การผลิตอาร์เอ็นเอสายคู่จำเพาะสองชนิดที่ต้านไวรัสหัวเหลืองและไวรัสตัวแดงดวงขาวใน *Litopenaeus vannamei*

นายดามพ์ ชัยมงคล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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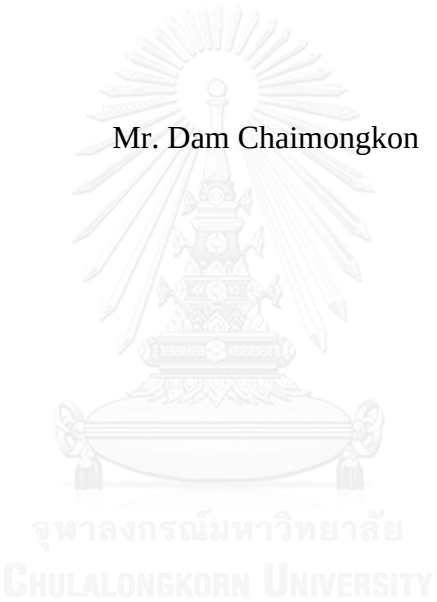
PRODUCTION OF TWO SPECIFIC DOUBLE-STRANDED RNAS AGAINST YELLOW HEAD AND WHITE SPOT SYNDROME VIRUSES IN *Litopenaeus vannamei*

Mr. Dam Chaimongkon



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Thesis Title	PRODUCTION OF TWO SPECIFIC DOUBLE-STRANDED RNAS AGAINST YELLOW HEAD AND WHITE SPOT SYNDROME VIRUSES IN *Litopenaeus vannamei*
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ดามพ์ ชัยมงคล : การผลิตอาร์เอ็นเอสายคู่จำเพาะสองชนิดที่ต้านไวรัสหัวเหลืองและไวรัสตัวแดงดวงขาวใน *Litopenaeus vannamei* (PRODUCTION OF TWO SPECIFIC DOUBLE-STRANDED RNAS AGAINST YELLOW HEAD AND WHITE SPOT SYNDROME VIRUSES IN *Litopenaeus vannamei*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร.วันชัย อัศวลาภสกุล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร.พงโสภี อัตศาสตร์, 89 หน้า.

กุ้งขาวเป็นสัตว์เศรษฐกิจสำคัญที่สร้างมูลค่าการส่งออกให้กับประเทศไทย อย่างไรก็ตามประเทศต้องสูญเสียรายได้อย่างมาก สืบเนื่องมาจากการระบาดของโรคที่เกิดจากไวรัสหัวเหลืองและไวรัสตัวแดงดวงขาว ในปัจจุบันได้มีการนำเทคนิค RNA interference (RNAi) มาใช้ในการควมคุมการแพร่ระบาดของไวรัสทั้งสองชนิดนี้ โดยการใช้อาร์เอ็นเอสายคู่ที่มีความจำเพาะกับยีน protease ของไวรัสหัวเหลือง และยีน ribonucleotide reductase small subunit (rr2) ของไวรัสตัวแดงดวงขาว ซึ่งให้ผลในการยับยั้งการติดไวรัสทั้งสองชนิดอย่างมีประสิทธิภาพ แต่ด้วยความจำเพาะในระดับนิวคลิโอไทด์ของเทคนิค RNAi นี้ ทำให้อาร์เอ็นเอสายคู่หนึ่งชนิดสามารถยับยั้งไวรัสได้เพียงชนิดเดียว ดังนั้นจึงจำเป็นที่จะต้องทำการสร้างอาร์เอ็นเอสายคู่เพียงชนิดเดียวแต่สามารถยับยั้งไวรัสได้ทั้งหัวเหลืองและตัวแดงดวงขาว ในการศึกษานี้ได้ทำการสร้างพลาสมิด 2 รูปแบบเพื่อให้สามารถผลิตอาร์เอ็นเอสายคู่ได้ 2 แบบคือ one-stem และ two-stem โดยอาร์เอ็นเอสายคู่นี้จะมีเป้าหมายทั้งต่อยีน protease ของไวรัสหัวเหลืองและต่อยีน rr2 ของไวรัสตัวแดงดวงขาว เมื่อทำการผลิตอาร์เอ็นเอสายคู่จากพลาสมิดทั้งสองในเซลล์แบคทีเรีย พบว่า one-stem มีสัดส่วนตามน้ำหนักของ อาร์เอ็นเอสายคู่ต่อยีน protease ต่ออาร์เอ็นเอสายคู่ต่อยีน rr2 เป็น 1:1 แต่ขณะที่ two-stem เป็น 1:4 เมื่อเปรียบเทียบประสิทธิภาพของ one-stem และ two-stem ในการยับยั้งไวรัสทั้งสองชนิด โดยทำการฉีดอาร์เอ็นเอสายคู่ทั้งสองรูปแบบเข้าสู่กระแสเลือดของกุ้งก่อนทำให้กุ้งติดไวรัส พบว่าอาร์เอ็นเอสายคู่จากทั้งสองรูปแบบสามารถยับยั้งการเพิ่มจำนวนของไวรัสหัวเหลืองและไวรัสตัวแดงดวงขาวในกุ้งได้ (ทั้งติดเชื้อไวรัสแต่ละชนิดและติดเชื้อไวรัสสองชนิดพร้อมกัน) แต่อาร์เอ็นเอสายคู่ในรูปแบบ one-stem ให้ผลในการยับยั้งไวรัสตัวแดงดวงขาวได้ดีกว่า two-stem และพบอีกว่าในการยับยั้งไวรัสหัวเลือง อาร์เอ็นเอสายคู่รูปแบบ one-stem สามารถคงประสิทธิภาพในการยับยั้งไวรัสได้ดีถึงแม้จะอยู่ในกระแสเลือดของกุ้งนานถึง 5 วันก่อนที่จะได้รับไวรัส ในขณะที่ two-stem จะสูญเสียประสิทธิภาพไป ผลที่ได้จากการศึกษานี้จึงนับเป็นข้อมูลที่จำเป็นในการออกแบบเพื่อให้ได้อาร์เอ็นเอสายคู่ที่มีประสิทธิภาพในการยับยั้งการแสดงออกของยีนหลายเป้าหมายในกุ้ง และนับเป็นแนวทางเพื่อนำไปใช้ควบคุมไวรัสหัวเหลืองและตัวแดงดวงขาวในระบบฟาร์มเลี้ยงกุ้งต่อไป

ภาควิชา	จุลชีววิทยา	ลายมือชื่อนิสิต	----------
สาขาวิชา	จุลชีววิทยาทางอุตสาหกรรม	ลายมือชื่อ อ.ที่ปรึกษาหลัก	----------
ปีการศึกษา	2558	ลายมือชื่อ อ.ที่ปรึกษาร่วม	----------

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DAM CHAIMONGKON: PRODUCTION OF TWO SPECIFIC DOUBLE-STRANDED RNAS AGAINST YELLOW HEAD AND WHITE SPOT SYNDROME VIRUSES IN *Litopenaeus vannamei*. ADVISOR: ASST. PROF. WANCHAI ASSAVALAPSAKUL, Ph.D., CO-ADVISOR: PONGSOPEE ATTASART, Ph.D., 89 pp.

Pacific white shrimp (*Litopenaeus vannamei)* is one of important aquatic animals in Thailand making a high income per year. However, outbreaks of diseases caused by yellow head virus (YHV) and white spot syndrome virus (WSSV) infection result in tremendous economic loss. Nowadays, dsRNA-mediated RNAi becomes the most promising technique to control these viruses. It has been shown that the best target genes for efficient inhibition of YHV and WSSV are protease and ribonuleotide reductase small subunit (rr2), respectively. With the sequence specificity of this RNAi technique, one dsRNA can suppress only one virus. Therefore, production of one molecule of dsRNA (multi-targeted dsRNA) to effectively inhibit both YHV and WSSV is needed. In this study, two types of recombinant plasmid (pET17b-dspro-rr2 and pET17b-dspro-dsrr2) that can produce two different forms of multi-targeted dsRNA (one-stem and two-stem) in *E. coli* were constructed. The multi-target dsRNA was designed specifically to both protease gene of YHV and rr2 gene of WSSV. After production of dsRNA in HT115 for 4 hours, weight ratio of dsRNA-protease and dsRNA-rr2 from the one-stem was 1:1 while that of two-stem was 1:4. The potency of each multi-targeted dsRNA on viral inhibition and shrimp mortality reduction were investigated in shrimp. Shrimp were injected with one-stem or two-stem into hemolymph before receiving YHV or WSSV. The results showed that one-stem and two-stem could inhibit both viruses (either separate or dual infection) however one-stem was more effective than two-stem when shrimp were infected by WSSV. One-stem could be maintained in shrimp hemolymph at least 5 days without loss of inhibitory effect whereas two-stem lost suppression level against YHV when dsRNA was 5 days pre-injected into shrimp. This study provides an essential information to design and achieve an effective multi-targeted dsRNA for multiple genes silencing in shrimp and reveals the potential anti-YHV and WSSV strategy for further application in the shrimp farm.

Department:	Microbiology	Student's Signature .....
Field of Study:	Industrial Microbiology	Advisor's Signature .....
Academic Year:	2015	Co-Advisor's Signature .....

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LIST OF ABBREVIATIONS

°C	degree Celsius
µg	microgram (s)
µl	microliter (s)
bp	base pair (s)
cDNA	complementary deoxyribonucleic acid
DNA	deoxyribonucleic acid
dsDNA	double-stranded DNA
dsRNA	double-stranded RNA
E. coli	*Escherichia coli*
kDa	kilodalton (s)
kb	kilobase pair (s)
L	liter (s)
mg	milligram (s)
mM	millimolar
mRNA	messenger RNA
No.	number
OD_{600}	optical density at 600 nanometer
ORFs	open reading frames
p.i.	post injection
PCR	polymerase chain reaction
ppt	part per thousand
rbs	ribosome binding site
RdRp	RNA dependent RNA polymerase
RISC	RNA-induced silencing complex

LIST OF ABBREVIATIONS (cont.)

RNA	ribonucleic acid
RNAi	RNA interference
RNase A	ribonuclease A
RNase III	ribonuclease III
rpm	revolution per minute
rr2	ribonucleotide reductase small subunit
RT-PCR	reverse transcription-polymerase chain reaction
sec	second (s)
shRNA	short hairpin ribonucleic acid
siRNA	small interfering RNA
ssRNA	single-stranded RNA
v/v	volume by volume
w/v	weight by volume
WSSV	white spot syndrome virus
YHV	yellow head virus

CHAPTER I
INTRODUCTION

Penaeid shrimp farming is an important aquaculture industry in Thailand, which makes a high value of income per year. Since 2012, the shrimp production was dramatically decreased from outbreaks of diseases caused by bacteria or viruses. Among shrimp viruses, the yellow head virus (YHV) and white spot syndrome virus (WSSV), causing high shrimp mortality rate (70-100%) within a very short period of time (3-10 days) after the onset of symptom, are the most devastating pathogens [1, 2].

