MECHANISMS OF RICE ENDOPHYTIC BRADYRHIZOBIAL CELL DIFFERENTIATION AND ITS ROLE ON NITROGEN FIXATION

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กลไกการเปลี่ยนแปลงรูปร่างเซลล์ของแบรดดีไรโซเบียมในเนื้อเยื่อข้าว และบทบาทต่อการตรึงไนโตรเจน

นางสาวตีรณา กรีธาธร

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

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Bradyrhizobium sp. สายพันธุ์ SUTN9-2 สามารถอาศัยอยู่ร่วมกับพืชตระกูลถั่ว และพืชที่ไม่ใช่พืชตระกูลถั่ว เช่น ข้าว เป็นต้น นอกจากนี้ยังมีรายงานว่า SUTN9-2 ช่วยส่งเสริมการเจริญเติบโตของพืช โดยมีคุณสมบัติในการสร้างโมเลกุลที่มีประโยชน์ต่อการเจริญเติบโตของพืชมากขึ้น เช่น indole-3-acetic acid (IAA), เอนไซม์ 1-amino-cyclopropane-1-carboxylic acid (ACC) deaminase, และการตรึงไนโตรเจน การศึกษานี้จึงได้ทำการตรวจสอบประสิทธิภาพของ SUTN9-2 ในการส่งเสริมการเจริญเติบโตของข้าวที่ระยะการเจริญเติบโตแตกต่างกันร่วมกับการใส่ N-free และ NH_4NO_3 ภายใต้สภาวะ *in vivo* พบว่า ข้าวที่มีการใส่เชื้อ SUTN9-2 ร่วมกับ N-free และ NH_4NO_3 มีน้ำหนักแห้ง และปริมาณคลอโรฟิลล์เพิ่มขึ้นโดยเฉพาะอย่างยิ่งที่ระยะต้นกล้า (7 และ 14 วันหลังการใส่เชื้อ) ผลจากการวิเคราะห์ในเชิงปริมาณของ IAA และ เอนไซม์ ACC deaminase พบว่าไม่สอดคล้องกับผลการแสดงออกของยีนที่เกี่ยวข้องกับการผลิต IAA (*nit*) and ACC deaminase (*acdS*) โดยผลเหล่านี้แสดงให้เห็นว่า IAA และ ACC deaminase ที่ผลิตโดย SUTN9-2 ไม่ได้ส่งผลโดยตรงต่อการเจริญเติบโตของข้าว แต่อาจมีปัจจัยอื่นๆ ที่เกี่ยวข้องในการผลิต IAA และ ACC deaminase ในข้าวด้วย นอกจากนี้ยังพบการแสดงออกของยีนที่เกี่ยวข้องกับการตรึงไนโตรเจนของ SUTN9-2 ในข้าว ซึ่งแสดงให้เห็นว่าการเติมแหล่งไนโตรเจน NH_4NO_3 และการตรึงไนโตรเจนของ SUTN9-2 อาจมีส่วนช่วยในการส่งเสริมการเจริญเติบโตของข้าวได้ด้วย นอกจากนี้ในด้านการศึกษากลไกการเปลี่ยนแปลงรูปร่างของเซลล์เมื่ออาศัยอยู่ในเนื้อเยื่อต้นข้าว ที่ส่งผลให้เซลล์มีการขยายขนาด (cell differentiation) จากการตรวจสอบพบว่า SUTN9-2 เมื่อถูกกระตุ้นด้วย rice extract ทำให้เซลล์มีขนาด ปริมาณ DNA และประสิทธิภาพในการตรึงไนโตรเจนเพิ่มขึ้น นอกจากนี้ยังพบว่า rice extract สามารถกระตุ้นการแสดงออกของยีนต่าง ๆ ใน SUTN9-2 ที่เกี่ยวข้องกับการแบ่งเซลล์ และการตรึงไนโตรเจน โดยจากวิธีการทำให้เกิดการกลายพันธุ์ในเชื้อ SUTN9-2 พบว่า ทั้งยีน *bclA* และ *nifV* ส่งผลกระทบต่อประสิทธิภาพการตรึงไนโตรเจน และเมื่อทำการวิเคราะห์ transcriptome ของ SUTN9-2 พบว่า rice extract และการกลายพันธุ์ของยีน *bclA* มีผลต่อการเปลี่ยนแปลงการแสดงออกของยีนต่าง ๆ ของ SUTN9-2 อย่างมีนัยสำคัญทางสถิติ โดยยีนที่แสดงออกอย่างมีนัยสำคัญคือยีนที่เกี่ยวข้องกับการสังเคราะห์เอนไซม์ oxidoreductase ซึ่งทำปฏิกิริยากับอะตอมของออกซิเจน โดยอาจมีบทบาทต่อการลด และควบคุมสภาวะออกซิเจนให้เหมาะสมต่อการตรึงไนโตรเจน และยีนที่ใช้ในการสังเคราะห์ GroESL chaperonins ซึ่งมีความสำคัญต่อ proteins folding และการทำงานของเอนไซม์ไนโตรจีเนส ผลเหล่านี้แสดงให้เห็นถึงความเป็นไปได้ที่การตรึงไนโตรเจนของ SUTN9-2 มีความสัมพันธ์กับ rice extract นอกจากนี้

ยังพบการแสดงออกของยีนที่เกี่ยวข้องกับ antimicrobial peptides transporter (*sapADF*) ของ SUTN9-2 โดยส่งผลให้เกิดการเปลี่ยนแปลงรูปร่างของเซลล์ แม้ว่าจะทำการกลายพันธุ์ยีน *bclA* (*sapDF*) แล้วก็ตาม นอกจากนี้ยังพบว่าข้าวสามารถผลิต defensin-like antimicrobial peptides (DEFs) ซึ่งคล้ายคลึงกับ NCR peptide ที่พบในพืชตระกูลถั่ว ส่งผลต่อการเปลี่ยนแปลงรูปร่างของเซลล์แบคทีเรีย โดยมีขนาดยาวขึ้นเมื่ออาศัยอยู่ในเนื้อเยื่อพืช

สาขาวิชาเทคโนโลยีชีวภาพ
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ลายมือชื่อนักศึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษา_____
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TEERANA GREETATORN : MECHANISMS OF RICE ENDOPHYTIC BRADYRHIZOBIAL CELL DIFFERENTIATION AND ITS ROLE ON NITROGEN FIXATION. THESIS ADVISOR : PROF. NEUNG TEAUMROONG, Dr.rer.nat., 128 PP.

RICE/ENDOPHYTE/CELL DIFFERENTIATION/NITROGEN FIXATION

Bradyrhizobium sp. strain SUTN9-2 is rice endophytic diazotrophic bacterium that can live in symbiotic and endophytic associations with legume plants and non-legume plants such as rice. SUTN9-2 was reported as a rice growth promotion agent, showing the capability of plant growth promotion charecteristics, such as indole-3-acetic acid (IAA), 1-amino-cyclopropane-1-carboxylic acid (ACC) deaminase productions and nitrogen fixation. In this study, the ability of SUTN9-2 to stimulate rice growth was investigated at different stages with N-free and NH_4NO_3 under *in vivo* condition. The rice dry weight and chlorophyll content could be enhanced when SUTN9-2 was inoculated in N-free, and NH_4NO_3 especially at seedling stage (7 and 14 dai). The results of the quantitative analysis of IAA and ACC deaminase were inconsistent with the expression of genes involved in IAA (*nit*) and ACC deaminase (*acdS*) productions. This inconsistency could imply that IAA and ACC deaminase produced from SUTN9-2 did not directly affect rice growth, but other factors resulting from the production of IAA and ACC deaminase could be involved. Moreover, the expression of genes involved in nitrogen fixation (*nifH* and *nifV*) of SUTN9-2 was also induced in rice tissues. This finding suggested that rice growth promotion may be supported by NH_4NO_3 together with nitrogen fixation by SUTN9-2. On the other hand,

this study revealed the presence of cell differentiation (enlarged size) and increased DNA content and nitrogen fixation ability of SUTN9-2 in response to rice extract. Moreover, rice extract also induced the expression of genes involved in cell cycle and nitrogen fixation of SUTN9-2. Based on the mutation of genes *bclA* and *nifV* in SUTN9-2, it was found that these two genes were involved in nitrogen fixation efficiency. The transcriptome results suggested that SUTN9-2 was affected by rice extract and Δ*bclA* mutant, according to differentially expressed genes (DEGs) were observed. The upregulated DEGs encoding the class of oxidoreductase, acting with oxygen atoms, which might play a role in reducing and controlling the oxygen level to be appropriate for nitrogenase activity, followed by GroESL chaperonins, require proteins folding and nitrogenase function. These results indicated the possibility that nitrogen fixation of SUTN9-2 might be induced by the rice extract. Also, the sensitivity to antimicrobial peptides transporter (*sapADF*) was upregulated, resulting in cell differentiation even the *bclA* (*sapDF*) was mutated. Interestingly, this implied similarities in the production of defensin-like antimicrobial peptides (DEFs) by rice and nodule-specific cysteine rich (NCR) peptides in legume plants, which affect bacterial cell elongation.

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LIST OF ABBREVIATIONS

ºC	=	Degree Celsius
µE	=	Microeinsteins
µm	=	Micrometer
µmol	=	Micromoles
ACC deaminase	=	1-amino-cyclopropane-1-carboxylic acid deaminase
AMPs	=	Antimicrobial peptides
ARA	=	Acetylene reduction assay
bB	=	SUTN9-2 Δ*bclA* in response to BNM-B
BclA	=	*Bradyrhizobium* bacA-like
BNF	=	Biological nitrogen fixation
BNM-B	=	Buffered nodulation medium B minimal medium
bRB	=	SUTN9-2 Δ*bclA* in response to BNM-B + rice extract
CAMP	=	Cationic antimicrobial peptide
CFU	=	Colony forming unit
CRPs	=	Cysteine-rich peptides
dai	=	Days after inoculation
DAPI	=	4′,6-diamidino-2-phenylindole
DEFs	=	Defensin-like antimicrobial peptides
DEGs	=	Differentially expressed genes
DI	=	Deionized water

LIST OF ABBREVIATIONS (Continued)

dpi	=	Days post-inoculation
EPS	=	Exopolysaccharide
Et alia (and other)	=	et al.
FDR	=	False discovery rate
FM4-64	=	*N*-(3-Triethylammoniumpropyl)-4-(6-(4-(Diethylamino) Phenyl) Hexatrienyl) Pyridinium Dibromide)
FS	=	Forward scatter
g	=	Gram
h	=	Hour
IAA	=	Indole-3-acetic acid
IAAld	=	Indole-3-acetaldehyde
IAM	=	Indole-3-acetamide
IAN	=	Indole-3-acetonitrile
IPA	=	Indole-3-pyruvate
IPDC	=	Indole-3- pyruvate decarboxylase
IRLC	=	Inverted repeat-lacking clade
logCPM	=	Log count per million
logFC	=	Log_2 fold change
LPS	=	Lipopolysaccharide
LTP	=	Lipid transfer proteins
mg	=	Milligram

LIST OF ABBREVIATIONS (Continued)

min	=	Minute
ml	=	Milliliter
mm	=	Millimeter
mmol	=	Millimoles
MPN	=	Most probable number
NCR	=	Nodule-specific Cysteine Rich
NI	=	Non-inoculation
nm	=	Nanometer
nsHbs	=	Non-symbiotic hemoglobins
PGP	=	Plant growth promoting traits
PGPR	=	Plant growth promoting rhizobacteria
PHB	=	Polyhydroxybutyrate
PI	=	Propidium iodide
pmol	=	Picomole
P value	=	Probability value
qRT-PCR	=	Quantitative Reverse Transcription PCR
RND	=	Resistance nodulation and cell division family
SAM	=	S-adenosyl-L-methionine
Sap	=	Sensitivity to antimicrobial peptides transporter
SEM	=	Scanning electron microscopy
SRI	=	System of rice intensification

LIST OF ABBREVIATIONS (Continued)

TAM	=	Tryptamine
TBD	=	Terminal bacteroid differentiation
TEM	=	Transmission electron microscope
THIONs	=	Thionins
Trp	=	Tryptophan
TSO	=	Tryptophan side-chain oxidase
vol	=	Volume
WB	=	SUTN9-2 WT in response to BNM-B
WRB	=	SUTN9-2 WT in response to BNM-B + rice extract
YEM	=	Yeast extract-mannitol

CHAPTER I

INTRODUCTION

1.1 Rationale and background

Bradyrhizobium spp. can associate as symbiont and endophyte with legumes and non-legumes such as rice, namely *Oryza breviligulata* (Chaintreuil et al., 2000) *O. sativa* L. ssp. *indica* and *japonica* (Piromyou et al., 2015) and also can facilitate plant growth and development. *Bradyrhizobium* sp. strain SUTN9-2 has been reported to capable of symbiotic and endophytic associations with legume plants and rice plants. Also, the production of indole-3-acetic acid (IAA), increasing plant cell division and elongation (Tivendale et al., 2014) and 1-amino-cyclopropane-1-carboxylic acid (ACC) deaminase, decreasing plant ethylene levels have been demonstrated, facilitating plant growth promotion (Glick, 2014).

The biological nitrogen fixation (BNF) by endophytic bradyrhizobia in rice may be caused by the nitrogenase enzyme activity, encoded by the gene *nifH* (the nitrogenase structural component). Another gene, *nifV*, is involved in the biosynthesis of the homocitrate synthase, to activate the nitrogenase Fe protein in free-living diazotrophs (Howard and Rees, 1994) and *Bradyrhizobium* sp. (Pagan et al., 1975). However, this gene is not mostly displayed in *Rhizobium* spp. that performs symbiotic nitrogen fixation only legume plants (Hakoyama et al., 2009). However, the *nifV* gene was found in SUTN9-2 (Noisangiam et al., 2012; Hashimoto et al., 2019). These bacterial capabilities make it a potential candidate for using as a biofertilizer or

bioinoculant. However, no information is available especially, molecular mechanisms that play a key role in plant colonization and growth promotion of rice endophytic bacteria. Therefore, the potential of plant growth promotion characteristics of SUTN9-2 on rice growth promotion at the different stages was investigated before applying in the system of rice intensification (SRI) for which healthy rice seedling is necessary (Dobermann, 2004).

Recently, SUTN9-2 was observed with elongated cells in rice tissues at 7 days post-inoculation (dpi) by scanning electron microscopy (SEM). Interestingly, elongated cells were observed in rice root tissues when compared between 3 (≅ 1-2 micrometer (μm)) and 7 (≅ 3 μm) dpi (Piromyou et al., 2017). This result suggested the hypothesis that the interaction between rice plant and bacterial factors may be speculated to involved in cell size enlargement and increase in nitrogen fixation efficiency of SUTN9-2 in rice plants, similar to bacteroid differentiation in the legume plants. Coutinho et al. (2015) used the RNAseq analysis to investigate the response of endophyte *Burkholderia kururiensis* M130 to rice extract. These experiments revealed that 27.7% of its open reading frames were differentially expressed in the presence of the rice extract, including genes for membrane transporters and secretion systems, motility, chemotaxis, and adhesion. This work suggesting that the exchange of molecules is an important aspect of bacterial endophytic growth and adaptation in rice plants.

A terminal bacteroid differentiation (TBD) process occurs regarding producing bacterial cells elongation, polyploidy that performed endoreplication (Alunni and Gourion, 2016). TBD is determined by host plant factors, including defensin-like antimicrobial peptides (DEFs) consisting of nodule-specific cysteine-rich (NCR)

peptide, produced in large families of IRLC and Delbergioid legume clades, together with BacA transporter protein in the microbes (Mergaert et al., 2003; Alunni et al., 2007). The bacteroid also evolved function for the response to NCR peptide. The formation of the differentiated and polyploidy cell is under the control of NCR peptide and BacA transporter protein. The BacA of *Sinorhizobium meliloti* provides the protection against the antimicrobial activity of NCR peptide in *Medicago* nodules (Haag et al., 2011).

NCR peptides have been reported to conduct the polyploidy of *S. meliloti* in *Medicago* nodules by affecting the process involved in sequential changes in cell cycle genes expression and cell size increment (De Nisco et al., 2014; Penterman et al., 2014). Master cell-cycle genes expression of *S. meliloti*, including *ctrA*, *gcrA,* and *dnaA* were moderated in NCR-treated cells, indicating cell division was blocked by sublethal NCR treatment (Penterman et al., 2014). In addition, the effect sublethal levels of cationic NCR peptides were detected *in vitro* ex-planta in *S. meliloti*, which increase bacterial cell size and ploidy levels (Van de Velde et al., 2010; Haag et al., 2011).

In the genus *Bradyrhizobium*, BacA-like transporter (BclA) have been identified in *Bradyrhizobium* sp. strains ORS285, carrying three genes (BRAO285v1_250005, BRAO285v1_950010 and, BRAO285v1_1320006) for cell differentiation and functional bacteroids in NCR-producing *Aeschynomene* legumes (Guefrachi et al., 2015). These genes were characterized by the transmembrane domain consisting of ABC_membrane_2 or SbmA_BacA. However, only gene BRAO285v1_1320006 that plays an important role in symbiotic phenotype in the host plants, indicating that this gene encodes BacA-like proteins in ORS285. However, the BacA or the homolog SbmA of *Escherichia coli* differ from *Bradyrhizobium* proteins

by the presence of a C-terminal cytosolic ATPase domain typical for canonical ABC transporters. Thus, *Bradyrhizobium* bacA-like (BclA) was named (Guefrachi et al., 2015). Remarkably, the small bacteroid size (1 – 2.5 μm) in Alfalfa (*M. sativa*) nodules had low nucleic acid content also had low acetylene reduction. On the other hand, the enlarged bacteroid size (5 – 7 μm) had high nucleic acid content and very active in acetylene reduction (Paau and Cowles, 1978). This result indicated the correlation between cell size and nitrogen fixation efficiency. Thus, the changes in cell size and nitrogen fixation ability of SUTN9-2 occurring during its interaction with the rice extract were analyzed using confocal microscopy, flow cytometry, and acetylene reduction assay. Also, an overview of gene expression in response to rice extract was investigated in order to have a better understanding of the mechanisms and factors involved in cell differentiation and nitrogen fixation in this model.

1.2 Hypothesis

1.2.1 SUTN9-2 had the potential for plant growth-promoting (PGP) traits to facilitate rice growth.

1.2.2 SUTN9-2 could enlarge cell size and fix nitrogen in endophytic association with rice plants similar to symbiotic bacteroids in legume plants.

1.2.3 The mechanisms and factors involved in cell differentiation and nitrogen fixation of endophytic SUTN9-2 in rice plants may share different or similar manner to symbiotic bacteroids in legume plants.

1.3 Research objectives

1.3.1 General objective

To examine the potential of endophytic SUTN9-2 on rice growth promotion at different stages and reveal the mechanisms and factors involved in cell differentiation and nitrogen fixation of endophytic SUTN9-2 in rice plants, which affected rice growth promotion

1.3.2 Specific objectives

1.3.2.1 To examine the potential for plant growth-promoting (PGP) traits of endophytic SUTN9-2 on rice growth promotion at different stages

1.3.2.2 To determine the increment in cell size, DNA content, and nitrogen fixation ability of SUTN9-2 in rice plant and response to rice extract

1.3.2.3 To reveal the mechanisms and factors involved in cell size enlargement and nitrogen fixation of SUTN9-2 in rice plant and response to rice extract, which different or similar to bacteroids in legume plants

1.4 Scope and limitation of the study

The potential of SUTN9-2 for rice growth promotion at the early rice seedling growth stage was investigated. The increment in cell size and nitrogen fixation ability of SUTN9-2 occurring during its interaction with rice plant and rice extract were determined. The similarity in the mechanisms and factors involved in cell differentiation and nitrogen fixation between an endophytic cell in rice and symbiotic cell in legume plants was demonstrated.

1.5 References

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CHAPTER II

LITERATURE REVIEW

2.1 Endophytic bacteria

The common inhabitants of bacteria can be both the surface and internal tissues of the host plant. Bacteria from plant surfaces are called epiphytes, while bacteria from internal plant tissues are known as endophytes. These bacteria inhabit without harmful to the host plant (Petrini et al., 1989; Azevedo et al., 2000). The endophytic bacteria are divided into (1) passenger endophytes (red cells) (Fig. 2.1) which invade plant tissues randomly and almost invaded to the root cortex, (2) opportunistic endophytes (blue cells) (Fig. 2.1) which invade plant tissues using a chemotactic response to colonize the rhizoplane and almost invade at the lateral root and root tip through cracks formed, (3) competent endophytes (yellow cells) (Fig. 2.1) which can manipulate plant metabolism and harmonious and can invade specific plant tissues then spread throughout the host plant (Hardoim et al., 2008). Endophytic bacteria can promote plant growth and development and also may have diverse effects on host plant such as the competition for nutrients, iron and, phosphorus, induction of systemic resistance in the host plant, phytohormones production, and nitrogen fixation (Glick, 1995; Shishido et al., 1999; Kinkel et al., 2000; Sturz et al., 2000).

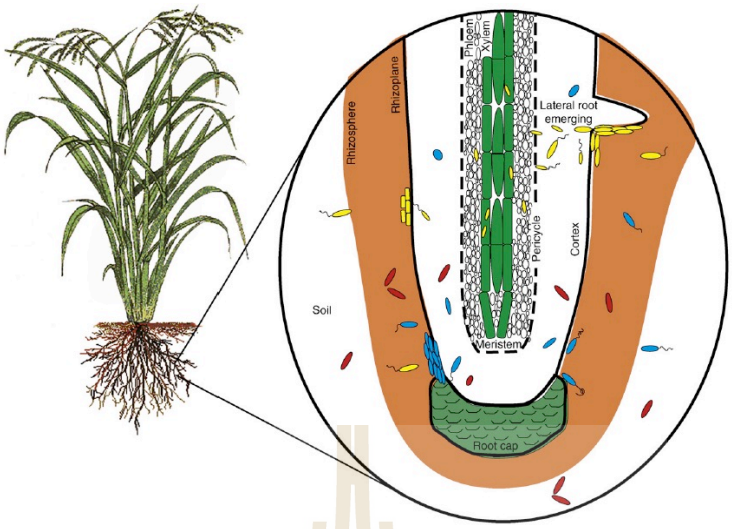

Figure 2.1 Types of endophytes and their root colonization process (Hardoim et al., 2008).

2.2 Endophytic bacteria colonize in rice plants

Endophytic bacteria, including *Azospirillum*, *Bradyrhizoium, Burkholderia*, *Herbaspirillum*, *Methylobacterium*, *Pantoea*, and, *Pseudomonas* have been reported as endophytic bacteria in rice plants and also promote rice growth and rice production and without harmful effects on the host plants (Table 2.1) (Mano and Morisaki, 2008; Piromyou et al., 2015a). Several endophytic bacteria can facilitate plant growth via plant growth-promoting (PGP) traits such as IAA, ACC deaminase, and nitrogen fixation (Etesami et al., 2014).

Table 2.1 Various endophytic bacteria colonized in rice plants.

Rice species	Endophytic bacteria	Rice response to the endophyte	Reference
Oryza. sativa L. cv. Yuefu	*Pantoea agglomerans*	Promotes host rice plant growth and affects allocations of host photosynthates.	Feng et al. (2006)
Oryza sativa L. cv. CO-43	*Methylobacterium* sp.	Improved the plant growth and lateral root formation	Senthilkumar et al. (2009)
Oryza sativa L. cv. Nipponbare	*Azospirillum* sp.	Induce disease resistance in rice	Yasuda et al. (2009)
*Oryza sativa*L. cvs. IR42 and IR72.	*Herbaspirillum seropedicae*	Increases in N content in grains and fresh weight.	James et al. (2002) Divan Baldani et al. (2000)
Oryza sativa L.	*Burkholderia kururiensis*	Promoting both plant growth and rice grain yield.	Mattos et al. (2008)
	Burkholderia vietnamiensis	Increased rice grain yield	Govindarajan et al. (2008)
	Pseudomonas putida and *Pseudomonas fluorescens*	Promote rice growth	(Etesami et al., 2014)
Oryza breviligulata	*Bradyrhizobium* sp.	Enhance cultivated rice production	(Chaintreuil et al., 2000)
Oryza sativa L. subsp. *indica* *Oryza sativa* L. subsp. *japonica*	*Bradyrhizobium* sp.	Promote rice growth	(Piromyou et al., 2015b)

2.3 Bradyrhizobia

The Rhizobiaceae are a family of proteobacteria, which includes several species consisting of the genera *Allorhizobium, Azorhizobium*, *Bradyrhizobium, Mesorhizobium, Neorhizobium, Rhizobium,* and *Sinorhizobium* (Mousavi et al., 2014) *Bradyrhizobium* spp. are soil proteobacteria, Gram-negative bacilli (rod-shaped), which can fix atmospheric nitrogen especially in symbiotic with legume plants. In addition, they can also survive under low-nutrient environments such as soil or water lacking organic substances (Hattori, 1984; Jaeggi and Schmidt-Lorenz, 1990). For example, nitrogen-fixing oligotrophic *B. oligotrophica* (*Agromonas oligotrophicum*) S58 and *Bradyrhizobium* sp. S23321 were isolated from paddy field soil, which lacking organic materials (Delamuta et al., 2013; Okubo et al., 2013). Thus, the nitrogen-fixing oligotrophic trait might also provide possibilities to apply as inoculum for plants under low nutrients conditions.

2.3.1 Endophytic bradyrhizobia

Although *Bradyrhizobium* spp. have symbiotic relationships with leguminous plant species, it may also occur as endophytes in the roots of the non-leguminous plants such as sugarcane (*Saccharum officinarum*) (Rouws et al., 2014), sweet potato (*Ipomoea batatas* L.) (Terakado-Tonooka et al., 2013) and rice (*Oryza breviligulata*) (Chaintreuil et al., 2000), *O. sativa* L. subsp. indica and japonica (Piromyou et al., 2015b) and can promote their growth and productivity. In sugarcane, both the proliferation of N_2 fixers and the expression of the *nifH* gene are required for N_2 fixation *of Bradyrhizobium* sp. and may contribute to the growth and yield production (Thaweenut et al., 2011). In sweet potato, nitrogen fixation in *Bradyrhizobium* sp. strain AT1 was also reported. The expression of genes involved in

nitrogen fixation was detected, indicating metabolites including carboxylic acids in sweet potatoes were used for nitrogen fixation (Terakado-Tonooka et al., 2013). In rice, photosynthetic *Bradyrhizobium* sp. strain ORS278 could fix nitrogen in *O. breviligulata* and significantly increases in both shoot and grain yields, suggesting their capability to enhance rice production (Chaintreuil et al., 2000). Also, It has been reported that Thai bradyrhizobial strains could promote Thai rice growth better than that of Japanese bradyrhizobial strains, suggesting the characteristics rice cultivars may facilitate or govern the associations of rice and endophytic bacteria (Piromyou et al., 2015b) In addition, *Bradyrhizobium* sp. strain SUTN9-2 promoted the total rice dry weight (*O. sativa* L. cultivar Pathum Thani 1) (Piromyou et al., 2015a) via the productions of IAA and ACC deaminase. Besides, nitrogen fixation and nitric oxide detoxification in rice by SUTN9-2 were also reported (Piromyou et al., 2017).

