DEVELOPMENT OF RECOMBINANT scFv ANTIBODY FOR POINT OF DEMAND (POD) DETECTION OF *Bradyrhizobium* STRAIN SUTN9-2 IN RICE-LEGUME CROPPING SYSTEM

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biotechnology

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การพัฒนาแอนติบอดีปรับแต่งพันธุกรรมชนิดเส้นเดี่ยว เอสซีเอฟวี (scFv) เพื่อการตรวจวิเคราะห์ เชื้อแบคทีเรียแบรดดี้ไรโซเบียม สายพันธุ์ SUTN9-2 (*Bradyrhizobium* strain SUTN9-2) ในระบบการปลูกพืชหมุนเวียนข้าวสลับถั่ว

นางสาวเจ๊าะ เค คาย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
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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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เจ๊าะ เค คาย : การพัฒนาแอนติบอดีปรับแต่งพันธุกรรมชนิดเส้นเดี่ยว เอสซีเอฟวี (scFv) เพื่อการตรวจวิเคราะห์ เชื้อแบคทีเรียแบรดดี้ไรโซเบียม สายพันธุ์ SUTN9-2 (*Bradyrhizobium* strain SUTN9-2) ในระบบการปลูกพืชหมุนเวียนข้าวสลับถั่ว (DEVELOPMENT OF RECOMBINANT scFv ANTIBODY FOR POINT OF DEMAND (POD) DETECTION OF *Bradyrhizobium* STRAIN SUTN9-2 IN RICE-LEGUME CROPPING SYSTEM) อาจารย์ที่ปรึกษา : ศาสตราจารย์ ดร.ภญ.มณฑารพ ยมาภัย, 132 หน้า.

แบรดดี้ไรโซเบียมเป็นแบคทีเรียชนิดหนึ่งที่อาศัยในดินมีความสามารถในการตรึงไนโตรเจนจากบรรยากาศมาให้กับพืชตระกูลถั่วที่เชื้ออยู่อาศัยร่วมกันแบบภาวะพึ่งพาได้ โดยสามารถเปลี่ยนก๊าซไนโตรเจนเป็นแอมโมเนียเพื่อใช้เป็นปุ๋ยให้แก่พืช ภาวะแบบพึ่งพาอาศัยกันนี้กลายเป็นปัจจัยสำคัญประการหนึ่งในการเกษตรเนื่องจากเป็นกลไกที่นำแหล่งไนโตรเจนปริมาณมหาศาลกลับสู่ชีวมณฑลของโลก จากคุณสมบัติที่เป็นประโยชน์นี้ แบคทีเรียชนิดนี้ถูกใช้เป็นปุ๋ยชีวภาพสำหรับระบบการปลูกพืชหมุนเวียนข้าวสลับพืชตระกูลถั่วอย่างยั่งยืน ทั้งนี้การตรวจสอบติดตามและการศึกษากระบวนการทางชีวภาพของแบคทีเรียตรึงไนโตรเจนที่อยู่อาศัยร่วมกันกับพืชนี้เป็นสิ่งจำเป็นสำหรับการศึกษาวิวัฒนาการของจุลินทรีย์ที่มีปฏิสัมพันธ์ต่อพืช ไมโครไบโอมในดิน ตลอดจนการควบคุมคุณภาพของแบคทีเรียที่ใช้เป็นปุ๋ยชีวภาพในการ ทำเกษตรอินทรีย์ ในขณะที่เทคโนโลยีการแสดงแอนติบอดีบนผิวเฟจมีการใช้กันอย่างแพร่หลายในการผลิตแอนติบอดีปรับแต่งพันธุกรรม เพื่อวัตถุประสงค์ทางการแพทย์ แต่มีรายงานการใช้เทคโนโลยีนี้น้อยมากในภาคเกษตรกรรม ในวิทยานิพนธ์นี้ แอนติบอดีปรับแต่งพันธุกรรมชนิดเส้นเดี่ยว เอสซีเอฟวี (scFv) ที่มีความจำเพาะต่อเชื้อแบคทีเรียแบรดดี้ไรโซเบียม สายพันธุ์ SUTN9-2 (โคลน yiN92-1e10) และ สายพันธุ์ DOA9 (โคลน yiDOA9-162) ที่ถูกคัดเลือกมาจากคลังแอนติบอดีบนผิวเฟจของมนุษย์ ซึ่งความจำเพาะในการจับและการวิเคราะห์ปฏิกิริยาการจับแบบข้ามสายพันธุ์แบคทีเรียของเอสซีเอฟวี ได้นำมาทดสอบโดยใช้หลักการ อีไลซ่า (ELISA) และเทคนิคการถ่ายภาพคอนโฟคอล-อิมมูโนฟลูออเรสเซนซ์ (confocal-immunofluorescence) ทั้งนี้การใช้วิธีที่ไม่ใช่จีเอ็มโอนี้ สามารถระบุตำแหน่งของเชื้อ SUTN9-2 ได้ทั้งในรูปแบบเอนโดไฟต์ติก (endophytic) ภายในเนื้อเยื่อข้าว และแบคทีเรียที่เปลี่ยนแปลงรูปร่างไปแล้ว (bacteroid) ในปมรากพืชตระกูลถั่วได้ นอกจากนี้ได้มีการเปรียบเทียบการใช้แอนติบอดีปรับแต่งพันธุกรรม ในการศึกษาการแข่งขันเข้าครอบครองปมรากของแบคทีเรียเปรียบเทียบกับวิธีการย้อมสี GUS แบบมาตรฐาน (Gus-staining method) ซึ่งพบว่าวิธีการใหม่นี้ให้ผลการทดลองไม่แตกต่างจากวิธีมาตรฐาน ดังนั้นผลการศึกษานี้จึงเป็นครั้งแรกที่แสดงถึงศักยภาพของการใช้แอนติบอดีมนุษย์บนผิวเฟจแบบเอสซีเอฟวี ในการถ่ายภาพและการตรวจติดตามปุ๋ยชีวภาพ

แบรดดี้ไรโซเบียมที่สามารถนำไปใช้สำหรับการตรวจสอบติดตามหัวเชื้อแบคทีเรียในระบบการปลูกพืชหมุนเวียนข้าวสลับถั่วได้ จากนั้นเอสซีเอฟวีได้ถูกพัฒนาให้เป็นแอนติบอดีเต็มรูปแบบ (IgG) และประสบผลสำเร็จในการผลิตในเซลล์มนุษย์ (Expi293F™) ซึ่งแอนติบอดีเต็มรูปแบบนี้สามารถพัฒนาเป็นสารตรวจสอบในการตรวจติดตามเชื้อแบคทีเรียแบรดดี้ไรโซเบียมในปุ๋ยชีวภาพเชิงพาณิชย์ และในดินจากพื้นที่เกษตรกรรม ทั้งในเชิงคุณภาพ และปริมาณด้วยการวิเคราะห์แบบแถบ (lateral flow) และเทคนิค flow cytometry ได้ในอนาคต นอกจากนี้ได้ทำอิพิโทปแมปปิ้ง (epitope mapping) โดยวิธีการไบโอแพนนิ่ง (bio-panning) จากคลังเฟจแสดงเปปไทด์แบบสุ่ม (SUT 12) ซึ่งเปปไทด์เลียนแบบ (mimotope) ที่ระบุได้นี้ สามารถใช้ในการทำนายแอนติเจนที่อยู่บนผิวของแบคทีเรีย อีกทั้งสามารถนำไปพัฒนาสำหรับใช้เป็นตัวควบคุมในชุดตรวจสอบสายพันธุ์แบรดดี้ไรโซเบียม ได้ในอนาคต

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2563 ลายมือชื่ออาจารย์ที่ปรึกษา_____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

KYAUT KAY KHAING : DEVELOPMENT OF RECOMBINANT scFv ANTIBODY FOR POINT OF DEMAND (POD) DETECTION OF *Bradyrhizobium* STRAIN SUTN9-2 IN RICE-LEGUME CROPPING SYSTEM. THESIS ADVISOR : PROF. MONTAROP YAMABHAI, Ph.D., 132 PP.

RECOMBINANT ANTIBODY/BACTERIOPHAGE DISPLAY/MONOCLONAL ANTIBODIES/BRADYRHIZOBIUM/ENDOPHYTES/RICE-LEGUME/ NITROGEN FIXATION/BIOFERTILIZERS/SYMBIOSIS

Bradyrhizobium is one of the soil bacteria that can fix atmospheric nitrogen in symbiosis with specific legumes and convert nitrogen gas into ammonia to be used as fertilizer in plants. This symbiosis becomes one of the significant factors in agriculture because it supplies huge quantity of nitrogen to the world biosphere. In order to take this advantage, this bacterium is currently used as biofertilizer for sustainable legume-rice rotational cropping system. Monitoring and bioimaging of this nitrogen fixing bacterium is essential for the study of plant-microbe evolution, soil microbiome, as well as quality control of its used in organic farming. While phage display antibody technology has been widely used to generate recombinant antibody for numerous medical purposes, very few of its application in agricultural sector has been reported. In this thesis, recombinant single-chain variable fragments (scFv) against *Bradyrhizobium* strains SUTN9-2 (yiN92-1e10) and DOA9 (yiDOA9-162) were isolated from human phage display antibody library. The binding specificity and cross-reactivity of the isolated soluble scFv was evaluated by ELISA and confocal-immunofluorescence imaging techniques. By using this non-GMO method, SUTN9-2

localization in both endophytic and bacteroid forms could be observed inside rice tissue and plant nodule, respectively. Moreover, successful application of the recombinant antibody for the evaluation of nodule occupancy was demonstrated in comparison with standard Gus-staining method. The results of this study revealed for the first time the potential use of human phage display scFv antibody for imaging and monitoring of *Bradyrhizobium* biofertilizer and thus could be further applied for point-of-detection of bacterial inoculum in the legume-rice rotational crop system. In addition, the scFv fragment was engineered to generated full length IgG, successfully expressed in human cell (Expi293F™). This IgG format has potential to be used as detecting reagent for lateral flow and flow cytometry immunoassays to detect *Bradyrhizobium* qualitatively and quantitatively in commercial biofertilizers and soil in agricultural field in the future.

Moreover, epitope mapping was performed by bio-panning of phage display random peptide library (SUT-12). The identified peptide mimotope can be used to predict the antigen on the surface of the bacteria and develop as a control for the point of demand (POD) diagnosis of *Bradyrhizobium* strains in the future.

School of Biotechnology

Academic Year 2020

Student's Signature_____

Advisor's Signature_____

Co-Advisor's Signature_____

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Kyaut Kay Khaing

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LIST OF ABBREVIATIONS

Amp	=	Ampicillin
bp	=	Base pairs
BSA	=	Bovine serum albumin
cfu	=	Colony forming units
CDR	=	Complementary determining region
CH1	=	Constant heavy chain 1
CH2	=	Constant heavy chain 2
CH3	=	Constant heavy chain 2
DNA	=	Deoxyribonucleic acid
dNTP	=	Deoxynucleotidyl triphosphates
E. coli	=	*Escherichia coli*
ELISA	=	Enzyme-linked immunosorbent assay
et al.	=	et alia (and other)
Hr	=	hour
HRP	=	Horse radish peroxidase
IgG	=	Immunoglobulin class
IMAC	=	Immobilized metal affinity chromatography
mM	=	millimoles
OD	=	Optical density
PAGE	=	Polyacrylamide gel electrophoresis
PBS	=	Phosphate buffer saline

LIST OF ABBREVIATIONS (Continued)

pH	=	Log of hydrogen ion concentration
RT	=	Room temperature
scFv	=	Single chain Fv antibody derivative
SDS	=	Sodium dodecyl sulphate
UV	=	Ultraviolet
µg	=	Microgram
kDa	=	(kilo) Daltons
µl	=	Microlitre
µM	=	Micromoles
°C	=	degree celsius
g	=	grams
g	=	times gravity
h	=	hours
Kg	=	kilograms
L	=	litre
m	=	meter
M	=	molar
mg	=	milligram
min	=	minute
ml	=	milliliter
rpm	=	revolutions per minute
sec	=	seconds

LIST OF ABBREVIATIONS (Continued)

v/v	=	volume per unit volume
w/v	=	weight per unit volume

CHAPTER I

INTRODUCTION

1.1 Significant of this study

Edible cereal grains, including rice, are the most important sources of energy for human beings. Large amounts of chemical nitrogen fertilizers based on Haber-Borsch process are used every year for promoting plant growth and productivity of cereals (Rosenblueth et al., 2018). Chemical nitrogen, on the other hand, faces a number of environmental and societal issues, such as the use of a huge amount of fossil fuel in the production process, which contributes to greenhouse effects and global warming. Besides, human and aquatic animals could be harmful by algae blooms due to nitrogen pollution because of excessive nitrogen fertilizer usage (Rosenblueth et al., 2018). Moreover, farmers in impoverished areas are facing the obstacles concerning with accessibility of chemical fertilizer regularly. The diazotrophic bacteria generate biofertilizers by biological nitrogen fixation (BNF), and these biofertilizers are alternative source for chemical fertilizer in cereal production (Zaim et al., 2017). This method is more effective and environmentally friendly than the chemical methods. Nevertheless, rhizobium-plant interactions are deeply complicated, particularly for nitrogen-fixing symbiosis with legumes since rhizobia are phylogenetically, metabolically, and genetically diverse (Masson-Boivin et al., 2009). Among diazotroph diversity, *Bradyrhizobium* is a genus of slow-growing Gram-negative soil bacteria that can fix nitrogen symbiotically with specific legumes by converting nitrogen gas into

ammonia, which is then used as fertilizer in plants (Zaim et al., 2017). This symbiotic interaction becomes one of the significant factors in agriculture, supplying enormous amount of nitrogen to the global ecosphere (Masson-Boivin et al., 2009). *Bradyrhizobium* can live in both leguminous and non-leguminous plants by symbiotically and endophytically associations. *Bradyrhizobium* sp. strain SUTN9-2 live as endophytes in rice tissues that can fix nitrogen as well as produce plant hormone (indole acetic acid; IAA) and the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, both of which can diminish plant stress and promote plant growth (Piromyou et al., 2017). As a result, this bacterium has the potential to be used as a biofertilizer/biostimulant in a rice-legume crop rotation system. Because rhizobium-plant host symbiosis is highly specific (Masson-Boivin et al., 2009), it is necessary to distinguish and choose a superior strain to be used as rhizobium inoculants in a biofertilizer for specific crops (Gunnabo et al., 2020). After application in rice-legume cropping systems, monitoring rhizobium inoculant is also essential to determine its persistence and efficiency in promoting plant growth (Piromyou et al., 2017). Additionally, the precise identification of specific diazotrophic bacteria in different plant hosts will be applicable for the study of plant-microbe interactions, the evolution of symbiotic nitrogen-fixing bacteria, and the control of nodulation and intracellular infection in plant hosts (Masson-Boivin et al., 2009).

At present, for identifying and monitoring rhizobia, the enzyme-linked immunosorbent assay (ELISA) has been extensively applied among various immunological methods. Despite this, polyclonal antibodies showed cross-reaction with other rhizobial strains within the same species Olsen et al., (1998). This cross-reaction is a crucial concern when using polyclonal antibodies to detect and monitor

specific rhizobia. The monoclonal antibody exhibited high specificity for detection of *Rhizobium trifolli* 162x95 by indirect ELISA (Wright et al., 1986). Traditional methods of producing monoclonal antibodies, on the other hand, are extremely laborious, time consuming, and costly for production. Additionally, all of the assays rely on the animal-derived-antibodies (Ahmad et al., 2012).

Because of cost-effective for long-term production and possessing the ability to acquire for diverse biosensor formats, permitting easy accessibility to a wide range of ultimate users, recombinant antibodies become popular to accompany or substitute hybridoma technology for monoclonal antibody production (Brichta et al., 2005). Phage display technology applies libraries of recombinant bacteriophages that expose the antibody on their surface, allowing for the isolation of recombinant antibodies with the desired binding affinity against the antigen via the repeated selection procedure (Burioni et al., 2012). The main advantage of this technology is the direct linkage between phage phenotype and its encapsulated genotype (Hoogenboom et al., 1998), which allows for further engineering or mass production of recombinant antibodies from *Escherichia coli* or other proper expression hosts without any utilization of experimental animals (Pansri et al., 2009). In 2018, this technology has been awarded a Nobel Prize in chemistry (Barderas & Benito-Peña, 2019).

A single chain fragment variable (scFv) recombinant antibody format is highly desirable among the various recombinant antibody formats. A scFv molecule is made up of a variable region of heavy and light chains linked by a flexible peptide linker. The good points of scFv molecules include their compact size, long storage stability, and ability to be engineered and allowing for scale-up of their production (Ahmad et al., 2012). The specific peptides and monoclonal antibodies against a variety of targets

such as pathogens, viruses, parasites, and mycotoxins were successfully isolated by using phage antibody display technology (Huang et al., 2012; Min & Yamabhai, 2021; Rangnoi et al., 2011; Sidhu, 2001).

High specificity and affinity of probes is the fundamental core of a successful diagnostic system (Scott & Smith, 1990). Chemical/physical stability is required to apply these probes to a wide spectrum of diagnostic methods (Kim et al., 2006). Although scFv recombinant antibody has been extensively used as a standard platform for biological detection system (Stockwin & Holmes, 2003), accumulating reports have identified nucleic acids or peptides as alternative probes with comparable (or higher) binding and specificity. Phage display of randomized peptide libraries is a standard technology for selecting peptides that target specific molecules, and this approach has been extensively used in various pharmaceutical biotechnologies (Balass et al., 1993; Fong et al., 1994; Gough, Cockburn, et al., 1999; Saggio & Laufer, 1993).

In the rhizobial research aspects, phage-display of rabbit scFv antibody technology was applied for the specific detection *Bradyrhizobium* sp. strain DOA9 (Vu et al., 2017). Rabbit antibody, on the other hand, necessitates animal immunization and sacrifice, and recombinant antibodies from rabbit are generally difficult to express (Ayyar et al., 2015). A naive human phage display antibody library is an interesting alternative source of recombinant antibody against rhizobium because possibly that human population has been exposed to several rhizobia in the environment (Pansri et al., 2009). Up until now, phage display with human antibody libraries has been mostly used for therapeutic purposes. The use of phage display technology to generate specific human recombinant scFv antibodies and to screen polypeptides binding to recombinant human IgG antibody against beneficial soil bacteria have never been reported yet. In

this study, the specific human recombinant scFv antibodies against two *Bradyrhizobium* strains, DOA9 and SUTN9-2 have been successfully produced by phage-display antibody technology. Application of the recombinant antibody for detection, bio-imaging and monitoring of this beneficial rhizobium in symbiosis with and endophytic associations in leguminous (mung bean) and non-leguminous (rice) hosts is demonstrated. The effectiveness of this new method is compared with the standard method that employed β-glucuronidase (GUS) reporter system. In addition, the scFv fragment was engineered to generated full length IgG, and the mimotope matched with protein sequences of *Bradyrhizobium* sp. SUTN9-2 was successfully identified from the phage display of a 12-mers random peptide library (SUT12) by using this reformatted full-length IgG as target. These specific ligands could further be applied as target instead of bacterial cell antigen for detection of *Bradyrhizobium* strains in agricultural fields in the future.

1.2 Research objectives

To develop scFv-based immunodetection of *Bradyrhizobium* strain SUTN9-2 for the study of plant-microbe interaction and applications in agricultural field.

Specific objectives for this study are:

1. To evaluate the binding property of scFv by different methods of immunodetection, i.e., ELISA, Immunofluorescence assay
2. To apply the scFv-based immunodetection method for monitoring rhizobia in rice-legume cropping system and determining percentage of nodule occupancy
3. To identify the mimotopes that are capable of binding to antibody against *Bradyrhizobium* strains

1.3 Hypothesis

This study hypothesized that the binding property of scFv could be properly evaluated by ELISA and immunofluorescence staining. The scFv-based immunodetection methods could be applied for monitoring rhizobia in rice-legume cropping system, and nodule occupancy percentage.

1.4 Scope of this study

The binding property of human scFv antibodies was examined by ELISA, Immunofluorescence assay. The source of antibody genes was from human origin. Experiments on the binding affinity and application of scFv antibody in rice-legume cropping system and quantification of nodule occupancy percentage. The high affinity and specificity ligands were successfully isolated against recombinant human IgG from phage display peptides library, used as point of demand (POD) detection reagent for further application in different biosensor formats.

CHAPTER II

LITERATURE REVIEWS

2.1 Nitrogen fixation

Nitrogen is a fundamental component of amino acids, nucleic acids, and numerous other biologically important organic and inorganic compounds and, consequently, a crucial element for the growth of all living organisms on the earth (Ferguson, 1998; Smil, 2002). Although this nitrogen elements exists as huge amount in the earth's atmosphere (78 % of dry air), the powerful triple bond between this nitrogen atoms makes N_2 biologically unavailable to most organisms (Jia & Quadrelli, 2014; MacKay & Fryzuk, 2004). To be utilized for organic processes, nitrogen must be fixed into more biologically accessible forms. The phenomenon of transformation of free nitrogen (molecular and elemental) into nitrogenous compounds (to make it accessible to the plants for absorption) is called nitrogen fixation. There are three common forms of nitrogen fixation. These include biological, atmospheric, and industrial nitrogen fixation. In total, approximately 380 tetragrams ($1x10^9$ kg) of nitrogen per year (Tg N/year) are fixed by these processes (Galloway et al., 2008).

2.1.1 Lightning activity

Most atmospheric nitrogen fixation takes place as a consequence of lightning action in a thunderstorm. Energy from lightning discharge is able to acquire responses that form nitrogen compounds from atmospheric N_2 (Noxon, 1976). Nitrogen dioxide (NO_2) is actually by far the most widely assessed product of that

responses (Noxon, 1976). The reaction is as follow:

$N_2 + O_2$ Lightning → Thunder 2NO (Nitric Oxide); $2NO + O_2 \rightarrow 2NO_2$

This NO_2 incorporates with rainwater related with thunderstorms during the rain to make nitrous acid (HNO_2) and nitric acid (HNO_3). The acids drop on the soil along with rainwater and react with the alkaline radicals to create water soluble nitrates (NO_3^-) and nitrites (NO_2^-).

$2NO_2 + H_2O \rightarrow HNO_2 + HNO_3$; HNO_3 + Ca or K salts → Ca $(NO_3)_2$ or K $(NO_3)_2$

The plant roots can easily uptake these soluble nitrates. Nitrogen fixation by atmosphere is estimated to contribute around 5 Tg N/year into worldwide nitrogen cycles (Galloway et al., 2008).

2.1.2 Haber-Bosch process

The discovery of the Haber-Bosch process in early 1900s and its successive industrialization has greatly expanded the total amount of nitrogen that is converted from atmospheric N_2 to NH_3 (Galloway et al., 2008). The Haber-Bosch process is an absolutely energy-intensive process that merges $3H_2$ and N_2 to produce $2NH_3$. It is the outcome of two reactions: the formation of H_2 and CO_2 from methane (CH_4) and steam (H_2O) utilizing a nickel catalyst and the transformation of N_2 and $3H_2$ to NH_3 using high pressure, heat, and an iron catalyst (Ertl, 1991). In 2005, it was estimated that inorganic nitrogen was adding 121 Tg N/year into global nitrogen cycles (Galloway et al., 2008).

2.1.3 Biological process

From the advancement of nitrogenase, around two billion years ago (Boyd et al., 2011) up until the extensive application of the Haber-Bosch process in the 1950s, all life acquired N from biological nitrogen fixation, with atmospheric nitrogen fixation

process representing an insignificant supporter to the supply of fixed nitrogen (Thamdrup, 2012). Since the increase in application of the Haber-Bosch process, the biological and industrial processes contribute comparably to N_2 fixation (Canfield Donald et al., 2010; Gruber & Galloway, 2008; Thamdrup, 2012).

Nitrogen fixation has a great agronomic, economic, and ecological impact due to the point that the accessibility of fixed nitrogen belongs to the factor that most often limits agricultural production all over the world (Smil, 2002). In fact, almost half of the current human population could not exist without utilization of the Haber-Bosch process for manufacturing of nitrogen fertilizers (Canfield Donald et al., 2010). Provided that over half of the fixed nitrogen supply that sustains earth's population is supplied biologically, there has been great interest in understanding how the nitrogenase enzyme achieves the difficult function of N_2 fixation at ambient temperature and pressure (Burgess & Lowe, 1996; Eady, 1996). An understanding of biological N_2 fixation might additionally function as the foundation for obtaining two highly advisable, though so far unfulfilled, goals: genetically providing higher plants with the capacity to fix their own nitrogen (Beatty & Good, 2011; Rubio & Ludden, 2008) and developing the better synthetic catalysts depend on the biological mechanism (MacLeod & Holland, 2013).

2.2 Biological nitrogen fixation (BNF)

The demand of agriculture production rises steadily resulting significantly greater fertilizer utilization (Wood, 1996). Nitrogen (N) utilized in fertilizer or manures which is not absorbed by crops can be leaked into the atmosphere as nitrogenous greenhouse gases (Flechard et al., 2007) or leached into ground water (Stout & Chaminade, 2007), with ensuing environmental implications. Instead of depending solely on utilization of

N fertilizer, substitutive resources are necessary to help build more sustainable agriculture system. Leguminous crops possess potentiality to satisfy this necessity because of their distinctive capacity to fix N biologically from the atmosphere, benefiting not only the legumes themselves but also the intercropped or successive crops. Biological nitrogen fixation is the transformation of atmospheric nitrogen into ammonia by symbiotic, associative and free- living bacteria (Dixon & Kahn, 2004).

Biological nitrogen fixation, a mechanism used only by definite prokaryotes, is catalyzed by a nitrogenase complex (Yan et al., 2010). Ammonia and a molecule of hydrogen gas is produced by activation of nitrogenase enzyme through the concurrent reduction of one N_2 and 2 H^+.