To control these diseases, RNA interference (RNAi) is the most promising technique nowadays. It is known as post-transcriptional silencing mechanism activated by double-stranded RNA (dsRNA). The dsRNA will be cleaved by Dicer to generate small interfering RNA or siRNA and then loaded into RNA-induced silencing complex (RISC). The RISC will keep the anti-sense strand of siRNA for guiding the protein complex to specific mRNA target, resulting in gene silencing by nucleolytic degradation of the targeted mRNA. It leads to no protein synthesis [3]. Introduction of long dsRNA that has sequence specifically to viral gene into shrimp prior to virus challenge shows suppression of that particular virus as well as reduction of shrimp mortality [4]. In addition, it has been shown that the best target genes for efficient inhibition of YHV and WSSV are the YHV protease [5-7] and WSSV ribonuleotide reductase small subunit (rr2) [8], respectively.

Nowadays, outbreaks of YHV and WSSV (either separate or co-infection) have been recorded frequently (personal communication). Moreover, with the sequence specificity of this RNAi technique, one dsRNA can suppress only one virus. Therefore, production of one molecule of dsRNA to effectively inhibit both YHV and WSSV (multi-targeted dsRNA) is needed. Two types of recombinant plasmid were constructed in order to produce two different forms of multi-targeted dsRNA in *Escherichia coli* (*E. coli*). The multi-targeted dsRNA was designed specifically to both protease gene of YHV and rr2 gene of WSSV. The potency of each multi-targeted dsRNA on YHV and WSSV inhibition and shrimp mortality reduction were investigated in pacific white leg shrimp (*Litopenaeus vannamei*).

CHAPTER II
LITERATURE REVIEW

2.1 Background of *Litopenaeus vannamei*

Litopeaeus vannamei, formerly called *Penaeus vannamei*, is known as the white leg shrimp or pacific white shrimp. It is classified into an order Decapoda and genus *Litopenaeus* following the taxonomic serial number 551682 [9, 10]. In the early of 1990s, the specific pathogen-free *L. vannamei* stocks and subsequent breeding to get the Taura syndrome virus resistant shrimp have been developed in the United States of America. With their potential higher profit and reliability production, the cultivation of shrimp in Thailand was changed from *P. monodon* to *L. vannamei* since 2002-2003 [11, 12].

2.2 The viral pathogen of *Litopenaeus vannamei*

Shrimp farming industry in Thailand has suffered from several viral diseases. Among them, two viruses which are considered as the causative pathogens of highest mortality rate in shrimp are yellow head virus and white spot syndrome virus.

2.2.1 Yellow head virus (YHV)

Yellow head virus (YHV) is identified firstly in Thailand in 1990 [13]. It causes yellow head disease in penaeid shrimp. The infected shrimp shows cellular necrosis in connective tissue, haemocytes, nerves, abdominal muscle, midgut, hepatopencreas, heart, gills and lymphoid organs resulting in weakness of its immune response. Outbreak of this disease leads to high and rapid shrimp mortality rate in the farm and consequently high economic loss.

YHV is classified in the genus *Okavirus*, family *Roniviridae* and order *Nidovirales* [14, 15]. Its virion is an enveloped bacilliform (approximately 40-50 x 150-210 nm) with prominent spike-like projection and inner helical nucleocapsid [16, 17]. It has a linear single stranded RNA genome of positive polarity containing 5' cap structure and 3' poly (A) tail. It composes of only four open reading frames (ORFs) dividing to ORF-1a, ORF-1b, ORF-2, ORF-3 and ORF-4, respectively. The largest ORF-1a and ORF-1b are overlapped occupying two-thirds of the genome. The

translation of ORF-1b is directed by fraction of ribosome using initiator AUG from the ORF-1a by -1 ribosomal frame shift (RFS) signal. The ORF-1a encodes as long polypeptide, which requires protease cleavage for generation of functional ORF-1a [18]. The ORF-1b encodes replication enzymes including helicase, RNA dependent RNA polymerase (RdRp). The ORF-2 encodes a single 20 kDa nucleocapsid protein (p20) which shows predominance of basic properties and high proportion of proline residues. The ORF-3 encodes the viral structural transmembrane glycoprotein (gp116 and gp64) synthesized as polyprotein then underwent post-translational proteolytic cleavage and glycosylation. Lastly, function of the ORF-4 is still not known [19, 20].

2.2.1.1 YHV replication cycle

The replication cycle of YHV starts after the attachment of the viral particles to cell membrane and entering into the cytoplasm of host cell by receptor mediated endocytosis. It is uncoated in the endosome complex and released into the cytoplasm by breaking down of endosomal membrane. Then, the released viral genome (positive RNA strand) might be used as the template for constructing negative RNA strand, which would in turn act as the template for a new positive strand. Further replication could produce multiple copies of positive RNA strands that could also act as mRNA for the translation of capsid proteins on the non-membrane bound ribosomes of the infected cells. Eventually, the completely enveloped viral particles are packaged in the secretory vesicles and released by exocytosis at the cell membrane [16].

2.2.2 White spot syndrome virus (WSSV)

WSSV was first detected in Taiwan in 1992, and it spreads to almost all Asian countries since 1993 in *P. japonicas* in Japan [21] and in 1994 in Thailand. It has a wide host range including crustaceans especially shrimp. It causes white spot disease (WSD) in shrimp. The infected shrimp shows reddish and pinkish-red discoloration of the body including obvious white spots inside the carapace, appendages and the body. Outbreak of this disease leads to high mortality in cultured shrimp up to 100% within 3-10 days [2].

WSSV is a double stranded DNA (dsDNA) virus classified into genus *Whispovirus* and family *Nimaviridae* [14, 15]. The virion is enveloped rod shape

containing the nucleocapsid ranging from 80-120 x 250-380 nm and 330-350 x 58-67 nm [22]. The dsDNA genome has a circular shape with size about 300 kb. It contains 181 open reading frames and 36 ORFs have already been identified to encode functional proteins [23]. According to their temporal expression profile, WSSV genes can be classified into three groups; immediate-early (IE), early and late genes [24]. IE genes are the first viral genes to be expressed during infection and their transcription does not require *de novo* viral protein synthesis. They often encode regulatory proteins that are essential for the initiation of viral primary infection or the switch from latency to lysis [25]. Early genes usually encode for an essential protein that are the key factors responsible for viral DNA replication as well as virus proliferation such as ribonucleotide reductase large (rr1) and small (rr2) subunits [26]. The ribonucleotide reductase genes encode enzymes that are involved in nucleotide metabolism by reducing ribonucleotides into deoxyribonucleotides as immediate precursors of DNA [27]. However, almost half of the WSSV late genes are expressed after the onset of viral DNA replication. They usually involve the expression of structural proteins that are generally participated during virus–host interactions such as cell entry and systemically spread [28].

2.2.2.1 WSSV replication cycle

Attachment of envelope proteins of the WSSV virion to specific cell receptor is required before entering into the host cell. The WSSV envelope protein probably fuses with the endosomal membrane then the naked nucleocapsid is released to the cytoplasm. Then the naked WSSV nucleocapsid attaches to the nuclear membrane and the WSSV genome is eventually released into the nucleus, where replication of the viral DNA and transcription of the viral genes occur. Before releasing out of the cell, the nucleocapsids are assembled in the virogenic stroma. A viral nucleosome is also observed as a filamentous structure in the virogenic stroma. This structure contains proteins that will form the nucleocapsid. New WSSV particles are assembled in the nucleus within an electron-dense inclusion. The empty envelopes are filled with a nucleocapsid. In cytoplasm, organelles become disintegrated and the cellular and nuclear membranes are disrupted. The WSSV virions are completely

formed and ready to be released from the disrupted cell to begin the cycle in other susceptible cells [29].

2.3 RNA interference (RNAi)

RNAi is an innate immune response firstly described in nematode (*Caenorhabditis elegans*) in 1998 [3]. The RNAi pathway has been identified in various kingdoms, however they are called in different names such as RNAi in animal, Post Transcription Gene Silencing in plant and quelling in fungi [4, 30, 31].

2.3.1 RNAi mechanism

The mechanism of RNAi can be divided into two main steps: the initiation step and the effector step. In the initiation step, long dsRNA or short dsRNA is cleaved by Dicer, a Ribonuclease III (RNase III) enzyme, to generate small interference RNA (siRNA) of 21-23 nucleotides with 5' phosphates and 2 nucleotides overhang at 3' end [32]. For an effective step, siRNA is incorporated into RNA induced silencing complex (RISC) and subsequently unwinded by helicase activity of RISC leaving only the guide strand of siRNA. The 5' end of the guide siRNA bound to the Piwi domain (possessing RNase H activity) and 3' OH bound to PAZ domain (bind to single stranded RNA). The target mRNA is recognized by the guide siRNA and forming of the RNA-RNA duplex. Finally, the mRNA target will be degraded by RNase H endonuclease of RISC [33] (Figure 1).

2.3.2 RNAi for antivirus in shrimp

The RNAi becomes a powerful technique to counteract viral infection in shrimp [34, 35]. The viral replication of both RNA and DNA viruses can be effectively suppressed by pre-introduction of specific dsRNA. Consequently, the shrimp mortality caused by these viruses infection is dramatically reduced. The dsRNA can be designed specifically to the viral gene (structural gene or non-structural gene) or corresponding to an endogenous gene of shrimp (viral transport gene or viral receptor gene) (Table 1).