2.4 Plant growth promoting (PGP) traits

2.4.1 Indole-3-acetic acid (IAA)

IAA directly improves plant root development below the soil by stimulating plant cell elongation, enhance photosynthesis mechanism, and biosynthesis of numerous sorts of pigment above the soil, indirectly by affecting bacterial ACC deaminase activity (Pedraza et al., 2004; Tivendale et al., 2014). In several studied microorganisms, multiple IAA biosynthetic pathways are present and active in a single microorganism (Spaepen and Vanderleyden, 2011). Production of IAA also has been reported in rice endophytic bacteria such as *Pseudomonas putida* and *Pseudomonas fluorescens*, which significantly increase in root length, colonization, and promotion of rice seedling growth (Etesami et al., 2014; Etesami et al., 2015), and also several strains

of *Bradyrhizobium* sp., showing a significant increase in total rice dry weight (Piromyou et al., 2017). Tryptophan (Trp) is the main precursor for the IAA synthesis. In bacteria, at least five different pathways were defined for the IAA synthesis from tryptophan, and most pathways are similar to those IAA synthesized by plants, consisting of indole-3-acetamide (IAM), indole-3-acetonitrile (IAN), indole-3-pyruvate (IPA), tryptamine (TAM), or tryptophan side-chain oxidase (TSO) pathway, each pathway includes distinct step as illustrated in Fig. 2.2 (Dosselaere and Vanderleyden, 2001; Merino et al., 2008). IAM pathway is found in many phytopathogens such as *P. savastanoi*, and *P. syringae*, producing high IAA quantities may encourage the virulence of these strains (Jameson, 2000). Also, IAM is found in symbiotic nitrogen-fixing *Rhizobium* and *Bradyrhizobium* (Sekine et al., 1988; Sekine et al., 1989) such as *Rhizobium* sp. strain NGR234 (Theunis et al., 2004). IPA pathway may be proven in several bacteria such as plant beneficial bacteria (*Azospirillum*, *Bacillus*, *Enterobacter cloacae*, *Paenibacillus*, *Pseudomonas*, *Rhizobium*, and *Bradyrhizobium*), phytopathogens (*P. agglomerans*), and also cyanobacteria (Spaepen and Vanderleyden, 2011). TAM pathway is present in *Bacillus cereus* and *Azospirillum* sp. and, also in plants (Perley and Stowe, 1966; Hartmann et al., 1983). Similarly, IAN pathway has been suggested in both plants and bacteria such as *Agrobacterium* and *Rhizobium* spp. (Kobayashi et al., 1995). However, TSO pathway has only been reported in *P. fluorescens* CHA0 (Oberhansli et al., 1991).

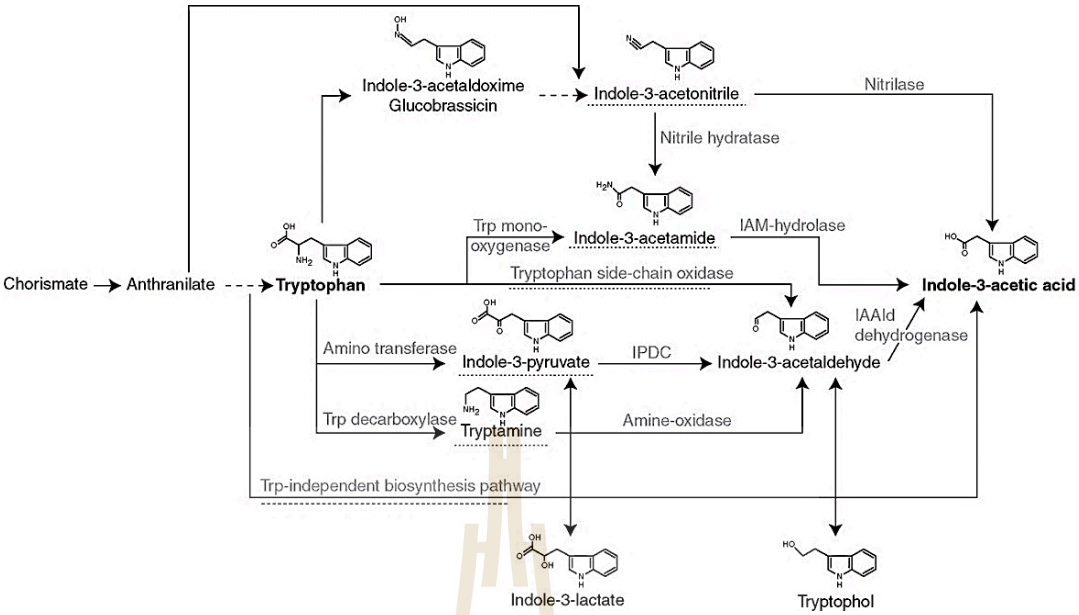

Figure 2.2 Overview of IAA biosynthetic pathways in bacteria (Spaepen and Vanderleyden, 2011).

2.4.2 1-amino-cyclopropane-1-carboxylic acid (ACC) deaminase

ACC deaminase can promote plant growth by suppression of ethylene level through enzyme hydrolyzes ACC to ammonia and α-ketobutyrate (Laslo et al., 2012), decreasing the suppressive impact of ethylene on root elongation, and hence promoting plant growth (Glick et al., 1998) (Fig. 2.3). ACC deaminase-containing bacteria treated plants promote longer roots (Belimov et al., 2001) and have a resistant to the stress effects of both biotic and abiotic ethylene on the plant such as heavy metal (Burd et al., 2000), both bacterial and fungal pathogens (Wang et al., 2000), flooding (Grichko and Glick, 2001), salt (Bal et al., 2013), and drought (Zahir et al., 2008) (Fig. 2.3). Several problems were observed in rice cultivated under constant flooding conditions such as flooding, pathogens, salinity, and drought. ACC deaminase is a beneficial trait of *P. fluorescens* strain REN1 to promote rice roots colonization and

elongation of rice seedling under flooded conditions (Etesami et al., 2014). Besides, PGP trait of endophytic *P. putida* and *P. fluorescens*, producing IAA and ACC deaminase may be required for rice seedlings in endophytic and rhizospheric association when compared to other PGP traits under constant flooding conditions (Etesami et al., 2015). In addition, ACC deaminase-producing *Bradyrhizobium* spp. have been reported in endophytic association with rice plants, which showed a significant increase in rice dry weight and also promote rice growth (Piromyou et al., 2017).

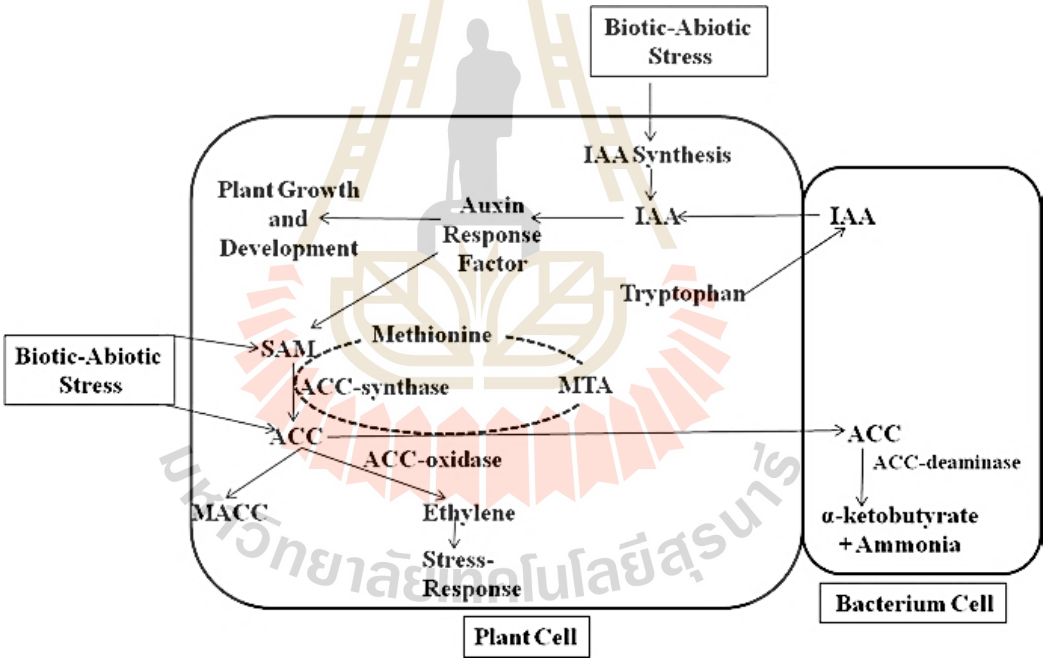

Figure 2.3 A schematic diagram of ACC-deaminase producing rhizobacteria facilitating plant growth in correlation with IAA. MACC; 1-malonyl-ACC, MTA; 5 methylthioadenosine, SAM; S-adenosyl methionine (Soni et al., 2018).

2.4.3 Biological nitrogen fixation (BNF)

BNF is performed by the nitrogenase enzyme activity of several microorganisms, converting atmospheric nitrogen into ammonia and can be used as a nitrogen source available for plant (Deacon, 1997). Rice receives a significant proportion of its nitrogen requirements from BNF. This is partly provided by free-living photosynthetic diazotrophs that live in soil and floodwater (Merrick, 2004) and heterotrophic diazotrophs bacteria in the rhizosphere (Wartiainen et al., 2008). Another significant source of fixed nitrogen is the endophytic diazotroph, which appears to live within the tissues of the plants and these endophytic diazotrophs are responsible for much of the nitrogen fixation (James et al., 2000) such as *Azoarcus* spp. (Engelhard et al., 2000), *Herbaspirillum seropedicae* (Brusamarello-Santos et al., 2012), *P. stutzeri* (Desnoues et al., 2003), *R. leguminosarum* bv. *trifolii* (Yanni et al., 1997), *Serratia marcescens* (Gyaneshwar et al., 2001), and *Bradyrhizobium* spp. (Piromyou et al., 2017). Also, *Pantoea agglomerans* has been evaluated for plant colonization and growth promotion as rice endophytic diazotrophic bacteria in deep water which can spread, persist, and fix nitrogen inside the rice tissues (Verma et al., 2001). Several endophytic diazotrophic bacteria can both inhibit and promote nitrogen fixation in rice plants. Some isolates were closely related to *Brevundimonas aurantiaca, Enterobacteriaceae, E. dissolvens*, *P. agglomerans*, and *Pseudomonas* spp. (Prakamhang et al., 2009). It is far recognized that endophytic diazotrophs are able to fix nitrogen more efficiently than that of rhizospheric diazotrophs, this may be because plants directly offer the endophytic diazotroph with their nutrient requirement and competition with other soil microbes is not necessary. In turn, bacteria provide fix nitrogen and plant growth-promoting compounds to the host plant directly.

2.5 Terminal bacteroid differentiation (TBD)

TBD has occurred especially in an inverted repeat lacking clade (IRLC) and Dalbergoid legumes clades that are characterized by cell morphological changes, polyploidy, and inactivity in reproductive. Bacteroids in this type of legume nodule, the morphology and physiology may vary among species and host legume clades. The morphological of bacteroids in legume is divided into (i) unaffected (U morphotype) is a type of small and rod-shaped like their free-living cell. In addition, they also undergo extreme morphological changes such as (ii) elongated (E morphotype) or (iii) spherical cells (S morphotype). E and S morphotypes develop in IRLC and Dalbergoid legumes, E morphotype could be performed in *Medicago*, *Pisum*, *Vicia*, *Galega*, *Astragalus*, and, in certain *Aeschynomene* species. For S morphotype can be found in *Arachis*, *Crotalaria*, and in certain *Aeschynomene*. Whereas, U morphotype are formed in other legumes, for example, in *Phaseolus*, *Glycine*, *Vigna*, *Lotus*, and *Sesbania* species (Kondorosi et al., 2013). U morphotype is considered to be the ancestral state of bacteroid and the other morphotypes have regarded later in evolution (Oono et al., 2010). Since IRLC and Dalbergoids are distantly associated legume clades, indicating the mechanisms of bacteroid cell differentiation may have independently evolved in these clades.

2.6 Plant and bacterial peptides affect the bacteriod differentiation

Two model systems of bacteroid differentiation have been mostly investigated in *Mesorhizobium loti – L. japonicus* symbiosis (non-IRLC legume) and *S. meliloti* - *M. truncatula* symbiosis (IRLC legumes). Bacteroid of *M. loti* formed U morphotype which had the same size and DNA content as free-living cells and maintained their reproductive capacity. They were capable of reverting to the free-living lifestyle. On the other hand, the formation of *S. meliloti* E morphotype in IRLC legumes is under the control of nodule-specific cysteine-rich (NCR) peptides, producing in the symbiotic nodules, which are targeted by the secretory pathway to the endosymbionts and induced polyploid bacteroid cell differentiation (Van de Velde et al., 2010) (Fig. 2.4). The polyploidy of the bacteroids, resulting from the inhibition of bacterial cell cycle, and cell division while DNA replication continues by the NCR peptides, targeting the bacterial master cell cycle regulator genes *dnaA*, *gcrA*, and *ctrA* (Penterman et al., 2014). The high DNA content of *S. meliloti* bacteroids formed E morphotype was >24-fold higher than that of cultured bacteria (Fig. 2.4) (Mergaert et al., 2006). However, BacA transporter is also required for the bacteroid cell differentiation and persistence of *S. meliloti* in the NCR-producing symbiotic cells, providing protection against this antimicrobial activity of NCR peptide in *Medicago* nodules (Haag et al., 2011).

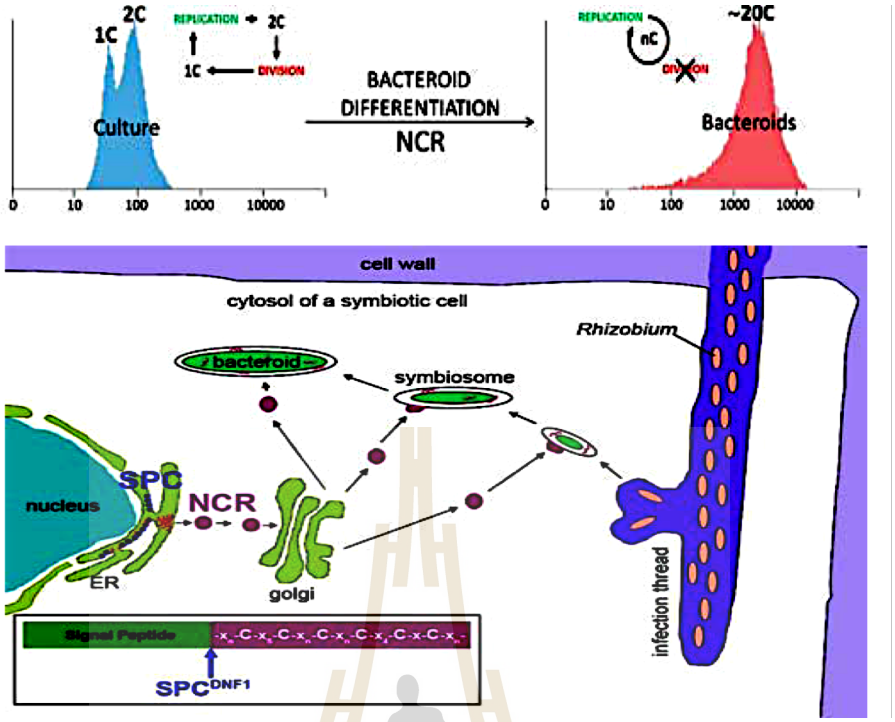

Figure 2.4 NCR-mediated differentiation and polyploidy bacteroids in IRLC legumes.

2.6.1 Nodule-specific cysteine-rich (NCR)

NCR peptide is a small peptide which almost expressed within the symbiotic root nodule, in which they manipulate cell differentiation of the symbiotic nitrogen-fixing bacteroids (Farkas et al., 2017). More than 700 NCR peptides have been coded from the genomes of *Medicago* plants (Fig. 2.5) (Fedorova et al., 2002). Also, NCR peptides of several legume plants in IRLC and Dalbergoids have been reported such as *Aeschynomene* plants (Fig. 2.5) (Czernic et al., 2015). The structure of NCR peptides similar to defensins, belonging to plant antimicrobial peptides (AMPs). NCR peptides have been demonstrated to be cationic, neutral, or anionic. However, cationic NCR peptides were reported to have a strong activity of antimicrobial peptide (Tiricz et al., 2013), interacting with bacterial cell surface electronegativity, which contributes

to cell lysis via bacterial membrane disruption leading to pore formation, and peptide transportation inside the cell (Jenssen et al., 2006). When entering the cells, AMPs can interact with multiple intracellular targets such as heat-shock protein (DnaK), DNA gyrase, ATPase, and peptidoglycan precursor lipid II, which implies an interference of nucleic acids, proteins or lipids, and inhibition of DNA, RNA, protein or cell wall synthesis (Brogden, 2005). The interference of AMPs was also reported in the cell cycle of fungal cells (Lobo et al., 2007). These demonstrated that the cationic peptides are efficient natural antimicrobials, killing several kinds of microorganisms such as bacteria and fungi rapidly (Farkas et al., 2017).

2.6.2 Defensin-like antimicrobial peptides

Most plant's antimicrobial peptides (AMPs) are small cysteine-rich peptides (CRPs) that contain antimicrobial activities. Based on disulfide bond patterns formation, AMPs can be divided into several kinds. Many plants encode genes in distinct classes of AMPs that do not appear to have homologs outside the plant kingdom, such as defensins (DEFs), thionins (THIONs), lipid transfer proteins (LTP), snakins, hevein-type peptides, and knottin-type peptide (Broekaert et al., 1997). Several uncharacterized AMPs have been suggested in rice (Silverstein et al., 2007) and genes encoding defensin like-antimicrobial peptides were also detected in rice plants, inhibiting the growth of bacterial pathogen but weakly active against photogenic fungi (Tantong et al., 2016). Defensins AMPs have activities of broad-spectrum for both anti-bacteria and fungi. Moreover, they also have diversity in antimicrobial activities, a specificity of bacterial plasma membrane target, and antibiotic resistance (Hancock and Scott, 2000). The alignment of defensin-like AMPs highlighted a consensus sequence of six cysteine residues, close to a group of NCR-like AMPs of *Aeschynomene* spp. and

Medicago spp., but spacing among the first three Cys residues is different (Fig. 2.5). In addition, two extra Cys residues (motif 2) of these NCR-like peptides were defined, leading to a typical defensin signature that similar to NCR-like AMPs of *Aeschynomene* spp. (Fig. 2.5) (Czernic et al., 2015).

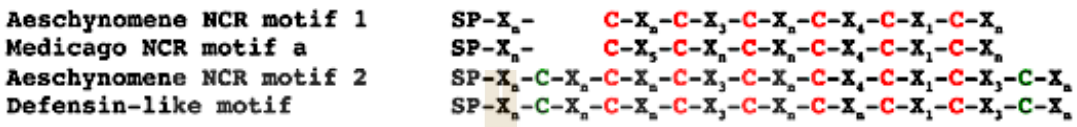

Figure 2.5 Comparison of defensin-like antimicrobial peptides (AMPs) of the Cys-rich motifs of *Aeschynomene* spp. with those of *Medicago* spp. NCR peptides. Red and green Cys residues correspond to the NCR and defensin signatures, respectively (Czernic et al., 2015).

2.6.3 Bacterial BacA protein

The ATP-binding cassette (ABC) transporter superfamily, consisting of an integral membrane protein is encoded by the *bacA* gene (known as *sbmA* in enterobacteria). This gene is conserved in many bacteria, and orthologous sequences were described not only in rhizobia but also in *E. coli* (human commensals) (Laviña et al., 1986) and pathogens such as *Brucella* spp. (Laviña et al., 1986) and *Mycobacterium* (LeVier et al., 2000). The BacA proteins seem to be essential during pathogenic and symbiotic interactions between bacteria and eukaryotic partners that produce antimicrobial peptides (AMPs). BacA is critical to provide protection against NCR AMPs, which promote membrane permeability and bacterial killing by reducing the amount of NCR AMPs in legume symbiosis (Haag et al., 2011). Besides, BacA may also be necessary for membrane integrity, that critically important during bacterial

differentiation within plant cells at the early stages (Ichige and Walker, 1997). A model of BacA was predicted base on the Klein predictions, BacA is integral to the membrane. The upstream of *TnphoA* (transposon that causes alkaline phosphatase expression) is the insertion site of the first segment in the periplasmic space, which is the places that cytoplasmic alkaline phosphatase is inactive. This segment possibly serves to anchor the fusion protein within the membrane in an orientation that the alkaline phosphatase is located (Fig. 2.6) (Glazebrook et al., 1993).

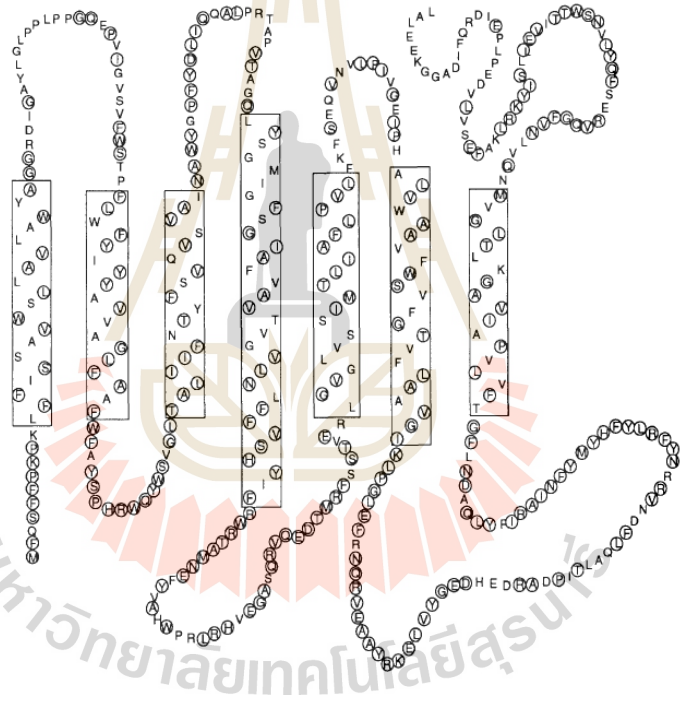

Figure 2.6 BacA in the inner membrane base on a model of the topology. Drawn at lower left is the amino terminus, and upper right is the carboxyl terminus. Based on Klein predictions, membrane-spanning segments are indicated in boxed regions. Periplasmic are proposed to be segments drawn above the boxed regions, and cytoplasmic are below. BacA are identical in residues that are circled (Glazebrook et al., 1993).

2.6.4 Bradyrhizobial BacA-like transporter (BclA)

BacA-like transporter has been identified in bradyrhizobia, the genomes of analyzed *Bradyrhizobium* spp. ORS285, carrying three genes (BRAO285v1_250005, BRAO285v1_950010, and BRAO285v1_1320006) encoding proteins homologous BacA of *S. meliloti*, ORS285 can nodulate several *Aeschynomene* spp. performing either S- or E-morphotype bacteroids (Bonaldi et al., 2010). These genes were identified by the transmembrane domain of ABC_membrane_2 or SbmA_BacA (Fig. 2.7). However, Only BRAO285v1_1320006 plays an important role in symbiotic phenotype on the host plants including *A. indica* and *A. afraspera*. It produced abnormal nodules size, form, color, and reduced nitrogen fixation activity. These nodules could not promote plant growth under nitrogen limiting conditions, showing leaves chlorosis and reduced in shoot mass. The other two genes formed normal nodules and nitrogen fixation, indicating that these genes are not important for ORS285 in *Aeschynomene* spp symbiosis. These indicated that BacA-like proteins in ORS285 were encoded by BRAO285v1_1320006. However, proteins of *Bradyrhizobium* sp. differ from proteins BacA and SbmA homologs of *E. coli* by canonical ABC transporters containing a C-terminal cytosolic ATPase domain. Thus, *Bradyrhizobium* bacA-like (BclA) was named (Fig. 2.7) (Guefrachi et al., 2015).

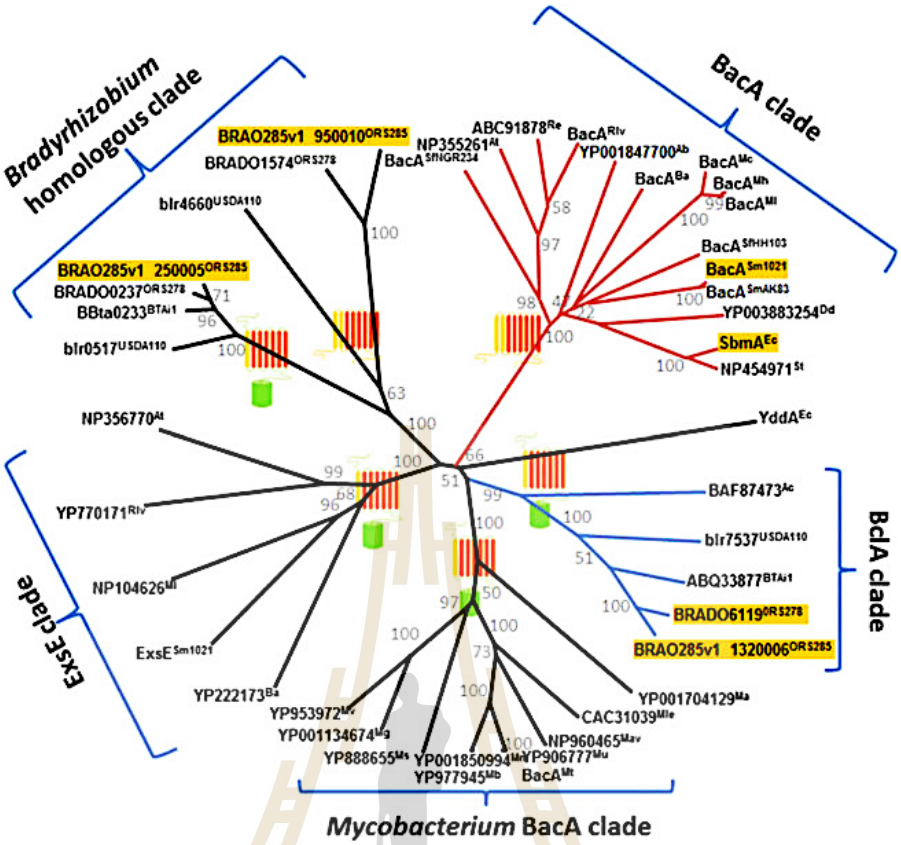

Figure 2.7 Phylogenetic tree of SbmA_BacA domain proteins. BclA clade and *Bradyrhizobium* homologous clade are separated in *Bradyrhizobium* sp. when compared to *Rhizobium* sp. and other bacteria in BacA clade and ExsE clade, respectively. In some branche, the ATPase domain (green) is only present. The ATPase domain is lacking in proteins of the BacA clade while the ATPase domain of BclA clade is present for ABC transporters (Guefrachi et al., 2015).

