the bacterium to the plant cell. The VirD2-T-DNA complex is recognized by the VirD4 protein, which facilitates its transfer to the T-pilus, forming a T-complex.
- Transfer and integration of T-DNA: The T-complex is translocated through the T-pilus and delivered into the plant cell. Once inside the plant cell, the VirD2 protein aids in nuclear import of the T-DNA. The T-DNA integrates into the plant genome, usually via a site-specific recombination event.
- Expression of T-DNA genes: After integration, the T-DNA genes can be transcribed and translated in the plant cell. These genes may include oncogenes (such as auxin and cytokinin biosynthesis genes) and other genes that manipulate plant cell physiology, leading to the formation of characteristic tumor-like galls.

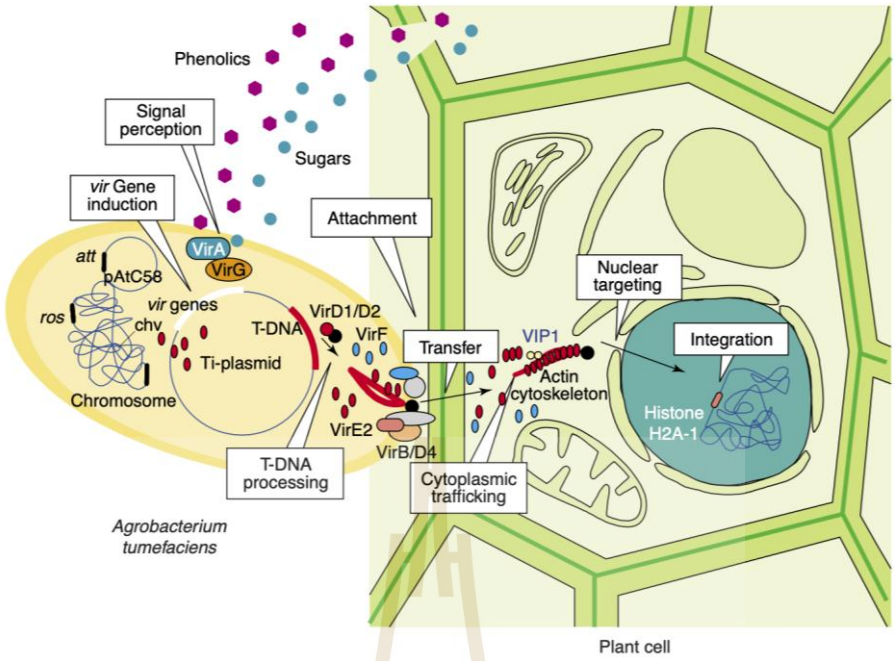

Figure 2.5 A comprehensive model is proposed to illustrate the *Agrobacterium*-mediated transformation (Gelvin, 2003).

2.4.2.2 The secretion systems associated with symbiosis process

The symbiotic relationship between rhizobia and legumes can undergo a pathogenic shift if the legumes fail to regulate the total number of nodules formed or if the rhizobia produce non-nitrogen-fixing nodules. The existence of these secretion systems in evolution indicates that while rhizobia and legumes have co-evolved a mechanism for establishing and sustaining symbiosis, there has also been a co-evolution of a relationship resembling that of a pathogen interacting with a plant (Nelson & Sadowsky, 2015). The specific microbial-plant relationship exhibits variability influenced by external factors, such as environmental changes as well as intrinsic factors inherent to both organisms. Communication between the host and microbe plays a pivotal role in both pathogenic and mutualistic interactions, predominantly relying on signal exchange as a fundamental mechanism (Tseng et al., 2009). In addition to nod factor mechanisms, some of rhizobia employ a secretion system that shares homology with those found in bacterial pathogens to initiation symbiosis mechanism with their host (Deakin & Broughton, 2009a). Rhizobia have been found to possess multiple secretion system mechanisms, including T3SS,

T4SS, and T6SS, symbiotic plasmids (pSym) or within symbiotic islands located on the chromosome (Figure 2.6). They have ability to translocated protein pass through both the inner membrane (IM) and outer membrane (OM) in one step, without forming stable intermediates in the periplasmic space (Fauvart & Michiels, 2008). In addition, these systems play a crucial role in symbiosis by facilitating the translocation of effector proteins during the symbiotic process (Sugawara et al., 2013; Nelson & Sadowsky, 2015).

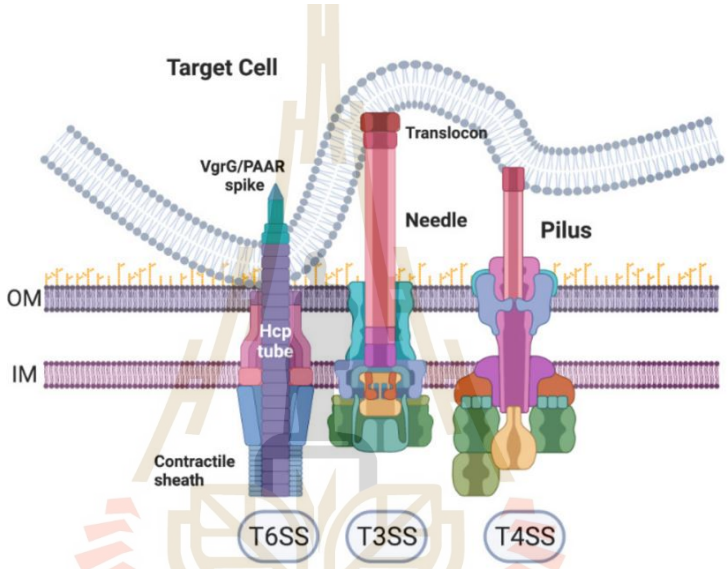

Figure 2.6 Bacterial secretion systems associated with symbiosis interaction between rhizobium and plant host. Various secretion systems are capable of directly injecting proteins, effectors, or toxins into target cells (Filloux, 2022).

2.4.2.2.1 The role of T3SS in symbiotic interactions

The T3SS is a complex structure composed of several subunits, approximately 20 bacterial proteins which can secrete bacterial proteins into the extrabacterial environment (Galán & Collmer, 1999). The T3SS system is typically associated with pathogenic bacteria and plays a critical role in host-pathogen interactions. Pathogenic bacteria utilize this system to directly inject effector proteins from the bacteria into host cells, crossing the cellular membrane in the process (Ghosh, 2004). Pathogenic bacteria employ the system to deliver a class

of proteins known as T3SS effectors (T3Es) (Büttner & Bonas, 2003). In rhizobia, the core machinery genes responsible for secretion are referred to as *rhc* (Rhizobium conserved) family which encode the key component including needle-like structure, transmembrane basal body, export apparatus and effector protein called nodulation outer proteins (Nops). These effector proteins modulate plant cell processes and signaling pathways, promoting the formation of nitrogen-fixing nodules on legume roots (Viprey et al., 1998; Deakin & Broughton, 2009). The production of NF can impact the expression of rhizobial T3SS. NodD, acting as an activator triggers the expression of the *ttsI* gene. *TtsI* serves as a transcriptional activator that is responsible for coding the T3SS apparatus and T3Es (Marie et al., 2004; Jiménez-Guerrero et al., 2022). T3SS is a well studied secretion system found in many rhizobia, the interaction between rhizobium and legume symbiosis was first identified in symbiotic plasmid of *Sinorhizobium* sp. NGR234 (Viprey et al., 1998) which is a broad host range nodulates more than 110 genera of legumes as well as the non-legume *Parasponia andersonii* (Broughton et al., 1986). The influence of T3SS and T3SEs on symbiotic phenotypes in legumes is highly dependent on the specific legume species and the corresponding rhizobia involved. The outcome can range from positive facilitation of symbiosis to neutral effects or even negative interference with the symbiotic relationship (Jiménez-Guerrero et al., 2022). For example, *Bradyrhizobium* ORS285 represent positive effect on *A. afraspera* but have neutral effect on A. *evenia* (Okazaki et al., 2015). However, the roles of the T4SS and T6SS in symbiosis are less well studied than those of the T3SS.

2.4.2.2.2 The role of T4SS in symbiotic interactions

Beside T3SS, some of rhizobia deriver molecules via T4SS during symbiosis process. The T4SS is a pathogenic machinery extensively used by *A. tumefaciens* C58 which purposes of conjugation (Cascales & Christie, 2003). T4SS belong to *virB/D4* complex have been identified in several rhizobia including *Sinorhizobium* spp. (Lloret et al., 2007; Sugawara et al., 2013; Nelson et al., 2017), *Mesorhizobium* sp. (Hubber et al., 2007), *Rhizobium* sp. and *Bradyrhizobium* sp. (Nelson & Sadowsky, 2015). In rhizobia, the T4SS has been identified in both plasmids and chromosomes, and it shows homology to the T4SS found in *A. tumefaciens*. There are two main types of T4SS found in rhizobia including the *virB/D4* and *tra/trb*

clusters which responsible for effector protein translocation and conjugative transfer (Hubber et al., 2007; Paço et al., 2019). These T4SS clusters are typically located within a genomic region known as the symbiosis island, which is a region of the genome that contains genes involved in symbiotic interactions with plants. The *virB/D4* T4SS has been extensively studied and is known to play a crucial role in the symbiotic interaction between rhizobia and plants. It is responsible for the transfer of various symbiotic signals such as Nod factors, into the plant cells (Hubber et al., 2007). In the symbiosis interaction T4SS represent both positive negative and neutral effect on nodulation (Nelson & Sadowsky, 2015). During symbiosis, *M. loti* R7A used T4SS to transport effector proteins, which in turn has a host dependent impact on the symbiotic interaction (Hubber et al., 2007). The deletion mutant of T4SS in *S. meliloti* KH46c and *S. medicae* M2 exhibited distinct nodulation patterns when interacting with various *Medicago* host plants. Interestingly, the impact of T4SS on symbiosis varied depending on the host, demonstrating both positive and negative effects. These findings strongly suggest that T4SS in *Sinorhizobium* likely plays a crucial role in determining host specificity (Sugawara et al., 2013). Furthermore, the deletion of the T4SS apparatus in *S. meliloti* KH46c resulted in reduced competitiveness when co-inoculated with wild type strain. The first identified T4SS effector protein in *Sinorhizobium* spp., TfeA, is crucial for the establishment of symbiosis with *Medicago truncatula* (Nelson et al., 2017). In contrast, T4SS have no effector for symbiosis interaction between *S. meliloti* 1021 and alfalfa (Jones et al., 2007).

On the other hand, the *tra/trb* T4SS operon in rhizobia has been less studied in the context of symbiosis. The induction of *traG* expression suggests that the *tra/trb* T4SS may be involved in the communication and interaction between *M. mediterraneum* Ca36T and the chickpea plant during the early stages of nodule formation. Moreover, *traG* gene also associated horizontally transferred together with the symbiosis genes (Paço et al., 2019). While, T4SS systems are commonly found in various bacteria and can have important roles in symbiosis, the specific involvement and significance of the T4SS in *Bradrhizobium* sp. symbiosis have not been reported or well-studied so far.

2.4.2.2.3 The role of T6SS in symbiotic interactions

The structure of the T6SS has primarily been studied in plant and animal pathogens (Shneider et al., 2013). T6SS functions as a contractile nanosyringe, bearing similarities to inverted phage puncturing tails, and acts as an efficient machinery for translocating proteins and DNA across lipid membranes into target cells (Leiman et al., 2009). The structural biosynthesis of the T6SS consist of the Tss (type six secretion) gene cluster which responsible for encoding 13 core structural proteins known as TssA-M. Additionally, the system utilizes *tag* (type six accessory gene) to encode accessory proteins that contribute to the overall functionality of the T6SS (Silverman et al., 2012). T6SS has been identified and characterized in in five different species of rhizobia including *R. leguminosarum*, *B. japonicum*, *M. loti, S. saheli,* and *S. fredii* (Nelson & Sadowsky, 2015; Sousa et al., 2021). The role of T6SS in the symbiotic interaction between *R. etli* Mim1 and common beans has been investigated, as this strain is known for its ability to generate effective nodules. Deletion of T6SS in *R. etli* Mim1 resulted in a reduction in nodule size and plant dry weight, highlighting the significance of T6SS in this process (Salinero-Lanzarote et al., 2019). As same as *Bradyrhizobium* sp. LmicA16 (A16) plays a critical role in establishing efficient nodulation with *Lupinus micranthus* and *L. angustifolius*. A mutant *vgrG* gene, which encodes a component of the T6SS nanostructure exhibited reduced nodule formation and smaller plant size compared to the wild type strain. Moreover, during coinoculation with the wild type strain, the *vgrG* mutant demonstrated decreased competitiveness (Tighilt et al., 2022). In contrast, the absence of the T6SS in *Azorhizobium caulinodans* ORS571 does not impact symbiotic competition, as evidenced by similar nodule phenotype, nodule size, and nitrogenase activity compared to the wild-type strain (Lin et al., 2018). This finding suggest that T6SS is essential for symbiotic performance and competitiveness depending on rhizobia species and plant host.

2.5 *Bradyrhizobium* sp. SUTN9-2

Bradyrhizobium sp. SUTN9-2, a strain known for its broad host range was first isolated from the *A. americana* (Thai) cultivar in the northern part of Thailand (Noisangiam et al., 2012). It has been observed that *Bradyrhizobium* sp. SUTN9-2 exhibits rice endophytic properties (Piromyou et al., 2015). Typically, *Bradyrhizobium* sp. SUTN9-2 requires NFs for infection and nodulation to establish symbiotic interactions. Based on legumes, in *Aeschynomene* species which exhibit the capacity to form both nitrogen-fixing root nodules and stem nodules in association with *Bradyrhizobium*. Interestingly, certain legume species can establish symbiotic interactions with specific strains of *Bradyrhizobium* that do not produce the common NF. *Aeschynomene* species are categorized into three distinct cross-inoculation (CI) groups based on their symbiotic characteristics including CI group 1: root nodulation by non-photosynthetic bradyrhizobia containing NF (ex. *A. americana* and *A. uniflora*), CI group 2: stem and root nodulation by both non-photosynthetic strains and photosynthetic which lacking NFs (ex. *A. afraspera and A. nilotica*), CI group 3: stem and root nodulation by only photosynthetic bacteria (ex. *A. evenia* and *A. indica*) (Mornico et al., 2011; Chaintreuil et al., 2013). Interestingly, *Bradyrhizobium* sp. ORS285, a photosynthetic bacterium, contains *nod* genes and is capable of nodulating both CI Group 2 and CI Group 3 legumes. However, when the *nod* genes are absent in *Bradyrhizobium* sp. ORS285, it loses the ability to nodulate CI Group 2 legumes while still maintaining its nodulation ability on CI Group 3 legumes (Mornico et al., 2011). While, *Bradyrhizobium* sp. SUTN9-2 displayed the ability to form nodules in all three CI groups, including *A. americana* (CI Group 1), *A. afraspera* (CI Group 2), and *A. indica* (CI Group 3). Notably, CI Group 3 is typically limited to photosynthetic bacteria and lacks NFs. This discovery suggests that *Bradyrhizobium* sp. SUTN9-2 may employ an alternative mechanism to establish symbiotic interactions with *A. indica* (Noisangiam et al., 2012). For the secretion systems, *Bradyrhizobium* sp.

2.5.1 The secretion systems of *Bradyrhizobium* sp. SUTN9-2

SUTN9-2 possesses symbiotic secretion systems, namely T3SS and T4SS, along with nodulation genes on its chromosome. Interestingly, the impact of T3SS on symbiosis varies, as it has been observed to have positive, negative, and neutral effects. Specifically, T3SS does not affect the original hosts *A. americana* (Thai) and

Vigna radiata (V4718, V4758, and V4785). However, it exhibits negative effects on several cultivars of *V. radiata* (KPD2, SUT4), *M. atropurpureum*, and *Lotus* spp. Conversely, a positive effect is observed in *V. radiata* CN72 (Piromyou et al., 2015; Piromyou et al., 2019; Hashimoto et al., 2020; Jiménez-Guerrero et al., 2022). These findings suggest that T3SS plays a role in restricting the host specificity of *Bradyrhizobium* sp. SUTN9-2.

However, the role of T4SS in *Bradyrhizobium* sp. SUTN9-2 and its interaction with legume symbiosis have not been reported. Interestingly, *Bradyrhizobium* sp. SUTN9-2 possesses two T4SS clusters belonging to the *tra/trb* operon (Piromyou et al., 2015). The *tra/trb* operon has been found to be crucial for the conjugative transfer of the Ti plasmid in *A. tumefaciens* C58 (Paço et al., 2019). Cluster 1 T4SS of *Bradyrhizobium* sp. SUTN9-2 exhibits significant similarities in amino acid sequences and gene organization with *B. diazoefficiens* USDA110, while cluster 2 shows high similarity to the T4SS of *Bradyrhizobium* sp. BTAi1 which belong to non-photosynthetic and photosynthetic *Bradyrhizobium*, respectively (Piromyou et al., 2015). Therefore, the objective of this study is to investigate the role of T4SS in *Bradyrhizobium* sp. SUTN9-2 and its potential impact on legume symbiosis.

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CHAPTER III

The type IV secretion system (T4SS) mediates symbiosis between *Bradyrhizobium* sp. SUTN9-2 and legume

3.1 Introduction

Typically, rhizobia stimulate the nodulation process using Nod factors (NF), to communicate with Nod factor receptors (NFRs) in host plants and lead to nodule formation. However, some rhizobia lack nodulation *(nod)* gene encoding for NF production; they use an alternative mechanism to form nodules known as secretion systems (Okazaki et al., 2016). Many pathogenic and symbiotic bacteria translocate the compounds, such as virulence genes, effector proteins, DNA, and DNA complexes into their host via secretion systems with similar signaling molecules (Deakin & Broughton, 2009). The secretion system involves bacterial membrane-embedded nanomachines translocating effector proteins into the cytoplasm of the host eukaryotic cells (Cascales & Christie, 2003). Rhizobia genera such as *Rhizobium*, *Mesorhizobium, Sinorhizobium,* and *Bradyrhizobium,* contain genes encoding for secretion systems located on both their chromosome and plasmid, depending on the species. The type of secretion system in rhizobia involved in symbiosis has been identified as type III (T3SS), IV (T4SS), and VI (T6SS) secretion systems (Sugawara et al., 2013; Nelson & Sadowsky, 2015). In rhizobia-legume symbiosis, T3SS is important for plant immune response and nodulation (Okazaki et al., 2013; Nelson & Sadowsky, 2015). T3SS has both positive and negative effects on symbiotic interactions, depending on the host (Okazaki et al., 2013a; Piromyou et al., 2019). T3SS and T4SS are injecting machine related to symbiotic interactions in several rhizobia. T4SS is well characterized in *Agrobacterium* and is a standard model for T4SS localization, structural components, and gene arrangement in bacteria (Aguilar et al., 2010). Typically, *A. tumefaciens* C58 transfers T-DNA, effector proteins, and virulence genes to plant cells via T4SS machinery in the pTi (tumor-inducer) plasmid. The pTi plasmid carries several T4SS genes, including virulence (*vir*), avirulence homolog

(avh), and *trb* genes. The *trb* genes are annotated for conjugal transfer, while *vir* and *avh* are essential for T-DNA transfer (Sugawara et al., 2013; Christie et al., 2014).

Gene encoding for T4SS in *Rhizobium*, *Mesorhizobium*, *Sinorhizobium,* and *Bradyrhizobium* have been found on chromosomes and plasmids. Most rhizobia contain *vir*B/D4 and *tra/trb* operons that encode the putative proteins involved in secretion apparatus production. In addition, T4SS is important to symbiotic relationships and host specificity. For example, the *vir* gene mutant strains of *M. loti* R7A and MAFF303099 displayed decreased and increased nodule formation depending on the host (Hubber et al., 2004) Similarly, the host-dependent nodulation was observed in *S. meliloti* KH46c and *S. medicae* M2 (Sugawara et al., 2013). The *S. meliloti* KH46c T4SS mutant strains showed less competition for nodulation when co-inoculated with wildtype strain in equal cell numbers on *Medicago truncatula* (Nelson et al., 2017). In contrast, the T4SS of *S. meliloti* 1021 is unnecessary for symbiosis but required for conjugation (Kobayashi et al., 2007). Interestingly, most bradyrhizobia containing T4SS components harbored *tra/trb* operon on the chromosome, such as *Bradyrhizobium* sp. SUTN9-2 (Piromyou et al., 2019), *B. diazoefficiens* USDA110, and *B. japonicum* USDA6 (Kaneko et al., 2011), while *vir* operon was found on symbiotic plasmids of *Bradyrhizobium* sp. DOA9 (Okazaki et al., 2015) and *Bradyrhizobium* sp. BTAi1 (Cytryn et al., 2008).

Here, *Bradyrhizobium* sp. SUTN9-2 that was isolated from *Aeschynomene americana* was focused. *Bradyrhizobium* sp. SUTN9-2 is a broad host range strain establishing symbiotic nodule with several legume genera. Based on 16S rRNA and housekeeping gene (*dnaK, recA,* and *glnB*) phylogenetic trees, *Bradyrhizobium* sp. SUTN9-2 belongs to the same clade as *B. yuanmingense* (Noisangiam et al., 2012). Furthermore, the secretion systems of *Bradyrhizobium* sp. SUTN9-2, including T3SS and T4SS, were observed on the chromosome. T3SS of *Bradyrhizobium* sp. SUTN9-2 plays a negative effect on *Vigna radiata* and *Macroptilium atropurpureum* symbiosis while having no effect on the original host, *A. americana* (Piromyou et al., 2015). This phenomenon implied that T3SS is one of the symbiosis determinants for *Bradyrhizobium* sp. SUTN9-2 (Piromyou et al., 2019). In contrast, the information on T4SS in most species of *Bradyrhizobium,* including *Bradyrhizobium* sp. SUTN9-2, is still unclear. In this study, 2 copies of T4SS were identified on the chromosome of

Bradyrhizobium sp. SUTN9-2 with different location. Both T4SS belong to the *tra/trb* operon. The T4SS copies 1 and 2 ($T4SS_1$ and $T4SS_2$) of *Bradyrhizobium* sp. SUTN9-2 showed high similarity with *B. diazoefficiens* USDA110 and *Bradyrhizobium* sp. BTAi1, respectively (Piromyou et al., 2015). As a result, this study specifically investigated $T4SS_1$ in *Bradyrhizobium* sp. SUTN9-2, a strain closely similar to *B. yuanmingense*. In subtropical region, *B. yuanmingense* is the main rhizobia associated with *V. unguiculata* and *V. radiata* plants (Zhang et al., 2008).

