การประเมินการผลิตเพปไทด์ต้านจุลชีพจากปาดด้วยระบบการแสดงออกของแบคทีเรียและพืช

นางสาวพัชราวลัย วงศ์ศิริ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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EVALUATION OF TREE FROG ANTIMICROBIAL PEPTIDE PRODUCTION BY BACTERIAL AND PLANT EXPRESSION SYSTEM

Miss Patcharawalai Whongsiri

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Zoology

Department of Biology

Faculty of Science

Chulalongkorn University

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Thesis Title	EVALUATION OF TREE FROG ANTIMICROBIAL PEPTIDE PRODUCTION BY BACTERIAL AND PLANT EXPRESSION SYSTEM
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พัชราวลัย วงศ์ศิริ : การประเมินการผลิตเพปไทด์ต้านจุลชีพจากปาดด้วยระบบการแสดงออกของแบคทีเรียและพืช (EVALUATION OF TREE FROG ANTIMICROBIAL PEPTIDE PRODUCTION BY BACTERIAL AND PLANT EXPRESSION SYSTEM) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ.ดร.พงชัย หาญยุทธนากร, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : ดร.ภัทรดร ภิญโญพิชญ์, 118 หน้า.

เพปไทด์ต้านจุลชีพทำหน้าที่เกี่ยวข้องกับระบบภูมิคุ้มกันของร่างกาย ในสิ่งมีชีวิตทุกชนิด Rhacoporin-2 เป็นเพปไทด์ต้านจุลชีพที่ได้จากเมือกของปาดดอยอินทนนท์ *Rhacophorus feae* มีคุณสมบัติสามารถยับยั้งการเจริญของเชื้อแบคทีเรียทั้งแกรมบวกและแกรมลบ แต่มีผลต่อการแตกตัวของเม็ดเลือดแดงของสัตว์เลี้ยงลูกด้วยนมในระดับต่ำ ดังนั้น Rhacoporin-2 จึงเป็นเพปไทด์ที่มีแนวโน้มในการนำมาประยุกต์ใช้ต่อไป

SUMO fusion system ถูกนำมาประยุกต์ใช้เพื่อเพิ่มปริมาณการผลิตเพปไทด์ Rhacoporin-2 ดังนั้น งานวิจัยนี้จึงมีวัตถุประสงค์เพื่อประเมินการผลิตเพปไทด์ลูกผสม, SUMO-Rhacoporin2, ในระบบของการแสดงออกของแบคทีเรียและพืช

การผลิตเพปไทด์ลูกผสมใน *Escherichia coli* พบว่า *E. coli* สามารถผลิตเพปไทด์ Rhacoporin-2 ได้ที่ปริมาณ 131 μg/L มีความบริสุทธิ์ 85% และที่สำคัญ เพปไทด์ที่ผลิตได้ยังสามารถยับยั้งการเจริญของแบคทีเรีย Salmonella Typhimurium ที่ระดับความเข้มข้น 8 μM ดังนั้น SUMO fusion system สามารถใช้ในการผลิตเพปไทด์ต้านจุลชีพในระบบแบคทีเรียได้ สำหรับการผลิตเพปไทด์ลูกผสมในระบบของพืช 2 ชนิด ได้แก่ แหนเล็ก *Lemna minor* และ *Arabidopsis thaliana* พบว่าแหนเล็กผลิตเพปไทด์ได้ในปริมาณต่ำเพียง 44.8 μg จากแหนเล็กสด 100 mg แต่การใช้ SUMOstar fusion tag ไม่ประสบความสำเร็จใน *Arabidopsis* ดังนั้น SUMO fusion system จึงไม่เหมาะสมสำหรับการผลิตเพปไทด์ต้านจุลชีพในระบบพืชทั้งสองชนิด อย่างไรก็ดี เมื่อเปรียบเทียบทางด้านต้นทุนการผลิต พบว่าระบบพืชเป็นระบบที่มีต้นทุนต่ำกว่าระบบของแบคทีเรีย ดังนั้น ระบบพืชจึงยังคงเป็นระบบที่น่าสนใจในการผลิตโปรตีนทางการแพทย์ต่อไป

ภาควิชา ชีววิทยา ลายมือชื่อนิสิต .....

สาขาวิชา สัตววิทยา ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ปีการศึกษา 2555 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

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KEYWORDS : ANTIMICROBIAL PEPTIDE / AMPHIBIAN / RECOMBINANT PEPTIDE PRODUCTION / SUMO FUSION SYSTEM

EVALUATION OF TREE FROG ANTIMICROBIAL PEPTIDE PRODUCTION BY BACTERIAL AND PLANT EXPRESSION SYSTEM. ADVISOR: ASST. PROF. PONGCHAI HARNYUTTANAKORN, Ph.D., CO-ADVISOR: PATARADAWN PINYOPICH, Ph.D., 118pp.

Antimicrobial peptides play a role in innate immune system in all living organisms. Rhacoporin-2, a novel AMP from the skin secretion of *Rhacophorus feae*, shows an antimicrobial activity against both Gram-positive and Gram-negative bacteria. Contrastingly, it is relatively unharmful to mammalian red blood cell. Thus, it has a potential to be used as pharmaceutical agent.

SUMO fusion system is employed for Rhacoporin-2 production. Therefore, the objective of this study is to evaluate the recombinant peptide, SUMO-Rhacoporin2, production by bacterial expression system and plant expression system.

In *Escherichia coli* system, the total 131 μg/L of Rhacoporin2 with 85% purity was produced and the peptide exhibited the antibacterial activity against Salmonella Typhimurium at 8 μM. Hence, SUMO fusion system was effective for antimicrobial peptide production by bacterial system. For the recombinant peptide production in 2 plant hosts, *Lemna minor* and *Arabidopsis thaliana*, the results showed that the very low amount of recombinant peptide, 44.8 μg, was synthesized from 100 mg fresh duckweed, whereas, SUMOstar fusion tag for peptide production was unsuccessful in *Arabidopsis*. Therefore, SUMO fusion system was ineffective in these two species. However, plant system is still promising due to the low cost of production for therapeutic agents.

Department : Biology Student's Signature .....

Field of Study : Zoology Advisor's Signature .....

Academic Year : 2012 Co-advisor's Signature .....

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CONTENTS

Page

ABSTRACT IN THAI iv

ABSTRACT IN ENGLISH v

ACKNOWLEDGEMENTS vi

CONTENTS vii

LIST OF TABLES viii

LIST OF FIGURES ix

CHAPTER I INTRODUCTION 1

1.1 Background and rationale 1

1.3 Objectives 5

CHAPTER II LITERATURE REVIEW 6

2.1 Antimicrobial peptides, the effector molecules in innate immune system 6

2.2 Mode of action of antimicrobial peptides 9

2.3 Antimicrobial peptides in clinics 13

2.4 Amphibian antimicrobial peptides 16

2.5 Recombinant production for antimicrobial peptides 19

CHAPTER III MATERIALS AND METHODS 36

CHAPTER IV RESULTS AND DISCUSSION FOR BACTERIAL SYSTEM 54

CHAPTER V RESULTS AND DISCUSSION FOR PLANT SYSTEM 65

CHAPTER VI CONCLUSION 82

REFERENCES 85

APPENDIX 113

BIOGRAPHY 118

LIST OF TABLES

Page

Table 1 MIC values of Rhacoporin-2 against bacterial strains 38

Table 2 IC_{50} values of Rhacoporin-2 against cancer cell lines 38

Table 3 HC_{50} values of Rhacoporin-2 against red blood cell 38

Table 4 The codon usage of *Lemna minor* 40

Table 5 The codon usage of *Escherichia coli* 41

Table 6 Codon optimization of Rhacoporin-2 gene for *Escherichia coli* 54

Table 7 The absorbance of standard BSA 55

Table 8 The total protein yield from various concentrations 59

Table 9 The amount of peptide calculated by Bradford assay 61

Table 10 Codon optimization of Rhacoporin-2 gene for *Lemna minor* 65

Table 11 The identity of SUMO gene sequences 79

Table 12 The evaluation of suitable system for Rhacoporin-2 production 84

LIST OF FIGURES

Page

Figure 1 Structure of genes and peptides of antimicrobial peptides 7
Figure 2 Classification of antimicrobial peptides 8
Figure 3 The basic mechanisms of antimicrobial peptides 12
Figure 4 The section of *Rana esculata* dorsal skin 18
Figure 5 The regulation of T7 promoter in pET plasmid 24
Figure 6 The features of pET SUMO vector 43
Figure 7 The features of pCAMBIA1305.2 44
Figure 8 The features of pUC57 45
Figure 9 2-fold dilution method for MIC assay 53
Figure 10 Standard curve and standard equation from BSA 56
Figure 11 The amplification of Rhacoporin-2 gene 57
Figure 12 SDS PAGE of temperatures and IPTG concentrations 59
Figure 13 SDS PAGE of induction duration of Rhacoporin-2 60
Figure 14 SDS PAGE of peptide purification procedure 62
Figure 15 GFP expression of transformed *Lemna minor* 68
Figure 16 SDS PAGE of transformed *Lemna minor* 70
Figure 17 Callus formation of *Lemna minor* 71
Figure 18 Nodule formation of *Lemna minor* 73
Figure 19 GFP amplification of GFP gene in *Arabidopsis thaliana* 75
Figure 20 GFP expression in transgenic *Arabidopsis* 76
Figure 21 SDS PAGE of protein extraction from *Arabidopsis thaliana* 77
Figure 22 SDS PAGE of purified peptide from *Arabidopsis thaliana* 78
Figure 23 DNA sequence alignment of SUMO genes 80

CHAPTER I

INTRODUCTION

Since the first discovery of penicillin by Alexander Fleming in 1928, antibiotics have been developed for the treatment of pathogen infections in human and animals. Due to the diversified characteristics of bacteria, hence, there are both susceptible and resistant strains to antibiotics use (Fleming, 1945 and Wiley, 1997). The antibiotic resistance in pathogens has become the most concerned problem in public health worldwide. The biochemical mechanisms of antibiotic resistance can be divided into 4 basic types including bacterial enzymatic inhibition, antibiotic efflux, bypass pathway and the most importance mechanism, genetic mutation. The mutant traits are resistant to antibiotics and their propagation has become rapidly populated (Hawkey, 1998 and Wiley, 1997). Thus, the novel antibiotics are expected to address this issue. And antimicrobial peptides from many living organisms are promising.

Antimicrobial peptides or AMPs are cationic and amphiphilic peptides that play a key role in innate immune system in all organisms including vertebrates, invertebrates and plants (Marshall and Arenas, 2003). The mode of action of AMPs depends on their characteristics. Cationic peptides can strongly interact with negative charge on microbial membranes and lead to cell damage by membrane permeabilization (Kamysz et al., 2003 and Marshall and Arenas, 2003). Hence, AMPs are defined to be a

therapeutic agent since they exhibit the antibacterial, antiviral and anticancer activity (Hancock and Patrzykat, 2002 and Silva et al., 2011). The clinical application of antimicrobial peptides has been developed for various infectious diseases such as oral and cutaneous infections. To date, various antimicrobial peptides have been in advanced stages of development in the form of tropical cream, mouth wash, aerosols, gels, and also intravenous route (Andrès, 2012).

In addition to the clinical application, antimicrobial peptides are valuable for livestock rearing. For instance, the dietary supplementation of antimicrobial peptides can promote the growth performance and enhance the immune function in weanling pig (Wang et al., 2011 and Yoon et al., 2012). Moreover, antimicrobial peptides have a potential use in the extension of shelf-life and food preservation since they can prevent the growth of bacterium contaminated in food, for example, nisin is developed to improve shelf life of sausage and other meat product (Ananou et al., 2007, Cleveland et al., 2001 and Reunanen and Saris, 2004). Interestingly, antimicrobial peptides are also applied for material devices. The immobilized antimicrobial peptide with biomaterial, for example an acrylic coating system, is effective in the reduction of microorganism survival (Costa et al., 2011 and Fulmer et al., 2010). The potential uses of this strategy, the antimicrobial peptide-coated material, is beneficial for long-term use of medical

devices such as contact lens (Willcox et al., 2008) or use as food packaging to extend the shelf life of food and food preservation (Limjaroen et al., 2003).

As described above, antimicrobial peptides are abundantly beneficial for human living. Thereupon, the novel antimicrobial peptides have been indentified from many species of living organisms since they can be found in all organisms including bacteria, plant, invertebrates and vertebrates (Marshall and Arenas, 2003), especially the amphibians.

The Anuran families are the rich sources of AMPs because of their distinguish character. A moist habitat causes a greater chance to be infected by microorganisms. Therefore, amphibians have to synthesize various AMPs from skin gland to protect themselves from infection. There are about 160 defense peptides from 20 ranid species have been established worldwide (Govender et al., 2012). In Thailand, there are more than 130 amphibian species (Khonsue and Thirakhupt, 2001) but there has been no publication about AMPs from amphibian in Thailand yet. From our previous study, we identified a novel antimicrobial peptide from tree frog *Rhacophorus feae* from northern part of Thailand, called Rhacoporin-2 (unpublished). This interesting peptide shows an antimicrobial activity against both Gram-positive and Gram-negative bacteria, and as well as anticancer activity against some cancer cell lines.

Biochemical procedure is mainly used as a tool to increase the amount of interested AMPs for basic researches such as the biological activity, chemical structure and the clinical application of AMPs. Since the amount of AMPs from natural sources, such as frog skin secretion, is rather little, the recombinant peptide production is used profitably to increase amount of AMPs for their applications (Li, 2011 and Parachin et al., 2012). This technology is involved with the conjugation between the interested AMPs and fusion tag utilizing for peptide purification and peptide production such as polyhistidine tags (His-tag), maltose binding protein (MBP) and N-utilizing substance A (NusA). Nevertheless, SUMO fusion system has been evaluated to be an efficient fusion tag to significantly increase the recombinant peptide yield (Butt et al., 2005).

In addition to the fusion tag, heterologous host systems are crucial for peptide production as well. *Escherichia coli* host system is commonly used for recombinant protein or peptide production (Baneyx, 1999), whereas plant host system is valuable for therapeutic protein or peptide production due to its post-translational modification (Sharma and Sharma, 2009). In this study, we evaluated recombinant expression of Rhacoporin-2 conjugated with SUMO fusion tag, in two host systems: bacterial and plant.

Objectives:

1. To compare the amount of recombinant Rhacoporin-2 produced by bacterial and plant host system
2. To compare the biological function of recombinant Rhacoporin-2 produced by bacterial and plant host system
3. To evaluate the suitable system for the production of the recombinant Rhacoporin-2

CHAPTER II

LITERATURE REVIEW

Antimicrobial peptides, the effector molecules in innate immune system

An innate immune system is the defense mechanism against invading pathogens of all multicellular organisms. Antimicrobial peptides (AMPs) are the first barrier in host defense providing a rapid and effective response. Antimicrobial peptides are small cationic peptides containing hydrophobic and hydrophilic regions proposed to their mechanisms. AMPs are gene-encoded molecules which express constitutively or inducibly. A prepropedtide, the primary translational product, comprises of 3 regions: N-terminal signal region as a target for endoplasmic reticulum, the pro segment involving in peptide folding and the C-terminus as a mature peptide (Bals, 2000 and Gallo et al., 1998).

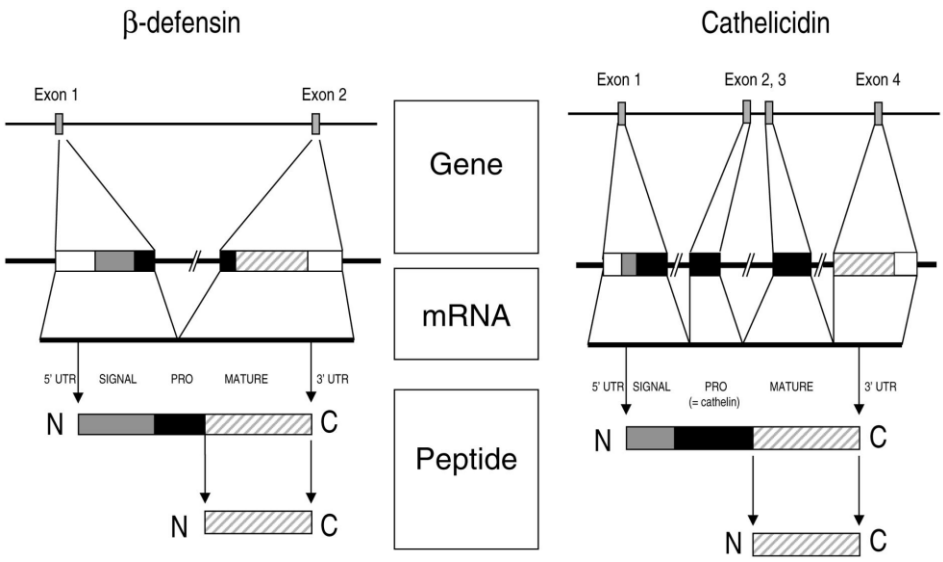

Figure 1 Structures of genes and peptides of the defensin and cathelicidin families. A prepropeptide contains signal sequence, pro segment and C-terminus with antimicrobial activity (Bals, 2000)

The mature peptides can be grouped into 3 major classes according to their conformational structure and amino acid structure. The classes include linear peptide without cysteines forming α-helical or β-sheet peptide, loop peptide or peptide with disulfide bridge and the extended peptide or peptide with an unusually high proportion of amino acids. (Bals, 2000 and Peters et al., 2010)