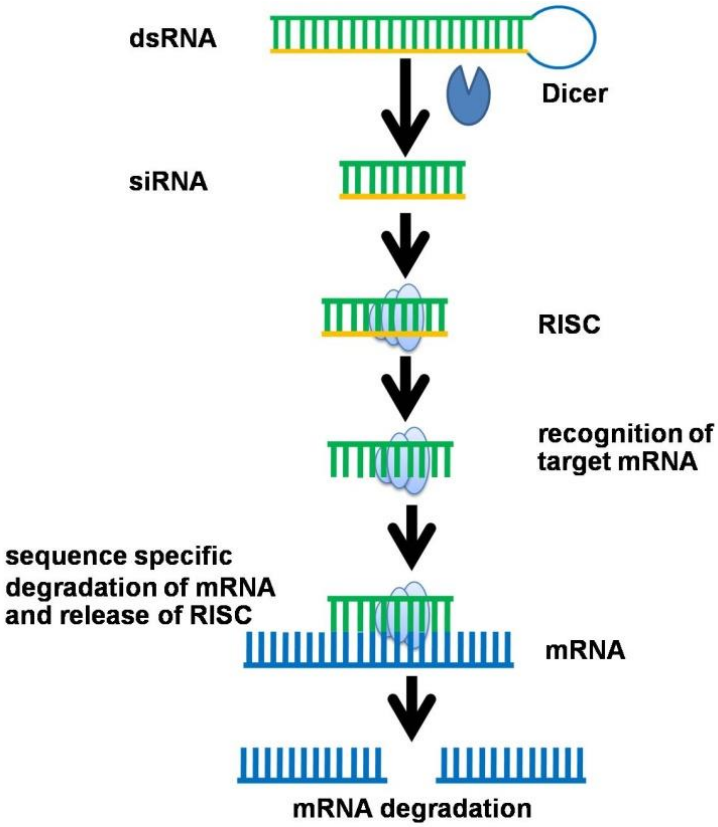


Figure 1 RNA interference pathway

Long dsRNA or short dsRNA is processed to generate ~ 21-23 nt siRNA by RNase III enzyme Dicer. The siRNA is then assembled into RNA induced silencing complex (RISC). The antisense strand helps the RISC to find its target mRNA which results in inhibition of gene expression by mRNA cleavage (Modified from [36]).

Table 1 RNAi application for anti-virus in shrimp

Virus	Target gene	dsRNA or siRNA	Result	Reference
YHV	Protease, Helicase, Polymerase and gp116	dsRNA	Protease was the best target gene for inhibiting YHV replication in shrimp primary cell culture.	[6]
	Protease	dsRNA	The inhibitory effect of dsRNA can last for at least 5 days in shrimp.	[7]
	Protease	dsRNA	Shrimp mortality was reduced from 100 to 5%.	[5]
	RdRp	dsRNA	Shrimp mortality was reduced from 100 to 30%.	[37]
	*Pm*YRP65 receptor	dsRNA	Shrimp mortality was reduced from 100 to 5%.	[38]
WSSV	DNA pol, rr2, and vp28	dsRNA	dsRNA-DNA pol, rr2 and vp28 and showed 44, 78 and 85% reduction of shrimp mortality, respectively.	[34]
	vp15 and vp28	siRNA	siRNA-vp15 and vp28 showed 42 and 50% reduction of shrimp mortality, respectively.	[39]
	vp28	siRNA	Shrimp mortality was reduced from 93 to 75%.	[40]
	DNA pol, rr2, TK-tmk, vp24 and vp28	siRNA	siRNA-DNA pol, rr2, TK-tmk and vp28 showed 33% reduction of shrimp mortality but siRNA-vp24 showed 17% reduction.	[41]
	vp28, vp281 and protein kinase (PK)	dsRNA	dsRNA-vp28,vp281 and PK showed 100, 33.3 and 93.7% reduction of shrimp mortality, respectively.	[42]
	ie1,ie3, rr2, DNA pol, vp26, and vp28	dsRNA	dsRNA-rr2 was the best target to suppress WSSV and showed 95% reduction of shrimp mortality.	[8]
	vp28, vp26	dsRNA	dsRNA-vp28 and vp26 showed 87 and 79% reduction of shrimp mortality, respectively.	[43]
	*Lv*Toll	dsRNA	Shrimp mortality was reduced from 86.7 to 73.3%.	[44]
	vp28, vp281, rr1 and DNA pol	dsRNA	dsRNA-vp28, vp281, rr1 and DNA pol showed 90, 66.7, 93.3 and 77.6% reduction of shrimp mortality, respectively.	[45]
	*Lv*STAT	dsRNA	Shrimp mortality was reduced from 78 to 20 %.	[46]
YHV and WSSV	*Pm*Rab7	dsRNA	dsRNA-Rab7 can inhibit YHV and WSSV replication in shrimp.	[47]

According to the list of target genes and their efficacy for viral inhibition as shown in the table 1, the best target genes for efficient inhibition of YHV or WSSV are the YHV protease or WSSV ribonucleotide reductase small subunit (rr2), respectively.

2.4 Multi-targeted shRNAs

As the sequence specificity of the RNAi technique, one shRNA/dsRNA can suppress only one virus/gene. Therefore, production of shRNAs/dsRNA that can inhibit more than one gene/gene is required. To simultaneously knock down multiple genes/viruses, four different approaches have been demonstrated.

First, mixture of separate expression vectors was used for co-expression of an individual shRNA for simultaneous gene suppression (Figure 2 A) [48]. Second, multiple shRNAs were produced from a vector containing more than one shRNA expression cassette (Figure 2 B) [48-59]. For example, Ma and colleague [53], have constructed two multiple-shRNAs expressing plasmids (pMuslttishvp2/2 and pMuslttishvp6/7) to generate two shRNAs for suppression of vp2 of grass carp reovirus (GCRV) type 1 and 3 and suppression of vp6 of GCRV type 1 and vp7 of GCRV type 3, respectively. These two shRNAs can effectively inhibit the replication of the GCRV type 1 and 3 simultaneously. Third, a recombinant plasmid contained one expression cassette but the expressed shRNA can target to more than one genes. For instance, a plasmid (pCWN11) has constructed to expresses multi-targeted shRNA specific to the VP1 genes of Foot-and-mouth disease (FMDV) serotype A, Asia I and O (Figure 2 C). This shRNA can inhibit the replication of three serotypes of FMDV in BHK-21 cells and suckling mice [60]. The last one, multi-targeted shRNA was produced based on a single transcript composing of different hairpin domains (Figure 2 D) [48, 61]. Junn and colleague has constructed an expression vector (pAdlox (k)) under cytomegalovirus (CMV) promoter for expressing one single transcript but can fold linked multiple shRNAs. This multi-targeted shRNA can simultaneously suppress X-chromosome-linked inhibitor of apoptosis protein (XIAP), Akt and Bcl-2 of adenovirus leading to effectively suppress the replication of adenovirus [61].

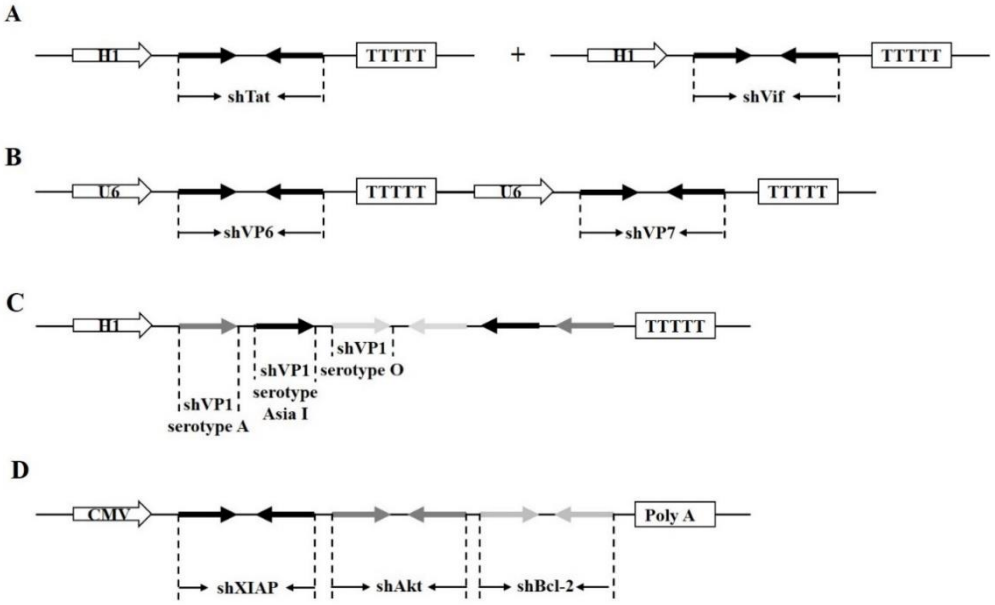


Figure 2 Different cloning pattern for producing multi-targeted shRNAs

The multiple shRNAs can be expressed from separate plasmid (A) or a plasmid containing multiple shRNA expression cassettes (B). The multi-targeted shRNA can be synthesized from a recombinant plasmid containing 1 expression cassette with one inverted repeat of multiple targeted sequences (C) or a recombinant plasmid containing 1 expression cassette with multiple inverted repeats of different target gene sequences (D) (Modified from [48, 53, 60, 61]).

CHAPTER III
MATERIAL AND METHODS

3.1 Bacterial host strains

Escherichia coli strain DH5α: *supE*44, Δ*lac*U169 (Φ80 *lac*Z ΔM15), *hsdR*17, *recA*1, *endA*1, *gyrA*96, *thi*-1, *relA*1 (obtained from GIBCO BRL) was used as a host cell for recombinant plasmid propagation.

Escherichia coli strain HT115: *F^-*, *mcrA*, *mcrB, IN(rrnD-rrE)1*, *lambda$^-$*, *rnc14:: Tn10(DE3 lysogen:lacUV5 promoter-T7 polymerase)* was used as a host cell for dsRNA production. This strain is deficient in the activity of Ribonuclease III that specifically cleaves dsRNA.