The evidence for the evolution of T4SS using gene comparison and phylogenetic tree analyses was demonstrated. Moreover, the role of T4SS in the symbiotic interaction of *Bradyrhizobium* sp. is elucidated in this study for the first time. The function of T4SS in *Bradyrhizobium* sp. SUTN9-2 was explored by gene mutation and complementation, and then investigated for nodulation efficiency in representative legumes belonging to Dalbergioids (*Aeschynomene americana*), Genistoids (*Crotalaria juncea*), and Millettiods (*V. radiata* cv. SUT4).

3.2 Materials and methods

3.2.1 Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 3.1. *Bradyrhizobium* sp. SUTN9-2 was cultured on Arabinose-Gluconate (AG) medium (Sugawara et al., 2010) at 28°C, and *Escherichia coli* strains were cultured in Luria-Bertani (LB) medium at 37°C. The media were supplemented with antibiotics at following concentrations (in μg/ml): streptomycin (sm), 200, nalidixic acid (nal), 20, kanamycin (Km), 50 and cefotaxime (cefo), 20.

3.2.2 Plasmid construction, gene deletion, and complementation

All primers used in this study are listed in Table 3.2. The scheme for T4SS mutation and complementation of *Bradyrhizobium* sp. SUTN9-2 is displayed in Figure 3.1. The construction of T4SS deletion mutants was achieved by double crossing over. Briefly, the fragments corresponding to the upstream and downstream flanking region of $T4SS_1$ (up-1049 bp and down-804 bp) were amplified and merged by overlapped extension PCR and cloned into the pNTPS129 plasmid harboring the *sacB* gene details provided in Appendix B.2 (Tsai & Alley, 2000), namely pNTPS129-$T4SS_1$. Then, a cefotaxime and streptomycin/spectinomycin resistance

cassette was introduced between the upstream and downstream flanking regions at the HindIII and BamHI sites of pNTPS129-T4SS$_1$. The resulting plasmids were transferred into *Bradyrhizobium* sp. SUTN9-2 via triparental mating using pRK2013 as a helper plasmid (Ditta et al., 1980). The single recombinant mutants were selected on AG agar supplemented with nal, 20, cefo, 20 and verified by PCR using two primer pairs amplifying the upstream and downstream flanking target genes. The double-crossed mutants were selected for on AG medium containing 10% sucrose supplemented with cefo, 20 for T4SS$_1$. The transconjugant colonies appearing on selective media were verified by PCR. The mutant was screened on antibiotic plates (cefo, 20) and verified by PCR using the primers described in Table 3.2. The complementation of ΔT4SS$_1$ was performed by introduced a 5,069 bp fragment contained T4SS$_1$ and its candidate promoter into pMG103-*npt2-sp/sm* at the XbaI and EcoRI sites. The constructed plasmid was transferred into ΔT4SS$_1$ using triparental mating and the transconjugant was confirmed by antibiotic resistant and PCR verification. The derivative mutant and complementation strains were further investigated for nodulation efficiency in various legumes.

3.2.3 Nodulation test and acetylene reduction assay (ARA)

The legume plants used in this study include *Aeschhynomene americana* (Shyleaf), *Vigna radiata* cv. SUT4 (Mung bean) and *Crotalaria juncea* (Brown hemp) for Dalbergioids, Millettiods, and Genistoids, respectively. All seeds were sterilized as previously described (Teamtisong et al., 2014), and then rinsed and soaked with sterilized water overnight at room temperature. The germinated seeds were placed on 0.85% water agar plates and incubated at 28°C for 1-2 days. After 1 week, seedlings were transferred into Leonard's jars containing sterilized vermiculite and inoculated with 1 ml bacterial cultures adjusted with sterilized distilled water to OD_{600} = 0.8. Plants were harvested 7, 14, and 21 days post inoculation (dpi) for nodule number evaluation and acetylene reduction assay (ARA). The nodules were incubated in 50 ml (Mung bean and Brown hemp) and 20 ml (Shyleaf) test tubes. Then, the air was withdrawn and replaced with pure acetylene at 10% (v/v). The samples were incubated for 1 h at 25°C. The conversion of acetylene (C_2H_2) to ethylene (C_2H_4) was measured by injecting 1 ml into Gas Chromatography (GC) using a PE-alumina-packed column at an injection temperature of 150°C and oven

temperatures of 200°C and 50°C for flame ionization detection (FID) (Renier et al., 2011). The nitrogenase activity is presented as nmol ethylene/h/plant dry weight (Somasegaran & Hoben, 1985).

Table 3.1 Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Reference or source
Strain		
Bradyrhizobium sp. SUTN9-2	*A. americana* nodule isolate (paddy crop)	Noisangiam et al., 2012
ΔT4SS$_1$	SUTN9-2 derivative, T4SS$_1$ (*copG, traG,* and *virD2*)::Cefo; Cefo^r	This study
Escherichia coli		
DH5α	*supE44 ΔlacU169 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Toyobo Inc.
Plasmid		
pRK2013	ColE1 replicon carrying *RK2* transfer genes; Km^r; Helper plasmid	Ditta et al., 1980
pNTPS129	Cloning vector harboring sacB gene under the control of the constitutive *npt2* (Appendix B.2) promoter; Km^r	Tsai & Alley, 2000
pNTPS129-ΔT4SS$_1$	pNTPS129-*npt2-sacB* containing the flanking region of T4SS copy 1 (T4SS$_1$)	This study
pMG103-*npt2-cefo*	Complementation vector harbors a cefotaxime resistance gene under the control of the constitutive *npt2* promoter; Cefo^r (Appendix B.4)	Giraud E, IRD, France
pMG103-*npt2-sm/sp*	Complementation vector harbors a Ω cassette; Sm^r, Sp^r resistant genes under the control of constitutive *npt2* promoter; Sm^r, Sp^r modified from pMG103-*npt2-cefo* (Giraud E, IRD, France), insert Ω cassette at HindIII site (Appendix B.3)	This study
pMG103-*npt2-sm/sp*-T4SS$_1$	pMG103-*npt2- sm/sp-npt2-gfp* carrying 5,069 bp T4SS copy1 (T4SS$_1$) fragment ; Sm^r, Sp^r	This study

Table 3.2 Primers and cloning strategies in this study.

Target genes	Primer names	Primer sequence (5'⟶3')	References
Deletion			
Upstream fragment of the ΔT4SS$_1$	Up.F.T4SS$_1$.XbaI	ACC CAG TCT AGA GAT AAC GCT CGA CCA ACT CTC A	This study
	Up.R.T4SS$_1$.overl	CTG CGT AAG TTC GAA GCT TTC TCT TCA TCC GCT TCT GGC T	This study
Downstream fragment of the ΔT4SS$_1$	Dw.F.T4SS$_1$.overl	GCG GAT GAA GAG AAA GCT TCG AAC TTA CGC AGT TCG AC	This study
	Dw.R.T4SS$_1$.BamHI	CAG AAT GGG ATC CAT CAT TTC CGC TTC ATC GTC TC	This study
Complementation			
T4SS1 complementation (T4SS$_{1compl}$)	Compl.T4SS$_1$.XbaI.F	CGC TGG TCT AGA ATC AGA TCC TCC GTC GCT GCT	This study
	Compl.T4SS$_1$.EcoRI.R	ACG ACA GGA ATT CTC GCA GCC ATC GTC CCT TT	This study
Gene expression			
Nodulation (*nod*) gene	S9-2.*nodA*.F	GTT CAA TGC GCA GCC CTT TGA G	This study
	S9-2.*nodA*.R	ATT CCG AGT CCT TCG AGA TCC G	This study
	S9-2.*nodC*.F	ATT GGC TCG CGT GCA ACG AAG A	This study
	S9-2.*nodC*.R	AAT CAC TCG GCT TCC CAC GGA A	This study
Structural T4SS$_1$ gene	S9-2.*traG*.F	TTC TCG ATC TGG TTC AGC GAC TG	This study
	S9-2.*traG*.R	TTG ACC GAG GAT CTT CAG GCC A	This study

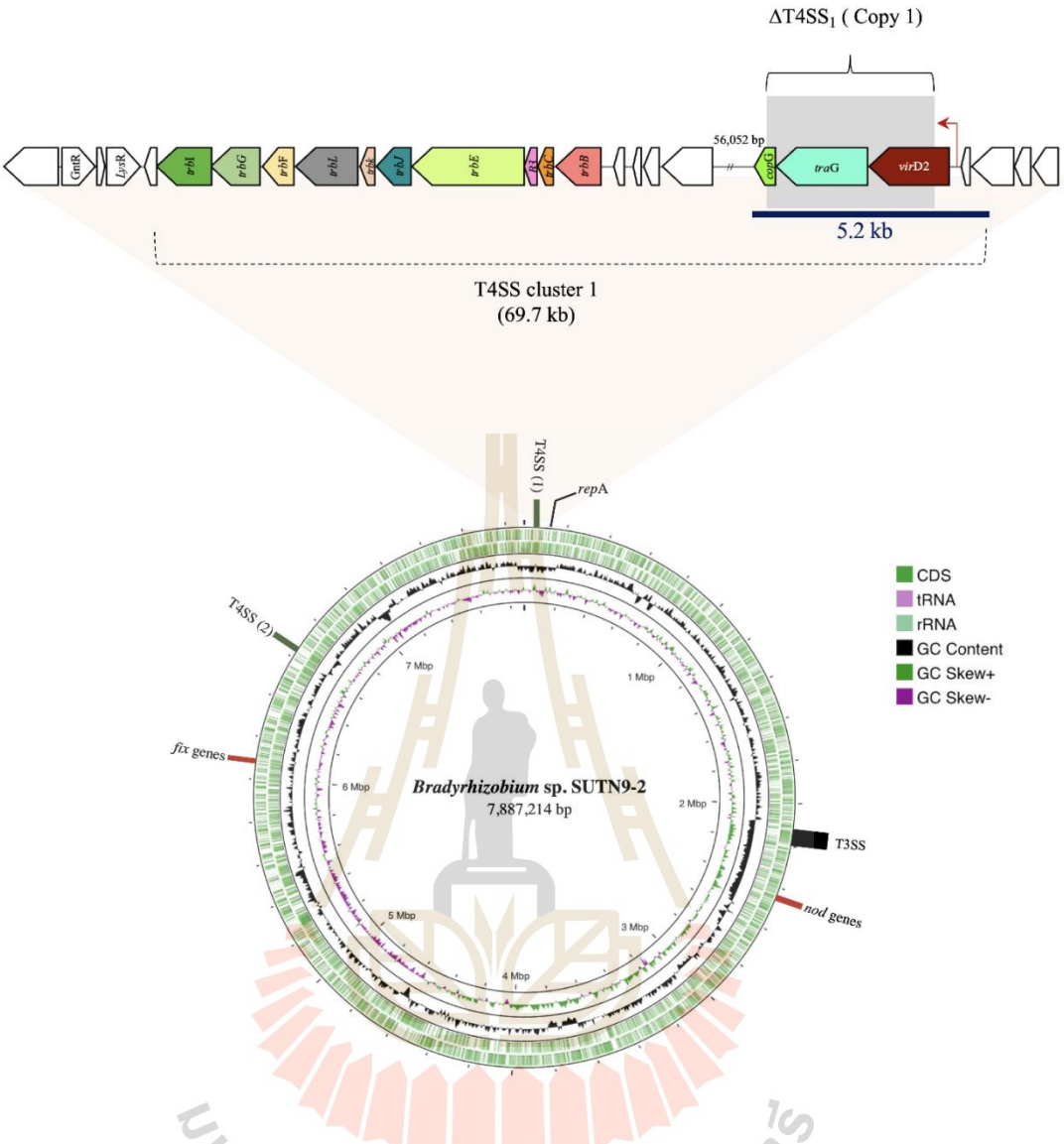

Figure 3.1 Scheme of T4SS mutation of *Bradyrhizobium* sp. SUTN9-2. The grey area represents the fragment of $\Delta T4SS_1$ (copy 1) and the dark blue bars under *copG*, *traG,* and *virD2* genes show the amplified region to complementation $\Delta T4SS_1$. The candidate promoter of T4SS copy 1 is indicated with a red arrow.

3.2.4 Microscopy

Nodule phenotypes were investigated under a stereomicroscope (LEIGA EZ4). The Nikon Inverted Eclipse Ti-E Confocal Laser Scanning Microscope investigated nodule development and live/dead cell bacteroids in symbiosomes. For this purpose, nodules were embedded in agarose (5%) and then sectioned at 40-50

μm by VT1000S vibratome (Leica Nanterre, France). Nodule sections were imaged under a compound microscope (CARL ZEISS/PRIMO STAR HD) and then stained with fluorophore SYTO9 (5 μM)/PI (30 μM) of live/dead cells staining for 30 min and stained with 1x calcofluor white stain in 1X PBS buffer for 20 min. Calcofluor was detected at 460-500 nm emission, while SYTO9 and PI were detected at 510-570 nm and 600-650 nm, respectively.

3.2.5 Gene annotation, Phylogenetic tree construction, CGView map, and integrative conjugative element (ICE) analysis

Two phylogenetic trees including *traG/trb* operon and *copG*, *traG* and *virD2* genes cluster were constructed by the Maximum Likelihood method at a confidence level of 1,000 replicates using MEGA X,10.0.5 versions (Kumar et al., 2018). *Bradyrhizobium* sp. SUTN9-2 genomes sequence and other bacterial strains were retrieved from the NCBI database (https://www.ncbi.nlm.nih.gov) and Genoscope (https://mage.genoscope.cns.fr). The circular CGView map of *Bradyrhizobium* sp. SUTN9-2 was generated by Proksee Genome Analysis (https://proksee.ca) (Stothard et al., 2019). Integrative and conjugative elements (ICEs) were identified with ICEfinder online program (https://bioinfomml.sjtu.edu.cn/ICEfinder/ICEfinder.html) (Gonçalves et al., 2022).

3.2.6 Bacterial induction

Mid-log phase bacterial cultures of SUTN9-2 (WT), and $\Delta T4SS_1$ strains (OD_{600}=0.4) were induced by 20 μM genistein (Sigma–Aldrich, USA) at 28°C for 24 h. Bacterial cells were collected by centrifugation (4,000 ×g, at 4°C) for further total RNA isolation.

3.2.7 RNA isolation and quantification of gene expression by qRT–PCR

Total RNA was isolated from bacterial pellets using RNeasy® Protect Cell Mini Kit (Qiagen, Chatsworth, CA, U.S.A.) according to the manufacture's instruction. Total RNA was treated with RNase-free DNase I (NEB, USA) for 30 min at 37°C. Complementary DNA (cDNA) was synthesized from 500 ng of total RNA using iScript reverse transcription Supermix (Bio–Rad, USA) according to the manufacturer's protocols. Fifty nanograms of cDNA was subjected to real-time PCR using specific primers for N-acyltransferase *nodA*, N-acetylglucosaminyltransferase *nodC* and

conjugal transfer gene *traG*. All primer sets used in the expression analysis are listed in Table 3.2.

For qRT–PCR, cDNA from each sample was briefly mixed with Luna® Universal qPCR Master Mix (NEB, USA) following the manufacturer's protocol, and thermal cycling was conducted in a QuantStudio™ 3 Real-Time PCR System (Thermo Fisher, USA). qPCR amplification was performed under the following cycling conditions: hold stage: 95 °C for 2 min; PCR stage: 40 cycles of 95°C for 15 sec and 60°C for 30 sec; melting curve stage: 95°C for 15 sec and 60°C for 1 min; and a final step of 95°C for 15 sec. Relative gene expression was analysed by the comparative Ct method (-ΔΔCT) and normalized to the endogenous 16S rRNA housekeeping gene. Three biological replicates were pooled and analysed. At least three replicates of PCR amplification were performed for each sample.

3.2.8 Statistical analysis

For statistical analyses, one-way analysis of variance (ANOVA) followed by post hoc tests (Tukey's tests at $P \leq 0.05$) were performed using IBM SPSS Statistics 22.0 software.

3.3 Results

3.3.1 Gene annotation of the type IV secretion system (T4SS) of the *tra/trb* operon in *Rhizobia* and *Agrobacterium*

The *tra/trb* operon was identified in several genera in the rhizobia group, such as *Bradyrhizobium, Rhizobium, Sinorhizobium, Mesorhizobium,* and *Agrobacterium*. The *tra/trb* complex could be observed in both chromosome and plasmid depending on bacterial strain. The *tra/trb* operon was found on the chromosome of *Bradyrhizobium* sp. SUTN9-2, *B. diazoefficiens* USDA110 and *Bradyrhizobium* sp. BTAi1, which is the same as in *Mesorhizobium*. The gene annotation of T4SS in the rhizobia group was compared with *A. tumefaciens* C58, which is widely recognized as a plant crown gall disease causative agent via T4SS. Interestingly, bradyrhizobia's *traG* gene is usually and uniquely flanked with the *copG* and *virD2* genes. Two copies of gene encoded for *tra/trb* operons on chromosome with different gene arrangement were observed in *Bradyrhizobium* sp.

SUTN9-2 (Figure 3.2). Copy 1 was inserted in gene fragment sizes ~56 kb between *trb* operon and *traG* gene (Figure 3.3). While both *M. loti* R7A and *M. loti* MAFF303099 lacked *virD2* gene on their chromosomes. Although the *tra/trb* operon of *Rhizobium* and *Sinorhizobium* were found on their plasmids, as in *Agrobacterium*. This suggests that the *tra/trb* operon in rhizobia differs from the conjugative function of the *tra/trb* operon in *Agrobacterium*.

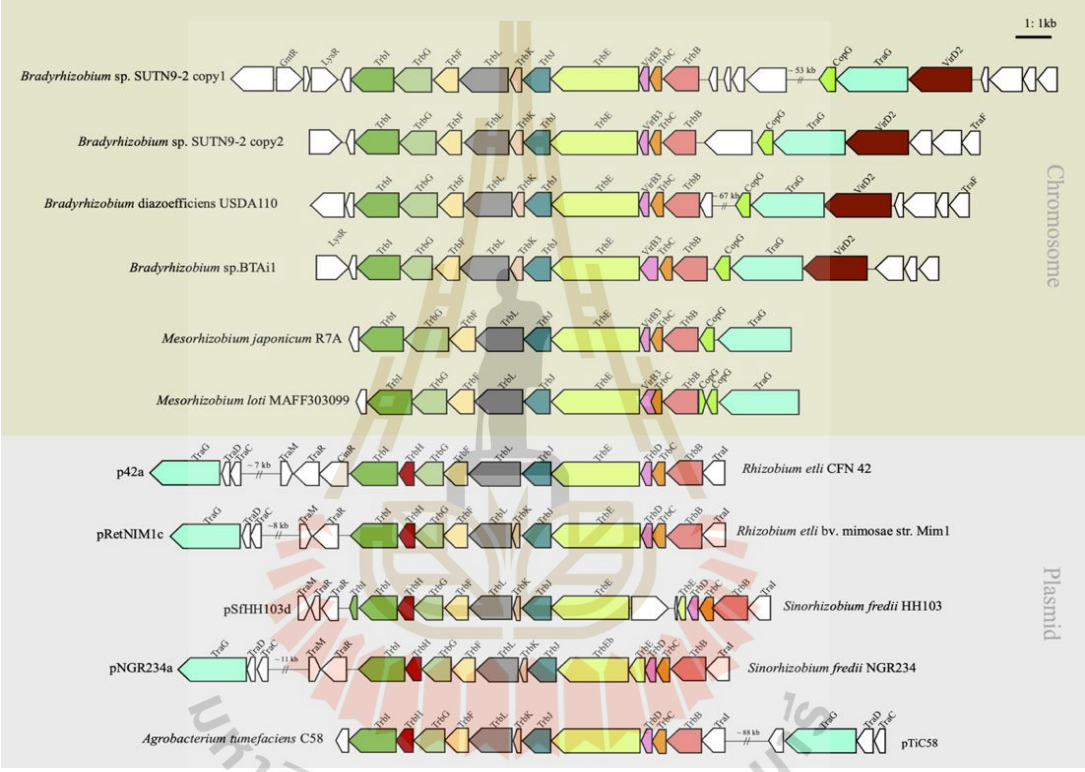

Figure 3.2 Genetic organization of T4SS clusters in strain *Bradyrhizobium*: T4SS genes organization of *Bradyrhizobium* sp. SUTN9-2, *Bradyrhizobium diazoefficiens* USDA110 and *Bradyrhizobium* sp. BTAi1; *Mezorhizobium: Mesorhizobium japonicum* R7A and *M. japonicum* MAFF 303099; *Rhizobium*: *Rhizobium etli* CFN 42 (plasmid p42a), *R. etli* bv. mimosae str. Mim1 (pRetNIM1c); *Sinorhizobium*: *Sinorhizobium fredii* HH103 (pSfHH103), *S. fredii* NGR234 (pNGR234a); *Agrobacterium*: *Agrobacterium tumefaciens* C58 (pTiC58). Orthologous T4SS structural genes are in the same color and in white are the surrounding genes.