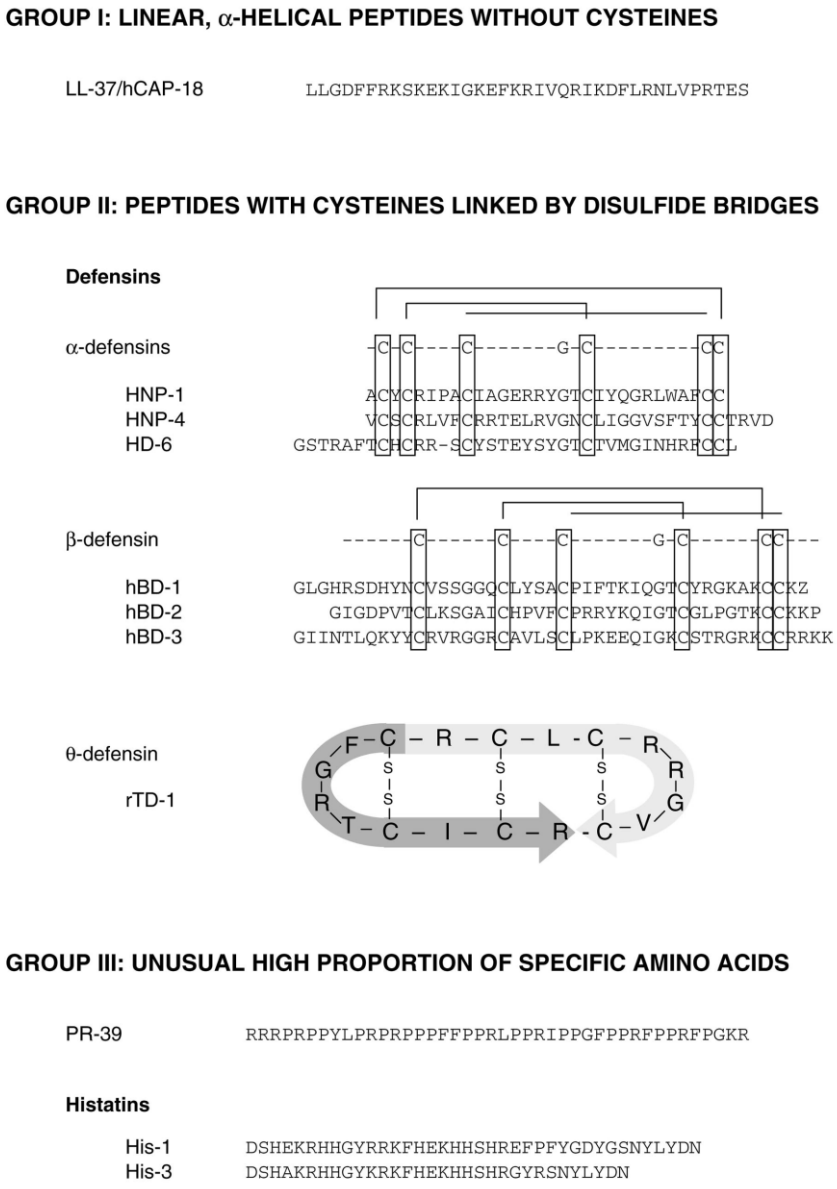

Figure 2 Classification of antimicrobial peptides according to amino acid sequences and structures (Bals, 2000)

Antimicrobial peptides are established as a key role in nonadaptive immunity in both plant and animal. In insect, AMPs are synthesized from fat body and released into

the haemolymph. AMPs expression in insect is induced by bacterial infection. Meanwhile, AMPs expression in higher eukaryotes is constitutive or inducible by infection or stress. In mammal, AMPs are synthesized in phagocytes or mucosal tissues such as respiratory tract and digestive tract. (Gazit et al., 1995, Nissen-Meyer and Nes, 1997) In plant, AMPs are induced by pathogens and constitutively expressed in infected tissues such as seeds and leaves. (Ganz and Lehrer, 1999, Maróti et al., 2011 and Zasloff, 2002)

Mode of action of antimicrobial peptides

The positive charges and the amphiphatic structure of AMPs are crucial factors for membrane permeabilization. The first step of mechanism is the electrostatic interaction between the positive charges of AMPs and the negative charges of target membrane. Afterwards, an amphiphatic structure conduces the interaction and insertion of AMPs into the membrane of pathogen. When the AMPs concentration on membrane surface reaches the threshold, peptides will start forming the membrane permeation. The models of membrane permeation pathway consist of firstly, the pore formation model via the Barrel Stave mechanism, secondly, the toroidal pore mechanism and lastly, the carpet mechanism (Andreu and Rivas, 1998, Shai, 2002 and Yeaman and Yount, 2003) as following:

I. *The Barrel Stave mechanism* In this model, the linear peptides (α-helix or β-sheet) form a variable number of transmembrane pores in lipid membrane of target cell. Following the electrostatic interaction, the hydrophobic surfaces of peptide monomers penetrate into the phospholipid layer forming the transmembrane pores on the target membranes which the hydrophilic surfaces face inside the pores. The size of transmembrane pores would increase according to the number of peptide monomers (Shai, 2002 and Yeaman and Yount, 2003).

II. *Toroidal pore or wormhole mechanism* The α-helical peptides insert into the phospholipid layer forming the channels like Barrel Stave mechanism. However, the difference between this model and Barrel Stave mechanism is the feature of pores. In the toroidal pores, phospholipids are intercalated with peptides whereas there are only peptides in the transmembrane channels of Barrel Stave mechanism. Due to the disintegration of pores, it may be a key mechanism of peptide translocation that peptides enter the cytoplasm of target cells inhibiting the DNA, RNA and protein systhesis (Yeaman and Yount, 2003).

III. *The carpet mechanism* This model is termed as a detergent-like mechanism. Unlike the Barrel Stave and Toroidal pore mechanisms, the carpet mechanism is not specific to peptide structure and there is no insertion of peptides into the phospholipid membrane. The positively charged peptides interact with the negatively charged target

membrane. The hydrophobic surfaces bind to the membrane whereas the hydrophilic surfaces face the solvent. The high density of peptides on membrane surfaces leads to the membrane disruption (Shai, 2002 and Yeaman and Yount, 2003).

These mechanisms exhibit the interaction between AMPs and cell membrane, exceptionally pathogenic membrane. Peptide selectivity is influenced by membrane composition. The fundamental components of membrane are a fluid mosaic of proteins and phospoholipids. The microbial and host membranes are significantly different in net charge due to the proportion of phospholipid elements such as phosphotidylglycerol (PG), phosphotidylcholine (PC), cardiolipin (CL), phosphotidylethanolamine (PE), and cholesterol. Bacterial membrane consisting of PG or CL tends to be highly negative charges. On the other hand, zwitterionic phospholipid bilayaer of mammal membrane composed of cholesterol elements, PG or PE is neutral in net charge, hence, negatively charged bacterial membrane are specific target for positively charged peptide or AMPs. (Yeaman and Yount, 2003)

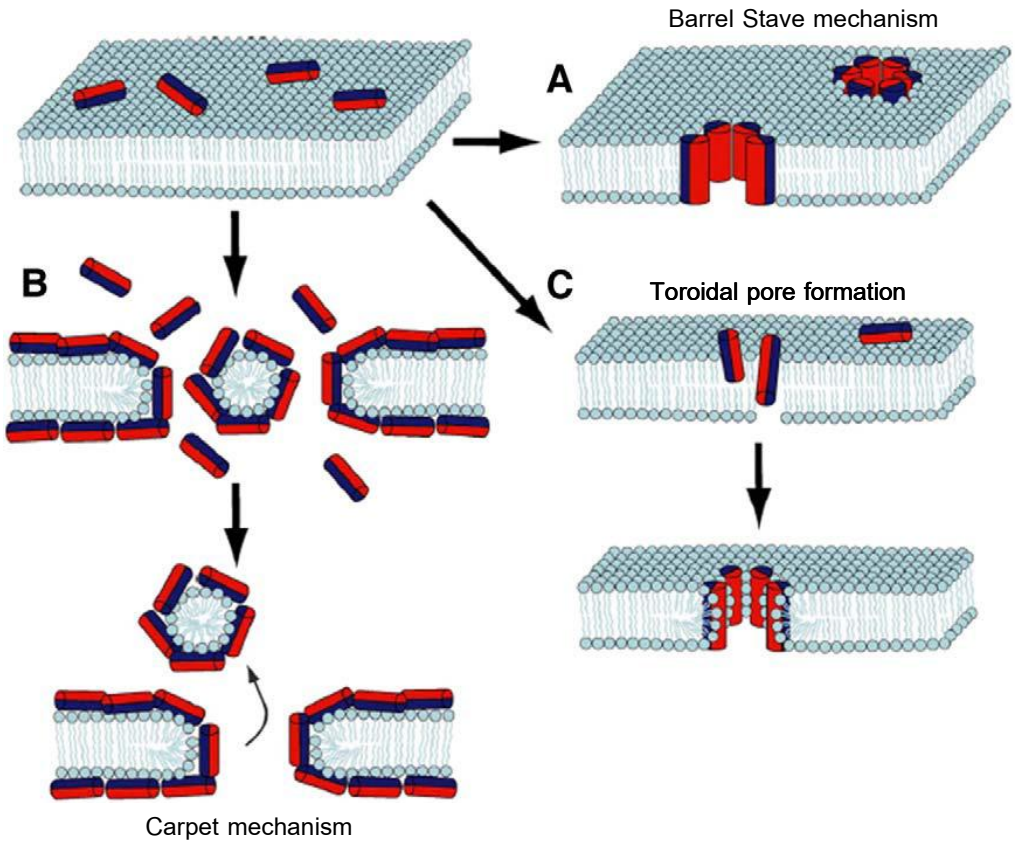

Figure 3 The basic mechanisms of AMPs, A: the Barrel Stave pore formation, B: the carpet mechanism, C: the toroidal pore formation (Rivas et al., 2009)

Antimicrobial peptides in clinics

a) *Antibacterial activity* The electrostatic interaction between the positively charged peptides and the negatively charged bacterial membranes leading to membrane permeabilization is the crucial mechanism of antibacterial activity. The cationic peptides can bind strongly to the lipopolysaccharide appearing on the outer membrane of Gram-negative bacteria inducing the leak of outer membrane, followed by the inner membrane disruption. For Gram-positive bacteria, permeabilization of membrane is considered as well. Due to the thicker membrane of Gram-positive bacteria, cationic peptides require the ability for insertion more deeply (Matsuzaki et al., 1997 and Torcato et al., 2013). Other than membrane permeabilization, AMPs can pass through bacterial membrane to target the cytosolic organelles conducting the intracellular killing mechanisms (Li et al., 2010). For instance, attacin from insect *Hyalophora cecropia* induces the membrane permeabilization and inhibits the outer-membrane synthesis of *Escherichia coli* (Carlsson et al., 1998), or human β-defensin exhibits the strong antimicrobial activity against both Gram-positive and Gram-negative bacteria (García et al., 2001).

b) *Antifungal activity* Antimicrobial peptides from many organisms exhibit a killing activity to fungi, for example, human lactoferrin and histatin 5 are effective against *Aspergillus fumigatus* (Lupetti et al., 2008). Generally, the cytoplasmic membrane is the

main target of antimicrobial peptides to kill fungi due to peptide hydrophobicity (Jiang et al., 2008). In addition, antimicrobial peptides involve with regulation of cellular ATP in fungi leading to cell death (Edgerton et al., 2000 and Tanida et al., 2006).

c) *Antiviral activity* In vitro study, antimicrobial peptides demonstrate the antiviral activity against enveloped viruses, for example, vaccinia virus, herpes simplex virus 1 and 2, adenovirus, rotavirus, cytomegalovirus, vesicular stomatitis virus and influenza virus A/WSN (Carriel-Gomes et al., 2007, Daher et al., 1986 and Mohan et al., 2010). Moreover, antimicrobial peptides did not inactivate the naked viruses; echovirus and reovirus. These results suggest that the lipid membrane on virus envelope may be the interaction site between antimicrobial peptides and virion (Daher et al., 1986). Interestingly, antimicrobial peptides, melittin and cecropin, exhibit anti-HIV activity by inhibiting the human immunodeficiency virus replication. It indicates that antiviral activity of antimicrobial peptides is not due to only interaction between peptides and virus envelope leading to cell lysis, but these peptides can also suppress the transcription of HIV gene to inhibit HIV replication (Lai et al., 2002 and Zhao et al., 2005).

d) *Antiparasitic activity* Cell membrane disruption of protozoa is the main mode of killing by various antimicrobial peptides. Mangoni et al., 2005 and Kulkarni et al., 2006 had studied the effect of various AMPs against *Leishmania*, parasitic protozoa involved with human Leishmaniacis. The results show that Temporin A, Temporin B and

Cathelicidins appear to be effective molecules against *Leishmania* via surface membrane disruption as shown by transmission electron microscopy. Moreover, the peptides may act as the triggering molecules to an intracellular signaling pathway, or they can pass through the membrane surfaces to directly bind to the mitochondrial membrane leading to mitochondrial membrane disruption (Kulkarni et al., 2006 and Mangoni et al., 2005).

e) *Anticancer activity* Cancer cell membranes and normal cell membranes are compositional difference. Many cancer cell membranes are composed of glycoprotein, *O*-glycosylated mucin, which increases negative charges on cancer cell surface. Increasing negative charges leads to stronger electrostatic interaction between cationic peptides and negatively charged cancer cells (Huang et al., 2011). Suttmann et al., 2008 evaluated the antitumor activity of Cecropin A and B against bladder cancer cells. They found that these peptides exert a cytotoxic efficacy against bladder cancer cells by cell membrane disruption resulting in cytolysis and cell destruction (Suttmann et al., 2008).

The drug resistance in pathogenic microorganisms has become an important health issue in human worldwide since the overuse of drugs in medicine. Identifying the new therapeutic technologies to overcome this issue is promising (Heinemann et al., 2000). Antimicrobial peptides are remarkable natural antibiotics. In addition to their

function that directly related to killing microorganisms, the microbial resistance against these peptides is rare. Antimicrobial peptides are ubiquitous. They are conserved elements in all organisms (Gabay, 1994). Exploring the novel antimicrobial peptides from numerous species, such as amphibian species, for pharmaceutical used is considerable.

Amphibian antimicrobial peptides

Amphibians are ancient vertebrate living in pathogen-rich and moist habitats, hence, the front-line of amphibian immune system is the skin. Amphibian skin is necessary for survival which involves with respiration, water regulation and defense mechanisms (Clarke, 1997). The defense mechanism of amphibian contains both innate and adaptive immune systems. However, the first barrier of defense mechanism is innate immunity, for example, antimicrobial peptides, macrophages and neutrophils, due to its rapid response (Apponyi et al., 2004, Conlon, 2011 and Rollins-Smith et al., 2005).

The important components in amphibian skin are two types of glands: mucous glands and granular glands (shown in figure 4). The other types are found in specific species and show the special functions. Mucous glands and granular glands are mainly located in the dorsal region that controlled by sympathetic nervous system associated

with the myoepithelial layer and stimulated by stress, injury or infection (Simmaco et al., 1998 and Toledo and Jared, 1995). The mucous glands secrete mucus to maintain a moist and slippery skin for respiration and water-loss prevention (Clarke, 1997). For the granular glands, the cytoplasm is full of granules that synthesize the secretions. The skin secretions of amphibians mainly include toxins and antimicrobial peptides (Clarke, 1997, Simmaco et al., 1998 and Toledo and Jared, 1995).

Antimicrobial peptides are biologically active elements in innate immune system which are vital to amphibian survival. The researchers studied whether AMPs affect *Batrachochytrium dendrobatidis*, chytrid fungus that concern with the amphibian global decline. The results suggested that AMPs are capable of the growth inhibition of chytrid fungus at zoospore stage. Therefore, AMPs are the protective elements in the amphibian innate immunity (Rollins-Smith et al., 2002, Rollins-Smith et al., 2003, Rollins-Smith, 2009 and Woodhams et al., 2007). In addition, Mangoni et al., 2001 investigated the antimicrobial peptide synthesis in amphibian skin glands. The result suggested that peptide synthesis is influenced by the presence of microorganisms. The antimicrobial peptides profile of amphibian depends on the microbial species that the frogs are exposed. These findings demonstrate the advantage of antimicrobial peptides that involved with the innate immunity preventing the pathogenic infection.

Figure 4 The section of *Rana esculenta* dorsal skin stained with haematoxylin-eosin, A: granular gland, B: mucous gland (Simmaco et al., 1998)

Frog skin has been used for a traditional medicine in some countries. Chan Su, medicine prepared from dried white secretion of skin glands of Chinese toad, is used for treating the heart diseases. Some active molecules in Chan Su demonstrate to maintain the cardiovascular and reproductive system, and induce apoptosis in human lung carcinoma cells (Bhuiyan et al., 2003 and Yun et al., 2009). Besides, frog skin is also used as a graft for wound healing with the advantage of some active molecules in frog skin including antibiotic substances and active substances that activate the healing mechanism (Piccolo et al., 2008 and Raghavan et al., 2010).

Identifying the active molecules in frog skin has been studied for decades. Various antimicrobial peptides are characterized from various amphibian species; maximin family from *Bombina maxima* (Lai et al., 2002), temporin and brevinin family from *Rana sakuraii* (Suzuki et al., 2007), buforin I from *Bufo bufo gargarizans* (Park et al., 1996), pleurocidin from *Pleuronectes americanus* (Cole et al., 1997), ranaruerin family from *Rana clamitans* (Halverson et al., 2000), cancrin from *Rana cancrivora* (Lu et al., 2008) and fallaxin from *Leptodactylus fallax* (Rollins-Smith et al., 2005). Interestingly, these demonstrate that amphibian skin secretion is the rich source of biologically active molecules. These antimicrobial peptides from frog skin secretions are potential pharmaceutical molecules that may be developed as novel antibiotics to overcome the multidrug resistance pathogens (Barra and Simmaco, 1995 and Rinaldi, 2002). However, large amounts of peptides are needed for basic researches and clinical trials. Hence, the recombinant peptide production is necessary for increasing the quantities of antimicrobial peptides for future studies (Li, 2011 and Parachin et al., 2012).

Recombinant production for antimicrobial peptides

Since sufficient amounts of antimicrobial peptides are needed for improvement and clinical trial, the recombinant production has been developed to remedy this issue (Li, 2011 and Parachin et al., 2012). Generally, three different methods are operated to

obtain enough peptides: direct isolation from natural sources, chemical synthesis and recombinant expression in heterologous organisms (Li et al., 2010 and Parachin et al., 2012).