2.7 Bacteroid cell differentiation and nitrogen fixation in leguminous root nodules

The correlation between bacteroid cell differentiation size and nitrogen fixation efficiency has been reported in alfalfa (*M. sativa* var. Buffalo) nodules, the morphology, nitrogen fixation capability, and nucleic acid content were investigated (Paau et al., 1978). In the nodule tip regions, small bacteroids (1 to 2.5 μmin length) had low nitrogen fixation efficiency (4.9 μmol C_2H_2 reduced/10^{10} bacteroids), and nucleic acid content. In contrast, bacteroids were greatly enlarged (5 to 7 μmin length) in the middle regions also had high nitrogen fixation efficiency (83.3 μmol C_2H_2 reduced/10^{10} bacteroids)and nucleic acid content. In addition, Vasse et al. (1990) have also studied for the correlation of nodule development, bacteroid differentiation, and acetylene reduction activity, showing that nitrogenase activity is associated with the differentiation of bacteroid cell, especially in the distal fixation zone of the nodule.

2.8 Bradyrhizobial cell enlarge size and nitrogen fixation in rice tissues

Bradyrhizobium sp. SUTN9-2 was observed by scanning electron microscope (SEM). The results revealed the enlarged cell size of SUTN9-2 at 7 days post inoculation (dpi) (~ 2.5-3 μm) when compared with 3 dpi (~2 μm) (Piromyou et al., 2017) (Fig. 2.8). Also, the expression of *nifH* gene was detected in rice leaf sheath and root inoculated with SUTN9-2 at 14, 28, 70, 84 dpi in response to N-free solution compared to uninoculated control (Piromyou et al., 2017). These results suggested the possibility that cell differentiation and nitrogen fixation by SUTN9-2 were performed in rice plants, which similar to bacteroid in legume plants.

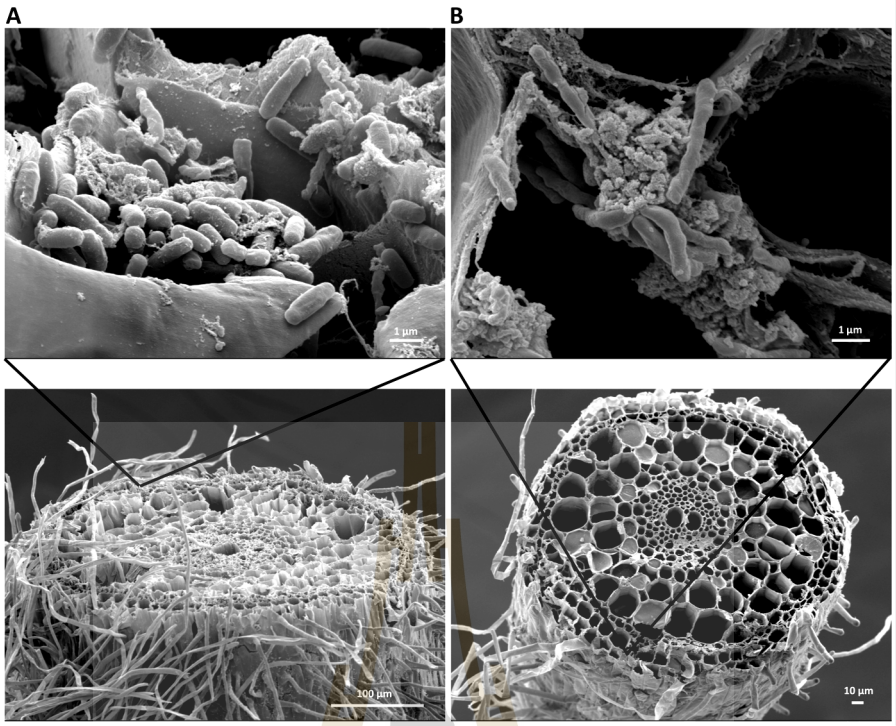

Figure 2.8 Scanning electron microscope (SEM) images of SUTN9-2 inside rice roots at 3 days (A) and 7 days (enlarged cell size) (B). The magnification (the enlargement of an image) is the same in panels A and B (bar = 1 µm) (Piromyou et al., 2017).

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CHAPTER III

Empowering rice seedling growth by endophytic *Bradyrhizobium* sp. SUTN9-2

3.1 Introduction

Bradyrhizobia have been reported to establish the endophytic diazotrophs with rice and also enhance rice growth (Piromyou et al., 2017). Some endophytic bacteria can facilitate plant growth via production of ACC deaminase by decreasing plant ethylene levels (Glick, 2014) and IAA by increasing plant cell division and elongation (Tivendale et al., 2014). *Bradyrhizobium* sp. SUTN9-2 has been recently reported to show the ability of IAA and ACC deaminase productions, and nitrogen fixation in their pure culture and rice endophytic association at 30 days after inoculation (dai) (Piromyou et al., 2017). Therefore, these characteristics of SUTN9-2 may affect rice growth promotion. However, the effect of SUTN9-2 on rice growth promotion at different stages have not been elucidated, especially the quantitative analysis and the expression of gene involved in rice growth promotion traits of SUTN9-2 in rice tissues. The present study was aimed at assessing the ability of SUTN9-2 for production of IAA and ACC deaminase, and nitrogen fixation in rice under N-free and 1 mmol l^{-1} NH_4NO_3 supplementation at different stages of rice growth. In addition, the expressions of genes were determined in rice tissues inoculated with SUTN9-2, using genes-specific primers designed for SUTN9-2. Those of observed genes involved in (1) IAA production;

nitrilase gene (*nit*) which converts indole-3-acetamide (IAM) to IAA, (2) ACC deaminase production; 1-aminocyclopropane-1-carboxylate deaminase gene (*acdS*) which reduces the ethylene concentration in plant, and (3) nitrogen fixation; dinitrogenase reductase gene (*nifH*) which encodes nitrogenase structural component and homocitrate synthase gene (*nifV*) which encodes a necessary component of the FeMo cofactor synthesis and activation of the nitrogenase Fe protein in free-living diazotrophs (Howard and Rees, 1994). The potential of SUTN9-2 for enhancing rice growth promotion at early rice seedling growth stage was also investigated prior to applying in the system of rice intensification (SRI) for which healthy rice seedling is necessary (Dobermann, 2004).

3.2 Objective

To examine the potential for plant growth promoting (PGP) traits by SUTN9-2 on rice growth promotion at different stages under N-free and NH_4NO_3 supplementation

3.2.1 Specific objective

3.2.1.1 To examine the efficiency of SUTN9-2 on the promotion of rice dry weight and chlorophyll content

3.2.1.2 To determine the ability of SUTN9-2 on the production of IAA and ACC deaminase in rice plants

3.2.1.3 To determine the expressions of genes involved in IAA, ACC deaminase production, and nitrogen fixation in rice tissues inoculated with SUTN9-2

3.3 Materials and methods

3.3.1 Plant and bacterial strain

Rice (*Oryza sativa* L. var. *indica* cv. Pathum Thani 1) was used in this study, and seeds were obtained from Lopburi Rice Seed Center, Thailand. The *Bradyrhizobium* sp. (LAXE00000000) strain SUTN9-2 was isolated from root and stem nodules of *Aeschynomene americana* grown in rice field areas in Thailand (Noisangiam et al., 2012). SUTN9-2 was grown in yeast extract-mannitol (YEM) broth medium (Somasegaran and Hoben, 1994) at 30 ± 2°C. The bacterial cells of exponential-phase were cultured for 4 days and measured at 600 nm by spectrophotometer (Spectronic 200, Thermo Scientific, USA) in order to adjust it to optical density of 10^8 CFU ml^{-1}, and used for inoculation in rice and other analyses (Piromyou et al., 2017).

3.3.2 Inoculation of SUTN9-2 in rice

The rice seeds were dehulled and then surface sterilized with 70% ethanol for 3 min, 10% hydrogen peroxide with 10 min for twice, 3% sodium hypochlorite for 1 h and washed 3 times with sterilized water. To obtain a small emerging root, surface-disinfected rice seeds were germinated on 0.85% agar of YEM medium for one day. Then, germinated seeds were soaked overnight in YEM broth containing SUTN9-2 (10^8 CFU ml^{-1}), and then three geminated seeds were transplanted into the glass test tubes (22 x 200 mm) containing sterilized aluminum net and N-free rice nutrient solution [(mmol l^{-1}): $NaH_2PO_4 \cdot 2H_2O$, 0.6; K_2SO_4, 0.3; $CaCl_2 \cdot 2H_2O$, 0.3; $MgCl_2 \cdot 6H_2O$, 0.6; EDTA-Fe, 0.045; H_3BO_3, 0.05; $MnSO_4 \cdot 5H_2O$, 0.009; $CuSO_4 \cdot 5H_2O$, 0.0003; $ZnSO_4 \cdot 7H_2O$, 0.0007; $Na_2MoO_4 \cdot 2H_2O$, 0.0001] and pH adjusted to 6.8 (Mae and Ohira, 1981). The nitrogen source of 1 mmol l^{-1} NH_4NO_3 was used by supplementing into the N-free solution. Rice was grown under controlled

environmental condition of 28 ± 2°C, 70% relative humidity on 16/8-h day/night cycle (full light, 639 microeinsteins [µE] · m^{-2} · S^{-1}). SUTN9-2 was tested in terms of bacterial colonization inside rice tissues (10^4 - 10^6 MPN g^{-1} inoculant g^{-1} rice fresh weight), and also showed rice growth promotion without causing pathological stress on them (Piromyou et al., 2017).

3.3.3 Rice dry weight and chlorophyll content

SUTN9-2 was inoculated onto rice and grown as described above. The rice shoots were harvested at 7, 14, 21 and 28 dai. The shoot samples were dried in an oven for 72 h at 65°C, and then dry weight was determined. The rice shoots from inoculated and non-inoculated conditions were harvested as described above. Then, three replicates (three plants replication^{-1}) of rice shoot samples were freeze-dried by lyophilizer (BETA 2-8 LD, Laurel USA). The dried shoot samples were macerated in a sterilized mortar and pestle with liquid nitrogen. Deionized (DI) water 100 ml was added into the test tubes containing shoot samples and the tubes were shaken for 10 min. Then, one ml of 95% ethanol was added into the shoot samples for 5 min. The samples were shaken and incubated in the dark place for overnight. The supernatant was collected by centrifugation at 13,000 x g for one min. The concentrations of chlorophyll a, chlorophyll b and carotenoid were measured by spectrophotometer at A648.6, A664.2 and A470, respectively. The concentrations of chlorophyll a, b and carotenoid were calculated and recorded together as a total chlorophyll content (Lichtenthaler, 1987).

3.3.4 Detection of the IAA, ACC deaminase and ethylene in rice

The rice samples (root + shoot) were harvested, sterilized with 70% ethanol for 1 min, 3% sodium hypochlorite for 3 min, and washed 3 times with

sterilized water. The samples were macerated separately with a sterilized mortar and pestle with liquid nitrogen. Then, the supernatant was collected by centrifugation at 10,000 x *g* for five min. The IAA production was observed as described by Costacurta et al. (1998). Pure IAA was used as a standard. The 50 mmol l^{-1} ACC was added into the supernatant of macerated rice samples and incubated at 30°C for 1 h then ACC deaminase activity was determined as described by Tittabutr et al. (2008). The test tubes (22 x 200 mm) containing rice plants (three plants test $tube^{-1}$) at 14 dai were tightly closed with a rubber septum and incubated for 5 days at 25˚C under light condition (639 $\mu E \cdot m^{-2} \cdot S^{-1}$). Accumulated ethylene gas (one ml) from headspace of the test tubes was injected into a gas chromatograph [6' x 1/8" S.S. Hayesep T column (Valco instruments Co. Inc.)]. Accumulated ethylene gas was calculated and compared with a standard curve of pure ethylene gas (Fukao et al., 2006).

3.3.5 Total RNA extraction and qRT-PCR analysis

The rice samples (shoot + root) were harvested, sterilized and macerated as described above. Total RNAs were directly isolated from plant samples using RNeasy Plant Mini Kit (QIAGEN, USA). RNAs were treated with the DNaseI to prevent contamination of genomic DNA, and then converted to cDNA using iScript™ cDNA Synthesis (BIO-RAD). Quantification of cDNA was carried out using the PowerUp SYBR Green Master Mix (Thermo Fisher Scientific). The *dnaK* was used as calibrator gene, and the results were analyzed (Dheda et al., 2004). The transcription levels were determined by qRT–PCR using Applied Biosystem, QuantStudio Design. Primers for amplification (*dnaK*, *acdS*, *nit, nifH* and *nifV*) are listed in Table 3.1. The PCR amplification was performed under the cycling condition as follows; an initial denaturation step at 95°C for two min; 35 cycles (two min at 95°C, 30 sec at the

annealing temperature of *dnaK, acdS* and *nit* (53°C), *nifH* and *nifV* (48 °C) followed by a final 5 min extension at 72°C. The relative gene expression was analyzed by comparative Ct method (-ΔΔCT) that was normalized to the endogenous housekeeping gene (*dnaK*). Three biological replicates were pooled and analyzed.

3.3.6 The statistical analysis

Statistical analysis of data sets was performed with the SPSS software (SPSS 16.0 for Windows; SPSS Inc., Chicago, IL) on data from three independent biological samples (each with three technical replicates) in each stage of rice growth. The experimental data were statistically analyzed according to Steel et al. (1980), and means were compared by Duncan's multiple range test ($P \leq 0.05$) (Duncan, 1955).

3.4 Results

3.4.1 Early stage rice growth promotion by SUTN9-2

Bradyrhizobium sp. strain SUTN9-2 has previously been confirmed as an endophyte in rice, and rice biomass could be enhanced when supplementing SUTN9-2 inoculation with N-free, KNO_3, NH_4NO_3, or urea (Piromyou et al., 2017). This result showed that SUTN9-2 obviously increased the rice dry weight at the early stage (7 dai) under 1 mmol l^{-1} NH_4NO_3 (21.90 mg plant dry weight) and 14 dai under N-free (34.33 mg plant dry weight). The highest rice dry weight under N-free and 1 mmol l^{-1} NH_4NO_3 was found at 28 dai and was also significantly higher than that of 21, 14 and 7 dai, respectively (Fig. 3.1A and B). The chlorophyll contents at 7 and 14 dai (6.65 and 3.69 chlorophyll mg g^{-1} plant fresh weight) under N-free of inoculated rice were significantly higher than that of non-inoculated rice (5.33 and 2.60 chlorophyll mg g^{-1} plant fresh weight) (Fig. 3.1C). However, chlorophyll content in N-free was significantly lowered

after 7 days in both inoculated and non-inoculated rice.

3.4.2 *In vivo* rice growth promoting properties by SUTN9-2

This experiment was explored the possibility of rice growth promotion characteristics derived from SUTN9-2. The previous study has shown that SUTN9-2 produced a small amount of IAA (0.75 IAA mg mg^{-1} protein h^{-1}) in the presence of L- tryptophan 100 µg ml^{-1} and ACC deaminase (3.48 µmol α-ketobutyrate mg^{-1} protein h^{-1}) under the free living stage (10^8 CFU ml^{-1}) (Piromyou et al., 2017). This study revealed that at 7 dai, SUTN9-2 showed that the level of IAA (18.93 IAA mg g^{-1} plant dry weight) andACC deaminase (0.0031 µmol α-ketobutyrate g^{-1} plant dry weight) under 1 mmol l^{-1} NH_4NO_3 was significantly higher than that of non-inoculated rice (10.76 IAA mg g^{-1} plant dry weight and 0.0019 µmol α-ketobutyrate g^{-1} plant dry weight) (Fig. 3.2). Therefore, it was not clear which factor contributed the most to rice growth promotion. However, there was no effect on rice growth from SUTN9-2 was found in other growth stages except at 21 dai of ACC deaminase activity under 1 mmol l^{-1} NH_4NO_3. In addition, ACC deaminase levels at 14 and 21 dai were significantly higher than that of 7 dai. The stress ethylene emission was also measured at 14 dai. The amount of ethylene from rice inoculated with SUTN9-2 (31.14 pmol ethylene g^{-1} plant fresh weight h^{-1}) was lower than that of non-inoculated rice (41.23 pmol ethylene g^{-1} plant fresh weight h^{-1}) (personal communication). This implied the effect of ACC deaminase of SUTN9-2 on ethylene suppression in rice. In contrast, IAA levels in inoculated rice at 14, 21 and 28 dai were significantly lower than that of 7 dai.

Table 3.1 Primers used in this study.

Target gene	Primer name	Gene description	Primer sequence (5'➔3')	Description of design and reference
Housekeeping				
dnaK	dnaK F	70 kDa chaperone protein	GAAGTGCTGCGCATCATCAA	Designed from *dnaK* of SUTN9-2(LAXE00000000)
	dnaK R		TCCTTCTGGAATTCGTCGGC	
IAA production				
nit	nit F	Nitrilase	CTCGCTCTACGATCCCTTCG	Designed from *nit* of SUTN9-2 (LAXE00000000)
	nit R		CTTCTCCCCGATCTGACTGC	
ACC deaminase				
acdS	acdS F	ACC deaminase	ACTACATCGTCGTCTGCACC	Designed from *acdS* of SUTN9-2 (LAXE00000000)
	acdS R		ATAGGCGGGATAGGCGTAGT	
Nitrogen fixation				
nifH	nifH F	Dinitrogenase reductase	TAACATCTCCAAGGGCATCC	Designed from *nifH* of SUTN9-2 (LAXE00000000)
	nifH R		CCGCCATTATTGTGAACCTT	

Cont.

Table 3.1 Primers used in this study.

Target gene	Primer name	Gene description	Primer sequence (5'➔3')	Description of design and reference
nifV	nifV F	Homocitrate synthase	CGTGCGATTCCTATGAACAA	Designed from *nifV* of SUTN9-2 (LAXE00000000)
	nifV R		GATGGCATATTTGCGGACTT	

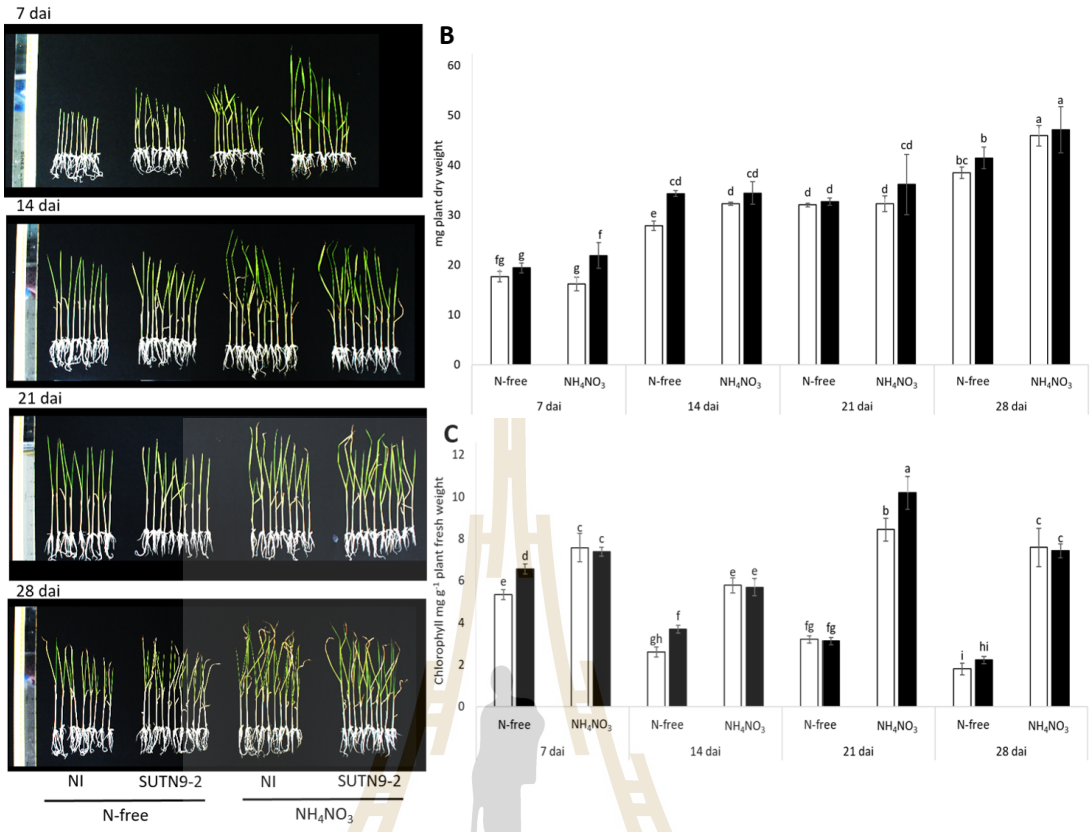

Figure 3.1 Rice growth phenotype (A), dry weight (B) and total chlorophyll (C) of rice inoculated with ■ SUTN9-2 and □ non-inoculation (NI) in N-free and 1 mmol l^{-1} NH_4NO_3 at 7 dai, 14 dai, 21 dai and 28 dai. Significant at $P \leq 0.05$ is indicated by mean standard error bar (n=3).

3.4.3 Expression of *nit* and *acdS* genes

The relative expression levels of *nit* and *acdS* genes were measured in rice inoculated with SUTN9-2. The inoculated rice under N-free demonstrated the higher *nit* and *acdS* expression levels at 7 dai (34.75 and 34.30 folds) and 14 dai (35.77 and 34.12 folds) than under 1 mmol l^{-1} NH_4NO_3 (Fig. 3.3). However, the expression levels at 21 dai were not significantly different between 1 mmol l^{-1} NH_4NO_3 and N-free (Fig. 3.3). Conversely, at 28 dai when nitrogen was consumed, the *nit* and *acdS*

expression levels of inoculated rice with 1 mmol l^{-1} NH_4NO_3 (23.55 and 21.89 folds) were significantly higher than those of N-free (11.40 and 11.36 folds) (Fig. 3.3). In addition, *nit* and *acdS* expression levels of inoculated rice with N-free were significantly decreased at 21 and 28 dai. In contrast with 1 mmol l^{-1} NH_4NO_3, the expression levels were significantly increased at 14, 21 and 28 dai, while detection of bacterial DNA in non-inoculated rice was close to zero.

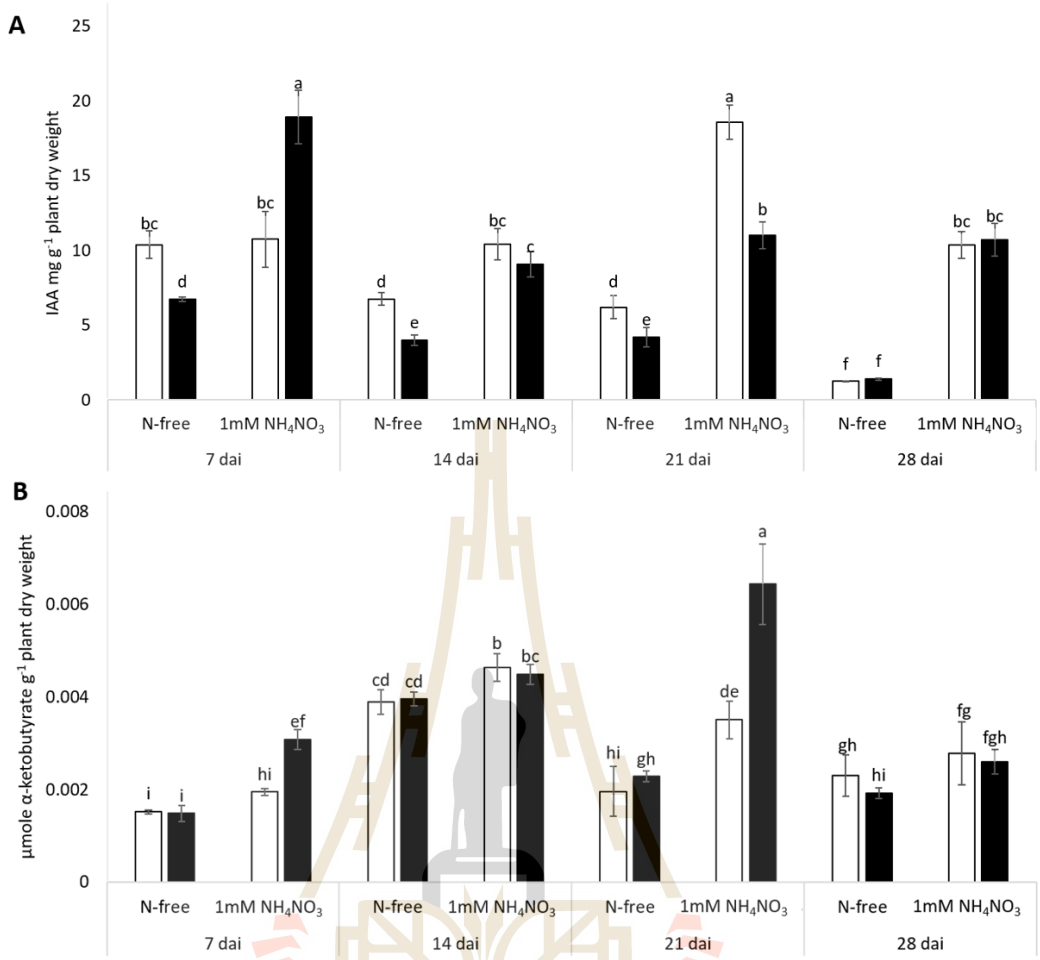

Figure 3.2 IAA content and ACC deaminase activity in rice inoculated with ■ SUTN9-2 and □ non-inoculation (NI) in N-free and 1 mmol l^{-1} NH_4NO_3 at 7, 14, 21 and 28 dai. Significant at $P \leq 0.05$ is indicated by mean standard error bar (n=3).