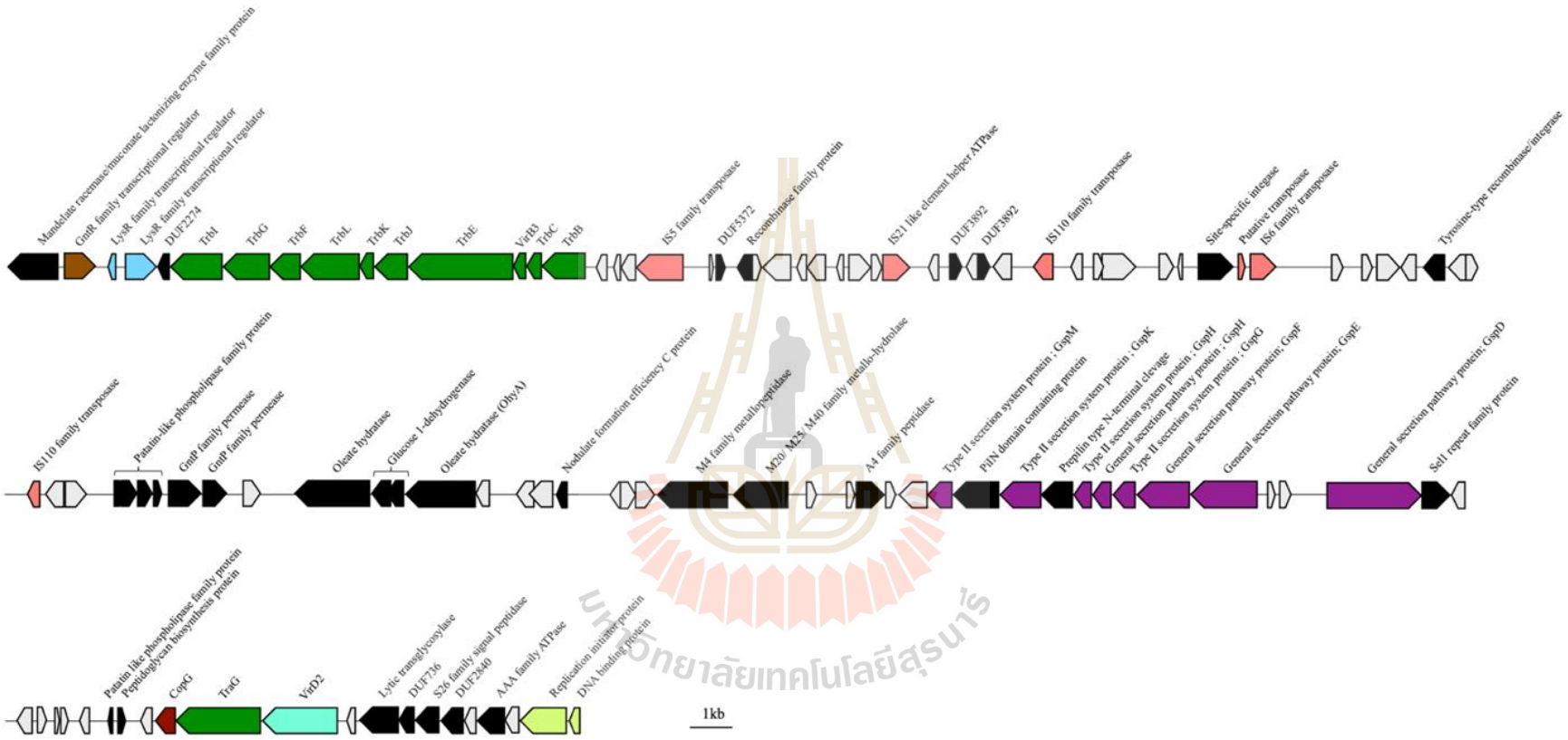

Figure 3.3 T4SS copy 1 full cluster containing ~56 kb inserted genes.

3.3.2 The phylogenetic tree of the T4SS structural gene (*traG/trb* operon)

To examine the evolutionary history of the two copies of *traG/trb* operon, the phylogenetic relationships of *traG/trb* operon homologous were constructed with various genera in the rhizobiaceae family, including *Bradyrhizobium, Rhizobium, Sinorhizobium, Mesorhizobium,* and *Agrobacterium*. *Cuprividus taiwanensis* LMG19424 was used as the outgroup strain. The phylogenetic tree could be divided into two clades (Figure 3.4). In clade 1 *Bradyrhizobium,* T4SS structural genes were genetically closely related to their homologous *Mesorhizobium* members. Both *Bradyrhizobium* sp. SUTN9-2 $T4SS_1$ and $T4SS_2$ were also located in this clade. Interestingly, at least one copy of the T4SS structural genes were found in bradyrhizobial strains, but *Bradyrhizobium* sp. SUTN9-2 contains two copies, which is unusual in *Bradyrhizobium*. Copy 1 showed similar sequences with *B. yuanmingense* BRP09 (copy 3), closely related to *B. japonicum*, *B. diazoefficiens,* and *B. elkanii* USDA61 (B1 clade). *B. elkanii* SEMIA938 and *B. . yuanmingense* BRP09 (copy 1) were closely related to copy .2*Rhizobium, Sinorhizobium,* and *Agrobacterium* members were included in cluster 2. Of note, *Bradyrhizobium* and *Mesorhizobium* were separated from other genera in the rhizobiaceae family, which implied that both T4SS of bradyrhizobia and mesorhizobia may have originated from the same ancestor. Thus, we hypothesized that the T4SS gene in bradyrhizobia evolved together with mesorhizobia upon horizontal gene transfer.

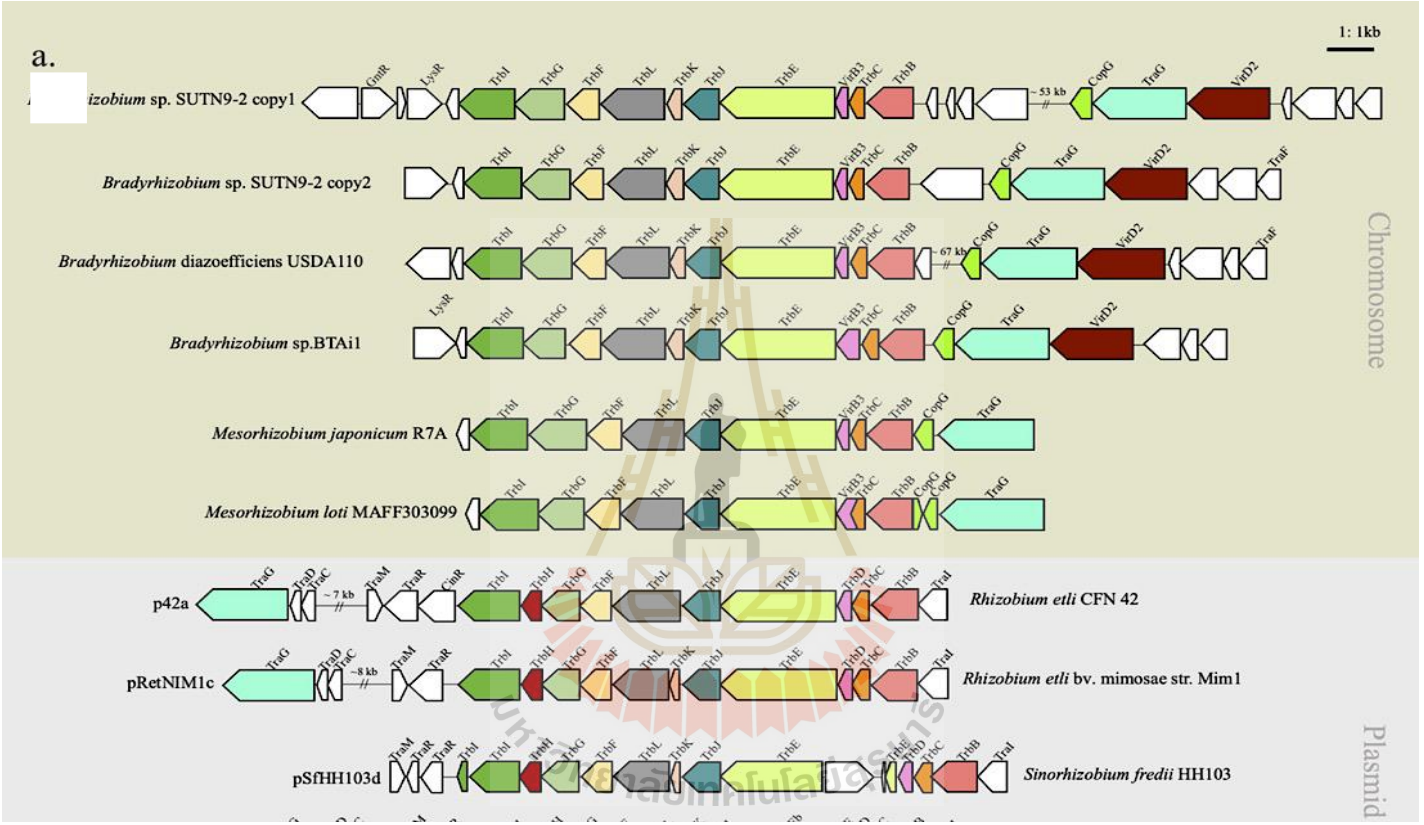

Figure 3.4 Phylogenetic tree of T4SS clusters. Maximum-likelihood phylogenetic tree based on *traG* gene sequences of *Bradyrhizobium* sp. SUTN9-2 with other reference strains of *Bradyrhizobium, Rhizobium,* and *Agrobacterium* bacteria. *Cupriavidus taiwanensis* LMG19424 was used as an outgroup. The phylogeny was inferred using the maximum likelihood method. Scale bar indicates 0.1 substitutions per site.

3.3.3 The phylogenetic tree of bradyrhizobia regarding *copG*, *traG, and virD2*

Phylogenetic tree analysis of concatenate the sequences of T4SS structural gene (*traG*) and T4SS-related genes (*copG* and *virD2*) of *Bradyrhizobium* sp. SUTN9-2 was constructed and compared with other T4SS containing *Bradyrhizobium* strains. The phylogenetic trees of each *traG*, *copG,* and *virD2* combination gene was generated using the same set of strains (Figure 3.5). Given that the T4SS of *Bradyrhizobium* sp. SUTN9-2 was identified in two clusters on the chromosome, and two copies of each gene were analyzed. As expected, the phylogenetic trees based on sequences of the *traG*, *copG,* and *virD2* combination gene showed similar topologies. The phylogenies were separated into two groups with different copies of the T4SS gene of *Bradyrhizobium* sp. SUTN9-2. The *traG*, *copG*, and *virD2* in copy 1 revealed a close evolutionary relationship with similar genes found in *B. yuanmingense* BRP09 (copy 3), *B. japonicum* E109, *B. japonicum* J5, *B. elkanii* USDA61, *B. diazoefficiens* USDA110, USDA122, and SEMIA5080. This copy was closely related to *B. yuanmingense* BRP09 (copy 3) with high bootstrap values. Copy 2 was closer to *Bradyrhizobium* sp. MOS004, *B. yuanmingense* P10 130, *B. elkanii* SEMIA938, *Bradyrhizobium* sp. Rc2d, *B. ottawaense* L2 and closely similar with *B. yuanmingense* BRP09 (copy 1). These results reflect that *Bradyrhizobium* has higher T4SS gene sequence similarity among *Bradyrhizobium* than other genera in the rhizobia group.

3.3.4 *Bradyrhizobium* sp. SUTN9-2 contains chromosomal integrative conjugative elements (ICEs)

This study aimed to identify the putative ICEs on chromosome of *Bradyrhizobium* sp. SUTN9-2. The 243 proteins were explored in putative ICE which containing integrase, T2SS, T4SS, transposase, and relaxase genes, among others. The putative ICE was located between 744 to 167,491 nucleotides (length 166,748 bp) with a GC content of approximately 59.45%. The putative ICE was located between nucleotides 774 and 167,491 and its insertion was facilitated by the site-specific DNA recombinase (SUTN92_v1_10001). The ICE was flanked with the conserved attachment (*att*) site, including *attL* (location: 774-758) and *attR* (location: 167,477-167,491) (Figure 3.6), Interestingly, the transposase, relaxase, and integrase were not observed $T4SS_2$ area. In addition, the putative ICE with different patterns

and numbers of elements were observed in other bradyrhizobia including *B. yuanmingense* CCBAU10071, *B. yuanmingense* CCBAU05623, *B. yuanmingense* 3051, *B. diazoefficiens* USDA 110, *Bradyrhizobium* sp. TSA, *Bradyrhizobium* sp. BTAi1, and *Bradyrhizobium* sp. OSR285 (Table 3.3).

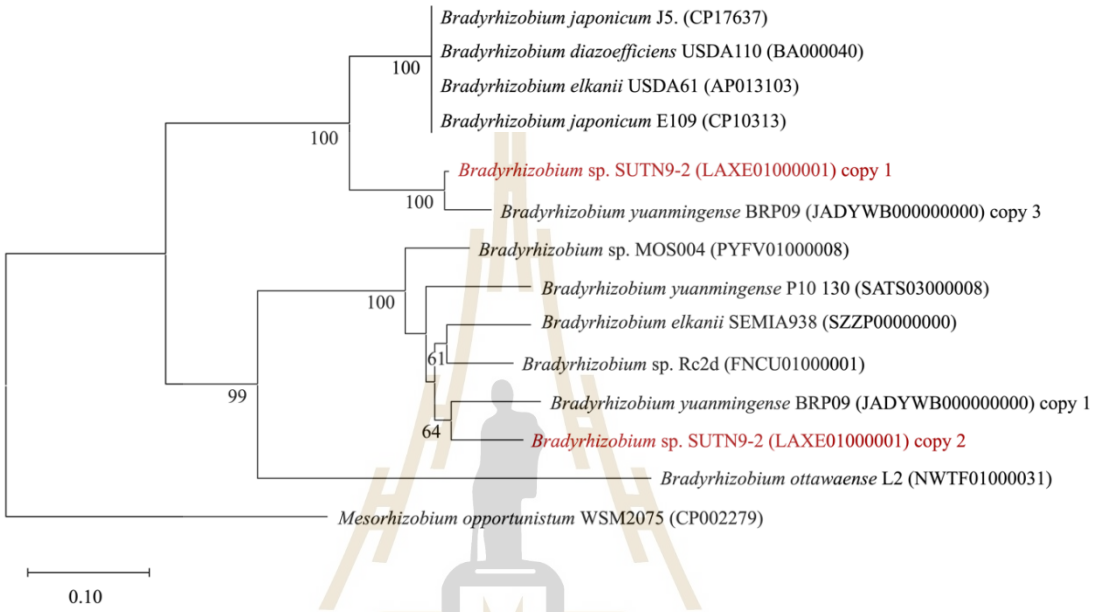

Figure 3.5 Phylogenetic relationship of *copG*, *traG* and *virD2* genes cluster**.** The tree was constructed by the maximum-likelihood method using MEGA10 software. The accession numbers are shown in brackets. *Mesorhizobium oppotunistum*WSM2075 was used as an outgroup. Bars: 0.10 estimated substitutions.

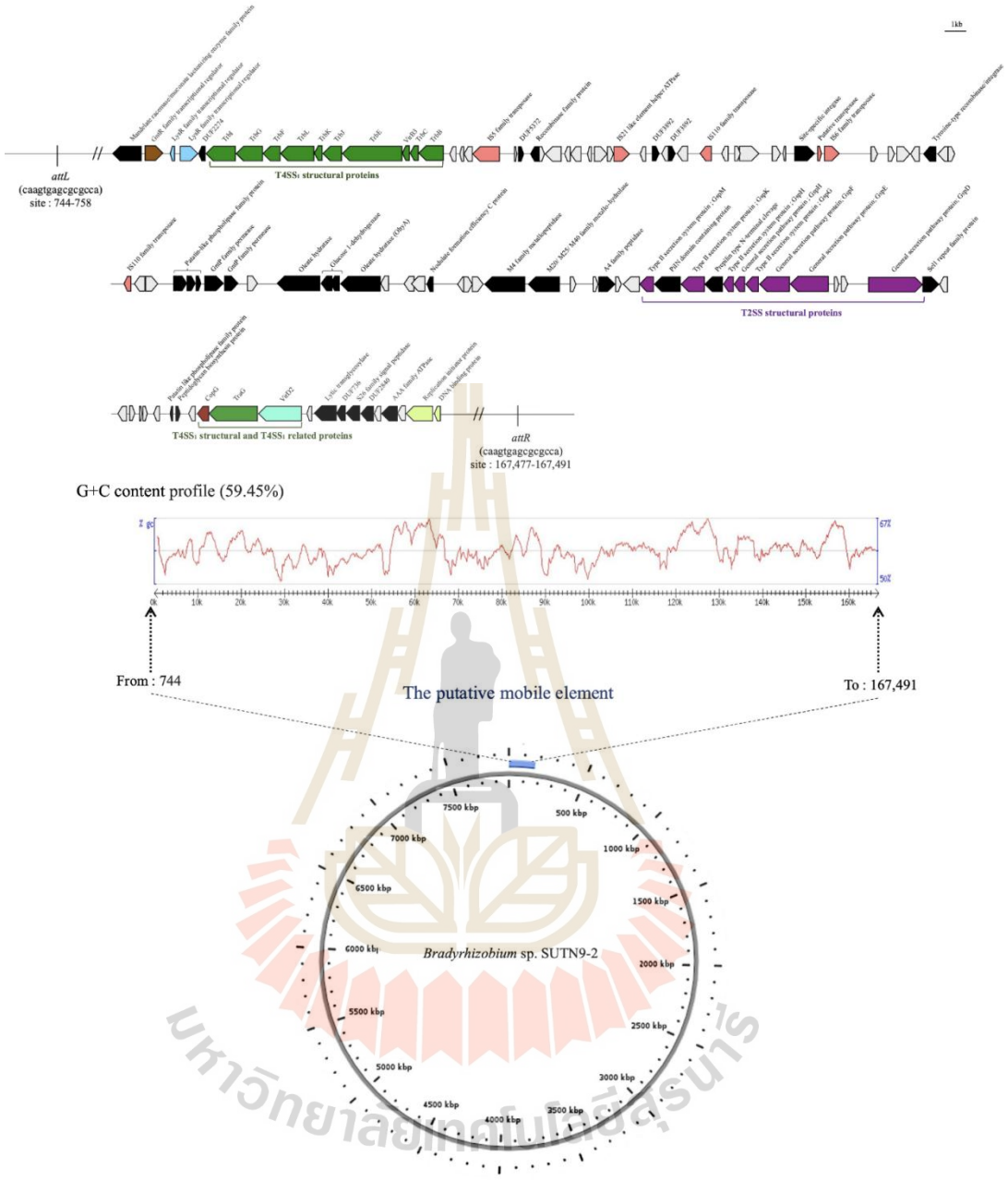

Figure 3.6 Integrative and conjugative element (ICE) identification on chromosome of *Bradyrhizobium* sp. SUTN9-2.

Table 3.3 Putative integrative and conjugative element (ICE) analysis in bradyrhizobia.

Strain	Number of element	Name	Location	Length/bp	GC content (%)	Type
Bradyrhizobium sp. SUTN9-2	1	Region1	744 - 167491	166748	59.45	Putative ICE with T4SS
Bradyrhizobium yuanmingense CCBAU 10071	4	Region1	2300382 - 2736784	436403	61.10	Putative ICE with T4SS
		Region2	6393092 - 6454383	61292	58.38	Putative ICE with T4SS
		Region3	6661095 - 7028779	367685	62.30	Putative ICE with T4SS
		Region4	8162440 - 8170535	8096	55.47	Putative IME
Bradyrhizobium yuanmingense CCBAU 05623	3	Region1	7182470 - 7254080	71611	62.86	Putative ICE with T4SS
		Region2	7679783 - 7716441	36659	61.49	Putative ICE without identified DR
Bradyrhizobium yuanmingense 3051	4	Region1	3398971 - 3698968	299998	60.70	Putative ICE with T4SS
		Region2	4882546 - 5135807	253262	64.76	Putative ICE with T4SS
		Region3	6613477 - 6685484	72008	61.85	Putative ICE with T4SS
		Region4	7830618 - 8045295	214678	64.10	Putative ICE with T4SS
Bradyrhizobium diazoefficiens USDA 110	2	Region1	5819 - 103921	98103	61.70	Putative ICE with T4SS
		Region2	2049053 - 2310150	261098	59.24	Putative ICE with T4SS
Bradyrhizobium sp. TSA1	4	Region1	1169945 - 1202372	32428	60.18	Putative ICE with T4SS
		Region2	1479038 - 1633542	154505	64.48	Putative ICE with T4SS
		Region3	3366004 - 3387901	21898	61.53	Putative IME without identified DR
		Region4	6321081 - 6488853	167773	62.29	Putative ICE with T4SS
Bradyrhizobium sp. BTAi1	5	Region1	434291 - 482263	47973	62.76	Putative IME
		Region2	1490463 - 1665320	174858	61.08	Putative ICE with T4SS
		Region3	6855606 - 6914635	59030	63.60	Putative IME
		Region4	7660819 - 7687258	26440	62.16	Putative IME
		Region5	8090405 - 8210585	120181	61.49	Putative ICE with T4SS
Bradyrhizobium sp. ORS285	4	Region1	284497 - 323900	39404	63.39	Putative ICE with T4SS
		Region2	2838212 - 2843244	5033	63.90	Putative IME without identified DR
		Region3	6483013 - 6636866	153854	62.23	Putative ICE with T4SS
		Region4	7086172 - 7099953	13782	61.19	Putative IME without identified DR

CE = Integrative and Conjugative Element

IME = Integrative and Mobilizable Elements (IMEs)

DR = Directed repeats (*attL* and *attR*)

3.3.5 The T4SS of *Bradyrhizobium* sp. SUTN9-2 is involved in symbiotic interaction

To examine the pivotal role of the T4SS in promoting nodulation in various leguminous plants, the nodulation-related organogenesis organs were determined at 7,14, and 21 dpi. The nodule formation of Shyleaf (original host) by *Bradyrhizobium* sp. SUTN9-2 was observed in the early stage of the nodulation process (7 dpi), while $\Delta T4SS_1$ formed nodule-like structures (Figure 3.7a), and the nitrogenase activity was lower than WT. In the case of mung bean, the plant inoculated with $\Delta T4SS_1$ showed drastically retarded nodulation (primordia formation) at 7dpi (Figure 3.6a), and nodule formation was recovered at 14 dpi (Figure 3.8b). However, the nodule number and nitrogenase activity was decreased compared with WT. As in mung bean, brown hemp generated nodules, which were quantified after inoculation with $\Delta T4SS_1$ at 14 dpi (Figure 3.9b). The dead bacteroid cells of $\Delta T4SS_1$ in the middle symbiosome at 7 dpi were observed, The WT generated the highest nodule number while $\Delta T4SS_1$ showed lower numbers of nodules; the nitrogenase activity level at 21 dpi was not much different among all treatments (Figure 3.9i). Interestingly, the numbers of nodules and ARA activity were decreased under inoculation of $\Delta T4SS_1$ when compared to the WT strain in all plants tested. The and the bacterial infection was lower than WT. Nevertheless, the derivative bacteroides derived by $\Delta T4SS_1$ become to multiply and increase live cells at 14 dpi. This result demonstrated that $T4SS_1$ represents a strong positive effect on nodulation, nitrogen fixation, and cell survival.