Due to a very low amount of antimicrobial peptides are presented in host organisms, the peptides directly isolated from the large numbers of natural sources may cause the ecological and environmental issues (Parachin et al., 2012). For example, strongylocin was firstly discovered from the hemolymph extraction of 66 individuals of the green sea urchin *Strongylocentrotus droebachiensis* and the higher amount of peptides were obtained from 500 individuals (Li et al., 2008, Li et al., 2010 and Parachin et al., 2012). The chemical synthesis of peptides is feasible. Nevertheless, for large-scale production, peptides that contain more than ten amino acid sequences or disulfide bridges are likely to be more complex and difficult for chemical synthesis, and therefore the costs of peptide production will increase certainly (Li et al., 2010 and Parachin et al., 2012). Alternatively, the recombinant production technology has been more approved for peptide production. This technology is capable of foreign gene insertion to specific vectors and expressed in heterologous host systems. It has been considered as the most cost-effective method and the greater amounts of peptides in large-scale production (Li et al., 2010 and Parachin et al., 2012).

For the scale-up production of antimicrobial peptides, peptides are produced outside of their natural host which termed as the heterologous host systems (Desai et al., 2010 and Mahmoud, 2007). The suitable properties of host systems are including forming the right conformation of heterologous peptides, high yield productivity, the ease of maintaining and providing the downstream processing (Desai et al., 2010). For recombinant expression in heterologous host systems, there are several expression systems that are utilized for antimicrobial peptides production. The main categories of expression systems comprise prokaryotic systems, such as bacteria, and eukaryotic systems, such as yeast, plant and mammalian cell. All expression systems possess different characteristics that should be considered to choose the best one. The selection of expression systems associates with peptide properties, such as size, folding or glycosylation pattern. Furthermore, the costs and the rapid scale-up of expression systems are extensively evaluated for the most suitable system for peptide production (Parachin et al., 2012 and Rai and Padh, 2001).

Heterologous host systems for antimicrobial peptide production

1. **Bacterial host system** For bacterial expression systems, *Escherichia coli* is notably among various bacterial platforms. *E. coli* contains well-characterized genetics with large number of cloning vectors. Besides, it rapidly grows on inexpensive medium

that benefit to low-cost and large-scale peptide production. These advantages of *E. coli* expression system cause it to remain the most attractive bacterial system for peptides which do not require post-translational modification (Baneyx, 1999 and Li and Chen, 2008).

E. coli system is noteworthy for studying in protein engineering and structural analysis. Therefore, the recombinant expression in *E. coli* has been designed to improve product quality and yields (Chen, 2012). The recombinant genes have been constructed and regulated under promoters. For instant, *lac* promoter and its relatives are used to regulate the expression of peptides that may be toxic to host cell. The expression of recombinant gene under *lac* promoter is performed by the induction of lactose analog, isopropyl-β-D-1-thiogalactopyranoside (IPTG). A little amount of IPTG, 50-100 μM, is adequate for fully induction. (Baneyx, 1999)

Currently, the pET vectors developed by Navogen have been more commercially popular (Baneyx, 1999 and Parachin et al., 2012). In this system, gene of interest is located downstream of the bacteriophage T7 promoter which regulated by T7 RNA polymerase (shown in figure 5). Hence, this vector requires a host strain that contains DE3 phage fragment under control of IPTG-inducible *lac*UV5 promoter to encode T7 RNA polymerase. IPTG induces the recombinant gene expression by binding to *Lac*I from *lac* operator, subsequently T7 RNA polymerase is transcribed from *E. coli* host

genome. Afterwards, T7 RNA polymerase binds to T7 promoter on pET plasmids initiating the transcription of gene of interest as shown in figure 5 (Baneyx, 1999, Lopez et al., 1998, Paul et al., 1997, Sørensen and Mortensen, 2005 and Williams et al., 1998). In addition, the genetic background of host strain is significantly important for recombinant expression. *E. coli* BL21(DE3) is one of the most common host for standard recombinant expression because it lacks ompT and lon proteases which relate to the recombinant protein degradation (Parachin et al., 2012 and Li, 2011).

Nevertheless, the recombinant antimicrobial peptides have been synthesized by different *E. coli* strains as well as different expression vectors. Amphibian antimicrobial peptides are successfully produced by *E. coli* host system. For instant, adenoregulin, from the arboreal frog *Phyllomedusa bicolor*, and brevinin-2GU, from the Asian frog *Hylarana guntheri*, are synthesized by *E. coli* conjugated with Trx fusion protein. After enzymatic cleavages, the intact peptides conserve their potency against microorganisms (Cao et al., 2005 and Zhou et al., 2009). Moreover, several antimicrobial peptides from various species can be synthesized from this system as well, such as snakin-1 and defensin from plants (Kovalskaya and Hammond, 2009), halocidin 18 from the ascidian *Halocynthia aurantium* (Wei et al., 2005), indolcidin, CM4, LL37 and dermcidin from human (Chen et al., 2008, Cipáková et al., 2006, Morin et al., 2006 and Li et al., 2006), cercopin from insects (Xu et al., 2007) and scygonadin from crabs (Peng

et al., 2010). These studies indicate that antimicrobial peptides can be produced by microbial host systems with conserved antibiotic activities.

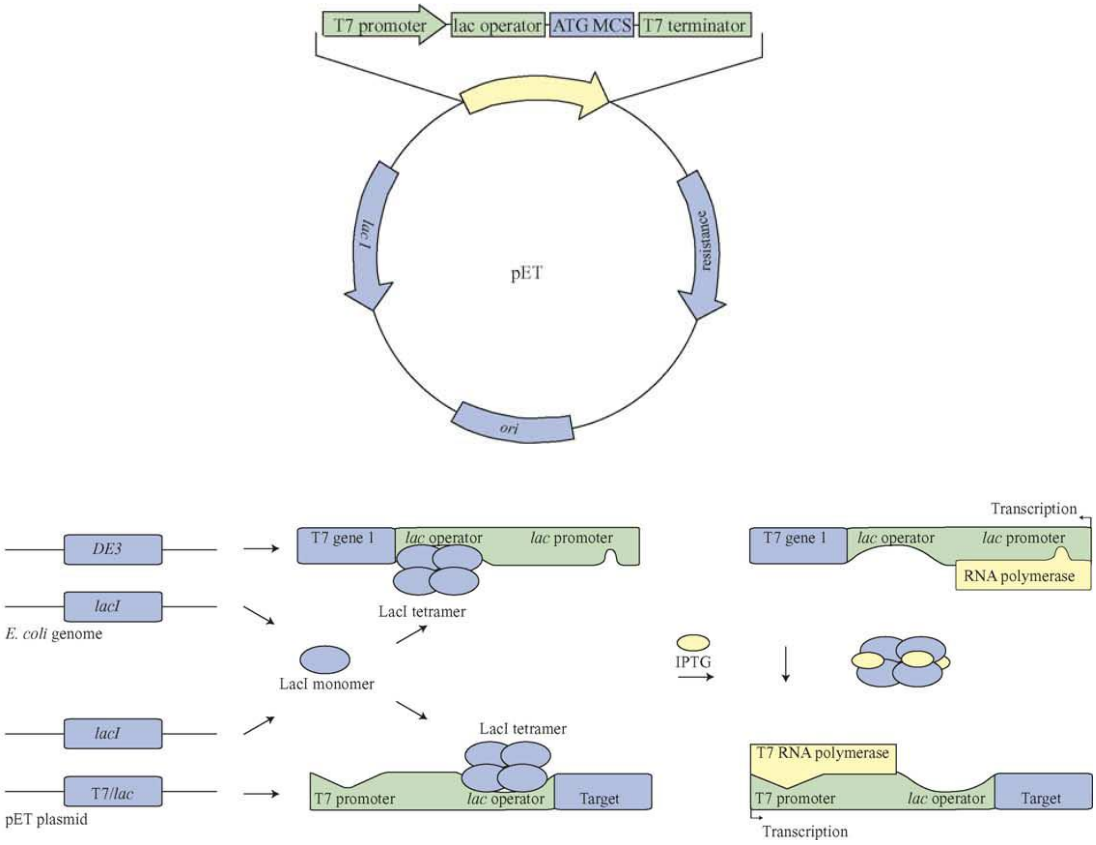

Figure 5 The regulation of T7 promoter in pET plasmid (Sørensen and Mortensen, 2005)

2. **Plant host system** Plants are the valuable sources for therapies since the world population uses them as a drug to cure health problems. Nowadays, plant biotechnology has becomes more important for the production of various therapeutic agents including nutritional components, vaccine antigens, therapeutic proteins and industrial products via transgenic plants (Sharma and Sharma, 2009).

A major advantage of plant system is low cost productivity comparing with other systems. The estimate cost of recombinant peptides from plant system is lower than mammalian systems, and as well as bacterial systems for 2-10% (Desai et al., 2010 and Twyman et al., 2003). Plant system provides a rapid scalability on inexpensive medium that is significant for low-cost and large-scale production. For pharmaceutical peptide production, plants are much safer than bacterial and animal cell systems because they are not host for human pathogenic microorganisms and are less likely to be contaminated with endotoxin from microbes (Desai et al., 2010, Twyman et al., 2003, Xu et al., 2012). Furthermore, peptides can be expressed in specific tissues such as seeds or some edible parts that provide easy and less expensive downstream processes. An edible plant vaccine is tested, for instance, transgenic tobacco that contain C4V3 recombinant protein are orally feeding in mice and it can activate the immune response against HIV. This study demonstrates that plants can be used as a candidate edible vaccine against human pathogens (Desai et al., 2010, Mason et al., 2002 and Rubio-

Infante et al., 2012). Plants can perform the post-translational modifications such as protein folding, disulfide bond formation and glycosylation. These mechanisms are very importance for complex eukaryotic proteins. The post-translational modifications are conserved in eukaryotic organisms including plants and animals, hence, proteins or peptides that synthesized from plant system appear to fold properly leading to efficiency bioactivity (Desai et al., 2010 and Sharma and Sharma, 2009).

On the molecular level of peptide production in plants, the expression level of gene encoding recombinant peptides is regulated by promoter which controls the transcription. The cauliflower mosaic virus 35S (CaMV35S) promoter is frequently employed to enhance the constitutive expression that performs the high expression levels in most tissues of plants, especially in dicotyledons (Jamal et al., 2009, Lessard et al., 2002, Lorence and Verpoorte, 2004) and in some monocotyledons (Pih et al., 1996).

To express the recombinant genes in plant host system, transformation methods enable to deliver the exogenous genes to plant cells. Plant gene transfer methods consist of two different strategies. First, transient transformation is fast and flexible method for plant cells to carry the recombinant genes. Secondly, stable transformation, which recombinant genes integrate into plant host genome, produces the transgenic lines that recombinant genes can be propagated to progeny (Mason et al., 2002 and Lorence and Verpoorte, 2004). The important tool to force the exogenous genes into

plant cells, for both stable and transient transformations, is plant pathogen *Agrobacterium tumefaciens* which causes crown gall disease infecting at wound site. Genetic transformation by *Agrobacterium* is driven by transferring T-DNA element from tumor-inducing (Ti) plasmid to plant host cells. Normally, T-DNA contains genes that involve in the cytokine and tumor-specific compounds that induce the formation of tumors in plants. For the application, these genes are removed and replaced by multiple cloning sites for gene of interest, as well as selectable markers and reporter genes. Therefore, *Agrobacterium* can transfer the modified T-DNA harboring the recombinant genes directly to plant cells. *Agrobacterium*-mediated transformation is capable for transformation in both dicotyledons and monocotyledons (Escobar and Dandekar, 2003, Lessard et al., 2002 and Lorence and Verpoorte, 2004).

The selection of plant host species is very important for recombinant peptide production because each plant displays its own genetic characters. Generally, tobacco is a plant model for recombinant production because it is easy to transform and manipulate (Boehm, 2007 and Sharma and Sharma, 2009), but there are several plant species which are developed to be host for recombinant peptide production.

2.1 ***Lemna minor*** *Lemna minor* is a small monocot and aquatic plant commonly called duckweed. Duckweed is one of a promising platform for peptide production referring to its rapid vegetative growth rate and convenience in maintaining. Duckweed

is free-floating plant on fresh water, so it can be readily cultivated in container or on open ponds by water or inorganic nutrients (Boehm, 2007 and Xu et al., 2012). Duckweed proliferates through vegetative budding of new fronds (leaves) from the meristematic region of parent fronds with doubling times of 1-2 days leading to increasing its biomass rapidly (Stomp, 2005).

Genetic transformation of duckweed has been investigated since 1990s by several laboratories. For transient transformation, recombinant genes can be transferred directly to the meristematic tissues of fronds. This strategy multiplies the recombinant genes by clonal reproduction system. However, to develop the stably transformed duckweed, tissue culture is employed. Duckweed fronds are able to establish the callus lines by callus induction medium. Yamamoto et al., 2000 have shown that the callus induction and frond regeneration are possible in *L. minor* and other *Lemna* species (Yamamoto et al., 2000). Both direct and indirect transformations are successful approaches to produce transgenic duckweed using *Agrobacterium*-mediated transformation method (Stomp, 2005 and Yamamoto, 2000).

Sun et al., 2007 expressed bacterial enzyme, endoglucanase E1 in transgenic *L. minor* using 35S promoter. The recombinant gene was transferred to duckweed callus by *Agrobacterium*-mediated transformation. The expression level of enzyme extracted from transgenic duckweed was estimated at 0.24% of total soluble protein and it

showed the endoglucanase activity (Sun et al., 2007). Besides, duckweed callus from *Lemna* species are applied for another pharmaceutical protein productions, for example monoclonal antibodies or mAbs (Cox et al., 2006) and avian influenza H5N1 hemagglutinin or HA protein to produce H5N1 HA antigen (Nguyen et al., 2012).

The application of duckweed expression system for pharmaceutical approach is already commercialized by BioLex (www.biolex.com) and LemnaGene (www.lemnagene.com). Both companies are focused on development of duckweed for recombinant protein production used as human therapies (Boehm, 2007 and Stomp, 2005). For instance, transgenic *L. minor* provided by BioRex was employed for studying in purification process of monoclonal antibody (Naik et al., 2012).

2.2 ***Arabidopsis thaliana*** *Arabidopsis thaliana* is a small mustard weed belonging to family Brassicaceae. This dicot has been determined as a plant model since 1943 by Friedrich Laibach due to its beneficial characters: small size, short regeneration time (5-6 weeks), large numbers of progeny (up to 10,000 seeds per plant) and the ease to grow under control conditions. These advantages are suitable for researches in plant biology. *Arabidopsis* has the smallest plant genome, so it is considered to be an excellent model for plant development, genetic mutation and plant biotechnology (Flavell, 2009, Koornneef and Meinke, 2010, Page and Grossniklaus, 2002 and Someville and Koornneef, 2002).

Genetic engineering in *Arabidopsis*, it can be easily transformed by *Agrobacterium*-mediated transformation. T-DNA insertion is powerfully occurred in meristem especially in dicotyledons. Consequently, *Arabidopsis* has been determined as powerful platform for recombinant protein production (Gepstein and Horwitz, 1995 and Someville and Koornneef, 2002), for example, the production of major outer membrane protein (MOMP) to be a potential vaccine candidate for human bacterial pathogen *Chlamydia trachomatis* (Kalbina et al., 2011), or to produce the viral-binding protein PmRab7 for using as in-feed vaccination against white spot syndrome in shrimp (Thagun et al., 2012). In addition to protein production, *Arabidopsis* host system can be used for antimicrobial peptide production such as the production of DmAMP1 from *Dahlia merckii* seeds and RsAFP2 from *Raphanus sativus* seeds (François et al., 2002) and the production of plant defensins (François et al., 2004).

Fusion expression for antimicrobial peptide production

Antimicrobial peptide production in *E. coli* system has to challenge with the natural properties of peptides that the cationic antimicrobial peptides may be toxic to the producing host. On the other hand, these small peptides may be the targets for proteolytic degradation leading to yield loss. To overcome these obstacles, the peptide of interest is conjugated with carrier protein or fusion protein (Li, 2011 and Parachin et

al., 2012). The anionic fusion proteins enable to neutralize the positively charged peptides preventing the toxicity to bacterial host cells. Additionally, the physical and structural properties of fusion proteins are advantageous to protect peptides from intracellular proteases (Parachin et al., 2012, Li et al., 2010 and Sørensen and Mortensen, 2005).

Unless the fusion proteins are desirable for recombinant expression, most proteins have been used as affinity tags for peptide purification. The most common affinity tag is polyhistidine tags (His-tag). Polyhistidine tags at N-terminus or at the junction between fusion partners allow effective purification by binding with immobilized metal affinity chromatography (IMAC) (Baneyx, 1999, Li et al., 2010 and Sørensen and Mortensen, 2005). Further, some fusion proteins act as solubility enhancing partner, such as maltose binding protein (MBP) and N-utilizing substance A (NusA), which are especially suitable for inclusion body prone proteins (Esposito and Chatterjee, 2006 and Sørensen and Mortensen, 2005).

For recombinant peptides, some amino acid sequences should be added for site-specific proteolysis to isolate the intact antimicrobial peptides after purification. For chemical cleavage, IEGR/X is the recognition site for Factor Xa or LVPR/G is recognized by thrombin. Another cleavage strategy is enzymatic cleavage that recombinant peptides are cut by some proteases. For example, enterokinase recognizes DDDK/X

whereas the tobacco etch virus protease (TEV) cleaves at ENLYFQ/G (Sørensen and Mortensen, 2005 and Schäfer et al., 2002). However, SUMO protease is one of the remarkable proteases that may be utilized for recombinant peptide production.