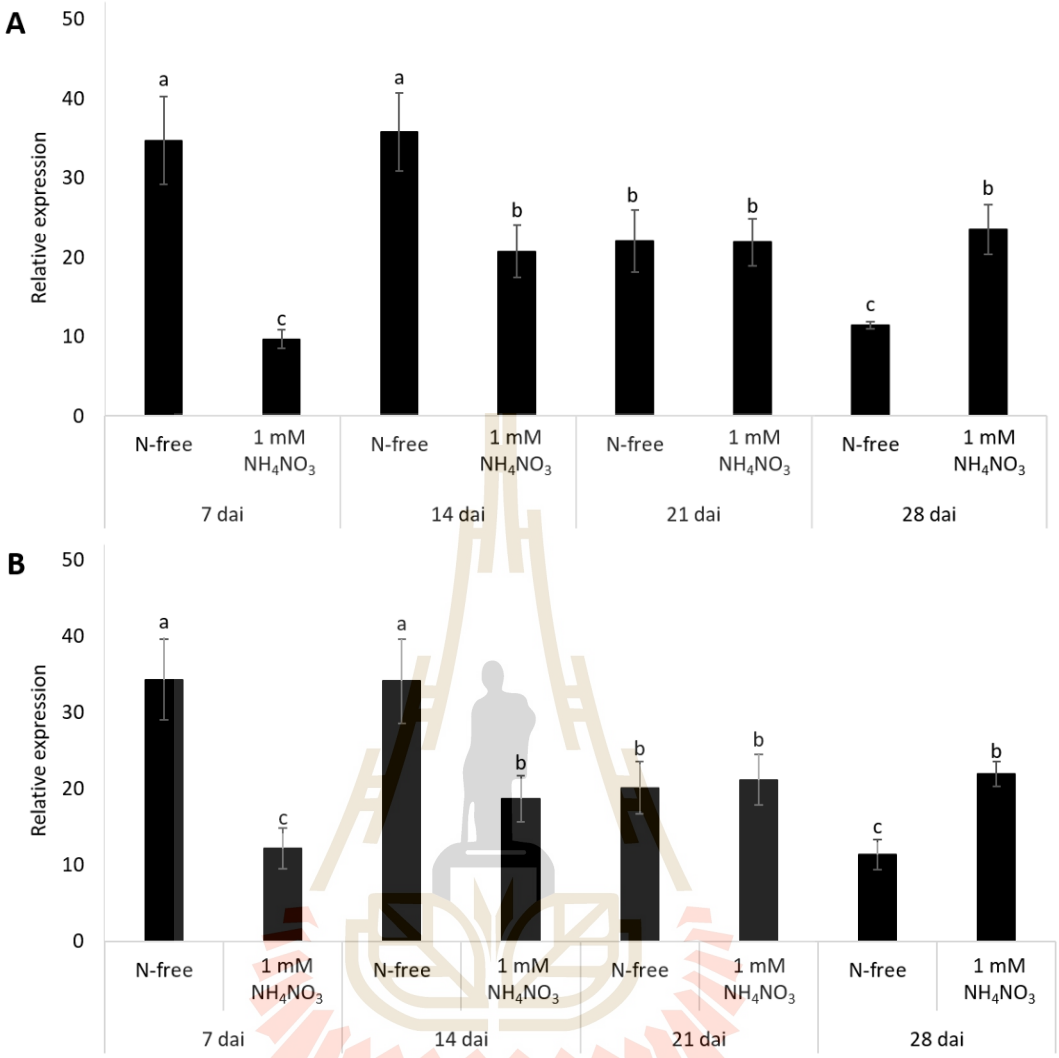

Figure 3.3 *In vivo* expression of bradyrhizobial *nit* (A) and *acdS* genes (B) in rice inoculated with SUTN9-2 in N-free and 1 mmol l^{-1} NH_4NO_3 at 7, 14, 21 and 28 dai. Significant at $P \leq 0.05$ is indicated by mean standard error bar (n=3).

3.4.4 Expression of *nifH* and *nifV* genes

The *nifH* and *nifV* expression levels in rice when treated with N-free at 7 dai (*nifH*, 38.35 and *nifV*, 27.88 folds) and 14 dai (*nifH*, 35.72 and *nifV*, 37.84 folds) were significantly higher than that in 1 mmol l^{-1} NH_4NO_3 at 7 dai (*nifH*, 4.28 and *nifV*, 7.61 folds) and 14 dai (*nifH*, 20.49 and *nifV*, 20.00 folds) (Fig. 3.4). However, the expression levels of *nifH* and *nifV* at 21 dai were not significantly different between 1 mmol l^{-1} NH_4NO_3 and N-free. In contrast, *nifH* and *nifV* expression levels at 28 dai in N-free condition (12.76 and 6.11 fold) were significantly lower than that in 1 mmol l^{-1} NH_4NO_3 (21.75 and 14.95 folds) (Fig. 3.4). In addition, *nifH* and *nifV* expression levels of inoculated rice with N-free were significantly decreased at 21 and 28 dai. In contrast with 1 mmol l^{-1} NH_4NO_3, the expression levels were significantly increased at 14, 21 and 28 dai. This suggested that rice growth promotion may partially be supported by NH_4NO_3 together with nitrogen fixation by SUTN9-2.

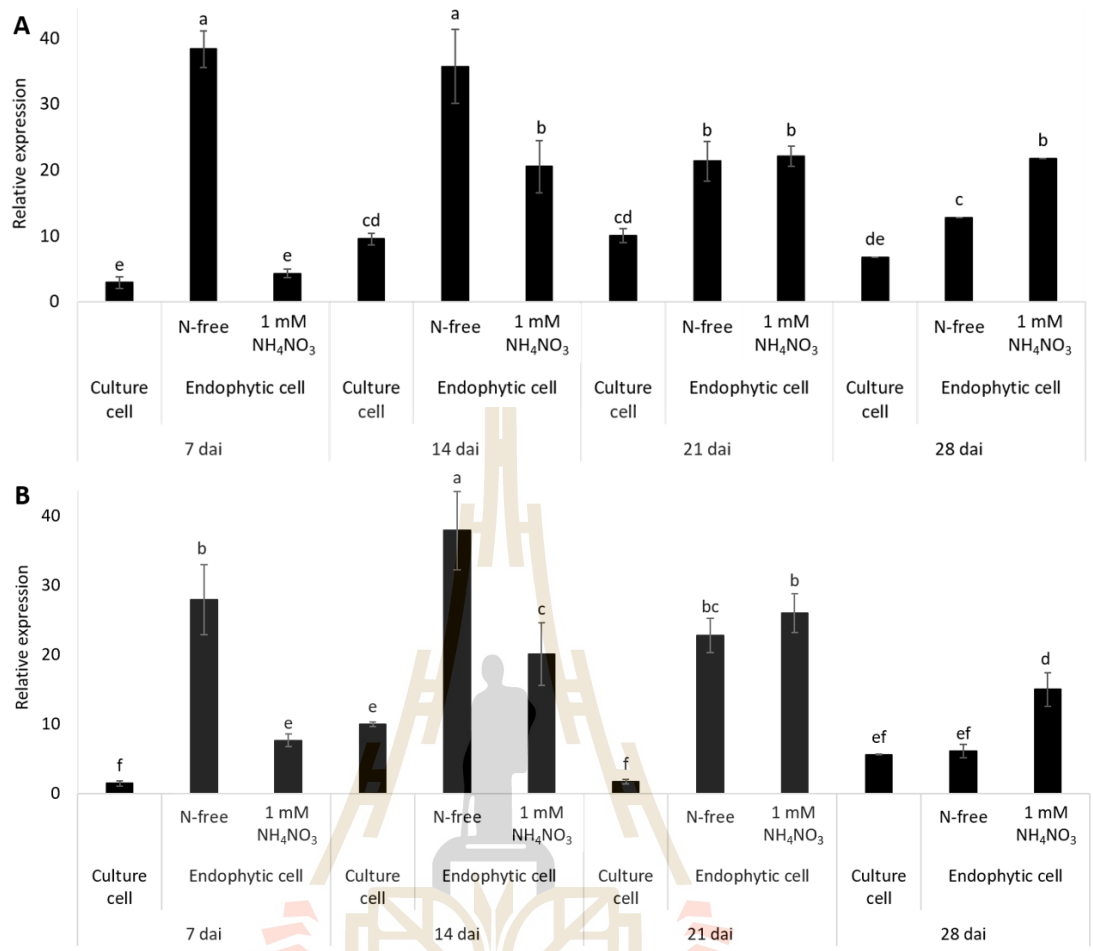

Figure 3.4 *In vivo* expression of bradyrhizobial *nifH* (A) and *nifV* genes (B) in both culture cell and endophytic cell SUTN9-2 inoculate rice with N-free and 1 mmol l^{-1} NH_4NO_3 at 7, 14, 21 and 28 dai. Significant at $P \leq 0.05$ is indicated by mean standard error bar (n=3).

3.5 Discussion

Rice plants responded to N-deficiency by decreasing light-harvesting capacity and by increasing thermal dissipation of absorbed energy (Huang et al., 2004). Das et al. (2007) reported that some sorghum rhizobacteria increased root, shoot-weight and biomass on early stage of 15 days-old sorghum seedlings which was contributed by

IAA production. However, SUTN9-2 might not be beneficial for rice growth at the late stages (21 and 28 dai) as there was no significant difference in dry weight between inoculated and non-inoculated rice (Fig. 3.1B). Root growth promotion might be helpful for quick establishment of seedlings and affects plant growth at early stage (Das et al., 2007). Therefore, to understand the plant growth promotion characteristics of SUTN9-2 in rice, IAA which affects to the root development and photosynthesis improvement and ACC deaminase which reduces the extent of ethylene growth inhibition and promotes root elongation were determined.

The population of SUTN9-2 was found in large amount at 7 dai in rice tissues [10^5 – 10^6 plant most probable number count (MPN) g^{-1} of inoculant g^{-1} rice fresh weight] and slightly decreased after 14 dai (Piromyou et al., 2017), these results implied that the population density may affect the detection of IAA and ACC deaminase in rice. In addition, other bacterial flora in rice tissues may also affect IAA and ACC deaminase levels in rice (Shahzad et al., 2017). Main function of IAA is to improve root development below the soil and improve photosynthesis mechanism, biosynthesis of various kinds of pigment above the soil (Tivendale et al., 2014). Meanwhile, ACC deaminase has been shown to increase root elongation, seedling survival, and stress tolerance by ethylene suppression, which inhibits root elongation (Glick, 2014). Therefore, the IAA and ACC deaminase from SUTN9-2 might be helpful for increasing dry weight, chlorophyll content, and rice growth especially, at seedling stage.

However, the *nit* and *acdS* expressions were inconsistent with the results of quantitative analysis of IAA and ACC deaminase produced in rice. IAA biosynthesis in bacteria can occur via multiple pathways as observed in plants such as indole-3-acetamide (IAM), indole-3-pyruvate (IPA), indole-3-acetonitrile (IAN), tryptamine

(TAM), or tryptophan side-chain oxidase (TSO) pathway (Spaepen and Vanderleyden, 2011). In this study, nitrilase gene (*nit*) which converts IAM to IAA was detected. However, in several *Rhizobium* spp., nitrile hydratase and amidase activity could be measured, suggesting the conversion of IAN to IAA via IAM (Kobayashi et al., 1995). In addition, IAM pathway was identified in the *Bradyrhizobium* spp. (Yang et al., 2018). These results suggested that IAA level in rice affected by SUTN9-2 might also be due to some other pathways. Moreover, the expression of IAA gene was also identified from rice (*O. sativa*) (Jain et al., 2006). Therefore, IAA can act as a reciprocal signaling molecule in plant-microbe interactions (Spaepen and Vanderleyden, 2011). It was also suggested that IAA is synthesized by plant growth promoting rhizobacteria (PGPR) and taken up by the plant which can stimulate the activity of the enzyme ACC synthase to convert S-adenosyl-L-methionine (SAM) to ACC (Glick, 2014). ACC may be exuded from plant roots or seeds, taken up by the bacterium and hydrolyzed by the enzyme ACC deaminase to α-ketobutyrate and ammonia which was used as a sole nitrogen source (Glick, 2014). Jaemsaeng et al. (2018) reported that endophytic *Streptomyces* sp. GMKU 336 enhances growth of rice (*O. sativa* L. cv. KDML105) by reduction of ethylene via the action of ACC deaminase. In addition, *Azospirillum lipoferum* strain AZm5 containing the gene *acdS* was reported to improve early growth of tomato seedlings under nitrogen deficiency by production of ACC deaminase, which favored leaf expansion and higher leaf N investment. (Esquivel-Cote et al., 2010). These may support the effect of ACC from rice and nitrogen source on the expression of *acdS* gene.

Bradyrhizobia expressed the *nifH* gene not only in the root nodules but also in sweet potatoes as diazotrophic endophytes (Terakado-Tonooka et al., 2008). In

addition, the *nifH* gene expression of SUTN9-2 in rice was induced under the treatment without nitrogen source (Piromyou et al., 2017). Thus, the nitrogen fixed by SUTN9-2 might be suspected as one of the factors involved in rice growth promotion at the early seedling stage. In addition, the expression trends of *nifH* and *nifV* were also similar to the expression of *nit* and *acdS* genes. A previous study showed an increase in nitrogen fixation associated with the bacterial IAA overproduction in endophyte–rice associations. Rice plants inoculated with IAA overproduction strain showed a significant upregulation of the *nifH* gene when rice infected with the wild-type strain was used as reference (Defez et al., 2017). Therefore, these results revealed that the interaction of plant growth promoting characteristics of endophytic bradyrhizobia (IAA production, ACC deaminase activity and nitrogen fixation) affected rice growth promotion. However, the contribution of nitrogen via *nifH* and *nifV* gene function of SUTN9-2 in rice plant will be further determined by gene deletion approach.

The results demonstrated the potential of using SUTN9-2 for empowering rice seedling growth promotion in the SRI system, an agro-ecological methodology that uses younger seedlings (8–15 days old) singly spaced, aiming at increasing the productivity of irrigated rice (Dobermann, 2004) and in the system of rice-legume crop rotation as well.

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CHAPTER IV

Mechanisms of rice endophytic bradyrhizobial cell differentiation and its role on nitrogen fixation

4.1 Introduction

Bradyrhizobium spp. can live in symbiotic and endophytic associations with legumes and non-legumes such as rice, namely *Oryza breviligulata* (Chaintreuil et al., 2000), *O. sativa* L. ssp. *indica* and *japonica* (Piromyou et al., 2015a). *Bradyrhizobium* sp. strain SUTN9-2 has been reported to capable of symbiotic and endophytic associations with legume plants and rice plants (Piromyou et al., 2017; Greetatorn et al., 2019). The biological nitrogen fixation (BNF) by endophytic bradyrhizobia in rice may be caused by the activity of nitrogenase enzyme, encoded by the gene *nifH* (the nitrogenase structural component). Another gene, *nifV*, is involved in the biosynthesis of the homocitrate synthase, to activate the nitrogenase Fe protein in free-living diazotrophs (Howard and Rees, 1994) and in *Bradyrhizobium* sp. (Pagan et al., 1975). Moreover, the *nifV* gene was found in *Bradyrhizobium* sp. SUTN9-2 (Noisangiam et al., 2012; Hashimoto et al., 2019). This gene is mostly not present in *Rhizobium* sp. that performs efficient nitrogen fixation only in symbiosis with legumes (Hakoyama et al., 2009). This result makes it a potential candidate for use as a biofertilizer or bioinoculant. However, no information is available for rice endophytic molecular mechanisms that play an important role in plant colonization and growth promotion.

Recently, SUTN9-2 was observed with elongated cells in rice tissues at 7 days post-inoculation (dpi) by scanning electron microscopy (SEM). Interestingly, elongated cells were observed in rice root tissues when compared between 3 (≅ 1-2 micrometer (μm)) and 7 (≅ 3 μm) dpi (Piromyou et al., 2017). This result suggested the hypothesis that the interaction between rice plant and bacterial factors may be involved in cell size enlargement and increase in nitrogen fixation efficiency by SUTN9-2 in rice plants, similar to bacteroid differentiation in the legume plants. Endophytic *Burkholderia kururiensis* M130 has been investigated in response to rice macerate using transcriptomic analysis. The results revealed 27.7% differentially expressed genes (DEGs) of its open reading frames in the presence of the rice macerate. These genes involved in membrane transporters and secretion systems, motility, chemotaxis, and adhesion, indicating the exchange of molecules is an important aspect of bacterial endophytic growth and adaptation in rice plants (Coutinho et al., 2015).

A terminal bacteroid differentiation (TBD) of *Rhizobium* and *Bradyrhizobium* spp. has been extensively studied, especially in an Inverted Repeat-Lacking Clade (IRLC) producing elongated polyploid bacterial cells that switched their cell cycle towards endoreduplication (Mergaert et al., 2006; Kondorosi et al., 2013; Alunni and Gourion, 2016). TBD is determined by host plant factors, including defensin-like antimicrobial peptides (DEFs) consisting of nodule-specific cysteine rich (NCR) peptide, produced in large families of IRLC and Delbergioid legume clades, together with BacA transporter protein in the microbes (Mergaert et al., 2006; Alunni and Gourion, 2016). NCR peptides and BacA transporter protein have been reported to mediate the polyploidy of *Sinorhizobium meliloti* in *Medicago* nodules by altering the process involved in sequential changes in expression of cell cycle genes and increasing

cell size (De Nisco et al., 2014; Penterman et al., 2014). The BacA-like transporter of *Bradyrhizobium* sp. strain ORS285 also provides protection against the antimicrobial activity of NCR peptide in *Aeschynomene* spp. nodules. BacA-like transporter has been identified in ORS285, carrying three genes (BRAO285v1_1320006, BRAO285v1_250005, and BRAO285v1_950010). These genes were characterized by the presence of the transmembrane domain pfam06472 (ABC_membrane_2) or pfam05992 (SbmA_BacA). However, only BRAO285v1_1320006 plays a key role in symbiotic phenotype in the host plants, providing protection against the antimicrobial activity of NCR peptide in *Aeschynomene* spp. nodules. The mutant produced small nodules size, undifferentiated bacteroids, reduced nitrogen fixation activity in *A. indica* and *A. afraspera*, and some dead cells were observed in *A. indica* nodules. The other two genes formed normal symbiosis nodules, differentiated bacteroid, and nitrogen fixation, indicating that these genes are not important for ORS285 in *Aeschynomene* spp. symbiosis (Guefrachi et al., 2015). These indicated that BacA-like proteins in ORS285 were encoded by BRAO285v1_1320006. However, *Bradyrhizobium* BacA-like (BclA), named according to the BacA or the *Escherichia coli* homolog SbmA, differ from *Bradyrhizobium* proteins by the presence of a C-terminal cytosolic ATPase domain typical for canonical ABC transporters (Guefrachi et al., 2015). In addition, the correlation between cell differentiation and nitrogen fixation ability was also revealed in alfalfa (*Medicago sativa*) nodules (Vasse et al., 1990). The small bacteroid size (1–2.5 μm) having low nucleic acid content, also had low acetylene reduction. On the other hand, the enlarged bacteroid size (5–7 μm), had high nucleic acid content and very active in acetylene reduction (Paau and Cowles, 1978). This result indicated the correlation between cell size and nitrogen fixation ability. Thus, the effect of DEFs

from rice plants and BclA of SUTN9-2 on cell size enlargement and nitrogen fixation efficiency, occurring during the interaction of SUTN9-2 with rice extract were analyzed. These results provided a better understanding of the mechanisms and factors involved in cell differentiation and nitrogen fixation in this model.

4.2 Objective

To reveal the similarities in the mechanisms and factors involved in cell differentiation and nitrogen fixation between endophytic SUTN9-2 cells in rice and symbiotic cells in legume plants

4.2.1 Specific objective

4.2.1.1 To determine cell size enlargement and increasing in DNA content of SUTN9-2 in rice plant and response to rice extract

4.2.1.2 To determine cell size enlargement and nitrogen fixation ability of SUTN9-2 in response to rice extract

4.2.1.3 To provide a global view of SUTN9-2 gene expression in response to rice extract

4.2.1.4 To define the mechanisms and factors involved in cell differentiation and nitrogen fixation of SUTN9-2 in response to rice extract

4.3 Materials and Methods

4.3.1 Plant and bacterial strain

Rice plants (*Oryza sativa* L. ssp. *indica* cv. Pathum Thani 1 and *O. sativa* L. ssp. *japonica* cv. Nipponbare) were used in this study. *Bradyrhizobium* sp. strain SUTN9-2 WT (LAXE00000000) was isolated from the root and stem nodules of

Aeschynomene americana, grown in rice field areas in Thailand (Noisangiam et al., 2012). SUTN9-2 DsRed-tagged (Piromyou et al., 2015b), Δ*nifV*, and Δ*bclA* were also used in this study. SUTN9-2 WT and mutants were cultured at 30 ± 2°C in yeast extract-mannitol (YEM) broth medium (Somasegaran and Hoben, 1994) for further analyses. The medium was supplemented with 200 µg/ml of streptomycin and spectinomycin for SUTN9-2 DsRed-tagged, 20 µg/ml of cefotaxime for Δ*nifV*, and 200 µg/ml of streptomycin for Δ*bclA*.

4.3.2 Rice growth and rice extract preparation

The rice seeds (*O. sativa* L. ssp. *indica* cv. Pathum Thani 1 and *O. sativa* L. ssp. *japonica* cv. Nipponbare) were dehulled and then surface sterilized with 70% ethanol for 3 min, twice with 10% hydrogen peroxide for 10 min, with 3% sodium hypochlorite for 1 h, and washed 3 times with sterilized water (Greetatorn et al., 2019). To obtain a small emerging root, surface-disinfected rice seeds were germinated on 0.85% agar with YEM medium for 1 day at 37°C in the dark. Then, 3 germinated seeds (no contamination on YEM) were transplanted into the glass test tubes (22x200 mm) containing sterilized aluminum net and N-free rice nutrient solution [(mmol L^{-1}): $NaH_2PO_4 \cdot 2H_2O$, 0.6; K_2SO_4, 0.3; $CaCl_2 \cdot 2H_2O$, 0.3; $MgCl_2 \cdot 6H_2O$, 0.6; EDTA-Fe, 0.045; H_3BO_3, 0.05; $MnSO_4 \cdot 5H_2O$, 0.009; $CuSO_4 \cdot 5H_2O$, 0.0003; $ZnSO_4 \cdot 7H_2O$, 0.0007; $Na_2MoO_4 \cdot 2H_2O$, 0.0001] and pH adjusted to 6.8 (Mae and Ohira, 1981). Rice plants were grown for 14 days under the controlled environmental conditions at 28 ± 2°C, 70% relative humidity on 16 8-h^{-1} day $night^{-1}$ cycle (full light, 639 microeinsteins [µE]$\cdot m^{-2} \cdot S^{-1}$).

The whole rice samples (root+ shoot; 0.5 g per plant) were harvested at 14 dpi, sterilized and macerated separately with a sterilized mortar and pestle in

sterilized buffered nodulation medium B (BNM-B) minimal medium and kept not over than 3 days at 4 ºC for further analysis. BNM-B is a synthetic plant growth medium (Ehrhardt et al., 1992) supplemented with succinate, glutamate, and a cocktail of vitamins (Renier et al., 2011). The rice extract was used by supplementing the BNM-B medium (20-25g of rice extract 400 mL^{-1} in BNM-B). The homogenate was passed through a three-layer of miracloth (22-25 µm), membrane filter (8 µm), and syringe filter (0.2 µm), to discard plant debris and the obtained rice extract for further incubated with 10^8 SUTN9-2 cells in 10 mL test tube with a tight cap and stable stage.

4.3.3 Construction of SUTN9-2 *bclA* and *nifV* mutant

The construction of the *bclA* deletion mutant (SUTN9-2 Δ*bclA*) was performed and standard molecular techniques were used for this study. The 700-bp upstream and downstream fragments of the *bclA* gene were amplified by PCR and the primers are listed in Table 4.1. The two regions were merged by overlap extension PCR and then digested by *Eco*RI/*Xba*I and were cloned into the plasmid pNPTS129. This plasmid cannot replicate in *Bradyrhizobium* strains and it carries the *sacB* and the kanamycin-resistance gene, which confers sensitivity to sucrose that induces bacterial death (Tsai and Alley, 2000). The spectinomycin and streptomycin cartridge from the pHP45Ω was digested by *Bam*HI and introduced into the upstream and downstream regions previously cloned in the pNPTS129 plasmid. Then, the plasmid was transferred into SUTN9-2 by triparental conjugation with the helper plasmid pRK2013 (Tamura et al., 2011). Single recombinant clones were obtained by antibiotic selection, and double recombinant clones were then obtained by growth on sucrose with spectinomycin and streptomycin, but without kanamycin. Candidate clones were checked for the loss of kanamycin-resistance from the pNPTS129 plasmid, and the deletion of the *bclA* gene

was verified by PCR. The mutation of *nifV* gene was constructed by Hashimoto et al. (2019).

4.3.4 Confocal laser scanning microscopy

SUTN9-2 DsRed-tagged was treated by BNM-B and BNM-B supplemented with rice extract (*O. sativa* L. ssp. *Indica*) for 7, 14, 21, and 28 days. While, SUTN9-2 WT, Δ*nifV*, and Δ*bclA* were treated by rice extract BNM-B and BNM-B supplemented with rice extract from *indica* and *japonica* for 7, 14, 21, and 28 days, and then were collected and stained with 30 µg/ml DAPI and 15 µg/ml FM4-64. All treated cells were observed for cell size, DNA content, and cell membrane by confocal laser scanning microscope (Nikon Model Ni-E, *Nikon* Instech Co., Ltd., Tokyo, Japan). The mean DNA content area was calculated from each cell (μm^2) using ImageJ analysis (Collins, 2007), and the average enlarged cell size in each treatment was calculated from 20 cells per 1 replication for 3 replications.