Previous results showed $\Delta T4SS_1$ decreased both nodule numbers and nitrogenase activity in plants tested. To ensure the function of $T4SS_1$, the $T4SS_1$ complementation ($T4SS_{1compl}$) was constructed and inoculated into legume plants (Figures. 3.7, 3.8, and 3.9). Nodulation efficiency was restored by $T4SS_{1compl}$, resembling those observed in WT depending on the host plant. In shyleaf at 7 dpi (Figure 3.7a), nodules were observed, and the nodule number of $T4SS_{1compl}$ was similar to WT. At 14 (Figure 3.7b) and 21 dpi (Figure 3.7c), nodule numbers descending order as follows WT, $T4SS_{1comp,}$ and $\Delta T4SS_1$. However, the nitrogenase activity of $T4SS_{1compl}$ was detected at 14 dpi (Figure 3.7h), which is near to WT, while the activity of nitrogenase was lower than WT at 21 dpi (Figure 3.7i). Young nodules

were formed on mung bean inoculated with $T4SS_{1compl}$ at 7 dpi (Figure 3.8a), and the nodule number was higher than that of plants nodulated by $\Delta T4SS_1$ but lower than WT. The efficiency of nitrogenase activity was decreased in this complemented strain. It was the same trend at 7, 14, and 21 dpi (Figures 3.8g, 3.8h, and 3.8i). As in shyleaf and mung bean, nodule formation of $T4SS_{1compl}$ was observed at 7dpi in brown hemp, while $\Delta T4SS_1$ formed the pseudo-nodules (Figure 3.9a). Nitrogenase activity was detected at 14 dpi (Figure 3.9h), which was higher than $\Delta T4SS_1$ but still lower than WT. However, at 21 dpi (Figure 3.9i), the nitrogenase activity level was the lowest. Since the $T4SS_{1compl}$ generates effective nodules on the host plant similar to WT, the ARA activity of each legume corresponded to the nodule phenotype. Confocal microscopy was performed to detect live/dead cells of bacteroids inside nodules. The results showed more living cells of $T4SS_{1compl}$ than dead cells in symbiosome when compared with $\Delta T4SS_1$ at 7 dpi (Figures. 3.7a, 3.8a, and 3.9a). Dead cells were present in mature nodules of $T4SS_{1compl}$ but still less than in $T4SS_1$ inoculation. In order to determine whether the secretion occur in the beginning when Nod factor is produced or later time when nodule is developed. The gene expression of nodulation genes (*nodA* and *nodC*) and T4SS gene (*traG*) in *Bradyrhizobium* sp. SUTN9-2 was conducted. The result showed that *nod* genes were expressed under genistein induction at 24 h (Figures 3.10a and 3.10b). However, *traG* was not induced under genistein induction but it was also expressed at 24 h (Figures 3.10c). In contrast, the expression of *nod* genes in $\Delta T4SS_1$ was less expressed than those of WT under genistein induction (Figures 3.10a and 3.10b). Therefore, it confirms that both of *nod* and T4SS genes were expressed at the early stage of nodulation. These results again indicate that T4SS in *Bradyrhizobium* sp. SUTN9-2 plays an important role in symbiotic interaction.

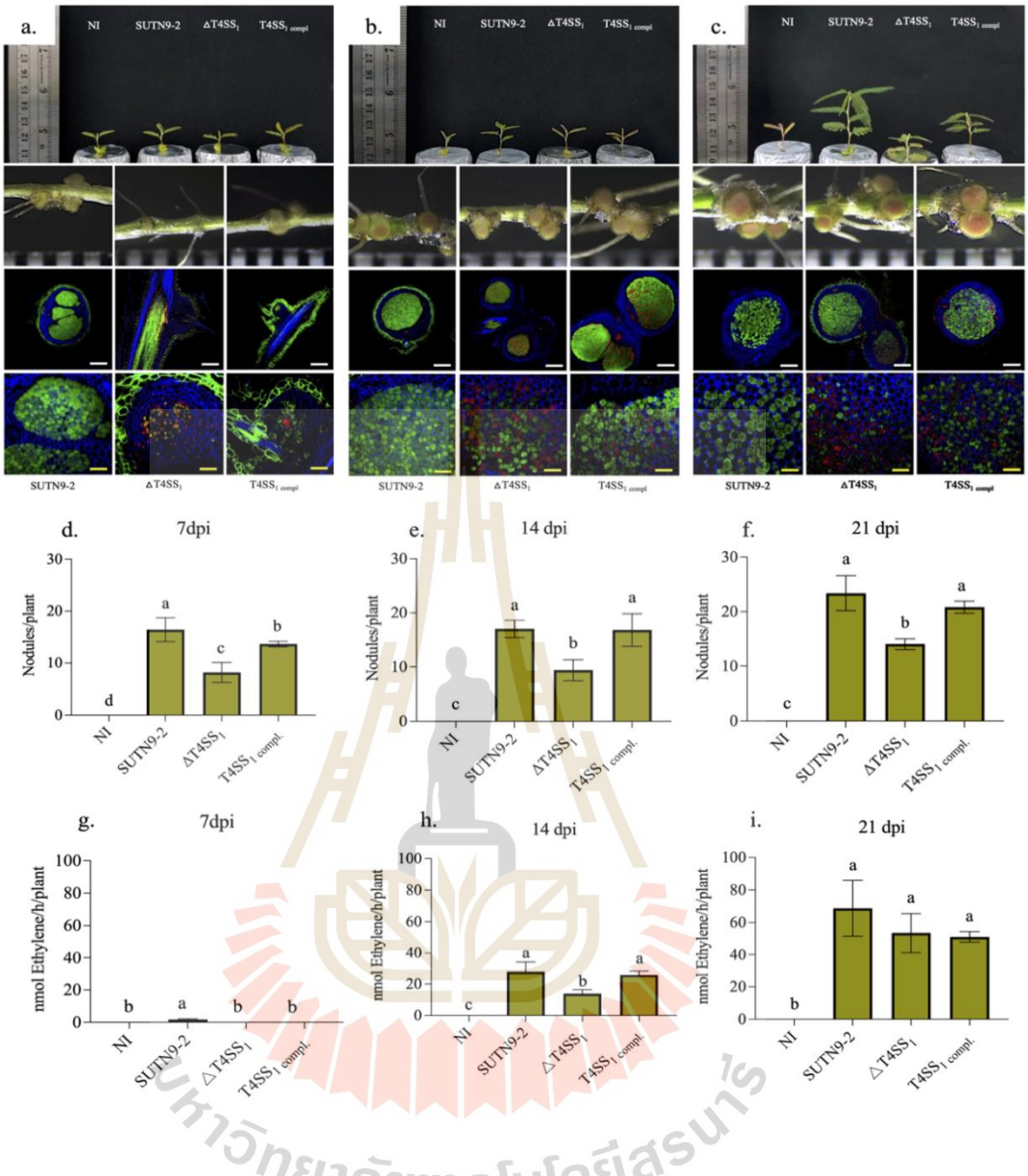

Figure 3.7 The symbiotic phenotype of *Aeschynomene americana* (Thai) inoculated with *Bradyrhizobium* sp. SUTN9-2, $\Delta T4SS_1$ and complementation ($T4SS_{1compl}$) strains. Plant growth, nodule phenotype, and bacteroid viability determined by live/dead staining of nodule section and confocal microscopy (a, b, c) nodule number (d, e, f) and nitrogenase activity (g, h, i) at 7, 14, and 21 days post inoculation (dpi). Scale bar white = 200 µm (10X) and yellow = 50 µm (40X). Error bar, mean (n =5) ± standard deviation. Different letters above bars indicate significant differences ($p < 0.05$) using Tukey's test.

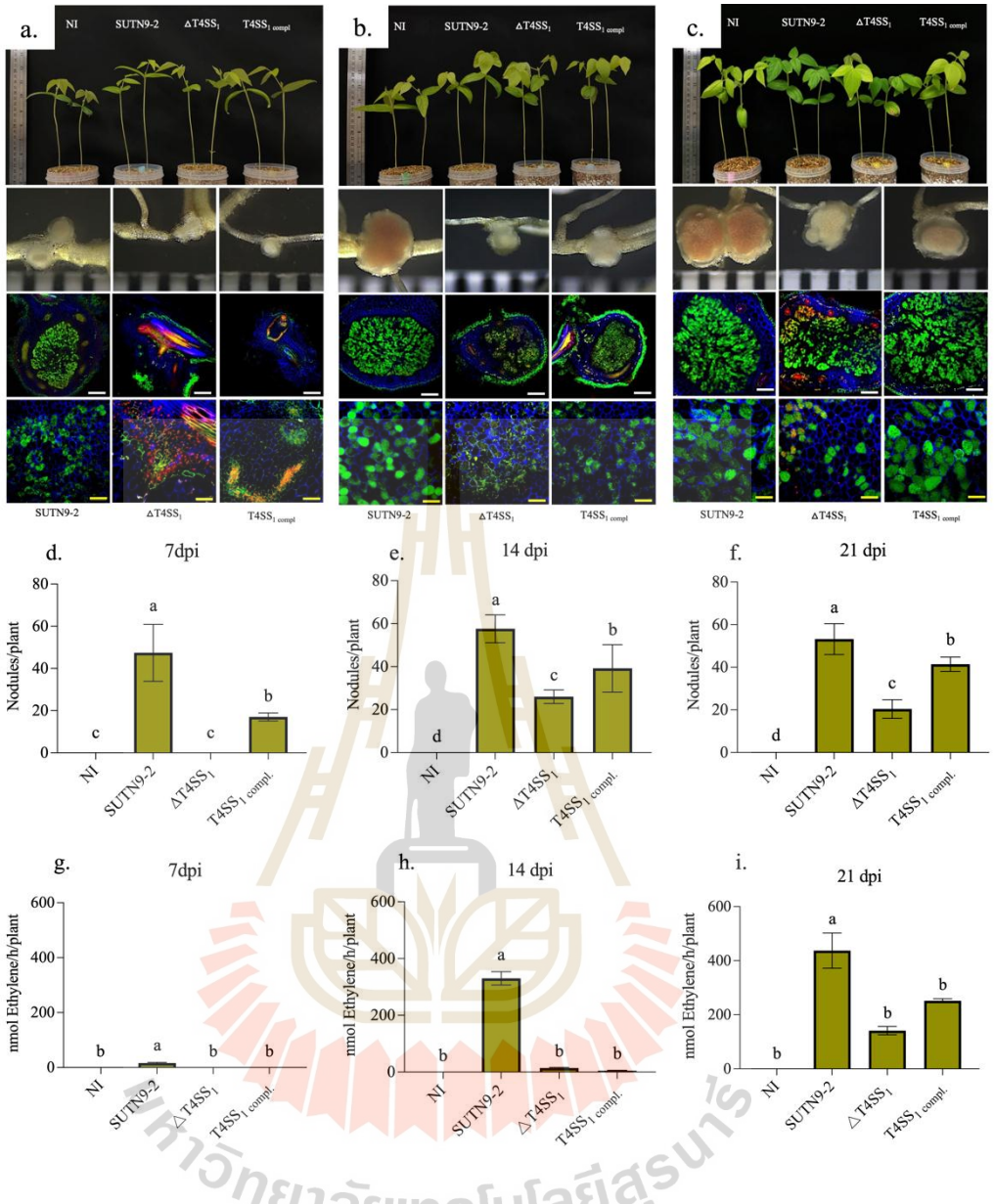

Figure 3.8 The symbiotic phenotype of *Vigna radiata* cv. SUT4 inoculated with *Bradyrhizobium* sp. SUTN9-2, $\Delta T4SS_1$ and complementation ($T4SS_{1compl}$) strains. Plant growth, nodule phenotype, and bacteroid viability determined by live/dead staining of nodule section and confocal microscopy (a, b, c), nodule number (d, e, f), and nitrogenase activity (g, h, i) at 7, 14, and 21 days post inoculation (dpi). Scale bar white = 200 µm (10X) and yellow = 50 µm (40X). Error bar, mean (n = 5) ± standard deviation. Different letters above bars indicate significant differences ($p<0.05$) using Tukey's test.

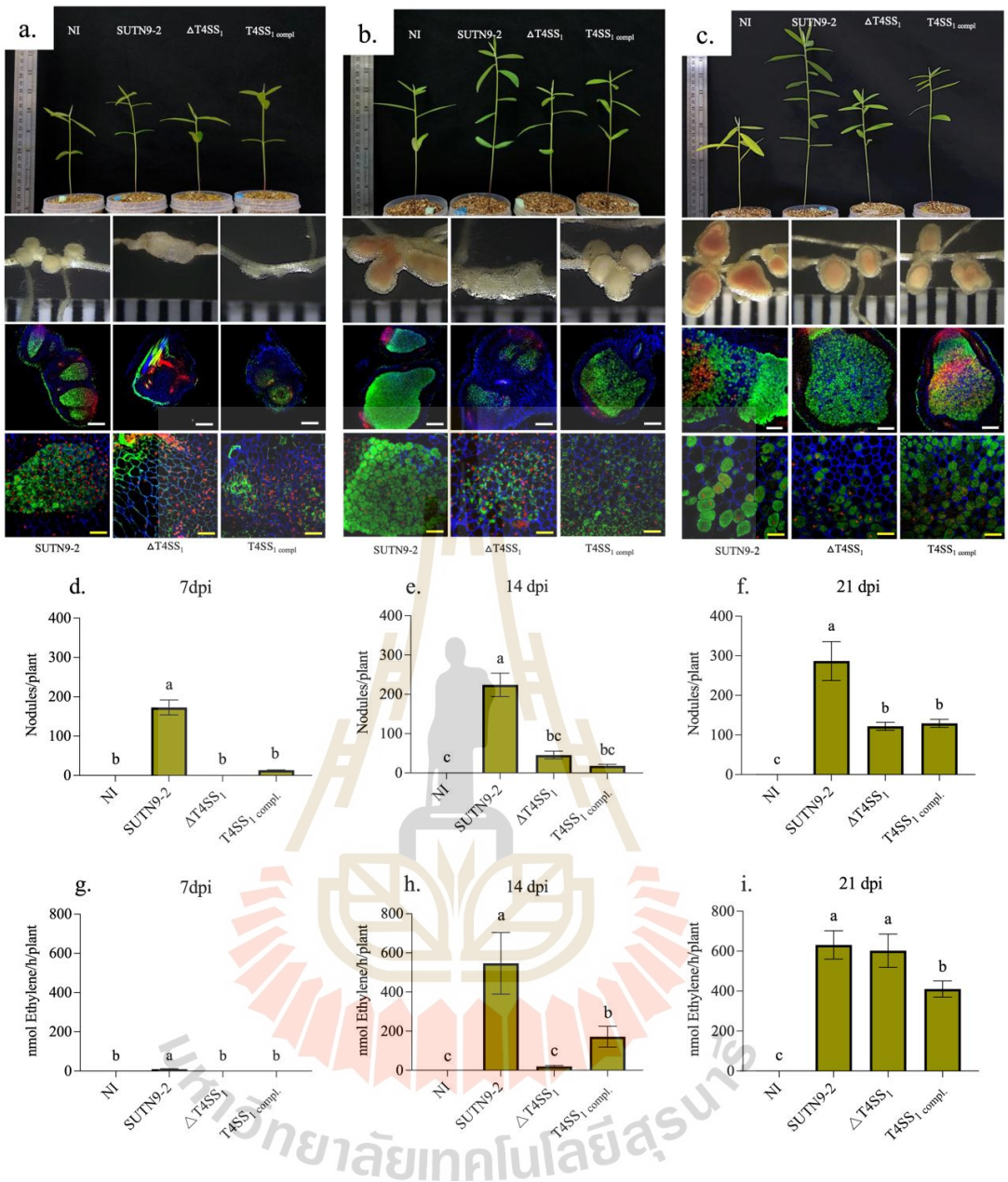

Figure 3.9 The symbiotic phenotype of *Crotalaria juncea* inoculated with *Bradyrhizobium* sp. SUTN9-2, ΔT4SS$_1$ and complementation (T4SS$_{1compl}$) strain. Plant growth, nodule phenotype, and bacteroid viability determined by live/dead staining of nodule section and confocal microscopy (a, b, c) nodule number (d, e, f) and nitrogenase activity (g, h, i) at 7, 14, and 21 days post inoculation (dpi). Scale bar white = 200 μm (10X) and yellow = 50 μm (40X). Error bar, mean (n =5) ± standard deviation. Different letters above bars indicate significant differences ($p < 0.05$) using Tukey's test.

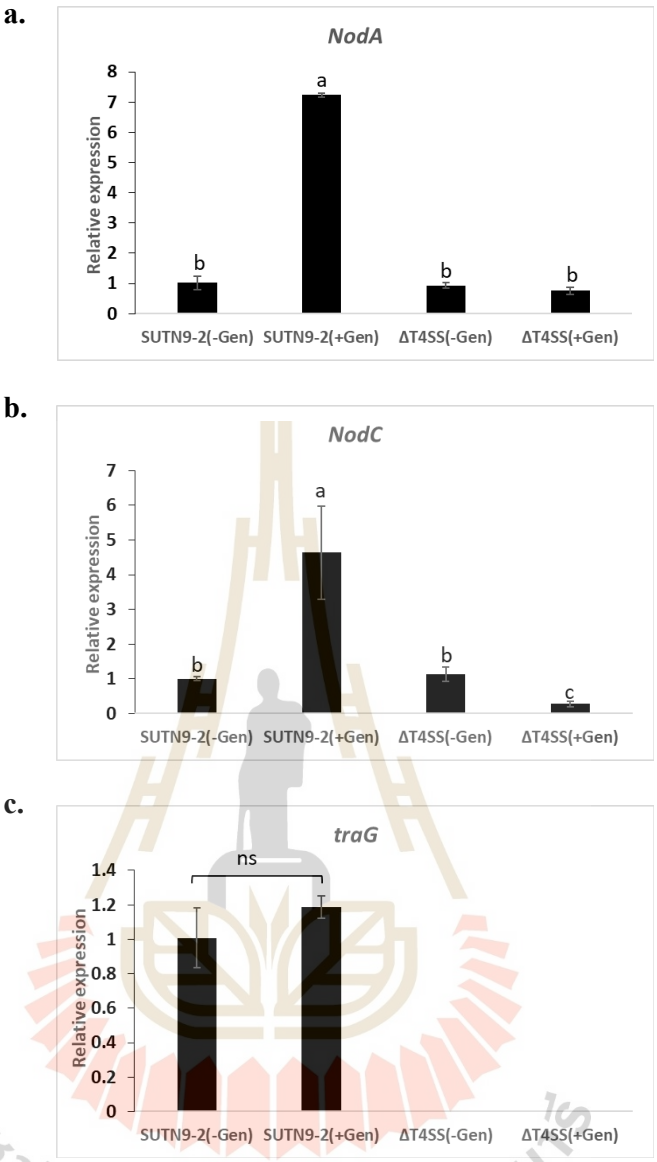

Figure 3.10 Relative expression of N-acyltransferase *nodA* (a), N-acetylglucosaminyl transferase *nodC* (b) and T4SS structural gene *traG* (c) of *Bradyrhizobium* sp. SUTN9-2 and $T4SS_1$ mutant strains ($\Delta T4SS_1$) after induced with 20mM genistein for 24 h (+Gen) comparing with non-induction cells. (-Gen). Data are expressed as means ±SD. Different letters above the error bars indicate significant differences at $P<0.05$ and ns = not significantly different.