SUMO fusion system

SUMO (small ubiquitin-related modifier) family proteins have been conserved in all eukaryotes but absent in prokaryotes. SUMO system is related to the attachment of SUMO to its substrates, similar to ubiquitination, and the reversible conjugation of SUMO by SUMO protease, called SUMOylation pathway (Dohmen, 2004). The family of SUMO proteins functions as the conjugated modifier of other proteins involving to protein transportation (Butt et al., 2005, Dohmen, 2004 and Wood et al., 2003), transcriptional regulation (Zheng and Yang, 2004), chromatin structure and cell division (Dasso, 2008 and Shiio and Eisenman, 2003), DNA repair (Matunis, 2002) and signal transduction pathway (Huang et al., 2003).

Since the discovery of SUMO system, it has been evaluated as an interesting fusion tag because of its advantages. Particularly for improving the expression of recombinant peptide in *E. coli*, SUMO protein is employed as fusion tag to increase the solubility of recombinant peptide (Butt et al., 2005). The exact mechanism of enhancing the solubility is unknown, but it has been hypothesized that the fusion tag, such as

maltose binding protein (MBP), may play a role as a chaperone to prevent self-association (Fox et al., 2001) or act as a nucleation site for peptide folding, such as ubiquitin. Thus, the insoluble peptide that fused to Ub, as well as SUMO, can fold properly and increase soluble avoiding the formation of inclusion bodies (Butt et al., 1989).

SUMO fusion tag enables to protect to proteolytic degradation from host cell that decreases the expression level of recombinant peptides. Since the recombinant peptides are susceptible to some proteases in cytosol, SUMO is helpful to translocate the recombinant peptide from cytosol to nucleus avoiding the degradation by proteases (Kishi et al., 2003). Contrarily, the fusion tags can neutralize the positive charge of peptides leading to decreasing the toxicity of peptide to the host cell (Li et al., 2010).

SUMO performs as a recognition site for SUMO proteases similarly to SUMOylation pathway. Other proteases that recognize the amino acid sequences for their cleavage sites and provide the extraneous amino acids at N-terminus that may interfere with the biological function of therapeutic peptides (Schäfer et al., 2002). Differentially, SUMO proteases recognize the tertiary structure of SUMO tag releasing the intact peptides without any extraneous amino acids. Approximately 100 SUMO fusions were observed and found that SUMO proteases cleaved these SUMO fusions without error (Butt et al., 2005).

Due to the lack of SUMO system in prokaryotes, SUMO fusion tag has been utilized for protein or peptide production in bacterial host systems with the cost-effective means for large-scale production (Bommarius et al., 2010). SUMO fusion tag improves the expression level of the difficult-to-express proteins, such as FLAP and SARS-CoV membrane proteins (Zuo et al., 2005), interleukin-1β (Kirkpatrick et al., 2006) and human tumor necrosis factor or TNF-α (Hoffmann et al., 2010). Comparing with many fusion systems, for example MBP, GST, TRX and NUS A, SUMO tag significantly increased the expression and solubility of target proteins and SUMO protease generated the intact proteins with native sequences (Marblestone et al., 2006). Hence, SUMO fusion system is the most attractive system for protein production. For antimicrobial peptide production, ABP-CM4 was fused to SUMO tag and expressed in *E. coli*. 24 mg of recombinant CM4 with 96% purity was produced and it showed the antimicrobial properties similar to the synthetic CM4 (Li et al., 2009). This demonstrates that SUMO fusion system can be applied for small therapeutic peptides as well.

For eukaryotic host systems, SUMO fusion tag can be cleaved by endogenous SUMO proteases because this system has been conserved in all eukaryotic organisms (Dohmen, 2004). SUMOstar fusion tag, the analogue SUMO tag containing the adaptive amino acid sequences, is developed for protein production in eukaryotes such as the

expression of dengue virus 2 NS1 and various proteins in baculovirus/insect cell system (Liu et al., 2008 and Rozen-Gagnon et al., 2012).

CHAPTER III

MATERIALS AND METHODS

1. Chemicals and reagents

1.1 **Reagents**: Acetosyringone, Agar powder, Bacteriological agar powder,Bovine serum albumin (BSA), Cefotaxime, Disodium hydrogen phosphate (Na_2HPO_4), Glucose, Glutaraldehyde, Hygromycin, Imidazole, IPTG, Kanamycin, Lysozyme, Methanol, MES hydrate, Potassium hydroxide (KOH), Sodium chloride (NaCl), Sodium dihydrogen phosphate (NaH_2PO_4), Sodium hydroxide (NaOH), Sucrose, SUMO protease, SUMOstar protease, Tween®20, Yeast extract powder

1.2 **Plant hormones**: 2,4-Dichlorophonoxyacetic acid (2,4-D), Benzyl adenine (BA),Thidiazuron (TDZ)

1.3 **Medium**: Lysogenic Broth (LB medium), Murashige and Skoog medium (MS medium), Mueller Hinton Broth (MHB medium), Schenk and Hildebrandt medium (SH medium)

1.4 **Kits**: 1 Kb Plus DNA Ladder, Amicon Ultra-0.5 mL Centrifugal Filters for Protein Purification and Concentration, Champion™ pET SUMO Expression System, DreamTaq™ PCR Master Mix, Dynabeads® His-Tag Isolation & Pulldown, FastBreak™ Cell Lysis Reagent, GeneJET™ Plasmid Miniprep Kit, Halt Protease Inhibitor Cocktail,

HisPur™ Cobalt Purification kit, NuPAGE® LDS Sample Buffer, NuPAGE® Novex 12% Bis-Tris Gel, PageBlue™ Protein Staining Solution, PageSilver™ Silver Staining Kit, P-PER Plant Protein Extraction Kit, Quick Start™ Bradford Protein Assay, TOPO® TA Cloning Kit

2. Dedicated gene

From previous study, Rhacoporin-2 or RF28 from tree frog *Rhacophorus feae* showed an antimicrobial activity against both Gram-positive and Gram-negative bacteria. It could kill bacterial cell including *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus* at 3.12 μM, 12.50-25 μM and 12.50-50 μM, respectively. Especially for Salmonella Typhimurium, Rhacoporin-2 could kill it at very low concentration, <0.78 μM. Moreover, Rhacoporin-2 also showed an anticancer activity against cancer cell lines, A459, MCF-7 and MRC-5 by 4.5 μM, 15 μM and 12 μM respectively. Nevertheless, at very high concentration of peptide, >300 μM, red blood cells of rat was normal. It showed that Rhacoporin-2 was harmless to mammalian red blood cells. Thus, Rhacoporin-2 was an attractive antimicrobial peptide for further study.

Table 1 MIC value of Rhacoporin-2 against bacterial strains

Bacterial strains	MIC (μM)
Escherichia coli	3.12
Salmonella Typhimurium	<0.78
Staphylococcus aureus	12.5 – 25
Bacillus cereus	12.5 – 50

Table 2 IC_{50} value of Rhacoporin-2 against cancer cell lines

Cell lines	IC_{50} (μM)
A549	4.5
MCF-7	15
MRC-5	12

Table 3 HC_{50} value of Rhacoporin-2 against mammalian red blood cell

Antimicrobial peptide	HC_{50} (μM)
Rhacoporin-2	>300

3. Host strains

3.1 Bacterial host strain *E. coli* TOP10 was used to manipulate the dedicated gene, Rhacoporin-2 or RF28, which fused with SUMO cleavage site in pET SUMO

vector. *E. coli* BL21(DE3) was an expression host which contained T7 RNA polymerase that could activate the expression of fusion gene. Both of these strains were grown in LB media containing 50 μg/mL kanamycin.

3.2 Plant host strain *Lemna minor* CM5 was selected as a host due to its property, high protein yield strain. It was cultivated in MS media containing 4 μg/mL TDZ with 18-hour-per- day light for 2 weeks.

4. Plasmid preparation

4.1 Codon optimization Naturally, Rhacoporin-2 gene is expressed in the animal host, tree frog *Rhacophorus feae*. To increase the expression level of Rhacoporin-2 gene in different hosts, *Escherichia coli* as a bacterial host and *Lemna minor* as a plant host respectively, genetic codes or codons were adjusted for each host using codon usage tables below. The optimized Rhacoporin-2 gene in pUC57 vector for both bacterial and plant systems was synthesized by GenScript (USA).

Table 4 Codon usage of *Lemna minor* (database from www.kazusa.or.jp)

Lemna minor [gbpln]: 4 CDS's (1597 codons)

Fields: [triplet] [frequency: per thousand] ([number])

UUU	17.5	(28)	UCU	13.8	(22)	UAU	8.8	(14)	UGU	5.0	(8)
UUC	36.3	(58)	UCC	17.5	(28)	UAC	15.7	(25)	UGC	14.4	(23)
UUA	5.6	(9)	UCA	14.4	(23)	UAA	0.0	(0)	UGA	1.9	(3)
UUG	13.8	(22)	UCG	13.8	(22)	UAG	0.6	(1)	UGG	16.3	(26)
CUU	15.7	(25)	CCU	11.9	(19)	CAU	6.9	(11)	CGU	4.4	(7)
CUC	25.7	(41)	CCC	15.7	(25)	CAC	16.9	(27)	CGC	18.2	(29)
CUA	5.0	(8)	CCA	11.3	(18)	CAA	10.0	(16)	CGA	6.3	(10)
CUG	21.3	(34)	CCG	14.4	(23)	CAG	22.5	(36)	CGG	10.6	(17)
AUU	18.8	(30)	ACU	9.4	(15)	AAU	13.8	(22)	AGU	10.0	(16)
AUC	19.4	(31)	ACC	17.5	(28)	AAC	21.9	(35)	AGC	15.0	(24)
AUA	1.9	(3)	ACA	5.0	(8)	AAA	15.7	(25)	AGA	20.7	(33)
AUG	20.7	(33)	ACG	10.0	(16)	AAG	35.7	(57)	AGG	17.5	(28)
GUU	15.0	(24)	GCU	25.0	(40)	GAU	20.0	(32)	GGU	8.1	(13)
GUC	25.0	(40)	GCC	22.5	(36)	GAC	26.3	(42)	GGC	21.9	(35)
GUA	6.3	(10)	GCA	14.4	(23)	GAA	26.3	(42)	GGA	16.9	(27)
GUG	30.7	(49)	GCG	18.2	(29)	GAG	40.1	(64)	GGG	18.2	(29)

Table 5 Codon usage of *Escherichia coli* (database from www.kazusa.or.jp)

Escherichia coli [gbbct]: 8087 CDS's (2330943 codons)

Fields: [triplet] [frequency: per thousand] ([number])

UUU	24.4	(56791)	UCU	13.1	(30494)	UAU	21.6	(50400)	UGU	5.9	(13662)
UUC	13.9	(32513)	UCC	9.7	(22537)	UAC	11.7	(27239)	UGC	5.5	(12777)
UUA	17.4	(40627)	UCA	13.1	(30502)	UAA	2.0	(4664)	UGA	1.1	(2674)
UUG	12.9	(30084)	UCG	8.2	(19071)	UAG	0.3	(751)	UGG	13.4	(31207)
CUU	14.5	(33816)	CCU	9.5	(22121)	CAU	12.4	(28919)	CGU	15.9	(37134)
CUC	9.5	(22074)	CCC	6.2	(14379)	CAC	7.3	(17117)	CGC	14.0	(32720)
CUA	5.6	(12951)	CCA	9.1	(21237)	CAA	14.4	(33607)	CGA	4.8	(11216)
CUG	37.4	(87261)	CCG	14.5	(33795)	CAG	26.7	(62329)	CGG	7.9	(18434)
AUU	29.6	(68942)	ACU	13.1	(30518)	AAU	29.3	(68348)	AGU	13.2	(30749)
AUC	19.4	(45213)	ACC	18.9	(44139)	AAC	20.3	(47233)	AGC	14.3	(33255)
AUA	13.3	(31065)	ACA	15.1	(35293)	AAA	37.2	(86726)	AGA	7.1	(16583)
AUG	23.7	(55356)	ACG	13.6	(31794)	AAG	15.3	(35652)	AGG	4.0	(9238)
GUU	21.6	(50261)	GCU	18.9	(44034)	GAU	33.7	(78663)	GGU	23.7	(55283)
GUC	13.1	(30515)	GCC	21.6	(50411)	GAC	17.9	(41619)	GGC	20.6	(47962)
GUA	13.1	(30461)	GCA	23.0	(53619)	GAA	35.1	(81727)	GGA	13.6	(31729)
GUG	19.9	(46309)	GCG	21.1	(49169)	GAG	19.4	(45154)	GGG	12.3	(28720)

4.2 Plasmid preparation for bacterial system pCR™4-TOPO vector containing Rhacoporin-2 gene extracted from *E. coli* TOP10 by GeneJET™ Plasmid Miniprep Kit was used as DNA template. Rhacoporin-2 gene was amplified by DreamTaq™ PCR Master Mix using ERHA1 (5'-GGCGCGTTTACCGATCTGCTGA-3'), forward primer, and ERHA2 (5'-TTAGCAGGTTTTCGCCAGTTTG-3'), reverse primer. The PCR reaction condition was composed of 30 cycles that preceded by denaturing at 94°C for 30 seconds, annealing at 53°C for 30 seconds, extension at 72°C for 30 seconds and final elongation at 72°C for 10 minutes. The size of PCR product was checked by agarose gel electrophoresis compared with 1 Kb Plus DNA Ladder running at 100 volt for 30 minutes. DNA fragment sized nearly 100 bp was ligated to pET SUMO vector by incubating with T4 DNA ligase at 15°C over night. Afterwards, pET SUMO vector that consisted of Rhaoporin-2 gene was transformed to host cell, *E. coli* BL21(DE3) by Heat-shock technique: cells were incubated on ice for 20 minute and suddenly heated at 42°C for 30 second. Cells were spread on LB plate containing 50 μg/mL kanamycin and incubated at 37°C over night for selection. Transformed cells that grew on antibiotic plate were selected and preserved in 15% glycerol stock at -80°C.

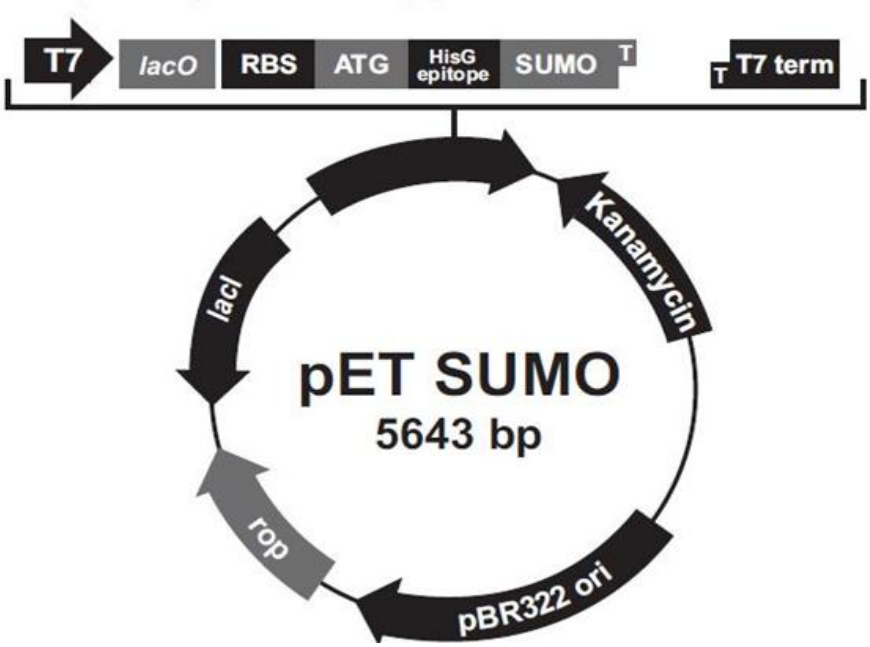

Figure 6 The features of pET SUMO vector, the crucial features contained T7 promoter, Lac operator, initiation ATG, poly histidine gene, SUMO gene, TA cloning site and kanamycin resistance gene.

4.3 Plasmid preparation for plant system The optimized and synthetic SUMOstar fused to Rhacoporin-2 gene in pUC57 was cleaved by HindIII and BamHI. The fusion gene, GFP-SUMOstar-Rhacoporin-2, was ligated to pCAMBIA1305.2 plasmid by T4 DNA ligase at 15^{o}C over night. Refer to pCAMBIA1305.2 plasmid, T-DNA region of this plasmid comprised GFP as a plant reporter gene, hygromycin and kanamycin-resistant gene as a selectable marker in plant and bacteria respectively. This pCAMBIA1305.2 plasmid with fusion gene construct was transformed into *Agrobacterium tumefaciens*

EHA105 by electroporation method. Cells were spread on LB plate containing 50 μg/mL kanamycin and incubated at 28°C 2-3 days for selection. Transformed cell containing pCAMBIA1305.2 that grew on antibiotic plate were selected and preserved in 15% glycerol stock at -80°C.

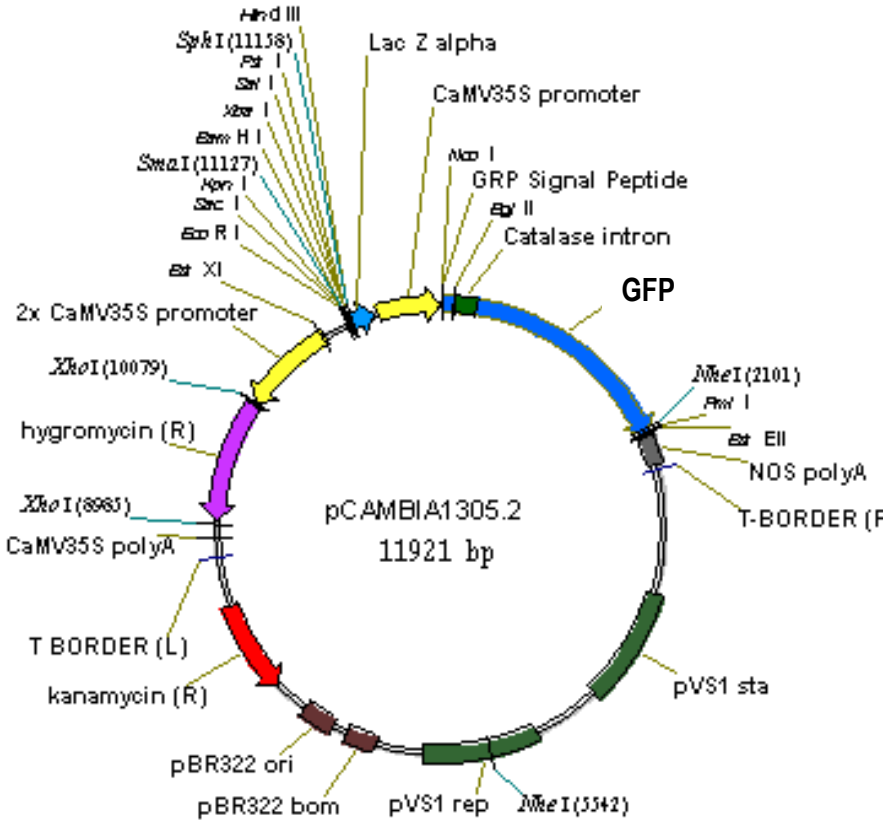

Figure 7 The features of pCAMBIA1305.2. the crucial features contained BamHI and HindIII restriction sites, hygromycin resistance gene, kanamycin resistance gene and GFP reporter gene.