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

2.2.1 ***Bradyrhizobium***

Rhizobia are soil bacteria that fix N_2 (diazotroph) after getting established inside root nodules of legumes (Fabaceae). They are gram-negative chemoheterotrophic organotroph bacilli that thrive freely in the soil. Rhizobia could form the specialized organs, called nodules, on roots or stems of the plants in Fabaceae family. The nodule inside bacteria could convert atmospheric nitrogen into ammonia, providing the nitrogen requirements of both rhizobia and their host plants. In feedback way, the plant feeds sugars, proteins, and oxygen for the bacteria (Sessitsch et al., 2002).

Rhizobia have many different species like *Rhizobium, Azorhizobium, Mesorhizobium, Allorhizobium*, *Sinorhizobium* and *Bradyrhizobium*. The current genera of Rhizobiaceae are summarized in Table 2.1. *Bradyrhizobium* is one of gram-negative bacilli with a single polar flagellum. *Bradyrhizobium* is nitrogen fixing soil bacteria among soil microorganisms in symbiosis with specific legumes and transform

nitrogen gas into ammonia to be utilized as fertilizer in plants. Like other rhizobia, lots of members of this particular genus possesses the capacity to fix atmospheric nitrogen into forms readily accessible for other organisms to use. These bacteria are also key constituents of forest soil microbial communities, where strains isolated from these soils are not generally able to fix nitrogen or form nodules (VanInsberghe et al., 2015). They are slow-growing in comparison to *Rhizobium* species, which are considered fast-growing rhizobia. In a liquid medium, *Bradyrhizobium* species take 3-5 days to produce a modest turbidity and 6-8 hours to double in population size. They have a tendency to develop perfect with pentoses as carbon sources. Some strains (for example, USDA 6 and CPP) are able to oxidized carbon monoxide aerobically (King, 2003). *Bradyrhizobium* can form either specific or general symbioses. What this means that one species of *Bradyrhizobium* may only be able to nodulate one legume species, although other *Bradyrhizobium* species could nodulate several legume species (Somasegaran & Hoben, 1994). Ribosomal RNA is extremely conserved in this particular number of microorganisms, producing *Bradyrhizobium* incredibly difficult to apply as an indicator of species diversity. DNA-DNA hybridizations are used instead and show more diversity. There are many species of *Bradyrhizobium* which are isolated from the various leguminous crops. For example, *B. betae* was isolated from tumor-like root deformations on sugar beets, *B. elkanii, B. diazoefficiens* and *B. liaoningense* set up symbiosis with soybeans, *B. japonicum* nodulates soybeans, cowpeas, mung beans, and siratro, *B. yuanmingense* nodulates *Lespedeza*, *B. canariense* is found in Lupin and Serradella nodules and nodulates genistoid legumes (Rivas et al., 2009).

Aeschynomene americana L. (American jointvetch) cv. Glenn is an erect-climbing, annual or short-lived perennial, shrub-like tropical legume (Tobisa et al.,

2014). This weed is also generally found in rice fields of Thailand between July and December (Piromyou et al., 2015). *Bradyrhizobium* strain SUTN9-2 produced nitrogen fixing nodules on the stem and root of *A. americana* which is originated in rice fields of Thailand as leguminous weed. This strain has ability to colonize well in the intercellular spaces of rice tissues as endophytic bacteria until rice harvesting season, and enhanced plant growth by increasing the nitrogen fixation ability producing IAA and ACC deaminase (Piromyou et al., 2015). In addition, *Bradyrhizobium* strain SUTN9-2 are always found to establish symbiosis with mung bean (*Vigna radiata*) and the leading adapted strain for *V. radiata* symbiosis in Thailand. Consequently, SUTN9-2 is a classic strain as symbiotic and endophytic partnership for *V. radiata* and *O. sativa* (Piromyou et al., 2017).

Table 2.1 Current taxonomy of rhizobia (Martínez-Romero et al., 2000).

Genus	**Species**	**Host**
Allorhizobium	*A. undicola*	*Nepunia natans*
Azorhizobium	*A. caulinodans*	*Sesbania rostrata*
Bradyrhizobium	*B. elkanii*	*Glycine max*
	B. japonicum	*G. max*
	B. liaoningense	*G. max*
Mesorhizobium	*M. amorphae*	*Amorpha fructicosa*
	M. cicero	*Cicer arietinum*
	M. huakuii	*Astragalus*
	M. loti	*Lotus japonicas*
	M. mediterraneum	*C. arietinum*
	M. plurifarium	*Acacia, Leucaena*
	M. tianshanse	*Glycyrrhiza, Sophora, Glycine* and others

Table 2.1 (continued).

Genus	Species	Host
Rhizobium	*R. etli*	*Phaseolus vulgaris, Mimosa affinis*
	R. galegae	*Galega*
	R. gallicum	*P. vulgaris*
	R. giardinni	*P. vulgaris*
	R. hainanense	*Stylosanthes, Centrocema, Desmodium*
	R. huantlense	*Tephorisa*
	R. leguminosarum	*S. herbaceae*
	R. monglense	*Vicia, Trifolium*
	R. tropici	*Medicago runthencia, P. vulgaris, Leucaena*
Sinorhizobium	*S. arboris*	*A. senegal, Prosopis chilensi*
	S. fredii	*G. max*
	S. kostiense	*A. senegal, P. chilensi*
	S. medicae	*Medicago* spp.
	S. melitoti	*M. sativa*
	S. sahelli	*Sesbina*
	S. terangae	*Sesbina, Acacia*
	S. xinjiangense	*G. max*

2.2.2 Legume-*rhizobium* symbiosis

Legume-*Rhizobium* symbiosis is a crucial facet of symbiotic nitrogen fixation which have been applied in sustainable agriculture. In this symbiosis, legume plant is macro-symbiont and *Rhizobium* is micro-symbiont. The macro-symbiont legume belongs to Leguminaceae, divided into three subfamilies containing 700 genera and 14,000 species (Beringer et al., 1979). Micro-symbiont *Rhizobium* is nitrogen

fixing motile prokaryote identified exclusively by their capacity to nodulate legumes (Brahmaprakash & Sahu, 2012).

The specific symbiosis between rhizobia and legumes outcomes in the development of specialized structures, called root nodules, in which bacteria are able to turn dinitrogen into ammonia and provide it to the host plant in exchange for carbohydrates (Young, 1992). This prokaryotic-eukaryotic intimacy is based on an intricate molecular crosstalk between both partners, that is set up through the secretion of flavonoids and other compounds to the rhizosphere by leguminous plants, inducing the rhizobial lipo-chito-oligosaccharides, the so-called Nod factors (Wang et al., 2012).

Moreover, this particular association is likewise crucial as legume crops are able to enhance the soil nitrogen accessibility to other crops, for example cereals and also in land remediation (Aslam et al., 2003). Several leguminous crops could fix almost 200 to 300 kg N ha^{-1} or approximately 70 million metric tons N $year^{-1}$ (Brockwell et al., 1995).

2.2.2.1 Infection process

The legume-rhizobia interaction occurs when plants release signals, such as flavonoids or isoflavonoids, which are recognize by bacteria using a positively acting transcription factor, usually encoded by *nodD* (Downie, 2010; Recourt et al., 1989) (Fig 2.1). The type of secreted signals, as well as the NodD protein usually differ depending on the plant or bacteria, respectively (Downie, 2010). Following NodD and flavonoids interaction, NodD binds to highly conserved bacterial promoters called nod boxes and induces the expression of several genes directly related with the nodulation process (as those involved in the synthesis of Nod factors) (Fig 2.1).

The NodD regulator belongs to the LysR family of transcriptor regulators. Constitutively expressed, NodD is responsible for the transcription of other nodulation genes (e.g. *nodABC*) in the presence of compatible flavonoids released by legume plants, consequently initiating the nodulation process (Kondorosi et al., 1989; Oldroyd, 2013; Spaink, 2000). In addition, NodD also regulates directly or indirectly several other symbiotic phenotypes in rhizobia, such as polysaccharide production, phytohormone synthesis, motility, quorum-sensing and the activation of the type-III secretion system (Krause et al., 2002; Lopez-Baena et al., 2008; Pérez-Montaño et al., 2011; Pérez-Montaño et al., 2014; Theunis et al., 2004; Vinardell et al., 2004). The number of *nodD* copies can vary depending on the rhizobial species and accordingly it was found that species harboring only one copy have the nodulation completely aborted when this gene is mutated, while species with multiple *nodD* copies, a complex interaction between the *nodD* genes seems to occur and the nodulation is not totally suppressed (Broughton et al., 2000; Garcia et al., 1996).

Besides determining which legumes, the bacteria is able to nodulate (Downie, 1998; Perret et al., 2000), the Nod factors are responsible for root hair curl, infection thread formation, induction of cell division and gene expression in the root cortex and pericycle, nodule development and the number of nodules (Garg & Geetanjali, 2007; Laranjo et al., 2014; Oldroyd, 2013; van Brussel et al., 2002).

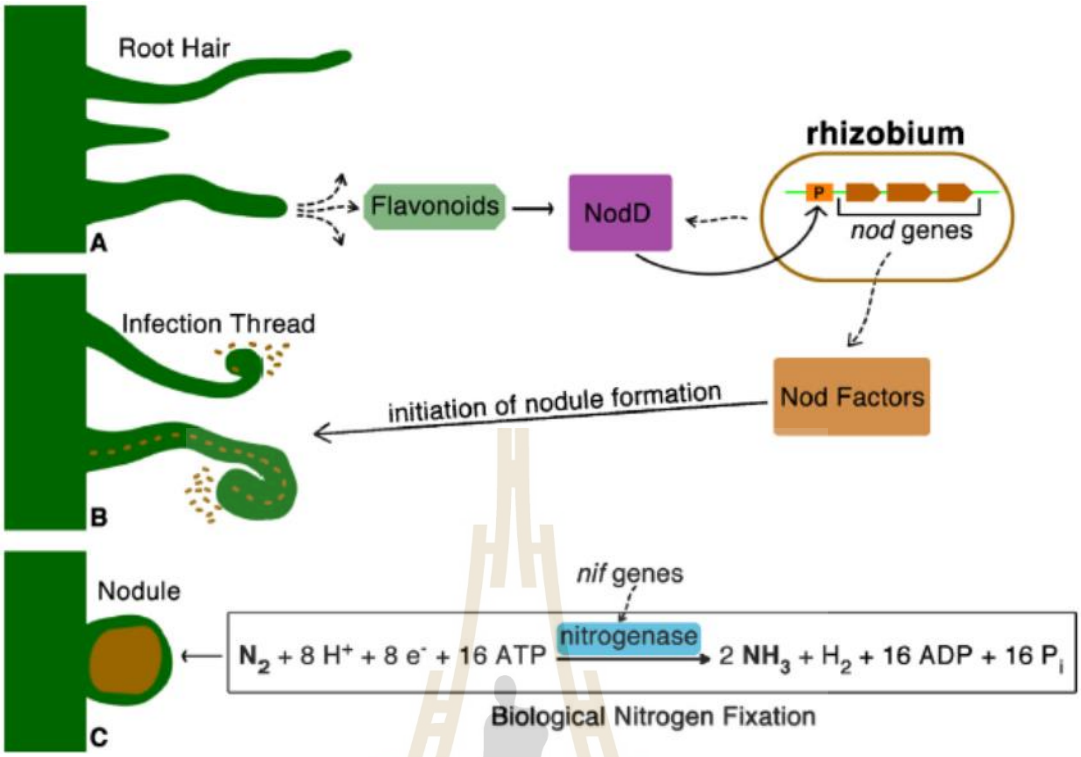

Figure 2.1 Schematic overview of the nodulation process and biological nitrogen fixation (Laranjo et al., 2014).

2.2.2.2 Nodule occupancy

The competition for nodule occupancy between strains of rhizobia in leguminous plants is a complex area of study of legume-*Rhizobium* symbiosis. The intrinsic characteristics of the rhizobia, environmental variables and genetic determinants of the host contribute to the failure or success of rhizobia strains to occupy a significant portion of nodules formed (Thies et al., 1992). A reliable and sensitive method of identifying specific strains in nodules is necessary for identification of rhizobia occupying nodules and assessment for competitiveness. This can be attained by several techniques (Aouani et al., 1997). Molecular markers, including the *gusA* gene expressing ß-glucuronidase (GUS) (Wilson et al., 1995) is powerful tools in the study of nodule occupancy. Nodules formed by Rhizobium marked with the *gusA* gene

turn blue upon treatment in buffer X-Gluc (5-bromo-4-chloro-3-indolyl-L-D-glucuronide cyclohexylammonium salt, while unmarked strains remain unstained. This process enables the visual detection of rhizobial occupants of nodules, because marked and unmarked strains can be easily distinguished. The simplicity of this system in identifying nodule occupants has many benefits to offer rhizobial ecology, because the assay is rapid and allows entire plant root systems to be tested (Wilson et al., 1999). Enzyme-linked immunosorbent assay (ELISA) and Polymerase chain reaction-Restriction fragment length polymorphism (PCR-RFLP) are the most used techniques for studying the symbiotic effectiveness of different rhizobia strains (Thies et al., 2001). Serological identification of rhizobia strains implies the application of antibodies generated against surface antigens of the test strain to detect the presence (or absence) of that strain in a suspension through agglutination, immunodiffusion, immunofluorescence or the enzyme-linked immunosorbent assay (ELISA) (Kishinevsky & Gurfel, 1980). The ELISA and immunofluorescence techniques are highly specific, reproducible, and frequently used to detect rhizobial strains directly from nodules. In addition, the method is precise, can detect antigens in small nodules, uses small amounts of reagents, is comparatively speedy, and allows the rapid screening of large nodule samples. It can also detect double strain occupancy of nodules (Spriggs & Dakora, 2009).

2.2.3 Rice -Legumes crops rotation

In rainfed lowland regions which are traditionally planted to only one crop of rice per year, land use can be optimized by using the pre- and/or post-rice wet period to grow-legume crops. Legumes are suitable rotational crops with rice because they: 1) can mature in 55-90 days, 2) can be grown as pre-rice crop when rainfall accumulation

reaches 100 mm/month or as post rice crop using the receding rain and residual soil moisture, 3) are acceptable crops because they are easy to prepare for consumption or to sell at the market, 4) are drought-tolerant and 5) are capable of using atmospheric nitrogen and contribute nitrogen to the soil (International institute of rural reconstruction, 1990).

Organic rice production has played an principal role in recent years in boosting the income of farmers in Northeast Thailand, due to expanding market demand in European countries since 2003 (Polthanee et al., 2008). One of its conditions at the production stage is that organic rice must be cultivated without chemical fertilizer and pesticides. With regard to soil fertility, compost, green manure and animal manure play an important role in improving the crop yield from organic rice farming. Legumes such as groundnut, mung bean (*Vigna radiata* (L.) Wilczek), soybean (*Glycine max* L.) and cowpea (*Vigna unguiculata* (L.) Walp) were well suited for rice-based cropping system in peninsular India (Gowda et al., 2001). Groundnut, because of its high oil and protein content was a major crop grow in the post-rainy or summer season crop in rice-fallows in India (Pratibha et al., 2013).

In Thailand, mung bean residues incorporated into the soil significantly increased plant height and tiller number per hill but had no significant effect on top dry weight per hill of the succeeding rice crop at panicle initiation stage (PI). At harvest, pre-rice mung bean significantly increased top dry weight per hill of rice. In addition, growing mung bean before rice provides the advantage of marketable grain of mung bean to 1.6 t/ha. The net economic return was found highest in growing mung bean alone with transplanted rice later (2,855US $/ha) (Polthanee et al., 2012).

2.2.4 Localization of rhizobia inside rice plant as endophytes

Beside rhizobia are able to fix atmospheric nitrogen by symbiotically with leguminous crops, they are able to endophytically colonize roots and stems of non-leguminous plants like corn, wheat, rice and canola (Biswas et al., 2000; Mia & Shamsuddin, 2010; Perrine-Walker Francine et al., 2007; R. K. Singh et al., 2006). These bacteria reside inside the roots and stems and promote growth of rice plant due to phytohormones, such as indole acetic acid (IAA) (Bhattacharjee et al., 2012; Chen et al., 2005), solubilization of phosphate (Bakhshandeh et al., 2014) , and ACC-deaminase production, which depletes levels of ethylene in roots (Chinnadurai et al., 2009). Vicariously, these bacteria prevent plants against pathogens by producing substances, like antibiotics and cell-wall degrading enzymes, or by inducing a systemic defense response (Dutta et al., 2008). It is known that rhizobia pierce cell walls of rice plant across radicular apertures in the secondary roots (Perrine-Walker Francine et al., 2007) and in this particular system, bacteria are able to pass through xylem to the plant aerial portion (Reddy et al., 1997; Yanni et al., 1997). Particularly in rice, rhizobia can enhance germination rates or seeds, stimulate the radicular growth and the aerial portion and increase the yield (Biswas et al., 2000; Yanni et al., 1997; Yanni et al., 2001; Yanni Youssef & Dazzo Frank, 2010). The major plant growth-promoting characteristics in rice is the indole acetic acid (IAA) production by four different pathways: indole-3-acetamide (IAM), indole-3-piruvate (IPyA), triptamide (TAM) and indole-acetonitrile (IAN) routes. Tryptophan is the key ancestor of IAA in almost all known pathways (Spaepen et al., 2007). The route IPyA, predominant in plants, were defined in bacteria of the genus *Bradyrhizobium* (Giraud et al., 2007). The enzyme tryptophan decarboxylase, involve in the route TAM was distinguished in

Mesorhizobium loti (Kaneko et al., 2000). Nitrile hydratase, possibly included in IAN route have been identified in *Rhizobim etli* (González et al., 2006) *Rhizobium leguminosarum* (Young *et al*., 2006), *Bradyrhizobium* sp. (Giraud et al., 2007).

Rice-legumes rotational cropping systems are useful for rice production in Thailand, since legumes can be planted after rice season and famer can earn some money from leguminous product. Moreover, legume-*Bradyrhizobium* can provide the nitrogen to soil fertility for zaanext rice cropping. Chaintreuil et al., (2000) reported that photosynthetic *Bradyrhizobium* strains, which are usually known to induce nitrogen-fixing nodules on the legume, are also natural true endophytes of the primitive rice *O. breviligulata* could significantly enhance cultivated rice production (Chaintreuil et al., 2000). Therefore, if we can select endophytic bradyrhizobia that can nodulated legume as well as can live inside the rice plant tissue, this selected *Bradyrhizobium* still persist in the soil (facilitated on the basis of oligotrophic characteristics) and perform symbiosis with legume or rice along the rotational cropping system to support the growth of plant. Thus, it is possible that rice stubble can be used as inoculum in field grown legumes rendering not necessary to supply chemical nitrogen fertilizer or often inoculate *Bradyrhizobium* to legume.

2.2.5 Antigens of rhizobia

Antigens of rhizobia can be categorized into somatic, flagella, and capsular, depending on their derivation. Somatic antigens are closely related to the rhizobial cell wall and are usually designated by the letter O. Some somatic antigens may be tightly bound to the cell wall, in which case they are not removed by washing of the cells. Therefore, these antigens are only detected when whole cells of rhizobia react with the antibody, as in agglutination or immunofluorescence. The somatic antigens that are

soluble and easily removed by washing are detected by precipitation in gel. Somatic antigens are also heat stable. They are the most specific of the three groups of antigens. The precipitating "internal antigens" are more widely shared and taxonomically significant. These are released from cells having fragile or broken walls. Because internal antigens are widely cross-reactive within and between species, they require recognition and interpretation in gel immunodiffusion. The flagella of the rhizobia are also antigenic and appropriately called flagella or H antigens. They are heat labile and are commonly detected by agglutination or immunofluorescence test. The extracellular antigens are surface antigens and are found outside the cell itself. They are usually designated by the letter K (Somasegaran & Hoben, 1994).

Generally, the antigens of cultures and nodules remain stable and antigenic stability is the main premise underlying the widely used serological practices reported formerly for serotyping root-nodules. Nevertheless, there are some differences between antigens of the nodule forms of *Rhizobium* and their parent cultures. The antigens between culture cells and nodule forms of 17 strains of *R. japonicum* were investigated by antisera against cultured cells (Means & Johnson, 1968). No detectable difference was observed between the two forms among 15 strains. Nonetheless, one strain nodule-bacteria cross reacted with a broad range of antisera than the parent culture. The reaction between boiled and non-boiled cells is the same (Means & Johnson, 1968).

Dudman (1971) used immunodiffusion to examine the antigens of cultures and nodule-bacteria of three strains of *R. japonicum*. Nodule-bacteria antigens from one strain lacked the full array of antigens of the cultured cells, and repeatedly formed spur reactions of partial identity with precipitin bands from the parent culture. Using this method, the ideal technique of autecology: the study of an individual organism in

its natural environment, could be used to the study of rhizobia, or to any other soil microorganism desired (Dudman, 1971).

2.3 Methods for detection and monitoring of rhizobia

Rhizobia which have extensive variations in such crucial characteristics as host specificity, invasiveness, and efficacy are inconspicuous from one another under the microscope. In addition, the rhizobial strain are very difficult to detect and monitor under the field condition (Somasegaran & Hoben, 1994).

2.3.1 The microbiological techniques

To characterize the rhizobial species, several morphological features, as well as biochemical and metabolic test were commonly utilized. This analysis consists of vitamin requirements, salt, acid and alkali tolerance, carbohydrate utilization, and resistance to antibiotics. Numerical analysis uses a wider range of biochemical and metabolic tests to differentiate among rhizobial species. The results obtained from numerical analysis support the proposal of several novel species of rhizobia. Rhizobial species vary in their enzymatic production and several enzymes have been found to be necessary for the symbiotic efficiency. Carbohydrate application properties are of taxonomic significance (Somasegaran & Hoben, 1994).

The possibility of low antibiotic resistance can also be used to characterize and identify rhizobial strains. When high density inoculant of a rhizobial strain is inoculated into media containing an antibiotic, a few cells may express resistance because of spontaneous genetic changes or mutations. The resistance of a rhizobial strain to a certain antibiotic is an applicable marker. If the mutant strain is used to inoculate a legume, then nodules occupied by that strain may be identified by plating nodule isolates on media containing the respective antibiotics. The mutant rhizobial

strain will grow on the antibiotic media and other bacteria will be suppressed. Streptomycin resistance is frequently used as a marker for rhizobia. Mutants resistant to this aminoglycoside are stable, have a low incidence of cross-resistance, and infrequently lose their symbiotic capacity. Besides streptomycin, spectinomycin and rifampicin have also been used (Olsen et al., 1996).

2.3.2 The DNA-based techniques

Today, the DNA based methods happen to be widely utilized for determining the genetic diversity of microorganisms. For rhizobial, various methods have been applied to determine genotypic diversity of rhizobial populations and to distinguish among rhizobial strains. These methods invlove random amplified polymorphic DNA (RAPD), two-primers RAPD (TP-RAPD), repetitive sequence-based PCR (rep-PCR) and amplified fragment length polymorphism (AFLP). The 3 main techniques of rep-PCR are enterobacterial repetitive intergenic consensus (ERIC)-PCR, repetitive extragenic palindromic (REP)-PCR and BOX-PCR. While RAPD utilizes a single primer to amplify the segments of DNA randomly throughout the genome, rep-PCR uses pairs of primers (for ERIC- and REP-PCR) or a single primer (for BOX-PCR) to amplify the intervals between conserved repeated sequences present in genome. In AFLP, total genomic DNA is digested and then ligated to oligonucleotide adapters. A pair of specific primer is utilized to amplify the product from restriction. RAPD, rep-PCR and AFLP are appropriate for distinguishing strains at species or below levels though they are less beneficial for taxonomic function. TP-RAPD happens to be created for taxonomic purpose as the patterns of strains in the same species have been discovered to be the same. The TP-RAPD patterns supported the proposal of novel species of rhizobia (Pongsilp, 2012).

Polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) is applied in figuring out the genetic relationships based upon PCR and restriction analysis. Specific genes, like small subunit ribosomal RNA gene (16S rDNA), large subunit ribosomal RNA gene (23S rDNA), 16S23S rRNA intergenic spacer (IGS) and symbiotic genes have been utilized in PCR-RFLP. The PCR-RFLP profile is utilized to estimate the genetic diversity of microorganisms. The PCR-RFLP method has been used fruitfully in the differentiation of rhizobial species (Pongsilp, 2012).

Restriction fragment length polymorphism (RFLP) furthermore has been used to analyze the genotypic diversity of bacteria. The procedure is based on restriction analysis and also hybridization, causing the fingerprint patterns. For rhizobia, symbiotic genes happen to be popular as probes for hybridization. Based on the same application, the insertion sequences (ISs) can be utilized as probes, causing the patterns called as "IS fingerprints". The application of ISs as probes for hybridization is able to offer high quality fingerprints of rhizobial strains. The ISs have been discovered to be plentiful in rhizobia. The distribution of ISs differs typically in both IS type and copy number. Several ISs are specific to rhizobial species (Pongsilp, 2012).

The identification of bacteria based on phenotypic characteristics is usually not precise because several species are extremely hard to always be distinguished phenotypically. For rhizobia, the present classification is primarily influenced by DNA sequences [especially a DNA sequence encoding small subunit ribosomal RNA (16S rDNA)], DNA homologies, phylogenetic relationships and the locations of symbiotic genes. The 16S rDNA is quite helpful for estimating the

evolutionary associations and determining bacteria. For bacterial identification, 16S rDNA sequencing is particularly crucial in the case of bacteria with uncommon phenotypic profiles, rare bacteria, slow-growing bacteria, uncultivable bacteria and culture negative infections. Probably the most remarkable development in microbial phylogeny and taxonomy is based on sequence analysis of 16S rDNA. These sequences mostly support the proposal of novel genera and species of rhizobia. In some instances, several genera are the same in 16S rDNA sequence analysis. The other regions, like large subunit ribosomal RNA gene (23S rDNA) as well as intergenic spacer between 16S and 23S rRNA sequences (16S23S IGS), are ideal options for classification and identification purposes. Multilocus sequence analysis (MLSA), which utilizes a set of nucleotide sequences involving 16S rDNA, housekeeping genes and symbiotic genes, has opportunity for rhizobial classification. Presently, sequence analysis is probably the most promising and useful technique for identification of rhizobial genera (Pongsilp, 2012).