4.3.5 Flowcytometry analysis

SUTN9-2 WT and DsRed-tagged cells were treated with BNM-B and BNM-B supplemented with rice extract (*O. sativa* L. ssp. *Indica*) for 7, 14, and 28 days. The SUTN9-2 WT treated cell were fixed overnight in 70% ethanol at 4°C, the fixed sample was digested with RNase (DNase free), and then stained with 50 µg/ml propidium iodide (PI) to analyze the DNA content (Deitch et al., 1982). The cell size of SUTN9-2 DsRed-tagged cells treated as mentioned above was determined. The analysis of cell size and DNA content was conducted by flow cytometer (BD FACSCalibur, BD Bioscience, Heidelberg, Germany) with Cyflowgic software.

4.3.6 Acetylene reduction assay

The effect of rice extract on nitrogen fixation efficiency of SUTN9-2 WT (DsRed-tagged), Δ*nifV*, and Δ*bclA* cells treated with rice extract was investigated using the acetylene reduction assay (ARA) (Chaintreuil et al., 2000). The reactions were carried out in a 10 ml test tube containing 2 ml of BNM-B medium and BNM-B medium supplemented with rice extract (*O. sativa* L. ssp. *indica* and *japonica*) as described previously. The reaction was incubated at 28 ± 2°C for 7, 14, 21, and 28 days. Acetylene was injected to reach a final concentration of 10% (vol/vol) of the gas phase in the headspace (Somasegaran and Hoben, 1994). After incubation, the gas from the vessel was injected into a gas chromatograph (6' x 1/8″ S.S. Hayesep T column; Valco instruments Co. Inc., Houston, TX]. Ethylene gas production was analyzed, as described in Renier et al. (2011). The total concentrations of cell suspension were evaluated using the plate count of the colony-forming unit (CFU) on the YEM medium. The medium was supplemented with 200 µg/ml of streptomycin and spectinomycin for SUTN9-2 DsRed-tagged cells, 20 µg/ml of cefotaxime for Δ*nifV*, and 200 µg/ml of streptomycin for Δ*bclA*.

4.3.7 Legumes cultivation, cell differentiation analysis, and Δ*nifV* complementation

The symbiotic characteristics and cell differentiation in the nodule of SUTN9-2 WT, Δ*nifV*, and Δ*bclA* were tested with *Stylosanthes hamata*. The seeds were surface sterilized by immersion in sulfuric acid for 10 min, followed by abundant washing with sterile distilled water, and then incubated overnight in sterilized water. The surface-disinfected seeds were germinated on 0.85% agar plates for 1 day at 37°C in the dark. The germinated seeds were transferred onto the top of 50 ml test tubes

covered by the aluminum foil for hydroponic culture in BNM-B. The plantlets were grown under the controlled environmental condition as described previously. After seven days, each seedling was inoculated with 1 ml of cell suspension resulting from a 5-day- old bacterial culture washed in BNM-B and adjusted to reach an optical density of 1.0 at 600 nm. For complementation studies, homocitrate (Sigma-Aldrich; 1 mM final concentration) was added to the 10 ml test tube covered by the aluminum foil containing the BNM-B medium. To observe the nodulation and cell differentiation, legume nodules were collected at 30 dpi and examined for bacteroid cell from nodule under a confocal laser scanning microscope. The experiment was carried out in 3 replications.

4.3.8 Rice cultivation and growth promotion

The rice seeds *O. sativa* L. ssp. *indica* were surface sterilized and germinated as described above. The germinated seeds were soaked overnight in YEM broth containing SUTN9-2 WT, Δ*nifV*, and Δ*bclA* (10^8 CFU per ml), and then three germinated seeds were transplanted into the glass test tubes (22 x 200 mm) containing sterilized aluminum net with N-free rice nutrient solution and N-free supplemented with 1mmol L^{-1} ammonium nitrate under the controlled environmental condition as previously mentioned. Rice plants were grown and harvested at 7, 14, 21, and 28 dpi, then the whole rice samples were dried in an oven for 72 h at 65°C, and then dry weight was determined.

4.3.9 RNA preparation for qRT-PCR

RNA isolation was carried out from three independent cultures of SUTN9-2 WT, Δ*bclA,* and Δ*nifV* incubated in BNM-B medium or BNM-B medium supplemented with rice extract (*indica*). The cultures were incubated in a 10 ml test

tube with a tight cap at 28 ± 2ºC for 21 days. RNA isolation was carried out from 10^8 cells using Plant RNA extraction kits (Qiagen, USA). The RNAs from rice plant samples (shoot + root) were harvested at 21 dpi, sterilized and macerated (Greetatorn et al., 2019). The method of total RNA extraction was done according to manufacturer procedure. RNAs were treated with the DNaseI (Qiagen, USA) at 28 ± 2°C for 30 min to prevent contamination of genomic DNA. The purity of RNA was assessed by PCR on total RNA (250 ng) with GoTaq polymerase (Promega Corp.) using *dnaK*_SUNT9-2 primers and *EF-1∝*_rice plant (Table 4.1). The RNA quality and concentration were assessed by Nanodrop (Thermo Scientific, Wilmington, DE, U.S.A.) and agarose gel electrophoresis.

4.3.10 qRT-PCR and analysis

The transcription levels were determined by qRT–PCR using Applied Biosystem, QuantStudio Design (Waltham, MA). Primers for amplification of genes involved in the cell cycle (*dnaK*, *gcrA*, *ctrA*, *dnaA*, and *GcrM*), nitrogen fixation (*nifH* and *nifV*), and rice hemoglobin (*EF-1∝*, *hb1*, and *hb5*) are listed in Table 4.1. PCR amplification was performed under the cycling condition as follows; an initial denaturation step at 95°C for two min; 35 cycles (2 min at 95°C, 30 s at the annealing temperature of all genes (50°C)) followed by a final 5 min extension at 72°C. The relative gene expression was analyzed by the comparative Ct method (-ΔΔCT) that was normalized to the endogenous housekeeping gene, *dnaK* for bacterial SUTN9-2 and *EF-1∝* for rice plants. Three biological replicates were pooled and analyzed.

4.3.11 RNA preparation for RNAseq analysis

RNA isolations were carried out from three independent cultures of SUTN9-2 WT and Δ*bclA* incubated in BNM-B medium and BNM-B medium

supplemented with rice extract (*indica*). The cultures were incubated in a 50 ml test tube with a tight cap at 28 ± 2°C for 21 days. RNA isolation was carried out from 3 x 10^8 cells using Plant RNA extraction kits (Qiagen, USA). The method of total RNA extraction was done according to manufacturer's procedure. The purity of RNA was assessed as described above.

4.3.12 RNA sequencing and analysis

To identify the bacterial genes that respond to rice extract, the comparative RNAseq analysis of SUTN9-2 was used. The RNA-Seq libraries were constructed from the RNA sample incubated with or without the rice extract (20-25 g of rice extract 400 ml^{-1} in BNM-B) for SUTN9-2 WT and Δ*bclA*. Three biological replicates were prepared for each treatment. The RNA samples were extracted following the same protocol for qRT-PCR. Eukaryotic rRNA from the samples was removed using the Ribo-Zero Magnetic Kit (Illumina, San Diego, CA) and stranded RNA-Seq libraries were constructed with TruSeq Stranded mRNA kit (Illumina). Paired-end sequencing (150 bp) of the libraries was performed by the NovaSeq platform (Illumina). The libraries were constructed and sequenced at Novogene Co. Ltd. (Beijing, China).

For gene expression analysis, the mapping of the obtained transcriptomic data was adapted to previously revealed SUTN9-2 genome. A total of 865M reads (R1 + R2) were obtained from the 12 libraries (Table 4.2). Adapter sequences and low-quality sequences were removed from the raw single-end reads using fastp (v0.20.0) [fastp: an ultra-fast all-in-one FASTQ preprocessor, (Chen et al., 2018)]. The trimmed paired-end reads to the SUTN9-2 genome (INSDC ID ASM312264v1) and calculated the TPM-normalized mapped read numbers by RSEM

(v1.3.1) [RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome, (Li and Dewey, 2011)] with --bowtie2 option (bowtie2 version 2.3.5.1) [Fast gapped-read alignment with Bowtie 2, (Langmead and Salzberg, 2012)]. The mapped reads were counted based on the gene models of SUTN9-2 (LAXE01000000) predicted by Piromyou et al. (2015a). The differentially expressed genes (DEGs) were detected based on the false discovery rate (FDR) (< 0.1) and $\log_2$ fold change (logFC) (> 0.1 or < -0.1) from the RSEM analysis. For enrichment analysis, the public gene models from LAXE01000000 were annotated with interproscan (v5.36-75) with --goterms option (for gene ontology annotation) and with KofamKOALA (downloaded on 13th Aug. 2019) [(KofamKOALA: KEGG Ortholog assignment based on profile HMM and adaptive score threshold (Aramaki et al., 2019) for KEGG Orthologs annotation)]. Enrichment analyses were performed by goseq (v1.38.0) on R (v3.6.1) [gene ontology analysis for RNA-seq: accounting for selection bias, (Young et al., 2010)].

4.3.13 qRT-PCR validation

To validate findings in the transcriptome analysis, a significantly DEGs were selected to determine their expression in SUTN9-2 WT in BNM-B with rice extract group compared to the SUTN9-2 WT in BNM-B alone, including biphenyl-2,3-diol 1,2-dioxygenase (*hpaD*), 3-(3-hydroxyphenyl) propanoate hydroxylase (*mhpA*), chaperonin GroEL (*groEL*), chaperonin GroES (*groES*), ABC transporter ATP-binding protein (*sapDF*), ABC transporter substrate-binding protein (*sapA*), and RND family efflux transporter (*cusF*). Primers for amplification of these genes are listed in Table 4.1. RNA preparation, qRT-PCR, and analysis were performed as described above.

4.3.14 SbmA_BacA domain proteins and phylogenetic analysis

The identified protein sequences of SbmA_BacA domain including BacA, *Bradyrhizobium* homologous, ExsX, and *Mycobacterium* BacA clades were obtained from Guefrachi et al. (2015). The BacA-related protein sequences of SUTN9-2 (BclA; PWE81210.1, SapA; PWE77331.1, and SapDF; PWE82048.1) were obtained from the National Center for Biotechnology Information (NCBI) database. The protein sequences were aligned using ClustalW program. The phylogenetic tree was constructed by the neighbor-joining method with the confidence levels for 500 replicates using MEGAX package (Saitou and Nei, 1987; Kumar et al., 2008).

4.3.15 The statistical analysis

Statistical analysis of data sets was performed with the SPSS software (SPSS 16.0 for Windows; SPSS Inc., Chicago, IL) on data from three independent samples (each with three technical replicates). The experimental data were statistically analyzed according to Steel and Torrie (1980), and means were compared by Duncan's multiple range test ($P \leq 0.05$) (Duncan, 1955).

Table 4.1 Primers used in this study.

Target name by type	Primer name	Gene description	Primer sequence (5'➔3')	Description of design and reference
House keeping				
dnaK	dnaK-9-2/858F	70 kDa chaperone protein	TCACGACTTCCTGGACCTTC	Designed from *dnaK* of SUTN9-2 (LAXE00000000)
	dnaK-9-2/1077R		ATCAACCTGCCCTTCATCAC	
EF-1α	EF-1α-Os03g08020F	Elongation factor 1α	GTCATTGGCCACGTCGACTC	(Caldana et al., 2007)
	EF-1α-Os03g08020R		TGTTCATCTCAGCGGCTTCC	
Nitrogen fixation				
nifH	nifH-9-2/489F	Dinitrogenase reductase	TAACATCTCCAAGGGCATCC	Designed from *nifH* of SUTN9-2 (LAXE00000000)
	nifH-9-2/758R		CCGCCATTATTGTGAACCTT	
nifV	nifV-9-2/850F	Homocitrate synthesis	CGTGCGATTCCTATGAACAA	Designed from *nifV* of SUTN9-2 (LAXE00000000)
	nifV-9-2/1107R		GATGGCATATTTGCGGACTT	
ABC trasporter				
bclA	bclA-9-2/349F	BclA-ABC transporter ATP-binding domain	CACTATCGGATGCAGCTCAA	Designed from *bclA* of SUTN9-2 (LAXE00000000)
	bclA-9-2/639R		GAGATTGACGAGAGGCGAAC	

Cont.

Table 4.1 Primers used in this study.

Target name by type	Primer name	Gene description	Primer sequence (5'➔3')	Description of design and reference
Master cell-cycle				
gcrA	gcrA-9-2/106F	Cell cycle regulator	GCCGCAGAAGAAGAAATCC	Designed from *gcrA* of SUTN9-2 (LAXE00000000)
	gcrA-9-2/3087R		AGCATATGATGCGGGTGAC	
ctrA	ctrA-9-2/372F	Cell cycle transcriptional regulator	ACGGCTTGGTGAGATAATCG	Designed from *ctrA* of SUTN9-2 (LAXE00000000)
	ctrA-9-2/607R		GGGCTACGAAATCCTGGAAG	
dnaA	dnaA-9-2/709F	Chromosomal replication initiator	GAGATCGTCGATGACAAGCA	Designed from *dnaA* of SUTN9-2 (LAXE00000000)
	dnaA-9-2/923R		TCAACCCGCTCTACATCCAT	
ccrM	ccrM-9-2/329F	DNA methyltransferase	GCCAGTTCATCGAAAAGCTC	Designed from *ccrM* of SUTN9-2 (LAXE00000000)
	ccrM-9-2/557R		AGACGGTGCGGAATTAACAG	
Hemoglobin				
hb1	hb1-indica/341F	Hemoglobin1	CATGTCCGTCTTCGTCATGG	Designed from *hb1* of *O. sativa* L. ssp. *indica* (U76029)
	hb1-indica/500R		TTGAGGGTGGTGTCTCTCAC	

Cont.

Table 4.1 Primers used in this study.

Target name by type	Primer name	Gene description	Primer sequence (5'➔3')	Description of design and reference
hb5	hb5-indica/456F	Hemoglobin5	GTCTTCGTCATGGCAAGCAA	Designed from *hb5* of *O. sativa* L. ssp. *indica* (EF061459)
	hb5-indica/594R		CTCCCTCACCTTCACGTCAC	
qRT-PCR validation				
hpaD	hpaD-9-2/15F	Biphenyl 2,3 dioxygenase	GCTCGGCTATATCGGAATCA	Designed from *hpaD* of SUTN9-2 (LAXE00000000)
	hpaD-9-2/233R		CTGCCAGCTTATCCAGTTC	
mhpA	mhpA-9-2/688F	3-3-hydroxyphenyl propionate hydroxylase	CCGAATGGAACGTGTAGACC	Designed from *mhpA* of SUTN9-2 (LAXE00000000)
	mhpA-9-2/906R		ACGACCTCGGTGATTACAGC	
groEL	groEL-9-2/536F	Molecular chaperone GroEL	TCGTAGTCCGAGGTGGTTTC	Designed from *groEL* of SUTN9-2 (LAXE00000000)
	groEL-9-2/750R		GAGGATATCGCGATCCTTACC	
groES	groES-9-2/37F	Molecular chaperone GroES	CCGAGAAGATCGCTTTCCTT	Designed from *groES* of SUTN9-2 (LAXE00000000)
	groES-9-2/241R		GCATCATCATTCCCGACAC	
sapA	sapA-9-2/10F	ABC transporter substrate-binding protein	CGTTCTGCGACATCGAACTA	Designed from *sapA* of SUTN9-2 (LAXE00000000)
	sapA-9-2/241R		CGTCGAAAACCGAAAAATGT	

Cont.

Table 4.1 Primers used in this study.

Target name by type	Primer name	Gene description	Primer sequence (5'➔3')	Description of design and reference
sapDF	sapDF-9-2/299F	ABC transporter ATP-binding protein	CTCGTCCATCAGCAGGATCT	Designed from *sapDF* of SUTN9-2 (LAXE00000000)
	sapDF-9-2/501R		TGGAGCAGAACATCGGCTAT	
cusF	cusF-9-2/92F	RND family efflux transporter	GCGAGGTCAAGAAGATCGAC	Designed from *cusF* of SUTN9-2 (LAXE00000000)
	cusF-9-2/296R		GCCTTCTGCATCTTGGTCAC	
bclA mutant construction				
bclA upstream region	up_EcoRI_for	Upstream region of BclA-ABC transporter ATP-binding domain	AGCGGGAATTCGCTCAATCTG TCCGACGATGCG	Designed from *bclA* of SUTN9-2 (LAXE00000000)
	up_ *BamH*I_rev		CCTCAGCGCGCGGATCCGATG TTGTTCACAGAGCTGG	
bclA downstraem region	dw_ *BamH*I_for	Downstream region of BclA-ABC transporter ATP-binding domain	TGTGAACAACATCGGATCCGC GCGCTGAGGCGAG CGCTGG	Designed from *bclA* of SUTN9-2 (LAXE00000000)
	dw_ *Xba*I_rev		CGGCGGTCTAGACATGAGCG ATTACG TCTTCCG	

Table 4.2 Total of 865M reads (R1 + R2) from the 12 libraries.

Sample	Raw data		
	Fragment #	**Read length**	**Total bases**
WB_1	39,626,861	150 x 2	11,888,058,300
WB_2	37,169,231	150 x 2	11,150,769,300
WB_3	31,573,758	150 x 2	9,472,127,400
WRB_1	33,521,269	150 x 2	10,056,380,700
WRB_2	39,326,307	150 x 2	11,797,892,100
WRB_3	32,584,299	150 x 2	9,775,289,700
bB_1	41,851,749	150 x 2	12,555,524,700
bB_2	49,348,149	150 x 2	14,804,444,700
bB_3	38,768,526	150 x 2	11,630,557,800
bRB_1	32,096,739	150 x 2	9,629,021,700
bRB_2	27,820,858	150 x 2	8,346,257,400
bRB_3	29,158,174	150 x 2	8,747,452,200
Total	432,845,920		129,853,776,000

4.4 Results

4.4.1 SUTN9-2 enlarged its cell size in rice (*indica*) plants

To confirm that SUTN9-2 cell size enlargement actually occurs within rice plants, red fluorescent-tagged SUTN9-2 (DsRed) was extracted from rice plant tissues (*indica*) at 21 and 28 dpi and visualized using confocal laser scanning microscopy. Elongated cells of SUTN9-2 were observed, with the average cell size of 5.6 µm and 5.8 µm at 21 and 28 dpi, respectively, when compared to free-living SUTN9-2 cells (2.8 µm) (Fig. 4.1). These results revealed that SUTN9-2 cell size enlarged within rice plants. However, low amounts of SUTN9-2 cells could be extracted from the rice plant tissues. Therefore, rice extract was prepared and subsequent experiments were carried out by incubating SUTN9-2 cells with the extract, to investigate the influence of rice and its derived molecules on cell differentiation of SUTN9-2.

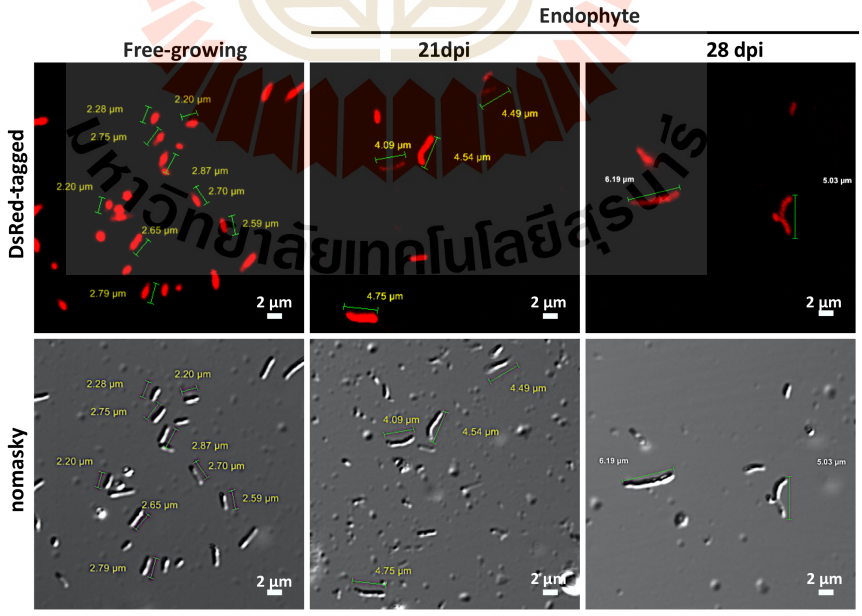

Figure 4.1 Cell size enlargement of extracted SUTN9-2 DsRed-tagged at 21 and 28 dpi from rice plant tissues by confocal laser scanning microscope.

4.4.2 SUTN9-2 increases cell size and DNA content in response to rice extract (*indica*) treatment

Increase in cell size and DNA content of SUTN9-2 in response to rice extract (*indica*) treatment were analyzed using a flow cytometer. The cell size of treated cell SUTN9-2 DsRed-tagged in buffered nodulation medium B (BNM-B) minimal medium and BNM-B supplemented with rice extract were analyzed. The forward scatter (FS) of the treated cell was related to their size. The BNM-B with rice extract-treated cells had a higher FS than BNM-B at 7, 14, and 28 days (Fig. 4.2A). Also, the DNA content of treated cell SUTN9-2 WT was measured with fluorescent staining of nuclei using propidium iodide (PI). The DNA content of treated cell SUTN9-2 in BNM-B with rice extract was higher than that of BNM-B alone. The DNA content of BNM-B treated cell was 1 to 2 genome complement (1C to 2C), similar to the ploidy level of free-growing cells (1C to 2C), whereas the BNM-B with rice extract treated cell reached 2C to 7C ploidy levels, similar to ploidy level of bacteroid cells (7C to 16C) (Czernic et al., 2015; Guefrachi et al., 2015) (Fig. 4.2A). The results confirmed the differentiated cell of SUTN9-2 affecting rice plants.

4.4.3 SUTN9-2 increases cell size and nitrogen fixation ability in response to the rice extract (*indica*)

To investigate whether enlarged cell size results in an increase in nitrogen fixation ability in response to rice extract, the SUTN9-2 DsRed-tagged cells in deionized water (DI), treated with BNM-B, and BNM-B with rice extract (*indica*), were analyzed. The longest cell size was observed in the BNM-B with rice extract group when compared to BNM-B alone and DI group. At 7, 14, 21, and 28 days, BNM-B with rice extract treated cells had the average longest cell size with 2.76 μm, 3.18

μm, 3.38 μm, and 3.79 μm, respectively (Fig. 4.2B), and the highest nitrogenase activity with 0.01, 0.02, 0.09, and 0.30 nmol C_2H_4 log_{10} CFU^{-1}, respectively (Fig. 4.2C). At 7, 14, 21, and 28 days, cells treated with BNM-B alone had the average cell size 2.52 μm, 2.74 μm, 2.42 μm, and 2.64 μm, respectively (Fig. 4.2B). The nitrogenase activity in this group was 0.01 nmol C_2H_4 log_{10} CFU^{-1} at 7, 14, 21 days and 0.02 nmol C_2H_4 log_{10} CFU^{-1} at 28 days (Fig. 4.2C). The average smallest cell size was observed in DI treated cells with 1.86 μm, 2.04 μm, 1.36 μm, and 1.37μm at 7, 14, 21, and 28 days, respectively (Fig. 4.2B), while having no nitrogenase activity, except at 14 days with 0.01 nmol C_2H_4 log_{10} CFU^{-1} (Fig. 4.2C). These results indicated the influence of rice extract on cell enlarged size and the nitrogen fixation efficiency of SUTN9-2. Moreover, these results also suggested the time-dependent increase of elongated cells in the BNM-B with rice extract group, while major changes were neither observed with BNM-B nor DI treatment (Fig. 4.2B and C).

4.4.4 SUTN9-2 WT and mutants undergo cell size enlargement and perform nitrogen fixation in the presence of rice extract (*indica* and *japonica*)

To understand the effects of rice species variation and *bclA* and *nifV* genes on cell differentiation and nitrogen fixation, SUTN9-2 WT, Δ*bclA,* and Δ*nifV* were treated with rice extract from 2 different species of rice (*indica* and *japonica*). The longest cell size and highest nitrogenase activity was observed in the BNM-B with rice extract (*indica*) group at 28 days with the average cell size of 3.28 μm, 3.22 μm, and 3.03 μm (Fig. 4.2D, E, and F), the mean DNA content areas of 0.43, 0.33, and 0.32 μm^2 (Fig. 4.3 and 4.4) and nitrogenase activity at 0.28, 0.07, and 0.00, nmol C_2H_4 log_{10} in

SUTN9-2 WT, Δ*bclA*, and Δ*nifV*, respectively (Fig 4.2D, E, and F). The BNM-B with rice extract (*japonica*) group showed the average cell size of 2.66 μm, 2.56 μm, and 2.60 μm (Fig. 4.2D, E, and F), the mean DNA content areas of 0.32, 0.33, and 0.28 μm^2 (Fig. 4.3 and 4.4), and nitrogenase activity at 0.06, 0.03, and 0.00, nmol C_2H_4 log_{10} for SUTN9-2 WT, Δ*bclA*, and Δ*nifV*, respectively (Fig 4.2D, E, and F). The average smallest cell size was observed at 28 days in BNM-B treated cell with an average cell size of 1.73 μm, 1.73 μm, and 1.63 μm (Fig. 4.2D, E, and F), the mean DNA content areas of 0.11, 0.15, and 0.15 μm^2 (Fig. 4.3 and 4.4), and nitrogenase activity at 0.05, 0.01, and 0.00, nmol C_2H_4 log_{10} in SUTN9-2 WT, Δ*bclA*, and Δ*nifV*, respectively (Fig 4.2D, E, and F). The results also showed a similar trend at 7, 14, and 21 days (Fig. 4.2D, E, and F). Besides, the effect of rice extract and Δ*bclA* and Δ*nifV* on enlarged cell size were observed by transmission electron microscope (TEM). Elongated cells of SUTN9-2 WT, Δ*bclA*, and Δ*nifV* were observed in response to rice extract (*indica*) treatment (Fig. 4.5).