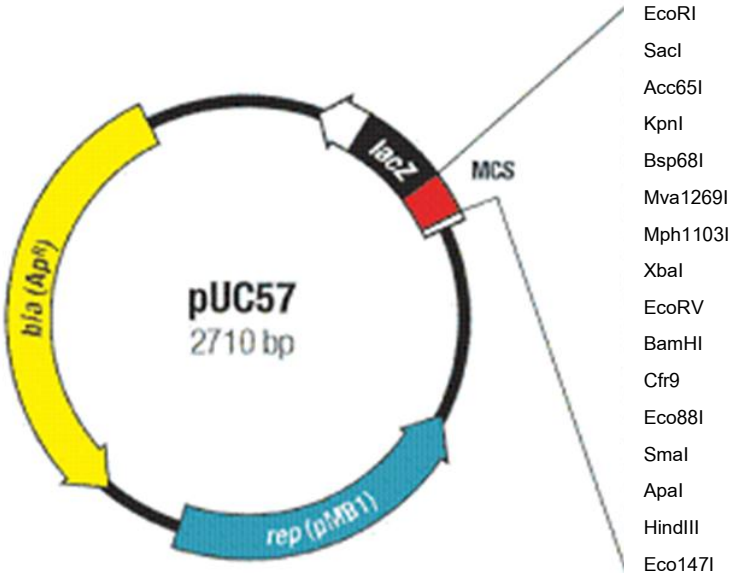

Figure 8 The features of pUC57 and the restriction sites including BamHI and HindIII.

5. Expression and purification in *E. coli* system

5.1 Pilot expression Pilot expression was a procedure used to optimize the peptide expression condition. First of all, cells were prepared by streaking on LB plate containing 50 μg/mL kanamycin and incubated at 37°C over night to isolate a pure strain. A single colony was selected and grew as a starter culture in LB broth containing 50 μg/mL kanamycin at 37°C with 100 rpm shaking over night. 1 mL of starter culture was added to 20 mL of LB culture, LB broth containing 50 μg/mL kanamycin, incubated at 37°C with 100 rpm shaking until cell number increased to log phase or OD_{600} reached at 0.4-0.6. Then, IPTG was added to culture to induce the peptide expression. The different final concentrations of IPGT: 0.5 mM and 1 mM, were examined. After adding IPTG, cells were continually shaking for various durations: 0, 40, 60, 120, 180 and 300

minutes to find out the optimized period for peptide induction. The optimum IPTG concentration and induction period was examined by SDS PAGE.

5.2 Peptide expression To produce Rhacoporin-2 in large scale culture, cells were prepared as above (5.1). 50 mL of starter culture was added to 1 L of LB broth containing 50 μg/mL kanamycin incubated at 37°C with 100 rpm shaking until OD_{600} reached at 0.4-0.6. After that, IPTG was added at 0.5 mM as a final concentration and additionally shook for 3 hours.

5.3 Peptide purification To purify Rhacoporin-2 from *E. coli*, firstly, cells were harvested by centrifugation at 4°C, 4000 rpm for 15 minutes and then were lysed by FastBreak™ Cell Lysis Rreagent which is comprised of Halt Protease Inhibitor Cocktail and lysosyme. Cell lysate was centrifuged to remove cell debris. The fusion peptide, SUMO-Rhacoporin-2, was purified from supernatant using Dynabeads® His-Tag Isolation & Pulldown. Supernatant was incubated and gently shaking with beads at room temperature for 10 minutes, washed the beads by wash buffer for 3 times and eluted the fusion peptide from beads by elution buffer. SUMO protease was used to cleave the fusion peptide and divide into two parts, SUMO fusion tag and Rhacoporin-2. 8 μg of fusion peptide was incubated with 8 units of SUMO protease at 30°C for 2 hours. After this reaction, the solution was desalted to remove imidazole using Amicon Ultra-0.5 mL Centrifugal Filters. Then, the desalted solution was purified repeatedly by Dynabeads to

part SUMO fusion tag and Rhacoporin-2. Every fraction in purification procedure was qualified by SDS PAGE and quantified by Bradford assay.

6. Expression and purification in plant system

6.1 Cell preparation *A. tumefaciens* EHA105 cells harboring expression plasmid were prepared by streaking on LB plate containing 50 μg/mL kanamycin for 2 days at 28°C to isolate the pure strain. A single colony was selected and grown in a starter culture or 5 mL LB broth containing 50 μg/mL kanamycin at 28°C with 100 rpm shaking over night. 5 mL of starter culture was transferred to 70 mL of LB broth containing 50 μg/mL kanamycin and was shaken at 100 rpm for 8-9 hours. Cell density was measured by the method OD_{600}. After that, cells were harvested by centrifugation at 4000 rpm, 28°C for 15 minutes. Supernatant was discarded and cells were resuspended in infiltration media or 1/2MS media containing 1% yeast extract, 100 μM acetosyringone and 2 μg/mL BA. Cell density or OD_{600} was adjusted to 0.8.

6.2 Transient transformation Transient transformation using *Agrobacterium*-mediated method was the simplest procedure for plant transformation, including duckweed *L. minor*, that gene was transferred directly to plant somatic cells. The procedures for transient transformation included pretreatment and inoculation steps. At

pretreatment step, there were 3 factors that we investigate to improve the transformation efficiency.

I. Plant medium Duckweed was cultured in different plant medium including MS media and SH media for 2 weeks. The growth of duckweed and GFP expression after transformation were observed.

II. Plasmolysis treatment After 2 weeks of cultivation, duckweed was incubated with 0.6 M manitol for 5 minutes to induce plasmolysis before co-cultivation. GFP expression after transformation was observed.

III. Cell wall degradation Duckweed was incubated with 0.1% cellulase for 30 minutes before inoculation. GFP expression after transformation was observed.

At inoculation step, 2-week-old *L. minor* was co-cultivated with cell suspension from 6.1 at 23^{o}C for a week. At this step, the different sugars including glucose, fructose, sucrose and sorbitol in infiltration media were examined. The effect of these sugars was observed by GFP expression after inoculation for a week. Finally, fronds, the leaves of duckweed that showed GFP expression were selected for peptide purification.

6.3 Stable transformation This procedure was to transform the fusion gene to host cell via callus, an unorganized parenchyma cell or somatic embryogenic cells.

I. Plant hormones proportion The proportion of plant hormones, between auxin and cytokinin, has affected to callus formation in plant tissue culture. To find out

the suitable plant hormone proportion for *L. minor*, 1 or 2-week-old *L. minor* were cultured in callus induction medium or MS agar containing various concentration of plant hormones: 1 μM 2,4-D + 2 μM BA, 2 μM 2,4-D + 2 μM BA, 2 μM 2,4-D + 1 μM BA, 0.5 μM TDZ + 1 μM 2,4-D, 0.5 μM TDZ + 5 μM 2,4-D and 0.5 μM TDZ + 10 μM 2,4-D. Plants were cultured for 6 weeks in 18-hour-per day light. Callus formation was observed by stereo microscope.

II. Callus transformation 1 or 2-week-old *L. minor* was cultures in callus induction medium or MS agar containing 0.5 μM TDZ and 5 μM 2,4-D for 6 weeks. After that, nodules were transferred to NPM, nodule promoting medium, or MS agar containing 1 μM 2,4-D and 2 μM BA for 2 weeks. At this step, callus could be maintained by subculturing in NPM every 2 weeks. For callus transformation, calluses were immersed with cell suspension from 6.1 for 5 minutes. Then, calluses were transferred to NPM-AS (NPM containing 100 μM acetosyringone) and co-cultivated at 23^{o}C in the dark for 2 days. After co-cultivation, transformed calluses were cultured in FRM (frond regeneration medium): SH media containing 500 μg/mL cefotaxime and 20 μg/mL hygromycin, shook at 100 rpm in 18-hours-per day light. Transformed calluses were subcultured every week.

6.4 Peptide purification The fusion peptide from transformed duckweed was extracted using P-PER Plant Protein Kit. Dynabeads was used to purify the fusion

peptide from total protein solution (according to 5.3). 10-100 μg of fusion peptide eluted from beads was cleaved by incubating with 1 unit SUMOstar protease at 4°C over night. After that, the solution was desalted and fusion peptide was purified repeatedly by Dynabeads to part SUMOstar fusion tag and Rhacoporin-2. Every fraction in purification procedure was qualified by SDS PAGE and quantified by Bradford assay.

6.5 The other host plant *Arabidopsis thaliana* is a flowering plant model that very popular in plant biology research, for example, plant development and plant transformation. From previous study of Khunpolwattana, 2012, the gene encoded for fusion peptide, SUMOstar-Rhacoporin-2, could be stably transfosrmed into the genome of *A. thaliana*. For this study, seeds from T3 generation and wild type were bleached using sterilization solution and planted in MS agar for 2 weeks. Total proteins from seedlings were extracted by P-PER Plant Protein Kit. The fusion peptide was purified by the same method as 6.4. Every fraction in purification procedure was qualified by SDS PAGE and quantified by Bradford assay comparing between *A. thaliana* wild type and *A. thaliana* T3 progeny.

7. SDS PAGE

SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis is a biochemical technique widely used to determine size and purity of the interested

protein. For this study, the quality of peptide from every fraction in peptide purification procedure was examined. Peptide was linearized by incubating with NuPAGE® LDS Sample Buffer at 70°C for 10 minutes. 1-2 μg of linearized peptide was loaded to NuPAGE® Novex 12% Bis-Tris Gel and run with NuPAGE® MES SDS Running Buffer at 120 volt for 70 minutes. After gel electrophoresis, polyacrylamide gel was fixed by 5% glutaraldehyde in 50% methanol and gently shaken for 1 hour to prevent the peptide diffusion. Finally, gel was incubated with PageBlue™ Protein Staining Solution and gently shaken overnight to stain peptide in polyacrylamide gel.

8. Bradford assay

Bradford assay is an analytical technique vastly used to measure total protein concentration based on an absorbance of protein dye complex at 595 nm.

8.1 Standard curve for microplate microassay A standard curve for Bradford assay is usually calculated from the association between known concentration of bovine serum albumin (BSA) and an absorbance at 595 nm. Firstly, BSA solution was prepared at various concentrations: 8, 26, 44, 62 and 80 μg/mL. 150 μL of samples was mixed with 150 μL of Quick Start™ Bradford Protein Assay and incubated for 5 minutes. Subsequently, the red color of solution turned to blue. The absorbance at 595 nm was measured for 3 replicates by microplate reader Multiskan EX (Thermo Scienctific). The

microassay standard curve and an equation for BSA concentration were calculated using Microsoft Excel 2007.

8.2 Peptide quantification Quick Start™ Bradford Protein Assay was used to dye peptide solution. Subsequently, the absorbance at 595 nm of the solution was measured by Multiskan EX (Thermo Scienctific). Peptide concentration was quantified comparing to standard curve from 8.1.

9. Minimum inhibitory concentration

Minimum inhibitory concentration or MIC, a monitor of antimicrobial agent activity, was the lowest concentration of antimicrobial agents that could either kill or inhibit the growth of microorganisms.

9.1 Bacterial strains Bacterial strains which related to gastrointestinal infections consisted of *Escherichia coli*, Salmonella Typhimurium, *Staphylococcus aureus* and *Bacillus cereus* were tested against Rhacoporin-2 at various concentrations.

9.2 MIC assay Cells were prepared by streaking on MHB agar plate and grew at 37°C over night. A single colony was selected and grew in MHB broth at 37°C. After that, 20 μL of starter culture was added to 10 mL of MHB broth and incubated at 37°C with 100 rpm shaking until OD600 reached at 0.1-0.3. Afterwards, cell suspensions were diluted to 0.002. 2-fold dilution method was used for this assay. 50 μL of MHB broth was

dispensed to wells in a row of 96-well cell culture polypropylene plate. 50 μL of peptide solution was mixed in the first well. Instantly, 50 μL of mixed solution was carried out and dispensed to the next well. The series of 2-fold dilution was continued for 5 times. 50 μL of cell suspension was added and mixed in wells, each row for each strain. Cell suspension without peptide solution was specified as a positive control, whereas, peptide solution without cell suspension was specified as a negative control. The 96-well cell culture polypropylene plate was incubated at 37°C and 100 rpm shaking over night. Afterwards, the lowest concentration that could inhibit the growth of bacteria was considered and specified as MIC value.

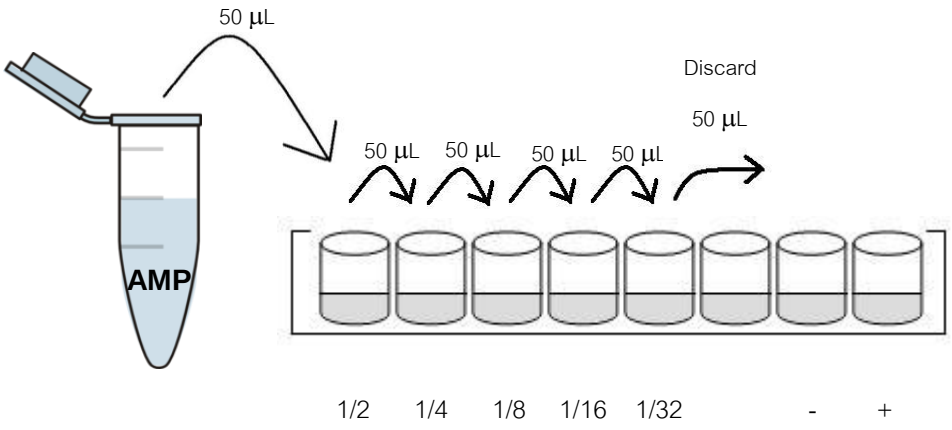

Figure 9 2-fold dilution method for MIC assay

CHAPTER IV

RESULTS AND DISCUSSION FOR BACTERIAL SYSTEM

1. Codon optimization

Targeted mutagenesis is one of approaches to remove rare codons and increase the efficiency of recombinant peptide production in heterologous host systems (Burgess-Brown et al., 2008). Some rare codons of 93 bp Rhacoporin-2 gene from *R. feae* was appropriately modified for *E. coli*.

Table 6 Codon optimization of Rhacoporin-2 gene for *Escherichia coli*

Hosts	Optimized codons
Orginal sequences (*Rhacophorus feae*)	ggagcctttactgatctactcaaaggtgtagccaagcaagcgggcataaaaat cctgggtattgctcaatgtaaacttgctaaaacatgttaa
Bacterial system (*Escherichia coli*)	ggcgcgtttaccgatctgctgaaaggcgtggcgaaacaggcgggcattaaaa ttctgggcattgcgcagtgcaaactggcgaaaacctgctaa

2. BSA standard curve

This standard curve was created for the estimation of protein or peptide yield in further procedures in both bacterial and plant system. The absorbance at 595 nm of standard BSA was measured (shown in table 7) to originate the standard curve and the standard equation (shown in figure 10).

Table 7 The absorbance at 595 nm of standard BSA at various concentrations

Concentrations (μg/mL)	Absorbance at 595 nm			
	1	2	3	Average
8	0.174	0.157	0.148	0.160
26	0.558	0.499	0.544	0.534
44	0.941	0.899	0.905	0.915
62	1.162	1.130	1.083	1.125
80	1.185	1.160	1.221	1.189

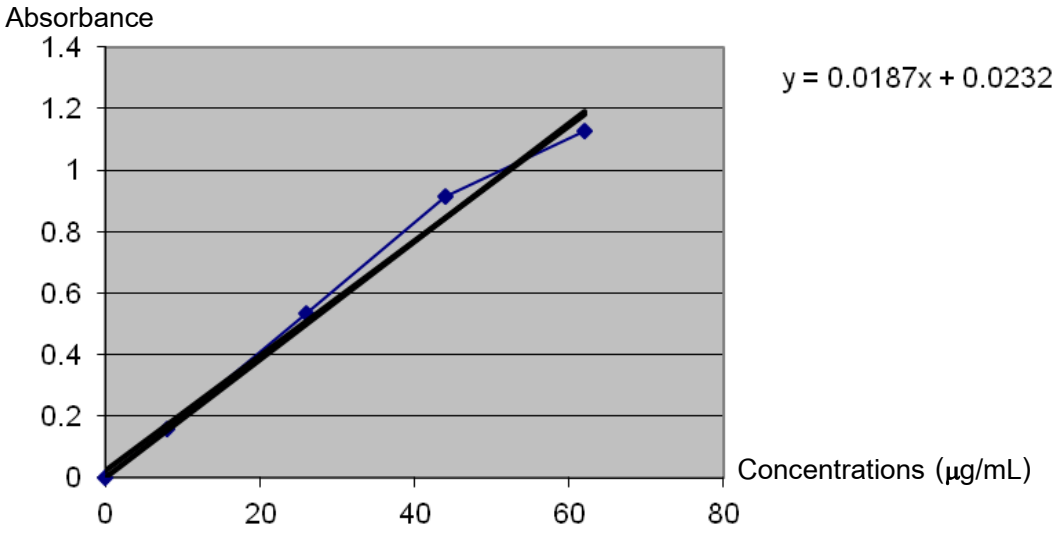

Figure 10 Standard curve and the standard equation from BSA concentration.