2.3.3 The immunological techniques

When bacteria (including rhizobia) are actually injected into a mammal, the animal produces antibodies which will bind antigens on the surface of the bacteria. As a consequence of antigen injections, complicated immunological reactions lead to the animal producing special proteins known as globular antibodies (immunoglobulins). The study of the reactions of the immune serum with the antigens outside the animal is recognized as serology. If the surface antigens on the rhizobia are relatively distinctive and unshared by other microorganisms the binding of the antibodies could be utilized to both detect and identify the target rhizobia. Antigen-antibody reactions are highly specific. The antibody reacts only with the antigen that

elicited its formation. A number of strategies based on antibody-antigen reactions and which can be applicable in the detection of rhizobial broth or inoculants have been developed. In rhizobia, both cultured cells and nodule antigens (bacteroides) are utilized for strain identification (Olsen et al., 1983; Somasegaran & Hoben, 1994).

Currently, several methods have been applied to study the phenotypic diversity of rhizobia, particularly numerical analysis, enzyme pattern and serological study. Serological techniques are the particular identification technique for rhizobia based on natural marker characteristics. Phenotypic characterization by serotyping has been commonly utilized to examine rhizobial populations in various geographical origins. Rhizobia in various serogroups have been found dominant among field populations (Pongsilp, 2012).

2.3.3.1 Agglutination

The process in which the antigens are linked together by their corresponding antibodies is called agglutination. The linked antigens might be microscopically or macroscopically able to be seen as clumps, agglutinates or aggregates. The agglutination reaction mainly depends on a firm structural relationship between an exposed bacterial antigen and the antibody. In case the antibodies do not bind to the cells, no agglutination happens. Agglutination is often used as a technique for determining specific bacterial antigens, and also in turn, the identity of such bacteria. The agglutination test was one of the first methods to be used to serological investigations of rhizobial bacteria. It is among the simplest of serological techniques to apply and it has been widely used in several taxonomic and ecologic investigations (Somasegaran & Hoben, 1994).

Advantages: the agglutination reaction is the simplest of all immunological tests and involves only primary antisera. The technique is fast and does not require specialized material and skill, and also secondary antibody or signal producing labels.

Limitations: The cross-reactivity between rhizobia is typical with non-adsorbed primary antisera. The agglutination test is not as sensitive as other immunological tests and does not demonstrate cell viability (Olsen et al., 1996; Somasegaran & Hoben, 1994).

2.3.3.2 Precipitation

In recent years, the precipitation tests of somatic antigens happen to be frequently utilized for rhizobia. The precipitation reaction occurs when certain soluble antigens are brought into touch with the corresponding antibody. Precipitation differs from agglutination in which the precipitating antigens are not whole bacterial cells (cellular) but are proteins or polysaccharide molecules in solution (Somasegaran & Hoben, 1994).

2.3.3.3 Immunodiffusion

The somatic antigens of many rhizobium strains diffuse steadily in the agar gels; they produce either no precipitin bands or only weak bands close to the antigen well as the location of bands relies on the relative concentrations of diffusible antigens and antibodies (Somasegaran & Hoben, 1994).

2.3.3.4 Immunofluorescence (IF)

One of the most sensitive of the serological methods available to study rhizobia is the fluorescent antibody (FA) method. It allows for the visualization and investigation of the antigens of individual cells with the fluorescent microscope

and requires just small quantity of both antigen and antibody. In comparison, both agglutination and immunodiffusion need large quantities of antigen and antisera to yield a visible reaction. Particular chemical dyes like fluorescein isothiocyanate (FITC) and lissamine rhodamine possess the property of fluorescing when excited by near UV light. Rhizobial antibodies originated in rabbits can be conjugated to these fluorescing chemical dyes or fluorochromes. In work with rhizobia, the chemical dye commonly utilized for labeling the specific antibody is FITC, which has an apple green fluorescence upon irradiation with blue light. There are two types of fluorescent antibody techniques, known as the direct and indirect immunofluorescence. In the direct method, the specific antiserum is conjugated and is utilized as a stain in the procedure. This is unlike from the indirect method, where the unconjugated (unlabeled) specific or primary antibody is first reacted with the antigen smear, and after adequate time is given for antigen-antibody reaction, the smear is then rinsed free of excess antiserum. This step is followed by staining with the FITC labeled secondary antibody. In serological work with rhizobia, the specific or primary antibody against the rhizobial strain is regularly produced in rabbits. The secondary antibody is produced by immunization of goats or sheep with purified rabbit immunoglobulins from a formerly unimmunized rabbit. As a result, the rabbit immunoglobulin serves as an antigen for immunization of the goat or sheep. Therefore, the antibody produced in the goat or sheep will not only react with the rabbit antiserum but will also react with rhizobial antigen with specific unlabeled rabbit antibody attached when the indirect procedure is employed. Though the results are the identical, the indirect method is considered more sensitive. The indirect method needs the labeling of only the immune serum from the goat or sheep and involves two reaction steps; the indirect method is also known to

give more nonspecific staining reactions. In the direct method, each rabbit antiserum developed against each rhizobial strain must be conjugated (Somasegaran & Hoben, 1994). Fluorescent antibody (FA) has been widely utilized in working with rhizobia, such as, used to identify strains of rhizobia, to identify the nodule bacteria, to detect doubly infected nodules, to study rhizobium in soil, to study population dynamics of *R. japonicum* in the rhizosphere, and to make quantitative studies of rhizobium in soil. The FA test is speedy and simple but needs fluorescent microscopy equipment and special skill (Olsen et al., 1996; Somasegaran & Hoben, 1994).

2.3.3.5 Enzyme-linked immunosorbent assay (ELISA)

Enzyme linked immunosorbent assay (ELISA) is one of the several enzyme immunoassays utilized in detecting antigens and antibodies. Basically, in an enzyme immunoassay, either the antibody or antigen is tagged with an enzyme (e.g., alkaline phosphatase). After finishing the assay, the existence or lack of the enzyme-labeled component is detected by adding an appropriate substrate (e.g., paranitrophenylphosphate) causing a colored product. In direct ELISA, the specific antibody (Abl) developed for a particular strain of rhizobia is immobilized in the wells of the plate. Excess unreacted Abl is rinsed off. The rhizobial antigen is then put to the Ab1coated wells. After an incubation period, excess unreacted antigen is removed by washing. This is followed by the addition of an enzyme Abl conjugate, which binds to its specific antigen. Excess enzyme Abl is washed off. The substrate is then put and the reaction is stopped following incubation; the colored product is measured colorimetrically by ELISA reader machine. In indirect ELISA, which is more popular with rhizobial workers, the antigen is immobilized first in the wells. This is followed by the addition of Abl, incubation, and washing. The next reactant added is enzyme

Ab2 conjugate. Ab2 is usually sheep or goat antibody against Ab1. The enzymeAb2 conjugate specifically binds to Ab1. After addition of the substrate, the reaction is completed as with direct ELISA. In both ELISA approaches, a 96well plastic microtiter plate (solid support) is utilized to immobilize the antigen or antibody. The advantages of ELISA are requirement tiny quantities of antiserum and no microscopic equipment is required. However, ELISA needs a purified antigen preparation, either from culture or from a root nodule (Somasegaran & Hoben, 1994).

2.3.3.6 Membrane immunoblot

The membrane immunoblot procedure is another enzyme immunoassay that has been developed to detect antigen or antibodies (proteins) immobilized (bound) onto a membrane support. This particular technique was widely utilized in inoculant quality control and ecological studies of rhizobia. The rhizobial cells (antigens) are blotted or put onto membranes construct from nitrocellulose or nylon. After incubating, the membrane-bound antigens with the homologous antibody (Abl) solution, and washing to get rid of excess unbound Abl, the membrane is immersed in a solution containing enzyme Ab2. As with ELISA, Ab2 is commonly sheep or goat antibody against Ab1. Ab2 has been conjugated with alkaline phosphatase enzyme and binds specifically to Ab1. The assay is completed by the addition of substrate reagents. These reagents are a mixture of 5-bromo-4-chloro-3 indolyl-phosphate (BClP) and Nitro Blue Tetrazolium (NBT). This technique is not complicated and requires no specialized equipment. However, like all immunological techniques for identifying rhizobia, the results of the spot blot test are only as good as the specificity of the anti-rhizobial antisera used. Also, the test does not demonstrate cell viability (Olsen et al., 1996; Somasegaran & Hoben, 1994)

2.3.3.7 Lateral flow assay (LFA)

LFA, immunochromatographic strip test, which can be constructed by Abs, aptamer, and nucleic acids, was described in the 1960s (Kohn, 1968) and also become a popular platform for speedy immunoassays since the mid-1980s (Ang et al., 2012). Depending on their formats, LFAs could be conveyed as dipstick assay, lateral flow device (LFD), point of care (POC) to bedside test, and lateral flow immunochromatographic assay (LFIA). LFAs are utilized to detect the presence or absence of a target analyte in sample and allow naked-eye analyses based on accumulation concepts (Ijeh, 2011). LFAs have many advantages compared to other detection assays. They are established mature technology, with processes already developed, relative ease of manufacture, and stable shelf lives of 12–24 months frequently without refrigeration; easily scalable to high-volume production; and integrated with various systems, having high sensitivity, specificity, relatively low cost, market presence, and minimal education required for users and regulators (O'Farrell, 2009).

The production of regular strip assay includes the preparation of colloidal gold conjugates, utilization of reagents onto the membrane and pads, lamination of the strip membranes onto a support backing, cutting the prepared master cards into strips of defined length and width, and strip packaging. Three types of pads, a sample pad, conjugate pad, and absorbent pad, and nitrocellulose membrane are applied for developing the strip assay. The test sample is applied onto the sample pad. Conjugate pad consists of Abs, aptamers, or nucleic acids specific to the target analyte which are commonly conjugated to colored particles, gold nanoparticles (GNPs), and latex beads. Capture reagents like anti-target Abs or aptamers are immobilized in a line

across the membrane which are nitrocellulose or cellulose acetate as a test line. It has also a control line including capture reagents like Abs or complementary nucleic acids specific for the conjugate Abs or aptamers present on the conjugate, respectively. The strip components are generally fixed to an inert backing material and might be placed in a plastic casing with a sample port, and reaction window showing the test and control line or strip can be produced as a simple dipstick format (Peruski & Peruski, 2003). After soaking of sample pad with analyte, it flows through the conjugate pad and nitrocellulose membrane via capillary action and ends on an absorbent pad. When the flow is continuing, the analyte bound by gold conjugate on conjugate pad is captured and piled on test line. The excess conjugate is also captured by a control line, and it should consistently be visible. If the test strip works precisely and it is positive, both the test and control lines are visible as red. If no colored capture lines or only a red color at the test line appears, the strip is invalid, and the test should be repeated (ÇAm & ÖKtem, 2017).

Most LFAs utilize several types of antibodies (monoclonal, polyclonal, HRP-conjugated, AP-conjugated, etc.) (Frenzel et al., 2013; Robinson et al., 2015). Morphine can be rapidly detected by gold nanoparticle conjugated single-chain fragment variable antibody (scFv) using lateral flow test strip (Gandhi et al., 2018).

2.4 Recombinant antibody

Antibodies are a modular defense system that identify and neutralize foreign objects as bacteria and viruses. The structure of most antibodies comprises of two heavy chains and a pair of light chains linked by noncovalent bonds and disulfide bridges (Figure 2.2). Each of them could recognize a specific antigen unique to its

target as they have the antigen-binding sites, a paratope (a structure analogous to a lock) located at the upper tips of the “Y” shaped antibody molecules. This paratope is displayed on the particular antigen, allowing these two structures to specifically bind together. Thus, this mechanism is able to permit an antibody to tag a microbe as well as an infected cell to be attacked by other parts of the immune system and also to directly neutralize its target (Mayilyan et al., 2008). Being a main part of the immune system, antibodies represent a powerful weapon system in defending our body against non-self-agents. Nevertheless, a vast number of different molecules carrying different specificities are needed for the interaction with many foreign structures.

This diversity can be developed through somatic recombinant and hyper-mutagenesis of a set of variant genes (Rogozin et al., 1991). Since 1975, Kohler and Milstein have developed the hybridoma technology which produced monoclonal antibodies in huge amount in the laboratory. Nevertheless, monoclonal antibodies from this technology face several problems, as they are almost absolutely murine in origin thus could create human anti-mouse antibody when introduced to human therefore limits their clinical applications (Klimka et al., 2000; Watkins & Ouwehand, 2000), and monoclonal antibody producing technology is quite laborious and time intensive.

Beginning in 1985, phage display technology, an attractive technology for the election of polypeptides with desired properties, was established by George Smith (Smith, 1985). The application of phage display technology for production of recombinant antibodies with desired specificities (McCafferty et al., 1990). This technology can replace hybridoma technology because of its higher specificity, unlimited source for long-term use, reduced manufacturing cost, and avoiding the use of experimental animals (McCafferty et al., 1990)

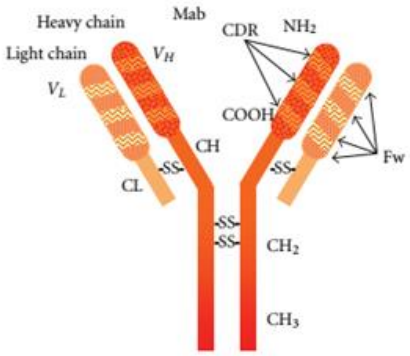

Figure 2.2 Antibody model showing subunit composition and domain distribution along the polypeptide chains (Ahmad et al., 2012).

Recombinant single chain antibody variable region fragments (scFvs)

In comparison to Fab fragments, which can also be produced by proteolytic cleavage, single-chain antibody variable region fragments (scFvs) are recombinant antibody fragments composed of the VH and VL domains connected by a 15 to 25 amino acid linker (Bird et al., 1988; Huston et al., 1996) that can be easily expressed in functional form in *E. coli*, allowing protein engineering to enhance the properties of scFv such as increase of affinity and alteration of specificity (Griffiths & Duncan, 1998). The variable fragment is the smallest unit of immunoglobulin molecule with function in antigen binding activities. The length of the flexible DNA linker used to link both of the V domains is critical in yielding the correct folding of the polypeptide chain. Previously, it has been estimated that the peptide linker must span 3.5 nm (35 Å) between the carboxyl terminus of the variable domain and the amino terminus of the other domain without affecting the ability of the domains to fold and form an intact antigen binding site. In addition to the linker peptides designed *de novo*, peptide sequences derived from known protein structure have been applied to provide a compatible length and conformational in bridging the variable domains of a without

serious steric interference. Aside from the length of the linker, their amino acid composition also plays an essential role in the design of a viable linker peptide. They must have a hydrophilic sequence in order to avoid intercalation of the peptide within or between the variable domains along with the protein folding. Currently, the most extensively used designs have sequences comprising stretches of Gly and Ser residues which meant for flexibility and or together with the charged residues such as Glu and Lys interspersed to improve the solubility (Ahmad et al., 2012).

The scFv antibodies have been constructed primarily from hybridoma (Singh et al., 2010), spleen cells from immunized mice (Hayhurst et al., 2003; Wang et al., 2006) and B lymphocytes from human (Pansri et al., 2009; Reiche et al., 2002). The scFv is a noncovalent heterodimer comprised of the VH and VL domains. For producing, mRNA is first isolated from hybridoma (or also from the spleen, lymph cells, and bone morrow) followed by reverse transcribed into cDNA to serve as a template for antibody genes amplification (PCR). With this method, large libraries with a diverse range of antibody VH and VL genes could be created (Pansri et al., 2009). In the scFv construction, most of them are constructed in a VH linker VL orientation (Ahmad et al., 2012). One of the most widely used methods is through PCR assembly which was first described by (Horton et al., 1990). In this method, it allows the V domains of antibody to be cloned without any prior information about the nucleic acid as well as amino acid sequence of the certain antibody. Furthermore, the V domains of antibody can be combined by *in vitro* recombination directly after the PCR of VH and VL genes into plasmid (Chaudhary et al., 1990) or phagemid (Ahmad et al., 2012). Numerous scFv have been constructed against hapten (Kobayashi et al., 2005), protein (Dai et al., 2003), carbohydrate (Sakai et al., 2007), receptor (Galeffi et al., 2006), tumor antigen

(He et al., 2002), and viruses (Saldarelli et al., 2005). All these scFv have excellent potential for use in many fields such as medical therapies and diagnostic applications (Ahmad et al., 2012).

Nowadays, scFv have been successfully isolated and displayed as fragments in a variety of expression systems such as mammalian cell and yeast (Ho et al., 2006), plant (Galeffi et al., 2006), and also insect cells (Choo et al., 2002). The scFv antibody can be expressed as correctly folded and directly active proteins or as aggregates requiring *in vitro* refolding to become active. Depending on the expression system, it varies in their ability to fold and secrete the scFv proteins. There are some general regulations to consider on the design of vectors and expression system used with the different hosts and each of this host has advantages and disadvantages for the production of active antibody (Verma et al., 1998). However, the bacterial expression system is most often applied for the production of scFv antibody fragments compared to the various expression strategies available (Frenzel et al., 2013).

2.5 Phage display antibody technology

2.5.1 Phage display technology

Phage display technology was established and developed by G. Smith in 1985 as a powerful tool for generation and selection of recombinant antibodies from phage antibody library that produces from immune or non-immune sources. In this technology, the antibody fragments are displayed on surface of filamentous bacteriophage in difference formats, including scFv fragment, Fab fragment, Fv fragment and nanobodies. Phage (also referred the filamentous bacteriophage) are a group of single-stranded DNA (ssDNA) viruses that infect a number of gram-negative bacteria, such as *Escherichia coli* cells (Figure 2.3). They involve members of the

Inoviridae family, of the genus *Inovirus,* such as, phage M13, f1 and fd (King, 2012). The bacteriophage strains M13, f1 and fd belong to Ff phage - the filamentous phage particles mainly utilized for display purposes. The Ff phage enclose a ssDNA genome of about 6400 nucleotides and consist of 11 genes (Table 2.2). The native phage particle is a thin, cylindrical shape, usually 930 nm long and 6-7 nm in diameter (Carmen & Jermutus, 2002; Willats, 2002).

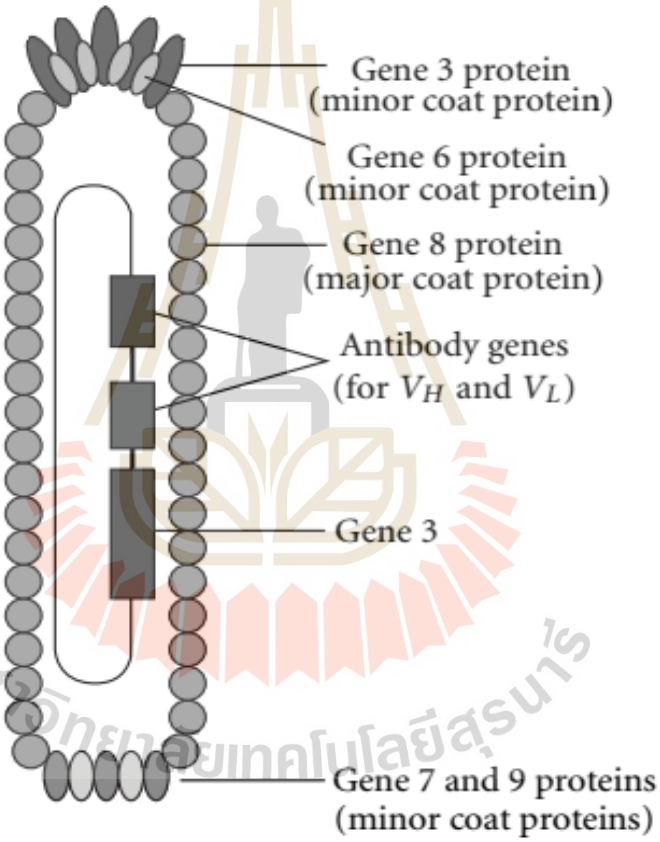

Figure 2.3 Schematic view of a filamentous phage particle (King, 2012).

Table 2.2 Genes and gene product of bacteriophage (Webster, 1996).

Gene	Function	Amino acid
I	Assembly	348
II	DNA replication	410
III	Minor capsid protein	406
IV	Assembly	405
V	Binding of ssDNA	87
VI	Minor capsid protein	112
VII	Minor capsid protein	33
VIII	Major capsid protein	50
IX	Minor capsid protein	32
X	DNA replication	111
XI	Assembly	108

In phage display technology, phagemid vectors are utilized as phage display systems for production of the fusion coat protein pIII (Type III) or pVIII (Type VIII) (Figure 2.4). A phagemid vector is a plasmid including a phage-derived origin of replication, a selective marker, an intergenic (IG) region, a gene of a phage coat protein, restriction enzyme recognition sites, a promoter, and a DNA segment encoding a signal peptide (Carmen & Jermutus, 2002; Paschke, 2006; Qi et al., 2012). The infection of phagemid (often called phage rescue) needs helper phage (Table 2.3) to provide the structural and functional proteins requirement for completing the life cycle of the phagemid (Scott & Barbas, 2001).

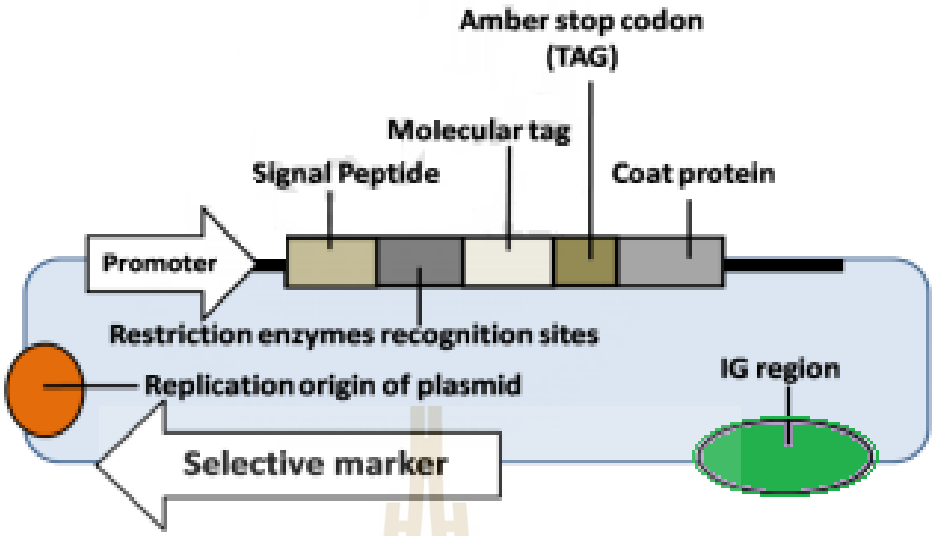

Figure 2.4 The scheme of phagemid vector chain (Carmen & Jermutus, 2002).

Table 2.3 Helper phage and their features (Paschke, 2006).

Helper phage	**Features**
Ex-phage pIII	carries amber stop codon
CT-Phage	N1–N2 deleted
Hyper-phage	pIII deletion, special packaging strain
R408d3	pIII deletion
M13Δ3.2	pIII deletion
Phaberge	pIII carries amber stop codon
KM13	protease site in pIII
M13 K07	replication-deficient
VCS M13	derivative of M13 K07

2.5.2 Phage-displayed antibody fragments

There are many different antibody formats which can be displayed on the surface of phage, including fragment of antigen binding (Fab fragment), Fv fragment, single chain Fv (scFv) fragment, and the variable fragment (VH or VHH) of single-domain heavy chain antibodies (HCAb) from camelids (Figure 2.5). The larger Fab fragment comprises of VH-CH and VL-CL segments linked by disulfide bonds. The smaller Fv fragment is consists of the VL and VH domains linked by a flexible peptide. The recombinant version of the Fv is termed the single-chain variable fragment (scFv) with with 15-17 amino acid long linker. In order to express the antibody fragments on the surface of the filamentous bacteriophage, phagemids must be converted to filamentous phage particles with the same morphology as Ff phage by co-infection (super-infecting) phagemid-carrying cells with the helper phages. Besides that, the genes of the antibody fragments can be fused either to the pVIII gene or the pIII gene (Figure 2.6). The most widely used antibody phage display systems are based on phagemid vectors encoding the antibody library (as Fv, scFv or Fab fragment libraries) fused to the minor coat protein pIII or its C-terminal (CT) domain (Carmen & Jermutus, 2002).

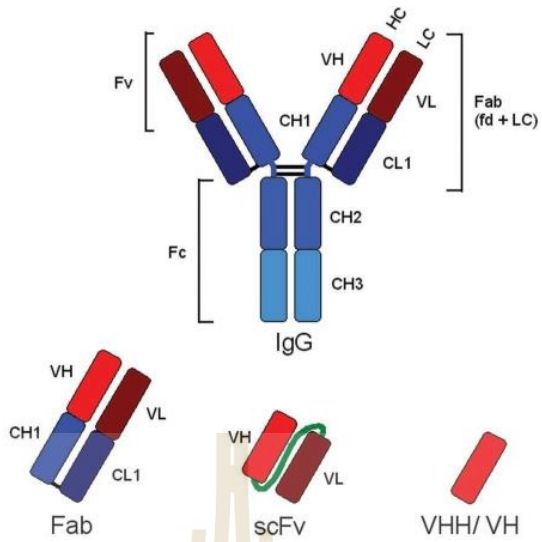

Figure 2.5 Antibody fragments. Immonoglobulin subtype G (IgG), ~150 kilodaltons (kD); Fragment of antigen binding (Fab), ~50 kD; Fv fragment, ~25 kD; Single-chain variable fragment (scFv), ~25-30 kDa; VHH antibody from camelids, ~15 kD; VH: variable heavy chain; VL: variable light chain; CH: constant heavy chain; CL: constant light chain (Carmen & Jermutus, 2002).