These results showed that the rice extract (*indica*) had more pronounced effects on cell elongation and nitrogen fixation ability than that of the rice extract (*japonica*) in WT and mutant SUTN9-2. The decrease in elongated cell size and nitrogenase activity was found in the Δ*bclA* compared with the WT strain. In addition, the nitrogenase activity could not be detected in Δ*nifV* and their cell size was smaller than that of WT and Δ*bclA* upon treatment with both the rice extracts. These results also revealed the effect of *bclA* and *nifV* genes on cell size enlargement and nitrogen fixation in the response to a variety of rice.

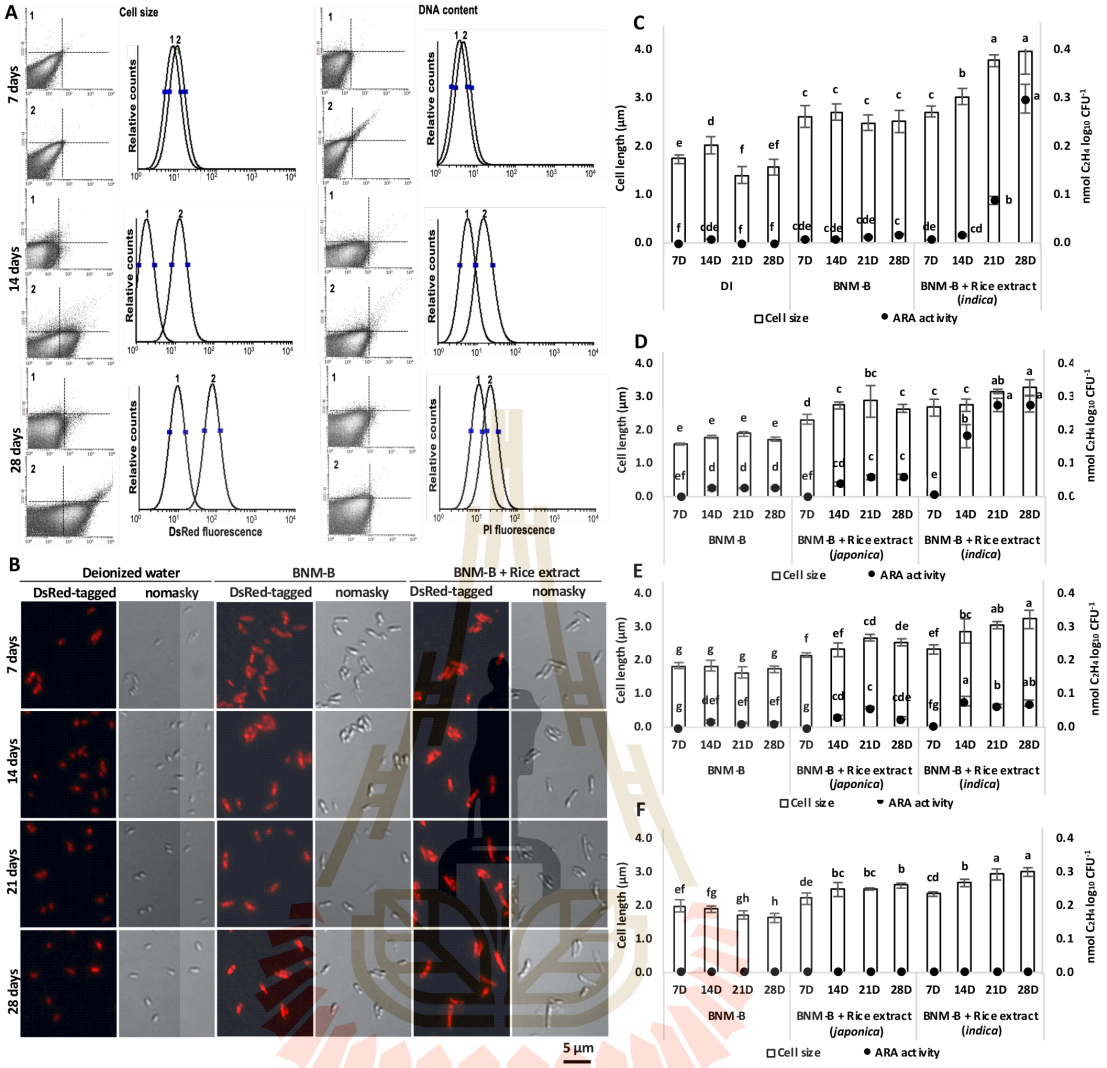

Figure 4.2 Cell size, DNA content, and nitrogenase activity of SUTN9-2 in response to rice extract. Cell size, enlarged size of DsRed-tagged cells, and of Propidium iodide (PI)-staining of DNA content was analyzed by flowcytometry (1 represent free growing, and 2 represent treated cell with rice extract) (A). Cell size and enlarged size of DsRed-tagged cells by confocal laser scanning microscope (B). Observation of cell size by confocal laser scanning microscope and nitrogenase activity by acetylene reduction assay of SUTN9-2 WT cell treated with DI, BNM-B, and BNM-B + rice extract (*indica*) (C), and SUTN9-2 WT (D), Δ*bclA* (E), and Δ*nifV*

(F) with BNM-B, BNM-B + rice extract (*japonica*), and BNM-B + rice extract (*indica*). Significant at $P \leq 0.05$ is indicated by the mean standard deviation (n=3).

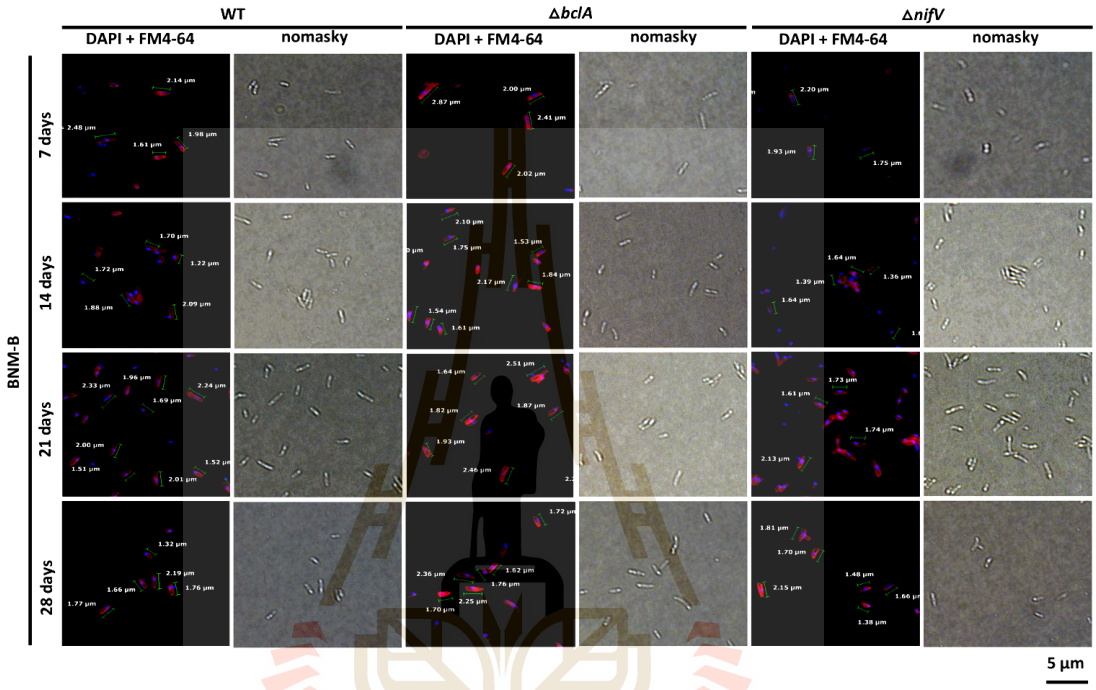

Figure 4.3 Cell size of SUTN9-2 WT, Δ*bclA*, and Δ*nifV* with DAPI-stained nucleoid and PI-stained membrane in response to BNM-B, BNM-B + rice extract (*japonica*), and BNM-B + rice extract (*indica*).

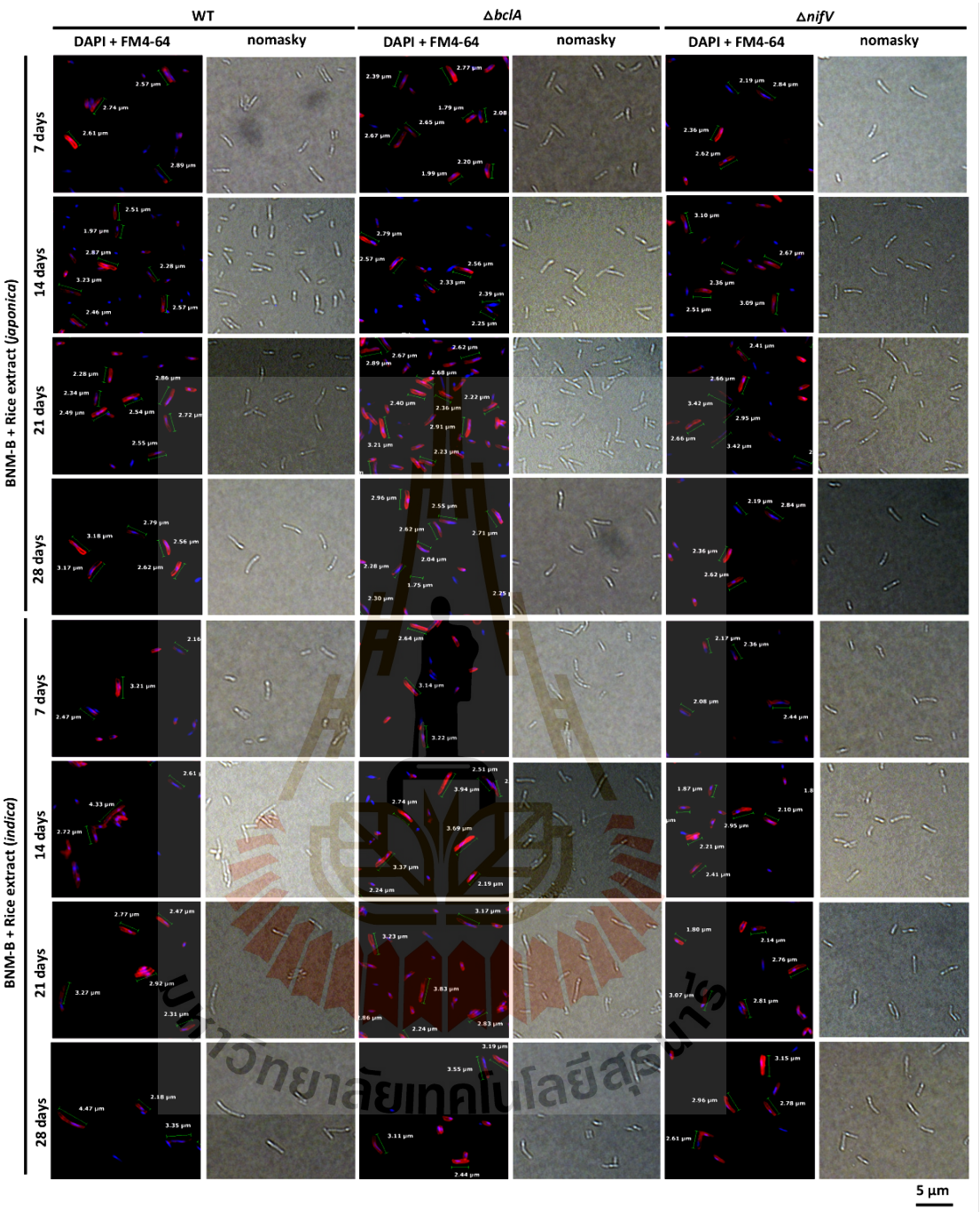

Figure 4.3 (Continued)

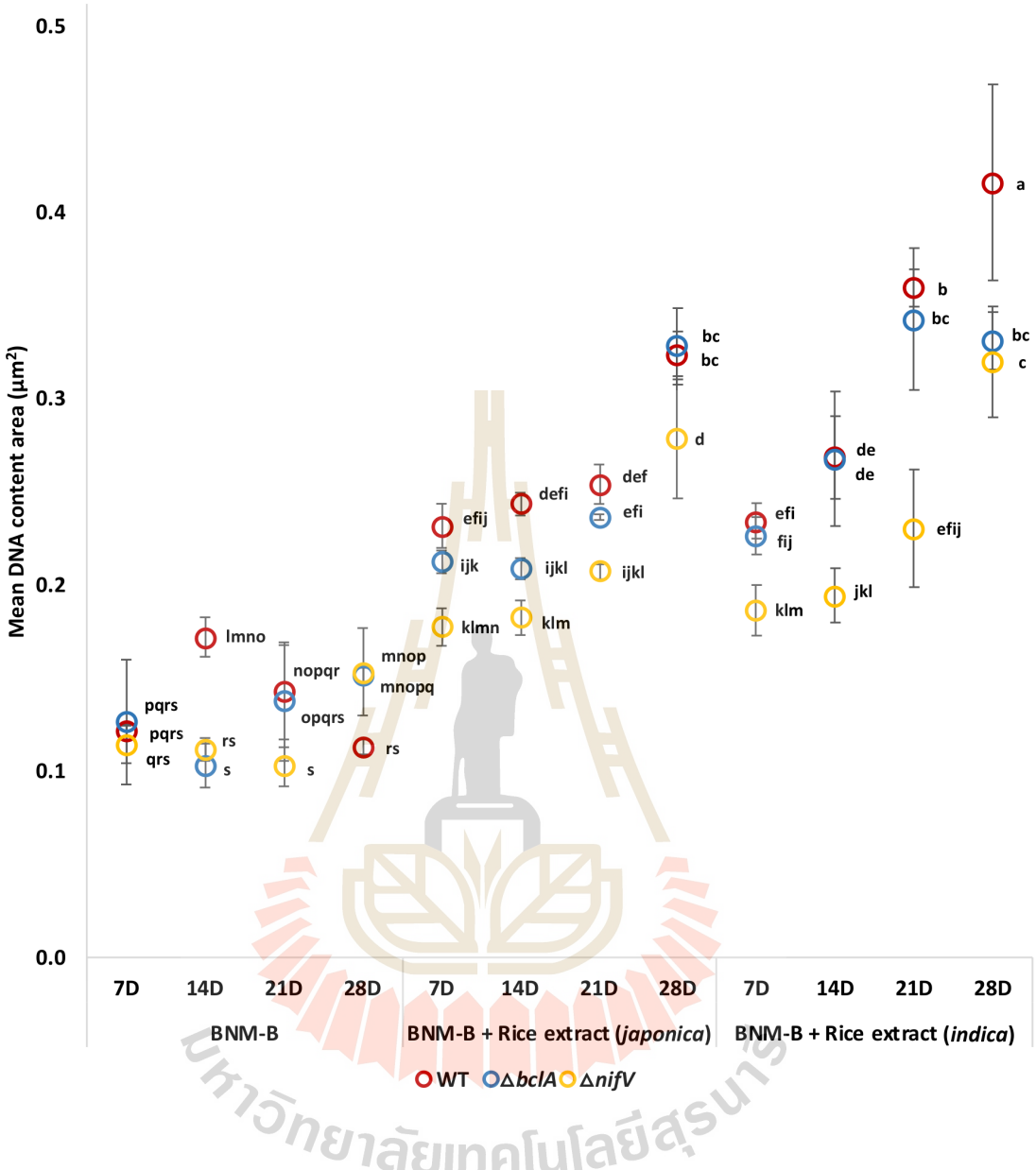

Figure 4.4. Mean DNA content areas (µm^2) from DAPI-stained nucleoid of SUTN9-2 WT, Δ*bclA*, and Δ*nifV* in response to BNM-B, BNM-B + rice extract (*indica*), and BNM-B+rice extract (*japonica*) at 7, 14, 21, and 28 days, means labeled with different letters are statistically different at a *P* value of ≤0.05 (n=3).

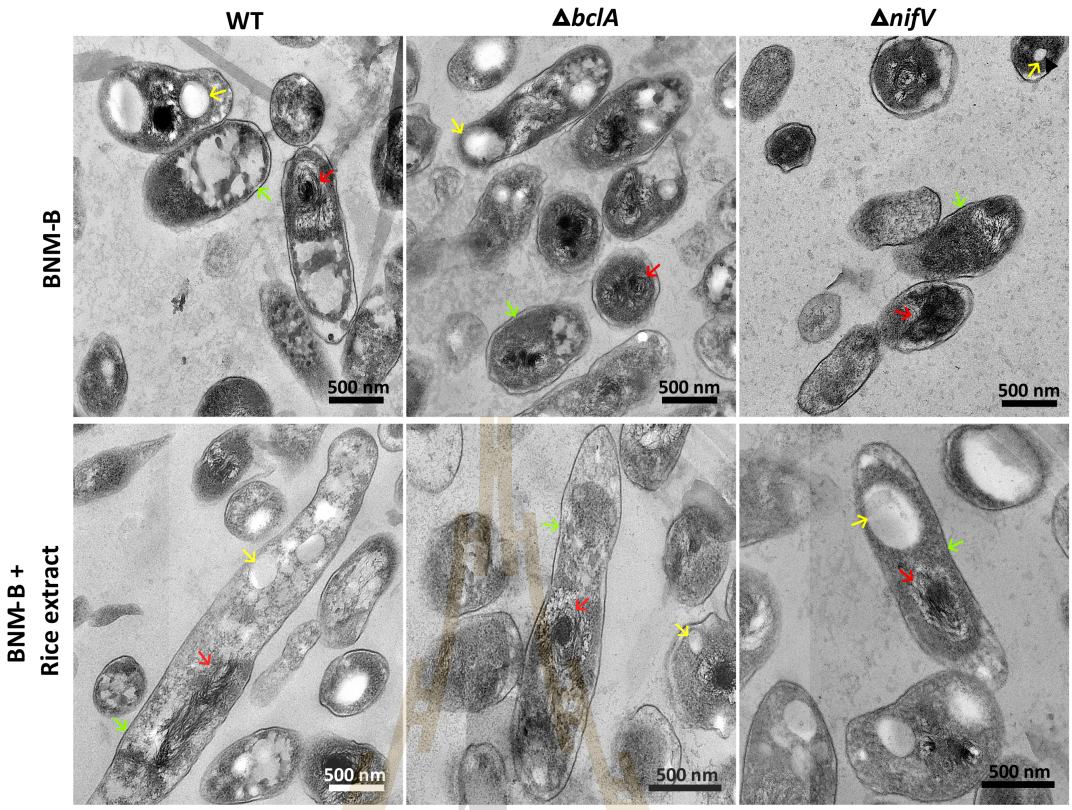

Figure 4.5 Cross-section of SUTN9-2 WT, Δ*bclA*, and Δ*nifV* by transmission electron microscope (TEM) in BNM-B and BNM-B + rice extract (*indica*) at 21 days. Enlarged cell size of WT, Δ*bclA*, and Δ*nifV* was observed in response to rice plant extract. Red arrows mark bradyrhizibial nucleoid, yellow arrows mark bradyrhizibial polyhydroxybutyrate (PHB), and green arrows mask cell membrane.

4.4.5 Wild type (WT) and mutant SUTN9-2 undergo differentiation in the symbiotic nodule of legume plants

It is important to evaluate that SUTN9-2 here performed cell differentiation in the nodule of legume plants, and also the effects of *bclA* and *nif* genes on cell differentiation were evaluated. The observation at 30 dpi using *Stylosanthes*

hamata as host plants, revealed dramatic growth suppression in Δ*nifV* when compared to WT and Δ*bclA*, However, there was no difference plant growth between WT and Δ*bclA* was observed (Fig. 4.6). Besides, cell differentiation of the spherical cells in *S. hamata* was still observed in SUTN9-2 WT, Δ*bclA* and Δ*nifV* (Fig. 4.6). Nevertheless, bacteroid cell size seems likely to reduce in the Δ*nifV* (Fig. 4.6). However, the addition of homocitrate to BNM-B growth medium restored the function of Δ*nifV*, increasing legume growth and SUTN9-2 Δ*nifV* cell size (Fig. 4.6). These results showed that SUTN9-2 WT and mutants are capable of cell differentiation in legume plants but Bcla transporter may be mutated incompletely, resulting in the differentiated cell was still observed in Δ*bclA*. Legumes plants inoculated with Δ*nifV* showed the effects of the absence of the homocitrate synthase NifV and displayed typical characteristics of nitrogen starvation, reducing plant growth and differentiated cell size when compared to legume plants inoculated with WT and Δ*bclA*.

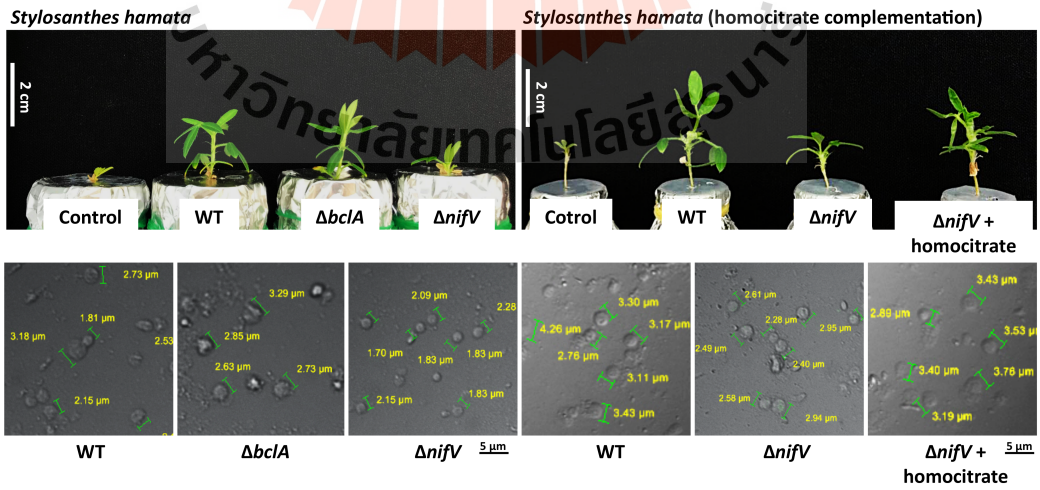

Figure 4.6 Effect of SUTN9-2 WT, Δ*bclA*, Δ*nifV*, and homocitrate complementation on nodulation and cell differentiation in *S. hamata* nodules.

4.4.6 Effect of WT and mutants SUTN9-2 on rice (*indica*) plant growth

SUTN9-2 has been reported to promote rice growth at the early seedling stage (Greetatorn et al., 2019). This experiment showed the similar results where SUTN9-2 WT, Δ*bclA*, and Δ*nifV* increased the rice dry weight, especially at the at 7 and 14 dpi, under N-free medium and ammonium nitrate-supplemented medium compared with the non-inoculated control (Fig. 4.7). The significant difference between inoculated rice plants with SUTN9-2 WT, Δ*bclA*, and Δ*nifV*, and non-inoculated control, was not observed at 21 and 28 dpi (Fig. 4.7). However, rice dry weight did not show a significant difference between the inoculated rice plant with SUTN9-2 WT, Δ*bclA*, and Δ*nifV* at 7, 14, 21, and 28 dpi (Fig. 4.7). These data indicated that SUTN9-2 could obviously promote rice plant growth at the early stage at 7 and 14 dpi under the presence or absence of nitrogen source supplementation. However, the effect of *bclA* and *nifV* genes on rice plant growth was not clearly observed.

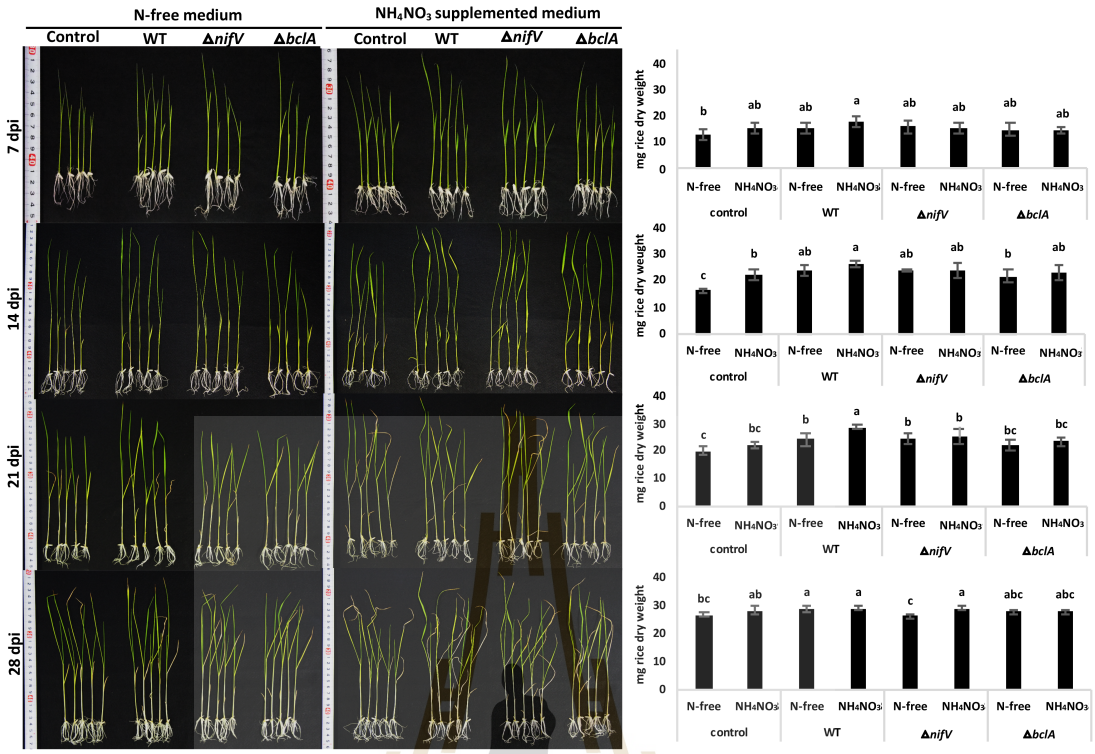

Figure 4.7 Effect of SUTN9-2 WT, Δ*bclA*, and Δ*nifV* on rice (*indica*) growth with non-inoculation control in N-free rice medium and N-free supplemented with 1mM ammonium nitrate at 7, 14, 21, and 28 dpi, means labeled with different letters are statistically different at a *P* value of ≤ 0.05 (n=3).