3.2 Bacterial growth mediums

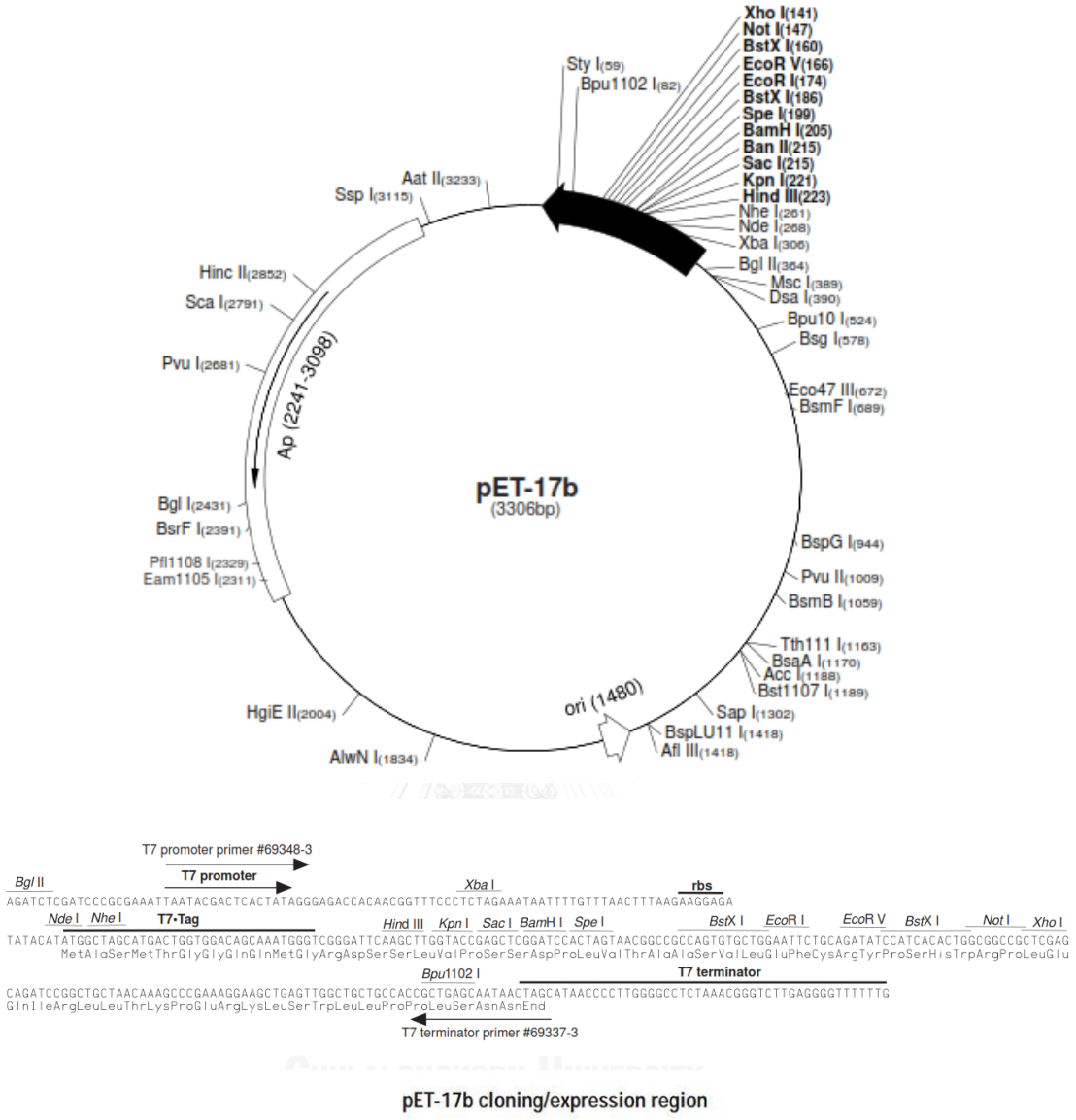
LB (Luria-Bertani) broth medium contains 1% (w/v) bacto-peptone, 0.5% (w/v) bacto-yeast extract and 0.5% (w/v) NaCl.

LB agar plate is LB broth containing 1% (w/v) bacto-agar.

2XYT medium consists of 1% (w/v) bacto-peptone, 1% (w/v) bacto-yeast extract and 0.5% (w/v) NaCl.

3.3 Plasmid DNA vector

pET-17b (Novagen) is an expression plasmid used for dsRNA synthesis. It contains multiple cloning sites for dsRNA expression cassette construction (Figure 3).



pET-17b cloning/expression region

Figure 3 Physical map of pET17b vector (Novagen)

The pET17-b vector contains T7 promoter, ribosome binding site (rbs), multiple cloning site, T7 terminator and ampicillin resistance gene

3.4 Virus sources

The YHV lysate was kindly provided by Mr. Poohrawind Sanitt [62].

The WSSV lysate was prepared from WSSV infected gill of *P. monodon*, size about 15-20 g. Fifty milligrams of gill tissue were ground in 500 µl of TN buffer (20 mM Tris-HCL, 400 mM NaCl, pH 7.4). The homogenate was centrifuged at 10,000 xg for 10 min at 4°C. The supernatant was transferred to a new 50 ml confocal tube and diluted with 150 mM NaCl at 1: 10 (v/v). Then, the solution was filtered through at 0.45 µm filter. Finally, the virus solution was aliquot and stored at -80°C until use.

3.5 Shrimp specimens

Post larva of white leg shrimp (*Litopenaeus vannamei*) were purchased from farm in Chonburi province. They were cultured in 500 L tank with seawater at 10 ppt salinity until their body weight reached 400 mg, an appropriate size in this work.

3.6 Restriction Enzymes

All restriction enzymes (FastDigest® enzyme) used in this thesis (table 2) were purchased from Fermentas.

Table 2 List of restriction enzymes

Restriction Enzyme	Recognition sequence (5' → 3')
*Hin*d III	a↓agctt
Kpn I	ggtac↓c
Nde I	ca↓tatg
Xba I	t↓ctaga
Xho I	c↓tcgag

↓ indicates the cleavage site

3.7 Oligonucleotide primers

The synthetic oligonucleotide primers were synthesized by Sigma-Aldrich® or Bio Basic Inc. All details of primers used in this thesis were shown in table 3

Table 3 List of oligonucleotide primers

No	Name	Sequence (5' → 3')	Size (bp)	Tm (°C)
1	Actin-F	ATGGCATCTCGCAAGAAGATT	21	65
2	Actin-R	TTAGCAAGAGCATGCATCCTG	21	65
3	YHV-Hel-F	CAAGGACCACCTGGTACCGGTAAGAC	26	72
4	YHV-Hel-R	GCGGAAACGACTGACGGCTACATTCAC	27	76
5	VP28-F	CCGCTCGAGACTCTTTCGGTCGTGTCGGCC	30	84
6	VP28-R	GGCACCATCTGCATACCAGTG	21	68
7	rr2-*Hin*d III–F	TTTTTaagcttGGAGCAAGCCAACCAAGTG	30	63
8	rr2-loop-393-*Nde* I-R	TTTTTTTcatatgCAGGCAGGGAAACTGTGAG	32	63
9	irr2-*Kpn* I-F	TTTTTTggtaccGGAGCAAGCCAACCAAGTG	31	66
10	irr2-*Nde* I-R	AAAAAAAcatatGTTCTCCGTAGACGTTGCC	31	62
11	rr2-*Xba* I-F	TTTTTtctagaGGAGCAAGCCAACCAAGTG	30	63
12	rr2-loop-139-*Nde* I-R	GGGGGGGcatatgACAATTAGTTCCGCCAAATCG	34	69
13	YHV-*Xho* I-F	GGGGGctcgagTCAGCGGCAAATTCCTCTAC	31	71
14	YHV-*Hin*d III-R	AAAAAaagcttGCCATACCTTGGGTGGAC	29	63
15	YHV-loop 124-*Hin*d III-R	CCCCCaagctTGGTCCAGTGTCTCGCAAC	29	72
16	iYHV-*Xba* I-F	GGGGGtctagaTCAGCGGCAAATTCCTCTAC	31	69
17	iYHV-*Kpn* I-R	TTTTTTggtaccGCCATACCTTGGGTGGAC	30	66

3.8 Miscellaneous

Lambda DNA/*Hin*d III	Promega
100 bp DNA Ladder	Sib EnZyme
Needle	B-D Ultra-Fine™
Ampicillin	Bio Basic Inc
Tetracycline	Bio Basic Inc
Isopropyl-β-D-thiogalactopyranoside (IPTG)	USB
DEPC	Bio Basic Inc

3.9 Plasmid DNA extraction

3.9.1 Cetyltrimethylammonium bromide (CTAB) plasmid DNA mini-preparation

A single colony of *E. coli* DH5α was inoculated into 3 ml LB medium containing 100 µg/ml ampicillin and incubated at 37°C with shaking. Next, the bacterial cell suspension was transferred to a 1.5 ml microtube and centrifuged at 2,700 xg for 1 min at room temperature. The cell pellet was resuspended in 350 µl of STET buffer (8% sucrose, 0.1% Triton X-100, 50 mM EDTA, and 50 mM Tris, pH8.0) and subsequently added with 25 µl of lysozyme (10 mg/ml). Then, the mixture was gently mixed by inversion and incubated at room temperature for 5-10 min. After incubation, the mixture was boiled at 100°C for 45 sec and immediately quick cooled at on ice for 30 sec then subsequently centrifuged at 17,900 xg for 10 min at room temperature. The cell debris was removed using toothpick. Next, 37.5 µl of 5% (w/v) CTAB was added into solution and mixed by vortex. The mixture was centrifuged at 17,900 xg for 10 min at room temperature. The supernatant was discarded and DNA pellet was dissolved in 300 µl of 1.2 M NaCl containing 10 µg/ml RNaseA and then incubated at 37°C for 30 min. After incubation, an equal volume of chloroform was added and subsequently mixed by vortex. Then, the mixture was centrifuged at 17,900 xg for 10 min at room temperature. The upper aqueous phase was transferred to a new 1.5 ml microtube and 2 volumes of absolute ethanol were added. The solution was mixed by vortex, incubated at room temperature for 30 min and centrifuged at 17,900 xg for 10 min at room

temperature. The DNA pellet was rinsed with 200 µl of 70% (v/v) ethanol and centrifuged at 17,900 xg for 10 min at room temperature. Finally, the DNA pellet was air dried and then dissolved in distilled water.

3.10 Total RNA extraction from shrimp infected tissue by Ribozol

Gill tissue from the YHV-infected moribund shrimp (approximately 50 mg) was ground in 500 µl Ribozol™ reagent (Amresco®) and incubated at room temperature for 5 min. Then, 200 µl of chloroform was added and subsequently mixed by vortex for 15 sec. The solution was incubated at room temperature for 5 min and then centrifuged at 12,000 xg for 15 min at 4°C. The aqueous phase was transferred to a new 1.5 ml microtube. Total RNA was precipitated by mixing with 250 µl of isopropanol and stored at -20°C for 10 min. The solution was subsequently centrifuged at 12,000 xg for 10 min at 4°C. The RNA pellet was washed with 500 µl of 70% (v/v) ethanol followed by centrifuged at 7,500 xg for 5 min at 4°C. Then, the RNA pellet was air dried and resuspended in RNase-free sterile distilled water (DEPC-treated water). The RNA solution was completely dissolved by incubation at 55°C for 10 min. Finally, the dissolved RNA was stored at -20°C until used.

The concentration of extracted RNA was determined by measuring the absorbance at 260 nm (A_{260}). The Total RNA concentration could be calculated by the following equation.

$$\text{RNA concentration (µg/ml)} = 40 \times A_{260} \times \text{dilution factor}$$

Ratio of A_{260}/A_{280} was applied to determine the RNA purity. The RNA sample with the ratio about 1.8-2.0 was used in the next process.