3.4 Discussion

The genes encoding for T3SS and T4SS of *Bradyrhizobium* sp. SUTN9-2 are located on the chromosome, whereas T6SS components were unidentified. We further investigated the role of T4SS of in *Bradyrhizobium* sp. SUTN9-2 controlling symbiosis with various leguminous plants. Typically, the pTi plasmid of *Agrobacterium* is the best study model for T4SS (Cao et al., 2018). The pTiC58 plasmid of *A. tumefaciens* C58 contains three types ofT4SS genes, *vir, avh,* and *trb,* which are required for effector protein translocation and DNA conjugal transfer (Chen et al., 2002; Sugawara et al., 2013). The *virB/D4* and *tra/trb* operons are important for protein transfer and conjugation processes that enable translocating protein, DNA, or protein DNA complex into eukaryotic host cells (Kregten et al., 2009; Aguilar et al., 2010; Lang & Faure, 2014). These operons are associated with symbiotic interaction effector protein translocation, limiting immune response and host specificity in eukaryotic host plants (Jaboulay et al., 2021). Besides the *Agrobacterium* genus, T4SS has been identified in some rhizobia (Hubber et al., 2004; Cytryn et al., 2008; Noisangiam et al., 2012; Nelson & Sadowsky, 2015; Okazaki et al., 2015). Moreover, the effector proteins of T4SS have been reported in some rhizobia that represent the effect on symbiosis, for example, the Msi059 and Msi061effector proteins of *M. loti* R7A (Hubber et al., 2004). Likewise, TfeA is a similar protein in *Sinorhizobium* spp. that exhibits 59% amino acid homology with the Msi061 protein from *M. loti* R7A and 25% identity with VirF protein of *A. tumefaciens* C58 (Nelson et al., 2017). Previous works reported that the T4SS belonging to *vir*B/D4 operon was regulated by NodD, which is the main regulator of *nod* gene expression (Hubber et al., 2004; Hubber et al., 2007). This assumes that T4SS is associated with the early steps of the legume-rhizobia symbioses (Soto et al., 2006; Deakin & Broughton, 2009), but *nodD* expression was not observed on the *tra/trb* operon (Paço et al., 2019). The *tra/trb* operon was also observed on the pTiC58 plasmid of *A. tumefaciens* C58 (Cho & Winans, 2007), and the gene organization was closely similar to *tra/trb* operon on a symbiotic plasmid (pSym) of *Rhizobium* and *Sinorhizobium.* The *trbH* and *trbK* genes were not observed on the T4SS cluster of *Mesorhizobium,* and *trbH* was also not found in

Bradyrhizobium (Figure 3.2a). Moreover, several *Bradyrhizobium* possess T4SS in the *tra/trb* operon on the chromosome (Banerjee et al., 2019). *Bradyrhizobium* with the *tra/trb* operon is often found on a broad range of host strains, such as *Bradyrhizobium* sp. SUTN9-2 (Piromyou et al., 2015), *B. diazofficiens* USDA110 (Kaneko et al., 2011), *B. japonicum* CPAC 15, and *B. diazoefficiens* CPAC 7 (Siqueira et al., 2014). In contrast, *virB/D4* operon was observed only in the plasmid of *Bradyrhizobium* strains without *virD2* (Banerjee et al., 2019), such as *Bradyrhizobium* sp. BTAi1 (Cytryn et al., 2008) and *Bradyrhizobium* sp. DOA9 (Okazaki et al., 2015).

Interestingly, the gene arrangement of T4SS structural (*traG*) and T4SS-related genes (*copG* and *virD2*) presented a unique characteristic of the *Bradyrhizobium* genus (Figure 3.2a). Although the gene arrangement of the T4SS structural gene of *Bradyrhizobium* sp. SUTN9-2 are conserved well with bradyrhizobia, mesorhizobia, rhizobia, sinorhizobia, and agrobacteria, but T4SS-related genes (*copG* and *virD2*) represent a unique characteristic of *Bradyrhizobium* genes (Figure 3.2a). However, *copG* and *virD2* genes arrangement inside the T4SS cluster was slightly diverse among bradyrhizobial group. The T4SS apparatus appears to have the same evolutionary origin as other rhizobia. Therefore, a unique T4SS gene organization (*copG*, *traG*, and *virD2*) may have evolved independently.

Likewise, the phylogenetic tree of *copG, traG,* and *virD2* gene cluster indicated that the coevolution of T4SS structural and T4SS-related genes might be specific to bradyrhizobia (Figure 3.5). Based on 16S rRNA gene sequence similarity, *Bradyrhizobium* sp. SUTN9-2 was phylogenetically closely related to *B. yuanmingense* (Appendix B.1) (Noisangiam et al., 2012; Piromyou et al., 2015). Our results from the phylogenetic analysis of T4SS (*copG*, *traG,* and *virD2*) in copy 1 were mainly congruent with the phylogenetic tree relationship of the 16S rRNA gene. $T4SS_1$ was similar to *B. yuanmingense* BRP09 (copy 3) while $T4SS_2$ was similar to *B. yuanmingense* BRP09 (copy 1) with different clade. The *Mesorhizobium* sp. *traG* gene is a substrate receptor for T4SS expressed in early nodule formation generated by *M. mediterraneum* $Ca36^T$. Perhaps, the T4SS belonging to *tra/trb* operon is important for the mutualistic and symbiotic interaction (Paço et al., 2019). In addition, it was also found that the evolution of

rhizobia involved lifestyle diversity that depended on bacteria-host interaction and eukaryotic host diversity. The genes associated with bacterium-host interactions, such as T3SS, T4SS, and T6SS, were derived in a lineage-specific form (Wang et al., 2020).

As in the *Mesorhizobium* genus, the *tra*G gene could horizontally be transferred between strains because they could establish nodules with the same plant. The TraG protein is also associated with the efficiency of symbiosis gene transfer among strains in this genus (Paço et al., 2019). Based on the T4SS structural genes phylogenetic tree of the rhizobia group, including *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium*, *Rhizobium,* and *Agrobacterium* demonstrated that the T4SS structural genes of *Bradyrhizobium* may have evolved from the same ancestor with *Mesorhizobium*, while *Sinorhizobium* and *Rhizobium* were closely similar with *Agrobacterium* (Figure 3.2b). These results corresponding to the hypothesis that *Bradyrhizobium* might be the ancestor of all rhizobia genera. This hypothesis has been confirmed by a molecular study, which concluded that *Bradyrhizobium*'s large genome acted as a broad host range strain responsible for effective nodules in several legumes (Green & Mecsas, 2016). Moreover, *Bradyrhizobium* also have the ability to generate nodules on primitive non-legume *Parasponia* plants (Ormeño-Orrillo & Martínez-Romero, 2019). Similarly, *Mesorhizobium* has the ability to nodulate the chickpea, a primitive legume, and limit the plant host that restricts rhizobia infection (Laranjo et al., 2008). Thus, the presence of T4SS in *Bradyrhizobium* sp. SUTN9-2 could be a tool to overcome plant host inhibition and effectively nodulate and fix N_2 in several legumes.

Integrative and conjugative elements (ICEs) are mobile genetic elements that can integrate into the bacterial chromosome and transfer themselves to other bacteria through horizontal gene transfer. In the Gram-negative bacteria conjugation machinery encoded by T4SS (Gonçalves et al., 2022), the ICEs consist of the core gene associated with conjugation and excision components (Ramsay et al., 2022). Moreover, the ICEs are able to spread large gene clusters, which provide competitive advantages to a particular group (Guglielmini et al., 2011). Normally, the symbiosis genes were observed on a large symbiotic plasmid of *Rhizobium* and *Sinorhizobium* while, in *Mesorhizobium, Azorhizobium* and

Bradyrhizobium were found in ICEs called ICEsym (Tang & Capela, 2020). In rhizobia species, ICEs were first described in *Mesorhizobium* spp. carried genes required for symbiosis, such as *M. loti* R7A, was explored ICE*Ml*Sym^{R7A}. ICE*Ml*Sym^{R7A} was transferred to non-symbiotic mesorhizobia in New Zealand soils and can convert non-symbiotic into symbiotic *Lotus corniculatus* (Sullivan & Ronson, 1998; Colombi et al., 2021). In this study, we identified one putative ICE on a chromosome of *Bradyrhizobium* sp. SUTN9-2 (ICE$^{SUTN9-2}$) with low CG content containing 243 proteins, such as T2SS, T4SS, relaxase, transposase, and integrase proteins, essential for gene transfer (Figure 3.6). Interestingly, T4SS$_1$ was a part of ICE$^{SUTN9-2}$ which is a core conjugative element. At least one of the large ICEs were found in the agent of bradyrhizobia species (Table 3.3). Based on *copG, traG,* and *virD2* gene cluster phylogenetic tree (Figure 3.5), *B. yuanmingense* was closely similar to *Bradyrhizobium* sp. SUTN9-2 and ICEs in a similar pattern were found on the chromosomes of both strains. Therefore, these results demonstrated that the horizontal gene transfer of *Bradyrhizobium* sp. SUTN9-2 is most likely performed by an ICE specific to *B. yuanmingense* group. Therefore, T4SS$_1$ was transferred by horizontal gene transfer together with ICE.

Plants inoculated with *Bradyrhizobium* sp. SUTN9-2 ΔT4SS$_1$ at 7dpi showed delayed nodulations in mung bean and brown hemp while shyleaf formed small nodules on root (Figure 3.7, 3.8, and 3.9). This displayed similar patterns as in the T4SS (*vir* cluster) mutant of *M. loti* R7A with *L. corniculatus* when compared with WT that consist of the secreted proteins facilitating host infection (Hubber et al., 2004). In addition, the dead cells in the ΔT4SS$_1$ symbiosome were observed even in the original host, indicating the survival of cells within host plants associated with T4SS *Bradyrhizobium* sp. SUTN9-2. Although, when ΔT4SS$_1$ was restored, nodules were generated after 7 dpi but the nitrogenase activity was lower than WT. T4SS of *M. loti*, which suggests a symbiotic relationship with the eukaryotic host. Moreover, the *M. loti* R7A T4SS mutant positively affected *L. corniculatus* and negatively affected *L. leucocephala* (Hubber et al., 2004). Taken together, these findings support that T4SS$_1$ had a positive effect via symbiotic interactions with legume plants. However, the effect of T4SS on symbiosis depends on the species of the host plant. Moreover, T4SS of *Bradyrhizobium* sp. SUTN9-2 may affect cells' survival during infection, which

is important for host specificity of the symbiotic interaction. As in *M. mediterraneum* Ca36^{T}, the *traG* gene was expressed in early infection and symbiotic interaction (Hubber et al., 2004).

The different gene organizations of T4SS$_1$ and T4SS$_2$ were explored. The T4SS$_1$, *trb* operon, and *traG* genes were inserted with ~56 kb fragment consisting of 84 genes (Figure 3.3), such as putative T2SS, transposase, peptidase, permease, tyrosine-type recombinase/integrase, and hypothetical proteins (Table 3.3). In addition, the *replication A* (*repA*) gene was found in the upstream *copG*, *traG,* and *virD2* cluster, while T4SS$_2$ contains complete *tra/trb* operon gene organization. Interestingly, a lower GC content and fewer transposase elements were found in the T4SS clusters, especially in the T4SS$_1$ cluster. This is one possible reason for the variation of genes in this region being more diverse than other regions and making different gene organization of both T4SS. To ensure the efficiency of T4SS$_1$ on symbiosis, we constructed T4SS$_1$ complement strain (T4SS$_{1compl}$) and tested it as in previous experiments (Figure 3.7, 3.8, and 3.9). This result confirmed that, besides nod factors and T3SS, *Bradyrhizobium* sp. SUTN9-2 used the T4SS pathway to establish symbiosis with several host plants. However, many aspects of T4SS's involvement in symbiotic interactions remain unknown. For example, the function of the *copG*, *traG,* and *virD2* genes warrant further investigation in legumes.

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CHAPTER IV

The influence of the *copG* gene in T4SS cluster of *Bradyrhizobium* sp. SUTN9-2 during symbiotic interaction

4.1 Introduction

Bradyrhizobium and legume symbiosis required signal exchange during symbiosis interaction between partners (Dénarié et al., 1996; Hanin et al., 1997). Nodulation mechanism was generated by two different mode including nod factor (NF)-dependent and NF-independent. For NF-dependent required NF to trigger nodule organogenesis (Geurts & Bisseling, 2002). While NF-independent, bradyrhizobia was penetrated host cells via crack-entry which found in *Aeschynomene* spp. and *Arachis hypogaea* (Madsen et al., 2010; Okubo et al., 2012; Guha et al., 2022). However, some of bradyrhizobia used an alternative mechanism to deliver the effector molecules directly to their host, called the secretion system (Okazaki et al., 2013). The secretion systems including type III, type IV and type VI (T3SS, T4SS and T6SS) secretion system have been identified rhizobia genera and involved in symbiosis interaction during infection process (Sugawara et al., 2013). Both positive effect, negative effect or no effect of T3SS were observed on *Bradyrhizobium* symbiosis interaction depending on host plant. In T6SS of *Bradyrhizobium* sp. LmicA16 (A16), *Lupinus micranthus* and *L. angustifolius* inoculated with *vgrG* mutant (T6SS component gene) were decreased nodule number and nodulation competitive efficiency comparing to wild type (Tighilt et al., 2022).

While, less study about the role of T4SS of *Brayrhizobium* on symbiosis process have been reported. *Bradyrhizobium* sp. SUTN9-2, a broad host range strain possesses multiple secretion systems involved in symbiotic interactions (Noisangiam et al., 2012; Piromyou et al., 2015). In its genome, two types of secretion systems have including T3SS and T4SS. The role of the T3SS in symbiosis interactions has recently been examined, revealing distinct effects on different host plants. Interestingly, it has a neutral effect on the original host, *A. americana*, positive effect

on *V. radiata* cv CN72. On the other hand, it has a negative effect on most of *Bradyrhizobium* sp. SUTN9-2 nodulating legumes. Another secretion system known as T4SS is also present in two copies on the chromosome of *Bradyrhizobium* sp. SUTN9-2 and these copies are located at different positions. Both T4SS copies belong to the *tra/trb* operon and exhibit a unique gene arrangement specific to the bradyrhizobia genera. Based on a phylogenetic tree analysis, it was observed that the two copies of the T4SS in *Bradyrhizobium* sp. SUTN9-2 are categorized into distinct groups within the bradyrhizobia genus. Indeed, an intriguing observation in *Bradyrhizobium* sp. SUTN9-2 is the gene arrangement of $T4SS_1$, where a gene fragment of approximately 56 kb is inserted between the *trb* operon and the *traG* gene. In contrast, copy 2 of the T4SS represents the complete T4SS cluster without any gene fragment insertion. This distinction in gene arrangement highlights the genetic diversity and complexity of the T4SS in this strain. The T4SS of bradyrhizobia show a distinctive gene arrangement characterized by the presence of *copG*, *traG*, and *virD2* genes within the T4SS cluster. This unique gene arrangement suggests that these T4SSs in bradyrhizobia have a specific organization. A deletion mutant referred to as $\Delta T4SS_1$ was generated in *Bradyrhizobium* sp. SUTN9-2 by removing a specific portion of the *copG*, *traG*, and *virD2* genes from copy 1 ($copG_1$, $traG_1$, and $virD2_1$). This mutant was inoculated into different legume species including Dalbegioids (*A. americana*), Millettiods (*V. radiata* cv. SUT4), and Genistoids (*Crotalaria juncea*). Similar to the T3SS, the T4SS in $\Delta T4SS_1$ did not show any significant impact on the symbiotic interaction with the original host, *A. americana*. However, a notable positive effect of the T4SS was observed in *V. radiata* cv. SUT4 and *C. juncea* with delayed nodulation at first week after inoculation (Wangthaisong et al., 2023). To further explore the roles of $copG_1$, $traG_1$, and $virD2_1$ in symbiotic interactions, individual deletions of these genes were generated in *Bradyrhizobium* sp. SUTN9-2. The impact of each gene deletion on symbiotic interactions was assessed using *V. radiata* cv. SUT4 as a plant model. These investigations could provide a better understanding of the functional significance of these genes in the context of symbiosis and their impact on plant-microbe interactions.

4.2 Materials and Methods

4.2.1 Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 4.1. *Bradyrhizobium* sp. SUTN9-2 was grown in Arabinose-Gluconate (AG) medium details provided in Appendix A.1 (Sadowsky et al., 1987). The derivative mutants Δ*copG*$_1$, Δ*traG*$_1$ and Δ*virD2*$_1$ were supplemented with streptomycin 200 µg per milliliter (sm 200 µg/ml). *Escherichia coli* strains were grown at 37°C in Luria–Bertani medium (LB) details provided in Appendix A.2. Antibiotic was added to the medium as required following concentrations: kanamycin (km), 50 µg/ml, nalidixic acid (nal), 30 µg/ml and streptomycin (sm), 200 µg/ml.

4.2.2 Plasmid construction, gene deletion and complementation

The deletion mutant of *copG*$_1$, *copG*$_2$, *traG*$_1$ and *virD2*$_1$ genes in *Bradyrhizobium* sp. SUTN9-2 (GeneBank accession number LAXE00000001) were obtained as follow. The upstream and downstream regions of *copG*$_1$ (up-575 bp, dw-841 bp) , *copG*$_2$ (up-874 bp, dw-1,043 bp), *traG*$_1$ (up-1,060 bp, dw-921 bp) and *virD2*$_1$ (up-944 bp, dw-738 bp) genes were obtained by PCR using primer listed in Table 4.2. The target deletions genes in *Bradyrhizobium* sp. SUTN9-2 were obtained by double crossing-over. PCR fragments corresponding to the upstream and downstream flanking regions gene of interest were merged by overlap extension and introduced into pNTS129 plasmid, harboring the *sacB* gene (Tsai & Alley, 2000). Then, a Ω casstte fragment (spectinomycin/streptomycin resistance gene) from pHP45 (omega) (Blondelet-Rouault et al., 1997) was introduced between upstream and downstream flanking region which already cloned into pNTS129. The restriction site for antibiotic insertion was HindIII for *copG*$_1$ and BamHI for *copG*$_1$, *traG*$_1$ and *virD*$_2$, respectively. Subsequently, the resulting plasmids were transferred into *Bradyrhizobium* sp. SUTN9-2 by triparental mating using pRK2013 as a helper plasmid (Ditta et al., 1980) as previously described (Wangthaisong et al., 2023). Single recombinant clone was obtained by antibiotic selection and PCR verification. Double recombinant clones were selected by cultured on AG supplemented with 10% sucrose and 200 µg/ml sm. Candidate clones were verifying the loss of *sacB* gene from pNTPs129 and the replacement of Ω cassette was verified by PCR. In order to complementation of *copG*$_1$ mutant, a DNA fragment of 510 bp containing *copG*$_1$ gene was amplified by

PCR with primers as listed in Table 4.2 and cloned into pMG103-*npt2-cefo* generating pMG103-*copG*$_1$. Conjugation of pMG103-*copG*$_1$ to Δ*copG*$_1$ was performed by triparental mating. All mutant strains were further investigated for nodulation efficiency in *V. radiata* cv. SUT4.

4.2.3 Nodulation test and acetylene reduction assay (ARA)

Vigna radiata cv. SUT4 seeds were surface-sterilized and germinated as previous described (Teamtisong et al., 2014) and placed on 0.85% water agar at 28°C for overnight. One-day-old, germinated seedlings were transferred into Leonard's jars containing sterilized vermiculite and liquid Buffered Nodulation Media (BMN) details provided in Appendix A.3 (Ehrhardt et al., 1992). Seven days after gemination, seedlings were inoculated with bacterial suspension 1 ml per seedling of *Bradyrhizobium* sp. SUTN9-2 or mutants (adjusted to OD_{600}=0.8). The symbiotic phenotypes and nitrogenase activity using acetylene reduction assay (ARA) at 7, 14 and 21 days post inoculation (dpi). To evaluate the nitrogenase activity by acetylene reduction assays (ARA) method, the root sample was transferred into test tubes and closed with a plastic stopper. Then, the samples were incubated with 10% (v/v) pure acetylene instead of the air that withdrawn for 1 h at room temperature. 1 ml of sample was injected into Gas Chromatography (GC) using a PE-alumina-packed column to measure the conversion of acetylene (C_2H_2) to ethylene (C_2H_4). The detector was detected at an injection temperature of 150°C and oven temperatures of 200°C and 50°C for flame ionization detection (FID) (Renier et al., 2011). The experiment was done with five biological replicates for minimum. The nitrogenase activity is presented as nmol ethylene/h/plant dry weight (Somasegaran & Hoben, 1985). The symbiotic profiles of Δ*copG*$_1$ comparing to *Bradyrhizobium* sp. SUTN9-2 was performed with several leguminous plants including; *Aeschynomene americana* cv. Thai, *Arachis hypogaea* cv. Thainan-9, *A. hypogaea* cv. Khonkaen 5, *Crotalaria juncea*, *Indigofera tinctoria*, *Macroptilium atropurpureum*, *V. radiata* cv. SUT1, *V. radiata* cv. CN72, *V. radiata* cv. KUML4, *V. radiata* cv. CN36, *V. radiata* cv. KPS1, *V. mungo* cv. U thong 2 and *V. subterranean* with growth pouch test. Seeds were surface-sterilized and germinated as previous described (Teamtisong et al., 2014; Phimphong et al., 2023). Pouches were prepared (Somasegaran & Hoben, 1994) and supplemented with BMN medium. Seedlings were grown two plants per pouch and

inoculated with 1 ml per plant of a suspension containing OD_{600}=0.8. The symbiotic phenotypes were examined at 21 dpi.

4.2.4 Microscopy

The nodule phenotypes and cross sections of representative nodules generated by WT or mutants were examined under stereomicroscope (LEIGA EZ4). In situ live/ dead cell staining, the nodules (Haag et al., 2011) were harvested and embedded with 5% agarose. Nodules section of 40-50 µm were prepared with a VT1000S vibratome (Leica Nanterre, France) and incubated with live/dead staining solution (5 µM SYTO9 and 30 µM PI in PBS pH 7.0 buffer) for 30 min, then staining with 1X calcofluor white stain for 20 min. Sections were washed from staining solution and mounted in 10% glycerol in 1X PBS buffer. Nodules after live/dead staining were observed by confocal microscopy using a Nikon Inverted Eclipse Ti-E Confocal Laser Scanning Microscope. Calcofluor was detected at 460-500 nm emission, while SYTO9 and PI were detected at 510-570 nm and 600-650 nm, respectively.

4.2.5 Bacterial induction, RNA isolation and qRT-PCR analysis of gene expression

For bacterial induction, mid-log phase of bacteria culture including *Bradyrhizobium* sp. SUTN9-2 and Δ*copG*$_1$ (OD_{600}=0.4) were induced by 10 µM naringenin at 28°C for 24 h. Then, bacterial pellets were collected by centrifugation (4,000 ×g, at 4°C) for further total RNA isolation. Total RNA was isolated from bacterial pellets using RNeasy® Protect Cell Mini Kit (Qiagen, Chatsworth, CA, U.S.A.) according to the manufacture's instruction. Total RNA was treated at 37°C for 30 min with RNase-free DNase I (NEB, USA). cDNA was synthesized by iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories, Inc.) according to the manufacturers' protocols. The cDNA concentration, 50 ng/ml was subjected to real-time PCR using specific primers for nodulation genes including *nodD1, nodD2*, *nodA* and *nodC*, T4SS structural genes including *traG*$_1$ and *trbE*$_1$ (Table 4.2). For qRT-PCR reactions were performed with Luna® Universal qPCR Master Mix (NEB, USA) following the manufacturer's protocol, and thermal cycling was conducted in a QuantStudio™ 3 Real-Time PCR System (Thermo Fisher, USA). The reactions were operated in triplicate for each of the three biological replicates. Relative gene

expression was analysed by the comparative Ct method (-ΔΔCT) and 16S rRNA was used as a reference gene. Three biological replicates were pooled and analyzed.