4.4.7 Expression of cell cycle genes in WT and mutant SUTN9-2 in response to rice extract (*indica*)

The SUTN9-2 cell differentiation-related genes were selected from their role in the master cell cycle, including *gcrA*, *ctrA*, *ccrM*, and *dnaA*. A similar trend for expression of all genes was found between SUTN9-2 WT and Δ*bclA*. However, Δ*bclA* showed some suppression of expression for the above listed genes. The expression levels of *gcrA* and *ctrA* in BNM-B with rice extract (*indica*) group were lower than that in BNM-B alone, with 0.01 and 0.09-fold in WT and 0.008 and 0.01-fold in Δ*bclA*,

respectively (Fig. 4.8A). However, there was no statistically significant difference between BNM-B alone and BNM-B with rice extract group for *gcrA* expression. In contrast, the relative expression levels of *ccrM* and *dnaA* in BNM-B with rice extract group were higher than in BNM-B alone, with 0.20 and 0.22-fold for WT and 0.10 and 0.10-fold for Δ*bclA*, respectively (Fig. 4.8A). Despite this, the relative expression of all genes in Δ*nifV* in the BNM-B with rice extract group was several folds lower than that in BNM-B alone (Fig. 4.8A). These results suggested that cell size enlargement might be also affected by *nifV* gene, supporting the observation of cell enlargement under the microscope. Besides, these results might indicate that *bclA* gene was disrupted but other *bclA*-associated genes are still active and are required for cell enlargement, showing a similar pattern of the gene expression as WT. Taken together, these show that some factors form rice extract may affect the master cell-cycle regulators by suppressing the expression of *ctrA* and promoting *ccrM* and *dnaA* expression, resulting in the enlargement of cell size and DNA content.

4.4.8 Nitrogen fixation and expression of BclA transporter genes in WT and mutant SUTN9-2 in response to rice extract (*indica*)

To evaluate the effect of rice extract on BclA transporter and nitrogen fixation-related gene expression in SUTN9-2, their relative expression levels were measured in SUTN9-2 WT, Δ*bclA*, and Δ*nifV*. The expression levels of *nifH*, *nifV*, and *bclA* genes were significantly increased in WT (0.12, 0.12, and 0.11-fold, respectively) and Δ*bclA* (*nifH*; 0.14, and *nifV*; 0.14-fold) in the presence of rice extract (Fig. 4.8B). In contrast, the expression levels of these genes were significantly decreased in Δ*nifV* (*nifH*; 0.06, *bclA*; 0.008-fold) in the presence of rice extract (Fig. 4.8B). However, the expression of *bclA* and *nifV* genes could not be detected in SUTN9-2 mutants lacking

bclA and *nifV*, respectively (Fig. 4.8B). The results suggest a similarity of the expression pattern between these genes and genes involved in the master cell-cycle, thus indicating the effect of interaction between the rice extract and BclA transporter, on cell differentiation and nitrogen fixation in SUTN9-2.

4.4.9 Expression rice (*indica*) hemoglobin genes in response to WT and mutants SUTN9-2

To assess a potential link of hemoglobin gene expression with the nitrogen fixation efficiency by SUTN9-2 in rice plant, *hb1* and *hb5* genes expression levels were assessed in rice plant inoculated with SUTN9-2 WT, Δ*bclA*, and Δ*nifV*. The results show that *hb1* and *hb5* expression were induced in rice inoculated with SUTN9-2 WT (0.2 and 0.03-fold), Δ*bclA* (0.02 and 0.007-fold), and Δ*nifV* (0.06 and 0.01-fold), compared to non-inoculated control, respectively (Fig. 4.8C). The expression of these genes was suppressed in Δ*bclA* and Δ*nifV* inoculated rice, where the expression level of the *hb5* gene was lower than that of the *hb1* gene (Fig. 4.8C). These results indicated that the activity of hemoglobin in rice plants might be induced in response to SUTN9-2 inoculation and might function as an oxygen scavenger or oxygen stock to facilitate nitrogen fixation of the endophytic state of SUTN9-2.

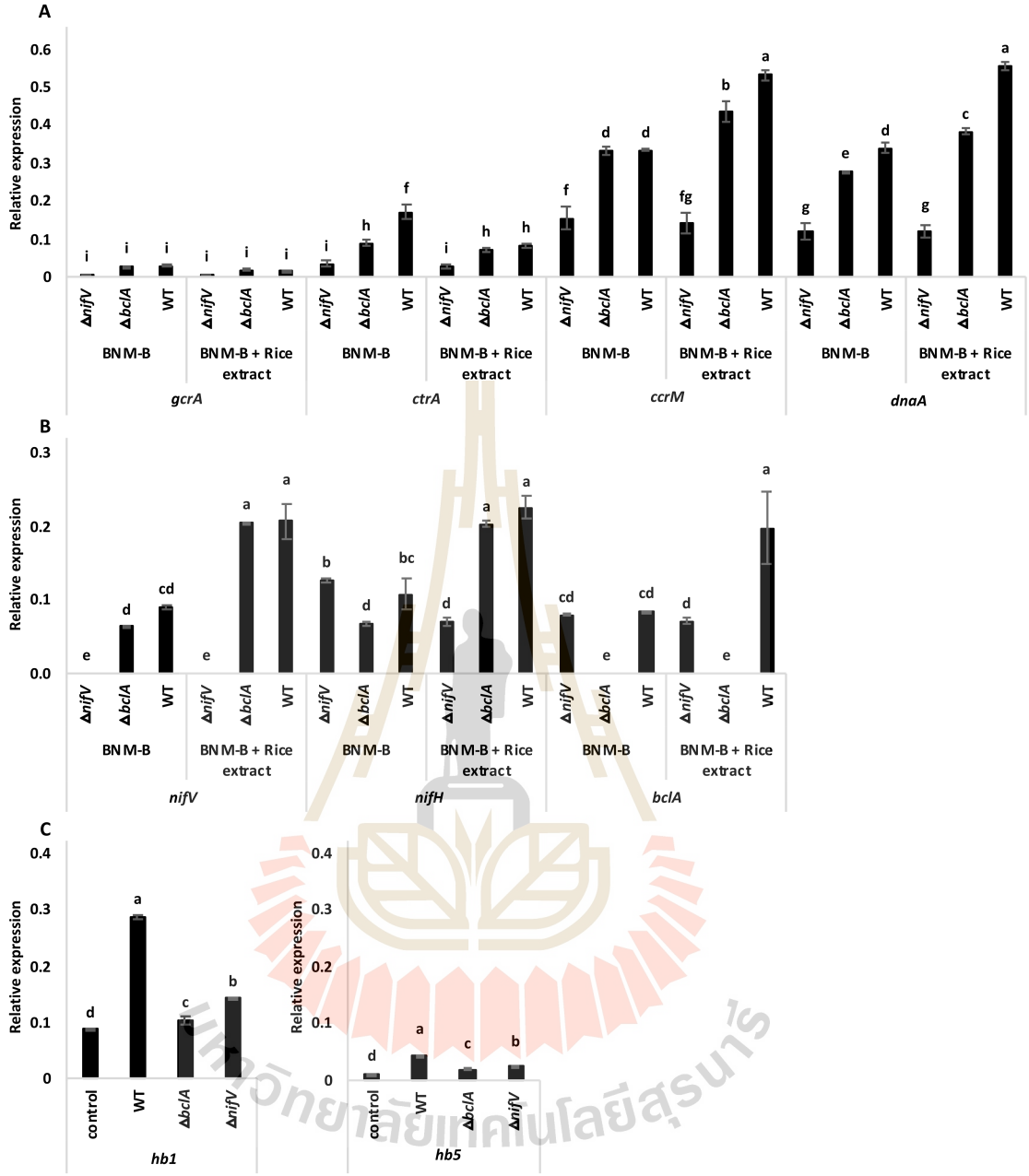

Figure 4.8 Relative expression of genes involved in the master cell cycle (A), nitrogen fixation and BclA transporter (B) of SUTN9-2 Δ*nifV*, Δ*bclA*, and WT in response to rice extract (*indica*) at 21 days. Relative expression of rice hemoglobin gene (*hb1* and *hb5*) in response to SUTN9-2 Δ*nifV*, Δ*bclA*, and WT at 21 dpi (C). Significant at $P \leq 0.05$ is indicated by the mean standard deviation (n=3).

4.4.10 SUTN9-2 transcriptome in response to the rice extract (*indica*)

To analyze the RNAseq transcriptome, total RNA was purified from SUTN9-2 incubated in BNM-B medium in the presence or absence of rice extract. The main reason for performing the transcriptome analysis was to define the mechanisms and factors involved in cell differentiation and nitrogen fixation by endophytic SUTN9-2 in rice plants. The results showed that the expression of a large number of genes was significantly altered (FDR value ≤ 0.1) in response to rice extract, with 365 genes being significantly differentially expressed, representing 63.8% of the differentially expressed genes (DEGs) (Fig. 4.9A). The highest percentage of the differentially expressed gene was 42.1 % in the treated cells of SUTN9-2 WT in BNM-B with rice extract group compared to the SUTN9-2 WT in BNM-B alone (Fig. 4.9A). Besides, the impact of Δ*bclA* in rice extract was also found, as 42.1% of genes were differentially regulated in the treated cells of WT SUTN9-2 in BNM-B with rice extract compared to Δ*bclA* in BNM-B with rice extract (Fig. 4.9A). These results revealed the influence of rice extract and the *bclA* gene on the expression of genes in SUTN9-2.

The highest differentially upregulated genes of SUTN9-2 WT in response to rice extract corresponded to biphenyl-2,3-diol 1,2-dioxygenase (PWE78131.1; 2.33 $\log_2$ fold change (logFC)) and the second was 3- (3-hydroxyphenyl) propanoate hydroxylase (PWE78129.1; 1.85 logFC) (Fig. 4.9D and E). Both these genes belong to the class of oxidoreductase catalytic enzymes, involved in the degradation of plant-related compounds and acting with the oxygen atoms, which incorporate into the substrate. The third and fifth were molecular chaperon GroEL (PWE76243.1; 1.42 logFC) and GroES (PWE81524.1; 1.34 logFC), which are required for the proper folding of many proteins and the function of nitrogen fixation regulatory

protein NifA. The results from RNAseq analysis indicated that genes involved in nitrogen fixation and nitrogen metabolism were not significantly differentially expressed in response to rice extract (Fig. 4.9E), whereas the qRT-PCR results showed that the nitrogen fixation genes (*nifH* and *nifV*) were upregulated (Fig. 4.8B), as the expression was detected in rice plants (Piromyou et al., 2017; Greetatorn et al., 2019). However, there is a key difference among the two experiments to consider. The experimental set-up for transcriptome analysis was performed on a bigger scale than the experimental set-up for qRT-PCR to obtain the high enough amount of the SUTN9-2 cells in rice extract for RNA purification and transcriptome analysis. This might be affected by the different oxygen levels in the two experimental set-ups, which perturbed the *nif* genes expression in the transcriptome experiment.

The differentially upregulated genes in response to rice extract were also found to code for a protein involved in the cationic peptide transport system ATP-binding process. These include genes involved in the ABC transporter ATP-binding protein (PWE82048.1; 0.84 logFC) and ABC transporter substrate-binding protein (PWE77331.1; 7.5 logFC) (Fig. 4.9D, E, and Table 4.3). The gene coding for efflux pumps was also found to be differentially upregulated in the presence of rice extract. This gene belongs to resistance nodulation and cell division family (RND) efflux system (PWE80966.1; 1.15 logFC) (Fig. 4.9D, E, and Table 4.3), that might be involved in bacterial defense against toxic-plant metabolites. These suggesting the presence of cationic antimicrobial peptide (CAMP) in rice plants which has toxicity and effect on bacterial cell differentiation. The significantly differentially downregulated expressed genes of SUTN9-2 WT were related to flagella (PWE80412.1, PWE80420.1, and PWE80424.1) in the response to rice extract (Fig.

4.9E). In contrast, these genes were upregulated in SUTN9-2 Δ*bclA* (PWE80424.1) in the response to rice extract (Fig. 4.9D), indicating the influences of rice extract and Δ*bclA* on cell motility.

4.4.11 qRT-PCR validation

qRT-PCR was performed for significantly DEGs, involving biphenyl-2,3-diol 1,2-dioxygenase (*hpaD*), 3-(3-hydroxyphenyl) propanoate hydroxylase (*mhpA*), chaperonin GroEL (*groEL*), chaperonin GroES (*groES*), ABC transporter ATP-binding protein (*sapDF*), ABC transporter substrate-binding protein (*sapA*), and RND family efflux transporter (*cusF*) in SUTN9-2 WT in BNM-B with rice extract group compared to the SUTN9-2 WT in BNM-B alone. The results showed that most of the genes were upregulated in response to rice extract similar to those that were observed in transcriptome analysis. The expression of *mhpA* seem to be more obviously upregulated in response to rice extract (0.09-fold) followed by *sapDF* (0.07-fold), *cusF* (0.07-fold), *groEL* (0.06-fold), *hpaD* (0.05-fold), *sapA* (0.04-fold), and *groES* (0.01-fold), respectively (Fig. 4.10).

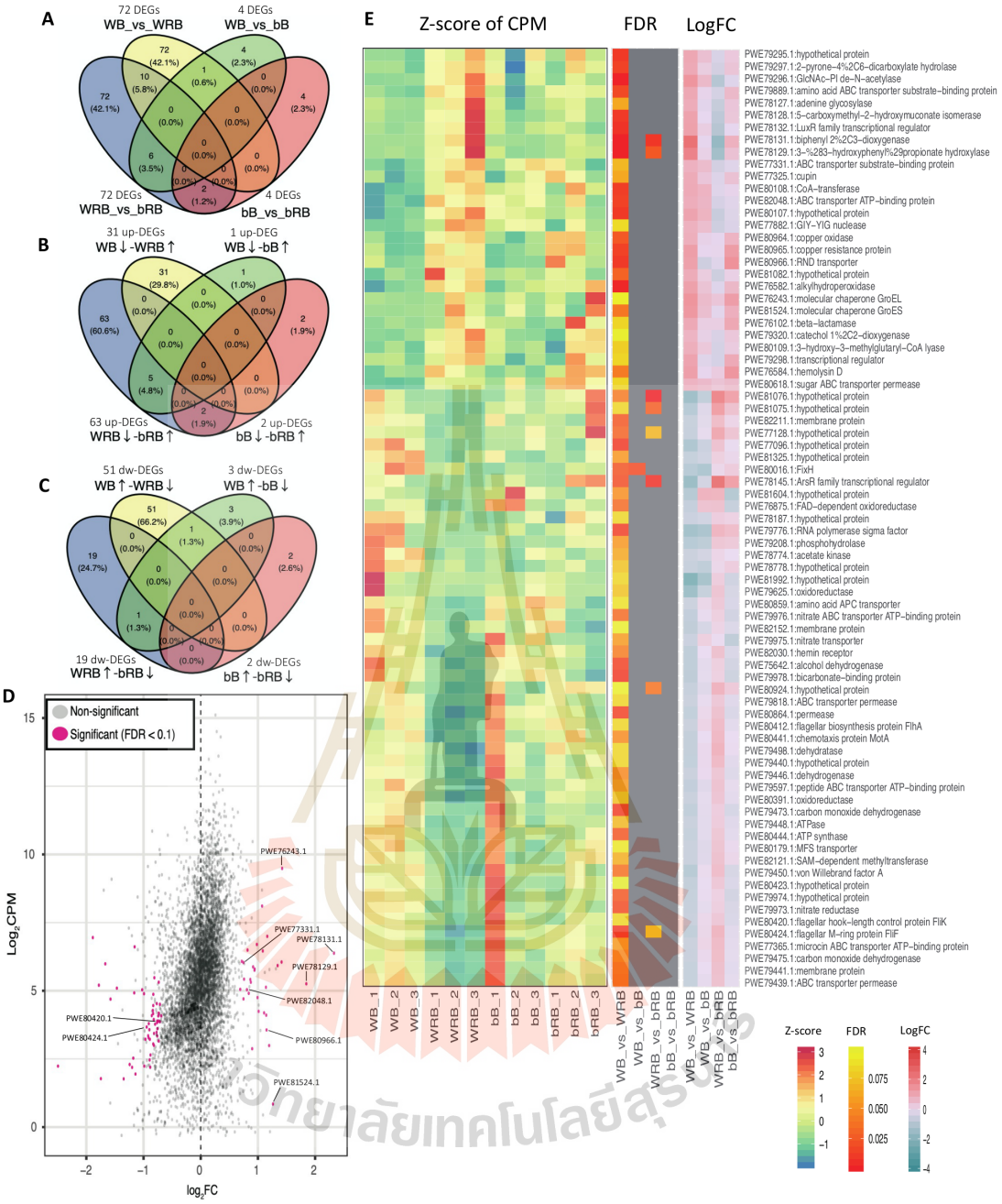

Figure 4.9 Differentially expressed genes (DEGs) of SUTN9-2 WT versus Δ*bclA* in response to BNM-B (WB and bB) and BNM-B + rice extract (WRB and bRB). Venn diagram of all DEGs of WB_vs_WRB, WB_vs_bB, WRB_vs_bRB, and bB_vs_bRB (A), showing the upregulated genes (B), and downregulated genes (C). MA plot of DEGs upregulated and downregulated genes of WB_vs_WRB and genes of interest were labeled

(D). Expression pattern of DEGs of WB, bB, WRB, and bRB. FDR (≤ 0.1) and logFC of DEGs of WB_vs_WRB, WB_vs_bB, WRB_vs_bRB, and bB_vs_bRB. The color scale bars for the normalized expression (E).

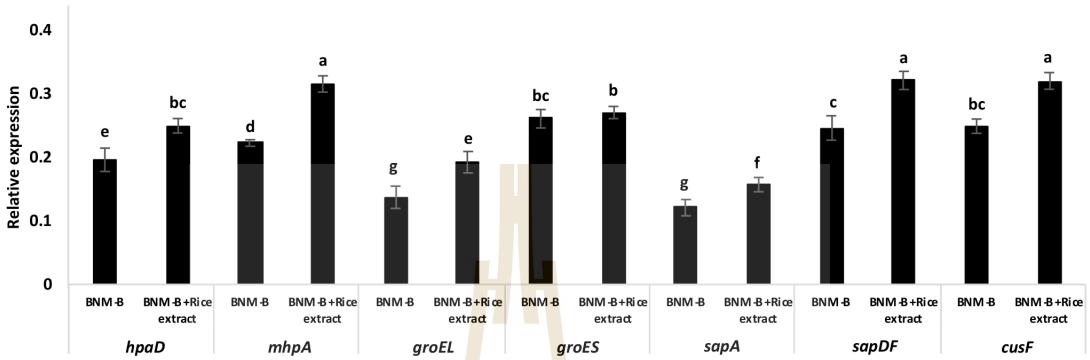

Figure 4.10 qRT-PCR analysis data for significantly DEGs of SUTN9-2 WT in response to BNM-B alone compared to BNM-B with rice extract. Significant at $P \leq 0.05$ is indicated by the mean standard deviation (n=3).

Table 4.3 Differentially upregulated gene expression of SUTN9-2 WT in response to rice plant extract by transcriptome analysis.

Protein_ID	Predicted function	logFC	logCPM	*P* value	FDR
PWE78131.1	biphenyl 2, 3-dioxygenase	2.33	6.38	1.24E-12	8.23E-09
PWE78129.1	3-3-hydroxyphenyl propionate hydroxylase	1.85	5.26	3.45E-09	1.07E-05
PWE76243.1	molecular chaperone GroEL	1.42	9.49	0.00	0.09
PWE79296.1	GlcNAc-PI de-N-acetylase	1.41	6.05	4.85E-09	1.07E-05
PWE81524.1	molecular chaperone GroES	1.34	5.92	2.27E-04	0.04
PWE80965.1	copper resistance protein	1.20	2.99	4.14E-05	0.01
PWE76584.1	hemolysin D	1.16	7.00	2.53E-04	0.04
PWE80966.1	RND transporter	1.15	3.56	1.93E-05	0.01
PWE78132.1	LuxR family transcriptional regulator	1.14	4.11	6.92E-06	0.01
PWE76582.1	alkylhydroperoxidase	1.14	5.13	7.73E-07	0.00
PWE78128.1	5-carboxymethyl-2-hydroxymuconate isomerase	1.11	4.19	1.95E-05	0.0

Cont.

Table 4.3 Differentially upregulated gene expression of SUTN9-2 WT in response to rice plant extract by transcriptome analysis.

Protein_ID	Predicted function	logFC	logCPM	*P* value	FDR
PWE80107.1	hypothetical protein	1.09	6.46	1.14E-05	0.01
PWE79298.1	transcriptional regulator	1.07	8.11	9.09E-04	0.08
PWE79295.1	hypothetical protein	0.99	4.75	1.48E-05	0.01
PWE79889.1	amino acid ABC transporter substrate-binding protein	0.98	6.70	6.40E-05	0.02
PWE79297.1	2-pyrone-4%2C6-dicarboxylate hydrolase	0.94	5.80	4.62E-05	0.01
PWE80108.1	CoA-transferase	0.92	5.88	2.73E-05	0.01
PWE77325.1	cupin	0.90	2.87	0.00	0.06
PWE81082.1	hypothetical protein	0.88	5.31	1.42E-04	0.03
PWE78127.1	adenine glycosylase	0.87	5.41	5.31E-04	0.06
PWE82048.1	ABC transporter ATP-binding protein	0.84	4.91	3.80E-05	0.01
PWE80109.1	3-hydroxy-3-methylglutaryl-CoA lyase	0.82	6.49	7.62E-04	0.08

Cont.

Table 4.3 Differentially upregulated gene expression of SUTN9-2 WT in response to rice plant extract by transcriptome analysis.

Protein_ID	Predicted function	logFC	logCPM	*P* value	FDR
PWE80964.1	copper oxidase	0.81	5.04	1.01E-04	0.02
PWE77882.1	GIY-YIG nuclease	0.76	5.43	9.81E-04	0.09
PWE77331.1	ABC transporter substrate-binding protein	0.75	6.04	6.17E-04	0.07
PWE80618.1	sugar ABC transporter permease	0.72	6.08	8.02E-04	0.08
PWE79320.1	catechol 1, 2-dioxygenase	0.69	5.05	0.00	0.10

Log_2 fold change (logFC) represent upregulation in the presence of rice extract

Log count per million (logCPM) represent differentially expression degree

Probability value (*P* value)

False discovery rate (FDR) value for RNAseq

4.5 Discussion

The results presented here indicate that SUTN9-2 undergoes major changes in cell size and nitrogen fixation efficiency in response to rice extract (Fig. 4.2). The statistically significant increase in the elongated cell size of SUTN9-2 was associated with a considerable increase in the nitrogen fixation efficiency. Similarly, a terminal bacteroid differentiation (TBD) process occurs before effective nitrogen fixation is established. These bacteroids are enlarged and polyploid and have lost their capacity to produce progeny (Mergaert et al., 2006; Alunni and Gourion, 2016). The small bacteroid size (1 – 2.5 μm) in Alfalfa (*M. sativa*) nodules had low nucleic acid content, and are inactive in acetylene reduction (4.9 μmol C_2H_2 reduced/10^{10} bacteroids). The enlarged bacteroid size (5 – 7 μm) had relatively high nucleic acid content and were very active in acetylene reduction (83.3 μmol C_2H_2 reduced/10^{10} bacteroids) (Paau et al., 1978). The bacteroid of bradyrhizobia in *A. afraspera* and *A. indica* have a differentiation along with a high DNA content, such that the mean DNA content of free-growing *Bradyrhizobium* sp. ORS285 was 1 to 2 genome complement (1C to 2C) and the bacteroid was 7C to 16C ploidy levels (Guefrachi et al., 2015).

Cell differentiation of *Bradyrhizobium* bacteroids occurred by the interaction of defensin-like antimicrobial peptides (DEFs) found especially in Inverted Repeat-Lacking Clade (IRLC) and Dalbergioids legume families with the action of BclA transporter (Guefrachi et al., 2015). Based on a phylogenetic tree of SbmA_bacA domain proteins, three genes of *Bradyrhizobium* sp. strain ORS285 were revealed. Two genes (BRAO285v1_250005 and BRAO285v1_950010) are in *Bradyrhizobium* homologous clade and one gene (BRAO285v1_1320006) is in BclA clade (Guefrachi et al., 2015). However, only the mutant in gene BRAO285v1_1320006 had abnormal

(undifferentiated) bacteroid cells, and a strongly reduced in nitrogen fixation activity on the host plants *Aeschynomene* spp. (Guefrachi et al., 2015). Similarly, SUTN9-2 Δ*bclA* in this study belongs to the BclA clade. This gene was disrupted according to high homology with BclA of ORS285 (Fig. 4.11). From the KO [KEGG (Kyoto Encyclopedia of Genes and Genomes) Orthology] annotation in the reconstruction pathway, the ABC transporter involving cationic antimicrobial peptide resistant (CAMP) of SUTN9-2 Δ*bclA* was perturbed by the deletion of ABC transporter ATP-binding domain (*bclA*; PWE81210.1) belonging to the sensitivity to antimicrobial peptides transporter (SapDF) (Lopez-Solanilla et al., 1998). However, transcriptomic analysis showed that ABC transporter substrate-binding protein consisting of SapA (PWE77331.1) and ABC transporter ATP-binding protein consisting of SapDF (PWE82048.1) were observed as the significantly differentially upregulated gene in response to rice extract (Fig. 4.9D and E, Table 4.3). A phylogenetic analysis based on the sequence of the SbmA_BacA domain proteins shows that SapA (PWE77331.1) and SapDF (PWE82048.1) identified in SUTN9-2 are distant from the BclA and *Bradyrhizobium* homologous clades identified in *Bradyrhizobium* spp. However, both of these genes showing their 97% similarity to these clades (Fig. 4.11) (Guefrachi et al., 2015). These results indicated that SapA (PWE77331.1) and SapDF (PWE82048.1) may play an important role together with SUTN9-2 Δ*bclA* (PWE81210.1; data not shown) for cell differentiation, nitrogen fixation in symbiosis, and response to rice extract, as cell differentiation of SUTN9-2 Δ*bclA* was still maintained in both systems of bacteroid in legume plants (Fig. 4.6) and bacteria in response to rice extract (Fig. 4.3 and 4.5). However, a decrease in elongated cell size and nitrogen fixation ability of Δ*bclA* and Δ*nifV* was also observed. These indicated the effects of *bclA* and *nifV* genes

on cell size and nitrogen fixation ability of SUTN9-2 cell in response to rice extract. The *sap* mutant pathogenic bacterium *Erwinia chrysanthemi* was sensitive to killing by antimicrobial peptides (AMPs) (wheat α-thionin and snaking-1) from potato tubers (Lopez-Solanilla et al., 1998). The Sap transporter consists of the SapABCDFZ, which share homology to a family of 'ATP-binding cassette' (ABC) family of transporters that are diverse in substrate binding and uptake (Hiles et al., 1987; Abouhamad et al., 1991; Parra-Lopez et al., 1993). SapA is predicted to function as a periplasmic solute-binding protein; SapB and SapC as inner membrane permease proteins; SapD and SapF as ATPase subunits; whereas the function of SapZ remains unknown (Mason et al., 2006). Moreover, previous studies have identified several genes encoding cysteine-rich peptides (CRPs), also suggesting several uncharacterized AMPs in rice plants (Silverstein et al., 2007), and genes encoding DEFs were also detected in rice plants (Tantong et al., 2016; Li et al., 2017). These results implied to the production of DEFs in rice plant interacting Sap transporter of SUTN9-2, affecting cell enlarged size and high DNA content in SUTN9-2 as in response to rice extract (Fig. 4.12), similar to cell differentiation and polyploidy of bacteroid in legume plants affected by NCR peptides.