3. The amplification of Rhacoporin-2 gene

After PCR reaction, Rhacoporin-2 gene sized 93 bp was amplified (shown in figure 11). The PCR product was ligated to pET SUMO vector and then transformed into an expression host cell, *E. coli* BL21(DE3). After transformation, a single colony of transformed cell that could grow under kanamycin selection medium was selected for a large-scale production.

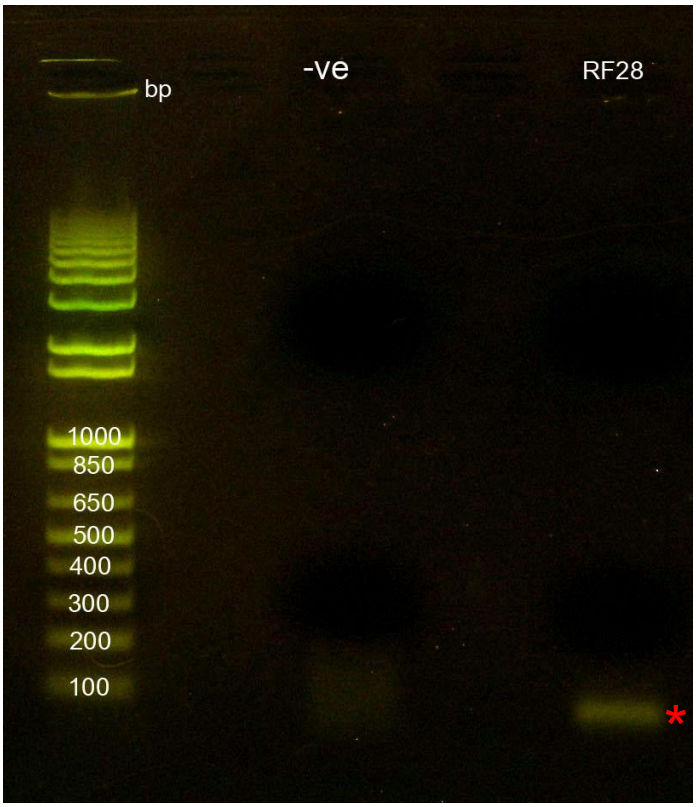

Figure 11 The amplification of Rhacoporin-2 gene, the first lane is DNA marker starting at 100 bp and the second lane is negative control. The fragment of gene sized 93 bp is indicated by star.

4. Pilot expression of Rhacoporin-2

To optimize the expression condition of antimicrobial peptide Rhacoporin-2, temperature, IPTG concentration and the induction duration were investigated.

When the cell density reached at log phase, IPTG was added to the culture for recombinant peptide induction. The final concentrations of IPTG, 0.5 and 1 mM, and the temperature of culture were examined. Temperature is one of the key roles for the formation of inclusion bodies in protein production by *E. coli*. There is a study indicated

that the inclusion bodies are formed in *E. coli* cell culture under 37°C cultivation, whereas it was rarely occur under 28°C cultivation (Wagner et al., 1992). Thus, *E. coli* cultivation under 28°C and 37°C were compared in this study. SDS PAGE from figure 12 indicated that peptide yield from cell cultured at 37°C was greater than 28°C. Because the optimized temperature for *E. coli* is 37°C, hence, it is preferable to grow at 37°C leading to higher amount of peptide production (Nguyen, 2006). The table 8 showed the total amount of proteins from cell cultured under various conditions. Cell under 37°C culture produced higher amount of total proteins comparing with 28°C. Combining with the final concentration of IPTG, transformed cell that induced by 0.5 mM IPTG synthesized the higher amount of protein in both 37°C and 28°C culture. This indicated that the higher concentration of IPTG may affect the growth rate of bacterial cell (Malakar and Venkatesh, 2012) caused the decreasing amount of peptide production. Thus, the optimum condition for Rhacoproin-2 production is to culture under 37°C and induced by 0.5 mM IPTG.

Table 8 Total protein yield from various temperatures and IPTG concentrations

Conditions	Total proteins yield (μg)
37°C, 1 mM IPTG	5,632.0
37°C, 0.5 mM IPTG	7,232.0
28°C, 1 mM IPTG	3,648.0
28°C, 0.5 mM IPTG	4,576.0

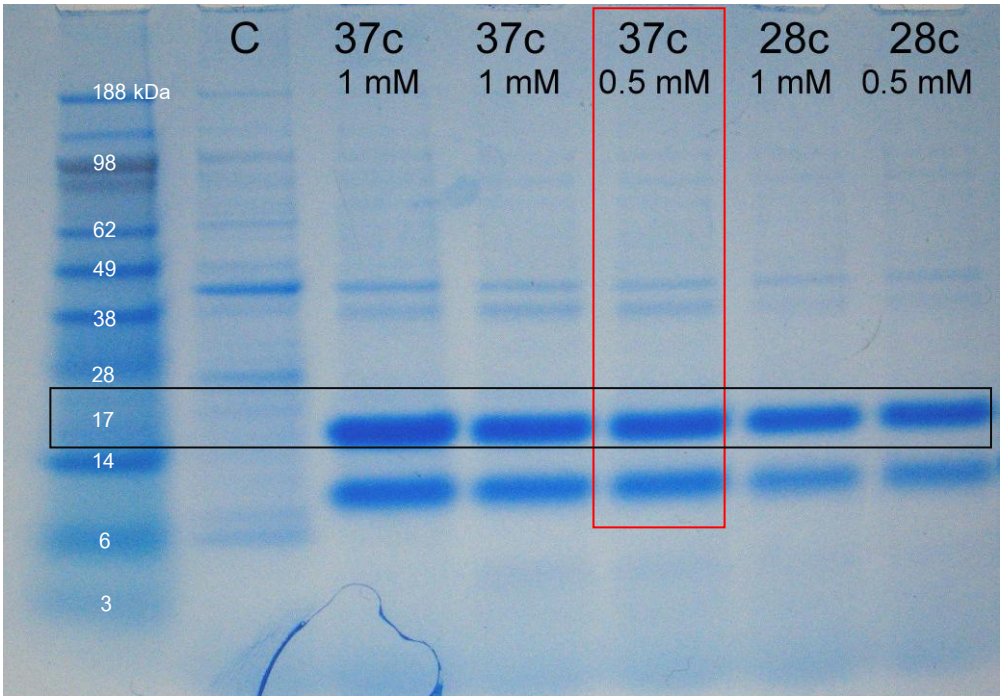

Figure 12 SDS PAGE of temperatures and IPTG concentrations, C; control cell (no induction). The black square indicates the recombinant peptide sized 16.5 kDa. The red square indicates the optimum condition for Rhacoporin-2 production.

The effect of duration of induction at 6 different time points: 0, 40, 60, 120, 180 and 300 minutes is shown in figure 13. SDS PAGE showed that the recombinant peptide was initially synthesized after induction for 40 minutes after that the amount of peptide increased continuously and reached very high amount after 180 minutes. This indicated that the suitable duration for peptide induction is 3 hours. Taken together, Thus, referred to previous result, the optimum condition for Rhacoporin-2 production is 37°C culture which induced by 0.5 mM IPTG for 3 hours.

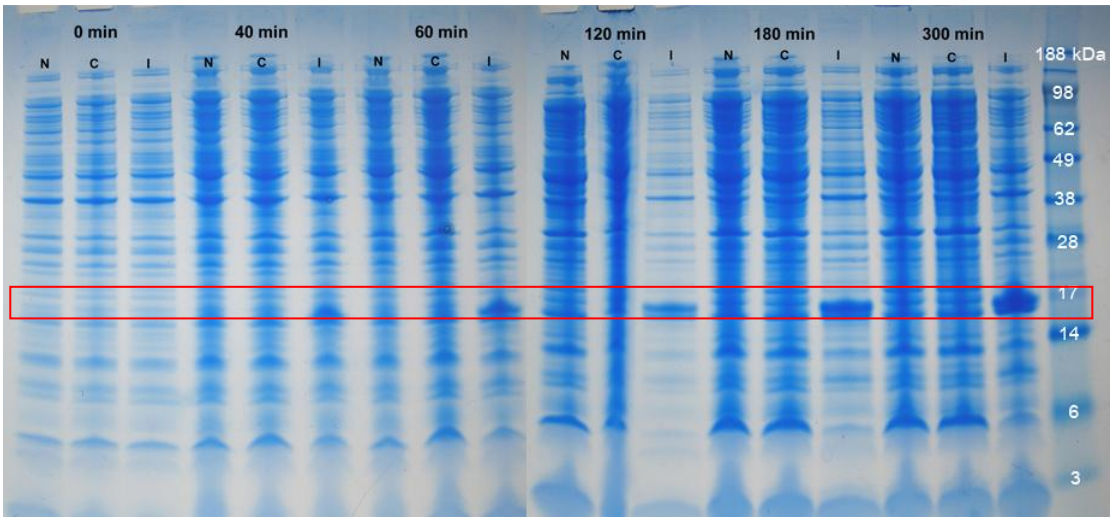

Figure 13 SDS PAGE of induction duration of Rhacoporin-2, N: non-transformed cell, C: transformed cell with no induction, I: transformed cell induced by 0.5 mM IPTG

5. Large-scale production

E. coli BL21(DE3) containing pET SUMO vector was cultured in 1 L kanamycin selection medium. After 3 hours of induction, the reagent was used for cell break releasing 26,880 μg of total proteins (shown in table 9). Then, the fusion peptide, SUMO-Rhacoporin2, was purified by Dynabeads® His-Tag Isolation & Pulldown kit. The fusion peptide sized 16.5 kDa was eluted from magnetic beads (shown in figure 14, lane 4). From Bradford assay, the amount of fusion peptide was 248.9 μg. After tag removal by SUMO protease, the fusion tag was released from the antimicrobial peptide (shown in figure 14, lane 5). Followed by repeatedly purification, the purified Rhacoporin-2 sized 3 kDa was eluted from magnetic beads (shown in figure 14, lane 7). The amount of purified peptide was 131 μg with 85% purity determined by band intensity using Adobe Photoshop. SDS PAGE shown in figure 14 demonstrated that this procedure could be employed for fusion peptide purification.

Table 9 The amount of protein and peptide calculated by Bradford assay

Fractions	Protein/peptide amount (μg)
Cell lysate	26,880.0
SUMO-Rhacoporin2	248.9
Rhacoporin-2	131.0

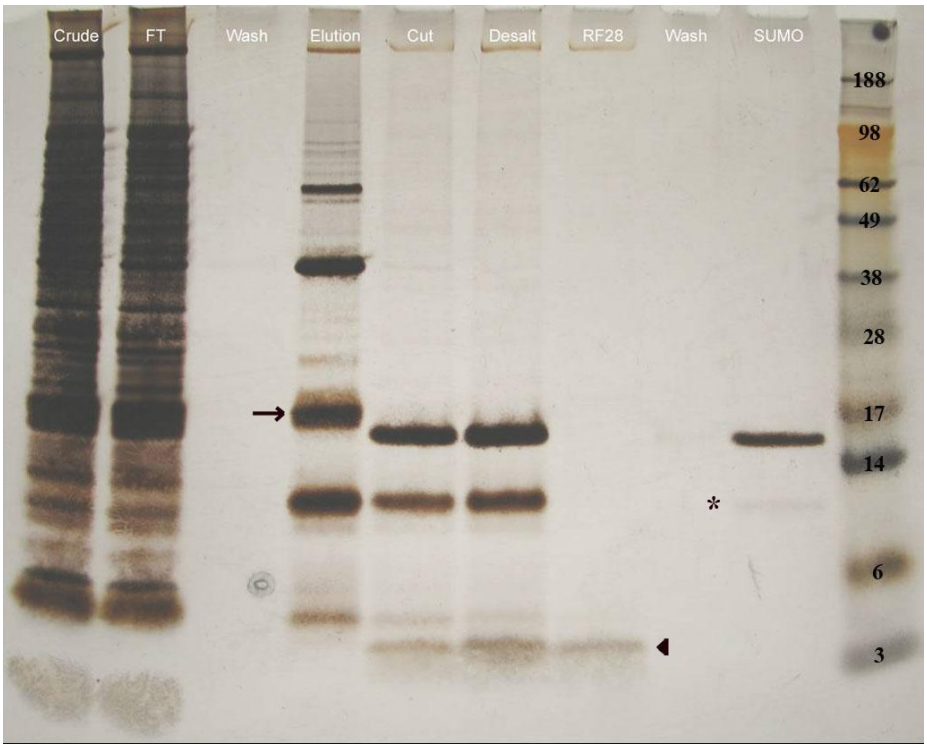

Figure 14 SDS PAGE of peptide purification stained by silver staining. Arrow: the fusion peptide sized 16.5 kDa, arrow head: the purified Rhacoporin-2 sized 3 kDa, star: SUMO fusion protein that separated from peptide

6. Biological function of purified peptide

Previous studies of biological activity of the synthesized Rhacoporin-2, showed that it exhibited very strong bactericidal activity against Gram-negative bacteria. Hence, in this study, the purified Rhacoporin-2 was examined against Gram-negative bacteria, *Escherichia coli* and Salmonella Thyphimurium, which cause the gastrointestinal

infection in human. The purified peptide exhibited no antibacterial activity against *E. coli*, whereas, it showed the bactericidal activity against S. Thyphimurium at 8 μM.

The results indicate that SUMO fusion protein is an effective fusion tag for bacterial host system. It plays a role as a solubility tag and also affinity tag that is beneficial for peptide purification procedure. Besides, it prevents the proteolytic degradation of peptide from host cell. Contrarily, it also decreases the peptide toxicity to host cell that leading to yield loss (Butt et al., 2005).

The total 131 μg of purified Rhacoporin-2 from 1 L of LB culture is rather low for peptide production. Ideally, SUMO fusion tag is utilized for enhancing the expression and solubility of peptide (Butt et al., 2005). However, this result indicated that increasing solubility of peptide by SUMO fusion tag may be protein- or peptide-dependent (Xie et al., 2013). The purified Rhacoporin-2 displays 85% purity and it shows antimicrobial activity against S. Typhimurium at 8 μM. Comparing to the synthesized peptide (95% purity) that showed antibacterial activity against *E. coli* at 3.12 μM and S. Thyphimurium at <0.78 μM, the antibacterial activity of purified Rhacoporin-2 is nearly 10-fold lower.

It is possible that the impurities may interfere with the biological function of the peptide. Alternatively, incorrect folding of the peptide may contribute to the partial loss of biological activity. For further study, the expression and purification procedure should be optimized for higher purity and yield. For instance, the tandem repeat of interested

genes technique is capable of increasing the higher yield of peptides (Li et al., 2009 and Li et al., 2011). Optionally, the collaborative fusion tag may be beneficial for higher yield of peptide production such as thioredoxin-SUMO dual fusion strategy (Li, 2013 and Xie et al., 2013). To improve the purity of peptide, in addition to the optimization of purification procedure, the extracellular secretion pathway, for example using outer membrane protein as a dual fusion tag, may increase higher purity of peptide since the fusion peptide can be purified efficiently from growth medium (Kotzsch et al., 2011).

Nevertheless, this partial purified Rhacoporin-2 showed bactericidal activity indicating that SUMO fusion protein system can be used for amphibian antimicrobial peptide production in *E. coli* host system.

CHAPTER V

RESULTS AND DISCUSSION FOR PLANT SYSTEM

1. Codon optimization

Some rare codons of 93 bp Rhacoporin-2 gene from *R. feae* were optimized for plant host system, *Lemna minor*.

Table 10 Codon optimization of Rhacoporin-2 gene for *Lemna minor*

Hosts	Optimized codons
Orginal sequences (*Rhacophorus feae*)	ggagcctttactgatctactcaaaggtgtagccaagcaagcgggcataaaaat cctgggtattgctcaatgtaaacttgctaaaacatgttaa
Plant system (*Lemna minor*)	ggcgctttcaccgacctcctcaagggcgtggctaagcaggctggcatcaagat cctcggcatcgctcagtgcaagctcgctaagacctgctag

2. Transient transformation and peptide purification of *Lemna minor*

From previous study (unpublished), Duckweed was treated by 4 μg/mL of thidiazuron in MS media for 2 weeks. Later, the treated duckweed was co-cultivated with *Agrobacterium tumefaciens* EHA105 containing fusion gene for 7 days. Fronds of duckweed that displayed GFP expression were selected for further study.

In this study, the factors that may enhance the expression efficacy of recombinant gene in *L. minor* CM5 were investigated. At pretreatment step, to prepare the most readily plant for genetic transformation, duckweed was cultured in plant medium containing 4 μg/mL Thidiazuron (TDZ) for 2 weeks. TDZ is a plant growth regulator that displays the cytokinin activity. It is involved in cell division and growth in plants and induces plant to be more readily for genetic transformation (Schulze, 2007). There were 3 approaches that we studied for duckweed pretreatment including plant medium, plasmolysis treatment and cell wall degradation. For plant medium, MS media was compared to SH media to find out the suitable source for plant culture. Fronds that grow in SH media were larger than in MS media, but after transformation, the expression level of GFP was lower. Hence, MS media was more suitable for duckweed culture. However, SH media may be proper for leaf induction and proliferation in plant tissue culture.

The plasmolysis treatment induced by 0.6 M manitol for 5 minutes was also investigated. Plasmolysis treatment is the process in cell wall owing to the water loss through osmosis in hypertonic solution. It involves with the alteration of cell wall compostition which enhances the frequency of somatic embryogenesis in plant (Tao et al., 2012). There is an interesting report demonstrated that it increased both transient and stable transformation efficiency in Citrus species (Kayim and Koc, 2005). Dissimilarly, in this study, there was no significantly increasing expression level for transgenic *L. minor*. However, the manitol concentration and incubating time should be adjusted in further study. Similar to plasmolysis treatment, cell wall enzymatic degradation by 0.1% cellulase for 30 minutes incubating did not increase the transformation efficiency in *L. minor* comparing to basic method.

