PRODUCTION OPTIMIZATION OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME (PRRS) VIRUS NUCLEOCAPSID PROTEIN IN *NICOTIANA BENTHAMIANA*

Mr. Boonyasit Porngarm

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmaceutical Sciences and Technology
Common Course
FACULTY OF PHARMACEUTICAL SCIENCES
Chulalongkorn University
Academic Year 2021

การหาภาวะที่เหมาะสมของการผลิตโปรตีนนิวคลีโอแคปซิดของไวรัสพีอาร์อาร์เอสในต้น *Nicotiana benthamiana*

นายบุณยสิทธิ์ พรงาม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเภสัชศาสตร์และเทคโนโลยี ไม่สังกัดภาควิชา/เทียบเท่า
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2564

Thesis Title	PRODUCTION OPTIMIZATION OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME (PRRS) VIRUS NUCLEOCAPSID PROTEIN IN *NICOTIANA BENTHAMIANA*
By	Mr. Boonyasit Porngarm
Field of Study	Pharmaceutical Sciences and Technology
Thesis Advisor	Associate Professor WARANYOO PHOOLCHAROEN, Ph.D.

Accepted by the FACULTY OF PHARMACEUTICAL SCIENCES, Chulalongkorn University in Partial Fulfillment of the Requirement for the Master of Science

..... Dean of the FACULTY OF PHARMACEUTICAL SCIENCES
(Assistant Professor RUNGPETCH SAKULBUMRUNGSIL, Ph.D.)

THESIS COMMITTEE

..... Chairman
(Associate Professor SORNKANOK VIMOLMANGKANG, Ph.D.)

..... Thesis Advisor
(Associate Professor WARANYOO PHOOLCHAROEN, Ph.D.)

..... Examiner
(Professor KANNIKA KHANTASUP, Ph.D.)

..... External Examiner
(Associate Professor Anchanee Kubera, Ph.D.)

บุณยสิทธิ์ พรงาม : การหาภาวะที่เหมาะสมของการผลิตโปรตีนนิวคลีโอแคปซิดของไวรัสพีอาร์อาร์เอสในต้น *Nicotiana benthamiana*. (PRODUCTION OPTIMIZATION OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME (PRRS) VIRUS NUCLEOCAPSID PROTEIN IN *NICOTIANA BENTHAMIANA*) อ.ที่ปรึกษาหลัก : รศ. ดร.วรัญญู พูลเจริญ

ไวรัสพีอาร์อาร์เอส (PRRS)เป็นเชื้อก่อโรคที่สำคัญที่ก่อให้เกิดโรคในสุกรและส่งผลกระทบทางเศรษฐกิจต่ออุตสาหกรรมสุกรทั่วโลกรวมทั้งประเทศไทย การตรวจสอบการติดเชื้อไวรัสพีอาร์อาร์เอสอย่างสม่ำเสมอเป็นสิ่งที่จำเป็นสำหรับการเฝ้าระวังและควบคุมโรคได้อย่างมีประสิทธิภาพ วิธีการตรวจสอบที่ใช้กันอย่างแพร่หลายคือการใช้โปรตีนนิวคลีโอแคปซิด (N) ของไวรัสในการตรวจหาแอนติบอดีต่อโปรตีนนั้นในเลือดสุกร ฉะนั้นการศึกษานี้จึงมีวัตถุประสงค์เพื่อผลิต โปรตีนนิวคลีโอแคปซิด (N) ของไวรัสพีอาร์อาร์เอส โดยใช้พืช *Nicotiana benthamiana* เป็นแหล่งผลิต โปรตีนนิวคลีโอแคปซิด (N) จะถูกผลิตใน 4 รูปแบบ คือ มีและไม่มี plant signal peptide รวมถึงตำแหน่งที่ของ poly His-tag ที่ต่างกันและถูกใส่เข้าไปในเวคเตอร์ geminiviral (pBYR2e-K2Md) สำหรับการแสดงออกชั่วคราวใน *N. benthamiana* สภาวะของการแสดงออก เช่น โครงสร้างยีน ระยะเวลาในการเก็บเกี่ยวใบ (dpi) และความหนาแน่นของเซลล์ Agrobacterium (OD_{600}) จะถูกปรับให้เหมาะสมสำหรับการผลิตโปรตีนให้ได้ปริมาณมากที่สุด นอกจากนี้การวิเคราะห์ด้วย western blot ของโครงสร้างยีนทั้งหมดบ่งชี้ว่า นิวคลีโอแคปซิด (N) มีน้ำหนักโมเลกุลประมาณ 38 kDa อย่างไรก็ตามโครงสร้างยีนที่ประกอบด้วย plant signal peptide และมี poly His-tag ในตำแหน่งปลายด้านหมู่อะมิโนอิสระ ให้ผลการแสดงออกในระดับที่สูงกว่าเมื่อเปรียบเทียบกับโครงสร้างยีนอื่นๆ จากนั้นโครงสร้างยีนนี้ได้นำมาหาสภาวะที่เหมาะสมสำหรับการแสดงออกเพื่อให้ได้ปริมาณโปรตีนที่สูงที่สุดคือเก็บเกี่ยวใบวันที่ 4 หลังการฉีดเชื้อและความหนาแน่นของเซลล์ Agrobacterium ที่ 0.6 ดังนั้นการพิสูจน์แนวคิดนี้บ่งชี้ว่า นิวคลีโอแคปซิด (N) ที่ผลิตจากพืชนั้นสามารถใช้เป็นทางเลือกหนึ่งในการผลิตโปรตีนรีคอมบิแนนท์แอนติเจนไวรัสพีอาร์อาร์เอส เพื่อใช้สำหรับการวินิจฉัยการติดเชื้อไวรัสพีอาร์อาร์เอส

สาขาวิชา เภสัชศาสตร์และเทคโนโลยี ลายมือชื่อนิสิต .....

ปีการศึกษา 2564 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

6176103333 : MAJOR PHARMACEUTICAL SCIENCES AND TECHNOLOGY
KEYWORD: PRRS virus, Transient expression, Nicotiana bemthamiana

Boonyasit Porngarm : PRODUCTION OPTIMIZATION OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME (PRRS) VIRUS NUCLEOCAPSID PROTEIN IN *NICOTIANA BENTHAMIANA*. Advisor: Assoc. Prof. WARANYOO PHOOLCHAROEN, Ph.D.

Porcine Reproductive and Respiratory Syndrome virus (PRRSV) is a considerable pathogen to occur disease in pigs and effect to economic on the swine industry around the world including Thailand. Routine observation, rapid diagnosis of PRRSV infection is essential for effective monitoring and disease control. The serological assays using highly conserved viral nucleocapsid (N) protein are widely used for the detection of antibodies to the PRRSV. Accordingly, this study was aimed to produce the N-protein of the PRRSV in the plant system by using *Nicotiana benthamiana*. The N-protein gene was designed into four constructs that contained with and without the plant signal peptide including the different locations of poly His-tag and cloned in a geminiviral vector, pBYR2e-K2Md, for transient expression in *N. benthamiana*. The conditions of expression such as effective gene construct, leaf harvesting time (dpi), and Agrobacterium cell density (OD_{600}) were optimized for maximal protein production. Further, by using a western blot assay, the results of protein expression of all constructs indicated N-protein size with an approximate molecular weight of 38 kDa. However, the construct that consists of the plant signal peptide with poly his-tagged in the N-terminus shows a higher-level expression compared to the other constructs. Subsequently, this construct was optimized for the high-level expression at 4 dpi and 0.6 of the Agrobacterium cell density. Therefore, this proof-of-concept study indicated that

Field of Study: Pharmaceutical Sciences and Technology Student's Signature .....
Academic Year: 2021 Advisor's Signature .....

ACKNOWLEDGEMENTS

I would like to sincerely thank the thesis advisors. Associate Professor Waranyoo Phoolcharoen, Ph.D. for her advice and expertise during research experiments and writing. Her passion, patience, and unwavering encouragement have kept me engaged in order to complete the thesis.

I am also grateful to the members of the Research Unit for Plant-Produced Pharmaceuticals (RU-PPP) for helping me with my laboratory work, giving valuable comments and it helps me to keep improving myself throughout the development of my thesis work. To my friend, thank you for creating a good atmosphere, providing optimism and motivation throughout this study.

To my parents, I would like to extend my love and genuine for giving me continued support of what I wanted to do, and my constant encouragement enabled me to reach the conclusion of the project.

I would like to express my thanks to Professor Hugh Mason (Arizona State University) for providing geminiviral vectors and the Department of Pharmacognosy and Pharmaceutical Botany for allowing me to work in the laboratory and to use their research facilities. I am also grateful to work with the B.F. feed company and thank them for providing samples and staff for their technical and laboratory assistance.

Lastly, I would like to acknowledge the important supporter, Research and Researchers for Industries (RRI)-Thai Science Research and Innovation (TSRI) (MSD62I0091), the 90th Anniversary of Chulalongkorn University Scholarship, Graduate School, Ratchadapisek Somphot Fund for financial support.

Boonyasit Porngarm

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is an infective swine illness, which affects pigs in respiratory illness and reproductive failure in pregnant sows. It causes widespread economic loss by decreases productivity in the swine industry worldwide including Thailand (1, 2). This virus was initially found in the North American countries in 1987, a few years later in the European countries (3) and it was an outbreak since 1989 in Thailand (4). The PRRSV has been described as two different genotypes, European (type 1), and North American (type 2). In 2004, PRRSV in Thailand was reported that virus type 1 was found more frequently than the virus type2 (5). In 2006, the highly pathogenic PRRSV (HP-PRRSV) outbreak caused extremely damages in most swine herds in China (6) and expanded to neighbor countries including Vietnam, Lao PDR in 2008 (7) and Thailand in 2010. After 2010, Thai-PRRSV can be divided into 2 groups, type 2 and HP-PRRSV strain (8).

PRRS virus (PRRSV) is a positive-stranded enveloped RNA virus that belongs to the genus *Arterivirus*. The PRRSV genome RNA is 15 kb in length and contains at least 11 known open reading frames (ORFs). The replicase gene composes of the immense ORFs 1a and 1b, which locates in the 5'-terminal three fourths of the polycistronic genome. Of these, ORF1 a and 1 b regions encode two large nonstructural polyproteins (pp), pp1 a and pp1 ab. the pp1a, and pp1ab replicase polyproteins processed into at least 14 nonstructural proteins (nsps). The ORFs 2–7 encode to eight structural proteins, consisting minor envelope proteins, major envelope proteins, and the nucleocapsid protein (N protein) (9).

ORF7 of the viral genome, 123-128 amino acid, produced nucleocapsid protein (N protein). The infected cell is plentiful of N protein, which is the most immunogenic. The anti-N antibodies are non-neutralizing and nonprotective. The N protein can separate into two parts, including N-terminal and C-terminal. The N-

terminal part contains a large number of positive charges, which is logical with function in the RNA-binding. The C-terminal part is the dimerization domain (10). This protein is enclosed by a lipid envelope comprising the viral membrane (11). In the previous study, N protein has ever been produced from several production systems, for example, bacteria (12-15), plants (16), and mammalian cells (17-19). The study of PRRSV N protein has been applied to use as a vaccine and detector.

Using plants to protein production system is a new technology. It has advantages over other systems, for example, cheap manufacturing cost, rapid production, protein modification by the addition of protein glycosylation for similar to the human protein, low contamination of pathogens that cause disease in humans and animals and not need laboratory skill. The plant-produced recombinant protein has already sold as a commercial product in the market, such as Taliglucerase alpha (20), Zmapp. The production of recombinant proteins in plants can produce in several ways. The traditional method is used to produce genetically modified plants or transgenic plants, but it takes a long time to produce, and protein production is low. Currently, the new technology that can be used in the production of proteins makes temporary the proteins produced in plants is a transient expression (21-23). This method has many advantages. They are ease of management, short production period, low production cost, and high production yield. The plant is used to produce proteins in the transient method are *Lactuca sativa*, *Arabidopsis thaliana* or tobacco (*Nicotiana tabacum*). Recently, *Nicotiana benthamiana* has been used as a source for transient producing protein because it is accessible to transferred the genes and higher protein production (24).

The financial capabilities for product manufacturing is associated on the final yield. Multiple aspects directly affect the product yields, including the mechanism of transgene expression and targeted protein secretion, physiological properties of the

host, and the surronding (25).To enhance protein production capasity, combination of affecting factors, for example, cloning methods for optimization of gene constructs can be utilized (26-29). For cloning techniques, the yields of protein is increased following the addition of stabilizing sequences such as the endoplasmic reticulum (ER) retention tag, which can induce protein storage in the intracellular compartment (29). Thus, this phenomenon demonstrates the improvement on recombiant protein production as provided by the localization capacity for recombinant protein storage and enhanced stability of storaged protein.

This study aims to optimize the condition of plants-produced recombinant nucleocapsid protein of porcine reproductive and respiratory syndrome virus (PRRSV) by transient expression technique.

1.1 Research Hypotheses

1. *Nicotiana benthamiana* can produce Porcine Reproductive and Respiratory Syndrome Virus nucleocapsid protein transient expression technique.