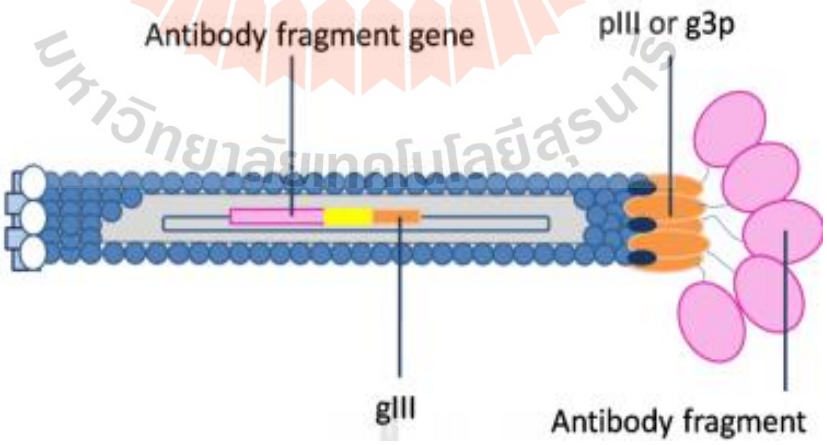

Figure 2.6 Phage displayed antibody (Carmen & Jermutus, 2002).

Antibody fragments can be developed in different forms (soluble antibody fragment or phage displayed antibody fragment) by phagemid vectors which are engineered by inserting an amber stop codon between the antibody fragment gene and pIII gene. This amber stop codon is suppressed in a suppressor host and not suppressed in a non-suppressor host. Thus, antibody fragments will be secreted out as soluble antibody fragments when the phage is growth in a non-suppressor host, in comparison to phage displayed antibody fragments which is produced when the phage is growth in non-suppressor host (Winter et al., 1994).

2.5.3 Phage display antibody libraries

Phage display libraries are classified into three different types: immune, naive, and synthetic. These libraries are constructed for different purposes depending on the nature of antigen panned during selection procedure together with the affinity and number of antibodies expected. Firstly, immune libraries have been constructed by cloning antibody genes of B cells from different immunized animals such as mice, chickens (Andris-Widhopf et al., 2000), rabbits, camels, sheeps, and also humans (Kramer et al., 2005). Secondly, naive libraries, derived from nonimmunized donors of B cells that have been constructed from a pool of V-genes of IgM mRNA. Thirdly, the synthetic libraries which also derived from non-immunized sources as their ranges were prepared synthetically by combining germ line gene sequences together with randomized complementary determining regions (CDRs) that are responsible for antigen binding. The majority of synthetic human antibody libraries produced focused on randomizing the CDR3 regions, which are usually most diverse and essentially responsible for antigen binding (Li et al., 2001).

2.5.4 Affinity selection of phage display antibody (Bio-panning)

Bio-panning is a method for selection of antibody fragments from phage display antibody libraries by using their affinity binding activity as shown in Figure 2.7. The affinity selection of ligands includes five fundamental steps: (i) preparation of a primary library or amplification of an existing library and target is immobilized, (ii) exposure of the phage particles to a target (immobilized protein/cell surface protein/vascular endothelium) for which specific ligands are planned to be identified, (iii) removal of non-specific binders (washing/perfusion), (iv) recovery of the target bound phage by elution or direct bacterial infection and amplification of the recovered phage, (v) back to step (i) from two to four times. In most cases, several rounds (approximately 2-4 rounds) of selection and amplification should be repeated to select the best binders from the phage display library. Furthermore, this procedure can be adjusted to enhance the optimal condition in selection of the specifically binding clones (Arap, 2005).

Even though the principle of biopanning technology is simple, the results can vary due to many factors, like library complexity, nature of the target, binding affinity and avidity, and other multiple experimental parameters. Even with an excellent selection strategy, the experiment will fail if the desired peptide/antibody is not present in the library. Binding affinity and avidity are other factors that need to be taken into consideration (Huang et al., 2012).

Selection using immobilized antigens: Phage libraries are selected by flowing through an affinity column with the immobilized antigen of interest (Clackson et al., 1991). Following washing of the column to remove nonspecific clones, specific binders are eluted and amplified in *E. coli*. Selection can also be performed against

antigen adsorbed onto plastic surfaces such as immunotubes or enzyme-linked immunosorbent assay (ELISA) plates (Marks et al., 1991). Alternatively, antigen may be immobilized on chips of BIA-core sensors (Malmborg et al., 1996).

It should be noted that selection of the immobilization method must take into consideration the conformational integrity of the immobilized antigen. Some phage antibodies selected against an adsorbed antigen may not be able to recognize the native form of the antigen. One way to solve this problem is to use indirect antigen coating through the application of antigen-specific capture antibodies (Sanna et al., 1995).

Selection using antigens in solution: This technique permits solution binding and overcomes problems with conformational changes that are encountered upon coating antigens on solid surfaces. The use of labeled soluble antigens also permits a more precise amount of the antigen used during selection and accordingly enhances the ability to use lower concentrations of the antigen to favor selection of high-affinity phage antibodies. Following incubation of phage-antibodies with biotinylated antigen, phage bound to the labeled antigen are recovered with avidin or streptavidin-coated paramagnetic beads. Specific phages are then dissociated from the antigen and characterized (Hawkins et al., 1992). One disadvantages of this technique is that anti-streptavidin antibodies will also be isolated. However, this problem can be solved by a depletion step using streptavidin-coated beads (Azzazy & Highsmith, 2002).

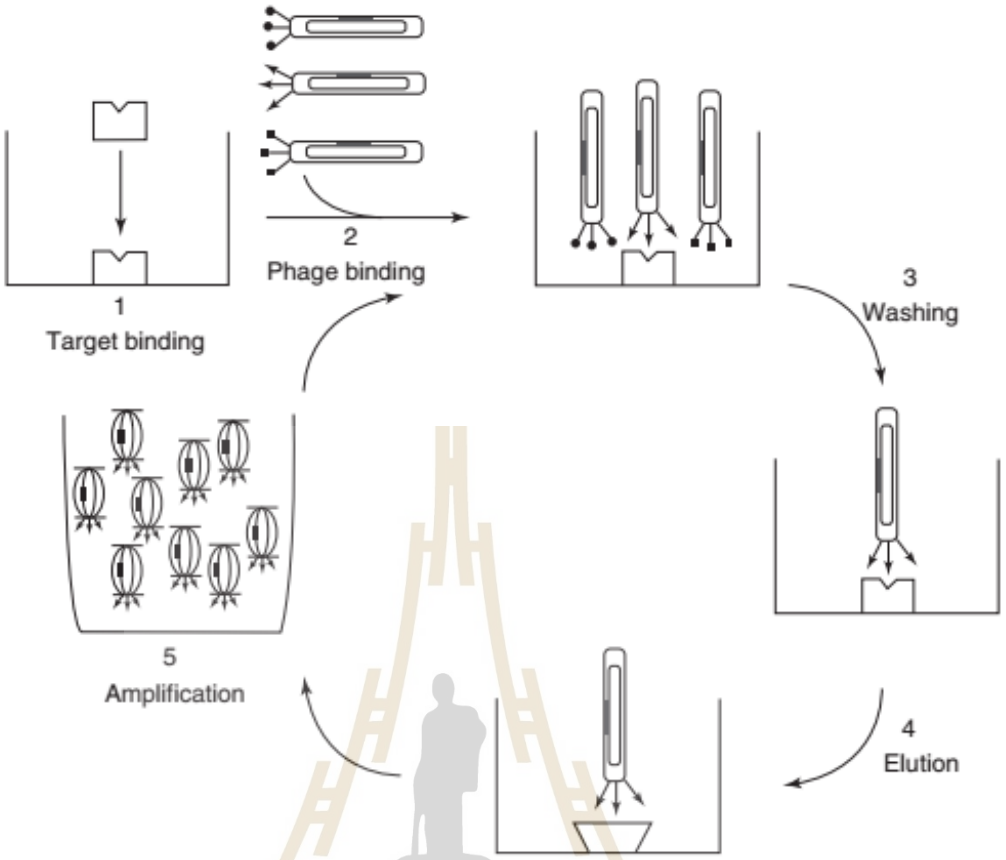

Figure 2.7 Schematic overview of Bio-panning method (Arap, 2005).

Selection on whole cells: Direct selection of antibodies against markers on cell surfaces may be performed on either monolayers of adherent cells or on cells in suspension. Unbound phage can be rinsed by washing tissue culture flasks (monolayers) or centrifugation (cell suspension). In order to optimize the isolation of antigen-specific binders and minimize the binding of irrelevant binders, a simultaneous positive and negative selection may be applied. In this technique, a competition is set up between a small number of antigen-positive target cells and an excess of antigen-negative “absorber” cells to bind antibodies of phage library; the absorber cells serve as a sink for the nonspecific adherence of irrelevant binders. A fluorescently labeled antibody against an irrelevant antigen present only on the target cells is added and FACS (Fluorescence-activated cell sorting) is used to isolate the target cells binding

the specific phage antibodies (de Kruif et al., 1995). Similar approaches can be used to identify putative tumor-specific antigens and produce a rapid high-yield approach for isolating self-replicative antibody fragments directed against novel or conformationally dependent cell surface markers. Another group subjected a scFv library to three rounds of positive selection on human melanoma cells and negative selection on human peripheral blood mononuclear cells (Kupsch et al., 1999). Selections may also be carried out on tissue sections as well as whole tissues (Azzazy & Highsmith, 2002).

The advantages of whole-cell phage display are easy to see. In the case of when antigen is unavailable or the antigen is unstable under immobilization conditions, whole-cell phage display panning is generally the best choice. Moreover, it is also applicable in the detection of unknown antigens. The biopanning procedure commonly requires no prior knowledge of the cell surface biomarkers, allowing for the isolation of targeting peptides for cell types for which little is known about the cellular profile. For whole-cell screening, the cellular targets are identified in a two-part process. First, peptides or antibodies are first identified by screening whole cells against a phage display library. Second, the binding peptides or antibodies are tested individually in functionally based screens. In all cases, activity is confirmed in functional assays; one does not need to either purify or identify a particular receptor in advance. Since whole cells are used as the affinity matrix, the receptors are likely to be in their native conformation, and a large variety of receptors are being screened at one time. It should be noticed that the cell surfaces would share a high degree of similarity and that the peptides would be recognizing abundant, common receptors. Thus, additional negative selections are necessary to avoid unexpected cell specificity of selected peptides (Huang et al., 2012).

The in vivo selection: In this method phage repertoires are directly injected into animals and then tissues are collected and examined for phage bound to tissue-specific endothelial cell markers as was revealed for peptide phage. Pasqualini and Ruoslahti (1996) were the first to isolate phage-displayed peptides that home to selective vascular beds *in vivo*. *In vivo* panning has numerous advantages: (i) the isolated phage-displayed peptides home selectively to "intact" targets of interest; (ii) an inherent blocking step is included where most of the phage-displayed peptides that recognize ubiquitous plasma and cell surface proteins are eliminated; (iii) these peptides may be applicable for the functional analysis of new receptors and potential identification of novel drug target candidates because some of the isolated peptides have been found to bind to endothelial receptors expressed in the vasculature of specific tissues (Pasqualini & Ruoslahti, 1996).

2.5.5 Application of phage display

Applications for phage-displayed peptide libraries: Phage display of random peptides: Synthetic oligonucleotides with a constant length but with unspecified codons, randomized via site-directed mutagenesis using degenerate oligodeoxynucleotides, are cloned as fusions to one of the coat proteins of M13 phage where they are expressed as peptide-capsid fusion proteins (Azzazy & Highsmith, 2002). Phage-displayed peptide libraries could be applied to isolate peptides that bind with high specificity and affinity to virtually any target protein. These binding peptides can be utilized as reagents to understand molecular recognition, as minimized mimics for receptors, or as lead molecules in drug design (Sidhu, 2000).

Mapping antibody epitopes: Fragments of DNA that encode parts of the protein antigen are fused to a gene encoding one of the capsid proteins. Phage particles

displaying antigenic peptides can be applied for mapping epitopes of monoclonal and polyclonal antibodies (Hill & Stockley, 1996). Phage display libraries of random peptides have also approved useful for identifying antibody epitopes in cases in which the antigen is unavailable or even not yet known (Cortese et al., 1994).

Phage-antibody applications: Phage-displayed recombinant antibodies have many advantages over monoclonal antibodies generated by hybridoma technology. Antibody genes can be cloned directly from spleen cells using rapid recombinant DNA methods in contrast to the time-consuming and labor-intensive cell screening processes of hybridoma production. Generation of a large natural display library from variable gene repertoires can terminate animal immunization and largescale cell culture for hybridoma development and permit isolation of antibodies with high affinity against any antigen. Phage display is especially applicable in cases where monoclonal antibodies could not be obtained by classical hybridoma technique such as antibodies against nonimmunogenic or toxic antigens. Phage displayed antibodies have stable genetic source. Phage antibody technology can also be used to clone and rescue monoclonal antibodies from genetically unstable hybridomas. Phage antibody genes can be easily sequenced, mutated, and screened to improve antigen binding. Finally, soluble recombinant antibodies (not displayed on phage) can be produced rapidly and economically and can be used as *in vitro* diagnostic reagents (Azzazy & Highsmith, 2002).

Phage display technique is popular for identification of specific antibodies against pathogen targets. These targets are commonly subdivided into two categories: (i) molecular targets, such as replication/cell division enzymes and host-pathogen virulence factors, and (ii) whole bacterial cells. In comparison with specific molecular

targets, cell-based screening has the advantage in that it is an assumption-free strategy with the potential to recognize cell surface structures that may not have been considered targets using genomic-based approaches or that have not yet been identified. Using live pathogens as the target also has the advantage that all "druggable" targets on the cell surface are screened simultaneously in their native physiological context, thus allowing for the selection of potential antimicrobial activity from the outset. Antigens on the cell surface of pathogens are appealing targets for biologics because they provide potential binding sites for molecules to interfere with bacterial division (Lock & Harry, 2008) colonization, and virulence (Rasko & Sperandio, 2010). Both strategies have been widely used for developing novel diagnostic tools and therapeutic treatments for infectious diseases (Huang et al., 2012).

Phage enzymes: Several enzymes have been displayed on M13 bacteriophages and retained their catalytic activities. These include alkaline phosphatase (McCafferty et al., 1991), trypsin (Corey et al., 1993), and β-lactamase (Siemers et al., 1996). In theory, any enzyme that can be expressed in *E. coli* may also be displayed on M13 phage. Phage display libraries based on suitable enzymes can improve diagnostics by enhancing the stability and catalytic activities of enzymes, and probably enabling the engineering of catalysis that is modifiable by antigen binding (Siemers et al., 1996).

Application of phage display technology in detection and monitoring of microorganisms: Conventional bacterial detection, diagnostic methods, especially pathogenic bacteria and virus, largely rely on microbiological and biochemical analysis which can be sensitive but overly time consuming, cost-ineffective and nonamenable to integration for onsite diagnosis (Singh et al., 2013).

Today, phage display is recognized as a powerful tool for selecting novel peptides and antibodies that can bind to a broad range of antigens, ranging from whole cells to proteins and lipid targets. Phage display has been applied widely for identification of specific peptides and antibodies against pathogen targets. These targets are generally subdivided into two categories: (i) molecular targets, such as replication/cell division enzymes and host-pathogen virulence factors, and (ii) whole bacterial cells. Both strategies have been widely applied for developing novel diagnostic tools and therapeutic treatments for infectious diseases (Huang et al., 2012).

Staphylococcus aureus, one of the most important human pathogens, has become a major threat to human health. Soykut and colleagues (2008) have utilized a peptide-phage display library to identify peptides binding to Staphylococcal enterotoxin B produced by *S. aureus*, is a pyrogenic toxin responsible for staphylococcal food poisoning in humans and has been an attractive choice of biological aerosol weapon due to its inherent stability and high intoxication effect (Soykut et al., 2008).

Listeria monocytogenes is a severe foodborne pathogen that causes life-threatening listeriosis. To avoid infection by *L. monocytogenes*, it is important to detect low levels of the pathogen in food samples. Paoli and colleagues used phage display to identify a scFv antibody that can only bind to *L. monocytogenes* (Paoli et al., 2004). Later, in 2007, a surface plasmon resonance (SPR) sensor was developed based on the scFv antibody by Nanduri and colleagues (2007) (Nanduri et al., 2007). *L. monocytogenes*-specific scFv-displayed phage was immobilized on the sensor surface to detect *L. monocytogenes* at a detection limit of $2x10^6$ cfu/ml.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

All chemical reagents were of molecular biology grade. Yamo I, a human non-immunized phage display scFv library was constructed in our laboratory using B-lymphocytes from 140 healthy individuals in the Northeastern Thailand (Pansri et al., 2009). *Escherichia coli* TG1 was obtained from MRC, Cambridge, and was used for cloning and amplification of phages. *E. coli* SHuffle® T7 Express (NEB, USA) was used for protein expression. Bovine albumin serum (BSA) was natural purified protein obtained from Fluka, USA. The anti-M13/HRP and His probe-HRP detection kits were purchased from Amersham-Pharmacia Biotech (Uppsala). Anti-His Dylight 488 secondary antibody and Calcofluor white stain were purchased from Abcam and Sigma, respectively. *Bradyrhizobium* strains SUTN9-2, DOA9 and other *Bradyrhizobium* strains were obtained from School of Biotechnology, Suranaree University of Technology. The phagemid pMOD I vector containing scFv fragments DNA, pTT28 vectors containing constant heavy chain and Kappa (κ) light chain were obtained from Molecular Biotechnology Laboratory, School of Biotechnology, Suranaree University of Technology.

3.2 Methods

3.2.1 Production of soluble scFv antibodies

3.2.1.1 Cloning of scFv antibodies

The gene encoding scFv antibody clones: yiN92-1e10 and yiDOA9-162 were inserted into pET-21d (+) vector (Figure 3.1) (New England Biolabs, NEB, USA) between *NcoI* and *NotI* sticky ends. The DNA of scFv fragments and pET-21d (+) vector were digested with the *NcoI* (20U/µl, NEB, USA) and *NotI* (20U/µl, NEB, USA) enzymes. The digestion reactions of scFv fragments and pET-21d (+) vector were performed separately, each in a total volume of 50 and 100 µl, respectively. The reaction mixtures consist of 10 µg of insert DNA, 2 µg of vector DNA, 1x Cutsmart buffer, 20U of *NcoI* (20U/µl, NEB, USA) and 20U of *NotI* (20U/µl, NEB, USA). The reactions were incubated at 37°C for 16 hr and heat inactivated at 80°C for 20 min. Afterwards, the digested DNA of vector was dephosphorylated by adding 1 µl of CIP enzyme (10U/µl, NEB, USA) and incubated at 37°C for 1 hr. The inserts and vector were separated from stuffer fragments by gel electrophoresis and followed by Wizard clean up kit (Promega, USA). The DNA of scFv antibodies were ligated into pET-21d (+) vector at a 3:1 ratio and incubated at 16°C for 16 hr. The ligation reaction was then transformed into *E. coli* DH5α by chemical transformation. Subsequently, the transformed cells were spread on LB plate containing 100 µl/ml of ampicillin and incubated overnight at 37°C. The single colony was picked and analyzed by double digestion with *NcoI* and *NotI* restriction enzymes. Plasmids were purified from each clone (QIAgen spin Miniprepkit, USA). The integrity of the constructs was confirmed by automated DNA sequencing (Macrogen, Korea), using universal primer (T7 promotor and T7 terminator).

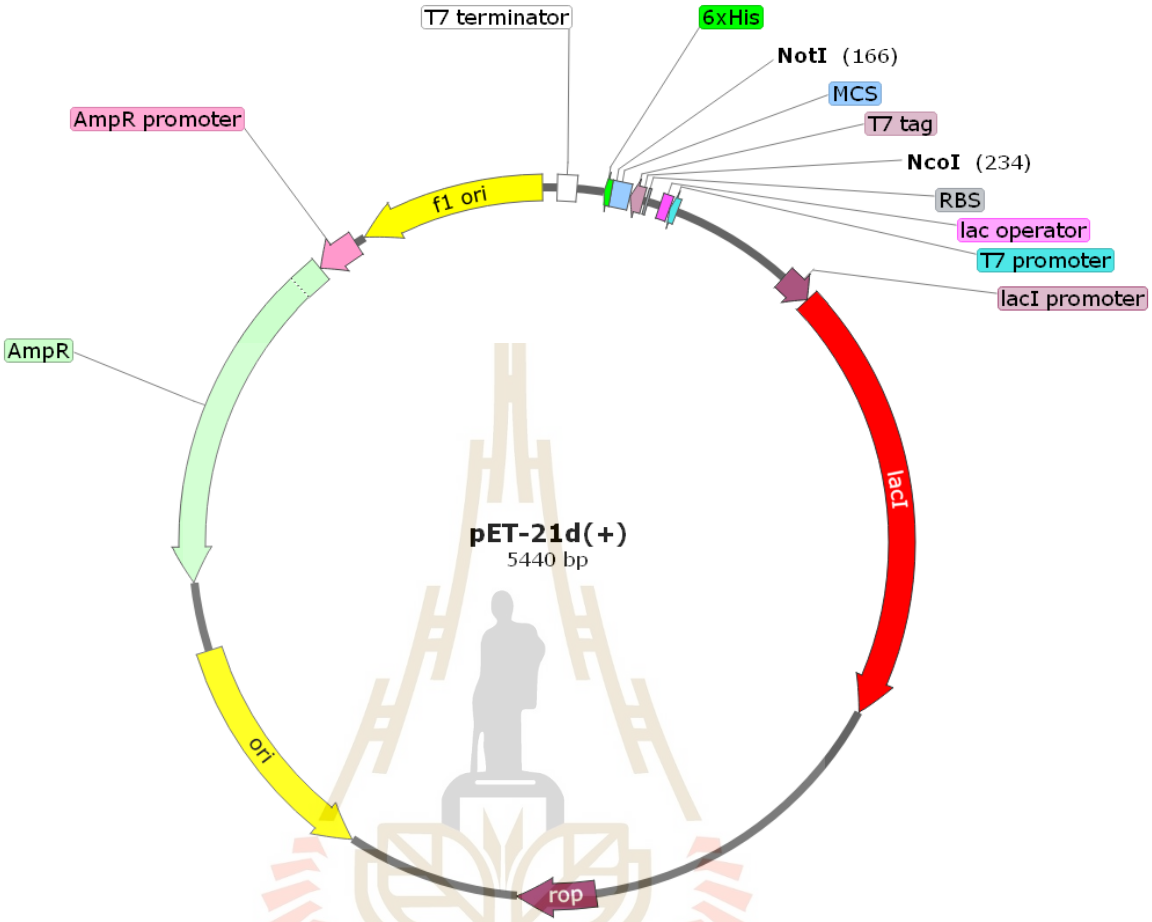

Figure 3.1 Map of pET21d+vector. The arrow shows the position of T7 promoter and ampicillin resistance genes.

3.2.1.2 Expression of scFv antibodies

To produce yiN92-1e10 and yiDOA9-162 soluble scFv, pET21d+/yiN92-1e10 and pET21d+/yiDOA9-162 plasmids were transformed into *E. coli* SHuffle® T7 Express and spread on LB plate containing 100 µl/ml of ampicillin and incubated for overnight at 37°C. Then, a single colony containing each plasmid was grown into 5 ml of LB media containing 100 µg/ml of ampicillin. The culture was incubated overnight with shaking (250 rpm) at 30°C. Two percent (v/v) of each overnight culture was used to inoculate into 400 ml LB medium supplemented with

100 μg/ml of ampicillin. Then, cells were cultured at 30°C until the OD_{600} nm reach 1.0 followed by inducing with 1.0 mM isopropyl-b-D-thiogalactopyranoside (IPTG) at 16°C for 24 hr before cell harvest.

To harvest and purify the recombinant antibodies, the cell pellets were collected by centrifugation at 10,000 *g* for 10 min, which were resuspended in 20 ml of ice-cold lysis buffer (20 mM sodium phosphate, 500 mM NaCl and 45 mM imidazole, pH 7.4) containing 1 mg/ml of lysozyme. Cells were disrupted by intermittent sonication for 10 min on ice using 30 sec pulse and 30 sec break for cooling, followed by centrifugation at 4°C for 30 min at 10,000 *g*. The retained soluble fractions were further processed for protein purification. The supernatant was applied to 1 ml His-Trap column (GE Healthcare, USA) pre-equilibrated with the binding buffer (20 mM sodium phosphate, 500 mM NaCl and 45 mM imidazole, pH 7.4). Elution by a 250 mM imidazole in elution buffer (pH 7.4) was performed. Fractions containing yiN92-1e10 and yiDOA9-162 scFv were subjected to buffer exchange by snake-skin dialysis bag with PBS buffer at 4°C. The samples were collected and kept at 4°C. The soluble fractions and purity of the samples were assessed by SDS-PAGE. Then, the total protein concentrations were determined by Bicinchoninic acid (BCA) assay (Smith et al., 1985). The affinities of soluble scFv antibodies were determined by the scFv ELISA and immunostaining assay with both types of antigen, i.e., pure culture (boiled and non-boiled) and nodule samples.

3.2.2 Detection of SUTN9-2 and DOA9 with scFv antibodies by ELISA

3.2.2.1 In pure culture antigen

Pure culture antigen preparation: *Bradyrhizobium* strains SUTN9-2 and DOA9 were sub-cultured on Yeast extract Mannitol (YM) Agar medium. After 5 days, the single colony was picked into 20 ml YM broth and the bacteria were incubated at 28˚C, 180 rpm for 7 days. The cells were harvested aseptically in sterile saline buffer (0.85%, w/v, NaCl), then washed three times in sterile saline by centrifugation at 3,300 *g*, 4˚C for 15 min. Cell pellets were resuspended in saline buffer and were adjusted to 1×10^9 cells/ml. This could be estimated by reading the optical density of the suspension on a spectrophotometer at an optical density of 1 (OD_{600} nm). Then, the cell suspension was split up and centrifuged at 3,300 *g*, 4˚C for 15 min and treated under boiled, and non-boiled (whole cell) conditions. For boiled cell treatment, the cells were suspended in 0.85% (w/v) NaCl of the same volume and boiled in water bath at 100˚C for 1 hr. For non-boiled condition, the cells were suspended in 100 mM $NaHCO_3$ of the same volume used for washing. Then, the total protein was measured by Bicinchoninic acid (BCA) assay (Smith et al., 1985). The stock of pure antigen was stored at -20˚C.