3.11 First strand cDNA synthesis

Total RNA (1 µg) was used for first strand cDNA synthesis in the mixture containing 2 µM of random primer and DEPC-treated sterile distilled water. After heating the mixture at 70°C for 5 min and the solution was immediately quick cooled on ice, the primer was allowed to anneal with mRNA for 5 min. After that, 1X Improm-II™ reaction buffer, 0.5 mM dNTPs, 1 µl of Improm-II™ Reverse transcriptase, and DEPC-treated sterile distilled water were added into the previous solution. The mixture was gently mixed and processed at 25°C for 5 min, 42°C for 60 min and 72°C for 15

min to synthesize first strand cDNA. Two microliters of synthesized cDNA were used for PCR amplification.

3.12 Total DNA extraction from shrimp infected tissue by DNAzol

Ten milligram of gill tissue was collected from the WSSV-infected shrimp and ground in 500 µl DNAzol® reagent (Invitrogen). Then, the solution was centrifuged at 10,000 xg for 10 min at 4°C and the aqueous phase was transferred into a new 1.5 ml microtube. Total DNA was precipitated by adding 250 µl of absolute ethanol and stored at room temperature for 5 min. The mixture was centrifuged at 10,000 xg for 10 min at room temperature. The DNA pellet was washed twice with 500 µl of 70% (v/v) ethanol followed by centrifuged at 10,000 xg for 5 min at room temperature. Next, the ethanol was removed and the DNA pellet was air dried and resuspended in RNase-free sterile distilled water (DEPC-treated water). The DNA solution was completely dissolved by incubation at 55-60°C for 10 min. Finally, the dissolved DNA was stored at -20°C until used.

The concentration of extracted DNA could be by measuring the absorbance at 260 nm (A_{260}). The Total DNA concentration could be calculated by the following equation.

$$\text{DNA concentration (µg/ml)} = 50 \times A_{260} \times \text{dilution factor}$$

Approximately 200 ng of DNA was further used as template for PCR analysis.

3.13 Polymerase chain reaction (PCR)

To amplify the DNA target, the reaction was performed by using 2 µl of cDNA, or 200 ng of total extracted DNA, or part of single colony of transformed clones was picked by a sterile toothpick and resuspended in the mixture of PCR reaction, or 5-10 ng of plasmid DNA. The components for PCR reaction in a total volume 25 µl contain 0.2 µM of each primer, 0.4 mM of dNTPs, 1X PCR buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl, pH 8.8, 2 mM $MgSO_4$ and 0.1% Triton X-100), 1 unit of Vent DNA polymerase, and sterile distilled water. In case of *Taq* DNA polymerase (Homemade), the PCR condition was reduced of each primer to 0.2 µM, 0.4 mM of dNTPs, 2 mM of $MgCl_2$,1X PCR buffer (75 mM Tris-HCl, pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20), 1 unit of *Taq* DNA polymerase and sterile distilled water. The

temperature profile for PCR amplification was performed by holding 94°C for 2 min, denaturation at 94°C for 10 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min. Then, the temperature was held at 72°C for 5 min. Finally, the PCR product was analyzed by agarose gel electrophoresis.

3.14 Recovery and purification

3.14.1 Purification of DNA fragment from agarose gel using QIAquick Gel Extraction Kit

The desired DNA band on agarose gel was excised under long-wave length UV light. The gel slice was weighed and transferred to 1.5 ml microtube. Then, 3 volumes of buffer QG were added to 1 volume of gel (100 mg ~ 100 µl). The solution was incubated at 50°C with shaking for 10 min to dissolve the gel. After incubation, the sample was transferred to QIAquick column placed in collection tube and centrifuged at 17,900 xg for 1 min at room temperature. The flow-through solution was discarded and 500 µl of buffer QG was added to the column and subsequently centrifuged at 17,900 xg for 1 min at room temperature as described. After centrifugation, PE solution was added into column and centrifuged at 17,900 xg for 1 min at room temperature. The flow-through was removed and then the empty column was additionally centrifuged at 17,900 xg for 1 min at room temperature. To elute DNA, an appropriate volume of buffer EB was added to the center of the column placed into a new 1.5 ml microtube and incubated at room temperature for 10 min. Finally, the microtube containing column was centrifuged at 17,900 xg for 1 min at room temperature and DNA solution was collected in the microtube.

3.14.2 Purification of DNA fragment from PCR using QIAquick PCR Purification Kit (QIAGEN)

In order to purify PCR products, 5 volumes of buffer PBI was added of one volume of samples. The mixture solution was transferred to a QIAquick spin column subsequently centrifuged at 17,900 xg for 1 min at room temperature. Then the flow-through solution was removed and the column was additionally centrifuged at 17,900 xg for 1 min at room temperature. After centrifugation, the column was transferred to a new 1.5 ml microtube. To elute DNA, a suitable volume of buffer EB was added to

the center of the column placed into a 1.5 ml microtube and incubated at room temperature for 10 min. Finally, the 1.5 ml microtube containing column was centrifuged at 17,900 xg for 1 min at room temperature and DNA solution was collected in the microtube.

3.14.3 Precipitation of DNA fragment by sodium acetate (NaOAc)

To remove enzyme and buffer in DNA solution, the solution was added with sterile distilled water to 100 µl and 100 µl of chloroform then mixed vigorously. Then the mixture was centrifuged at 17,900 xg for 5 min at room temperature. After centrifugation, the supernatant was transferred into new microtube and added 100 µl of sterile distilled water into the previous tube and mixed then centrifuged again. After that the supernatant was taken and combined into the same tube before adding 10% (v/v) of 3M NaOAc pH 5.2 and 2 fold (v/v) of absolute ethanol. The solution was mixed and stored at -20°C for 20 min and centrifuged at 17,900 xg for 10 min at room temperature. The DNA pellet was washed with 200 µl of 70% (v/v) ethanol then centrifuged at 17,900 xg for 5 min at room temperature. Finally, DNA pellet was resuspended in sterile distilled water.

3.15 DNA cloning strategy

3.15.1 Restriction endonuclease digestion

The digestion reaction composing of 1 µg of plasmid DNA, 1X FastDigest® buffer, 1 µl of FastDigest® enzyme and sterile distilled water was incubated at 37°C for 5 min. In case of PCR product, the reaction containing 200 ng of PCR product, 1X FastDigest® buffer, 1 µl of FastDigest® enzyme and sterile distilled water was incubated at 37°C for 20 min. Finally the digested product was analyzed by agarose gel electrophoresis.

3.15.2 Dephosphorylation of 5' end of DNA

To remove 5'-phosphate groups from DNA, the reaction in final volume of 50 µl containing of 1-10 picomoles of DNA termini, 1X reaction buffer (0.01 M Tris-HCl pH 7.5, 0.01 $MgCl_2$ and 0.1 mg/ml of BSA), 1 U of Shrimp Alkaline Phosphatase and sterile distilled water was incubated at 37°C for 30 min. Finally, the reaction was inactivated by heating at 65°C for 15 min.

3.15.3 Blunting of DNA end

To fill in 5' overhang of DNA end, the reaction composed of 1 µg of DNA, 1X reaction buffer (67 mM of Tris-HCl pH 8.8, 6.6 mM of $MgCl_2$, 1 mM of DTT and 16.8 mM of $(NH_4)_2SO_4$), 0.1 mM of dNTPs, 1 U of T4 DNA polymerase and sterile distilled water to final volume of 20 µl was incubated at 11°C for 20 min. The reaction was then stopped by incubation at 75°C for 10 min.

3.15.4 DNA ligation (PCR product, ligated fragment/ vector)

The linearized DNA vector was joined with insert by consideration of molar ratio of both fragments at 1:4. The molar ratio was calculated based on size and concentration of vector and insert. The following equation indicated the calculation of molar ratio.

$$\text{Molar of DNA} = \frac{\text{amount of DNA (g)}}{660 \times \text{size of DNA (bp)}}$$

The ligation reaction was composed of digested plasmid vectors, purified fragments, 1X ligase reaction buffer (40 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 10 mM DTT and 0.5 mM ATP), 1 U of T4 DNA ligase and sterile distilled water in final volume of 10 µl. In case of PCR fragments of blunt-end ligation, the reaction composed of both fragments at 1:1 molar ratio, 1X Fast Digest® buffer, 0.5 mM ATP and 5 U of T4 DNA ligase in final volume of 10 µl. Finally, the ligation reaction was incubated overnight at 16°C.

3.15.5 Competent cell preparation by calcium chloride ($CaCl_2$)

A single colony of *E.coli* DH5α or HT115 was inoculated into 5 ml of LB medium and incubated at 37°C overnight with shaking at 250 rpm. The overnight culture was diluted (1:100) in LB medium 100 ml and incubated at 37°C with shaking at 250 rpm until OD_{600} reached 0.3-0.5. The culture was transferred to a 500 ml tube and was chilled on ice 10 min. Then, the culture was centrifuged at 3,000 xg for 10 min at 4°C. The supernatant was discarded and the pellet was resuspended in 50 ml of ice-cold 100 mM $CaCl_2$ and stored on ice for 10 min. The solution was centrifuged at 3,000 xg for 10 min at 4°C. After centrifugation, the pellet was resuspended in 4 ml of ice-

cold 100 mM $CaCl_2$ and stored on ice for 10 min. Next, Dimethyl sulfoxide (DMSO) was slowly added with stirring to give 7% (v/v) final concentration. Finally, 200 µl of the competent cells was aliquoted into 1.5 ml microtube, immediately frozen in liquid N_2, and stored at -80°C until used.

3.15.6 Plasmid DNA transformation by heat shock method

To transform the ligated product to the competent cells of *E. coli* DH5α or HT115 were transformed with 25 ng of plasmid DNA. The ligation reaction was diluted for 2 folds before subsequently transformed to the competent cells and added into 200 µl of competent cells. The mixture was gently mixed and chilled on ice for 30 min. Next, the mixture was incubated at 42°C for exactly 90 sec and then immediately cooled on ice for 5 min. After that, the transformed cells were diluted in LB medium to 1 ml of final volume and slowly shaked at 37°C for 60 min. Finally, 200 µl of transformed cells were spreaded onto LB agar plate containing 100 µg/mg of ampicillin and subsequently incubated at 37°C for 16-18 hours.

3.16 Recombinant clone screening

3.16.1 Rapid size screening

A single colony of each candidate clone was picked by a sterile toothpick and lysed in 30 µl of lysis buffer (100 mM NaOH, 60 mM KCl, 5 mM EDTA, 10% (w/v) D-sucrose, 0.25% (w/v) SDS and 0.05% (w/v) bromophenol blue). The lysate mixture was incubate at 37°C for 10 min and cooled on ice for 10 min. Then cooling, the mixture was centrifuged at 17,900 xg for 10 min at room temperature. Finally, 20 µl of supernatant were loaded on agarose gel.

3.16.2 DNA sequencing

The nucleotide sequence of insert DNA was determined by DNA sequencing of First BASE (Malaysia). After automated DNA sequencing, the sequences were compared with available sequences in database by Vector NTI Advance 10 (Align X).