Moreover, the gene coding for RND transporter efflux pumps (PWE80966.1) was also found to be differentially upregulated in SUTN9-2 WT when exposed to rice extract (Fig. 4.9D and E). Efflux pumps are transport proteins involved in the extrusion of toxic substrates into the external environment (Coutinho et al., 2015). RND efflux system has been recognized to play an important role in the successful colonization of the apple tree by the phytopathogen *Erwinia amylovora* (Burse et al., 2004). The RND efflux systems are highly upregulated in *B. kururiensis* M130 in the presence of rice extract (Coutinho et al. , 2015). This type of efflux system might be important in

bacterial survival defense against toxic-plant metabolite including DEFs (Fig. 4.12).

It was evaluated that flagellar biosynthesis is mostly downregulated in the symbiotic bacteroid cell. The transcriptome analysis of *Mesorhizobium loti* revealed that genes for flagellar formation were strongly repressed under the symbiotic condition because rhizobia under the symbiotic condition would not need motility (Uchiumi et al., 2004; Tatsukami et al., 2013). In addition, the relative expression level of SUTN9-2 flagella biosynthetic protein (*fliP*) in response to rice root exudate was lower than that without rice root exudate. Also, the expression level of *fliP* slightly decreased with increase in plant age (Piromyou et al., 2015b). Similarly, the differentially downregulated expressed genes of SUTN9-2 WT were related to flagellar biosynthesis (PWE80412.1, PWE80420.1, and PWE80424.1) in response to rice extract. In contrast, these genes were upregulated in Δ*bclA* (PWE80424.1) in response to rice extract (Fig. 4.9D). This upregulation has also been observed in the transcriptome experiments with *B. kururiensis* in response to rice extract, to allow bacteria to escape host defense responses (Coutinho et al., 2015). Taken together, the downregulation of flagellar biosynthesis might be supporting the cell differentiation of SUTN9-2 performed in response to rice extract, similar to bacteroids in the symbiotic condition. In contrast, Δ*bclA* lacking some parts of the transporter against plant AMPs, upregulate genes for flagellar biosynthesis, to allow bacteria to move faster in plant environment for escaping host defense responses (Fig. 4.12).

The rice extract (*O. sativa* L. ssp. *indica*) has the effects on cell elongation and nitrogen fixation ability in SUTN9-2, which are more pronounced than that of rice extract (*O. sativa* L. *japonica*), in all treated cells of SUTN9-2 WT, Δ*bclA*, and Δ*nifV* (Fig. 4.2D, E, F, and 4.3). The smallest elongated cell size was observed in Δ*nifV*

compared to WT and Δ*bclA* SUTN9-2. The decrease in elongated cell size was observed in Δ*bclA* compared to WT SUTN9-2 (Fig. 4.2D, E, F, and 4.3). When SUTN9-2 WT was inoculated into the Thai rice cultivar Pathum Thani 1 (*indica*) and Japanese rice cultivar Nipponbare (*japonica*), the SUTN9-2 WT cell number at 30 dpi in Thai rice root tissues (*indica*) was higher than that in Japanese rice (*japonica*), with 10^3 and 10^1 CFU g^{-1} root fresh weight, respectively (Piromyou et al., 2015b). The population density of SUTN 9-2 was larger in the Thai rice (*indica*) than in Japanese rice (*japonica*). Besides, Thai bradyrhizobial strain SUTN9-2 could promote total dry weight of rice (*indica*) better than that of Japanese bradyrhizobial strains (Piromyou et al., 2015a). On the other hand, rice (*indica*) responded positively only to putative Thai rice endophytic bradyrhizobia, while this phenomenon was not observed in Japanese rice (*japonica*) (Piromyou et al., 2015a; Piromyou et al., 2015b). In addition, type III secretion system (T3SS) of SUTN9-2 is involved in bradyrhizobial infections in rice plants. The densities of SUTN9-2 T3SS mutant in Thai rice (*indica)* were significantly lower than that of SUTN9-2 WT, this property was not detected in Japanese rice (*japonica*), indicating SUTN9-2 had the ability to overcome native host rice defense responses through the function of T3SS, and also rice developed a system to protect themself from non-native soil bacteria (Piromyou et al., 2015b). These results implied that rice cultivar and bacterial strain are the important factors that control the compatibility of the rice-bacterium association which may contribute to the bradyrhizobia-host evolution (Piromyou et al., 2015b). These results supported that there exists a preferable host for SUTN9-2 in rice species, which might also influence the increased ability of cell size enlargement and nitrogen fixation of SUTN9-2 in response to rice extract from *indica* than that of *japonica*.

The attenuated pattern of *ctrA* expression in NCR-treated cells during the cell cycle could be caused by reduced GcrA activity or by defects in the CtrA autoregulation pathway. DnaA likely is needed for the repeated rounds of DNA replication initiation during endoreduplication *in vivo* (Collier, 2012). CcrM DNA methyltransferase is present and active for a short time and essential for methylation at the start of DNA replication (Collier, 2012). In this study, the results suggested that AMPs in rice extract might affect the master cell-cycle regulators by suppressing the expression of *ctrA* and promoting *dnaA* and *ccrM* expression, resulting in the increase in cell size and DNA content (Fig. 4.8A, 4.3, and 4.4). In addition, *nifV* gene might also affect cell size enlargement when exposed to rice extract. The expression of genes involved in nitrogen fixation (*nifH* and *nifV*) of SUTN9-2 have been detected in endophytic association with rice plants (Piromyou et al., 2017; Greetatorn et al., 2019). Also, the expressions of *nifH*, *nifV*, and *bclA* in WT and Δ*bclA* SUTN9-2 were increased in response to rice extract. In contrast, the expression of these genes in SUTN9-2 Δ*nifV* was decreased in response to rice extract (Fig. 4.8B). The results suggest the similarity of the expression model between these genes and the genes involved in the master cell cycle, when exposed to rice extract. Furthermore, the effect of the interaction between the rice extract and BclA transporter on cell differentiation and nitrogen fixation was implied based on the expression of cell cycle and *nif* genes in response to rice extract (Fig. 4.12). Non-symbiotic hemoglobins (nsHbs) have been detected in several monocot plants. Rice (*Oryza sativa*) contains five copies of the *nsHb* gene, namely *hb1-hb5* (Lira-Ruan et al., 2002). The *hb1*, *hb2*, and *hb5,* express in both, the rice embryonic organs and vegetative organs, and their apparent function is an oxygen carrier or participate in some aspects of the oxygen metabolism (Garrocho-Villegas et al., 2008; Lira-Ruan et

al., 2011). Hormone and stress-response promoters exist upstream of the rice *hb5* gene and it was seen to be transcribed in rice organs. The amino acid sequence and the protein model structure of Hb5 are different from the rice Hbs 1 to 4 (Garrocho-Villegas et al., 2008), suggesting different expression levels between *hb1* and *hb5* (Fig. 4.8C). However, they are present in very low concentrations inside the host rice cell. In addition, the physiological functions of rice nsHbs have shown that they are not involved in oxygen transport, but more closely resemble known oxygen sensors (Goodman and Hargrove, 2001). This observation revealed that nsHbs in rice plants might function as a regulator to keep low oxygen partial pressure for nitrogenase activity to facilitate the nitrogen-fixing endophytic cell (Fig. 4.12).

The RNAseq experiment provided a global view of the gene expression profile in response to rice extract. The RNAseq results indicated that the SUTN9-2 endophytic cell was affected by rice extract and Δ*bclA* mutation as observed in the results for significantly differentially expressed genes (Fig. 4.9A). The expression of genes involved in cell cycle and nitrogen fixation were upregulated when SUTN9-2 was exposed to rice extract. These results are in disagreement with the observation from the transcriptome analysis of SUTN9-2 in the presence of rice extract. These genes did not show significantly differentially expressed genes in the response to rice extract. Bacteria capable of utilizing biphenyl and phenylpropanoid compounds as carbon and energy source are wildly distributed in natural environments, and they can originate from putrefaction of proteins in soil or as breakdown products of several constituents of plants, such as lignin, various oils, and resins (Ferrandez et al., 1997; Díaz et al., 1998; Wesche et al., 2005). The highest upregulated differentially expressed genes involved in biphenyl-2,3-diol 1,2-dioxygenase (PWE78131.1) and 3- (3-

hydroxyphenyl) propanoate hydroxylase (PWE78129.1) were observed. These genes belong to the class of oxidoreductase acting on the donors with oxygen and incorporation of the atoms of oxygen into the substrate (Díaz et al., 1998; Wesche et al., 2005), which may have a role in controlling the oxygen level to be appropriate for nitrogenase activity (Fig. 4.9D and E). This suggests the excess oxygen in the experimental set-up for transcriptome analysis experiment (50 ml tube), disturbing nitrogenase activity. A bigger scale (50 ml tube) was performed to obtain the high amount of the SUTN9-2 cell in rice extract for transcriptome analysis, bigger than the experimental set-up for qRT-PCR (10 ml tube). The nitrogenase enzyme complex is highly sensitive to molecular oxygen, which irreversibly inactivates the enzyme. The inhibition of *nif* gene expression by molecular oxygen at the nitrogen regulatory protein NifA post-transcriptional stage, was found in *B. japonicum* (Fischer and Hennecke, 1987; Kullik et al., 1989). Therefore, the suppression in the nitrogen fixation genes observed by transcriptome analysis might be due to the effect of excess oxygen on the nitrogenase sensitivity (Fig. 4.12).

The molecular chaperone GroEL (PWE76243.1) and GroES (PWE81524.1) were shown to be significantly differently upregulated genes in response to rice extract (Fig. 4.9D and E). GroESL chaperonins are required for the formation of a functional nitrogenase in *B. japonicum*, which is co-regulated together with symbiotic nitrogen regulatory gene *nifA* and transcribe by σ^{54} RNA polymerase (Fischer et al., 1993; Fischer et al., 1999). However, it has been also reported that the requirement of chaperonins for nitrogen fixation does not occur at the level of RegSR-NifA-σ^{54}- or FixLJ-FixK-dependent gene regulation (Fischer et al., 1999). This indicated that the nitrogen fixation of SUTN9-2 in response to rice extract might be affected by GroESL

chaperonins with or without Nif-dependent gene regulation. These reports implied the possibility that the nitrogen fixation of SUTN9-2 might be induced in response to rice extract, probably as the differentially upregulated genes of GroESL chaperonins were detected (Fig. 4.12).

Base collectively on the results and transcriptomic findings reported here, a proposed model of cell differentiation and nitrogen fixation of SUTN9-2 in response to rice extract was presented (Fig. 4.12). Rice plant was predicted to produce some AMPs that are recognized during interactions by AMPs recognition receptor (Sap ABC transporter family), promoting the import of AMPs and protecting SUTN9-2 cell against the antimicrobial activity of these peptides. Then, upon recognition and transduction of these AMPs, several DEGs are induced in SUTN9-2. These AMPs predictably modulate master cell cycle regulators, thereby causing cell differentiation. These interactions induced several processes, including, oxidoreductase, GroESL chaperonin, RND efflux system, and flagellar biosynthesis that might promote cell size enlargement, nitrogen fixation, and eventually the rice growth (Fig. 4.12). These observations implied the striking similarities in the mechanism and factors involved in cell differentiation and nitrogen fixation, between endophytic cells in rice plants and symbiotic cells in legume plants, which is based on similar mechanisms from both the bacterial side (BclA-like transporters) and the plant side (AMPs). It is important to understand the mechanism of regulation of the factors, molecules, and signal of the plant and bacterial cell involved in inducing cell differentiation and nitrogen fixation in endophytic cells required for *in-planta* survival and plant growth promotion. The specialized legume plant genes involved in symbiotic interaction may have arisen from a pre-existing non-symbiotic plant gene such as rice plants, suggest convergent coevolution in these distant plant species.

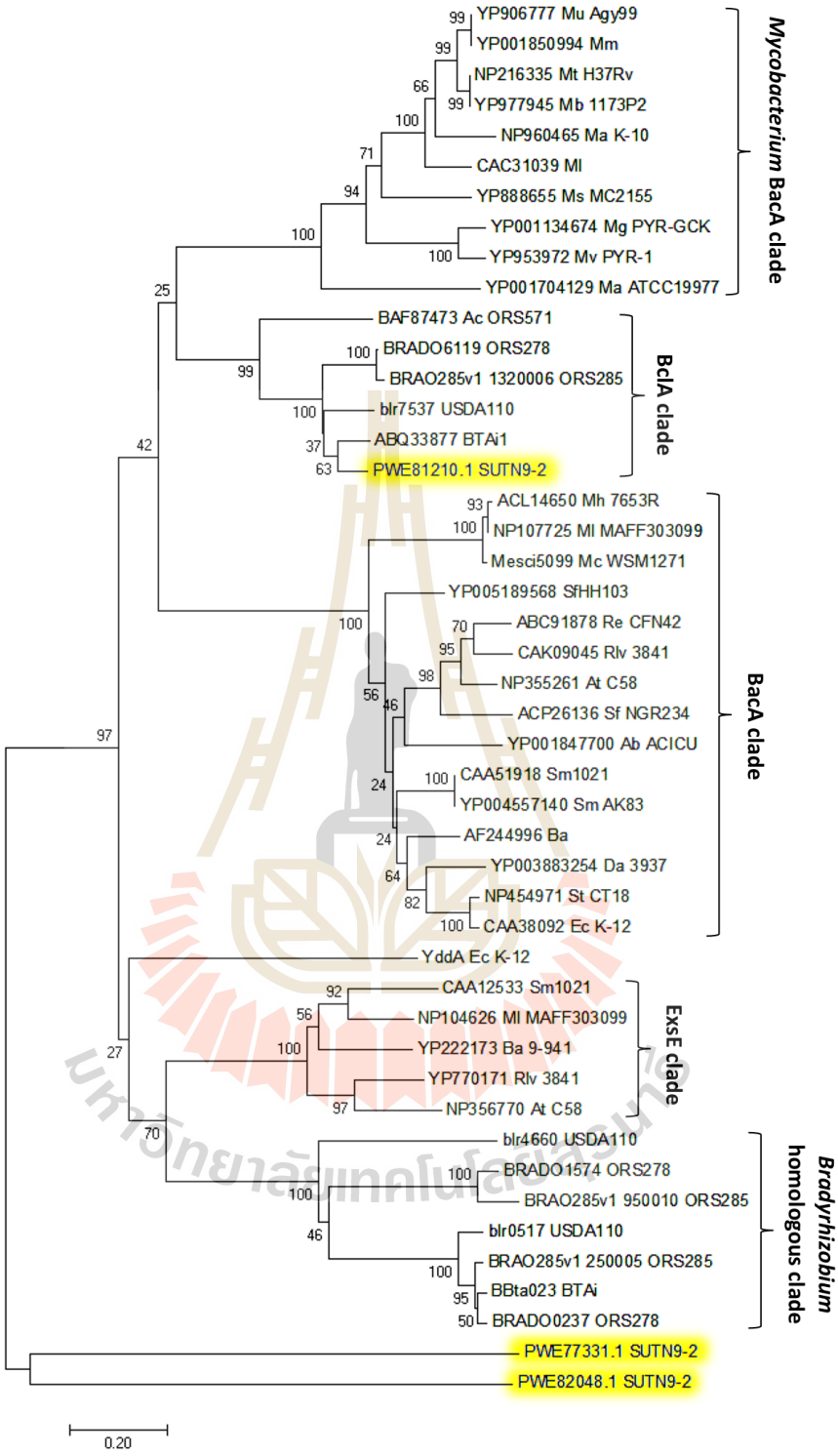

Figure 4.11 Phylogenetic tree base on SbmA_BacA domain proteins. The neighbor-joining tree was generated from a ClustalW alignment with bootstrap 500 replicates. The identified protein sequences including BacA,

Bradyrhizobium homologous, ExsX, and *Mycobacterium* BacA clades were obtained from Guefrachi et al. (2015). The BacA-related protein sequences of SUTN9-2 were obtained from the NCBI database.

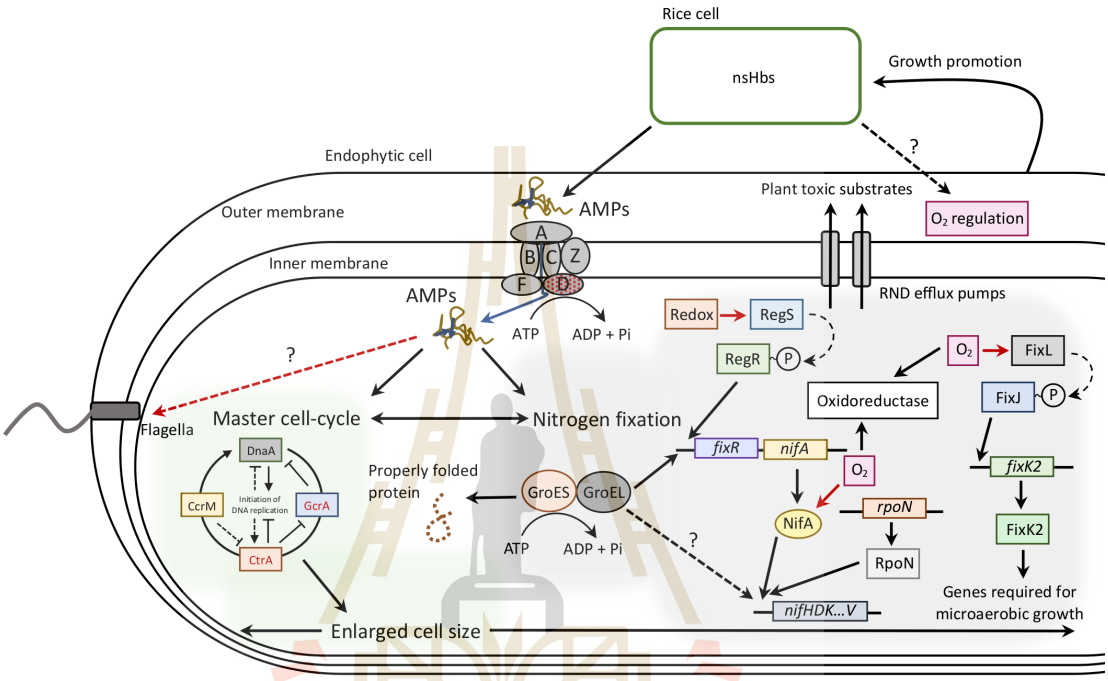

Figure 4.12 Working model of cell differentiation and nitrogen fixation by SUTN9-2 exposed to rice extract. The currently implied AMPs from rice plants are predictably recognized by BclA ABC transporter belonging Sap ABC transporter family-like AMPs recognition receptor. The AMPs are targeted to the cell membrane of bradyrhizobia, localizing BclA or Sap transporter, promoting the import of AMPs and providing protection against the antimicrobial activity of these peptides. The AMPs affect the master cell cycle regulators by reducing GcrA activity and CtrA autoregulation pathway, but promoting DnaA and CcrM for DNA replication initiation, resulting in an increase in DNA content and enlarged cell size. Later, a defect in flagellar activity during cell

differentiation was observed. This is followed by an increased nitrogen fixation ability correlating cell enlarged size. The excess oxygen perturbs nitrogen regulatory protein NifA and reduced nitrogen fixation ability. Oxidoreductase may have a role in controlling the oxygen level to be appropriate for nitrogenase activity. GroESL co-regulated with nitrogen regulatory gene *nifA* and *rpoN* RNA polymerase for the formation of a functional nitrogenase. The successful colonization of bradyrhizobia in rice plants is triggered by the RND efflux system and then induced rice plant hemoglobin to regulate low oxygen partial pressure and facilitate the nitrogen-fixing endophytic cell.

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APPENDIX

APPENDIX

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

Compounds	Volume (g)
D-Mannitol	10
K_2HPO_4	0.5
$MgSO_4 \cdot 7H_2O$	0.2
NaCl	0.1
Yeast Extract	0.5

Add distilled water to 1 L

Adjust pH to 6.8 with 0.1 N NaOH

N-free Rice Nutrient Solution (Mae and Ohira, 1981)

	Compounds	Chemical formula	MW	Stock conc. (M/100 ml)	Volume (ml/L)
1	Main Element	$NaH_2PO_4 \cdot 2H_2O$	119.89	0.6	1
		K_2SO_4	174.26	0.3	1
		$MgCl_2 \cdot 6H_2O$	203.30	0.6	1
2	Trance Element	H_3BO_3	61.83	0.05	1
		$MnSO_4 \cdot 5H_2O$	169.01	0.009	1
		$CuSO_4 \cdot 5H_2O$	249.68	0.0003	1
		$ZnSO_4 \cdot 7H_2O$	287.56	0.0007	1
		$Na_2MoO_4 \cdot 2H_2O$	241.95	0.0001	1
3		EDTA-Fe	385.05	0.045	1
4		$CaCl_2 \cdot 2H_2O$	147.01	0.3	1
5	Nitrogen source	NH_4NO_3	80.40	1	1

Adjust pH to 6.8

Buffered Nodulation Medium B (BNM-B) Minimal Medium solution stock (Ehrhardt and Atkinson, 1992)

Compounds	Chemical formula	Volume (g)
200X Nod major salts	KH_2PO_4	6.8
	$MgSO_4$, $7H_2O$	12.2
200X Nod minor Salts I	$ZnSO_4$, $7H_2O$	0.46
	H_2BO_3	0.31
	$MnSO_4$, H_2O	0.845
200X Nod minor salts II	Na_2MoO_4, $2H_2O$	0.025
	$CuSO_4$, $5H_2O$	0.025
	$CoCl_2$, $6H_2O$	0.025
200X Fe-EDTA (protect from light by cover with aluminum foils)	Na_2EDTA dihydrate	1.865
	$FeSO_4$, $7H_2O$	1.39

Add H_2O to 500 ml each

1000X Mixed Vitamin Solution Stock

Compounds	Volume (mg)
Biotin	1.1
Folic acid	1.1
PABA (para-amino)	110
Riboflavin	110
Pantothenic acid	110
Pyridoxine HCI	220
Thiamine HCl	220
Niacinamide	220

Adjust H_2O/50% EtOH to 1000 ml

Protect from light by cover with aluminum foils

BNM-B medium's plant preparation

Compounds	Volume
CaCl2, 2H2O	0.344 g
MES buffer	0.39 g
200X Nod major salts	5 ml
200X Nod minor Salts I	5 ml
200X Nod minor salts II	5 ml
200X Fe-EDTA	5 ml

Add H2O to 1L

Adjust pH at 6.5 with 2 or 5 N KOH

BNM-B medium's bacterial nitrogen fixing preparation

Compounds	Volume
HEPES	11.9 g
200X Nod major salts	5 ml
200X Nod minor Salts I	5 ml
200X Nod minor salts II	5 ml

Add before sterilize at 120 °C for 20 min

Add H_2O to 1L

Adjust pH at 6.5 with 2 or 5 N KOH

Compounds	Volume
1M Succinate	2 ml
0.4M $CaCl_2$	1 ml
200X 200X Fe-EDTA	1 ml
1000X Mixed vitamin solution	200 µl

Mix all solution and filtrate through 0.45 µm syringe filter

Add to sterilized BNM-B medium 200 ml

BIOGRAPHY

Ms.Teerana Greetatorn was born on September 12, 1989, at Ratchaburi, Thailand. She graduated with a Bachelor's Degree of Science with first-class honor from Crop Production Technology, Institute of Agricultural Technology, Suranaree University of Technology in 2012. In the same year, she obtained a scholarship for outstanding students from Suranaree University of Technology to continue her studied in the School of Biotechnology and graduated with a Master's Degree of Science in 2015. She was awarded a scholarship from the Royal Golden Jubilee Ph.D. program in 2015 to continue her Ph.D. degree in the same field. During her study, she presented research work in the 4thAsian Conference on Plant-Microbe Symbiosis and Nitrogen Fixation, October 16-19, 2016, Penang, Malaysia. Japanese Society of Plant-Microbe Interactions, the 28th annual meeting, September 19-21, 2018, Faculty of Agriculture, Tottori University, Tottori, Japan. The 5th Asian Conference on Plant-Microbe Symbiosis and Nitrogen Fixation, May 15–17, 2019, Tohoku University (Katahira Campus), Sendai, Japan (Best poster award; in "Mechanism of rice endophytic bradyrhizobial cell differentiation and its role on nitrogen fixation"). The TSRI Congress on Disruptive Technology for World Society, August 8-9, 2019, Siam Paragon, Bangkok, Thailand. She has the experience to improve her knowledge regarding cell differentiation and nitrogen fixation at Kagoshima University, Kagoshima, Japan for one year, and RNA sequencing analysis at National Institute for Basic Biology, Okazaki, Aichi, Japan for one month. In 2019 and 2020, she has published her research in a title of empowering rice seedling growth by endophytic *Bradyrhizobium* sp. SUTN9-2, and another in a title of mechanism of rice endophytic bradyrhizobial cell differentiation and its role on nitrogen fixation, respectively.