2. A construct containing plant signal peptide has a higher protein expression than another construct that is without plant signal peptide.

CHAPTER II LITERATURE REVIEW

1. Overview of Porcine Reproductive and Respiratory Syndrome (PRRS)

Porcine Reproductive and Respiratory Syndrome (PRRS) is a porcine infectious disease caused by the PRRS virus (PRRSV). It causes abnormal symptoms in pigs such as fever, conjunctivitis, depression, diarrhea, and anorexia. In addition, an acute respiratory failure in young piglets is more obvious. In breeder pigs, the reproductive system has serious problems, for example, the semen quality of the male pigs is low or contaminated with PRRSV that makes symptoms of miscarriage in pregnant sows (9, 30).

Initially, PRRSV emerged in the United States in 1987 and a few years later in Europe. With the outbreaks in different regions, the genomic identity is an approximately 50-60% between the two subtypes (10), so there is divided into two distinct genotypes: type 1 was found in Europe also known as Lelystad (LV) and Type 2 in the United States (ATCC VR2332) (7). The PRRSV strains that can be isolated in Thailand are genetically similar to the PRRSV genotype type 2 (8).

PRRSV is a member of the *Arteriviridae* family within the order *Nidovirales*, a recently recognized viral family genomically related to the *Coronaviridae* and *Roniviridae* families. The *Arteriviridae* family consists of five viruses and constitutes of single-stranded positive-sense (+) RNA genome which participate in a hallmark replication/transcription strategy (31). There characteristics are genetically and biologically similar, such as genomic organization, morphology, and a cellular tropism for the macrophage lineage (32) but differ in host species, disease phenotype genomic size, and encoded content (33). In length 14.9 to 15.5 kb of PRRSV genome expresses a range of accessory and structural proteins through a set of sub-genomic mRNA transcripts, each used for the translation of one open reading frame (ORF) *Figure 1*.

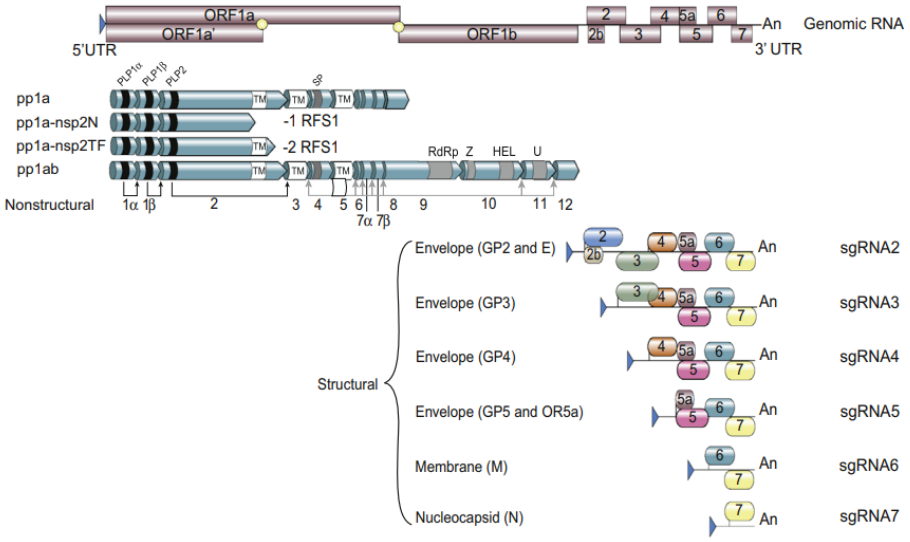


Figure 1. Porcine reproductive and respiratory syndrome virus (PRRSV) genome organization.

PRRSV replication progresses by a range of genetic and protein regulatory mechanisms. Expression of the first three-fourths of the 14.9–15.5 kb genome yields 4 known polyproteins (pp1a, pp1a-nsp2N, pp1a-nsp2TF, pp1ab). Canonical structural proteins are expressed exclusively through a set of subgenomic RNAs (sgRNA; 2–7) via a co-terminal discontinuous transcription strategy via a negative-sense strand intermediate (31).

The PRRSV genome has the large overlapping replicase ORF, the first and largest region of ORF for the non-structural proteins via ORF1a, ORF1a', and ORF1b. ORFs 2-7 can be encoded to make up 8 structural proteins; 4 types of glycosylated membrane protein (GP2-5), three of un-glycosylated membrane proteins (E, GP5a, M), and nucleocapsid protein (N).

In Thailand, the specific treatment of PRRS diseases for infected herds, or prevention methods other than vaccines are still incurable (34, 35). Moreover, the use of antibiotics is necessary to prevent the secondary infection by other viruses or bacteria. In the field, various management procedures have been implemented to

achieve farm and regional control of PRRSV infection; these include a PRRSV test for semen and gilt acclimation, the elimination of the seropositive animal, herd depopulation and repopulation. However, control and eradication of PRRSV within a relatively large region is much more complicated and is expected to require a much longer-term commitment. Therefore, effective PRRSV control and prevention methods are urgently needed (36).

2. Recombinant Protein Production via *Nicotiana benthamiana*

In the current production of recombinant proteins, it can be produced using various systems such as bacteria, yeast, insect cells, mammalian cells, and plants. Furthermore, the production of recombinant proteins in bacteria is used the most because it can be produced easily, quickly and the production price is relatively low (37). However, produced-recombinant proteins by bacteria are not the same as recombinant proteins produced by higher organisms such as yeast and mammalian cells because bacteria do not have the ability of post-translational modification, the addition of a sugar group (glycan) to a protein. In order that bacteria are non-processed of post-translation modification (38), which is necessary to many proteins such as antibodies (39) or other glycoproteins (40-42) because the sugar groups on proteins have a significant effect on the functioning of these proteins. Therefore, many proteins produced by bacteria cannot function effectively.

Another popular industrial production of recombinant protein is the use of mammalian cell cultures (43), which can produce good quality recombinant protein and similar to proteins in humans. Nevertheless, in this production system from mammalian cell cultures, the production cost is quite high because the production process must be controlled to have very high sterility and cell culture media is quite expensive. Therefore, the recombinant protein production system using mammalian cell cultures is not suitable for use in developing countries.

The production of recombinant proteins can be processed by using the platform that mention above. Plants are novel recombinant protein production platforms and have many advantages over other production systems such as low production costs, fast production time, the protein is enriched by adding a sugar group for similar to that of humans, free from pathogens that cause disease in humans and animals, and do not need laboratory skills workers. In addition, production can be scaled up at the factory level. Recently, many recombinant proteins are produced in plants and tested to use properly, such as monoclonal antibodies (44-46), subunit vaccines (42, 47, 48), cytokines (49, 50), biologically active peptides (51).

There are several methods for the production of recombinant proteins in plants. The traditional method used is transgenic plants were developed in a sterile condition and transfer the gene of interest into plant cells by using Agrobacterium. In order to transfer the genes into plant cells, the plant cells are cultured on selective medium and then only plant cells with the transfected genes that make the cells resistant to antibiotics. After the selection of cells containing the desired genes will change the media by adding hormones to stimulate roots and stems until when the plants grow and able to be planted in the soil. Then it is possible to select plants with the genes of interest (52). The process of producing genetically modified crops to produce the required proteins is shown in Figure 2.

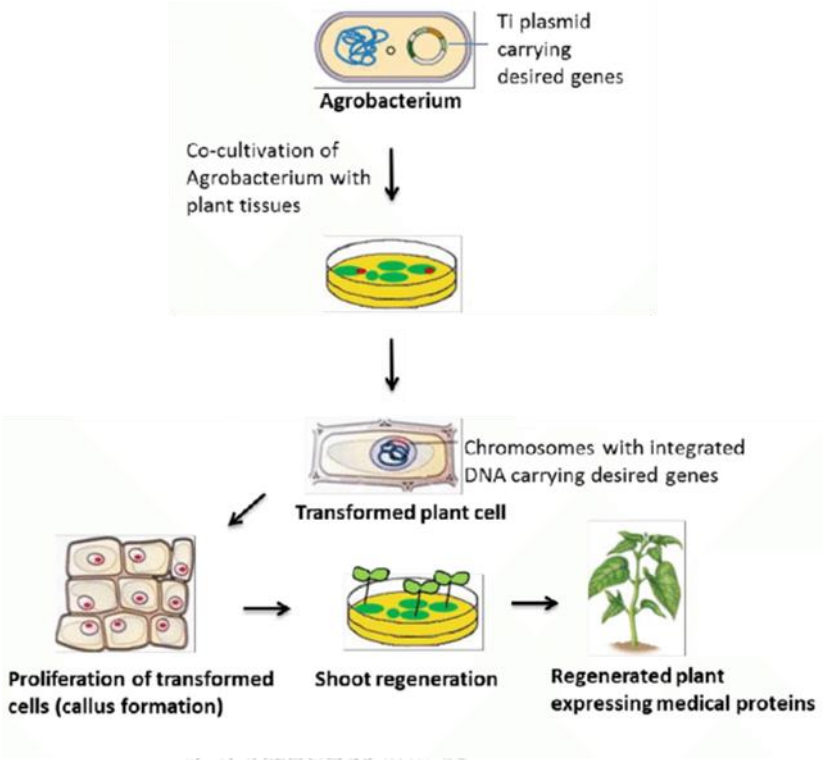


Figure 2. Schematic diagram showing major steps involved in the generation of transgenic plants using Agrobacterium-mediated transformation. (52)

Due to the transgenic plant will take a long time to develop and the amount of recombinant protein produced is low. Presently, the new technologies that can be used to produce protein in plants can be done without the need to change the plant's genetics or transient expression (21-23). The transgenic plant method requires considerable time and cost, and the level expression of target proteins is usually low, which results make a higher product cost due to the difficulty of protein purification (53, 54). Transient protein expression is a quick alternative method to genetically modified plants. The advantages of transient production ease of management short production time, low cost, and higher protein yield per weight of plant tissue/ fresh leaves.

There are several types of plants used in the transient proteins production, such as lettuce (*Lactuca sativa*), Arabidopsis (*Arabidopsis thaliana*) or tobacco (*Nicotiana tabacum*). Currently, *Nicotiana benthamiana* has been used as a

bioreactor to produce a large amount of transient protein. Because the genes can be easily transmitted to plant cells and can produce large quantities of recombinant proteins (24). As part of the transient recombinant protein production process in plants will be able to bring plants that are not genetically modified to put the genes by using Agrobacterium. After injecting bacteria into the leaves, the plants are planted for 4-12 days, and then the leaves can be extracted for the protein and purified proteins out of other plant proteins. This method of protein production requires a short production time and able to produce high amounts of protein. In addition, there is no genetic modification of plants. A method for the production of transient recombinant proteins in plants. is shown in Figure 3.

Figure 3. The general principle of transient expression in plants mediated by A. tumefaciens. (55)

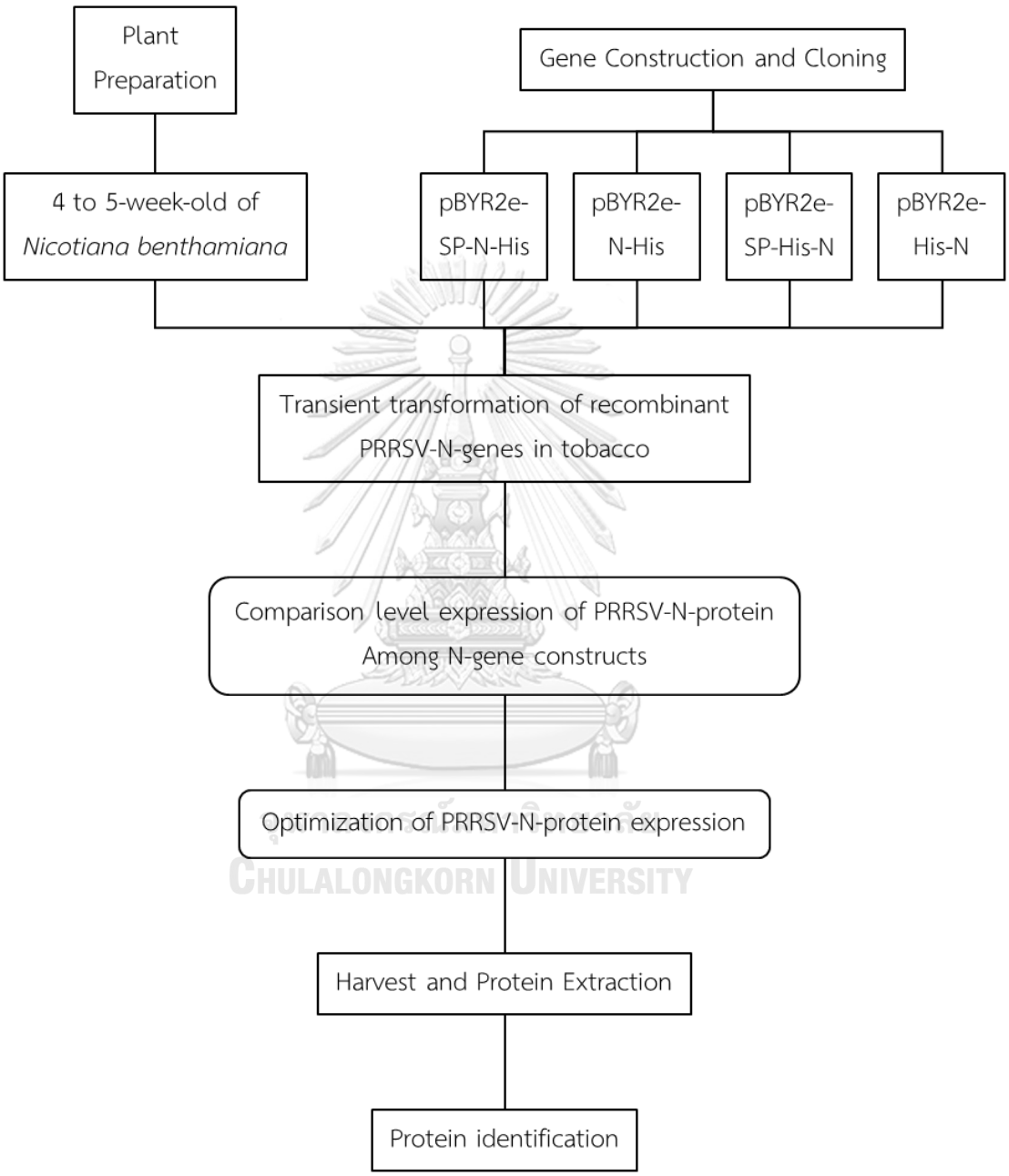
Genes encoding the recombinant protein of interest in a binary expression vector (1) are introduced into A. tumefaciens (2), which is cultivated (3) and infiltrated into plant tissue (4), facilitating the expression and accumulation of the recombinant protein in plant cells (5).