3.17 Production of double-stranded RNAs

3.17.1 Expression of dsRNA in bacterial cells

The recombinant plasmid containing dsRNA expression cassette was transformed into *E. coli* HT115 bacterial host. Then, a single colony of *E. coli* HT115 harboring recombinant plasmid was grown in 5 ml of 2XYT medium containing 100 µg/ml of ampicillin and 12.5 µg/ml of tetracycline at 37°C overnight. The bacterial starter culture was diluted (0.5 OD_{600}/ 15 ml) with 2XYT medium containing the same antibiotic as mention earlier. The culture was incubated at 37°C until OD_{600} reached 0.4. The dsRNA was expressed by adding 0.4 mM IPTG to induce T7 RNA polymerase. Next, the bacterial culture was further incubated at 37°C for 4 hours. Finally, the bacterial cells were harvested by centrifugation at 8,000 xg for 5 min at 4°C and the supernatant was discarded then stored cells pellet at -20°C until used.

3.17.2 dsRNA extraction from bacterial cells by Ribozol

One OD_{600} of cell pellet (3.17.1) was resuspended in 50 µl of PBS containing 0.1% SDS and boiled for 2 min to break the cell then incubated on ice for 30 sec. Next, 1 µg of RNase A was added into the solution containing 1X RNase A buffer (300 mM sodium acetate, 10 mM Tris-Cl PH 7.5, and 5 mM EDTA) and added 160 µl of Ribozol™ reagent (Amresco®) then incubated at room temperature for 5 min. After that, 20 µl of chloroform was added and subsequently mixed by vortex then following the manufacturer's protocol. Finally, the total RNA solution was dissolved with 150 mM NaCl and stored at -20°C until used.

3.17.3 Total RNA extraction from bacterial cells by Ribozol

One OD_{600} of cell pellet (3.17.1) was resuspended in 130 µl of PBS containing 0.1% SDS and 160 µl of Ribozol™ reagent (Amresco®) was immediately added and mixed then incubated at room temperature for 5 min. Next, 20 µl of chloroform was added and subsequently mixed by vortex then following the manufacturer's protocol. Finally, total RNA was dissolved with 150 mM NaCl and stored at -20°C until used.

3.17.4 Verification of dsRNAs

In order to investigate the formation of dsRNAs, digestion with RNase A (specific with ssRNA) and RNase III (specific with dsRNA) ribonuclease enzyme were performed. The digestion reaction containing 1 µg of total RNA, 1X RNase III buffer (10 mM Tris-Cl, 0.1 mM $CaCl_2$ and 2.5 mM $MgCl_2$), 1X $MnCl_2$, 0.5 ng of RNase A or 0.5 U of RNase III and sterile distilled water to 10 µl of final volume was mixed with loading dye. Finally, the mixture was loaded onto agarose gel.

3.18 Viral infection in shrimp

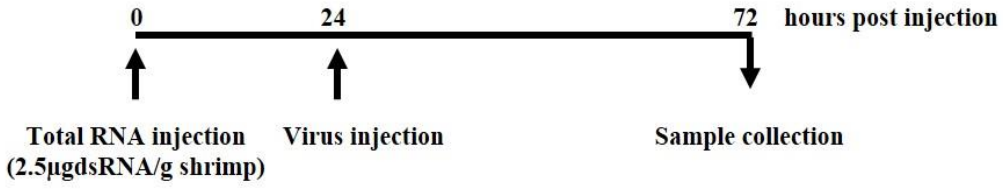
3.18.1 Introduction of YHV or WSSV into shrimp by injection

To viral infectivity test, shrimp were injected with 20 µl of YHV lysate or WSSV lysate diluted in 150 mM NaCl into haemolymph and cultured in 5 L tank with seawater at 10 ppt salinity at 28°C. The mortality was observed for 7 days.

3.19 Determination of inhibitory effect of dsRNAs on YHV and WSSV infection

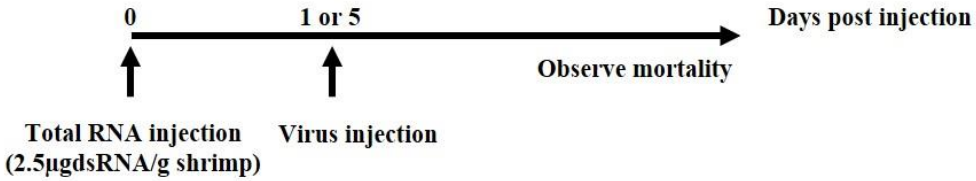
3.19.1 Suppression test

Shrimp with body weight about 400 mg were injected with 20 µl of total RNA including 1 µg of dsRNA 24 hours before challenging with virus YHV (at $5x10^{-7}$ or 10^{-6} dilution) and WSSV (at 10^{-3} dilution) (these dilutions showed 100% of viral-infected shrimp at 48 hours after injection) (first preparation). At 48 hours after viral injection, shrimp were sacrificed and their gill were isolated for RNA extraction for determination of YHV infection or DNA extraction for WSSV infection. The injection plan was shown in the diagram below.



3.19.2 Mortality test

Shrimp were injected with 1µg of dsRNA 1 or 5 days before challenging with YHV ($5x10^{-7}$ dilution) or WSSV ($5x10^{-4}$ dilution) or mixture of YHV ($2.5x10^{-7}$ dilution) and WSSV ($2.5x10^{-4}$ dilution) (these dilutions showed 80-90% death of viral-infected shrimp within 5-7 days) (second preparation). Shrimp were cultured at the same condition in 3.18.1. Shrimp mortality was observed up to 14 days. The injection plan was shown in the diagram below.



3.20 Statistical analysis

The statistical analysis of relative level of viral genome normalized by the shrimp's actin was performed by using 1 way ANOVA test. The probability (*p*) value of < 0.05 (*) and < 0.01 (**) was accepted as statistically significance.

CHAPTER IV
RESULTS

To establish a multi-targeted dsRNA for simultaneous inhibition of YHV and WSSV in shrimp, three main steps were performed. Construction of dsRNA expression plasmid was firstly performed. Secondly, the dsRNA was synthesized in *E. coli* and characterized by RNase treatment. Lastly, efficiency of the produced dsRNA for suppression of viral replication and shrimp mortality reduction was investigated.

4.1 Construction of dsRNA expression plasmid

In this study, two dsRNA expression plasmids were constructed for producing two different forms of dsRNA. One has single stem-loop whereas another has two stem-loop structures. According to the previous information [5, 7, 8, 63], the viral gene target for efficient inhibition of YHV and WSSV are protease gene and rr2 gene, respectively. Therefore, the dsRNA that has sequence specific to YHV protease gene located at 8710-9183 (474 nt) on YHV genome (Genbank accession No.FJ848675.1) (Figure 4) and specific to WSSV rr2 gene located at 146533-146987 (455 nt) on WSSV genome (Genbank accession No.AF369029.2) (Figure 5) were generated.

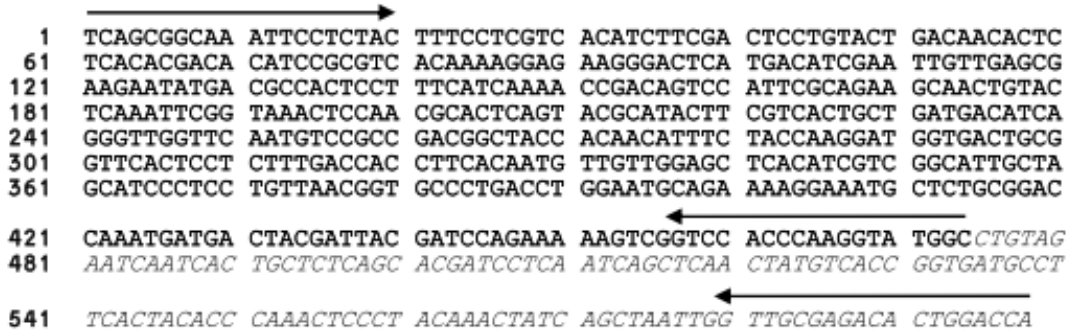


Figure 4 Nucleotide sequence of the protease region of YHV used for dsRNA synthesis

The nucleotide sequence of the protease used for dsRNA synthesis was located at 8710-9183 of YHV genome (GenBank accession No.FJ848675.1). The bold letters present the region for stem (455 bp) and the italic represents the region for loop spacer (124 bp for two-stem). Areas for primers are indicated by arrows.

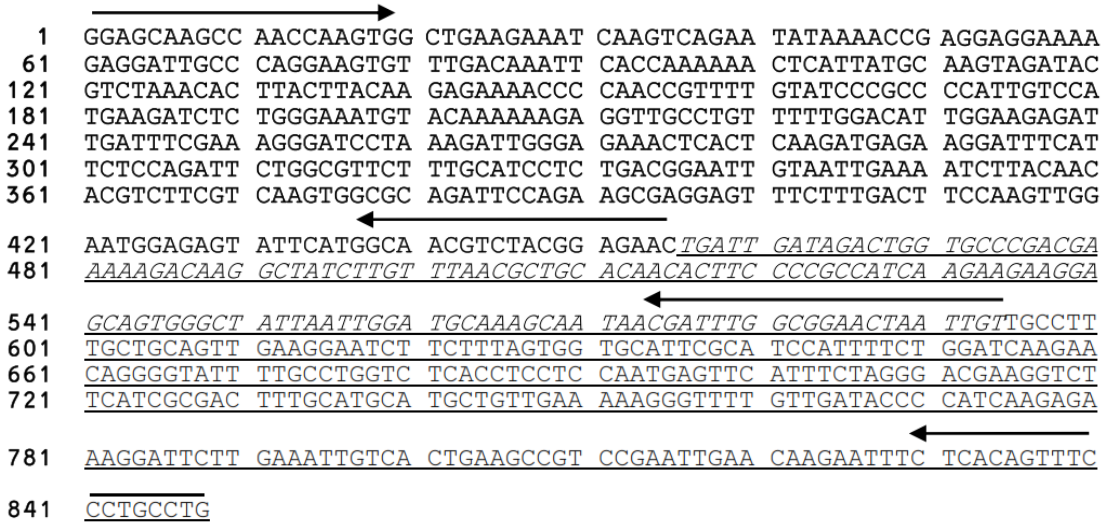


Figure 5 Nucleotide sequence of the rr2 region of WSSV used for dsRNA synthesis

The nucleotide sequence of the rr2 used for dsRNA synthesis was located at 146533-146987 of WSSV genome (Genbank accession No.AF369029.2). The bold letters present the region for stem (474 bp) and the underlined region represents the loop spacer of one-stem (393 bp) while the italic is the loop of two-stem (139 bp). Areas for primers are indicated by arrows.