Table 4.1 Bacterial strains and plasmids used in this study.

Strain or plasmid	**Relevant characteristics**	**Reference or** source
Strain		
Bradyrhizobium sp.		
SUTN9-2	*A. americana* nodule isolate (paddy crop)	
Δ*copG*$_1$	SUTN9-2 derivative containing an Ω cassette insertion at HindIII site, *copG* copy 1::sm/sp; Sm^r, Sp^r	This study
Δ*copG*$_2$	SUTN9-2 derivative containing an Ω cassette insertion at BamHI site, *copG* copy 2::sm/sp; Sm^r, Sp^r	This study
Δ*traG*$_1$	SUTN9-2 derivative containing an Ω cassette insertion at BamHI site, *traG* copy 1::sm/sp; Sm^r, Sp^r	This study
Δ*virD2*$_1$	SUTN9-2 derivative containing an Ω cassette insertion at BamHI site, *virD2* copy 1::sm/sp; Sm^r, Sp^r	This study
Escherichia coli		
DH5α	*supE44 ΔlacU169 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Toyobo Inc.
Plasmid		
pRK2013	ColE1 replicon carrying *RK2* transfer genes; Km^r; Helper plasmid	Ditta et al., 1980
pNTPS129	Cloning vector harboring sacB gene under the control of the constitutive *npt2* promoter; Km^r (Appendix B.2)	Tsai & Alley, 2000
pNTPS129-Δ*copG*$_1$	pNTPS129-*npt2-sacB* containing the flanking region of *copG* copy 1	This study
pNTPS129-Δ*copG*$_2$	pNTPS129-*npt2-sacB* containing the flanking region of *copG* copy 2	This study
pNTPS129-Δ*traG*$_1$	pNTPS129-*npt2-sacB* containing the flanking region of *traG* copy 1	This study
pNTPS129-Δ*virD2*$_1$	pNTPS129-*npt2-sacB* containing the flanking region of *virD2* copy 1	This study
pMG103-*npt2-cefo*	Complementation vector harbors a cefotaxime resistance gene under the control of the constitutive *npt2* promoter; Cefo^r (Appendix B.4)	Giraud E, IRD, France

4.2.6 Bioinformatics

Bradyrhizobium sp. SUTN9-2 genome sequences were obtained from NCBI database (https://www.ncbi.nlm.nih.gov) and Genoscope **(https://mage**. genoscope.cns.fr). Multiple Sequence Alignments were determined by CLUSTALW (2.1) (https://www.genome.jp/tools-bin/clustalw). The annotation features and whole genome sequences were analyzed by SnapGene Viewer version 6.2.1 (https://www. snapgene.com).

4.2.7 Statistical analysis

All data were received from experiments performed in triplicated. For statistical analyses, one-way analysis of variance (ANOVA) followed by post hoc tests (Tukey's tests at $P \leq 0.05$) were performed using IBM SPSS Statistics 22.0 software and GraphPad Prism statistical software (version 9.0).

Table 4.2 Primers used in this study.

Name	Sequences (5'-3')	Describe
Up.*copG*$_1$.XbaI.F	CCT TGA GAT CTA GAT GTA GTC TGC CCC GAA GTA GC	The deletion of *copG*$_1$ gene of *Bradyrhizobium* sp. SUTN9-2
Up.*copG*$_1$.overl.HindIII.R	GAG GCG GAC ATG AAA GCT TAA TGA AGG CGG ACG GCC ACT AG	
Dw.*copG*$_1$.overl.HindIII.F	GTC CGC CTT CAT TAA GCT TTC ATG TCC GCC TCA CAG TCC GA	
Dw.*copG*$_1$.EcoRI.R	AGA TCG GGA ATT CGT TGA CCG AGG ATC TTC AGG CCA	
Up.*copG*$_2$.XbaI.F	GCC GTT TCT AGA ATT GCG ACA ACG GAC CAG GGC AA	The deletion of *copG*$_2$ gene of *Bradyrhizobium* sp. SUTN9-2
Up.*copG*$_2$.overl.HindIII.R	GCG CGA CCG AAT GAA GCT TAA GCT GGT CAC GCT ATC GGC T	
Dw.*copG*$_2$.overl.HindIII.F	GCG TGA CCA GCT TAA GCT TCA TTC GGT CGC GCA TAT TGC C	
Dw.*copG*$_2$.EcoRI.R	CTG TCC GAA TTC ATG TCG TTC CTC GGG TTG TAC C	

Table 4.2 Primers used in this study (Cont.)

Name	Sequences (5'-3')	Describe
Up.*traG*$_1$.XbaI.F	TTC GGG TCT AGA TGT AGT CTG CCC CGA AGT AGC	The deletion of *traG*$_1$ gene of *Bradyrhizobium* sp. SUTN9-2
Up.*traG*$_1$.overl.BamHI	TCC CTC CAA TCA CGG ATC CAT CCT GGT GAC GAT CTC GGA C	
Dw.*traG1*.overl.BamHI	TCG TCA CCA GGA TGG ATC CGT GAT TGG AGG GAT CGT TCA CAG	
Dw.*traG1*.EcoRI	CCG GCT GAA TTC CTT GGA AAG CCT TGG TCT CG	
Up.*virD2*$_1$.XbaI.F	ACC GGC TTC TAG AAG ATG CGC AGT CCG CAT CAT C	The deletion of *virD2*$_1$ gene of *Bradyrhizobium* sp. SUTN9-2
Up.*virD2*$_1$.overl.BamHI	GAG GAG AAG GAA TGG ATC CTG AAC GAT CCC TCC AAT CAC CG	
Dw.*virD2*$_1$.overl.BamHI	GAG GGA TCG TTC AGG ATC CAT TCC TTC TCC TCA GCC ATG GC	
Dw.*virD2*$_1$.EcoRI	CCA TCG GAA TTC TTG TCG ATG CGG AGG AGG CAT C	
SUTN9-2.*nodA*.F	GTT CAA TGC GCA GCC CTT TGA G	*nodA* gene expression primer designed from *Bradyrhizbium* sp. SUTN9-2 chromosome
SUTN9-2.*nodA*.R	ATT CCG AGT CCT TCG AGA TCC G	
SUTN9-2.*nodC*.F	ATT GGC TCG CGT GCA ACG AAG A	*nodC* gene expression primer designed from *Bradyrhizbium* sp. SUTN9-2 chromosome
SUTN9-2.*nodC*.R	AAT CAC TCG GCT TCC CAC GGA A	
SUTN9-2.*nodD1*.F	ATT CGT CTC CTC AGA CCG TGC T	*nodD1* gene expression primer designed from *Bradyrhizbium* sp. SUTN9-2 chromosome
SUTN9-2.*nodD1*.R	TTC ATG TCG AGT GCG CAC CCT A	
SUTN9-2.*nodD2*.F	TGC TTA ACT GCA ACG TGA CCC	*nodD2* gene expression primer designed from *Bradyrhizbium* sp. SUTN9-2 chromosome
SUTN9-2.*nodD2*.R	ATG AGC ACG AGG AGC TTC TC	
SUTN9-2.*trbE*$_1$.F	GAT TGC AGG AGA ACC GTG AGG C	*trbE*$_1$ gene expression primer designed from *Bradyrhizbium* sp. SUTN9-2 chromosome
SUTN9-2.*trbE*$_1$.R	AAC AGC GCC GAG GAT TCA GTC T	
SUTN9-2.*traG*$_1$.F	TTC TCG ATC TGG TTC AGC GAC TG	*traG*$_1$ gene expression primer designed from *Bradyrhizbium* sp. SUTN9-2 chromosome
SUTN9-2.*traG*$_1$.R	TTG ACC GAG GAT CTT CAG GCC A	
16S.F	ATT ACC GCG GCT GCT GG	16S rRNA gene expressio primers
16S.R	ACT CCT ACG CGA GGC AGC AG	

4.3 Results

4.3.1 Symbiotic properties of Δ*copG*$_1$, Δ*traG*$_1$ and Δ*virD2*$_1$ on *Vigna radiata* cv. SUT4 symbiosis

From previous result of ΔT4SS$_1$ was deficient in nitrogen fixation and nodulation on *A. americana*, *M. atropurpureum* and *V. radiata* cv. SUT4. The nodulation was retarded in all plants tested at 7 dpi and the nodulation efficiency was recovered after 14 dpi, due to ΔT4SS$_1$ mutant was deleted a part of gene covered *copG*$_1$, *traG*$_1$ and *virD2*$_1$ (Wangthaisong et al., 2023). In order to determine the role of *copG*$_1$, *traG*$_1$ and *virD2*$_1$ on symbiosis interaction, mutation of these genes was constructed by gene deletion using a Ω cassette resistance gene. Differences between *Bradyrhizobium* sp. SUTN9-2, WT and mutants were observed in nodulation and nitrogenase activity in *V. radiata* cv. SUT4 (Figure 4.1). The Δ*traG*$_1$ and Δ*virD2*$_1$ induced a higher nodule number on plant tested than WT at 7 dpi (Figure 4.1m). The dead cells were monitored in sectioned nodules inoculated with Δ*traG*$_1$ and Δ*virD2*$_1$ at symbiosome area (Figure 4.1c and 4.1d). However, nodule number at 21 dpi was not different in Δ*traG*$_1$, Δ*virD2*$_1$ and WT (Figure 4.1n). The nitrogenase activity was different in each mutant, Δ*virD2*$_1$ represent low nitrogen fixation while, Δ*traG*$_1$ was not different from WT (Figure 4.1o). Interestingly, nodulation was abolished by Δ*copG*$_1$ in *V. radiata* cv. SUT4 (Figure 4.1f) and all *Bradyrhizobium* sp. SUTN9-2 nodulating legumes (Table 4.3). Although, nodule organogenesis was not observed in plant inoculated with Δ*copG*$_1$ but live and dead cells were determined in cortex and vascular tissue instead (Figure 4.1j). The result indicated that Δ*copG*$_1$ was able to infect to plant cell, but they lost ability to survive in host cells. Since, we found the cell density in cortex was less than vascular tissues especially, viable cells (data not shown). However, the nodule formation was not observed in the host plant.

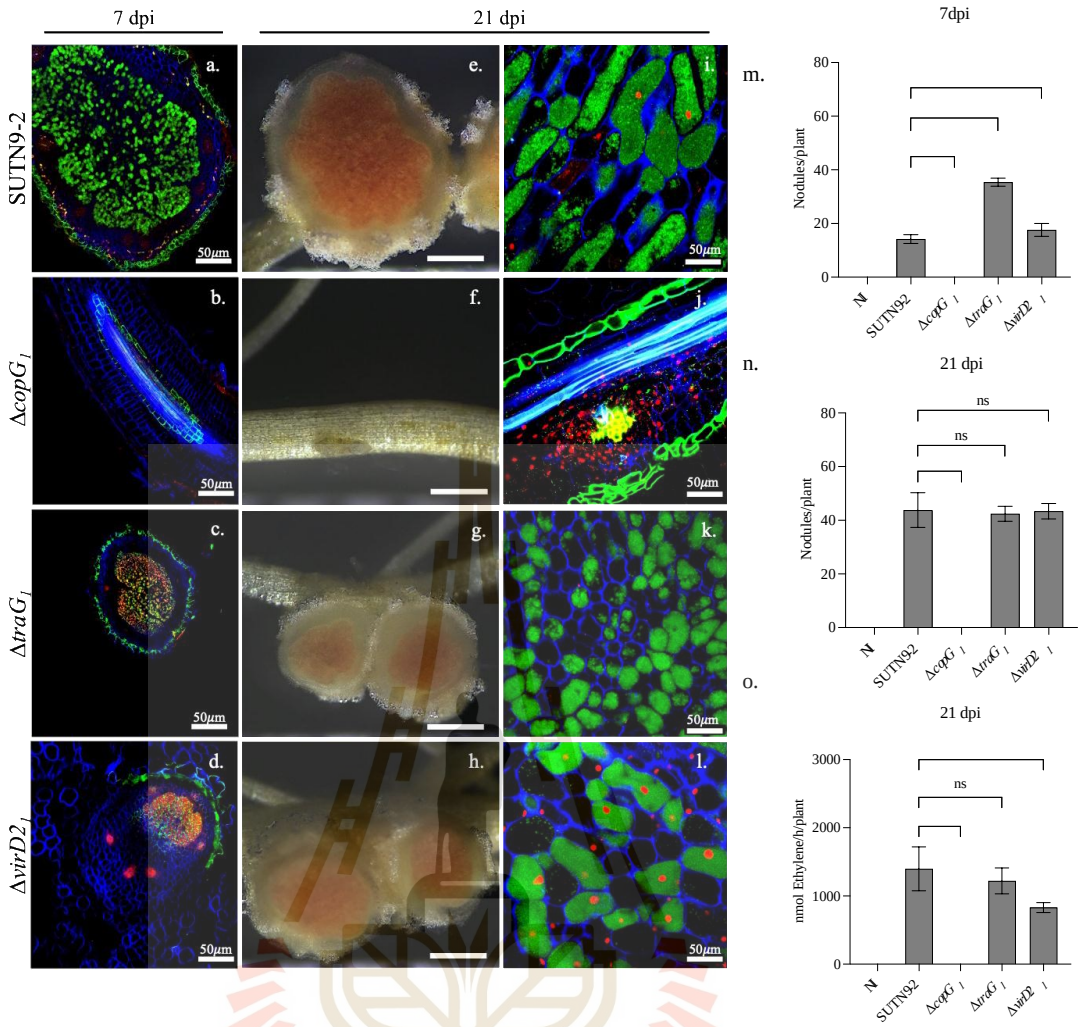

Figure 4.1 Symbiotic phenotype of *Bradyrhizobium* sp. SUTN9-2 and mutant strains including Δ*copG*$_1$, Δ*traG*$_1$, and Δ*virD2*$_1$ during symbiosis with *Vigna radiata* cv. SUT4. The nodule number at 7 dpi (m) and 21 dpi (n). Nitrogen fixation activity determined by the acetylene reduction assay on whole roots of nodulated plants infected with the indicated bacterial mutants at 21 dpi (o). Nodule phenotype at 21 dpi generated by *Bradyrhizobium* sp. SUTN9-2, WT (e), Δ*copG*$_1$ (f), Δ*traG*$_1$ (g) and Δ*virD2*$_1$ (h). Confocal microscopy was observed section of nodule infection with *Bradyrhizobium* sp. SUTN9-2, WT (a, i), Δ*copG*$_1$ (b, j), Δ*traG*$_1$ (c, k) and Δ*virD2*$_1$ (d, l) staining with PI, SYTO9 and calcofluor white at 7 dpi and 21 dpi, respectively. scale bars are indicated 1 mm. Values represent mean ± standard deviation (n = 5). *P* Value are based on one-way ANOVA, Tukey's test (ns, P > 0.05,*, P ≤ 0.05,***, P ≤ 0.001).

Table 4.3 Nodulation pouch test inoculated with *Bradyrhizobium* sp. SUTN9-2 and Δ*copG*$_1$ mutant in various legumes.

Plants	Nodulation [a]	
	SUTN9-2	Δ*copG*$_1$
Papilionoideae		
Genistoids		
Crotalaria juncea	(+)	(-)
Dalbergioids		
Aeschynomene americana cv. Thai	(+)	(-)
Arachis hypogaea cv. Thainan-9	(+)	(-)
A. hypogaea cv. Khonkaen 5	(+)	(-)
Millettioids		
Indigofera tinctoria	(+)	(-)
Macroptilium atropurpureum	(+)	(-)
Vigna radiata cv. SUT1	(+)	(-)
V. radiata cv. CN72	(+)	(-)
V. radiata cv. KUML4	(+)	(-)
V. radiata cv. CN36	(+)	(-)
V. radiata cv. KPS1	(+)	(-)
V. mungo cv. U thong 2	(+)	(-)
V. subterranean	(+)	(-)

[a] (+), positive effect for nodulation; (-), negative effect for nodulation

4.3.2 The *copG*$_1$ gene is involved in nodulation efficiency of *Bradyrhizobium* sp. SUTN9-2

Bradyrhizobium sp. SUTN9-2 carries two copies of *copG* gene where located at downstream of *traG* and *virD2* gene. *copG* gene function as a helix-turn-helix transcriptional regulator. In *Bradyrhizobium* sp. SUTN9-2 genome, *copG*$_1$ and *copG*$_2$ were located in different regions. These two genes, *copG*$_1$ (152 amino acids) and *copG*$_2$ (141 amino acids) have a relatively low similarity of 51.77% in terms of

amino acid identity (Figure 4.2) with differences observed in both the N-terminal and C-terminal regions. Through in silico analysis, it was discovered that the *copG*$_1$ gene in *Bradyrhizobium* sp. SUTN9-2 does not show similarity to other strains and remains uncharacterized. On the other hand, *copG*$_2$ demonstrates a high similarity to the *copG* gene of *B. diazoefficiens.* This study, Δ*copG*$_2$ mutant was inoculated into *V. radiata* cv. SUT4 and compared with Δ*copG*$_1$, *copG*$_{1compl}$ and WT (Figure 4.3). The results were collected at 7, 14 and 21 dpi. Unlike Δ*copG*$_1$, both Δ*copG*$_2$ and WT were capable of inducing nodule formation within the first week inoculated. The Δ*copG*$_2$ exhibited the highest number with small pink nodules (Figure 4.3c). However, upon confocal microscopy examination, it was observed that the Δ*copG*$_2$ sectioned nodules contained dead cells within the symbiosome, in contrast to the WT (Figure 4.3e). Furthermore, when compared to the WT, the Δ*copG*$_2$ displayed a statistically significant decrease in nitrogenase activity (Figure 4.3j and 4.3l). Despite this reduction in nitrogenase activity, the Δ*copG*$_2$ showed a higher number of nodules and this trend was consistent at 14 and 21 dpi (Figure 4.3i and 4.3k).

```
CLUSTAL 2.1 multiple sequence alignment

copG1           MTAPKRKAQMSVYLDPDVMKALSAFAARREQSLSLIAEAAIASFLSPDSDERREAAIAKR
copG2           -----MRDRMNVYFPPELLKQISELADRKKLSRSAIVEAAVASFLSPDGADRREAAFTRR
                     : :*.**: *:::* :* :* *:: * * *.***:*******. :*****:::*

copG1           LDQIDRRIARLERDVGISVETIALFIRFWLTTTPPLPEPAAKAARAQAGARYDNFVAALG
copG2           LDRLSRQMQRLERDLGLTAETLALFIRFWLTITPPLPNDAQAAAQAKGRERFEGFVEALG
                **::.*:: *****:*::.**:********* *****: *  **:*:.  *::.** ***

copG1           RRLSQGPKLRQEIPDDVESVQFSPETMKADGH
copG2           RRLQKGQSFLREIPEDIRSQEPVEET------
                ***.:* .: :***:*:.* :   **
```

Sequence format is Pearson
Sequence 1: *copG*$_1$ 152 aa
Sequence 2: *copG*$_2$ 141 aa
Sequences (1:2) Aligned score: 51.773

Figure 4.2 CLUSTALW (2.1) Multiple Sequence Alignments of *copG*$_1$ and *copG*$_2$ amino acid sequences in *Bradyrhizobiumm* sp. SUTN9-2. The definitions of the consensus colors are shown in black for identical sequences and red for not similar sequences. The protein sequences alignments were represented as; "*", conserved sequences (identical), ":", conservative mutation, "·", semi-conservative mutation and "—".gap.

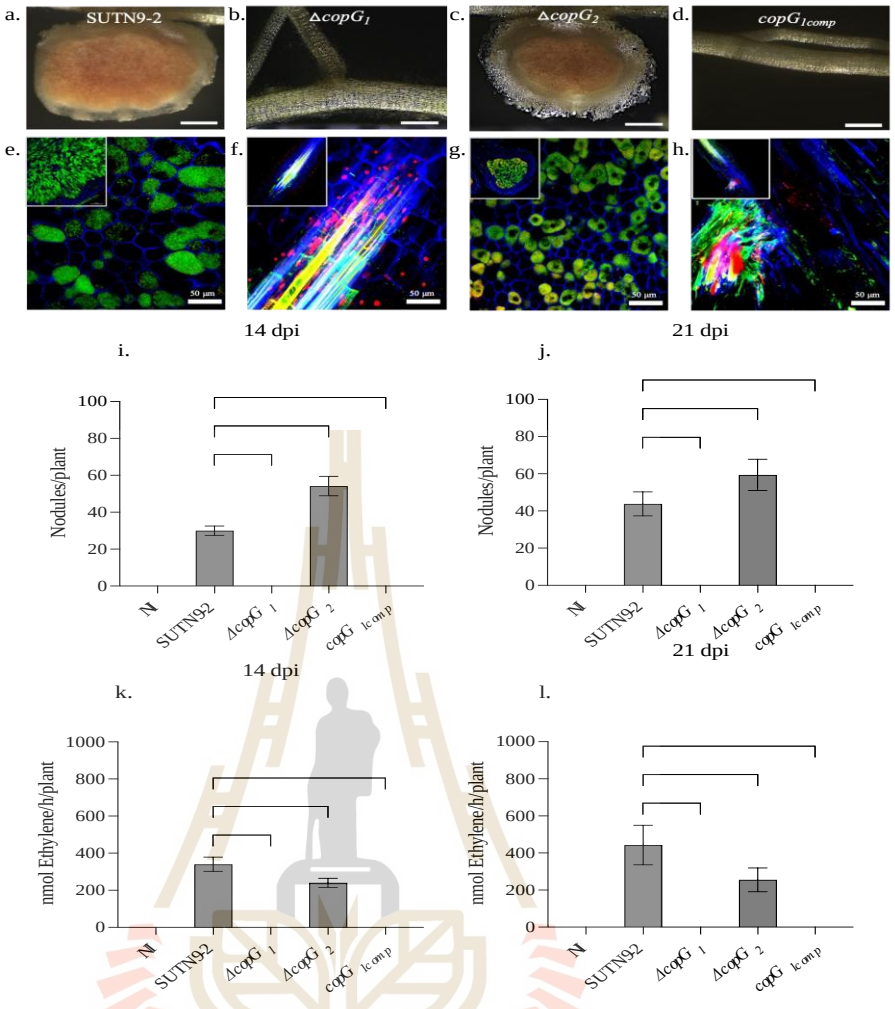

Figure 4.3 The derivative *copG* gene mutants of *Bradyrhizobium* sp. SUTN9-2 represent different symbiotic interaction with *Vigna radiata* cv. SUT4. Nodule phenotypes induced by *Bradyrhizobium* sp. SUTN9-2, WT (a), Δ*copG*$_1$ (b), Δ*copG*$_2$ (c) and *copG*$_{1comp}$ (d). Live/dead cells of section nodule infection with WT (e), Δ*copG*$_1$ (f) and Δ*copG*$_2$ (g) and *copG*$_{1comp}$ (h) at 14 dpi were observed under confocal microscopy, the bacteroids were staining with PI, SYTO9 and calcofluor-white. The nodule number at 14 dpi (i) and 21 dpi (j). Nitrogen fixation activity determined by the acetylene reduction assay on whole roots of nodulated plants infected with the indicated bacterial mutants at 14 (k) and 21 dpi (l). Scale bars are indicated 1 mm. Values represent mean ± standard deviation (n = 5). *P* Values are based on one-way ANOVA, Tukey's test,*, P ≤0.05,**, P ≤ 0.01 ***, P ≤ 0.001,****, P ≤ 0.0001).