ELISA was done according to the protocol described by Vu et al., (2017). Both boiled and non-boiled bacterial antigens of SUTN 9-2 and DOA9 were diluted in sodium bicarbonate buffer to achieve 5 µg of total protein per well. Bovine serum albumin 1% (w/v) BSA in PBS was used as blank. The bacteria samples were immobilized on the 96-well ELISA plate (MicroWell™, Nunc) and then the wells were sealed with tape to avoid evaporation and incubated overnight at 4˚C. Then, the wells were rinsed 2 times with PBS and blocked with 2% (w/v) MPBS for 1 hr at room

temperature with rotating. After blocking, the wells were washed 2 times with PBS. After that, 5 and 10 µg of two scFv antibodies were added into each well. The scFv antibody 3E3, against aflatoxin B1 (Rangnoi et al., 2018) was used as a negative control. The plate was incubated at room temperature for 1 hr with shaking. Then, the wells were washed 3 times with PBST (PBS with 0.05% Tween 20) followed by 2 times washing with PBS. Bound scFv antibodies were detected with His-probe horseradish peroxidase (HRP) (1:5,000 dilution PBS). After incubating at room temperature for 1 hr, the wells were washed again 3 times with PBST and 2 times with PBS. Color reaction was developed by adding 200 µl ABTS (2, 2- azino-di-3-ethyl-benzothiazoline-6-sulfonate) peroxidase substrate containing 0.05% H_2O_2. Detection was done by measuring the absorbance at 405 nm in an ELISA plate reader (Sunrise, TECAN, Austria). The assay was performed in triplicates.

3.2.2.2 In nodule sample

Nodule antigen preparation: The seeds of mung bean (*Vigna radiata*) were surface sterilized by soaking in 95% ethyl alcohol for 15 min. After that, the seeds were washed with sterilized water 3 times and then soaked in 3% sodium hypochlorite solution for 3 min, and then rinsed with sterilized water for 10 times and soaked in sterilized water overnight at room temperature under dark condition. For shyleaf (*Aeschynomene americana*), the seed were sterilized by soaking in concentrated sulfuric acid for 30 min. Afterwards, all seeds were rinsed with sterilized water 10 times and then soaked in sterilized water overnight at room temperature under dark condition. All seeds were germinated on sterilized 0.8% (w/v) water agar for 2 days in the dark conditions at room temperature. The germinated seeds of mung bean and shyleaf were then transplanted in plastic pouches and inoculated with

1 ml of 1 x 10^9 cells/ml of *Bradyrhizobium* strain SUTN9-2 and DOA9, respectively, at 2 days after transplanting. All plants were supplemented with N-free medium (Hoagland, 1950) and grown under controlled environmental condition of 28 ± 2˚C, 70% relative humidity on 16/8 hr day/night cycle for 1 month. The root nodules and bacteroid suspension were used for further analyses as antigen by immunostaining and ELISA techniques.

To prepare plant nodules for ELISA, the nodules was detached from *V. radiata* and *Aeschynomene americana* root inoculated with SUTN9-2 and DOA9, respectively. The nodule samples were washed with sterile water and then crushed and gently ground in sterilized small mortar and pestle. Nodules were calculated with 5 nodules/wells. Then, 1 ml sodium carbonate buffer (pH 8.5) was added to mortar. Finally, 200 µl of the bacteroid suspension was added to each well of an ELISA plate (Nunc, Denmark). The assay was performed in triplicate as described for pure culture samples.

3.2.3 Detection of *Bradyrhizobium* SUTN9-2 and DOA9 with scFv antibodies by immunofluorescent assay

The immunofluorescence analysis was performed according to previously published protocol (Min & Yamabhai, 2021). For pure culture, 1 ml of broth culture was centrifuged (3000 *g*, 5 min), washed one time with 0.85% (w/v) NaCl, followed by a thorough washing with PBS, and then resuspended in PBS. About 5 µl of bacterial suspension was spread into a smear on a glass slide and dried completely at 37°C. Thereafter, the smear was fixed with 4% paraformaldehyde and then blocked with 1%BSA-300 mM glycine-0.1% PBST for 30 min. After blocking, the cells were washed 3 times with PBS and then treated with 10 µg of 6xHis-tagged scFv antibodies

yiN92-1e10 and yiDOA9-162, respectively, at room temperature for 1 hr. The slides were rinsed 3 times with PBS and incubated with 5 µl of 1:500 dilution of Dylight 488-labeled anti-hexa-histidine mouse monoclonal antibody (Abcam #ab117512, UK) in PBS for 1 hr at room temperature. Unbound antibodies were removed by washing with PBS for 3 times. Subsequently, the cells were counterstained with 300 µM DAPI (4′,6-diamidino-2-phenylindole) for 5 min at room temperature and rinsed with PBS for 3 times. Finally, the stained cells were covered with SlowFade gold antifademountant (Invitrogen #S36936, U.S.A) and then the slides were sealed. The immunostained cells were examined with the confocal microscope (Nikon A1, Japan).

For nodule samples, the cross sections at 65 µm thickness were cut using the vibratome and placed on the slides. Then, sections were incubated in PBS containing Calcofluor white M2R (Sigma, Germany) to a final concentration of 0.01% (w/v) for staining of the plant cell wall. After washing with PBS, the procedure was performed as described above for pure culture samples. Finally, the stained nodule slices were examined with the confocal microscope (Nikon A1, Japan).

3.2.4 Determination of scFv cross-reactivity

To confirm binding specificity of recombinant scFv yiN92-1e10, ELISA was performed against *Bradyrhizobium* strain SUTN9-2 and other related 27 *Bradyrhizobium* strains, namely; PRC008, USDA 110, DOA9, DOA1, ORS3257, 194, CB1809, S23321, ORS278, TAL173, DASA02002, DASA02082, DASA02042, DASA02068, DASA02193, DASA02198, and peanut bradyrhizobia isolates no. 2, 3, 4, 6, 7, 8, 9, 10, 11, 12, and 13). The ELISA was conducted as described above. The boiled bacterial antigens of all tested strains were prepared as in the section 3.3.2.1 and then diluted in sodium bicarbonate buffer to achieve 5 µg of total protein per well. To

detect the binding, 10 µg of scFv yiN92-1e10 were added into each well. The next steps were the same as above procedure.

3.2.5 Application of scFv antibody for monitoring bradyrhizobial inoculum under rice-legume rotational cropping system

The experiment was conducted under pot trial condition. The plastic pots (10 L size, 25.5 cm diameter and 22.5 cm height) were filled with 5 kg of low-organic-matter non-sterile soil (pH 6.5; % organic matter 0.68%; phosphorus [P] 43.1 ppm; potassium [K], 139 ppm; calcium [Ca] 675 ppm). Rice seeds were dehulled and surface disinfected by washing with 95% ethyl alcohol for 30 s, 10% hydrogen peroxide for 10 min, 3% sodium hypochlorite for 3 min, and then 5 to 6 times with sterilized water. Seeds were germinated in the dark at 30°C for 2 days on plates containing 0.8% (w/v) water agar. The germinated seeds were soaked in the suspension of *Bradyrhizobium* sp. SUTN9-2 (10^8 cfu/ml) overnight. Next day, the seedlings were transplanted into the plastic pots and grown in the greenhouse until the seed maturation stage of rice. The plants without inoculation served as control. The plants were irrigated with tap water every 3 days. The scFv antibody was further applied for detection and monitoring in the following schemes.

3.2.5.1 Detection of rice endophytic bradyrhizobia by ELISA and immunofluorescence assay

The endophytic bradyrhizobia in rice tissues were monitored at 1, 2, 3, and 4-month. About 3 g of rice tissues were thoroughly rinsed with tap water, then surface sterilized with 70% ethanol for 1 min, followed by 3% sodium hypochlorite for 3 min and then rinse 3 times with sterilized water. Thereafter, the cleaned rice seeds, leaves, leaf sheaths and roots were chopped into small pieces and ground separately

with small, sterilized mortar and pestle. Then diluted with 3 ml sodium carbonate buffer at 1:1 dilution. Subsequently, the rice extracts were filtered with three layers comprising a miracloth (22-25 µm), membrane filter no.4 (25 µm) and no.1(11 µm) to separate the plant debris from the extracts. Then, 200µl of each rice extract including endophytic bradyrhizobial cell were detected by ELISA as above procedure.

For immunostaining, each rice tissues: leaves, leaf sheaths, roots and seeds, were cut cross section into 85 µm thickness using the vibratome and placed on the glass slides. Then, the plant cell walls were stained by incubating the sections were incubated in PBS containing Calcofluor white M2R (Sigma) to a final concentration of 0.01% (w/v). After washing 3 times with PBS, the immunostaining procedure was conducted as previously described.

3.2.5.2 Detection for the persistence of rice endophytic bradyrhizobia in soil samples

After harvesting, the rice inoculated with SUTN9-2, the remaining rice stubbles were immediately incorporated into the soil in each pot. Then, SUTN9-2 in the soil samples was detected at 0, 1, 2, 3, and 4 weeks after. About 5 g of soil samples were diluted in 5 ml of sterile sodium carbonate buffer and homogenized by vortexing for 5 min at full speed. This suspension was centrifuged at 130 *g* for 5 min to exclude the biggest soil particles. Then, the supernatant containing bradyrhizobial cell was detected with soluble scFv antibodies by ELISA as mentioned above. Soil sample in the pot containing non-inoculated rice was used as a negative control.

3.2.5.3 Detection of rice endophytic bradyrhizobia from nodules of mung bean

Following the decomposition of the rice-straw into the soil for 4 weeks, the mung bean was planted in each pot for 3 weeks under greenhouse conditions. After 3 weeks, the plants were uprooted, and the nodules were detached from the plant and detected with specific scFv yiN92-1e10 by ELISA and immunofluorescence assay as mentioned in the above experiments.

3.2.6 Quantification of nodule occupancy by scFv immunofluorescence staining

Nodule occupancy analysis was carried out by immunofluorescence and Gus-staining methods. Three inoculants: SUTN9-2 (wild type), PRC 008 (wild type), SUTN9-2 (WT) + PRC008 (co-inoculation; 1:1), and SUTN9-2 (Gus-tagged), PRC 008 (wild type), SUTN9-2 (Gus-tagged) + PRC008 (co-inoculation; 1:1) were prepared for immunostaining and Gus-staining, respectively. The mung bean seeds were surface sterilized and germinated as described in section 3.3.2.2. The bacterial suspension for all strains were prepared as mentioned in section 3.3.2.1. One milliliter of mixed inoculum was added to the 2-day-old seedlings. After that, plants were grown under controlled environmental condition of 28 ± 2°C, 70% relative humidity on 16/8 hr day/night cycle (full light, 639 microeinsteins [µE]/m2/S) for 1 month. There were 5 replicates for each treatment in both staining methods. The total number of nodules from each plant was enumerated after 1 month. Then, each nodule was cut into 65 µm thickness by cross sectioning. The immunofluorescence staining was conducted as mentioned in the previous experiments. For the detection of Gus-marked rhizobia in plant nodule, the nodule sections were immersed in a microtiter plate containing the

Gus assay solution (40 µl X-Gluc 20 mg/ml in N, N-Dimethyl-formamide, SDS 20 mg, methanol 2 ml, 1M sodium phosphate buffer 0.2 ml and distilled water 7.76 ml), in vacuum for 120 min before overnight incubation at 28°C (Manassila et al., 2007). Then, the stained nodule slices were examined under the light microscope and the occupancy percentage was calculated.

3.2.7 Reformatting of phage displayed scFv antibody fragments to full length IgG

3.2.7.1 Amplifying heavy and light chain sequences

The genes for variable regions of heavy chain and Kappa (κ) light chain, (VH and Vκ) were amplified separately. PCR was carried in a 50 µl reaction containing 1x Pfu DNA polymerase Buffer with MgSO4, 1 µM of forward and reverse primer, 10 mM dNTP mix, 1.5 U of Pfu DNA polymerase (Invitrogen), and 0.5 µg of DNA template and MilliQ water to 50 µl. PCR cycling are as following: 95°C for 2 min; 30 cycles of 95°C for 1 min, 58°C for 30 sec and 72°C for 2 min; and 72°C for 5 min. Subsequently, the amplified VH and VL were cloned into pKR-CH and pKR-CL vectors (PTT28) (Figure 3.2) at *NheI*/*NheI* and *NheI*/*BsiWI* sites, respectively.

After digestion the insert and vector with restriction enzymes, the vector and insert were ligated using T4 DNA ligase (400 U/µl, NEB, USA) at 16°C for 16 hr and then was transformed into *E. coli* Top 10 (Invitrogen, USA). The colonies were picked individually and incubated overnight for preparation of plasmid using QIAgen Spin Miniprepkit (USA) according to the manufacturer's protocol. The plasmids were confirmed by DNA sequencing. After checking sequence, the plasmid was amplified in *E. coli* DH5αF′ in 25 ml culture volume to prepare the high concentration of plasmid by using Hi Pure Plasmid Midiprep Kit (Invitrogen, USA).

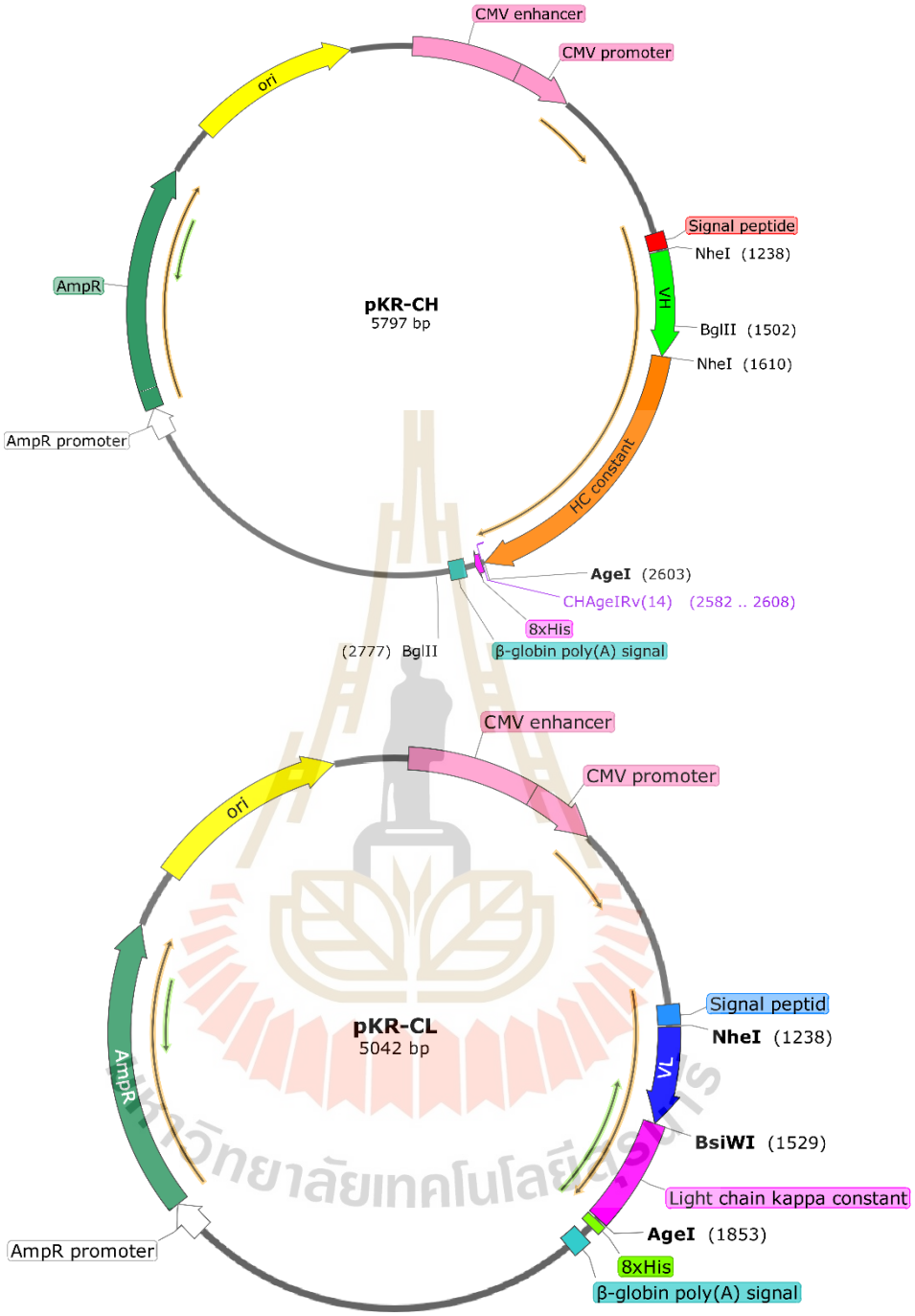

Figure 3.2 Map of pKR-CH and pKR-CL vectors (pTT28). The arrow shows the position of CMV promoter and ampicillin resistance genes.

Table 3.1 Oligonucleotides for construction of IgG vector.

Primer	Sequence
E10VH_Fw_PTT	CTGTGCGCTAGCGAGGTGCAGCTGGTGGAG
E10VH_Rv_PTT	GCACAGGCTAGCTGAGGAGACGGTGACCAGG
E10VL_Fw_PTT	CTGTGCGCTAGCGAAACGACACTCACGCAGTC
E10VL_Rv_PTT	GCACAGCGTACGTTTGATATCCACTTTGGTCCCTCCG

Sequences corresponding to *NheI* and *BsiWI* sites are underlined.

3.2.7.2 Expression and purification of antibody

Expi293F™ human cells were used for IgG expression. This cell was maintained in a shaker flask with orbital shaker at 37°C. Before, transfection, the cells were passaged and cultured Expi293™ Expression Medium until the cells reach a density of approximately 3 x 10^6 viable cells/ml.

The cell was transfected with plasmid in culture volume 25 ml Expi293™ Expression Medium using 125 ml flask. About 80 µl of ExpiFectamine was diluted into 1.4 ml of Opti-MEM™ I Reduced Serum Medium, incubated at room temperature for 5 min. Then 25 µg of plasmid DNA (HC:LC=1:2) was diluted with 1.5 ml of Opti-MEM™ I Reduced Serum Medium. Subsequently, the diluted ExpiFectamine™ 293 Reagent was mixed with diluted plasmid DNA, followed by incubated at room temperature for 15 min. Then, the Expi293F™ cells were transfected by adding complex solution of plasmid DNA. The cells were incubated at 37°C in 8% CO_2 orbital shaker. After 20 hr, 150 ul of ExpiFectamine™ 293 Transfection Enhancer 1 and 1.5 ml of ExpiFectamine™ 293 Transfection Enhancer 2 were added to the flask and then incubated at 37°C. After 6 days, the culture was spun at 4,000 *g* for 10 min. The IgG was found in the supernatant.

Human IgG was purified on a 1ml HiTrap® MabSelect™ PrismA column (Cytiva). Twenty-five milliliters of sample were diluted with 75 ml binding buffer (20 mM Sodium Phosphate, 0.15 M NaCl, pH 7.2), then filtered with 0.45 µm filter. The column was equilibrated with 5 ml binding buffer. Then, the filtered supernatant was loaded to the column at flow rate of 0.5ml/min. To remove loosely bound protein, the column was washed with 15 ml binding buffer. The scFv was eluted from the column with elution buffer (0.1 M Sodium Citrate, pH 3.0) gradient buffer programme. The eluted fractions were neutralized with 1 M Tris-HCl, pH 8.0. The antibody fraction was dialysed with PBS buffer by centrifugal dialysis using Amicon® Ultra4 Centrifugal Filter Devices (Mr 10,000 cutoff, Millipore, Ireland). The protein concentration was measured by Bicinchoninic acid (BCA) assay (Smith et al., 1985). To detect antibody purity, SDS-PAGE electrophoresis was used. To determine the efficiency of purification, the flow through fraction, wash fraction and eluted fractions were compared by SDS polyacrylamide gel.

3.2.7.3 Biopanning of polypeptides binding to yiN92-1e10 antibody from phage display peptide library

Phage display of a 12-mers random peptide library (SUT12), which was prepared in our laboratory (Khoushab et al., 2012), was used to screen for IgG antibody yiN92-1e10 binding peptides. The Immuno 96 microWell™ plate (Nunc, Denmark) was coated with 10 µg of yiN92-1e10 per well. The wells were sealed with plastic tape to protect evaporation and incubated at 4° C overnight. After that, 300 µl of blocking solution (1% BSA in 0.1 % PBST) was added to each well to block non-specific binding, incubated at room temperature for 1 hr. After blocking, the wells were rinsed 3 times with PBST (PBS-0.1% Tween 20). Biopanning was carried out by

incubating 25 µl of the SUT12 peptide library (10^{12} pfu/ml) in 125 µl of PBST to each well and then the plate was incubated at room temperature for 2 hr. After 5 times washing away the unbound phage with PBST (PBS-0.1% Tween 20), the bound phages were eluted by adding 100 µl of 50 mM glycine-HCl (pH 2.0) to each well. Then the plate was incubated at room temperature for 15 min. Subsequently, the solution was neutralized by adding 100 µl of neutralization solution (Solution A: 16 µl [0.2 M $NaH_2HPO_4H_2O$] and Solution B: 84 µl [0.2 M $Na_2HPO_4.7H_2O$]). Twenty microliters of the overnight culture of *E. coli* DH5αF′ were diluted in 2 ml 2x YT (1:100 dilution). Then, 100 µl of eluted phage from first round biopanning was added and the culture was incubated at 37°C for 8 hr at 250 rpm. Next day, the amplified phages were collected by spinning out cells at 4000 *g*, 4˚C for 10 min and the phage supernatant was transferred to a new tube. For second and third round biopanning, 5 µg and 2.5 µg of yiN92-1e10, respectively, were immobilized at 4°C overnight onto each well. The blocking and washing steps were the same as described for first round biopanning. After washing, 200 µl of amplified phages from first round were added to the wells of second round plate and then the plate was incubated ate room temperature for 1 hr. After 5 times washing with PBST, the bound phages were eluted as described for first round. Afterwards, 100 µl of eluted phage from the second-round panning was added to each well of third round plate, then the plate was incubated at room temperature for 2 hr and followed by washing 5 times with PBST. The bound phages were eluted as described for first round biopanning.

The bound phages from third round panning were diluted as 10-fold serial dilution in PBS. Then, 10 µl from each dilution were added into the tube containing 200 µl of overnight culture of *E. coli* DH5α, followed by mixed with 4 ml

of molten 0.8% Top agar containing 40 µl of 2% X-Gal (5-Bromo-4-Chloro-3-Indolyl β-D-Galactopyranoside) and 100mM IPTG (Isopropyl β-D-1-thiogalactopyranoside). Thereafter, the agar mixture was poured into the 2x YT plates. The plates were allowed to stand for a few minutes until the top agar hardens, and then incubated at 37°C for overnight. The blue isolated plaques of phage were picked and inoculated into 3 ml of *E. coli* DH5αF′ diluted 1:100 in 2x YT by using sterile wooden toothpicks. Then, the culture was incubated at 37˚C with shaking (250 rpm) for 8 hr. The amplified phages were collected by spinning out cells at 4000 *g*, 4˚C for 10 min and the phage supernatant were transferred to a new tube. The replicative form DNA was prepared from the bacterial cell pellet.

3.2.7.4 Confirmation of binding activity of affinity-selected phage clones by ELISA

The Immuno 96 microWell™ plate (Nunc, Denmark) was coated with 0.5 µg of target yiN92-1e10 IgG antibody, and 1% BSA using the same coating buffer as negative control for selection. Then, the plate was incubated at 4°C for overnight. Three hundred microliters of blocking solution (1% BSA in 0.1% PBST) were added to each well and then the plate was incubated at room temperature for 1 hr. After blocking, the wells were washed 3 times with PBST as described in selection. About 200 µl of each supernatant containing amplified phages were added into a separate pair (target/negative control) of wells and incubated at room temperature for 2 hr. After washing with PBST for 5 times, each well was incubated with 100 µl of 1:5000 dilution of HPR-anti M-13 in PBST at room temperature for 1 hr. Then, the wells were washed 5 times with PBST, followed by 200 µl of ABTS substrate and 0.05% H_2O_2 was added into each well. The plate was incubated at room temperature

for 30 min. The absorbance value was measured at 405 nm in an ELISA plate reader (Sunrise, TECAN, Austria).

3.2.7.5 Phage Peptide Sequence Analysis

The replicative form DNA was prepared from individual cell pellet of positive clones. Plasmids were purified from each clone by using QIAgen Spin Miniprepkit (USA). DNA sequences were determined by automated DNA sequencing (Macrogen, Korea), using the -96 gIII primer (5′-CCC TCA TAG TTA GCG TAA CG-3′).

3.2.7.6 Comparison of bound phage sequences with GenBank sequences

The sequences obtained were compared with sequences from GenBank, using Basic Local Alignment Search Tool (BLASTP) software (http://blast.ncbi.nlm.nih.gov/). Sequences sharing at least three amino acids (at the same position) within the 12-mers (regardless of their matching with known protein sequences) and appearing more than three times among the selected phage clones were classified as consensus sequences (CS) (Yang et al., 2005). The aligned amino acid sequences shared by three (or more) identical amino acids within the peptides were considered as being mimotopes of the matched protein sequences.