At inoculation step, *Agrobacterium* cell was re-suspended in infiltration media. The substances in infiltration media were examined. Some researches revealed that some monosaccharide when combined with acetosyringone can activate the virulence genes in *Agrobacterium* triggering the transference of bacterial T-DNA to plant cell (Shimoda et al., 1990). Some sugars in infiltration media, such as glucose, fructose, sucrose and sorbitol, were examined in this study. The result showed no difference expression level in different sugars.

There are many factors that we study to improve the transient transformation efficiency in *L. minor*. Unfortunately, every factor cannot enhance the expression level of GFP. In every transformed line, there was less than 1% of duckweed that displayed the GFP expression. When GFP expression was observed by fluorescence microscope, the observation revealed that low level of GFP expressed was found only in meristematic tissues (shown in figure 15). It indicated that the recombinant gene was transferred to the meristem. Nevertheless, the GFP expression in transgenic fronds was disappeared within 10 days after inoculation. It is possible that the recombinant gene cannot integrate into plant host genome and it is degraded by some cytosolic enzymes.

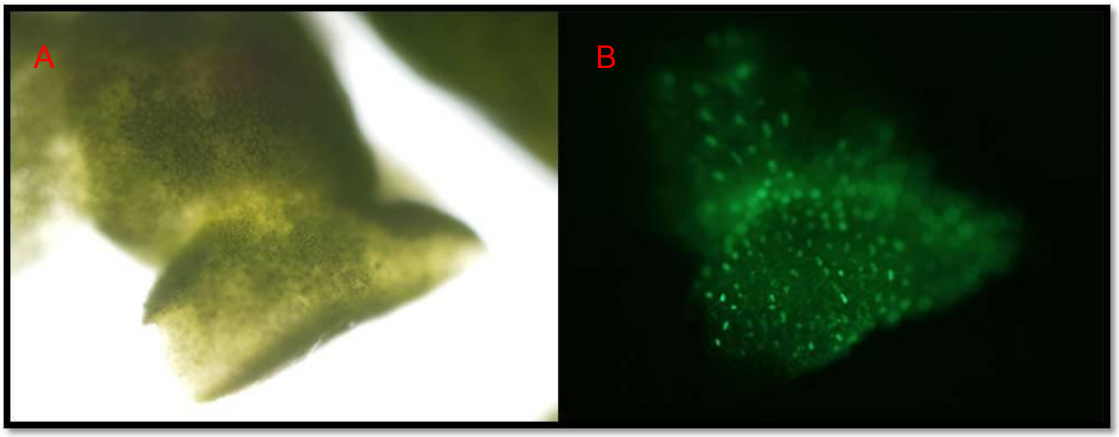

Figure 15 GFP expression of transformed *Lemna minor*, A: transformed fronds under light microscope, B: GFP expression in meristematic region in transformed fronds under fluorescence microscrope

The transformed duckweeds that acquire T-DNA region from *Agrobacterium* are able to synthesize GFP protein and the fusion peptide as well. Hence, the duckweeds that showed GFP expression were selected for fusion peptide extraction and purification. The protein extraction as well as purification was performed. The fusion peptide can be purified by Dynabeads because it contains polyhistidine tag for purification procedure. SDS PAGE (shown in figure 16) showed the fusion peptide sized 16.5 kDa was found in transformed duckweed but could not be found in wild type *L. minor* CM5. However, the amount of fusion peptide was very low comparing to the amount of fusion peptide from bacterial host system. The fusion peptide yield was only 44.8 μg from 100 mg transformed duckweed.

Because transient transformation of *L. minor* yields very low amount of fusion peptide. Stable transformation is attempted to increase the expression level in transgenic duckweed. The stable transformation of *L. minor* is performed through callus induction.

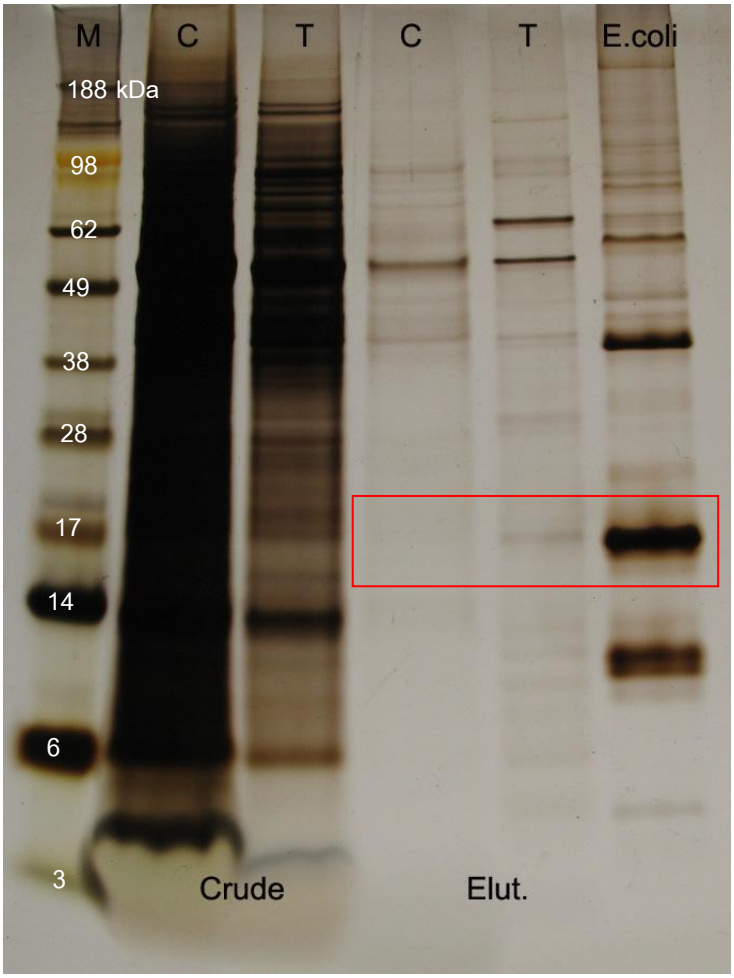

Figure 16 SDS PAGE of transformed *L. minor* CM5, the fusion peptide is indicated by red square. C: wild type *L. minor* CM5, T: transformed *L. minor* CM5

3. Stable transformation of *Lemna minor*

Somatic embryogenesis was employed for *L. minor* stable transformation. The proportion of plant hormone regulators, cytokinin and auxin, was varied to induce callus formation. It revealed that 0.5 μM TDZ combined with 5 μM 2,4-D was the most suitable proportion for callus induction of *L. minor*. Approximately, 80% of duckweeds in callus

induction medium containing 0.5 μM TDZ and 5 μM 2,4-D formed green and clear callus at meristem (shown in figure 17).

Figure 17 Callus formation of *Lemna minor*. Green and clear callus is formed at the meristematic tissue in the middle region of frond.

Callus induction in various geographical strains of *L. minor*, including CM5, HHK3-1, TAK3-1 and SING143-1, was investigated. The result showed that HHK3-1, TAK3-1 and SING143-1 strains were able to induce the callus formation, whereas CM5 was impracticable due to its short-lived.

Besides, the physical factors for callus induction were evaluated as well. Callus induction under dark and light was compared. For long period of induction, callus formation under light condition was higher efficiency and more healthy as indicated by

size and color. The callus was larger and deeper green. In addition, duckweeds were cultivated in 3 different tissue culture rooms: room 405 at MHMK building, Phytotron room at Botany building and plant tissue culture room at Botany building (both MHMK and Botany building were located at Faculty of Science, Chulalongkorn University). The observation disclosed that humidity was critical factor for duckweed cultivation. The humidity in phytotron room was rather low leading to lower growth rate and lower efficiency of callus formation. On the contrary, the humidity in plant tissue culture room was uncontrollable which was not proper to the cultivation of *L. minor* in agar plate. After 2 weeks of callus induction, the agar plates were full of water laeding to lacking of nutrient and undeveloped plant. 60-70% humidity in room 405 at MHMK building was the most suitable moisture for *L. minor* cultivation. Duckweeds that were grown in this room showed the highest growth rate, the most healthy callus and the highest frequency of callus formation comparing to the other rooms.

After 6 weeks of callus induction, healthy callus was transferred to nodule promoting media (NPM) containing 1 μM 2,4-D and 2 μM BA for 2 weeks. After 2 weeks, callus turned from green to white, yellow or black and was larger (shown in figure 18). Nodules can be maintained in NPM medium by subculture every 2 weeks. This nodule was ready for genetic transformation.

Figure 18 Nodule formation of *Lemna minor*, After 2 weeks of nodule promoting, callus had turned from green to white, yellow or black.

Similarly to transient transformation, *Agrobacterium*-mediated transformation was executed for stable transformation as well. After genetic transformation, selection and frond regeneration were managed by FRM containing 500 μg/mL cefotaxime and 20 μg/mL hygromycin. Cefotaxime is an antibiotic to kill the remaining *Agrobacteria* cells, whereas hygromycin is antibiotic for plant selection. Transformed plant contained the hygromycin-resistant gene can grow in selection medium. The regeneration procedure in agar FRM and broth FRM were compared. After 3-4 weeks of selection and

regeneration, no transformed nodules were remained in both agar FRM and broth FRM. It indicated that the efficacy of stable transformation of *L. minor* strain HHK3-1, TAK3-1 and SING143-1 was rather low. Consistent with previous study (unpublished), the efficacy of transient transformation in HHK3-1, TAK3-1 and SING143-1 strains were lower comparing to CM5. CM5 is the most promising strain for genetic transformation due to its high protein synthesis and higher efficiency of transient transformation. Unfortunately, the callus of *L. minor* CM5 cannot be induced because it is the shortest-lived strain as mentioned above. Thus, the stable transformation of *L. minor* to improve the recombinant peptide synthesis was unachievable. Therefore, we considered the other plant hosts for stable transformation for further study. *Arabidopsis thaliana* was selected as alternative plant model because it is one of the most popular plant model for genetic transformation.

4. Stable transformation of *Arabidopsis thaliana*

From previous study of Khunpolwattana, 2012, the fusion gene construct encoding GFP-SUMOstar-Rhacoporin-2 under control of 35S promoter, could stably integrate into the genome of *Arabidopsis* and the transgene was transferred to *A. thaliana* progenies. As shows in figure 20, GFP gene is expressed in every tissue.

In this study, T3 progenies of transgenic *Arabidopsis* were investigated. GFP gene from *Arabidopsis* wild type and T3 progenies was amplified by polymerase chain

reaction. The PCR products sized nearly 300 kb were found in positive control (plasmid from *A. tumefaciens* EHA105) and transgenic line including 1.3T3, 3.5T3 and 5.1T3 but were not found in wild type (shown in figure 19). This assured that the recombinant gene was inserted into plant host genome and transferred to *Arabidopsis* progenies.

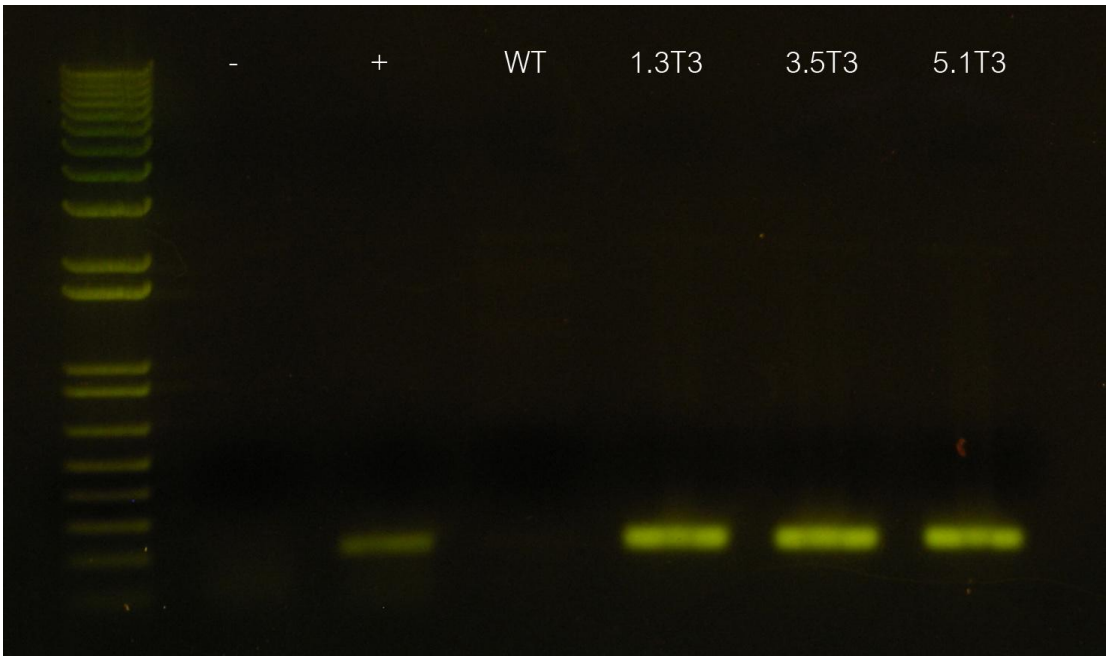

Figure 19 GFP gene amplification in *Arabidopsis*, +: positive control (plasmid from *A. tumefaciens* EHA105), WT: wild type, 1.3T3, 3.5T3, 5.1T3: transgenic lines from T3 progeny

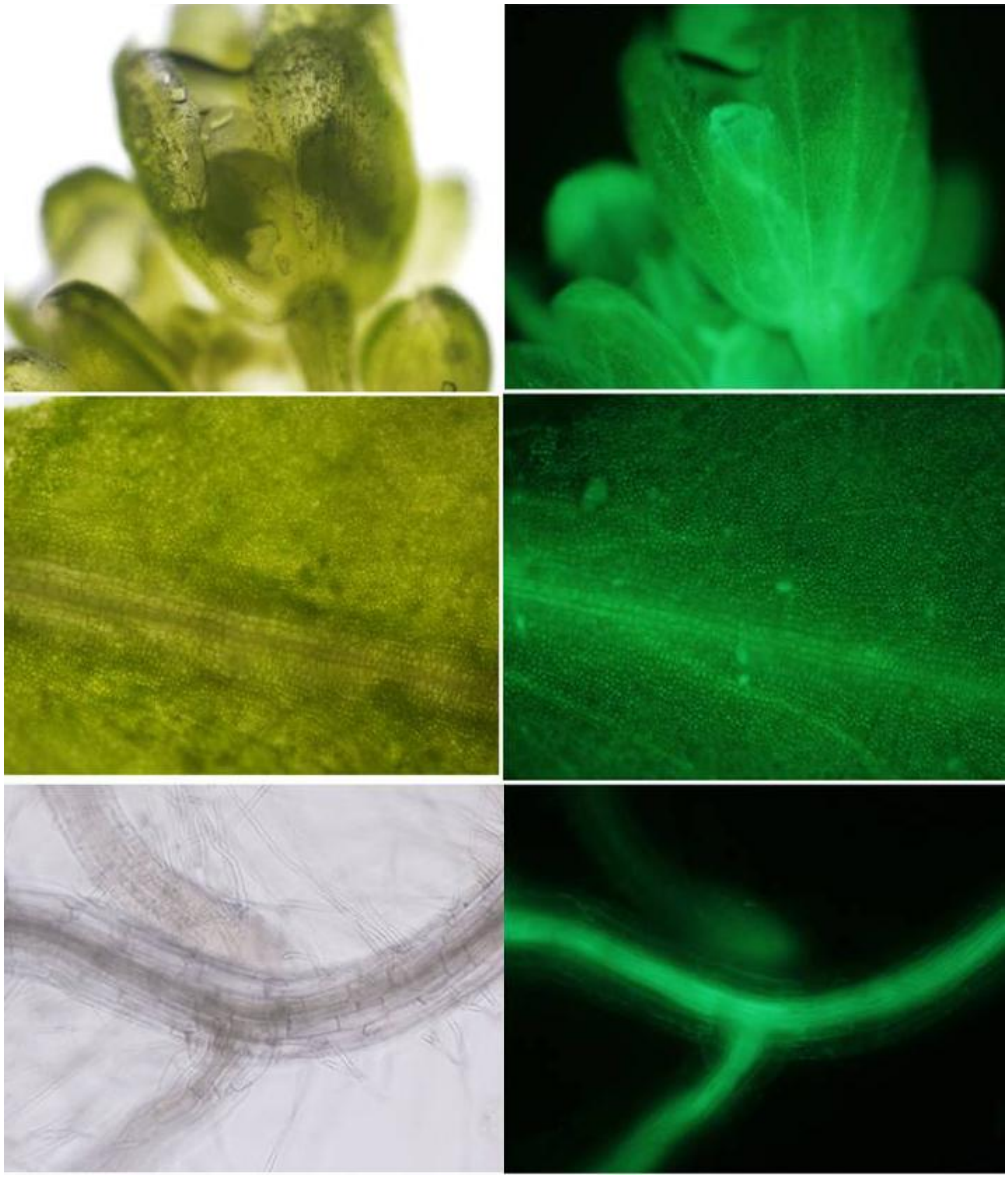

Figure 20 GFP expression in transgenic *Arabidopsis* in flower, leaf and root

For peptide purification, the total proteins from fresh *Arabidopsis* wild type and 3 lines from T3 progeny including 1.3T3, 3.5T3 and 5.1T3 were extracted and subsequently purified by Dynabeads. SDS PAGE in figure 21 showed the total proteins from *Arabidopsis*. Identically, in every sample, there were various sizes of proteins and peptides eluted from beads including a peptide that its size resembles the fusion peptide (shown in figure 22). To find out whether it was the interested fusion peptide, the elution fraction was subjected to digestion by SUMOstar protease. However, no digested band was detected. It is possible that the fusion peptide, SUMOstar-Rhacoporin2, might be cut already by endogenous SUMO protease in *Arabidopsis*.