4.1.1 Construction of plasmid pET17b-dspro-rr2 (one-stem)

4.1.1.1 Primer design

The specific primers were designed and synthesized (Table 3). Restriction enzyme recognition sequences were added into 5' end of each primer to provide directional cloning (Figure 6).

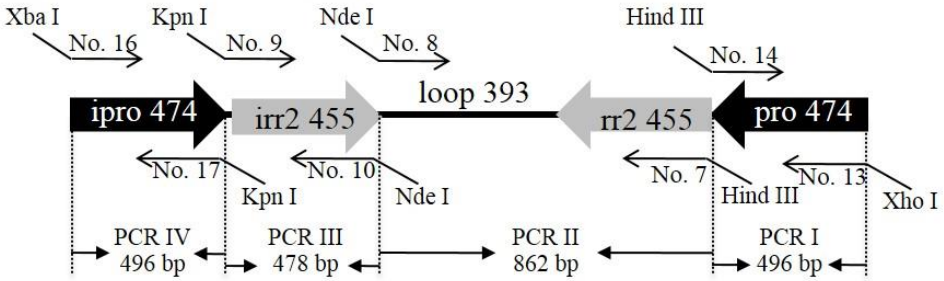


Figure 6 Diagram of an inverted repeat for one-stem construction and primers location

Primer No.13 + No.14 and No.7 + No.8 were used for the synthesis of PCR I (protease sense fragment) and PCR II (rr2 sense fragment with loop spacer), respectively. In case of PCR III (anti-sense fragment of rr2 (irr2)) and PCR IV (anti-sense fragment of protease (ipro)) were amplified by primer No.9 + No.10 and No.16 + No.17, respectively. The restriction enzyme sites providing the directional cloning are indicated at 5' end of each primer. The black arrow and gray arrow represent an inverted repeat of protease and rr2 target genes, respectively, while the solid line indicates the spacer between the inverted repeat.

4.1.1.2 Cloning strategy and recombinant clone screening

The cloning steps of the pET17b-dspro-rr2 were shown in Figure 7. The sense fragment of protease (pro) (496 bp) was amplified by using specific primers No.13 and 14 and rr2 including loop (rr2) (862 bp) was amplified by using specific primers No.7 and 8 (Figure 8). These two fragments were digested with *Hin*d III and ligated together. Then, the ligated fragment was double digested with *Nde* I and *Xho* I before cloning into the *Nde* I and *Xho* I digested pET17b vector. After transformation of the ligated product into *E.coli* strain DH5α the recombinant clones were screened by rapid size screening, colony PCR analysis and restriction enzyme digestion analysis. Each single colony was picked and ground in lysis buffer before loading on agarose gel electrophoresis. Six clones showed upper size when compared with the size of plasmid vector alone. Then, to perform colony PCR, specific primers (No.8 and 13) were used for amplification. All six clones presented the 1,336 bp amplified PCR product indicating that they contained sense (pro-rr2-loop) insert fragment. To confirm by restriction enzyme analysis, the plasmid DNAs (No.3, 8 and 23) were extracted and triple digested with *Hin*d III, *Nde* I and *Xho* I enzymes. The plasmid from three recombinant clones can generate three expected 3,306 bp, 862 bp and 474 bp DNA fragments (Figure 9). Finally, two positive clones (No.8 and 23) were confirmed by DNA sequencing. When comparing with the reference sequence (pro_rr2), the clone No.23 showed 100% match in the protease region and one nucleotide mismatch in the region of rr2 (Figure 10). This mismatch might not affect the production of dsRNA. So, the clone No.23 was chosen for further cloning with the anti-sense fragment. The anti-sense fragment of protease (ipro) (496 bp) was amplified by using specific primers No.16 and 17 and rr2 (irr2) (478 bp) was amplified by using specific primers No.9 and 10 (Figure 11). These two fragments were digested with *Kpn* I and ligated together. Next, the ligated fragment was double digested with *Nde* I and *Xba* I before cloning into the pET17b-pro-rr2 (No.23) vector to create the pET17b-dspro-rr2. Then, the ligated product was transformed into the competent cell DH5α. After that, the recombinant clones were verified by rapid size screening, colony PCR and restriction enzyme digestion. The result of size screening showed 7 positive clones. Then colony PCR by using specific primer to T7 promoter and primer No.10 showed 5 positive clones. They presented 929 bp amplified PCR product indicating that they contained

anti-sense (ipro-irr2) insert fragment. To confirm by restriction enzyme digestion analysis, the plasmid DNAs (No.46, 122 and 199) were extracted and double digested with *Hin*d III and *Kpn* I enzymes. The plasmid from three recombinant clones can generate two expected 3,870 bp and 1,303 bp DNA fragments (Figure 12). Finally, two positive clones (No.122 and 199) were confirmed by DNA sequencing. When comparing with the reference sequence (pro_rr2), all of them showed 100% match in the protease region and rr2 region (Figure 13).

The nucleotide sequences of sense and anti-sense strands of clone No.199 were aligned together in order to identify the similarity of both strands. The result showed one mismatch at 3' end of the stem region, which was in the rr2 sequence. Hence, the size of dsRNA-rr2 was reduced from 455 bp to 438 bp while the size of dsRNA-pro was similar as expected (474 bp) (Figure 14). At this step, we got the correct plasmid pET17b-dspro-rr2 (one-stem) (No. 199) for further dsRNA production.

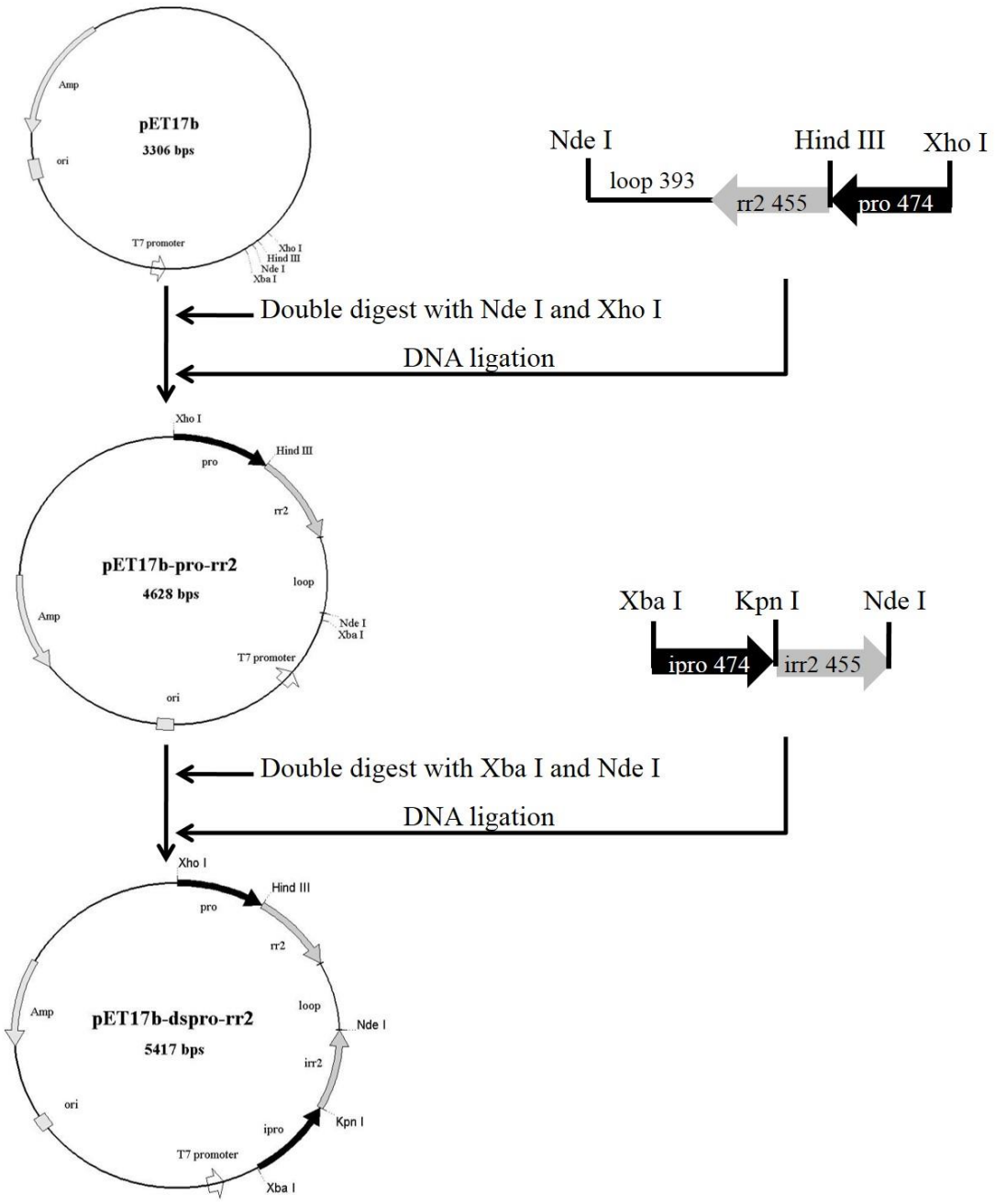


Figure 7 Schematic diagrams for construction of the pET17b-dspro-rr2

The pET17b vector was double digested with *Nde* I and *Xho* I before ligating with the sense fragment (pro-rr2). The resulting pET17b-pro-rr2 was then double digested with *Xba* I and *Nde* I before ligation with the anti-sense fragment (ipro-irr2) to generate the pET17b-dspro-rr2.

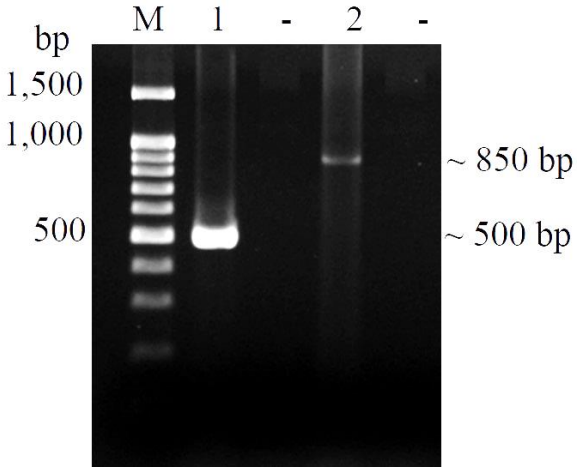


Figure 8 PCR amplification of protease and rr2-loop sense fragments

The YHV specific primers (No.13 and 14) were used for amplification of the protease sense fragment (496 bp). The WSSV specific primers (No.7 and 8) were used to amplify the rr2-loop sense fragment (862 bp). The PCR products were detected by agarose gel electrophoresis stained with ethidium bromide.