4.3.3 The *nod* genes production was abolished in Δ$copG_1$

The gene expression of *nodD* regulatory and nod factors genes was investigated (Figure 4.4). To examine the impact of 10 μM naringenin treatment, the relative expression of *nodD1*, *nodD2*, and representative nod factors (*nodA* and *nodC*) was analyzed. The gene expression of *nodD2*, *nodA* and *nodC* were decreased in Δ$copG_1$ which is unable to initiate nodulation on *V. radiata* cv. SUT4 (Figure 4.4b, 4.4C and 4.4d). It should be noted that *nodD1* acts as a constitutive expression in *Bradyrhizobium* sp. SUTN9-2 and therefore, statistically significant changes in expression were not observed for the *nodD1* gene (Fig 4.4a). However, the expression level was slightly decreased in Δ$copG_1$ when compared with WT. Interestingly, despite the lack of the $copG_1$ gene, the expression levels of *nodD2*, *nodA*, and *nodC* genes were not significantly affected even when induced with 10 μM naringenin. The findings indicate that $copG_1$ plays a role in the regulation of *nodD2*, *nodA,* and *nodC* expression, which are crucial for nodulation, while its absence does not directly impact the expression levels of these genes even under naringenin induction. In terms of the nod genes expression was not found in $copG_{1compl}$ as expected.

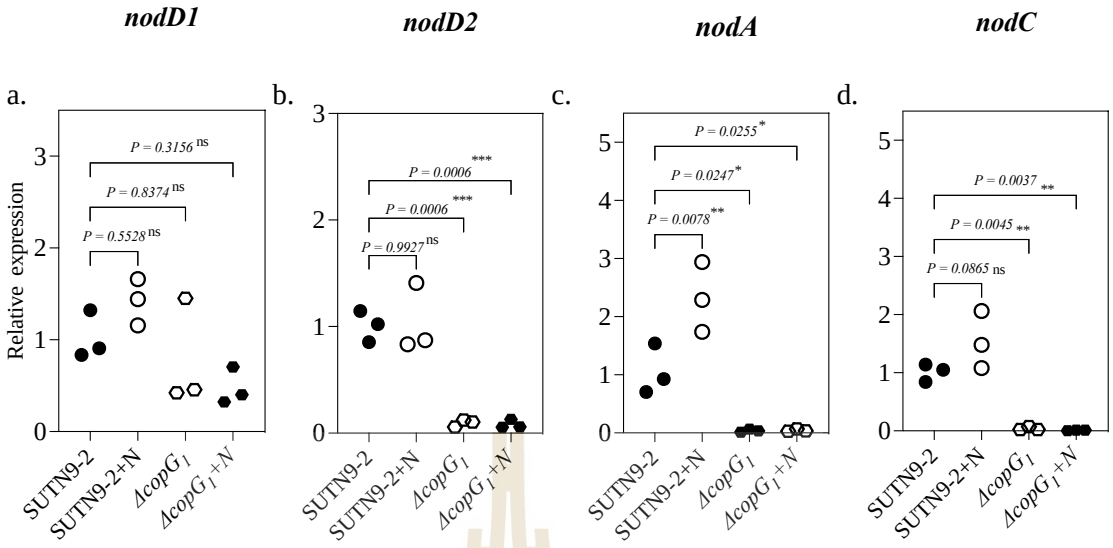

Figure 4.4 qRT-PCR analysis of expression of *nod* genes from *Bradyrhizobium* sp. SUTN9-2, WT and Δ*copG*$_1$ grown in absence and presence of naringenin. (10 µM). The expression of regulatory gene ; *nodD1* (a), *nodD2* (b) and structural genes; *nodA* (c), and *nodC* (d) Data were normalized in relation to the endogenous control (16S rRNA). Values represent mean ± standard deviation (n = 3). P Values are based on one-way ANOVA, Tukey's test (ns, P > 0.05,*, P ≤ 0.05,**, P ≤ 0.01,***, P ≤ 0.001).

4.3.4 *Bradyrhizobium* sp. SUTN9-2 *copG*$_1$ is involved in the repression of T4SS structural genes, *traG*$_1$ and *trbE*$_1$ genes

In addition to the loss of nodulation ability observed in the tested plants and the absence of nod gene expression in Δ*copG*$_1$, further investigations were conducted to explore the relationship between the *copG*$_1$ gene and the T4SS. The *copG*$_1$ gene was found to be located downstream of the *traG*$_1$ gene, which is a T4SS structural gene present in both T4SS clusters. To gain a better understanding of the T4SS and its relationship with the *copG*$_1$ gene, gene expression fold changes were analyzed. The results revealed that representative T4SS genes including *trbE*$_1$ and *traG*$_1$ exhibited higher expression levels in the absence of *copG*$_1$ compared to the WT (Figure 4.5a and 4.5b). Notably, the expression level of *traG*$_1$ was particularly elevated in the absence of the *copG*$_1$ gene (Figure 4.5b). These findings indicate that the *copG*$_1$ gene may function as a repressor of T4SS gene expression, specifically in relation to

the *traG*$_1$ gene located upstream of *copG*$_1$. Therefore, it can be inferred that the *copG*$_1$ gene plays a regulatory role in the expression of T4SS genes and its absence leads to increased expression of *traG1* and potentially other T4SS components. This suggests a possible relationship between the *copG*$_1$ gene and the regulation of the T4SS machinery in *Bradyrhizobium* sp. SUTN9-2.

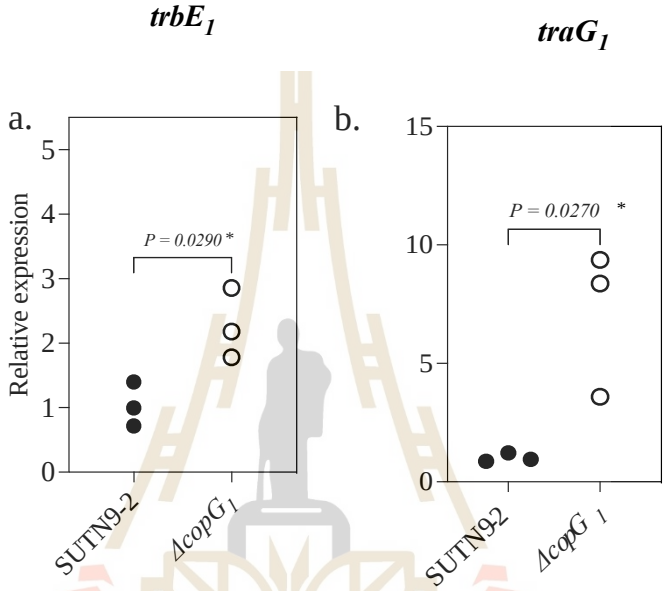

Figure 4.5 *Bradyrhizobium* sp. SUTN9-2 *copG*$_1$ gene act as a repressor of T4SS. Relative expression of representatives T4SS structural genes *trbE*$_1$ gene (a) and *traG*$_1$ gene (b) in WT and Δ*copG*$_1$. The expression level of various genes was determined by qRT-PCR. The 16S rRNA gene was used as an internal control. Values represent mean ± standard deviation (n = 3). *P* values were based on Student's t-test (*,P ≤ 0.05).

4.4 Discussion

Bradyrhizobia containing T4SS on chromosome belong to *tra/trb* operon were represented a unique gene arrangement of *copG*, *traG* and *virD2* cluster. Based on phylogenetic relationship of *copG*, *traG* and *virD2* genes cluster T4SS copy 1 (T4SS$_1$) and 2 (T4SS$_2$) were separated in two clades with different bradyrhizobia group. T4SS$_1$ was similar with *B. yuanmingense* BRP09 which is main rhizobia associated with *V. unguiculata* and *V. radiata* plants in subtropical region (Zhang et al., 2008). The

deletion of *copG*, *traG* and *virD2* copy 1 fragment of *Bradyrhizobium* sp. SUTN9-2 was constructed , named $\Delta T4SS_1$. The $\Delta T4SS_1$ was decreased the symbiosis efficiency both nodulation and nitrogen fixation in *V. radiata* cv. SUT4 and *M. atropurpureum*. The $\Delta copG_1$, $\Delta traG_1$ and $\Delta virD2_1$ were constructed and tested with *V. radiata* cv. SUT4. The *V. radiata* cv. SUT4 inoculated with $\Delta traG_1$ was increased the nodule formation while $\Delta virD2_1$ showed not significantly different nodule number with WT (Figure 4.1m). However, under confocal microscopy of sectioned nodules of 7 dpi, the symbiosome spcace elicited by $\Delta traG_1$ and $\Delta virD2_1$ were smaller than WT and dead cells were observed at symbiosome within infected nodules (Figure 4.1c and 4.1d).

The *traG* gene, commonly found in conjugative plasmids, plays a crucial role in horizontal gene transfer between bacteria. It encodes the TraG protein, which acts as a coupling receptor and is involved in binding translocation substrates such as DNA or effector proteins through the T4SS channel (Fronzes et al., 2009; Cascales et al., 2013; Christie et al., 2014). Previous studies have demonstrated the importance of TraG in DNA transfer during bacterial conjugation and its induction by root exudates and early nodules in *Mesorhizobium* spp., indicating its role in mutualistic symbiosis (Schröder et al., 2002; Paço et al., 2019). In the case of *Bradyrhizobium* sp. SUTN9-2, the $\Delta traG_1$ did not prevent nodule formation on *V. radiata* cv. SUT4, but the resulting nodules were smaller than those formed by the WT. Interestingly, the mutant nodules exhibited the presence of dead cells of bacteroids within the symbiosome, which was not observed in the WT nodules. However, as time progressed, the bacteroids within the mutant nodules showed a remarkable ability to recover. These findings suggest that the $traG_1$ gene may contribute to nodule size regulation and potentially play a role in maintaining symbiotic stability during early nodule development. Furthermore, in silico analysis of the TraG protein using the SMART database revealed specific domains, including Pfam:T4SS-DNA_transfer and Pfam:TraG-D. Pfam:T4SS-DNA_transfer are essential for bacterial DNA transfer, while Pfam:TraG-D is based on conserved TraG protein sequences. Additionally, TraD, another member of the TraG family of coupling proteins, was found to interact with relaxosome components through its C-terminal region (Figure 4.6a).

VirD2 gene in *Bradyrhizobium* sp. SUTN9-2 was classified as a relaxases/ mobilisation protein which were required for horizontal transfer of genetic information contained on plasmids that occurs during bacterial conjugation in *Agrobacterium* (Byrd & Matson, 1997). *virD2* gene encoded the VirD2 protein of T4SS. In *Agrobacterium* sp., required VirD2 for translocation and integration of T-strands into recipient plant cells (Ramsay et al., 2006). However, domain analysis presented VirD2 in *Bradyrhizobium* sp. SUTN9-2 possess a part of domain of unknown function 3363 (DUF3363) overlaped with relaxase domain (Figure 4.6b). A part of DUF in VirD2 was not required for transformation in *Agrobacterium* (Kregten et al., 2009). The deletion of *virD2* gene in *Bradyrhizoibium* sp. SUTN9-2 was not retard the nodulation as $\Delta T4SS_1$ but dead cells were monitored at early nodulation process and symbiosome spaces were smaller than WT. These results assumed that *Bradyrhizobium* sp. SUTN9-2 may require $traG_1$ and $virD2_1$ genes for symbiosis interaction at the early infection stage. In contrast to $\Delta traG_1$ and $\Delta virD2_1$, the $\Delta copG_1$ exhibited reduced nodulation efficiency and nitrogenase activity in *V. radiata* cv. SUT4. CopG was found in several bacteria such as *Pseudomonas aeruginosa* (Hausrath et al., 2020), *Streptococcus agalactiae* (Gomis-Ruth, 1998), *Vibrio cholerae* (Marrero et al., 2012), *Bradyrhizobium* sp. and *Mesorhizobium* sp. (Wangthaisong et al., 2023). CopG protein is a small transcriptional repressor containing a helix-turn-helix motif domain, which shares structural similarities with regulatory repressors such as Mnt, Arc, and MetJ (Costa et al., 2001). This protein was first characterized in streptococcal plasmid pMV158 act as a transcriptional repressor, CopG protein binds to the promoter region of *copG-repB* and regulates plasmid replication by influencing the activity of RepB which controlled plasmid copy number pMV158 (Gomis-Ruth, 1998; del Solar et al., 2002). In addition, copper resistance was also demonstrated to be influenced by CopG in *P. aeruginosa* and *V. cholerae* (Marrero et al., 2012; Hausrath et al., 2020). The CopG protein in *P. aeruginosa* plays a crucial role in copper resistance by regulating the redox properties of its structure to facilitate copper reduction (Hausrath et al., 2020). While, under anaerobic conditions, *copG* in *V. cholerae* was also demonstrated to play a significant role in copper resistance. Moreover, CopG also act as an antitoxin which a member of the type II toxin-antitoxin (TA) system that is prevalent in bacteria and archaea. In particular, the majority of antitoxins encoded by the *relBE* and *parDE* loci belong to

the MetJ/Arc/CopG family of dimeric proteins, which possess DNA-binding capabilities through N-terminal ribbon-helix-helix (RHH) motifs (Gerdes et al., 2005). A typical type II TA system comprises two genes located in an operon that encodes a stable toxin and a labile antitoxin. In addition, TA systems plays a role in coordinating the replication of both plasmids and chromosomes by facilitating the interaction between the antitoxin and the plasmid origin of replication (ori) (Ni et al., 2021). The number of plasmids present in bacteria can change throughout their life cycle, and the ability to regulate this number is crucial for conjugative plasmids to maintain a harmonious relationship with their host bacteria (Del Solar & Espinosa, 2002). In *Bradyrhizobium* sp. SUTN9-2 was observed two copies of *copG* (named, $copG_1$ and $copG_2$) gene in genome with unknown function. The $CopG_1$ protein was classified as uncharacterized conserved protein (Figure 4.6c), while $CopG_2$ was Pfam;RHH_1 (Figure 4.6d). The deletion of both *copG* gene of *Bradyrhizobium sp*. SUTN9-2 showed different nodulation efficiency. In the Δ$copG_1$ abolished nodule formation in *V. radiata* cv. SUT4, contrast to the Δ$copG_2$ showed a significantly higher nodule number albeit smaller in size compared to the WT. However, despite the lack of nodulation, the Δ$copG_1$ retained its ability to infect plants, as evidenced by the ability to monitor both live and dead cells within plant tissues. Intriguingly, living cells were predominantly observed in the vascular bundle tissue, resembling the behavior of endophytic bacteria. These findings suggest that $copG_1$ is potentially essential for *Bradyrhizobium* sp. SUTN9-2 in protecting the survival of bacterial cells within the host plant. To investigate the impaired nodulation phenotype of the Δ$copG_1$ in *V. radiata*, we examined the expression of nod genes including *nodD2*, *nodA* and *nodC*. Strikingly, our analysis revealed that *nodD2*, *nodA*, and *nodC* were not expressed even in induction of flavonoids, while $copG_1$ have no effect on *nodD1* expression. These results indicated that *nodD2* may require $copG_1$ gene for stimulate nod factors production that important for early step of nodule organogenesis in legume. These results indicated that $copG_1$ gene may mediate a positively regulate the expression of *nodD2* by binding to nod box to stimulate nod factor production that is important in early step of nodule organogenesis in legume.

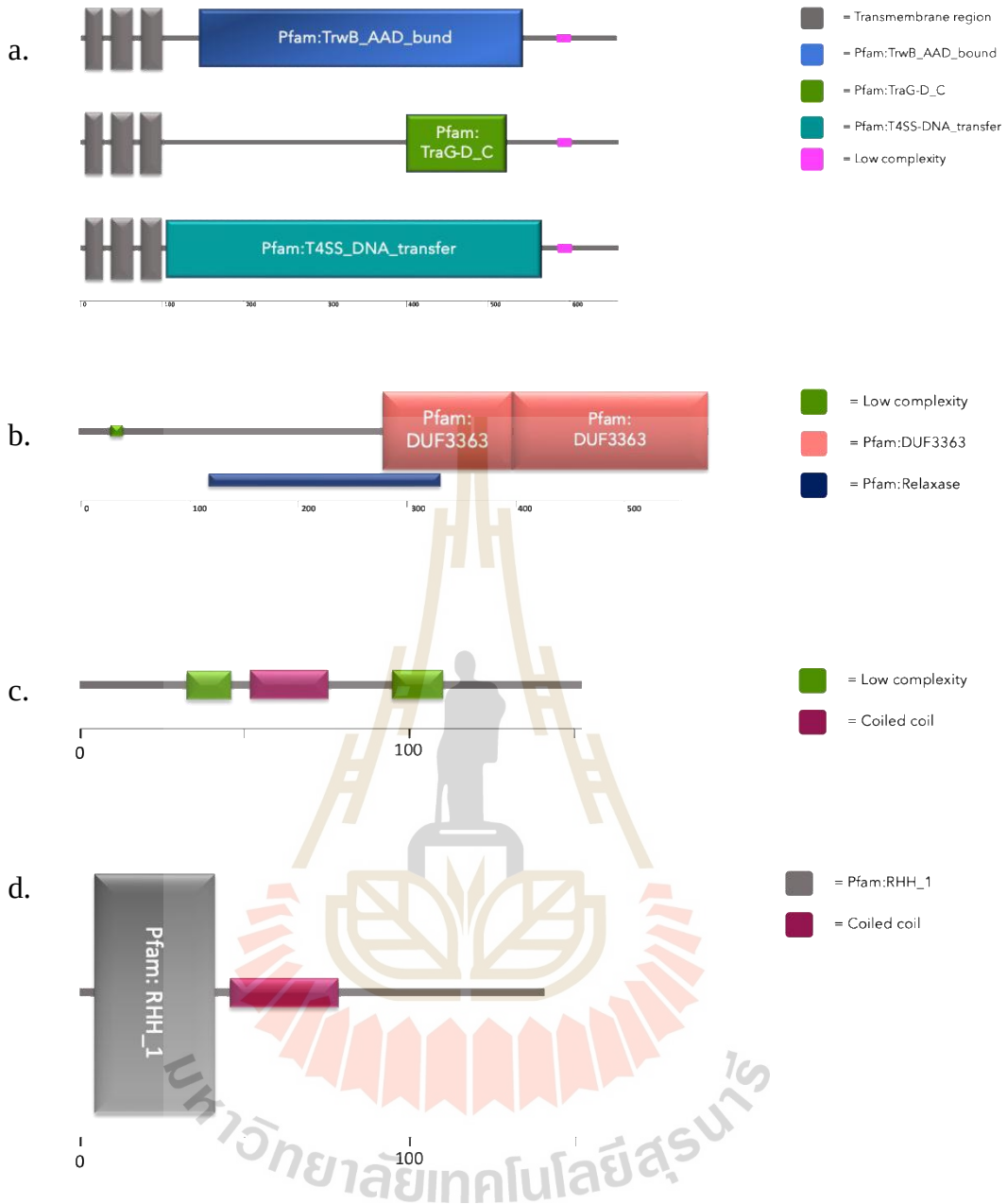

Figure 4.6 Domain architecture analysis using SMART program of $TraG_1$ (a), $VirD2_1$ (b), $CopG_1$ (c) and $CopG_2$ (d) protein sequences.

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CHAPTER V
CONCLUSION

Prior to understand the role of T4SS in *Bradyrhizobium*-legume symbiosis, first phylogenetic trees of the gene involved in T4SS were constructed. A unique arrangement of the *copG*, *traG,* and *virD2* gene cluster was found in T4SS specific to the *Bradyrhizobium* group. The phylogenetic trees implied that the horizontal transfer of T4SS genes was restricted to the rhizobia group. The symbiotic interactions between *Bradyrhizobium* sp. SUTN9-2 and legume plants were performed using T4SS deletion strains. The results showed $\Delta T4SS_1$ reduced nodule number, nitrogenase activity, and delayed nodulation in *V. radiata* cv. SUT4 and *C. juncea* but not in *A. americana*. In addition, dead cells were observed during the early stage of infection with $\Delta T4SS_1$. The result indicated that T4SS may be important for cell survival within plant host that associated with host specificity. This is the first report to demonstrate that the T4SS of *Bradyrhizobium* sp. SUTN9-2 represents another mechanism for symbiotic interaction with leguminous plants. Moreover, within the T4SS cluster, a distinctive gene arrangement comprises *copG*, *traG*, and *virD2* genes in T4SS copy 1. Among these genes $traG_1$ and $virD2_1$ play a crucial roles in the early stages of symbiosis in contrast to $copG_1$. Nodulation efficiency was compromised in the $\Delta copG_1$ mutant with no detectable expression of *nod* genes (*nodD2*, *nodA*, and *nodC*), while $T4SS_1$ genes ($traG_1$ and $trbE_1$) were repressed in the presence of the $copG_1$ gene. These findings suggest that T4SS genes, including $traG_1$ and $virD2_1$, are associated with early nodulation processes. Moreover, the $copG_1$ could potentially act as an alternative factor in facilitating the nodulation mechanism in the symbiosis between *Bradyrhizobium* sp. SUTN9-2 and legume plants. Notably, $copG_1$ also plays a role as a repressor for T4SS structural genes.