Advances in the expression of heterogeneous proteins from plant systems include strategies relevant to bioengineering, such as optimizing gene expression constructs that have some affecting factors to improve the potential of protein by adding promoter sequences, tag sequences, and retention signals (56-59). First, signal peptides (SP) are proteins tagged with an N-terminal secretion sequence in a short length of approximately 16-30 amino acid residues. The signal peptides are necessary for passing through the secretory pathway across the first membrane and for this

reason universally control the entry of all proteins to the secretory pathway in eukaryotes and prokaryotes (60). Moreover, the newly synthesized recombinant protein with secretion signal is recognized and translocated in the endoplasmic reticulum (ER)-Golgi pathway to allow protein processing and complete post-translational modifications. Previous studies have demonstrated that utilizing efficient signal peptides to subcellular localization can contribute to the yields of the recombinant proteins (61-63). Selection of the most suitable signal peptide has described a method to increase secretion of proteins and improve production levels of recombinant proteins. Second, poly-histidine (His) tag is a well-known small metal chelating tag which consists of 6 to 10 Histidine residues added either at the N- or C-terminus (64). Poly-His tag is widely used for detection and purification of recombinant proteins via immobilized metal affinity chromatography (65). Third, retention peptide tag, a C-terminal peptide (SEKDEL/KDEL), play as a major determinant for recombinant proteins structure and stability (66) by expressing proteins that go through to the endoplasmic reticulum (ER) for post-translational modification (PTM) and move to Golgi complex for Golgi-specific modifications (67). In addition, the SEKDEL-tagged protein can interact with the KDEL receptor, leave the ER, undergo PTMs in the cis-Golgi, and be successively retrieved back to the ER by retrograde transport (68). Furthermore, the SEKDEL-tagged possess essential features to assist recombinant protein in the proper folding because they are localized in ER that has become promising for protein folding and storage to presumably increase protein levels (69) Therefore, the potential of the SEKDEL tagging strategy in increasing yields of recombinant proteins suggests further investigations.

CHAPTER III EXPERIMENTAL

1. Experimental Design



2. Materials

2.1. Gene

The published PRRSV nucleocapsid gene sequence (N gene; Accession No. ABU87671.1) has been synthesized (Bioneer company, Korea)

2.2. Enzyme

Restriction enzyme *Xho*I (New England BioLabs, Ipswich, MA, USA)

Restriction enzyme *Xba*I (New England BioLabs, Ipswich, MA, USA)

Restriction enzyme *Sac*I (New England BioLabs, Ipswich, MA, USA)

Taq DNA polymerase (Vivantis, Malaysia)

Q5 DNA polymerase (New England BioLabs, Ipswich, MA, USA)

T4 DNA ligase (New England BioLabs, Ipswich, MA, USA)

2.3. Cloning and Expression vector

pGEMT-Easy Vector (Promega) (Appendix A)

pGEMT-SP Vector

pBYR2e-K2Md Vector (Appendix B)

2.4. Molecular Biology kits

AccuPrep Nano-Plus Plasmid Mini Extraction kit protocol (Bioneer, Korea)

AccuPrep Gel Purification Kit (Bioneer, Korea)

2.5. Chemical

Acrylamide (Himedia, India)

Agarose (Vivantis, Malaysia)

Ampicillin (ITW Reagents, Darmstadt, Germany)

Kanamycin (Bio Basic, Markham, ON, Canada)

Rifampicin (Thermo Fischer Scientific, Waltham, MA, USA)

Gentamicin (ITW Reagents, Darmstadt, Germany)

2-N-morpholino-ethanesulfonic acid (MES) (ITW Reagents, Darmstadt, Germany)

Magnesium Sulphate ($MgSO_4$) (Merck, USA)

Tris (Vivantis, Malaysia)

Enhanced Chemiluminescence (ECL) plus detection reagent (Abcam, UK)

Color reagent A (stabilized peroxide solution) and color reagent B (stabilized chromogen solution) (R&D Systems)

Nitrocellulose membrane (Bio-Rad)

0.45 µm S-Pak membrane filters (Merck, Massachusetts, USA)

Amintra Ni-NTA affinity resin (Expedeon, Cambridge, UK),

Visafe green dye staining (Vivantis, Malaysia)

2.6. Bacteria

Escherichia coli (DH10B strain)

Agrobacterium tumefaciens (GV3101 strain)

2.7. Antibodies

HRP-conjugated rabbit polyclonal anti-His antibody (Abcam, UK)

2.8. Buffer

2.8.1. DNA loading 6x dye

38% (w/v) Glycerol, 0.08% (w/v) Bromophenol blue, 0.08% (w/v) Xylencyanol

2.8.2. Z-buffer non-reducing dye

125 mM Tris HCl pH 6.8, 12% Sodium Dodecyl Sulphate, 10% Glycerol, 0.001%

Bromophenol blue

2.8.3. 1X Phosphate-buffered saline (PBS)

137 mM Sodium chloride (NaCl), 2.7 mM Potassium Chloride (KCl)

8.1 mM Sodium hydrogen phosphate (Na_2HPO_4)

1.5 mM Potassium dihydrogen phosphate (KH_2PO_4) pH 7.4

2.8.4. Phosphate-buffered saline-Tween (PBST)

1X PBS, 0.05% Tween 20

2.8.5. Coomassie® blue stain solution

0.1% (w/v) Coomassie Brilliant Blue R-250, 40% Methanol, 10% Glacial acetic acid, H2O

2.8.6. Destaining solution

10% Glacial acetic acid, 40% Methanol, H2O

2.8.7. 1X Running buffer

25 mM Tris, 192 mM Glycine, 1% SDS

2.8.8. 1X Transfer buffer

25 mM Tris, 192 mM Glycine, 15% Methanol

2.8.9. 1X Infiltration buffer

10 mM MES, 10mM $MgSO_4$ pH 5.5

2.8.10 Buffer for protein Isolation: Extraction buffer

20 M Tris-HCl pH 7.4, 50 mM NaCl, 5 mM Imidazole

2.8.11 Buffer for protein Isolation: Washing buffer

20 M Tris-HCl pH 7.4, 50 mM NaCl, 20 mM Imidazole

2.8.12 Buffer for protein Isolation: Eluting buffer

20 mM Tris-HCl pH 7.4, 50 mM NaCl, 250 mM Imidazole

2.9 Media

2.9.1. Luria Bertani Broth (LB broth)

1% NaCl, 0.5% Yeast extract, 1% Peptone

2.9.2. Luria Bertani Agar (LB Agar)

1% NaCl, 0.5% Yeast extract, 1% Peptone, 1.5% Agar

3. Method

3.1. Gene design and construction

3.1.1. Codon opimization for Nicotiana benthamiana

a. SP–PRRSV-N–His–SEKDEL

The published PRRSV nucleocapsid gene sequence (N gene) was selected from GenBank (Accession No. ABU87671.1). The nucleotide sequence was condon-optimized for *Nicotiana benthamiana* expression by geneart codon optimization (ThermoFisher Scientific, USA). The gene was combined with plant signal peptide, 8x histidine tag, SEKDEL sequence and restriction enzyme including *Xba*I, *Xho*I, and *Sac*I. The pattern of PRRSV N-gene construct was ***Xba*I - plant signal peptide – *Xho*I - PRRSV N-gene - 8x histidine tag – SEKDEL – *Sac*I** (Figure 4). This template was ordered for synthesis from a company (Figure 4A) and was inserted into geminiviral vector (pBYR2e) at gene of interest position (Figure 4B).

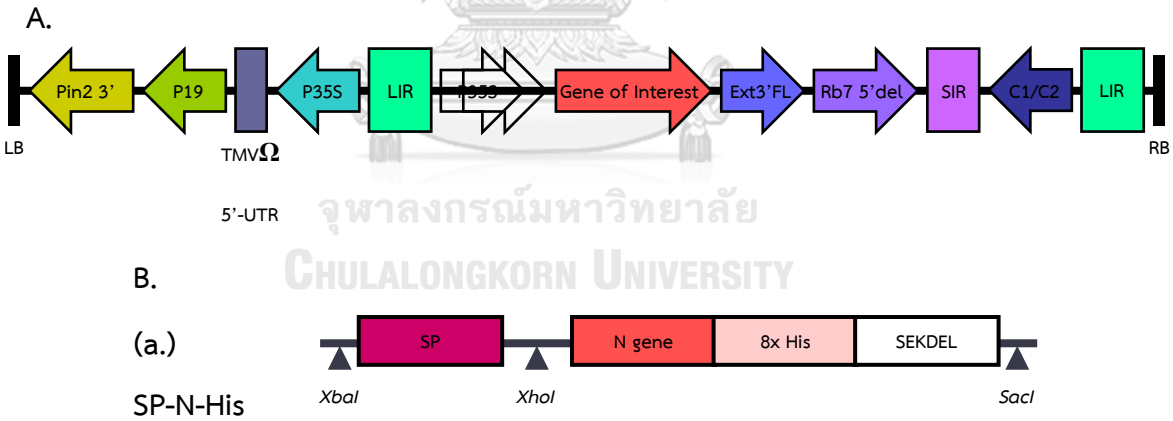


Figure 4. Schematic diagram of pBYR2e with PRRSV N-gene constructs (A.) Schematic diagram of pBYR2e used in this study. LB and RB, the left and right borders of the gene region transferred by Agrobacterium into plant cells; Pin2 3′: the terminator from potato proteinase inhibitor II gene; P19: the RNA silencing suppressor from tomato bushy stunt virus; TMVΩ 5′-UTR: 5′ untranslated region of

tobacco mosaic virus Ω; P35S: Cauliflower Mosaic Virus (CaMV) 35S promoter; LIR: long intergenic region of BeYDV; (B.) Gene of Interest: construct (a.) SP-N-His.

3.1.2. Primer designed and construction

For the other three constructs of PRRSV N-gene, **SP-PRRSV-N–His–SEKDEL**, which used as a template, was amplified with specific primer (Table 1.) and cut with a restriction enzyme.

Table 1. The designed primers used for amplification and cloning

Primer name	Primer sequence (5' to 3')	Direction
*Xba*I-SP-F	TCTAGAACAATGGGCTGG	Forward
*Sac*I-SEKDEL-R	CGAGCTCTCAAAGCTCATCCTTTT	Reverse
SP-His-PRRSV-F	CTCGAGCATCATCACCACCATCACCATCATATGGCCGGCAAGAACCAGTC	Forward
SEKDEL-PRRSV-R	GAGCTCTCAAAGCTCATCCTTTTCAGAACTAGCACCCTGTGAAGCAG	Reverse
T7-F	TAATACGACTCACTATAGGG	Forward
SP6-R	ATTTAGGTGACACTATAG	Reverse
2e-29e-F	TGATATCTCCACTGACGTAAGG	Forward
2e-29e-R	GCTTTGCATTCTTGACATC	Reverse

b. PRRSV-N–His–SEKDEL

For **PRRSV-N–8x histidine–SEKDEL** (Figure 5) was digested the **SP–PRRSV-N–His–SEKDEL**, used as a template, by using restriction enzyme *Xho*I and *Sac*I for 4 hours at 37°C.