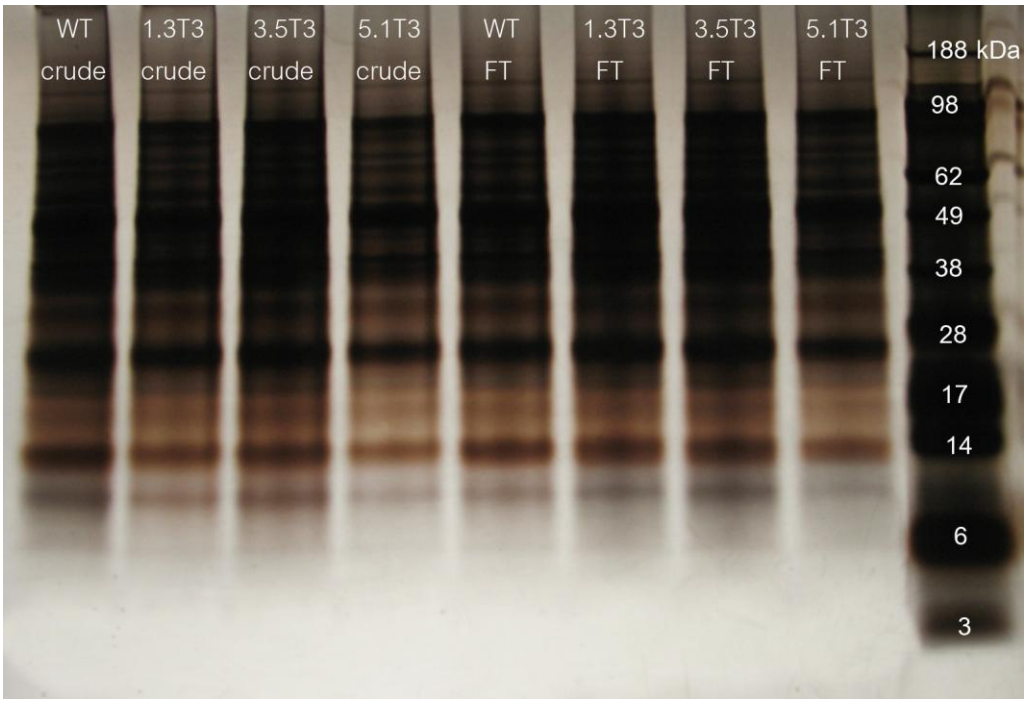

Figure 21 SDS PAGE of total protein extraction from *Arabidopsis thailiana*, WT: wild type, T3: T3 progeny, crude: total proteins, FT: flow through fraction

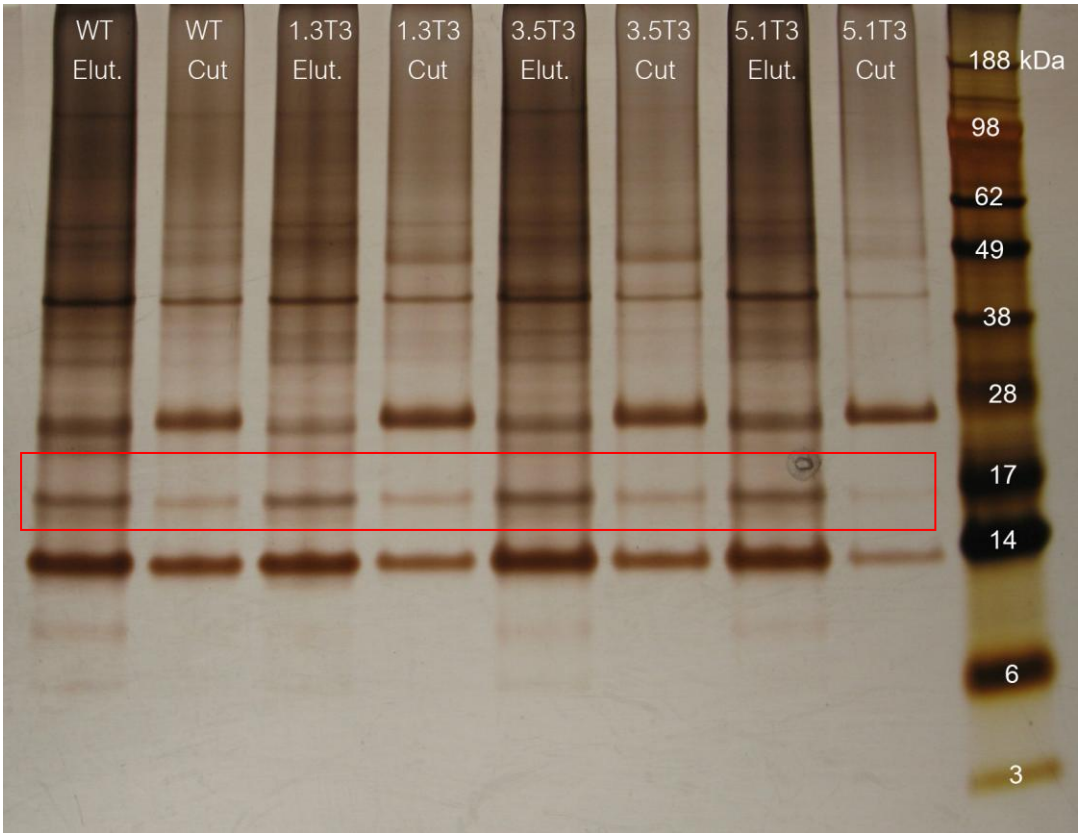

Figure 22 SDS PAGE of elution fraction from peptide purification in *Arabidopsis* and cleavage by SUMOstar protease, WT: wild type, T3: T3 progeny, Elut.: elution fraction, Cut: cleavage fraction

Kurepa and co-workers identified several encoded genes involved with SUMO pathway in *Arabidopsis*. The genome of *Arabidopsis* encodes 9 SUMOs including AtSUMO1 – AtSUMO9 but the expression of only 4 AtSUMOs (SUMO1, SUMO2, SUMO3 and SUMO5) could be detected by Northern blot and RT-PCR analyses (Kurepa et al., 2003). For this study, sequence alignment of SUMOstar gene (167 bp) to various SUMO

genes in *Arabidopsis* genome was carried out. The expressed sequence tags (ESTs) of *Arabidopsis thaliana* from the *Arabidopsis* Information Resource or TAIR (www.arabidopsis.org) were inspected and 4 isoforms of SUMO genes were found including SUMO1 (303 bp, accession number AT4G26840.1), SUMO2 (351 bp, accession number AT5G55160.2), SUMO3 (336 bp, accession number AT5G55170.1) and SUMO5 (327 bp, accession number AT2G32765.1). The % identity of all encoded gene sequences was calculated using AlignX program in Vecter NTI advanced 10 (Invitrogen).

Table 11 The identity of SUMO gene sequences

	SUMO1	SUMO2	SUMO3	SUMO5	SUMOstar
SUMO1		90	71	55	62
SUMO2			74	55	61
SUMO3				52	56
SUMO5					52
SUMOstar					

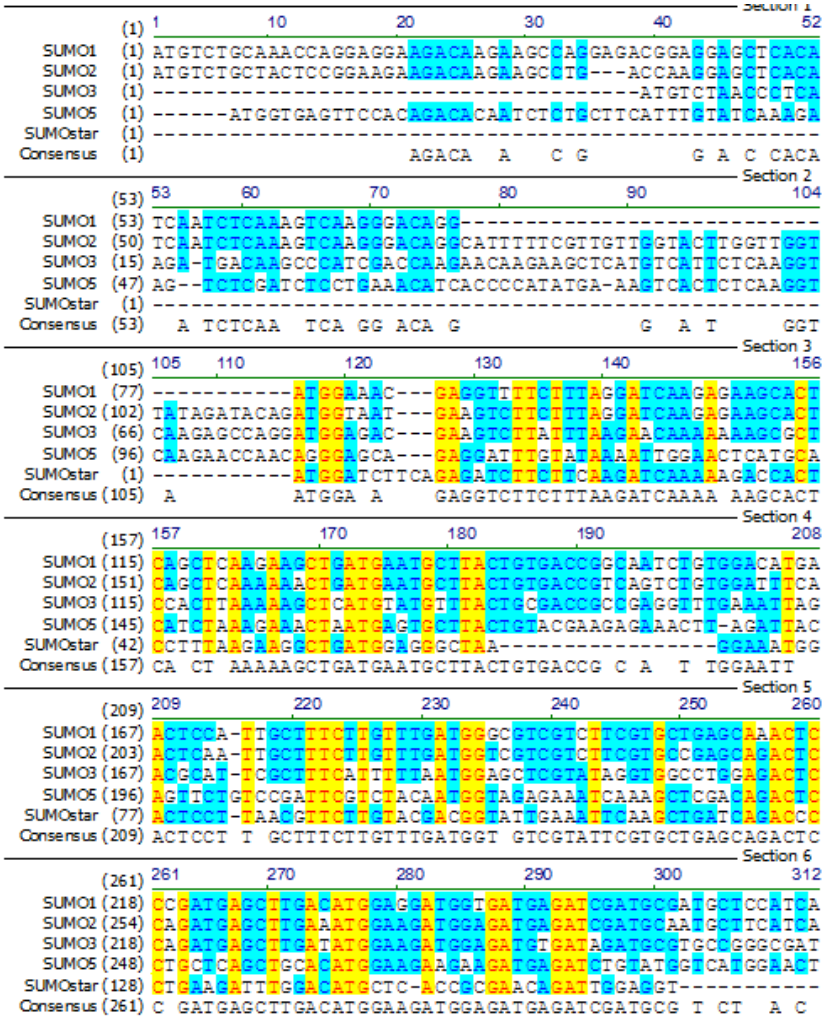

Figure 23 DNA sequence alignment of SUMO genes including SUMO1, SUMO2, SUMO3, SUMO5 and SUMOstar.

The result showed that SUMOstar DNA sequence shared 50-60% identity with SUMO genes that expressed in *Arabidopsis* indicating that it was rather related to AtSUMO gene family. In SUMO pathway, SUMO protease specifically cleaves the SUMO conjugation. Kurepa and co-workers also identified 12 genes encoded Ub-Like-

Protease or ULP (AtULP*1a* - AtULP*1d* and AtULP*2a* – AtULP*2h*) that attack various targets (Kurepa et al., 2003). This demonstrates the diversity of SUMOs and SUMO proteases in SUMO pathway in *Arabidopsis*. Our results suggest that SUMOstar fusion tag was possibly recognized and cleaved by some intracellular AtULP. Thus, SUMOstar fusion system is ineffective in *Arabidopsis* due to various SUMO protease families in plant and cannot be used for heterologous peptide expression.

Regarding to Rhacoporin-2, some approaches are needed to verify whether this peptide was cut and mingled with the total soluble protein (in FT fraction) such as western blot or ELISA analyses. On the other hand, this peptide may be degraded by some intracellular enzymes. However, some strategies can prevent the peptide degradation for recombinant peptide production. For instant, an endoplasmic reticulum (ER) retention signal, SEKDEL, is promising for small peptide production in plant by adding this signal to the N-terminus of interested peptide. *Agrobacterium*-mediated transformation was utilized to transfer the p24 gene conjugated with SEKDEL signal to *Arabidopsis*. The result indicated that p24 protein maintained its antigenicity although it was conjugated with SEKDEL signal (Lindh et al., 2009).

CHAPTER VI

CONCLUSION

The main purpose of our study is to produce the recombinant antimicrobial peptide, SUMO-Rhacoporin2. The large-scale production of this recombinant peptide in heterologous host system is alternative strategy for low cost production. Thereupon, bacterial host system and plant host system are promising.

For bacterial host system, the production of this recombinant peptide, SUMO-Rhacoporin2, in *Escherichia coli* BL21(DE3) was successful. The total 131.0 μg of purified Rhacoporin-2 was synthesized from 1 L culture and it showed the biological function against Gram-negative bacteria, Salmonella Typhimurium at 8 μM. It indicated that SUMO fusion system was effective for peptide production in bacterial host system. Contrastingly, plant host system was more complicated.

SUMOstar fusion tag (The modified SUMO protein) was employed for eukaryotic host system. The fusion peptide production from transient transformation of duckweed *Lemna minor* was very low. The total 44.8 μg of fusion peptide was synthesized from 100 mg transformed duckweed. Therefore, the increasing yield of fusion peptide from *L. minor* by stable transformation was expected. Unfortunately, the somatic embryogenesis in *L. minor* could not be induced and stable transformation of duckweed was unsuccessful leading to non-achievement in improving the fusion peptide production.

Ultimately, the other host plant, *Arabidopsis thaliana* was also considered. The transgenic *Arabidopsis* displayed the GFP expression in all transgenic lines indicating the successfulness of stable transformation of this recombinant peptide gene. However, the recombinant peptide could not be detected in transgenic *Arabidopsis* examined by SDS PAGE. It is assumed that SUMOstar fusion tag might be specifically recognized and cleaved by *Arabidopsis* SUMO protease since it was related with several SUMO isoforms in *Arabidopsis*. In conclusion, SUMO fusion system was efficient for antimicrobial peptide production in bacterial host system but it was inefficient for plant host system.

Nevertheless, the cost effective evaluation is also considered. As shown in table 12, although the media cost for bacterial system is lower but the fusion peptide yield from plant system is much higher. Therefore, the production cost in plant system is significantly lower than bacterial system.

Moreover, the peptide production by bacterial system needs the downstream processing including the operating tools for peptide production and peptide purification for therapeutic uses that may increase cost of peptide production. However, some plants can be cultured on land (*Arabidopsis*) or pond (duckweed) and modified as an edible vaccine which the downstream processing is extrinsic. Therefore, plant host system is considered to be the promising system for low cost production of therapeutic

agents, especially duckweed. Finally, the improvement of stable transformation in duckweed is recommended in further study for antimicrobial peptide production.

Table 12 The evaluation of suitable system for Rhacoporin-2 production

Host systems / Evaluations	*Escherichia coli*	*Arabidopsis* (Stable)	*Lemna minor*	
			Transient	Stable
Media cost	163.5 Baht/L	275 Baht/L	297 Baht/L	275 Baht/L
Fusion peptide yield	248.9 μg	NA	1344 μg	NA
Downstream processing	Need	Need	Need?	Need?
Time	1 week	2 weeks	3 weeks	2 weeks
Expansion	Fermentation	Land	Pond	Pond
Biological activity	√	X	X	X

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APPENDIX

The media formula for bacterial and plant culture

Murashige and Skoog (MS) broth medium for plant culture

MS basal salt mixture (Phytotech)	4.43	g/L
MES hydrate buffer	1	g/L
Sucrose	30	g/L
pH 5.6		

Murashige and Skoog (MS) agar medium for plant culture

MS basal salt mixture (Phytotech)	4.43	g/L
MES hydrate buffer	1	g/L
Sucrose	30	g/L
Agar	8	g/L
pH 5.6		

Schenk and Hildebrandt (SH) medium for plant culture

SH basal salt mixture (Phytotech)	3.2	g/L
MES hydrate buffer	1	g/L

Sucrose	10	g/L
pH 5.6		

Nodule Promoting Medium (NPM) for nodule induction

MS basal salt mixture (Phytotech)	4.43	g/L
MES hydrate buffer	1	g/L
Sucrose	30	g/L
BD Difco™ Bacto-agar	4	g/L
Gelrite	1.5	g/L
2,4-Dichlorophenoxyacetic acid (2,4-D)	1	μM
Benzylaminopurine (BA)	2	μM
pH 5.6		

Infiltration Media for *Agrobacterium* cell resuspension

MS basal salt mixture (Phytotech)	2.215	g/L
MES hydrate buffer	3	g/L
Sucrose	60	g/L

pH 5.6

LB broth for Agrobacterium culture

BD Difco™ LB broth powder	20	g/L
Sucrose	5	g/L

pH 7.0

Frond Regeneration Medium (FRM) for duckweed regeneration

SH basal salt mixture (Phytotech)	1.6	g/L
Sucrose	5	g/L
MES hydrate buffer	1	g/L

pH 5.6

Resuspension medium for plasmolysis treatment

MS basal salt mixture (Phytotech)	4.43	g/L
Manitol	109.3	g/L (0.6 M)

LB agar for kanamycin selection

BD Difco™ LB broth powder	20	g/L

BD Difco™ Bacto agar	15	g/L
Kanamycin	50	μg/mL
pH 7.0		

Muller Hilton Broth (MHB) medium for MIC assay

BD Difco™ Muller Hilton Broth powder	21	g/L
pH 7.0		

Solution S for cellulase dissolution

Potassium chloride (KCl)	340	mM
Calcium chloride (CaCl2)	1.4	mM
MES hydrate buffer	3	mM

The solutions for peptide purification

2X binding/wash buffer for peptide purification

Sodium phosphate buffer pH 8.0	100	mM
Sodium chloride (NaCl)	600	mM
0.02 % Tween20		

His-tagged elution buffer

Imidazole	300	mM
Sodium phosphate buffer pH 8.0	50	mM
Sodium chloride (NaCl)	300	mM
0.01% Tween20		

Gel fixative for peptide preservation in polyacryamide gel

50% Methyl alcohol

5% Glutaraldehyde

BIOGRAPHY

Miss Patcharawalai Whongsiri was born on October 5th, 1986. She received her Bachelor's Degree of Science, major Biology, from the Department of Biology, Faculty of Science, Chulalongkorn University in 2009. She has been the student under the Development and Promotion of Science and Technology Talents Project (DPST). Throughout the Master's degree program, she participated to the conferences for research presentation including:

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- Poster presentation at 16th Biological Sciences Graduate Congress on 12th-14th December 2011 at National University of Singapore, Singapore (won 2nd prize for poster presentation in theme II Biochemistry, Physiology and Biotechnology)
- Oral presentation at II International Conference on Antimicrobial Research on 21st-23rd November 2012 at University of Lisbon, Portugal
- Oral presentation at The 8th conference on Science and Technology for Youths on 22nd-23rd March 2013 in Bangkok (published the conference proceedings)