CHAPTER IV

RESULTS

4.1 Production of soluble scFv antibody fragments

In order to express the scFv fragment in *E. coli*, the scFv genes of yiN92-1e10 and yiDOA9-162 phage clones were inserted into pET-21d (+) vector (Figure 4.1). The nucleotide sequence of soluble scFv clones were determined, and sequence alignment indicated that identical with the original phage clones (Figure 4.2). Recombinant expression plasmids were transformed into *E. coli* SHuffle® T7 Express for protein expression. The expression of scFv-6xHistidine-tag-fusion-proteins in the resultant *E. coli* SHuffle® T7 Express clones were induced by IPTG. Subsequently, the cell pellets were collected and lysed by ultrasonic disruption after induction. The supernatant containing recombinant antibody was first purified by one-step immobilized metal affinity chromatography (IMAC). The most of unwanted co-eluting proteins was removed by purifying the eluted fractions for second time using the same column. The protein bands of scFv-hexahistidine fusion antibodies were observed in the elution fractions at the expected molecular weight of approximately 29 kDa on SDS-PAGE gel (Figure 4.3). The reactivity of recombinant antibodies after purification against strains SUTN9-2 and DOA9, both in the pure culture and bacteroid forms inside the nodule of *V. radiata* and *A. americana*, respectively, were tested by ELISA. The results were shown in Figure 4.4 and 4.5. The results demonstrated that free scFv antibodies

had specific binding activity against both forms of each target strain. The ELISA signals showed that 5 µg/well of antibody could be applied to detect 5 µg/well of total protein of target *Bradyrhizobium* cells, and 200 µl suspension of 5 nodules/ml of the bacteroid forms. There was no binding signal by the negative control 3E3 scFv antibody (Rangnoi et al., 2018) against either *Bradyrhizobium* strains SUTN9-2 or DOA9, in both pure culture (Figure 4.4) and bacteroid forms (Figure 4.5).

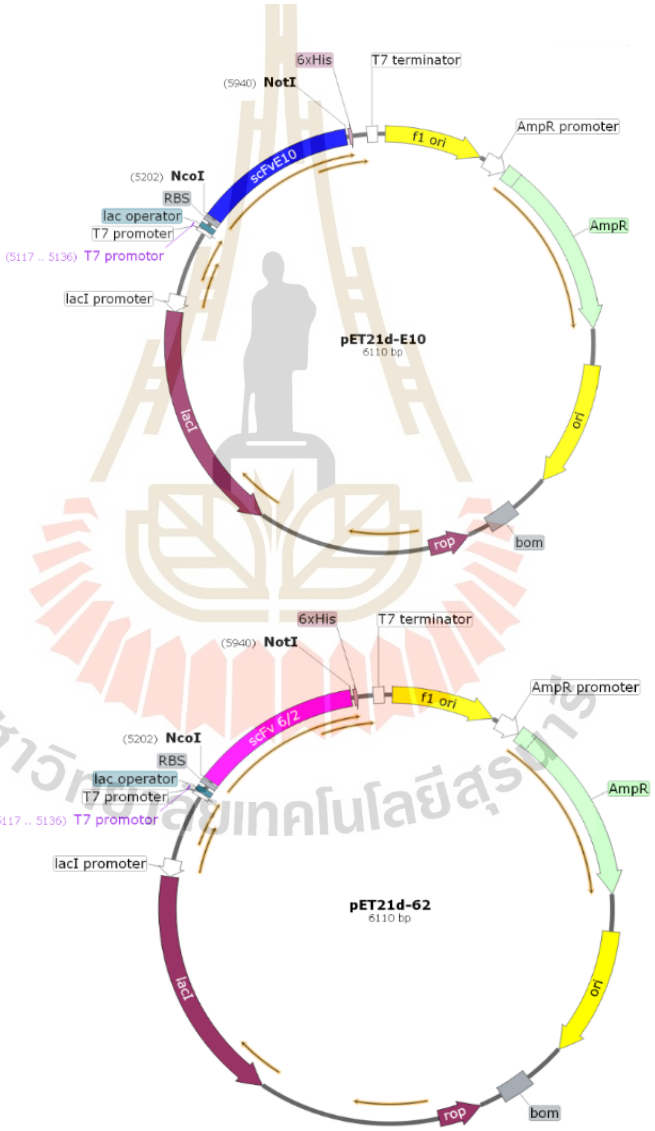

Figure 4.1 Map of pET21d+/yiN92-1e10 and pET21d+/yiDOA9-162 plasmids. The scFv fragments were subcloned into pET-21d (+) vector between *NcoI* and *NotI* sites.

(A)

Figure 4.2 Amino acid sequence analysis of soluble scFv clones yiN92-1e10 (A) and yiDOA9-62 (B). The amino acid sequences were aligned with original phage clones. Sequence identical was noted for both yiN92-1e10 and yiDOA9-162.

(B)

```
                                        VH/CDR1                 VH/CDR2
yiDOA9-162    MAQVQLQESGPGLVKPSETLSLTCTVSGDSITTYYWNWIRQPPGKGLEWIGYAHHTGNTN  60
Clone 3       MAQVQLQESGPGLVKPSETLSLTCTVSGDSITTYYWNWIRQPPGKGLEWIGYAHHTGNTN  60
Clone 4       MAQVQLQESGPGLVKPSETLSLTCTVSGDSITTYYWNWIRQPPGKGLEWIGYAHHTGNTN  60
              ************************************************************
                                                VH/CDR2
yiDOA9-162    LNPSLKGRAIISVDTSNNKFSLKVTSVTAADTAVYYCAKWSESLNAFDEWGQGTLVTVSS  120
Clone 3       LNPSLKGRAIISVDTSNNKFSLKVTSVTAADTAVYYCAKWSESLNAFDEWGQGTLVTVSS  120
Clone 4       LNPSLKGRAIISVDTSNNKFSLKVTSVTAADTAVYYCAKWSESLNAFDEWGQGTLVTVSS  120
              ************************************************************
              Linker sequence                   VL/CDR1
yiDOA9-162    GGGGSGGGGSGGGGSEIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAP  180
Clone 3       GGGGSGGGGSGGGGSEIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAP  180
Clone 4       GGGGSGGGGSGGGGSEIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAP  180
              ************************************************************
                VL/CDR2                                 VL/CDR3
yiDOA9-162    RLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSSPPLTFGGGTK  240
Clone 3       RLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSSPPLTFGGGTK  240
Clone 4       RLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSSPPLTFGGGTK  240
              ************************************************************

yiDOA9-162    LEIKRA   246
Clone 3       LEIKRA   246
Clone 4       LEIKRA   246
```

Figure 4.2 (continued).

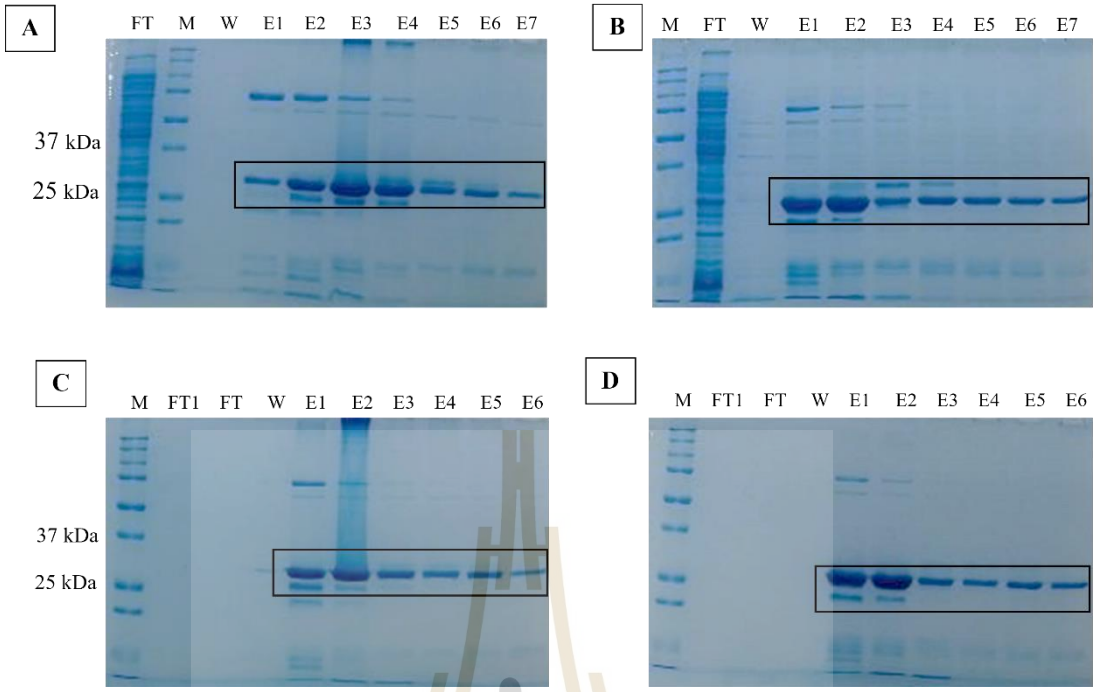

Figure 4.3 SDS-PAGE analysis of purified soluble scFv antibody. **(A)**The soluble scFv antibody against *Bradyrhizobium* strain DOA9 clone yiDOA9-162, and **(B)** soluble scFv antibody against *Bradyrhizobium* strain SUTN9-2 clone yiN92-1e10 were purified from cell lysate by IMAC. Lane M: protein molecular weight marker; lane FT: flow through fraction; lane W: wash fraction; lanes E1, E2, E3, E4, E5, E6, and E7 are the seven elution fractions. The soluble scFv antibody of approximately 30 kDa can be found in elution fractions E1 to E7. Due to a lot of co-eluted proteins, **(C)** all eluted fractions of scFv clone yiDOA9-162 and **(D)** yiN92-1e10 were purified again as second purification. FT1: flow through fraction exchange buffer from first purification.

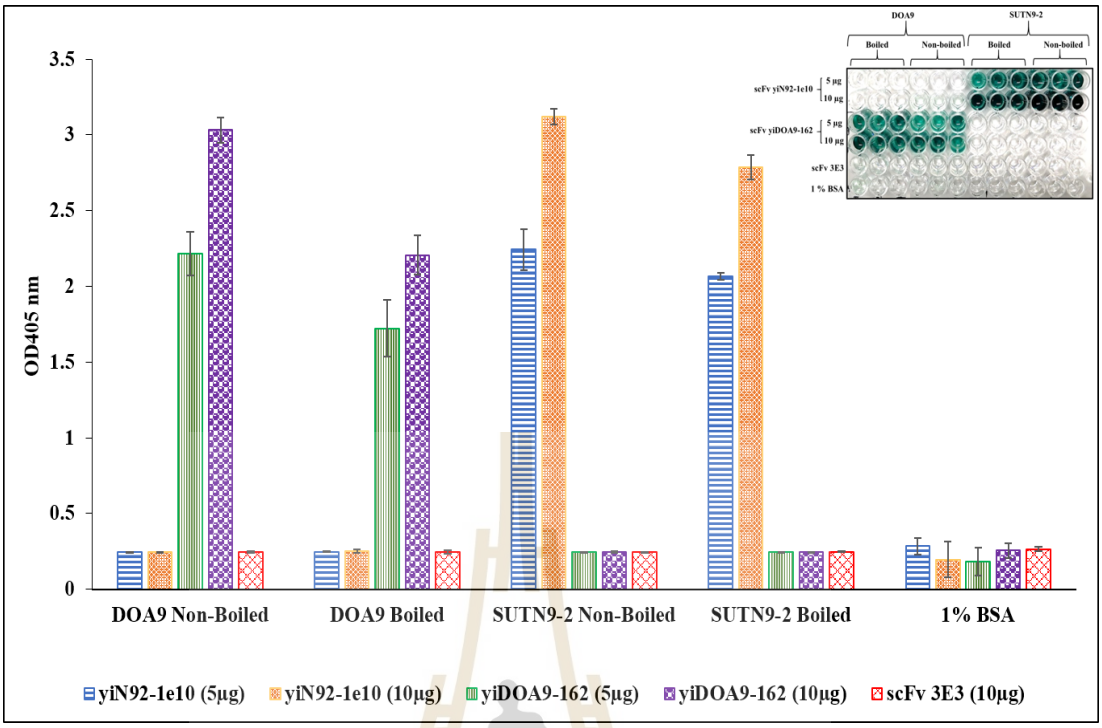

Figure 4.4 Binding property of free soluble scFv antibodies against pure culture cells. ELISA results of soluble scFv antibodies (yiN92-1e10 and yiDOA9-162) against *Bradyrhizobium* strains DOA9 and SUTN9-2 in pure culture. Values are the mean of triplicate reactions. Error bars show the standard deviation for each set of data. Negative control is 1%BSA.

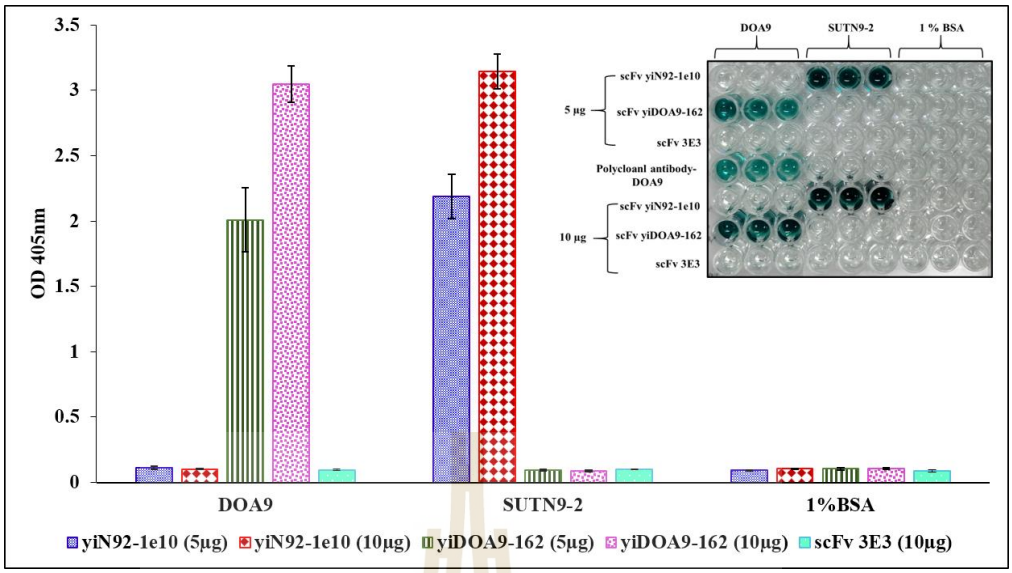

Figure 4.5 Binding property of free soluble scFv antibodies against bacteroid forms. ELISA results of soluble scFv antibodies (yiN92-1e10 and yiDOA9-162) against *Bradyrhizobium* strains SUTN9-2 and DOA9 obtained from the nodules of *V. radiata* and *A. americana*, respectively Values are the mean of triplicate reactions. Error bars show the standard deviation for each set of data. Negative control is 1%BSA.

Immunofluorescence staining of *Bradyrhizobium* strains in pure culture and nodule

Immunofluorescence analysis was also conducted to confirm the binding specificity of the free soluble scFv and to visualize the morphology of the bacteroid form inside plant nodule directly under the microscope. The scFv clone yiDOA9-162 and yiN92-1e10 had specific binding activity with DOA9 and SUTN9-2 target antigens, respectively, in both pure (Figure 4.6) culture and nodule sample (Figure 4.7). No cross reactivity was observed between the two scFv clones against strains DOA9 and SUTN9-2 in both pure culture and bacteroid forms. The morphology of the symbiosis nodule could be distinctly observed as the presence of green bacteroid

surrounded by blue plant tissue from Calcofluor staining, in comparison with the image obtained using bright field.

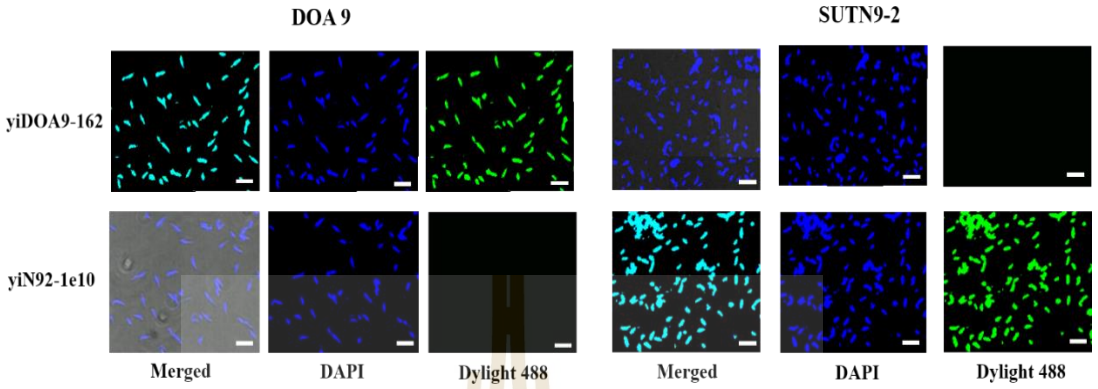

Figure 4.6 Confocal laser scanning micrographs of *Bradyrhizobium* strains SUTN9-2 and DOA9 cells from pure culture. The bacterial samples were incubated with two different scFv antibodies, i.e., yiN92-1e10 and yiDOA9-162, detected by Dylight 488-labeled anti-hexa-histidine mouse monoclonal antibody, counterstained with DAPI. Scale bars represent 10 μm at 600X mangnification. In DAPI panel, nucleoids of bacteria were stained blue. In Dylight 488 panel, only DOA9 was exhibited green fluorescence color by staining with scFv yiDOA9-162, and only SUTN9-2 was stained green by yiN92-1e10.

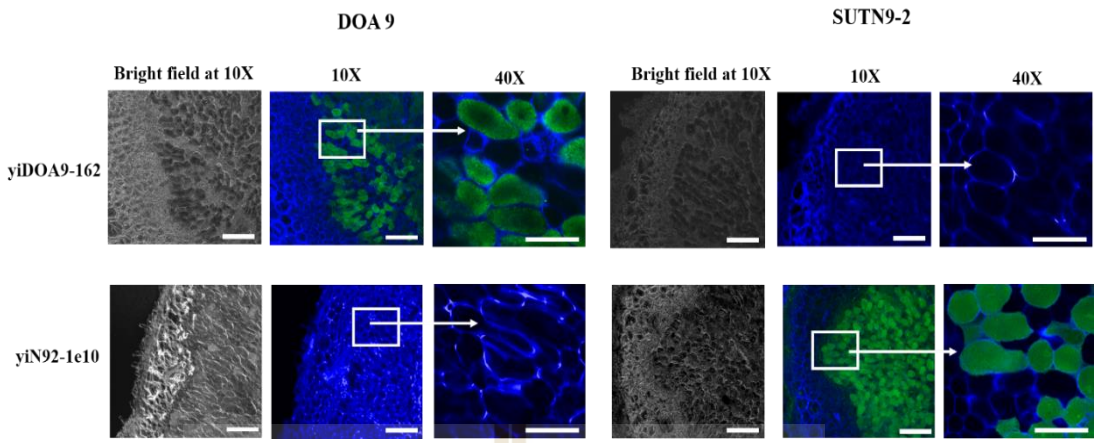

Figure 4.7 Confocal laser scanning micrographs of *Bradyrhizobium* strains SUTN9-2 and DOA9 in form of nodule bacteroid. The nodule slices were stained with two different scFv antibodies, i.e., yiN92-1e10 and yiDOA9-162. Green spot indicated the green-fluorescent staining of scFv antibody, using secondary antibody conjugated to anti-His Dylight 488. Plant cell walls were stained with a blue fluorophore (Calcofluor white M2R) and emitted blue color. The bacteroid forms are shown as green spots inside a blue plant cavity. Scale bars represent 100 µm at 100X magnification and 50 µm at 400X magnification.

4.2 Cross-reactivity analysis of *Bradyrhizobium* SUTN9-2 scFv antibody clone yiN92-1e10 against related *Bradyrhizobium* strains

The scFv antibody against SUTN9-2 has been selected to further study the possibility of using scFv antibody to monitor the bacteria in the ecosphere since many *V. radiata* legumes are able to form nodule symbiotically with SUTN9-2 (Piromyou et al., 2019), and that strain could be able to promote beneficially rice plant growth as endophytes (Piromyou et al., 2017), allowing a period of monitoring in legume-rice rotational crop system. Consequently, the specificity of anti-SUTN9-2 scFv antibody clone yiN92-1e10 was further checked against 27 other related *Bradyrhizobium* strains

by ELISA before applying in a rotational cropping system. The results demonstrated that yiN92-1e10 was specific with only SUTN9-2 and no cross reactivity with other related *Bradyrhizobium* strains tested (Figure 4.8), indicating that strain specific scFv antibody against *Bradyrhizobium* strain SUTN9-2 could be generated.

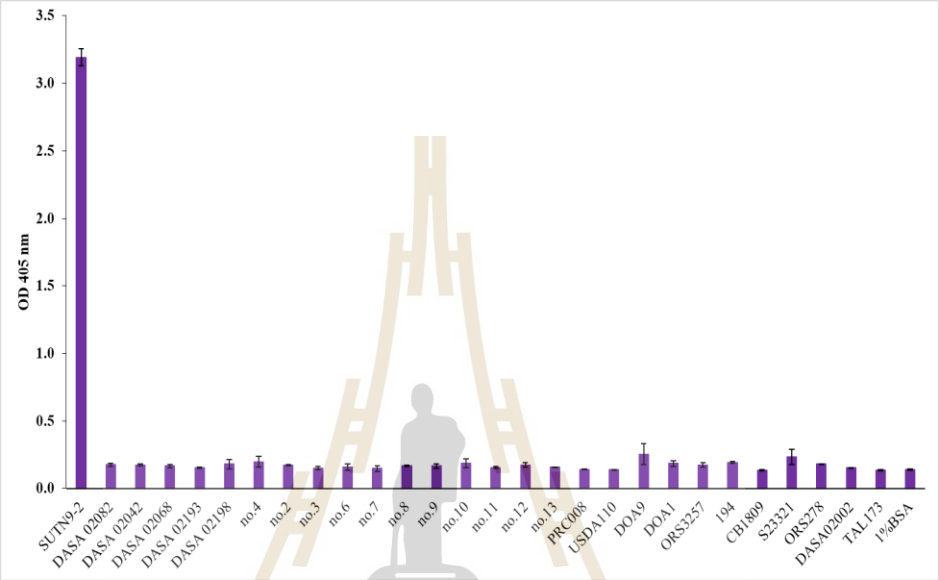

Figure 4.8 Cross reactivity of scFv yiN92-1e10 against related 27 *Bradyrhizobium* strains. The wells of microtiter plate were coated with 5 µg/well of each target antigen and detected with 5 µg of scFv yiN92-1e10. One percent BSA was served as negative control. Values are the mean of triplicate wells. Error bars exhibit the standard deviation for each set of data.

4.3 Detection of the persistence of SUTN9-2 in rice plant

To detect the persistence of SUN9-2 in rice grown in pots under greenhouse conditions, the inoculated rice seeds, leaves, leaf sheath and roots were investigated at different time intervals (1, 2, 3, and 4-months after inoculation) by ELISA and confocal immunofluorescence staining. As illustrated in Figure 4.9, *Bradyrhizobium* SUTN9-2 cells could be detected by ELISA with specific scFv yiN92-1e10 in the extract of rice seeds, leaves, leaf sheath and root at various time points (1^{st}, 2^{nd}, 3^{rd}, and 4^{th} month

after rice cultivation in the pots). Even though, the absorbance values from each rice tissue did not significantly different at various time points, the bacteria seemed to be especially prevalent in the leaf sheath. At the harvesting time (4th month after cultivation), the rice seeds were also harvested and the endophytic SUTN-2 in the seeds were also detected. The lowest ELISA signal was observed in the seeds when compared with other rice plant tissues at the harvesting time (4-month after planting).

Moreover, the rod-shaped bacteria were also observed in the intercellular and intracellular spaces of seeds, leaves, leaf sheath, and root of rice plant at different time points (Figure 4.10A, B, C, and D) when these tissues were immuno-stained with specific scFv yiN92-1e10 and checked under Confocal Laser Scanning Microscope. No bacterium was observed in the non-inoculated rice plant tissues.

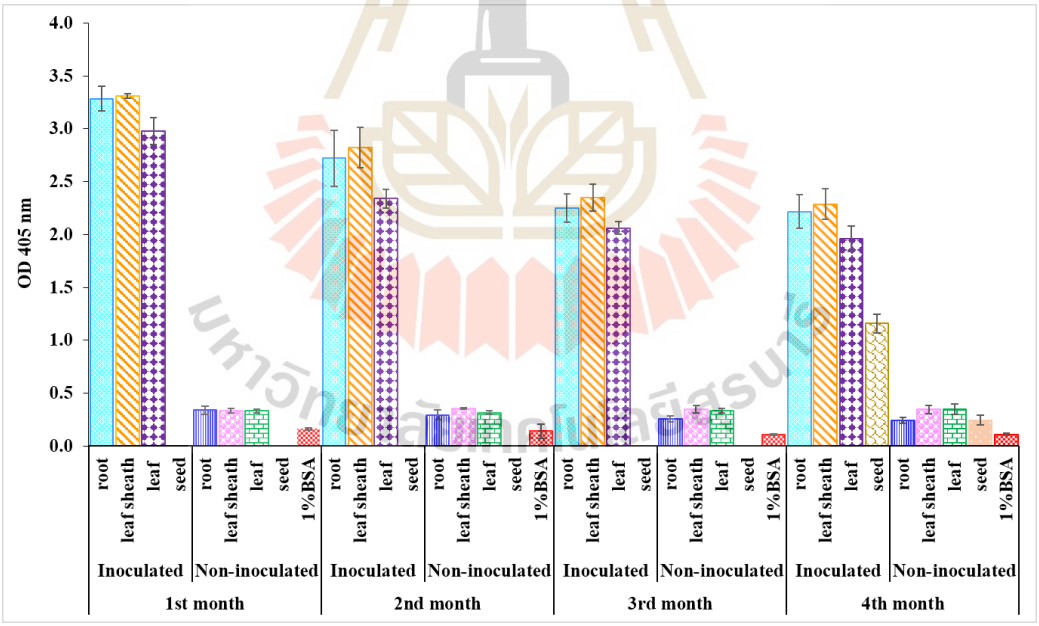

Figure 4.9 Detection of SUTN9-2 bacterial cells from extracts of rice root, leaf, leaf sheath and seeds by ELISA. The values are the mean of triplications. Error bars show the standard deviation for each set of data.