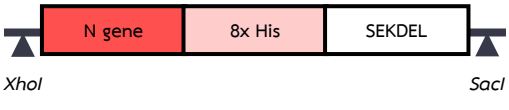


Figure 5. Schematic diagram of PRRSV N-gene constructs (b.)

c. His–PRRSV-N–SEKDEL

To make **8x histidine-PRRSV-N-SEKDEL** construct (Figure 6), the specific primer SP-His-PRRSV-F and SEKDEL-PRRSV-R were used for amplifying the template. This construct was amplified by polymerase chain reaction (PCR) with Q5™ High-Fidelity DNA polymerase which has a proofreading activity (3' to 5' exonuclease proofreading activity) and in final step was added Taq DNA polymerase for generating the DNA products have adenosine base overhang at 3'-terminus that useful in cloning with plasmid that has a T (Thymine) 3' overhang, pGem®-T cloning vector, so the DNA products were able to ligate into cloning vector.

Figure 6. Schematic diagram of PRRSV N-gene constructs (c.)

d. SP–His–PRRSV-N–SEKDEL

For **SP–8x histidine–PRRSV-N–SEKDEL** (Figure 7) was ligated into pGem®-T cloning vector that had already been fused with plant signal peptide.

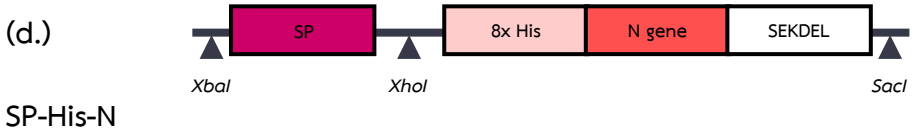


Figure 7. Schematic diagram of PRRSV N-gene constructs (d.)

3.2. Cloning and transformation of cloning vector

The synthetic gene (**construct a.**) with the cloning vector from a company was transformed into competent *Escherichia coli* (*E. coli*) cells strain DH10B by heat-

shocked transformation, which process introduces foreign DNA into a cell. The cells were mixed with the recombinant plasmid and incubated for at least 20 minutes on ice. The mixture cells were heat-shocked at 42°C for 45-90 seconds in the heating box. The heat-shocked cells were put back on the ice again for 2 minutes and incubated with Luria Bertani (LB) broth at 37°C for 45-60 minutes. The transformed cells were spread on LB agar overnight in -37 °C incubator. The recombinant cells grew up and were selected for confirming by PCR with specific primers, *Xba*I-SP-F and *Sac*I-SEKDEL-R (Table1.), and separated by 1% agarose gel electrophoresis. The gel visualized by Visafe green dye staining.

The completion amplified gene (**construct c.**) was ligated into pGem®-T cloning vector with T4 DNA ligase enzyme and incubated at room temperature for overnight. The ligated product was transformed into DH10B competent cells by the heat shock transformation. The transformed cells were spread plate on LB agar with 100 mg/L of ampicillin, 20 µl of 1M IPTG (Isopropyl β-d-1-thiogalactopyranoside), and 40 µl of X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside) and incubated at 37°C for 16-18 hours. The cells grew up in blue and white colonies because the cloning vector, pGem®-T, contains the coding sequence of *lac*Z gene which encodes of β-galactosidase enzyme for hydrolyzing X-gal and making blue-colored colonies. When a plasmid vector is inserted by foreign DNA, the β-galactosidase enzyme will not produce, therefore, the colonies will turn a white color. The white-colored colonies were selected and confirmed by PCR with forward primer, T7-F, and reverse primer, SP6-R, (Table1.). The PCR products were separated by 1% agarose gel electrophoresis and visualized by Visafe green dye staining.

The selected colonies were cultured in LB broth containing ampicillin 100 mg/L and incubated at 37°C, for 16-18 hours. The cultured cells were extracted from the recombinant plasmid by using a plasmid extraction kit. The extracted plasmids

contain the gene of interest (**construct a.**) was double digested with a restriction enzyme at *Xba*I- *Sac*I site and *Xho*I- *Sac*I site, which was for making **construct b.** and resolved from 1% agarose gel after agarose gel electrophoresis by using the Gel DNA Extraction kit.

The extracted plasmid (**construct c.**) was double digested with *Xho*I and *Sac*I restriction enzyme. The digested **construct c.** was ligated into pGem®-T cloning vector that had already been fused with plant signal peptide and transformed into *E. coli* by the heat-shocked transformation. The selected recombinant cells were checked by PCR with T7-F and SP6-R primer and cultured in LB broth containing ampicillin 100 mg/L at 37°C, for 16-18 hours with shaking incubator, 200 rpm. The cultured cells were extracted from the recombinant plasmid. The extracted plasmid was double digested with *Xba*I and *Sac*I restriction enzyme. The digested gene was a **construct d**. All the constructs were confirmed the sequence by sequencing analysis.

3.3. Cloning and tranformation of plant expression vector

This study was used plant expression vector that is a geminiviral vector named pBYR2e. The pBYR2e was double digested with *Xba*I and *Sac*I and *Xho*I and *Sac*I. The digested expression plasmids were ligated with the digested N gene (**construct a.-d.**) to make pBY-SP-N-His (**construct a.**), pBY-N-His (**construct b.**), pBY-SP-His-N (**construct c.**), and pBY-His-N (**construct d.**). The recombinant plasmids were transformed into *E. coli* by the heat-shocked transformation. The recombinant cells were spread on LB agar plates containing 50 mg/L of kanamycin and incubated at 37°C for 16-18 hours. The colonies were selected and verified by using PCR with specific primer, 2e-29e-F and 2e-29e-R, and double digestion with a restriction enzyme. The selected colonies were cultured in LB broth containing 50 mg/L of kanamycin at 37°C, for 16-18 hours with shaking incubator, 200 rpm. The recombinant plasmid was extracted from cells cultured.

After recombinant plasmid extraction, the plasmid was transformed into *Agrobacterium tumefaciens* strain GV3101 by electroporation, which technique uses an electric for the permeability of cell membrane is introduced DNA into the cells. The competent *Agrobacterium tumefaciens* was mixed with a recombinant expression vector on ice and transferred to a dry electroporation cuvette and electroporated using an electroporator. The electroporated cells were added 1 mL of LB broth immediately and incubated at 28°C for 90-120 minutes. The recombinant cells were spread plate on LB agar comprising of three antibiotics, 50 mg/L of kanamycin, gentamicin, and rifampicin, and maintained at 28°C for 48 hours. The chosen colonies were confirmed the inserted gene by PCR with primer, 2e-29e-F, and 2e-29e-R.

3.4. Plant inoculation and protein expression

3.4.1. Determination of protein expression level from each construct

Each construct of recombinant *Agrobacterium tumefaciens* was cultured in the LB media containing 50 mg/L of kanamycin, gentamicin, and rifampicin in condition at 28°C for overnight with shaking incubator, 200 rpm. The cultured cells were centrifuged in condition at 4,000 g for 15 minutes. The cell pellet was resuspended with 1x infiltration buffer (10 mM of MES and $MgSO_4$). Tobacco leaves, *Nicotiana benthamiana*, 6 to 8 weeks-old were infiltrated with the cell solution in optical density at wavelength 600 nm (OD_{600}) approximately 0.4 by syringe without a needle. Plants are grown in a controlled room with 16-hr light exposure per day at 28°C after infiltration. The infiltrated leaves were collected on days 4 post-infiltration (dpi).

For small-scale, infiltrated tobacco leaves were extracted by grind using liquid nitrogen with a mortar. The leaves were become too fine powder and added PBS buffer (phosphate buffer saline) as an extraction buffer in ratio 1 g of fresh leaves to

1 mL of extraction buffer. The solution was homogenized by vortex for at least 5 minutes. The crude was centrifuged at 26,000 g, 4°C for 20 minutes. The crude extract was determined by SDS-PAGE and western blot analysis.

3.4.2. Optimization of Protein expression

The highest protein expression level construct was chosen for optimizing condition including the concentration of cell solution and day post-infiltration. The recombinant cell was cultured at 28°C for overnight with shaking incubator and centrifuged at 4,000 g for 15 minutes. The cell pellet was resuspended with 1x infiltration buffer. The resuspended cells were prepared in various OD_{600} concentration of 0.2, 0.4, 0.6, 0.8, and 1.0. The prepared cell solutions were infiltrated into tobacco leaves and harvested at 2-5 dpi. Infiltrated tobacco leaves were extracted in small-scale extraction and determined by SDS-PAGE and Western blot analysis.

3.4.3. Large scale protein expression

The recombinant cell, the highest protein expression level construct, was cultured in a small volume (5-10 mL) of LB broth containing antibiotics at 28°C for overnight and cultured again in large volume (100-300 mL) of LB broth containing antibiotics at 28°C for overnight with shaking incubator. The cultured cell was centrifuged at 4,000 g for 15 minutes. The cell pellet will be resuspended with at least 2 L of 1x infiltration buffer to a final optimized OD_{600} concentration. The plants were infiltrated by using vacuum infiltration. The plants were placed upside down into Agrobacterium cell solution in vacuum chamber. The infiltrated leaves were harvested at optimized dpi and extracted with extraction buffer ratio 1:1 by using a blender. The homogenized solution was filtered through cheesecloth and centrifuged at 26,000 g, 4°C for 45 minutes. After centrifugation, the supernatant was filtered with

a 0.2-micron filter membrane and become crude extract. The crude extract will be purified by though into affinity chromatography.

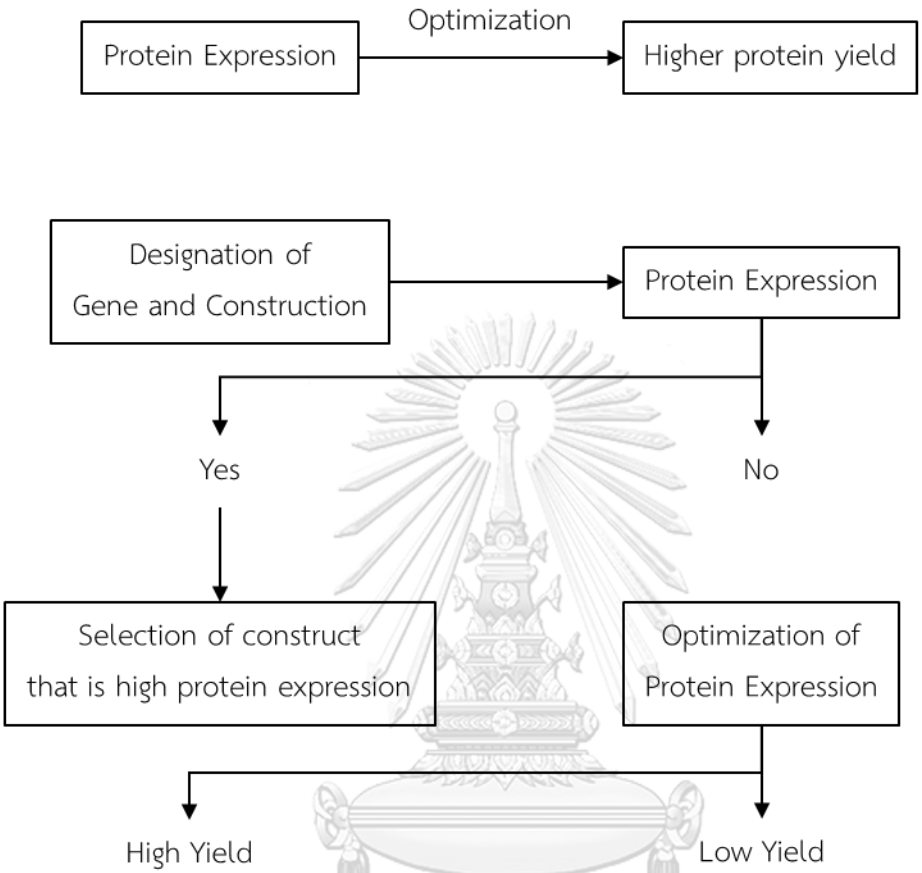
3.5. Protein purification

The affinity chromatography in this study used nickel column chromatography. To prepare the nickel column, Chelating Sepharose^{TM} was initially loaded onto the column and washed with 10 bed volumes of distilled water. Then, the bead was incubated with 5 column volumes (CV) of 1M $NiSO_4 \cdot 6H_2O$ solution for at least 5 minutes. Subsequently, the column was equilibrated with 10 CV of PBS, extraction buffer in preparation for sample addition. The filtered crude extract was loaded into the column. After that, the column was washed with 10 CV wash buffer (20mM imidazole, 20mM Tris-HCl pH7.4, 50mM NaCl), the his-tagged protein was eluted out of the column after the addition of elution buffer (250mM imidazole, 20mM Tris-HCl pH7.4, 50mM NaCl) and examined by SDS-PAGE and western blot.

3.6. SDS-PAGE and western blot

The proteins were subjected to denaturation under non-reducing condition by boiling at 95^{o}C for 5 minutes with Z-buffer as loading dye buffer and separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) (15% acrylamide). Protein separation was investigated by visualization using Brilliant Blue R250 Coomassie staining or electrophoretically transferred to 0.2 μm nitrocellulose membrane. The membrane was blocked with 5% skim milk in PBS buffer. The membrane was probed with anti-6xHistidine conjugated with HRP diluted at 1:5,000 in 3% skim milk in PBS buffer. The membranes were developed by chemiluminescence using ECL plus detection reagent.