APPENDICES

APPENDIX A

Growth medium and Buffered Nodulation Media (BMN)

Appendix A.1 Arabinose-Gluconate (AG) medium (Sugawara et al., 2010)

Constituents per liter;

1.	HEPES	1.3	g
2.	MES-Buffer	1.1	g
3.	Yeast Extract	1.0	g
4.	L-Arabinose	1.0	g
5.	Sodium-Gluconate	1.0	g

solution

1.	Na_2SO_4 (12.5 g/100ml)	2.0	ml
2.	$CaCl_2 \cdot 2H_2O$ (0.65 g/100ml)	2.0	ml
3.	$FeCl_3 \cdot 6H_2O$ (0.433 g/100ml)	2.0	ml
4.	$MgSo_4 \cdot 7H_2O$ (9 g/100ml)	2.0	ml
5.	Na_2HPO_4 (6.25 g/100ml)	2.0	ml
6.	NaCl (15 g/100ml)	2.0	ml
7.	Distilled Water	up to 1 liter	

Adjust the pH to 6.8 with 5 N NaOH.

Appendix A.2 Luria-Bertani (LB) medium (Bertani, 1951)

Constituents per liter;

1.	Tryptone	10.0	g
2.	NaCl	10.0	g
3.	Yeast Extract	5.0	g
4.	Distilled Water	up to 1 liter	

Adjust the pH to 7.0 with 5 N NaOH.

Appendix A.3 Buffered Nodulation Media (BMN) (Ehrhardt et al., 1992).

Constituents per liter;

1.	$CaCl_2 \cdot 2H_2O$	0.344	g
2.	MES-Buffer	0.390	g
3.	1000X Nod major salts	1.0	ml
4.	1000X Nod minor salts I	1.0	ml
5.	1000X Nod minor salts II	1.0	ml
6.	1000X Fe-EDTA	1.0	ml
7.	Distilled Water	up to 1 liter	

Adjust the pH to 6.5-6.8 with 2N KOH

Solution stock (500 ml) for BMN

1000X Nod major salts

1.	KH_2PO_4	34.0	g
2.	$MgSo_4 \cdot 7H_2O$	61.0	g

1000X Nod minor salts I

1.	$ZnSO_4 \cdot 7H_2O$	3.68	g
2.	H_3BO_3	2.48	g
3.	$MnSO_4 \cdot H_2O$	6.80	g

1000X Nod minor salts II

1.	$Na_2MoO_4 \cdot 2H_2O$	125.0	mg
2.	$CuSO_4 \cdot 5H_2O$	8.0	mg
3.	CaCl2	6.9	mg

1000X Fe-EDTA (0.01M)

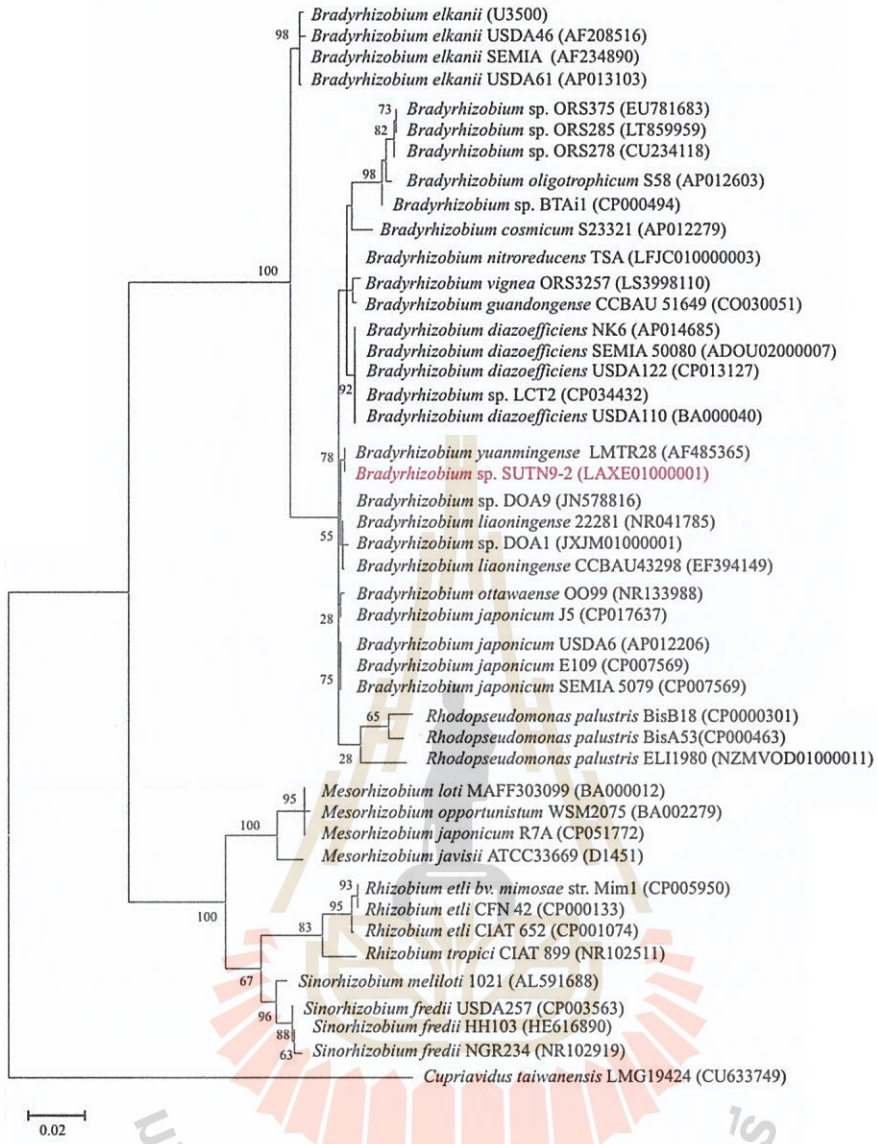
1.	NaFe-EDTA	9.176	mg

or

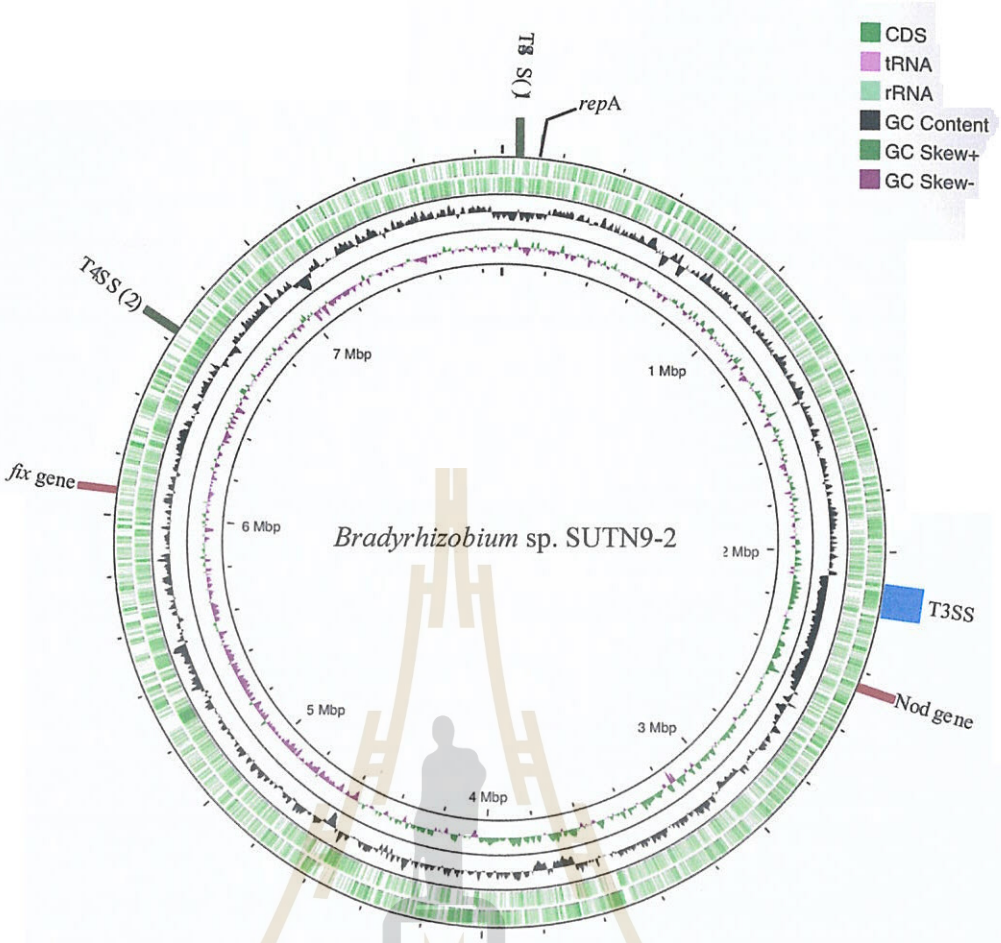
1.	Na_2EDTA	9.325	mg
2.	$FeSO4 \cdot 7H_2O$	6.950	mg

APPENDIX B

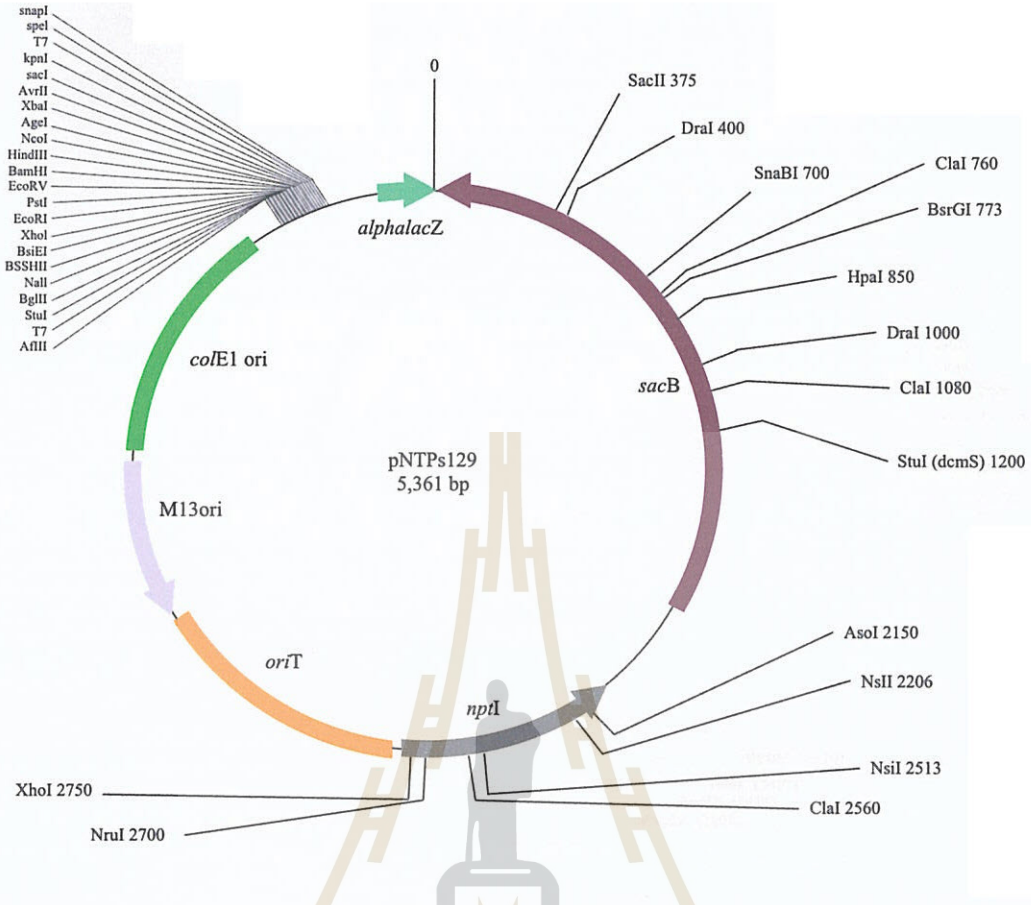
Phylogenetic tree, *Bradyrhizobium* sp. SUTN9-2 and Plasmid map for molecular cloning



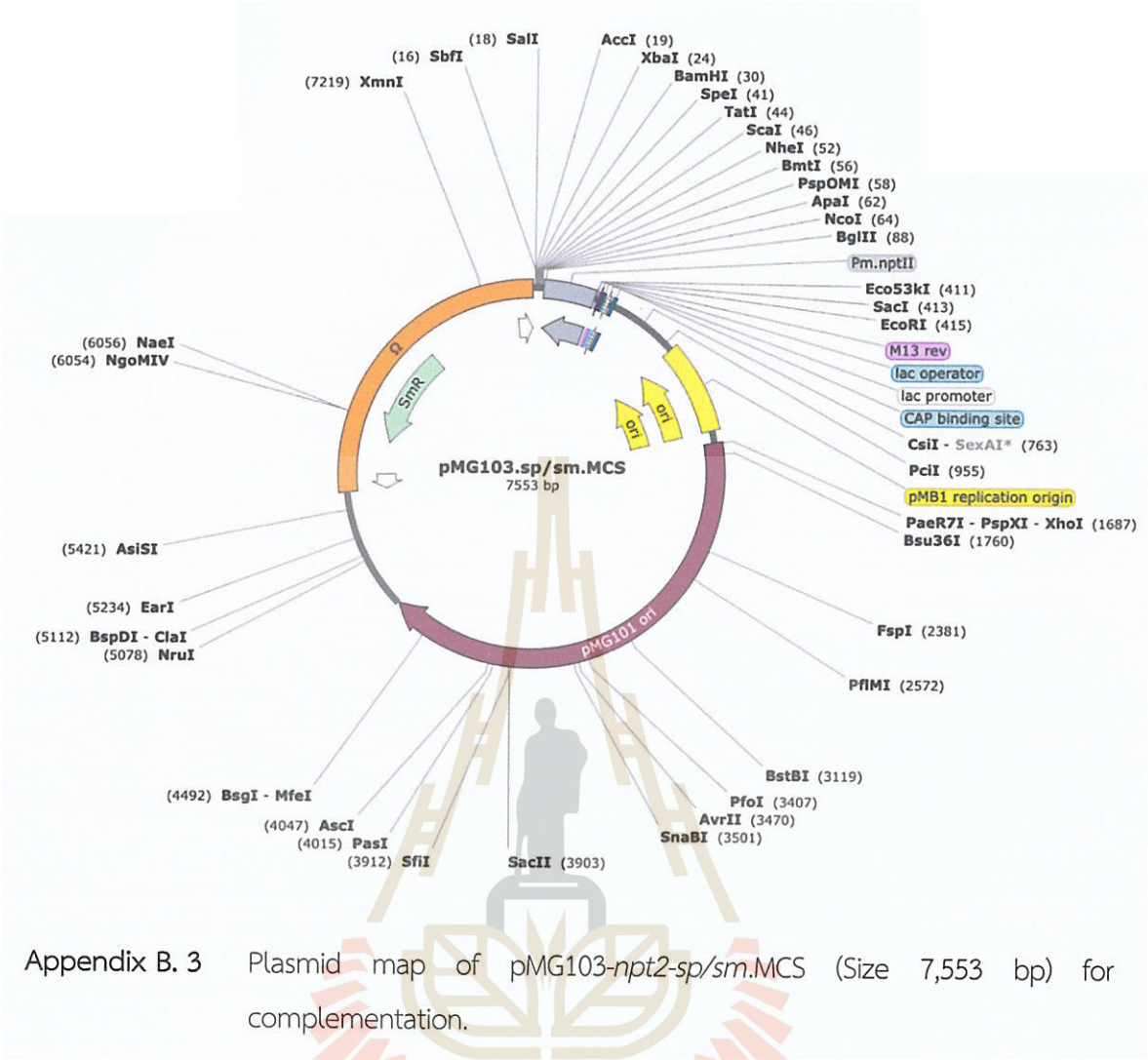
Appendix B.1 Maximum Likelihood tree based on 16S rRNA gene sequences of rhizobia. Percentage of bootstrap support (1000 replicates) is indicated on internal branches. *Cupriavidus taiwanensis* LMG19424 was used as an outgroup. Scale bar indicates 0.2 substitutions per site.



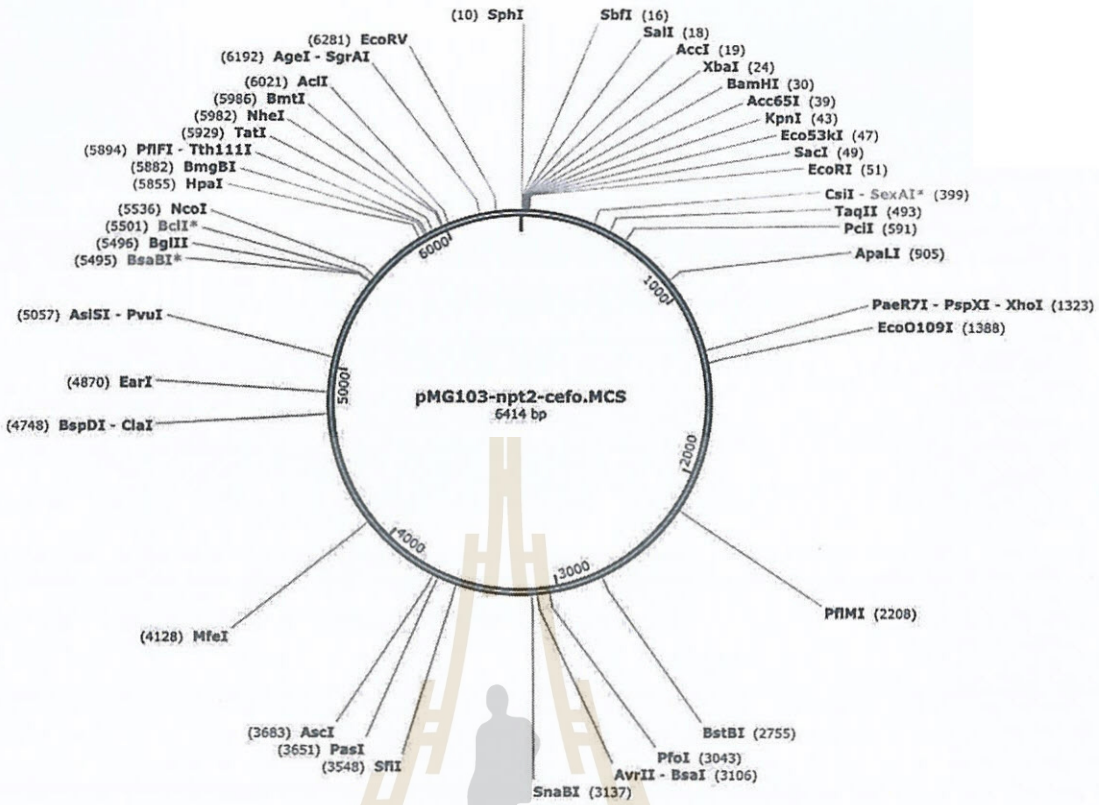
Appendix B.2 The genome structure of *Bradyrhizobium* sp. SUTN9-2.



Appendix B.2 Plasmid map of pNTPS129-*npt2-sacB* (Size 5,361 bp) for gene deletion.



Appendix B. 3 Plasmid map of pMG103-*npt2-sp/sm*.MCS (Size 7,553 bp) for complementation.



Appendix B. 4 Plasmid map of pMG103-*npt2-cefo*.MCS (Size 6,414 bp) for complementation.

BIOGRAPHY

Miss Praneet Wangthaisong was born on April 8th, 1992, in Nakhon Ratchasima, Thailand. She attended Bannon-Udomnoppadon Ooppathump School for primary education and Chumphuang Suksa School for high school. In 2013, she graduated from Suranaree University of Technology with a Bachelor's degree in Crop Production Technology. In 2014, she received a scholarship from the Thailand Research Fund (TRF) under the Royal Golden Jubilee Ph.D. Programme (RGJ) to pursue a combined Master-Doctoral degree in the School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, under the Supervised of Prof. Dr. Neung Teaumroong. During her enrollment in the doctoral degree program, she presented her research work titled "Role of Type IV (T4SS) secretion System on *Bradyrhizobium*-legume symbiosis" in a poster presentation at the 4th, Asian Conference on Plant-Microbe Symbiosis & Nitrogen Fixation held in Penang, Malaysia from October 16 to 19, 2016. She also participated in the RGJ-Ph.D. Congress 19, themed "Innovation Challenges toward Thailand 4.0: Research Inspiration, Connectivity and Transformation," held at the Jomtien Palm Beach Hotel & Resort in Pattaya, Thailand, from June 7 to 9, 2018 for poster presentation in the topic "Role of Type IV (T4SS) secretion System on *Bradyrhizobium* sp. SUTN9-2-legume symbiosis". Moreover, she obtained a scholarship for her short-term research position at Tokyo University of Agriculture and Technology in Japan. This scholarship was generously supported by the Japan Student Services Organization (JASSO) and Suranaree University of Technology from November 15, 2019 to January 21, 2020. Her research work in the topic of "The type IV secretion system (T4SS) mediates symbiosis between *Bradyrhizobium* sp. SUTN9-2 and legumes" has been published in Applied and Environmental Microbiology in 2023.