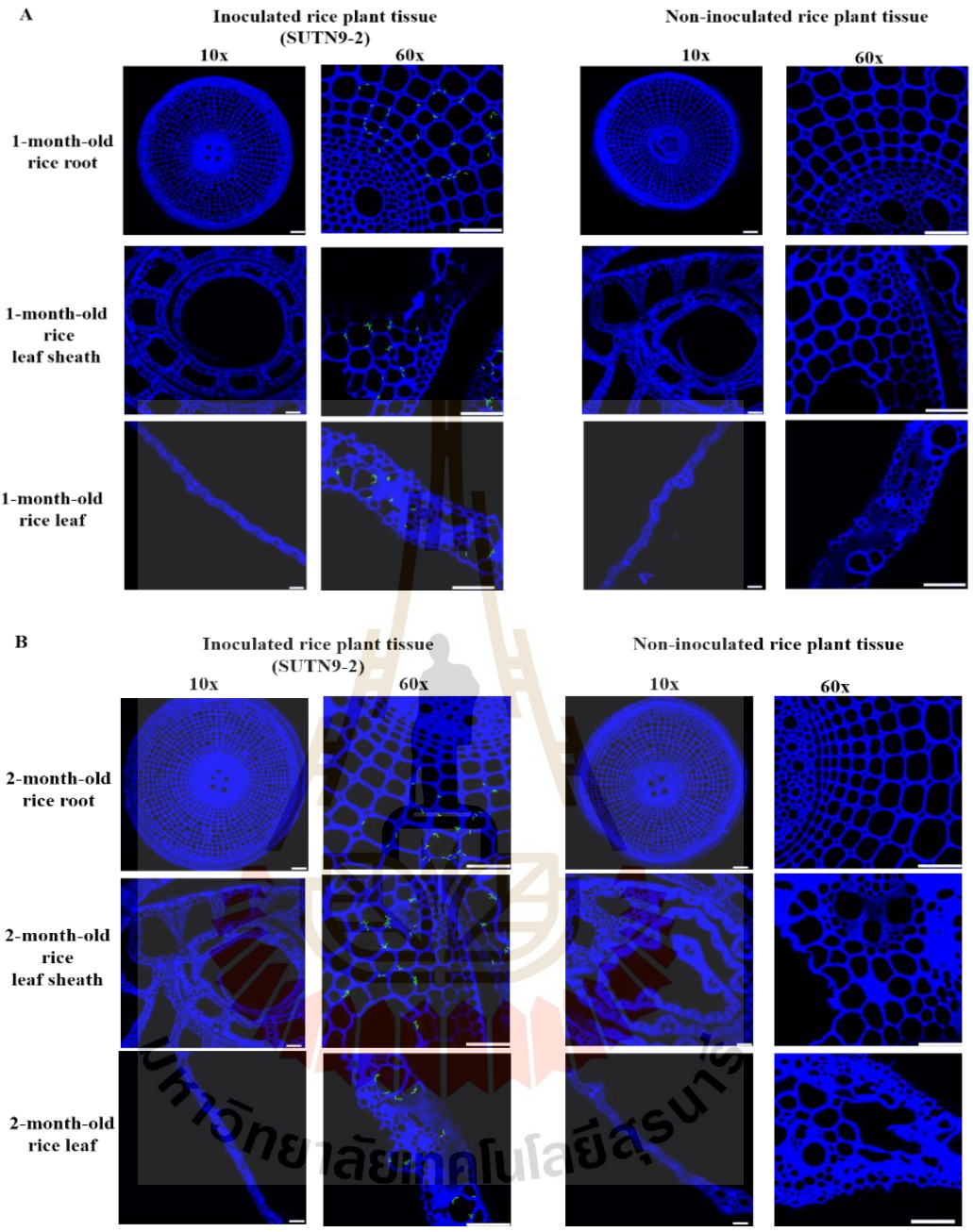

Figure 4.10 Confocal laser scanning micrographs of rice root, leaf, leaf sheath and seed inoculated with SUTN9-2 at 1st (B), 2nd (C), 3rd (D), and 4th (E) month after cultivation. Cross-section of the tap root, flag leaf, leaf sheath above the stem base and seeds were examined as indicated in each figure. Scale bar is 100 µm at 100x magnification, and 50 µm at 600x magnification.

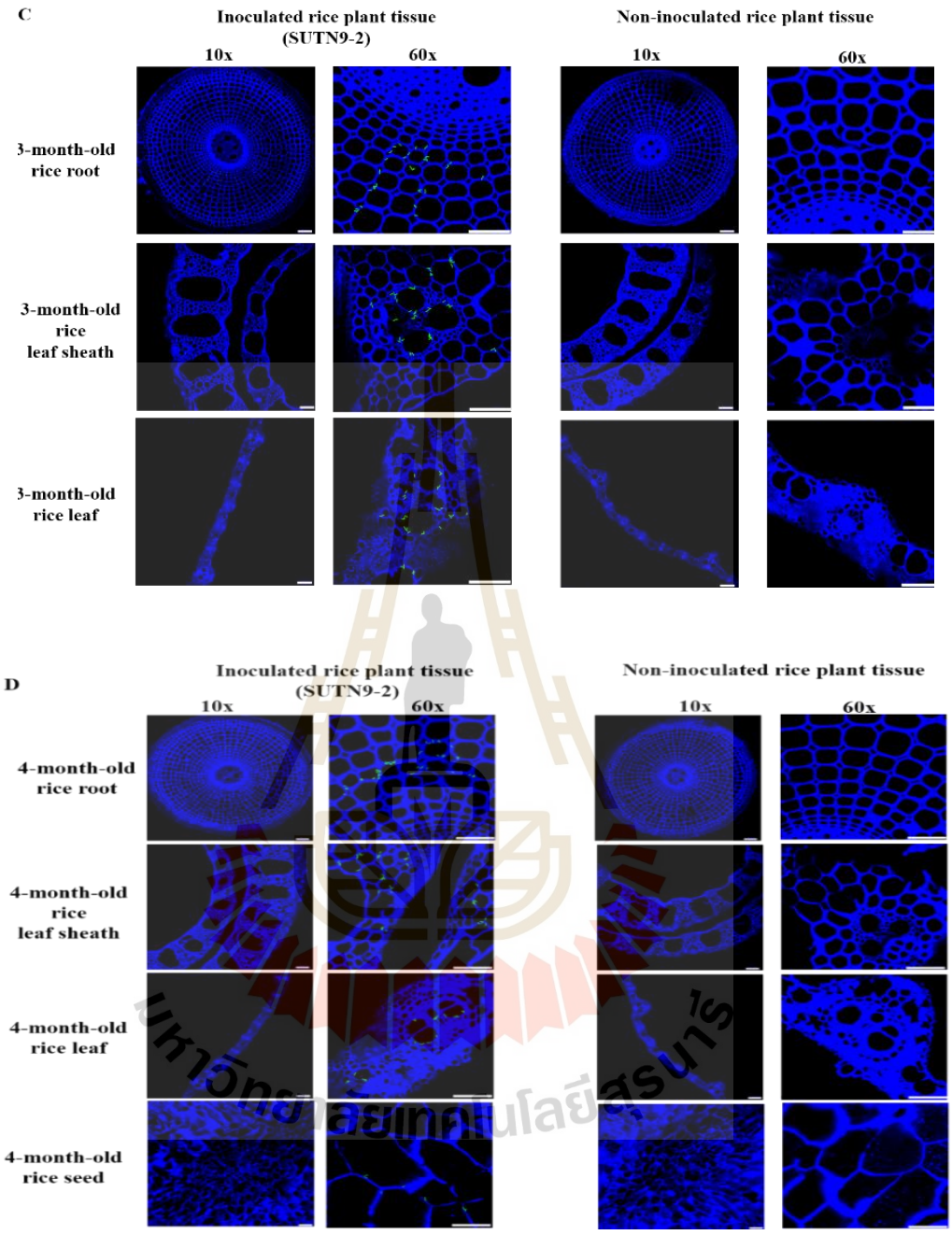

Figure 4.10 (continued).

4.4 Investigation of SUTN9-2 nodulation in mung bean using rice stubble as inoculum

The scFv yiN92-1e10 was applied to monitor SUTN9-2 in soil samples at various time points by ELISA to complete the observation of a legume-rice-legume cropping system (Figure 4.11). The population of SUTN 9-2 in soil steadily increased from the time of rice production until 4 weeks later, corresponding to an increase in the ELISA signal. Only background value was detected in non-inoculated soil. After 4 weeks, the mung bean seeds were grown into each pot for imitation of the rotational cropping system. The nodulation was found on the root of the mung bean at 28 days after cultivation. The symbiotic *Bradyrhizobium* strain SUTN9-2 inside the nodule could be detected with specific scFv yiN92-1e10 by ELISA (Figure 4.12) and confocal immunofluorescence staining (Figure 4.13). There was no signal when non-specific scFv yiDOA9-162 was applied in both assays. These results demonstrated that SUTN9-2 persisted in rice tissues until rice-harvest season and could be utilized as inoculum for mung bean in the following season in the rotational cropping system and the scFv yiN92-1e10 will be an applicable tool for monitoring the persistence of bradyrhizobial inoculum under this cropping system.

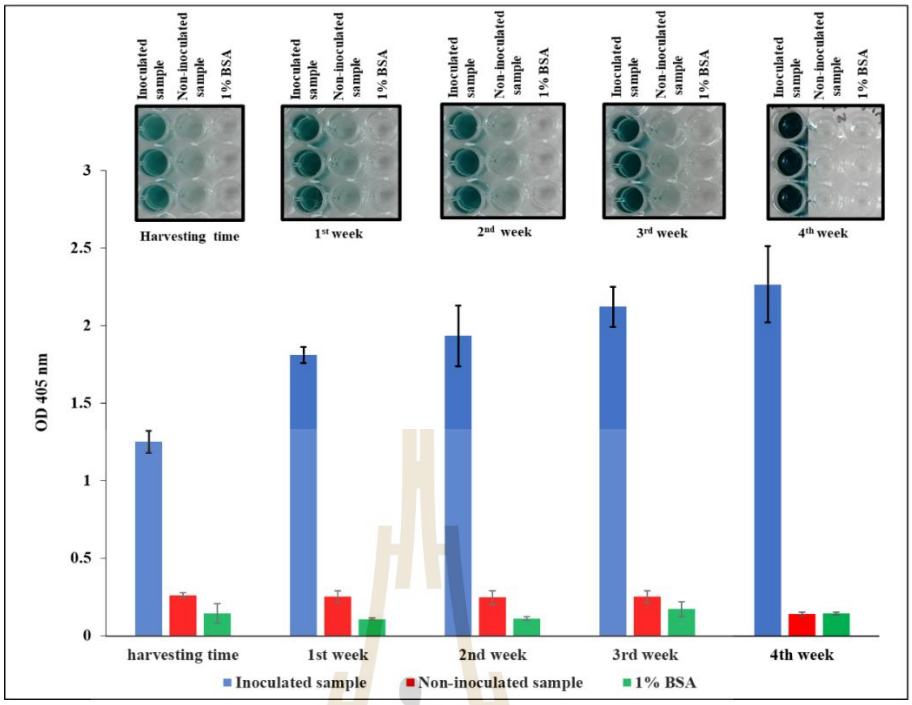

Figure 4.11 Detecting the persistence of *Bradyrhizobium* SUTN9-2 from rice stubbles in the soil by ELISA. The values are the mean of triplicates. Error bars represent the standard deviation for each set of data.

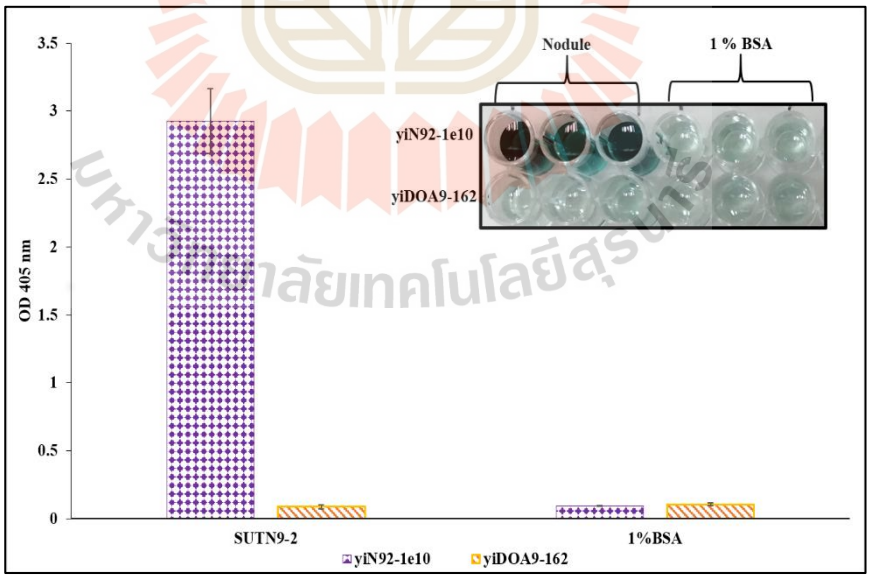

Figure 4.12 Detecting the nodulation of *Bradyrhizobium* SUTN9-2 from rice stubbles in mung bean by ELISA. The values are the mean of triplicate wells. Error bars represent the standard deviation for each set of data.

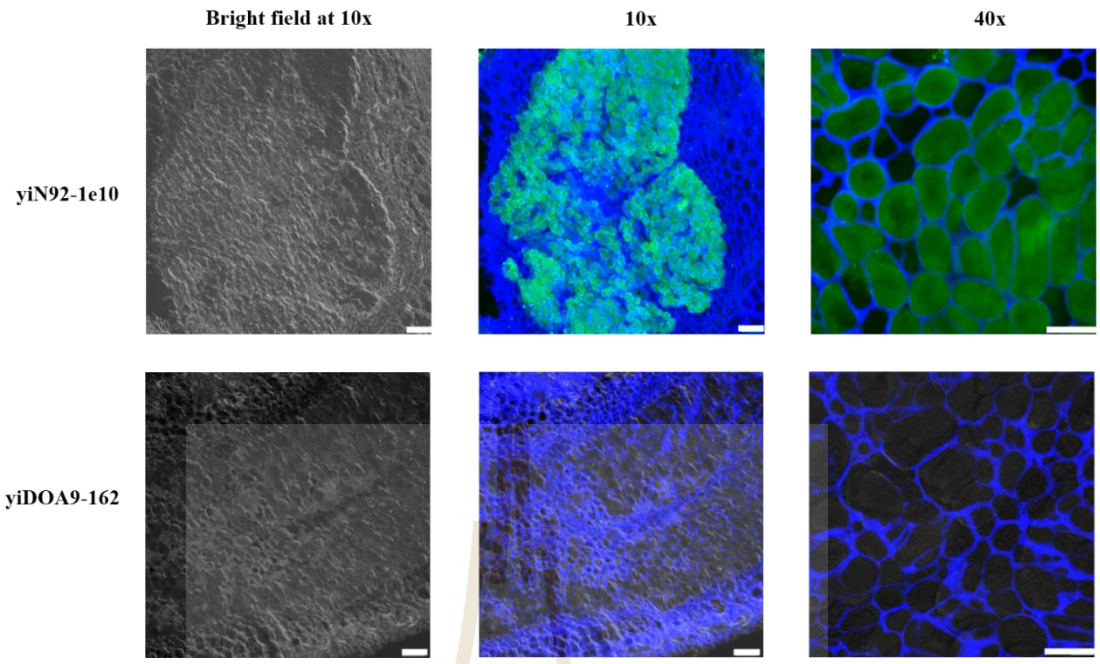

Figure 4.13 Confocal laser scanning micrographs of nodule of mung bean after cultivation of rice inoculated with SUTN9-2. The cross-sections of nodule were immunostained with scFv yiN92-1e10 and yiDOA9-162. Green spot indicated the green-fluorescent staining of scFv antibody, using secondary antibody conjugated with anti-His- Dylight 488. Plant cell walls were stained with a blue fluorophore (Calcofluor white M2R) and emitted blue color. The bacteroides are shown as green spots inside a blue plant cavity. Scale bar is 100 µm at 100x magnification, and 50 µm at 400x magnification.

4.5 Determination of nodule occupancy by scFv antibody immunofluorescence staining

At last, the potential application of recombinant scFv yiN92-1e10 antibody to study symbiotic nodule occupancy percentage was investigated in contrast to the standard Gus-staining assay. Mung bean plants were inoculated with *Bradyrhizobium* sp. SUTN9-2 wild type or Gus-tagged strain, and PRC008 wild type (single

inoculation) or a combination of SUTN9-2 wildtype or Gus-tagged and PRC008 at 1:1 ratio (co-inoculation). After 30 days, the nodules were checked under confocal laser scanning microscope for immunostaining of wild type samples or a light microscope for Gus-staining samples. Green spots revealed the green- fluorescent staining of scFv yiN92-1e10 antibody, detected by Dylight 488-labeled anti-hexa-histidine mouse monoclonal antibody. By this immunofluorescence staining, the nodule area occupied by SUTN9-2 (WT) exhibiting the green-fluorescent color was easily distinguished from the area occupied by PRC008 (without fluorescence) in the nodule occupied by dual strains (Figure 4.14, right panel). For Gus-staining assay, the area occupied by SUTN9-2 Gus-tagged strain developed blue color from the beta-galactosidase substrate X-Gluc. Assessment of nodule occupancy (Table 4.1) showed that 59.71 or 60.74% of the nodules were co-occupied by both *Bradyrhizobium* strains, when determined using scFv staining or Gus assay, respectively. Furthermore, the results also indicated that the PRC008 strain was more competitive than SUTN9-2 in nodule formation. The percentage of single nodule occupied by SUTN9-2 and PRC008 was 9.17 and 31.12%, respectively, when determined by immunostaining. For Gus-staining, the percentage of one single nodule occupied by SUTN9-2 and PRC008 was 13.18 % and 26.08 %, respectively. This finding might be due to the high sensitivity of scFv techniques, especially for mung bean. Based on these results, it could be concluded that recombinant scFv antibody is a valuable reagent for the study of symbiotic nodule formation in legumes.

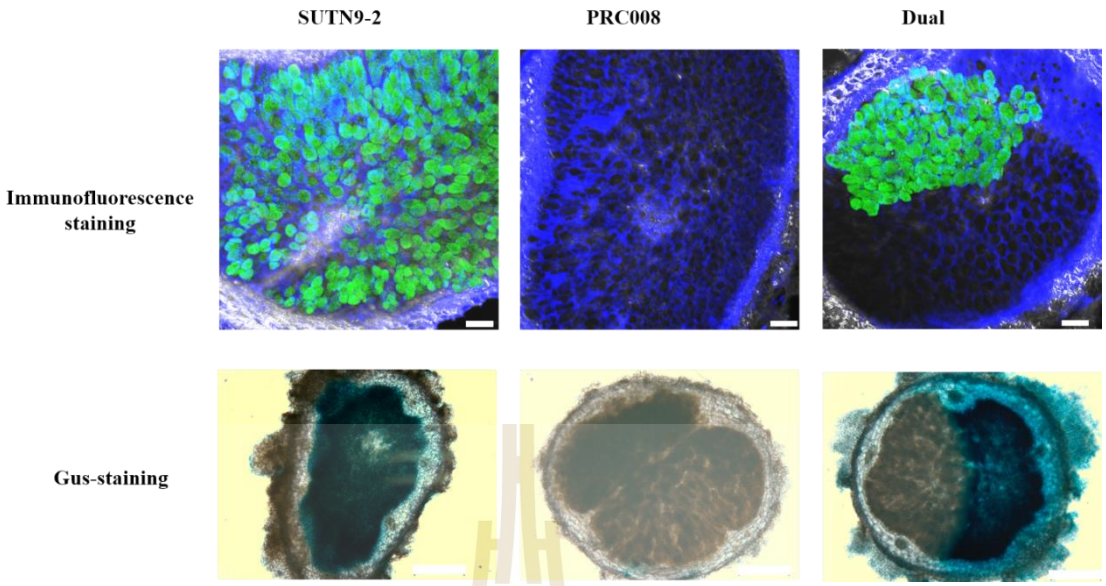

Figure 4.14 Bioimaging of nodule occupancy. Nodule phenotype after co-inoculation with SUTN9-2 (wild type) and PRC008 (wild type) for immunofluorescence staining (upper panel), and with SUTN9-2 (Gus-tagged strain) and PRC008 (wild type) for Gus-staining (lower panel). Green spots indicated the green-fluorescent staining of scFv yiN92-1e10 antibody, using secondary antibody conjugated with anti-His- Dylight 488. Blue color indicated Gus-staining of SUTN9-2 (SUTN9-2 Gus reporter gene-tagged strain) using the X-Gluc substrate. Scale bar is 100 µm and 500 µm at 100X magnification for immunofluorescence staining and Gus-staining, respectively.

Table 4.1 Immuno- and Gus- staining for the analysis of nodulation competition SUTN9-2 and PRC008 (1:1).

Plant inoculated with	Nodule occupancy %					
	Immunostaining			Gus-staining		
	SUTN9-2	PRC008	Co-occupied	SUTN9-2	PRC008	Co-occupied
SUTN9-2	100 ± 0	0 ± 0	0 ± 0	100 ± 0	0 ± 0	0 ± 0
PRC008	0 ± 0	100 ± 0	0 ± 0	0 ± 0	100 ± 0	0 ± 0
SUTN9-2 and PRC008 (1:1)	9.17 ± 2.96	31.12 ± 11.59	59.71 ± 9.87	13.18 ± 7.86	26.08 ± 15.50	60.74 ± 19.43

The values are the mean of five replications.

4.6 Biopanning of peptides binding to yiN92-1e10 IgG antibody from phage display peptide library

4.6.1 Mammalian expression vectors for expression antibodies

The mammalian IgG vectors pKR-CH and pKR-CL were used to express the IgG kappa by cloning the VH genes with *NheI*/ *NheI* sites to express the human IgG heavy chain and VL kappa gene via *NheI*/ *BsiWI* restriction sites to express kappa light chain (Figure 4.15).

4.6.2 Expression and purification of antibody

The scFv antibody was engineered to be IgG format by cloning into mammalian expression vector. IgG was produced in Expi293F™ cells in 25 ml scale. This antibody format was purified by protein A affinity chromatography. The purity and apparent molecular weight of purified antibodies was assessed by SDS-PAGE analysis. The molecular weights were observed approximately 50 and 25 kDa for heavy chain and light chain, respectively (Figure 4.16).

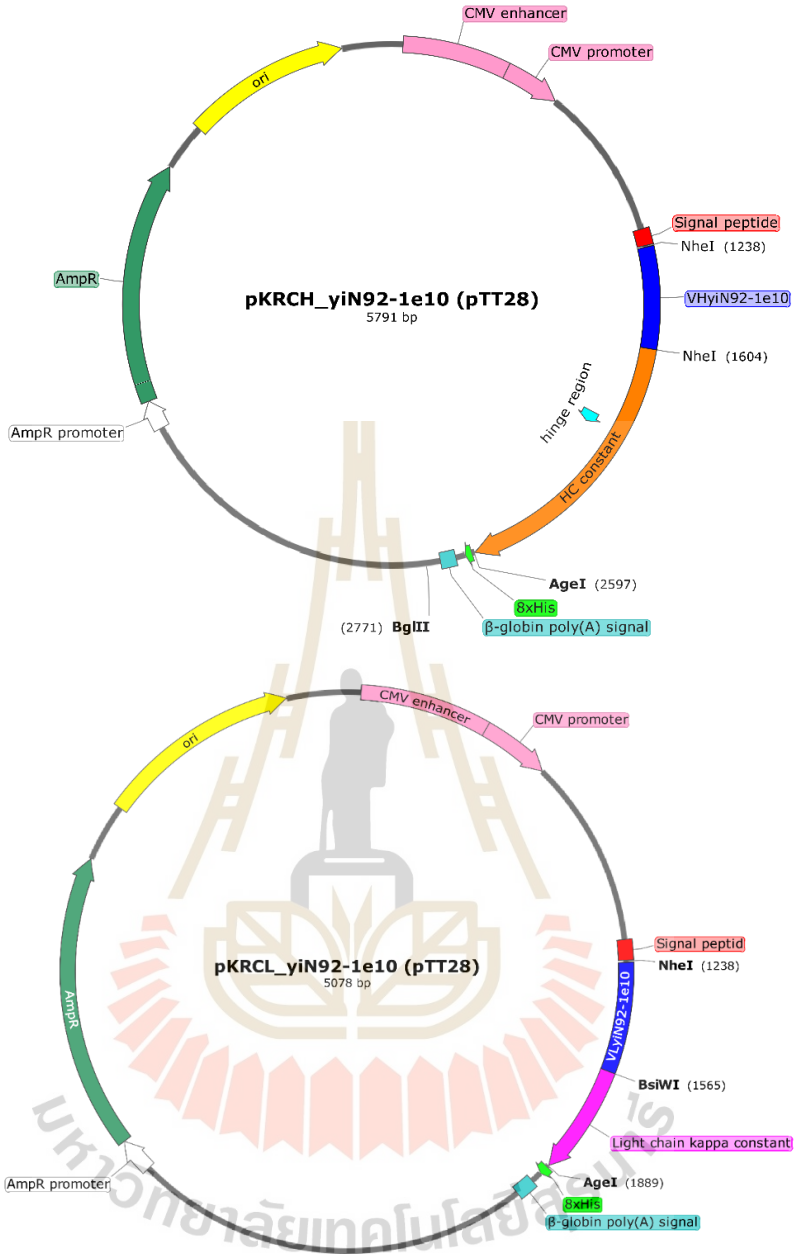

Figure 4.15 Map of pKRCH_yiN92-1e10 and pKR_CL-yiN92-1e10 vectors (pTT28). VH and VL genes were subcloned into the pKR-CH and pKR-CL vectors between *NheI*/ *NheI* and *NheI*/ *BsiWI* restriction sites, respectively.