6. Research Framework



CHAPTER IV RESULTS AND DISCUSSION

Porcine reproductive and respiratory syndrome (PRRS) is described to be the swine disease-causing to pig's health and productivity as a result has a significant economic and social impact. The PRRS virus (PRRSV) observation is necessary for early diagnosis, implementation of control measures, and disease handling (70). The N-protein stimulates the rapid humoral immune response after PRRSV infection. Since the N-protein is a numerous protein of the virion, and it is highly conserved and great antigenic. However, the N-protein of PRRSV has been generated in *E. coli* (71), the unseemly folding and aggregation of recombinant proteins may affect diagnostic utility and test sensitivity because viral proteins expressed in eukaryotic cells (72). On the other hand, the proteins expressed in bacteria do not have post-translational modifications so that such an approach may fail to produce clear results when relevant to the antigen complex (14). Therefore, the diagnostic tests are used to detect antibodies specific N-protein of PRRSV and are considered a potential biomarker for diagnosing early stage. In this study, the production optimization of PRRSV nucleocapsid protein in *Nicotiana benthamiana* was investigated. Recombinant proteins produced in plants have been shown to be optimally folded and used with fast scalability, safety, speed, and the most economical benefits. Plants are therefore an alternative protein production platform (73, 74).

1. *Nicotiana benthamiana*-produced nucleocapsid protein of PRRSV

1.1 Cloning of N-protein coding gene in plant expression vector

To obtain a recombinant bacterium expressing a codon-optimized nucleocapsid protein (N-protein), the gene that encoding N-protein of PRRSV (region on ORF7) was codon-optimized for expression in tobacco. The clone was developed by containing plant signal peptide, 8x histidine tag and three restriction enzymes (*Xba*I, *Xho*I, and *Sac*I) as showing in Figure 8. The gene sequence encoding N-protein of PRRSV was synthesized in different expression constructs as shown on Appendix A.

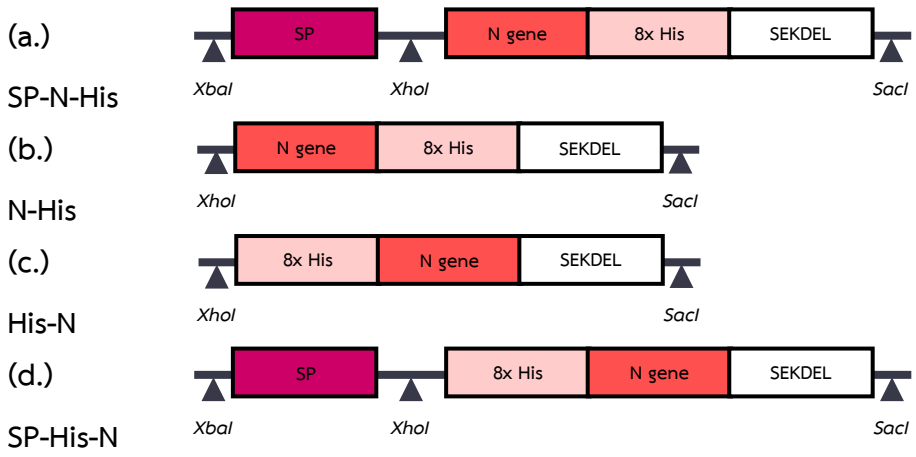


Figure 8. Schematic diagram of gene of interest PRRSV N-gene constructs Construct (a.): SP-N-His, Construct (b.): N-His, Construct (c.): SP-His-N, Construct (d.): His-N; SP: plant signal peptide, N: PRRSV N-gene, His: N or C terminal 8x histidine-tag.

First two expression vector constructs, SP-N-His (**construct a.**) and SP-His-N (**construct c.**), were constructed with plant signal peptide that located at N-terminal. Last two expression vector constructs, N-His (**construct b.**) and His-N (**construct d.**) were constructed without plant signal peptide. All of constructs were located of 8x histidine tag either C-terminal or N-terminal and included for detection by western blot analysis. The restriction sites used for cloning respective fragments into plant expression vector pBYR2e-k2Md are mentioned in the Figure 8. The expression vector contains p19 PTGS suppressor, ER retention signal SEKDEL and the gene of interests were cloned under the transcriptional control of CaMV 35S promoter. The results indicated positive of N-gene constructs at 519 bp for SP-N-His, at 450 bp for N-His, at 519 bp for SP-His-N, and at 450 bp for His-N as predicted.

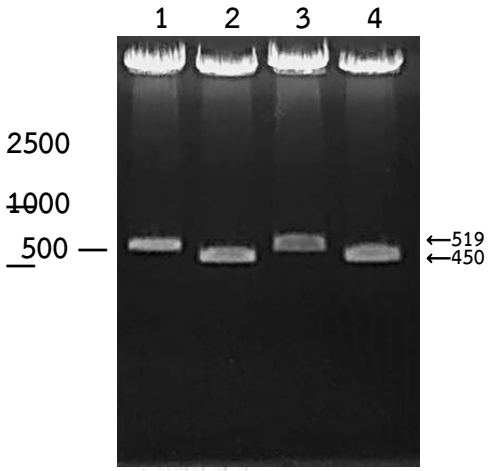


Figure 9. Amplification of PRRSV N-gene constructs

PCR amplification of Porcine Reproductive and Respiratory Syndrome (PRRS) virus gene constructs used in the study. Lane 1: SP-N-His; Lane 2: N-His; Lane 3: SP-His-N; Lane 4: His-N

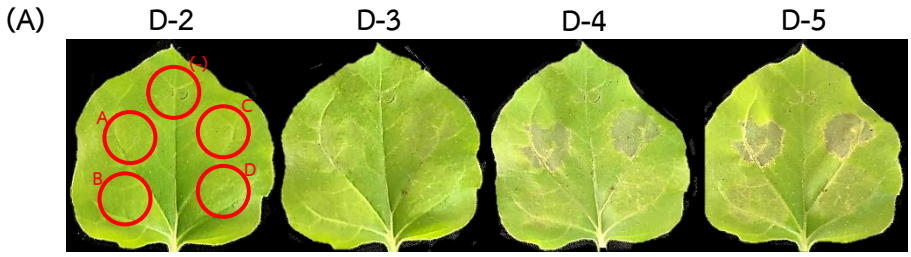
Accordingly, the recombinant genes were purified, ligated into pGEMT or pGEMT-SP vector and transformed into competent *Escherichia coli* cells strain DH10B for increasing a copy number of recombinant plasmids. The developed constructs were cloned into plant geminiviral vector (pBYR2e-k2Md) to get pBYR2e-SP-N-His (SP-N-His), pBYR2e-N-His (N-His), pBYR2eSP-SP-His-N (SP-His-N) and pBYR2e-His-N (His-N) as the expression plasmid and transformed into competent *Agrobacterium tumefaciens* cells strain GV3101. The geminiviral vector derived from the bean yellow dwarf virus (BeYDV) was used to produce multiple copies of the N-protein mRNA transcript (44). The transformed *Agrobacterium* cells were infiltrated into tobacco leaves (Figure 9).

In this way, the transformation by *Agrobacterium* provides numerous benefits, such as the simple system production, the utilization of the cost-effective and the well-developed mechanism of DNA integration into host cell (75, 76). It provides plant viral vectors carry with genes of interest to be immediately transient delivered to plant cells without loss of genes during transfection and allows for high protein

accumulation without expression of the infecting virus (77). These plant viruses are systematic proliferation that increases the level of the target gene's increase and able to release part of their genome into the host species, can result in efficiently produce recombinant proteins (78). There is substantial evidence showing that the possibility of viral antigen expression in plants could be used as a candidate for vaccines or diagnostic reagents (79-81). Consequently, the strategy relies on virus-based transient expression has been invented for the adaptable and efficient expression of PRRSV N-protein in *N. benthamiana*.

1.2. Optimization of Porcine Reproductive and Respiratory Syndrome (PRRS) virus nucleocapsid protein (N-protein) expression in *Nicotiana benthamiana*

In order to obtain the maximal protein production condition in *N. benthamiana*, the different PRRS N-protein expression constructs were evaluated. The protein expression levels of each construct were analyzed by the leaf harvesting time post infiltration on days 4 and the concentration of *Agrobacterium* cell suspension with optical density (OD_{600}) 0.4. According to the results, the research found the signs of necrosis on the infiltrated tobacco leaves that appear at 3 days post-infiltration (dpi) for all constructs (Figure 10A). The protein molecular weight of PRRSV N-protein is approximately 19 kDa in monomer form and 38 kDa after dimerization (18). Under non-reducing conditions, the result, via western blot using HRP-conjugated anti-His antibody, of PRRSV N-proteins were detected at 38 kDa, as expected. The results showed that the construct SP-His-N (**construct c.**) showed highest level expression when compared to other constructs (Figure 10B). Therefore, this construct was used for further experiments



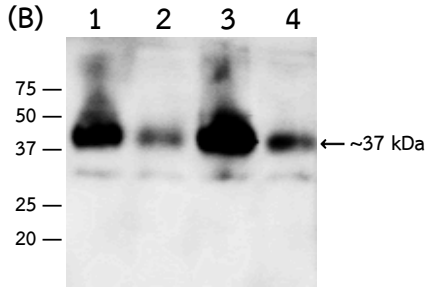


Figure 10. PRRSV N-protein expression in Nicotiana benthamiana

Expression comparison of PRRSV N-protein four constructs in N. benthamiana leaves. The expression levels of recombinant protein in plants infiltrated with different vector constructs were determined on 2, 3, 4 and 5 dpi. (A) The leaf necrotic symptoms observation on 2, 3, 4 and 5 dpi with different constructs. (B) Expression of PRRSV N-protein was shown for all constructs used. Western blot probed with anti-His antibody. Lane 1: SP-N-His (construct A.); Lane 2: N-His (construct B.); Lane 3: SP-His-N (construct C.); Lane 4 His-N (construct D.).

Agro-infiltration was performed as the same with the previous experiment by using the selected construct pBYR2e-SP-His-N. The infiltrated *N. benthamiana* leaves were collected on 2, 3, 4, and 5 dpi to obtain an optimal time course of expression. The expression of PRRSV N-protein was significantly represented from 2 dpi and the levels of recombinant protein expression at different time points were evaluated by western blot analysis. The result of crude extracts indicated that the strongest expression was detected on 4 dpi (Figure 10B). Correspondingly, the effect of *Agrobacterium* cell density was investigated during infiltration. The infiltration *Agrobacterium* cell suspensions that retain the construct pBYR2e-SP-His-N, at OD_{600} concentrations of 0.2, 0.4, 0.6, 0.8, and 1.0 were demonstrated by infiltration into the leaves of *N. benthamiana*. After infiltration, the infiltrated plant leaves were collected at 4 dpi and protein expression was analyzed by western blot analysis. The western blot data of the infiltrated samples collected at 4 dpi showed the highest

expression at OD_{600} at 0.6. The conditions optimized for the use of the expression of PRRS N-protein was limited due to the rapid development of necrosis which can affect protein expression level, protein stability and final biomass of leaves (82, 83). According to the results indicated that the expression level on 4 dpi is higher than 5 dpi However, even though the restricted of transfection time but achieved a high expression level of PRRS N-protein on the harvesting time at 4 dpi and optimal cell density of Agrobacterium infiltrated suspension (OD_{600}) at 0.6 as show in Figure 11.

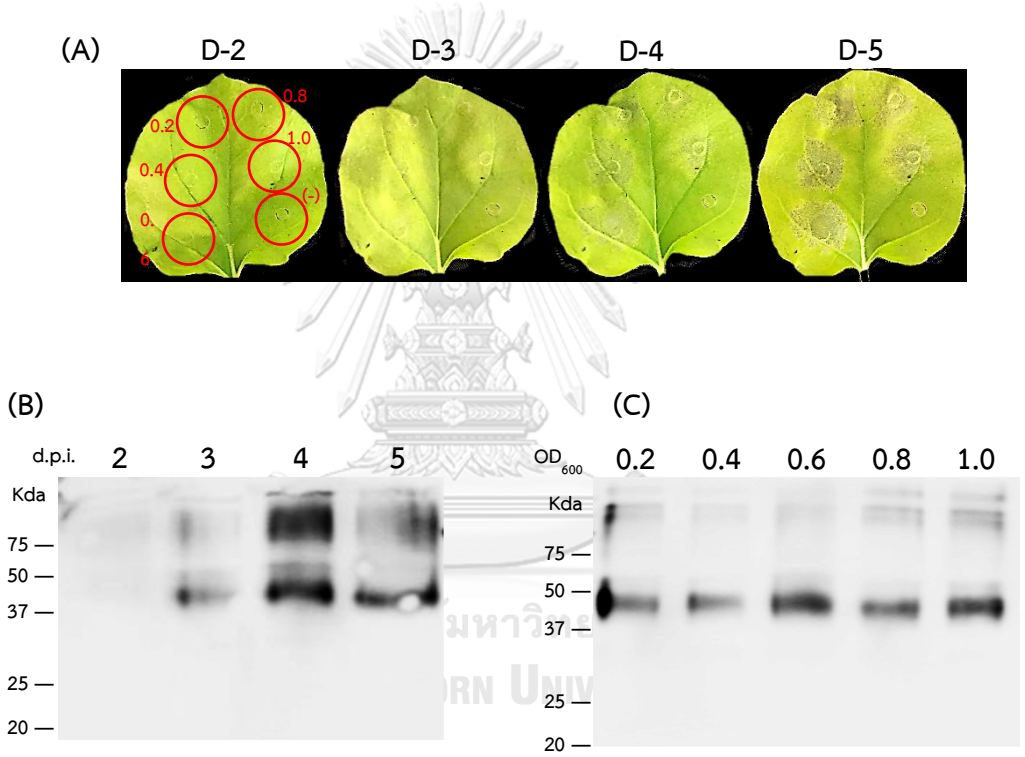


Figure 11.Expression optimization of selected PRRSV N-protein construct in Nicotiana benthamiana

Expression optimization of chosen PRRSV N-protein in N. benthamiana leaves. (A) The leaf necrotic symptoms notice in different concentration at 0.2, 0.4, 0.6 0.8 and 1.0 on 2, 3, 4 and 5 dpii. (B) Expression of PRRSV N-protein was shown in same concentration 0.6 at OD600 on 2, 3, 4 and 5 dpi. (C) The expression of PRRSV N-

protein was indicated with OD_{600} 0.2, 0.4, 0.6, 0.8 and 1.0 concentration of selected vector construct on 4 dpi. Western blot probed with anti-His antibody.

In comparison the same harvest time of protein expression among the four constructs, the expression of PRRSV N-protein in both of SP-N-His and SP-His-N constructs was higher expression level than other two constructs without signal peptide. In previous studies, the signaling localization peptide, SP, and ER retention peptide, SEKDEL, have been reported to increase protein production in plants (84, 85). The signal peptide has shown efficacy to lead the yield of recombinant protein to increase (61, 86). Likewise, the selection of optimal peptide signaling describes a method for increasing protein secretion and improving levels of recombinant protein production. Moreover, the SEKDEK-tagged, interact with the KDEL receptor in Golgi body, is convenient to direct the protein recovery to ER by retrograde transport and ensures proper folding and enhanced protein stability (68, 87). This phenomenon is related to the possible effects of overwhelming protein accumulation triggering ER stress (68). Thereby, as shown from two highest expressing constructs, SP-N-His and SP-His-N, inclusion of signal peptide sequence help in enhancing accumulation of PRRS N-protein expression.

1.3. Purification of plant-produced PRRS N-protein via Ni-NTA affinity chromatography.

In this study, a simple purification method was performed only using Ni-NTA affinity chromatography to purify extracted plant-produced PRRSV N-protein. The construct c. with the highest-level protein expression (SP-His-N) was transiently expressed and the crude extract was purified with Ni-NTA column. The plant-purified PRRS N-protein was determined by western blot using anti-histidine conjugated with HRP antibody.

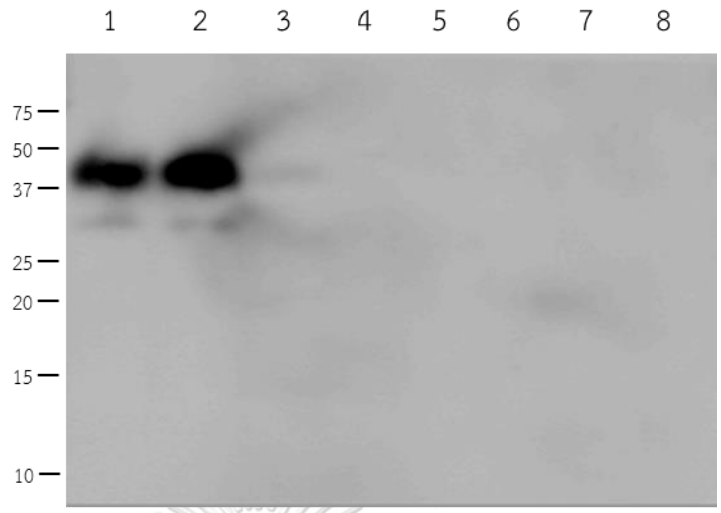


Figure 12. *Western blot of plant-produced PRRS N-protein*

The expression and purification of plant-produced PRRS N-protein from construct c. (SP-His-N). Purification of N-protein was determined by western blot probed with anti-His antibody. Lane1: plant crude extract, Lane2: flow through; Lane 3: washing fraction at 20 mM Imidazole; Lanes 4-8: elution fraction 1 to 5 (1 mL) at 250 mM Imidazole.

The result revealed that PRRSV N-protein, extracted SP-His-N, was not able to bind to immobilized nickel ion column because the finally recovered with a high concentration of imidazole represented the inaccessible binding of the plant-derived His-tagged PRRSV N-protein to the Ni column (Figure 12). Nevertheless, despite the apparent unbinding capacities of SP-His-N construct, this study was incapable to verify protein structure because it was not investigated. By chance, when a protein forms into a structure, the facilitated purification tag possible folds inside the structure. This occurrence may be the reason why the protein unable to be purified or the protein amount is quite small, resulting in undetectable in the western blot after protein purification. First, in order to solve the shortcoming may begin by changing the protein signaling localization to transport and accumulate protein in another organelle, such as nucleus or chloroplast (88). Second, the extraction buffer

used for protein extraction may be changed, including adjusting the pH of buffer or adding some additives (89). Before purifying protein, reduction of concentration of crude extract able to be made by ammonium sulfate precipitation method (89) or fractional precipitation with acetone method. However, protein denaturation with high concentration of salt and refolding can be done as well (88). Interestingly, the process of protein purification try to change to other protein purification method , for example, ion-exchange chromatography or gel filtration chromatography (90). In contrast, the protein purification by using affinity chromatography with poly-histidine, as a purification aid, may try to change the location of affinity tag to the amino- (N) or carboxyl- (C) terminus of a target protein (91) or coating metal to the Sepharose bead that able to bind with poly-histidine by coating with cobalt instead (89). In addition, another fusion tag can be used to purify the protein, such as glutathione S-transferase (GST) (92). At optimal expression and purification conditions, the results showed that PRRSV N-protein was not suitable to produce in *N. benthamiana* even though this protein could be expressed in this platform.

CHAPTER V CONCLUSION

In conclusion, this study demonstrates the plant transient expression system to produce the N-Protein of PRRSV in *N. benthamiana* by using geminiviral vector. Preliminary testing of plant-derived N-protein by western blot showed that the protein was detected by anti-his antibody. As the results, the optimal condition for expression N-protein on 4 dpi at 0.6 (OD_{600}) and the protein size of plant-produced PRRSV N-protein was approximately 38 kDa as predicted. Further study, the purification method for purifying PRRRV N-protein should be optimized conditions or procedure. This indicates the possible method to produce plant-derived antigens for PRRSV diagnosis. This proof-of-concept study enables the use of plant expression platforms for antigen production to diagnose infectious diseases.

REFERENCES

1. Yu JE, Ouh IO, Kang H, Lee HY, Cheong KM, Cho IS, et al. An enhanced immunochromatographic strip test using colloidal gold nanoparticle-labeled dual-type N proteins for detection of antibodies to PRRS virus. J Vet Sci. 2018;19(4):519-27.
2. Li J, Wang G, Yang D, Zhao B, Zhao Y, Liu Y, et al. Development of luciferase-linked antibody capture assay based on luciferase immunoprecipitation systems for antibody detection of porcine reproductive and respiratory syndrome virus. BMC Biotechnol. 2018;18(1):73.
3. Gilbert SA, Larochelle R, Magar R, Cho HJ, Deregt D. Typing of porcine reproductive and respiratory syndrome viruses by a multiplex PCR assay. J Clin Microbiol. 1997;35(1):264-7.
4. Amonsin A, Kedkovid R, Puranaveja S, Wongyanin P, Suradhat S, Thanawongnuwech R. Comparative analysis of complete nucleotide sequence of porcine reproductive and respiratory syndrome virus (PRRSV) isolates in Thailand (US and EU genotypes). Virol J. 2009;6:143-.
5. Thanawongnuwech R, Amonsin A, Tatsanakit A, Damrongwatanapokin S. Genetics and geographical variation of porcine reproductive and respiratory syndrome virus (PRRSV) in Thailand. Veterinary Microbiology. 2004;101(1):9-21.
6. Tong G-Z, Zhou Y-J, Hao X-F, Tian Z-J, An T-Q, Qiu H-J. Highly pathogenic porcine reproductive and respiratory syndrome, China. Emerg Infect Dis. 2007;13(9):1434-6.
7. Nilubol D, Tripipat T, Hoonsuwan T, Kortheerakul K. Porcine reproductive and respiratory syndrome virus, Thailand, 2010-2011. Emerg Infect Dis. 2012;18(12):2039-43.
8. Jantafong T, Sangtong P, Saenglub W, Mungkundar C, Romlamduan N, Lekchareonsuk C, et al. Genetic diversity of porcine reproductive and respiratory syndrome virus in Thailand and Southeast Asia from 2008 to 2013. Vet Microbiol. 2015;176(3-4):229-38.
9. Lunney JK, Fang Y, Ladinig A, Chen N, Li Y, Rowland B, et al. Porcine Reproductive and Respiratory Syndrome Virus (PRRSV): Pathogenesis and Interaction

with the Immune System. Annu Rev Anim Biosci. 2016;4:129-54.

10. Dokland T. The structural biology of PRRSV. Virus Res. 2010;154(1-2):86-97.

11. Doan DN, Dokland T. Structure of the nucleocapsid protein of porcine reproductive and respiratory syndrome virus. Structure. 2003;11(11):1445-51.

12. Cong Y, Huang Z, Sun Y, Ran W, Zhu L, Yang G, et al. Development and application of a blocking enzyme-linked immunosorbent assay (ELISA) to differentiate antibodies against live and inactivated porcine reproductive and respiratory syndrome virus. Virology. 2013;444(1-2):310-6.

13. Chen C, Fan W, Jia X, Li J, Bi Y, Liu W. Development of a recombinant N-Gp5c fusion protein-based ELISA for detection of antibodies to porcine reproductive and respiratory syndrome virus. J Virol Methods. 2013;189(1):213-20.

14. Wootton SK, Nelson EA, Yoo D. Antigenic structure of the nucleocapsid protein of porcine reproductive and respiratory syndrome virus. Clin Diagn Lab Immunol. 1998;5(6):773-9.

15. Zhang G, Li N, Chen Y, Zhou J, Liu H, Qi Y, et al. Identification of the B-cell epitopes on N protein of type 2 porcine reproductive and respiratory syndrome virus, using monoclonal antibodies. Int J Biol Macromol. 2019;130:300-6.

16. Vimolmangkang S, Gasic K, Soria-Guerra R, Rosales-Mendoza S, Moreno-Fierros L, Korban SS. Expression of the nucleocapsid protein of Porcine Reproductive and Respiratory Syndrome Virus in soybean seed yields an immunogenic antigenic protein. Planta. 2012;235(3):513-22.

17. Song BH, Kim JM, Kim JK, Jang HS, Yun GN, Choi EJ, et al. Packaging of porcine reproductive and respiratory syndrome virus replicon RNA by a stable cell line expressing its nucleocapsid protein. J Microbiol. 2011;49(3):516-23.

18. Zhao K, Li LW, Jiang YF, Gao F, Zhang YJ, Zhao WY, et al. Nucleocapsid protein of porcine reproductive and respiratory syndrome virus antagonizes the antiviral activity of TRIM25 by interfering with TRIM25-mediated RIG-I ubiquitination. Vet Microbiol. 2019;233:140-6.

19. Zhao K, Li LW, Zhang YJ, Jiang YF, Gao F, Li GX, et al. MOV10 inhibits replication of porcine reproductive and respiratory syndrome virus by retaining viral nucleocapsid protein in the cytoplasm of Marc-145cells. Biochem Biophys Res Commun.

2018;504(1):157-63.

20. Zumbrun EE. Mission critical: mobilization of essential animal models for Ebola, Nipah, and Machupo virus infections. Veterinary pathology. 2015;52(1):18-20.

21. Pogue GP, Lindbo JA, Garger SJ, Fitzmaurice WP. Making an ally from an enemy: plant virology and the new agriculture. Annu Rev Phytopathol. 2002;40:45-74.

22. Gleba Y, Klimyuk V, Marillonnet S. Viral vectors for the expression of proteins in plants. Curr Opin Biotechnol. 2007;18(2):134-41.

23. Lico C, Chen Q, Santi L. Viral vectors for production of recombinant proteins in plants. J Cell Physiol. 2008;216(2):366-77.

24. Goodin MM, Zaitlin D, Naidu RA, Lommel SA. Nicotiana benthamiana: its history and future as a model for plant-pathogen interactions. Mol Plant Microbe Interact. 2008;21(8):1015-26.

25. Sack M, Hofbauer A, Fischer R, Stoger E. The increasing value of plant-made proteins. Curr Opin Biotechnol. 2015;32:163-70.