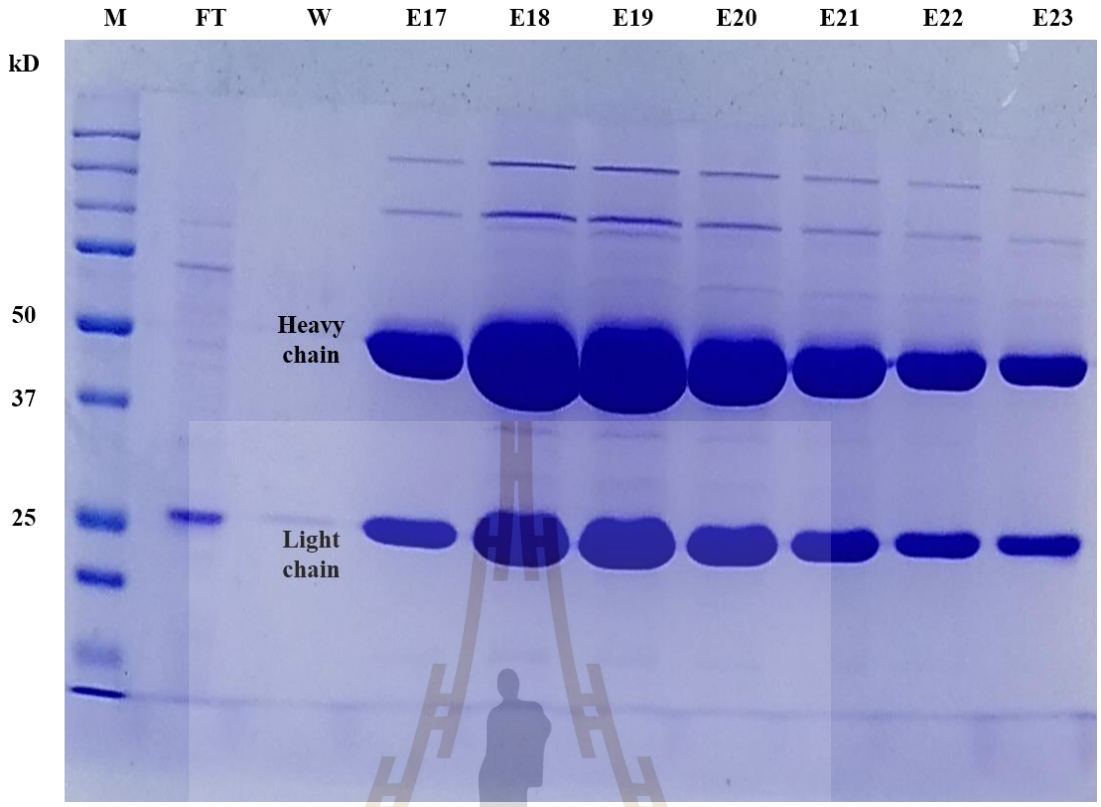

Figure 4.16 Protein-A affinity chromatography purified fractions from supernatant of transient expression of IgG in Expi293F™ cells. M, protein molecular weight marker (All blue), lane FT, flow-through fraction; lane W, wash fraction; lanes E17, E18, E19, E20, E21, E22, and E23 are the seven elution fractions. Twenty microliters per lane of each fraction were analyzed on 12% polyacrylamide gel and stained with coomassie blue. The size of IgG is shown about 50 and 25 kDa for heavy chain and light chain, respectively.

4.6.3 Binding analysis of individual phage with yiN92-1e10 using ELISA

After the 3rd panning round, a total of 12 phage clones were randomly selected and amplified. In order to test the binding ability of the selected phage clones, 12 independent phage clones encoding different amino acid sequences were selected and detected by ELISA using the purified recombinant yiN92-1e10 IgG as target. Among these yiN92-1e10 binding peptides, clone no. 2, 4, 7, 8, 9, 10, 11, and 12 could bind specifically to target protein (yiN92-1e10) but not to negative control 1% BSA. The binding ability of clone no. 1, 3, 5, and 6 are not significantly different from 1% BSA (Figure 4.17). Although there is no criterion for judging the positive clones, it is suggested that the clone no. 2, 4, 7, 8, and 9 might have a higher affinity to yiN92-1e10 IgG than the other clones.

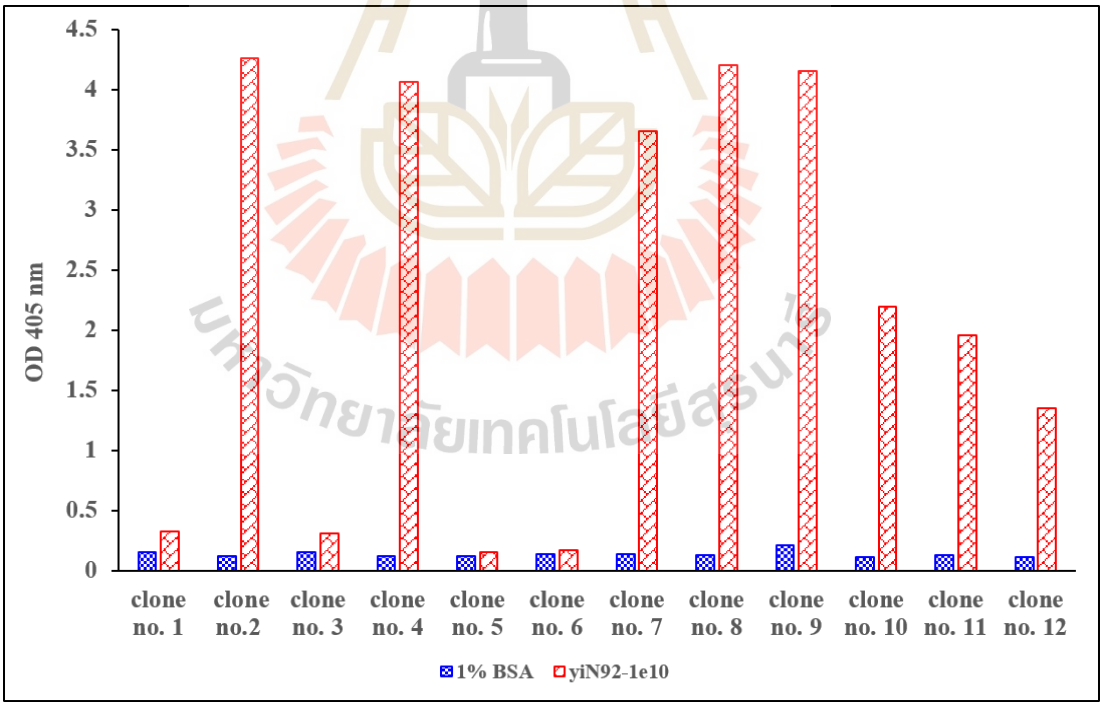

Figure 4.17 ELISA analysis of binding activities of the selected phage clones. Negative control is 1% BSA.

4.6.4 Amino acid sequence analysis of specific peptides

After the third panning round, a total of 12 phage clones were randomly selected and amplified. Seven phage clones that showed positive signals in ELISA were selected for automated DNA sequencing to determine their amino acid sequences. Among 7 phage clones sequenced, the peptides no. 9 and 10 shared the same consensus sequence RFxTGEWxGxPM (x is any amino acid residue). The clone no. 2 and 7 also has a consensus sequence WGA, and no. 7 bear WAWGxAF similarity with clone 11.

Table 4.2 The peptide sequences of selected phage clones.

Phage clone	Phage displayed peptide amino acid sequence
4	WNNPM***F W***DP***G***SI
2	GDP***F***VE***WGA***KML
7	DNIRE***WAWGAAF***
11	TAH***WAWG***WYD***AF***
8	EL***WA***NAANAT***G***L
9	***RF***M***TGEWA***GN***PM***
10	***RF***T***TGEW***V***G***S***PM***

The conserved amino acids are indicated in bold and italics.

4.6.5 Comparison of bound phage sequences with GenBank sequences

After comparing the mimotopes of these phages with protein sequences from GenBank database using BLASTP, interestingly, TGEWAGxPM mimotope was found to match with protein sequences of *Bradyrhizobium* sp. SUTN9-2 (Table 4.3). BLAST search for the TGEWAGxPM motif against all non-redundant GenBank, showed seven hits, out of which only one hit showed five-amino-acid-long sequence, whereas the other five sequences are composed of four amino acids, and there is only three-amino-acids in one hit.

Using PsortB software to predict protein localization in the cell, the following GenBank matched proteins were found to be located at cytoplasmic membrane of *Bradyrhizobium* sp. SUTN9-2: lytic murein transglycosylase (genebank accession number WP_109143668.1) that matched with mimotope -GxWAG---, nicotinamide adenine dinucleotide cofactor, NAD(P)-dependent Oxidoreductase (genebank accession number WP_109145137.1) that matched with mimotope -EWAGxxM, cyclic adenosine 3', 5'-monophosphate, cAMP-binding protein, partial (genebank accession number WP_146207794.1) that matched with mimotope ---WAGxP-, cupin domain-containing protein (genebank accession number WP_109141748.1) that matched with mimotope TGEW----, multidrug efflux MFS transporter (genebank accession number WP_109142319.1) that matched with mimotope -GxWAGx--, ATPases associated with diverse cellular activities, AAA family ATPase (genebank accession number WP_109141653.1) that matched mimotope -GEWA----, and protein adenylyltransferase SelO, YdiU family protein (genebank accession number WP_109145829.1) that matched with mimotope --EWAG---.

Table 4.3 BLAST search result against TGEWAGxPM motif.

Protein	Accession number	Align sequence[a]
Lytic murein transglycosylase (*Bradyrhizobium* sp. SUTN9-2)	WP_109143668.1	183 -GxWAG--- 187
Nicotinamide adenine dinucleotide cofactor, NAD(P)-dependent Oxidoreductase (*Bradyrhizobium* sp. SUTN9-2)	WP_109145137.1	289 --EWAGxxM 295
Cyclic adenosine 3', 5'-monophosphate, cAMP-binding protein, partial (*Bradyrhizobium* sp. SUTN9-2)	WP_146207794.1	338 ---WAGxP- 342
Cupin domain-containing protein (*Bradyrhizobium* sp. SUTN9-2)	WP_109141748.1	112 TGEW---- 115
Multidrug efflux MFS transporter (*Bradyrhizobium* sp. SUTN9-2)	WP_109142319.1	218 -GxWAGx-- 223
ATPases associated with diverse cellular activities, AAA family ATPase (*Bradyrhizobium* sp. SUTN9-2)	WP_109141653.1	23 -GEWA---- 26
Protein adenylyltransferase SelO, YdiU family protein (*Bradyrhizobium* sp. SUTN9-2)	WP_109145829.1	400 --EWAG--- 403

[a] Numbers indicate amino acid positions of the protein.

A dash indicates blank space (no amino acid at this position).

“x” is any amino acid residue

CHAPTER V

DISCUSSIONS AND CONCLUSION

Phage display is widely used to generate human monoclonal antibodies (Barderas & Benito-Peña, 2019). The recombinant antibodies produced by phage display technology offer several advantages, such as small amounts of antigen, large scale and cost-effective. Compared with other antibody formats, scFv format has many advantages, namely, more powerful and stable for display on phage coats (Michael et al., 1998), higher permeability due to nano size (Kasturirangan & Sierks, 2010), easy to express in *E. coli* (Sompunga et al., 2019) and other expression hosts (Verma et al., 1998). It can also be further engineered to suit several assay formats, including biosensor-based detection (Rangnoi et al., 2011). The research from this study emonstrates, for the first time, the benefits of applying a compact human phage display scFv antibody library for rhizobium research. The antibody library in this study was produced from a healthy population that had previously encountered the numerous antigens in the region of northeastern Thailand around 15 years ago, apparently from this study, including nitrogen-fixing soil bacteria (Pansri et al., 2009). Specific scFv antibodies from this library has been generated against a wide variety of targets such as mycotoxins (Rangnoi et al., 2011; Sompunga et al., 2019), venom (Kazemi-Lomedasht et al., 2019), pathogenic bacteria (Min & Yamabhai, 2021), as well as virus (Pruksametanan et al., 2012) and cancer biomarker (Duranti et al., 2018). From the previous study, the recombinant scFv antibody that could bind

specifically to *Bradyrhizobium* strain DOA9 was successfully generated (Vu et al., 2017), from a phage-displayed rabbit scFv antibody library, generated from an immunized rabbit. In this study, specific scFv antibodies against two *Bradyrhizobium* strain, DOA9 and SUTN9-2, could be obtained from a naïve human phage library without utilization of experimental animals. The library was based on the naïve human re-arranged V-genes and assembled through the use of a high diversity gene repertoire derived from 140 non-immunized donors (Pansri et al., 2009). Since the method does not depend on an animal's immune system, antibodies to a wide variety of antigens, including the molecules that cannot stimulate immune system of the animals such as nonimmunogenic, "self", cell surface or toxic antigens, can be generated (Bugli et al., 2008; Griffiths et al., 1994; Winter et al., 1994). In addition, specific antibodies have also been successfully generated from naïve human phage display libraries against several important plant pathogens such as *Phytophthora infestans* (Gough et al., 1999), *Ralstonia solanacearum* (Griep et al., 1998), *Alternaria alternata* (melanin) (Carzaniga et al., 2002). The recombinant scFv antibodies for bacteria expression in this study based on pET-21d (+) vector, and this vector was expressed in *E. coli* SHuffle® T7 Express, an *E. coli* host that promotes disulfide bond formation in the cytoplasm (Ren et al., 2016). Therefore, the recombinant proteins were extracted from the cell cytoplasm. The purified proteins from cell lysate contained unwanted proteins as shown in SDS-PAGE. Therefore, double purification was performed to get rid of co-eluted proteins (Figure 4.3). Nickel-nitrilotriacetic acid (Ni^{2+}-NTA) column IMAC purification with the pET21d hexa-His tag system exhibited the affinity of the tagged target protein for the Ni^{2+}-NTA column to be noticeably weak, give rise to both an undesirable loss of target protein in flow-through and very early dissociation upon

elution. This result was in accordance with previous report that hexa-his tagged protein showed weak affinity for the nickel column and was therefore lost to flow-through and overly contaminated with early co-eluting proteins (Jones et al., 2016). The reason why the protein could not be purified to apparent homogeneity could be because the His-tag was not very efficient for protein purification.

The isolated purified scFv antibody can bind to the bacterial cells in pure culture (both boiled and non-boiled), the bacteroid forms inside plant nodule, and endophyte inside the rice tissue. The antigen on the bacterial cell surface, which seems to be heat stable and dominant in all forms of this diazotroph will be identified in the next step. *Bradyrhizobium* sp. usually contain extracellular polysaccharide (EPS), capsular polysaccharides (CPS) or K-antigen, flagella proteins or H-antigen, and lipopolysaccharide (LPS) or O-antigen (Carlson, 1984). Since, K and H-antigens are heat labile (Somasegaran & Hoben, 1994), the candidate epitope of this selected antibodies is likely to be present on an O antigen or another strain specific cell surface molecule. This information will be valuable in understanding the mechanism of specificity in legume-rhizobium interactions and the evolution of rhizobium symbiosis. Even though, both *Bradyrhizobium* strains SUTN9-2 and DOA9 are rice endophytes, DOA9 cannot promote rice plant growth effectively (Piromyou et al., 2015). Therefore, strain SUTN9-2 was selected as inoculated strain in rice-legume rotational cultivation. Determination of cross-reactivity to related *Bradyrhizobium* strains (both native and reference strains) demonstrated that scFv yiN92-1e10 has high specificity with *Bradyrhizobium* sp. SUTN9-2. The high specificity of this recombinant yiN92-1e10 antibody indicated that it could be used to accurately distinguish *Bradyrhizobium* strain SUTN9-2 from other related strains in the environment. However, a minimum of 10^2-

10^3 cells are required in the environmental samples such as soil or peat inoculant for a detectable ELISA reaction, limiting the use of this technique when the number of rhizobia is low (Nambiar & Anjaiah, 1985). The phage scFv ELISA also had a minimum detection limit of 4-5 x 10^5 cells for pure culture of *Bradyrhizobium* sp. DOA (Vu et al., 2017). Therefore, the recombinant yiN92-1e10 antibody from this study should be suitable for routine quality control and monitoring persistence of SUTN9-2 inoculum as biofertilizer (at least 10^5 cells) for sustainable and precision agriculture of rice-legume rotational cropping system. Using the scFv yiN92-1e10 antibody, the strain SUTN9-2 could be detected as endophytes by ELISA and immunofluorescence assay in both the intercellular and intracellular spaces of rice tissues at different growth stages. This result was in accordance with previous report on the presence of endophytic *R. leguminosarum* bv. *trifolii*, recovered from surface-sterilized leaf sheaths, leaves, and roots (Chi et al., 2005). According to the previous study, the detectable number of SUTN9-2 cells in each inoculated rice plant tissue might be higher than 10^5 cells (Vu et al., 2017). The population of *Bradyrhizobium* strain NC 92 in peat containing 10^6, 10^7, 10^8, and 10^9 cells/g exhibited optical density values 0.6, 1.2, 1.8, 2.4, and 3 at OD_{405}nm, respectively. The number of rhizobia estimated by ELISA were close to the values obtained from the plate count and plant infection techniques (Nambiar & Anjaiah, 1985). According to this, the population of *Bradyrhizobium* SUTN9-2 cells in different rice plant tissues and soil at higher OD value might be higher than those at lower OD values. These results also confirmed previous observation that endophytic rhizobia persisted in rice throughout the rice growing season and could infect the rotational crop, mung bean (Piromyou et al., 2017). The colonization of healthy rice plant tissues by endophytic *gfp*-tagged rhizobia and

gus-tagged *Gluconacetobacter diazotrophicus* strain PAL5 were detected by plate count method, fluorescent microscopy, and bright field microscopy (Chi et al, 2005; Rouws et al., 2010). However, genetically modified organisms (GMOs) have always been considered as a threat to environment and human health. Consequently, these GMOs should not be applied in real filed condition (Prakash et al., 2011).

Previous studies on the evaluation of nodule occupancy was done using reporter genes such as *gfp* and *gusA* (Ramos et al., 2007). Although this method is effective, it requires to produce genetically modified organisms (GMOs) that express *gfp* and/ or *gusA* genes at regions that would not disrupt bacterial growth and nodulation capacity. Moreover, GMOs have always been considered a threat to the environment and human health (de Melo-Martín & Meghani, 2008) and need to be regulated under the general statutory authority of environmental, health, and safety laws. This study revealed that the effectiveness of immunofluorescence staining, and Gus-staining methods was not significantly different for the determination of nodule occupancy percentage (Table 4.1). The high rate of dual occupancy of nodules reported in *Parasponia andersonii* (Trinick & Hadobas, 1989a) grown in plant tubes also occurred in this study (Table 4.1). Dual occupancy with SUTN9-2 and PRC008 in both staining methods was high (59.71% in immunostaining and 60.74% in Gus-staining). Perhaps the environment provided by the plastic pouch together with the high populations of only the competing strains present in the rhizosphere influence the occurrence of dual occupancy. These results may not reflect the incidence of dual occupancy occurring in the more complex natural soil environments (Trinick & Hadobas, 1989b). Consequently, using specific recombinant scFv antibody that bind specifically against relevant *Bradyrhizobium* strains is a more appropriate method that could substitute for the use of *gus* reporter

system. Moreover, the functionality of the recombinant antibody can be further upgraded to suit various applications, including an increase in affinity, and stability (Sheedy et al., 2007). These improvements will also permit easy and rapid quantification of the population of *Bradyrhizobium* bacteria in an inoculum or ecosphere. In this way, additional data can be obtained on individual cell physiology and structure by staining of cells with specific fluorescent dyes. Combinations of dyes (LIVE/DEAD fluorescent dyes) could be frequently used to determine cell viability from individual cells and sub-populations (Wilkinson, 2016). The detection format could be in the form of rapid and simple test kit or biosensor-based, for commercialization as point-of-demand diagnosis in the agricultural fields (Barry et al., 2009).

In recent year, phage-display technology also provides an ideal approach of identifying peptides with affinities to specific proteins for diagnostic analysis and the discovery of mimic antibodies (Wang et al., 2011; Wang et al., 2013). Peptides have several advantages over antibodies or other small molecular probes for therapeutic and diagnostic purposes. Because the peptides are 10 to 50 amino acids in length, they are chemically and physically more stable than antibodies and can more strongly and specifically interact with their targets compared to small molecular probes (Park et al., 2011). In this study, recombinant scFv was successfully reformatted to full-length IgG, and phage display of a 12-mers random peptide library (SUT12) was used to identify yiN92-1e10 IgG. After three rounds of panning, deduced peptide amino acid sequences from 7 selected candidate phage clones exhibited binding affinity to yiN92-1e10 IgG according to ELISA.

The epitopes or “mimotopes” reacting with IgG yiN92-1e10 specific to *Bradyrhizobium* sp. SUTN9-2 were also observed from this study. Using BLASTP software to assess the similarity of mimotopes from the bacterial protein database, interestingly, TGEWAGxPM mimotope was found to match with parts of protein sequences of *Bradyrhizobium* sp. SUTN9-2. And the peptide sequences showed consensus motifs, indicating that the screening process was successful. Consequently, the mimotope from phages that bound to IgG yiN92-1e10 recombinant antibody could be used to forecast the antigen on the cell surface of the bacteria and applied as a target as a substitute for cell antigen of *Bradyrhizobium* sp. SUTN9-2. Using PsortB software to analyze and predict the protein localization in the cell, all were cytoplasmic-associated proteins. Among the matched proteins, ATPases associated with diverse cellular activities, AAA family ATPase proteins are involved in a wide variety of different functions in which the energy extracted from ATP hydrolysis is used in molecular remodeling events. They are involved in processes as diverse as protein unfolding and degradation, peroxisome biogenesis, bacteriochlorophyll biosynthesis, and DNA recombination, replication and repair (Snider et al., 2008). ATP hydrolysis is required in biological nitrogen fixation by diazotrophic bacteria including *Bradyrhizobia* (Shaw, 2017). Cyclic adenosine 3', 5'-monophosphate (cAMP) may also have an important role in the sequence of biological events regulating nodule formation and functioning. Upchurch and Dlkan (1978) suggested the involvement of cAMP in the regulation of ammonia assimilation in *B. japonicum* (Upchurch & Elkan, 1978).

In conclusion, a convenient method based on phage display antibody technology was successfully employed for the generation of specific recombinant antibodies: yiN92-1e10 and yiDOA9-162 for the detection of *Bradyrhizobium* strains SUTN9-2 and

DOA9, respectively. These two recombinant scFv antibodies could be used for precise detection of the Rhizobia both in symbiosis with legume and endophyte in rice tissue by ELISA and immunofluorescent staining. This methodology can be further employed for the study of other plant-microbe interactions, and monitoring of biofertilizer in diverse sustainable cropping systems as well as in precision agriculture. However, there is still a requirement for quickly and predictable detection for the presence or absence of rhizobia in field condition. The test for laboratory scale is very difficult to operate for the farmers who lack proper skills and expertise concerning with handling protocols for laboratory equipment. Even though, they are unfamiliar with advanced technologies, they might want to know the presence or absence of rhizobia in their fields with the obvious result within the short period (Holmes, 1996). As compared to ELISA and immunofluorescence assay, lateral flow immunoassay (LFIA) is a rapid and simple point-of-care method for the detection of various analytes (Dzantiev et al., 2014). The yiN92-1e10 IgG from this study which is more stable than scFv format could be conjugated with gold nanoparticle. This antibody conjugated with gold nanoparticle could be applied for the development of rapid and simple lateral flow test strips to detect *Bradyrhizobium* strain in the field condition. Additionally, the yiN92-1e10 IgG-binding peptides from this study could also be a useful alternative mimotope for point of demand (POD) detection of *Bradyrhizoibum* strain SUTN9-2 in the future.

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APPENDIX

LIST OF PATENTS

1. Thai Patent Application No. 2101003946

   Yamabhai M., Khaing K. K., Tittabutr P., Rangnoi, K.

   Title: Recombinant human anti-bacterial scFv antibodies against *Bradyrhizobium* strain DOA9

2. Thai Patent Application No. 2101003948

   Yamabhai M., Khaing K. K., Tittabutr P., Rangnoi, K.

   Title: Recombinant human anti-bacterial scFv antibodies against *Bradyrhizobium* strain SUTN9-2

BIOGRAPHY

Miss Kyaut Kay Khaing was born on November 25, 1985, in Sagaing Division, Sagaing Township, Union of Myanmar. She graduated with the Bachelor of Agricultural Science, Department of Plant Pathology, Yezin Agricultural University, Myanmar in 2008 and received her Master Degree (M. Agr. Sc.) in 2012 from Department of Plant Pathology, Yezin Agricultural University (YAU), Myanmar. After graduation, she joined Department of Plant Pathology, YAU, Myanmar as demonstrator in 2012. From 2015 to present, she is serving as Assistant Lecturer at YAU.

In 2018, she got an award for a PhD program from "The Office of the Higher Education Commission, Year (2018)" under the NRU project of Thailand. She studied in the field of antibody engineering at School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology from August 2018 to July 2021 with the thesis entitled "Development of recombinant scfv antibody for point of demand (POD) detection of *Bradyrhizobium* strain SUTN9-2 in rice-legume cropping system and biofertilizer production". She had presented research work in The 9th School of Biotech International colloquium March 26, 2021, Suranaree University of Technology, Nakhon Ratchasima, Thailand (Oral presentation; "Monitoring of nitrogen-fixing bacteria in legume and rice using recombinant human scFv antibody").