26. Engler C, Youles M, Gruetzner R, Ehnert TM, Werner S, Jones JD, et al. A golden gate modular cloning toolbox for plants. ACS Synth Biol. 2014;3(11):839-43.

27. Sarrion-Perdigones A, Palaci J, Granell A, Orzaez D. Design and Construction of Multigenic Constructs for Plant Biotechnology Using the GoldenBraid Cloning Strategy. In: Valla S, Lale R, editors. DNA Cloning and Assembly Methods. Totowa, NJ: Humana Press; 2014. p. 133-51.

28. Hood EE, Devaiah SP, Fake G, Egelkrout E, Teoh KT, Requesens DV, et al. Manipulating corn germplasm to increase recombinant protein accumulation. Plant Biotechnol J. 2012;10(1):20-30.

29. Takaiwa F. Increasing the production yield of recombinant protein in transgenic seeds by expanding the deposition space within the intracellular compartment. Bioengineered. 2013;4(3):136-9.

30. Denac H, Moser C, Tratschin JD, Hofmann MA. An indirect ELISA for the detection of antibodies against porcine reproductive and respiratory syndrome virus using recombinant nucleocapsid protein as antigen. J Virol Methods. 1997;65(2):169-81.

31. Kappes MA, Faaberg KS. PRRSV structure, replication and recombination: Origin of phenotype and genotype diversity. Virology. 2015;479-480:475-86.

32. Family - Arteriviridae. In: King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ, editors. Virus Taxonomy. San Diego: Elsevier; 2012. p. 796-805.

33. Order - Nidovirales. In: King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ, editors. Virus Taxonomy. San Diego: Elsevier; 2012. p. 784-94.

34. Meng XJ. Heterogeneity of porcine reproductive and respiratory syndrome virus: implications for current vaccine efficacy and future vaccine development. Vet Microbiol. 2000;74(4):309-29.

35. Mengeling WL, Lager KM, Vorwald AC, Clouser DF. Comparative safety and efficacy of attenuated single-strain and multi-strain vaccines for porcine reproductive and respiratory syndrome. Vet Microbiol. 2003;93(1):25-38.

36. Du T, Nan Y, Xiao S, Zhao Q, Zhou EM. Antiviral Strategies against PRRSV Infection. Trends Microbiol. 2017;25(12):968-79.

37. Rosano GL, Ceccarelli EA. Recombinant protein expression in Escherichia coli: advances and challenges. Front Microbiol. 2014;5:172-.

38. Tripathi NK, Shrivastava A. Recent Developments in Bioprocessing of Recombinant Proteins: Expression Hosts and Process Development. Frontiers in Bioengineering and Biotechnology. 2019;7(420).

39. He J, Lai H, Engle M, Gorlatov S, Gruber C, Steinkellner H, et al. Generation and analysis of novel plant-derived antibody-based therapeutic molecules against West Nile virus. PloS one. 2014;9(3):e93541.

40. Shoji Y, Prokhnevsky A, Leffet B, Vetter N, Tottey S, Satinover S, et al. Immunogenicity of H1N1 influenza virus-like particles produced in Nicotiana benthamiana. Human vaccines & immunotherapeutics. 2015;11(1):118-23.

41. Chen X, Liu J. Generation and immunogenicity of transgenic potato expressing the GP5 protein of porcine reproductive and respiratory syndrome virus. J Virol Methods. 2011;173(1):153-8.

42. Kim TG, Kim MY, Yang MS. Cholera toxin B subunit-domain III of dengue virus envelope glycoprotein E fusion protein production in transgenic plants. Protein Expr Purif. 2010;74(2):236-41.

43. Birch JR, Froud SJ. Mammalian cell culture systems for recombinant protein production. Biologicals. 1994;22(2):127-33.

44. Huang Z, Phoolcharoen W, Lai H, Piensook K, Cardineau G, Zeitlin L, et al. High-level rapid production of full-size monoclonal antibodies in plants by a single-vector DNA replicon system. Biotechnol Bioeng. 2010;106(1):9-17.

45. Hiatt A, Whaley KJ, Zeitlin L. Plant-Derived Monoclonal Antibodies for Prevention and Treatment of Infectious Disease. Microbiology spectrum. 2014;2(1):AID-0004-2012.

46. Both L, van Dolleweerd C, Wright E, Banyard AC, Bulmer-Thomas B, Selden D, et al. Production, characterization, and antigen specificity of recombinant 62-71-3, a candidate monoclonal antibody for rabies prophylaxis in humans. Faseb j. 2013;27(5):2055-65.

47. Gonzalez-Rabade N, McGowan EG, Zhou F, McCabe MS, Bock R, Dix PJ, et al. Immunogenicity of chloroplast-derived HIV-1 p24 and a p24-Nef fusion protein following subcutaneous and oral administration in mice. Plant Biotechnol J. 2011;9(6):629-38.

48. Thanavala Y, Yang YF, Lyons P, Mason HS, Arntzen C. Immunogenicity of transgenic plant-derived hepatitis B surface antigen. Proc Natl Acad Sci U S A. 1995;92(8):3358-61.

49. Zhang XH, Keating P, Wang XW, Huang YH, Martin J, Hartmann JX, et al. Production of functional native human interleukin-2 in tobacco chloroplasts. Molecular biotechnology. 2014;56(4):369-76.

50. Musiychuk K, Sivalenka R, Jaje J, Bi H, Flores R, Shaw B, et al. Plant-produced human recombinant erythropoietic growth factors support erythroid differentiation in vitro. Stem Cells Dev. 2013;22(16):2326-40.

51. Donini M, Lico C, Baschieri S, Conti S, Magliani W, Polonelli L, et al. Production of an engineered killer peptide in Nicotiana benthamiana by using a potato virus X expression system. Appl Environ Microbiol. 2005;71(10):6360-7.

52. Ma S, We L, Yang H, Deng S, Jevnikar A. Emerging technologies to achieve oral delivery of GLP-1 and GLP-1 analogs for treatment of type 2 diabetes mellitus (T2DM). Canadian Journal of Biotechnology. 2017;1.

53. Edelbaum O, Stein D, Holland N, Gafni Y, Livneh O, Novick D, et al. Expression of active human interferon-beta in transgenic plants. J Interferon Res. 1992;12(6):449-53.

54. Kusnadi AR, Nikolov ZL, Howard JA. Production of recombinant proteins in transgenic plants: Practical considerations. Biotechnol Bioeng. 1997;56(5):473-84.

55. Spiegel H, Boes A, Morales C, Rademacher T, Buyel J. Ready-to-Use Stocks of Agrobacterium tumefaciens Can Simplify Process Development for the Production of Recombinant Proteins by Transient Expression in Plants. Biotechnology Journal. 2019;14.

56. Moghaddam M, Aghamollaei H, Kooshki H, Azizi K, Mirnejad R, Choopani A. The development of antimicrobial peptides as an approach to prevention of antibiotic resistance. Reviews in Medical Microbiology. 2015;26:98-110.

57. Moghaddam MM, Abolhassani F, Babavalian H, Mirnejad R, Azizi Barjini K, Amani J. Comparison of in vitro antibacterial activities of two cationic peptides CM15 and CM11 against five pathogenic bacteria: Pseudomonas aeruginosa, Staphylococcus aureus, Vibrio cholerae, Acinetobacter baumannii, and Escherichia coli. Probiotics Antimicrob Proteins. 2012;4(2):133-9.

58. Fox MA, Thwaite JE, Ulaeto DO, Atkins TP, Atkins HS. Design and characterization of novel hybrid antimicrobial peptides based on cecropin A, LL-37 and magainin II. Peptides. 2012;33(2):197-205.

59. Cao Y, Yu RQ, Liu Y, Zhou HX, Song LL, Cao Y, et al. Design, recombinant expression, and antibacterial activity of the cecropins-melittin hybrid antimicrobial peptides. Curr Microbiol. 2010;61(3):169-75.

60. Mølhøj M, Degan FD. Leader sequences are not signal peptides. Nature Biotechnology. 2004;22(12):1502-.

61. Hemmerich J, Rohe P, Kleine B, Jurischka S, Wiechert W, Freudl R, et al. Use of a Sec signal peptide library from Bacillus subtilis for the optimization of cutinase secretion in Corynebacterium glutamicum. Microbial Cell Factories. 2016;15(1):208.

62. Kleiner-Grote GRM, Risse JM, Friehs K. Secretion of recombinant proteins from E. coli. Engineering in Life Sciences. 2018;18(8):532-50.

63. Schillberg S, Zimmermann S, Voss A, Fischer R. Apoplastic and cytosolic expression of full-size antibodies and antibody fragments in Nicotiana tabacum. Transgenic Research. 1999;8(4):255-63.

64. de Souza A, Hull PA, Gille S, Pauly M. Identification and functional characterization of the distinct plant pectin esterases PAE8 and PAE9 and their deletion mutants. Planta. 2014;240(5):1123-38.

65. Cheung RC, Wong JH, Ng TB. Immobilized metal ion affinity chromatography: a review on its applications. Appl Microbiol Biotechnol. 2012;96(6):1411-20.

66. Munro S, Pelham HR. A C-terminal signal prevents secretion of luminal ER proteins. Cell. 1987;48(5):899-907.

67. Béraud-Dufour S, Balch W. A journey through the exocytic pathway. J Cell Sci. 2002;115(Pt 9):1779-80.

68. Bao J, Huang M, Petranovic D, Nielsen J. Balanced trafficking between the ER and the Golgi apparatus increases protein secretion in yeast. AMB Express. 2018;8(1):37.

69. Lindh I, Wallin A, Kalbina I, Sävenstrand H, Engström P, Andersson S, et al. Production of the p24 capsid protein from HIV-1 subtype C in Arabidopsis thaliana and Daucus carota using an endoplasmic reticulum-directing SEKDEL sequence in protein expression constructs. Protein Expr Purif. 2009;66(1):46-51.

70. Nathues H, Alarcon P, Rushton J, Jolie R, Fiebig K, Jimenez M, et al. Cost of porcine reproductive and respiratory syndrome virus at individual farm level - An economic disease model. Prev Vet Med. 2017;142:16-29.

71. Seuberlich T, Tratschin JD, Thur B, Hofmann MA. Nucleocapsid protein-based enzyme-linked immunosorbent assay for detection and differentiation of antibodies against European and North American porcine reproductive and respiratory syndrome virus. Clin Diagn Lab Immunol. 2002;9(6):1183-91.

72. Peternel S, Komel R. Active protein aggregates produced in Escherichia coli. Int J Mol Sci. 2011;12(11):8275-87.

73. Rattanapisit K, Shanmugaraj B, Manopwisedjaroen S, Purwono PB, Siriwattananon K, Khorattanakulchai N, et al. Rapid production of SARS-CoV-2 receptor binding domain (RBD) and spike specific monoclonal antibody CR3022 in Nicotiana benthamiana. Sci Rep. 2020;10(1):17698.

74. Shanmugaraj B, Rattanapisit K, Manopwisedjaroen S, Thitithanyanont A, Phoolcharoen W. Monoclonal Antibodies B38 and H4 Produced in Nicotiana benthamiana Neutralize SARS-CoV-2 in vitro. Front Plant Sci. 2020;11:589995.

75. Chen Q, Lai H, Hurtado J Fau - Stahnke J, Stahnke J Fau - Leuzinger K, Leuzinger K Fau - Dent M, Dent M. Agroinfiltration as an Effective and Scalable Strategy of Gene Delivery for Production of Pharmaceutical Proteins. LID - 103 [pii].

76. Leuzinger K, Dent M Fau - Hurtado J, Hurtado J Fau - Stahnke J, Stahnke J Fau - Lai H, Lai H Fau - Zhou X, Zhou X Fau - Chen Q, et al. Efficient agroinfiltration of plants for high-level transient expression of recombinant proteins. LID - 10.3791/50521 [doi] LID - 50521. (1940-087X (Electronic)).

77. Mor TS, Moon YS, Palmer KE, Mason HS. Geminivirus vectors for high-level expression of foreign proteins in plant cells. Biotechnol Bioeng. 2003;81(4):430-7.

78. Timmermans MCP, Das OP, Messing J. Geminiviruses and Their Uses as Extrachromosomal Replicons. Annual Review of Plant Physiology and Plant Molecular Biology. 1994;45(1):79-112.

79. Rybicki EP. Plant-made vaccines and reagents for the One Health initiative. Hum Vaccin Immunother. 2017;13(12):2912-7.

80. Marques LEC, Silva BB, Dutra RF, Florean E, Menassa R, Guedes MIF. Transient Expression of Dengue Virus NS1 Antigen in Nicotiana benthamiana for Use as a Diagnostic Antigen. Front Plant Sci. 2019;10:1674.

81. Shanmugaraj B, CJ IB, Phoolcharoen W. Plant Molecular Farming: A Viable Platform for Recombinant Biopharmaceutical Production. Plants (Basel). 2020;9(7).

82. Rattanapisit K, Bhoo S, Hahn T, Mason H, Phoolcharoen W. Rapid transient expression of cholera toxin B subunit (CTB) in Nicotiana benthamiana. In Vitro Cellular & Developmental Biology - Plant. 2013;49:107-13.

83. Hamorsky KT, Kouokam JC, Jurkiewicz JM, Nelson B, Moore LJ, Husk AS, et al. N-glycosylation of cholera toxin B subunit in Nicotiana benthamiana: impacts on host stress response, production yield and vaccine potential. Sci Rep. 2015;5:8003.

84. Chen J, Gai Y, Fu G, Zhou W, Zhang D, Wen J. Enhanced extracellular production of α-amylase in Bacillus subtilis by optimization of regulatory elements and over-expression of PrsA lipoprotein. Biotechnology Letters. 2015;37(4):899-906.

85. Young CL, Raden DL, Robinson AS. Analysis of ER resident proteins in Saccharomyces cerevisiae: implementation of H/KDEL retrieval sequences. Traffic.

2013;14(4):365-81.

86. Kleiner-Grote GRM, Risse JM, Friehs K. Secretion of recombinant proteins from E. coli. Eng Life Sci. 2018;18(8):532-50.

87. Heidari HR, Bandehpour M, Vahidi H, Barar J, Kazemi B, Naderi-Manesh H. Improvement in the stability and functionality of Nicotiana tabacum produced recombinant TRAIL through employment of endoplasmic reticulum expression and ascorbate buffer mediated extraction strategies. Bioimpacts. 2014;4(3):123-32.

88. Structural Genomics C, China Structural Genomics C, Northeast Structural Genomics C, Graslund S, Nordlund P, Weigelt J, et al. Protein production and purification. Nat Methods. 2008;5(2):135-46.

89. Doonan S. Protein purification protocols. General strategies. Methods Mol Biol. 1996;59:1-16.

90. Sheehan D. Physical biochemistry: principles and applications: John Wiley & Sons; 2013.

91. Zhong M, Molday LL, Molday RS. Role of the C terminus of the photoreceptor ABCA4 transporter in protein folding, function, and retinal degenerative diseases. J Biol Chem. 2009;284(6):3640-9.

92. Falke JJ, Corbin JA. Methods | Affinity Tags for Protein Purification. In: Jez J, editor. Encyclopedia of Biological Chemistry III (Third Edition). Oxford: Elsevier; 2013. p. 701-5.

APPENDICES

APPENDIX A

PRRS virus N-protein gene expression construct sequences

1. SP–PRRSV-N–His–SEKDEL

TCTAGAACAATGGGCTGGTCCTGCATCATCCTGTTCCTTGTTGCTACTGCTACCGGCGTTCACT
CTGATGTTCAACTTCTCGAGATGCCGAACAACAACGGCAAGCAGCAGAACCGGAAGAAAGGTGA
TGGTCAGCCTGTGAATCAGCTGTGCCAAATGCTGGGCAAGATTATCGCTCAGCAGAATCAGAGC
AGAGGTAAAGGTCCGGGGAAGAAGAACAAGAAGAAAAACCCTGAGAAGCCGCACTTCCCACTT
GCTACTGAGGATGATGTGAGGCATCACTTCACTCCATCTGAGAGGCAGCTCTGCCTTAGCTCTA
TTCAGACCGCTTTCAATCAAGGCGCTGGTACTTGCACCCTTAGCGATTCTGGTAGGATCAGCTA
TACCGTCGAGTTCTCTCTTCCTACTCATCACACCGTGAGGCTTATTAGGGTTACCGCTTCTCCTT
CTGCTCATCATCACCACCATCACCATCATTCTGAAAAGGATGAGCTTTGAGAGCTC

2. PRRSV-N–His–SEKDEL

CTCGAGATGCCGAACAACAACGGCAAGCAGCAGAACCGGAAGAAAGGTGATGGTCAGCCTGTG
AATCAGCTGTGCCAAATGCTGGGCAAGATTATCGCTCAGCAGAATCAGAGCAGAGGTAAAGGTC
CGGGGAAGAAGAACAAGAAGAAAAACCCTGAGAAGCCGCACTTCCCACTTGCTACTGAGGATGA
TGTGAGGCATCACTTCACTCCATCTGAGAGGCAGCTCTGCCTTAGCTCTATTCAGACCGCTTTC
AATCAAGGCGCTGGTACTTGCACCCTTAGCGATTCTGGTAGGATCAGCTATACCGTCGAGTTCT
CTCTTCCTACTCATCACACCGTGAGGCTTATTAGGGTTACCGCTTCTCCTTCTGCTCATCATCAC
CACCATCACCATCATTCTGAAAAGGATGAGCTTTGAGAGCTC

3. His–PRRSV-N–SEKDEL

CTCGAGCATCACCACCATCACCATCATATGCCGAACAACAACGGCAAGCAGCAGAACCGGAAGA
AAGGTGATGGTCAGCCTGTGAATCAGCTGTGCCAAATGCTGGGCAAGATTATCGCTCAGCAGAA

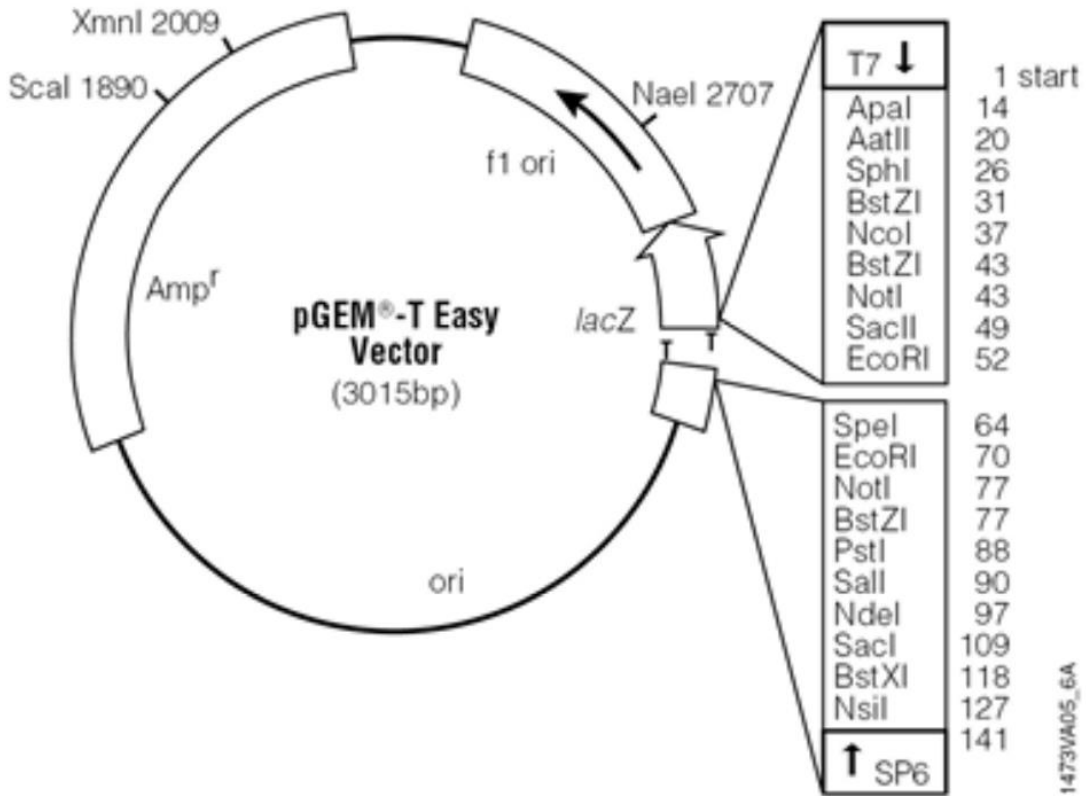
TCAGAGCAGAGGTAAAGGTCCGGGGAAGAAGAACAAGAAGAAAAACCCTGAGAAGCCGCACTT
CCCACTTGCTACTGAGGATGATGTGAGGCATCACTTCACTCCATCTGAGAGGCAGCTCTGCCTT
AGCTCTATTCAGACCGCTTTCAATCAAGGCGCTGGTACTTGCACCCTTAGCGATTCTGGTAGGA
TCAGCTATACCGTCGAGTTCTCTCTTCCTACTCATCACACCGTGAGGCTTATTAGGGTTACCGC
TTCTCCTTCTGCTTCTGAAAAGGATGAGCTTTGAGAGCTC

4. SP–His–PRRSV-N–SEKDEL

TCTAGAACAATGGGCTGGTCCTGCATCATCCTGTTCCTTGTTGCTACTGCTACCGGCGTTCACT
CTGATGTTCAACTTCTCGAGCATCACCACCATCACCATCATATGCCGAACAACAACGGCAAGCA
GCAGAACCGGAAGAAAGGTGATGGTCAGCCTGTGAATCAGCTGTGCCAAATGCTGGGCAAGATT
ATCGCTCAGCAGAATCAGAGCAGAGGTAAAGGTCCGGGGAAGAAGAACAAGAAGAAAAACCCT
GAGAAGCCGCACTTCCCACTTGCTACTGAGGATGATGTGAGGCATCACTTCACTCCATCTGAGA
GGCAGCTCTGCCTTAGCTCTATTCAGACCGCTTTCAATCAAGGCGCTGGTACTTGCACCCTTAG
CGATTCTGGTAGGATCAGCTATACCGTCGAGTTCTCTCTTCCTACTCATCACACCGTGAGGCTT
ATTAGGGTTACCGCTTCTCCTTCTGCTTCTGAAAAGGATGAGCTTTGAGAGCTC

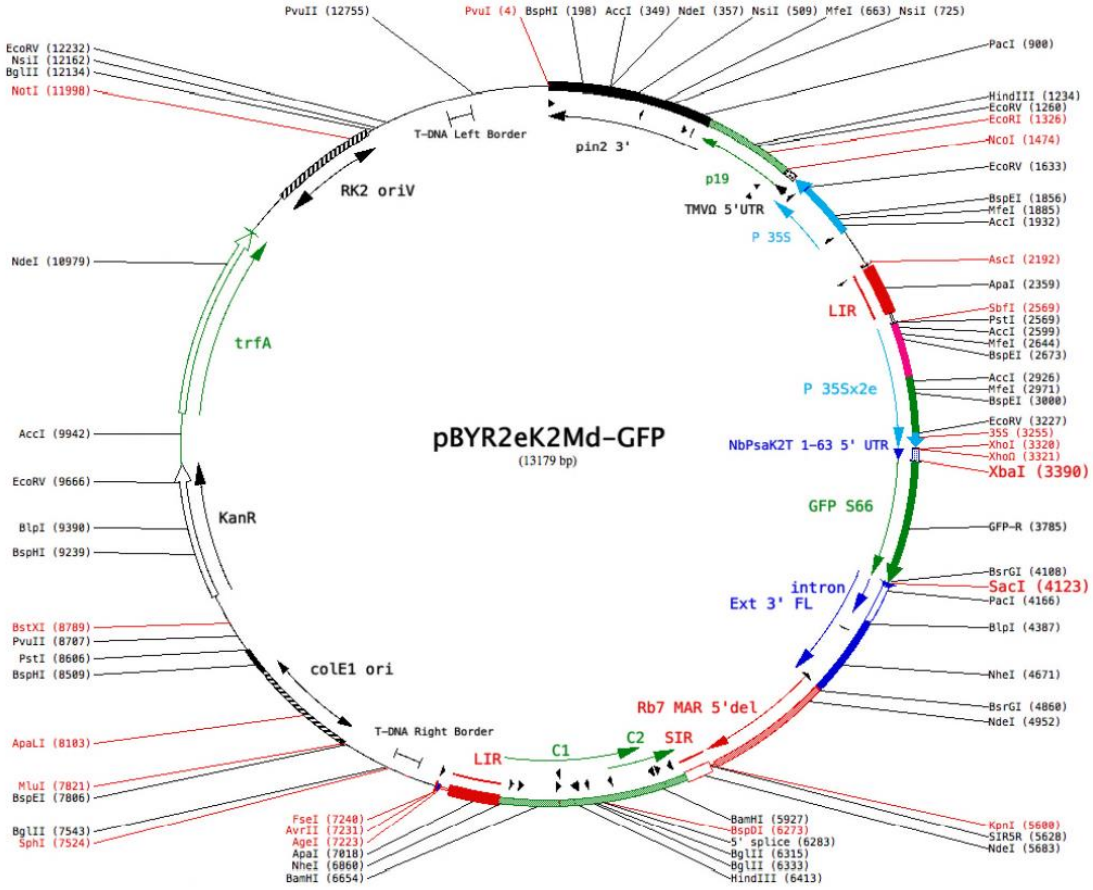
APPENDIX B

pGEM-T Cloning Vector



APPENDIX C

pBYR2e-K2Md Expression Vector



VITA

NAME	Boonyasit Porngarm
DATE OF BIRTH	11 October 1995
PLACE OF BIRTH	Bangkok, Thailand
INSTITUTIONS ATTENDED	Kasetsart University
HOME ADDRESS	141, Charoenkrung 85 lane; Watprayakrai; Bangkholeam; Bangkok; Thailand; 10120