Lane M: 100bp ladder

Lane 1: The amplified protease sense fragment at 496 bp

Lane 2: The amplified rr2-loop sense fragment at 862 bp

Lane -: PCR negative control (each pair of primers)

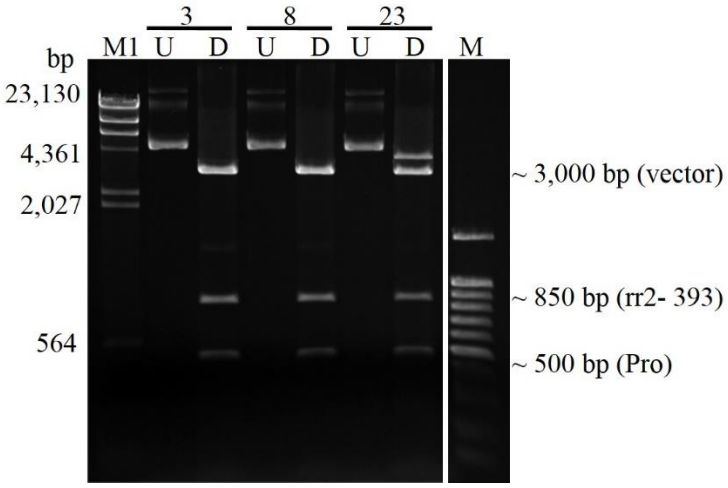


Figure 9 Verification of pET17b-pro-rr2 by restriction endonuclease analysis

The positive recombinant clones from rapid size screening and colony PCR analysis were verified by restriction enzyme digestion. The plasmid DNA isolated from the clone No.3, 8 and 23 were triple digested with *Hin*d III, *Nde* I and *Xho* I to verify the cloning. The expected DNA fragments at 862 bp of rr2-loop region, 474 bp of protease region and 3,306 bp of linearized pET17b vector were observed.

Lane M1: λ/*Hin*d III marker

Lane M: 100bp DNA marker

Lane U: Undigested plasmid DNA

Lane D: Digested plasmid DNA with *Hin*d III, *Nde* I and *Xho* I

Figure 10 Nucleotide sequence alignment of the sense fragment of protease and rr2-loop of pET17b-pro-rr2

Nucleotide sequences of the sense strands of clone No.8 and 23 were compared with the reference sequence (pro_rr2; the combined sequences of protease and rr2) by Vector NTI Advance 10 (Align X) program. Nucleotides at position 1 - 474 and 481 - 935 represent the protease region and rr2 region, respectively. Specific primer sequences are underlined. Box indicates restriction enzyme site (*Hin*d III) and arrow indicates the nucleotide mismatch position.

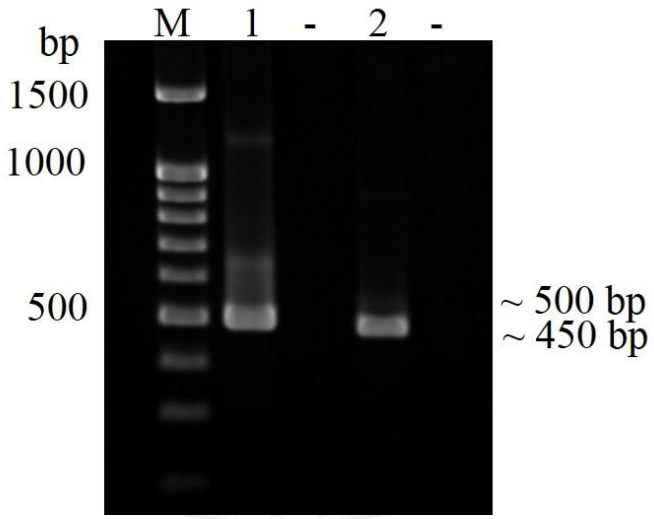


Figure 11 PCR amplification of protease and rr2 anti-sense fragments

The YHV specific primers (No.16 and 17) were used for amplification of the protease anti-sense fragment (496 bp). The WSSV specific primers (No.9 and 10) were used to amplify the rr2 anti-sense fragment (478 bp). The PCR products were detected by agarose gel electrophoresis stained with ethidium bromide.

Lane M: 100bp ladder

Lane 1: The amplified protease anti-sense fragment at 496 bp

Lane 2: The amplified rr2 anti-sense fragment at 478 bp

Lane -: PCR negative control (each pair of primers)

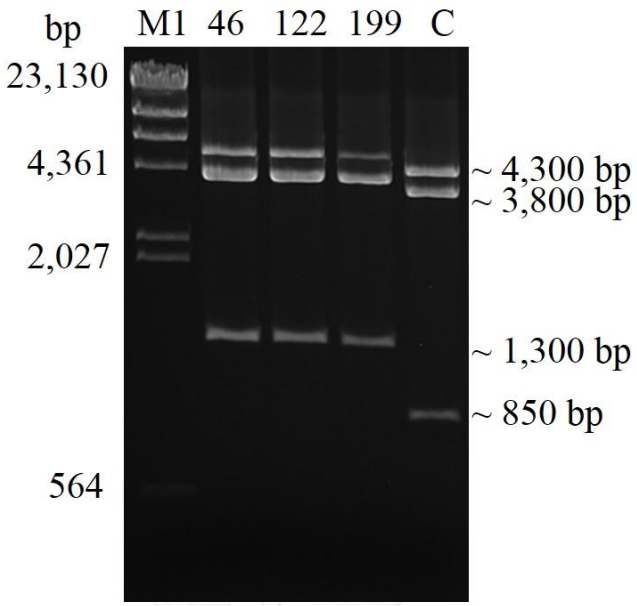


Figure 12 Verification of pET17b-dspro-rr2 by restriction endonuclease analysis

The positive recombinant clones from rapid size screening and colony PCR analysis were verified by restriction enzyme digestion. The plasmid DNA isolated from clone No.46, 122 and 199 were double digested with *Hin*d III and *Kpn* I to verify the cloning. The expected DNA fragments at 1,303 bp of rr2-stem including loop region, 3,870 bp of linearized pET17b-protease vector were observed.

Lane M1: λ/*Hin*d III marker

Lane C: pET17b-pro-rr2 (No. 23) digested with *Hin*d III and *Kpn* I

Lane 46, 122 and 199: Digested plasmid DNA of clone No. 46, 122 and 199, respectively with *Hin*d III and *Kpn* I

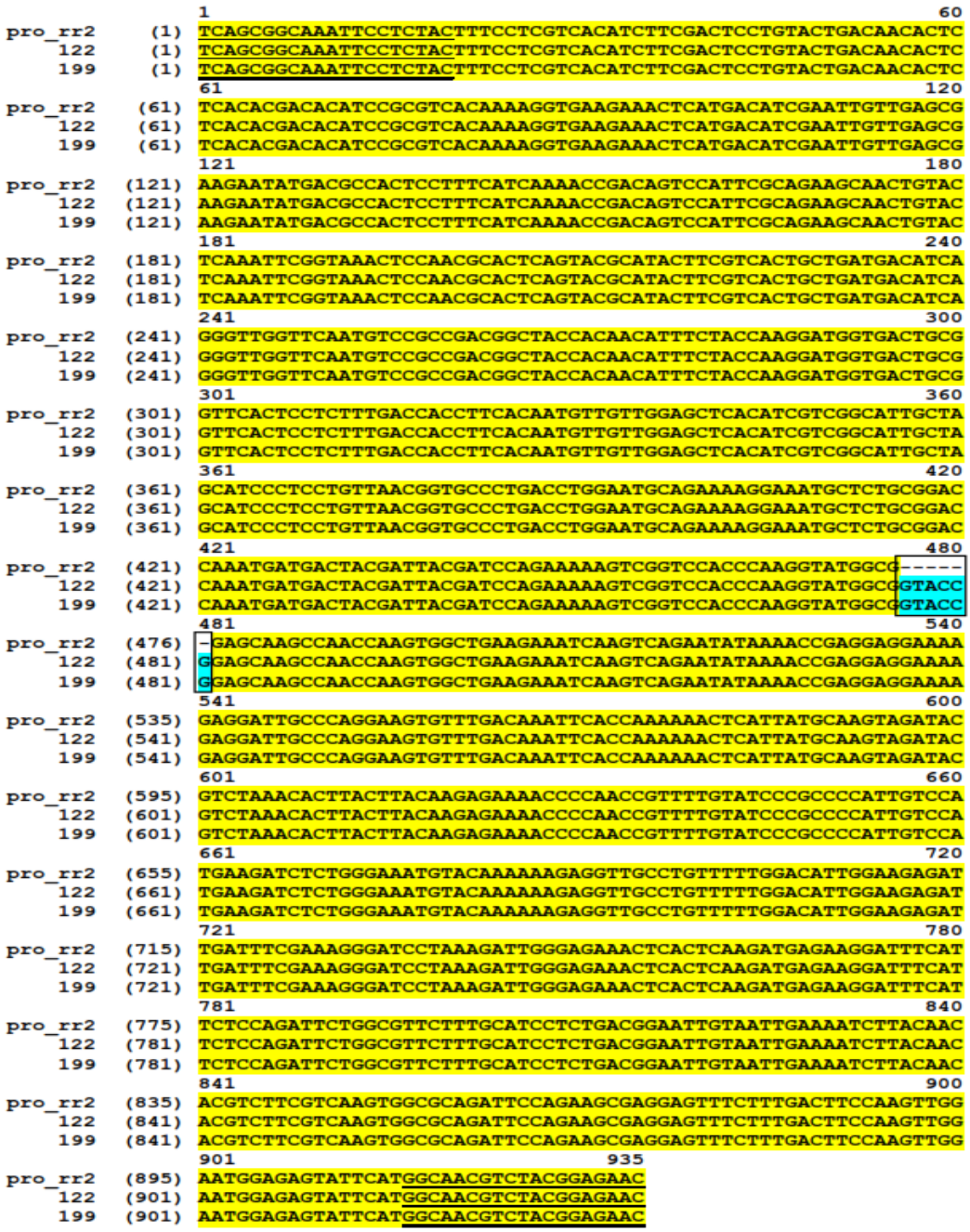


Figure 13 Nucleotide sequence alignment of the anti-sense fragment of protease and rr2 of pET17b-dspro-rr2

Nucleotide sequences of the anti-sense strands of clone No.122 and 199 were compared with the reference sequence (pro_rr2; the combined sequences of protease and rr2) by Vector NTI Advance 10 (Align X) program. Nucleotides at position 1 - 474 and 481 - 935 represent the protease region and rr2 region, respectively. Specific primer sequences are underlined. Box indicates restriction enzyme site (*Kpn* I).

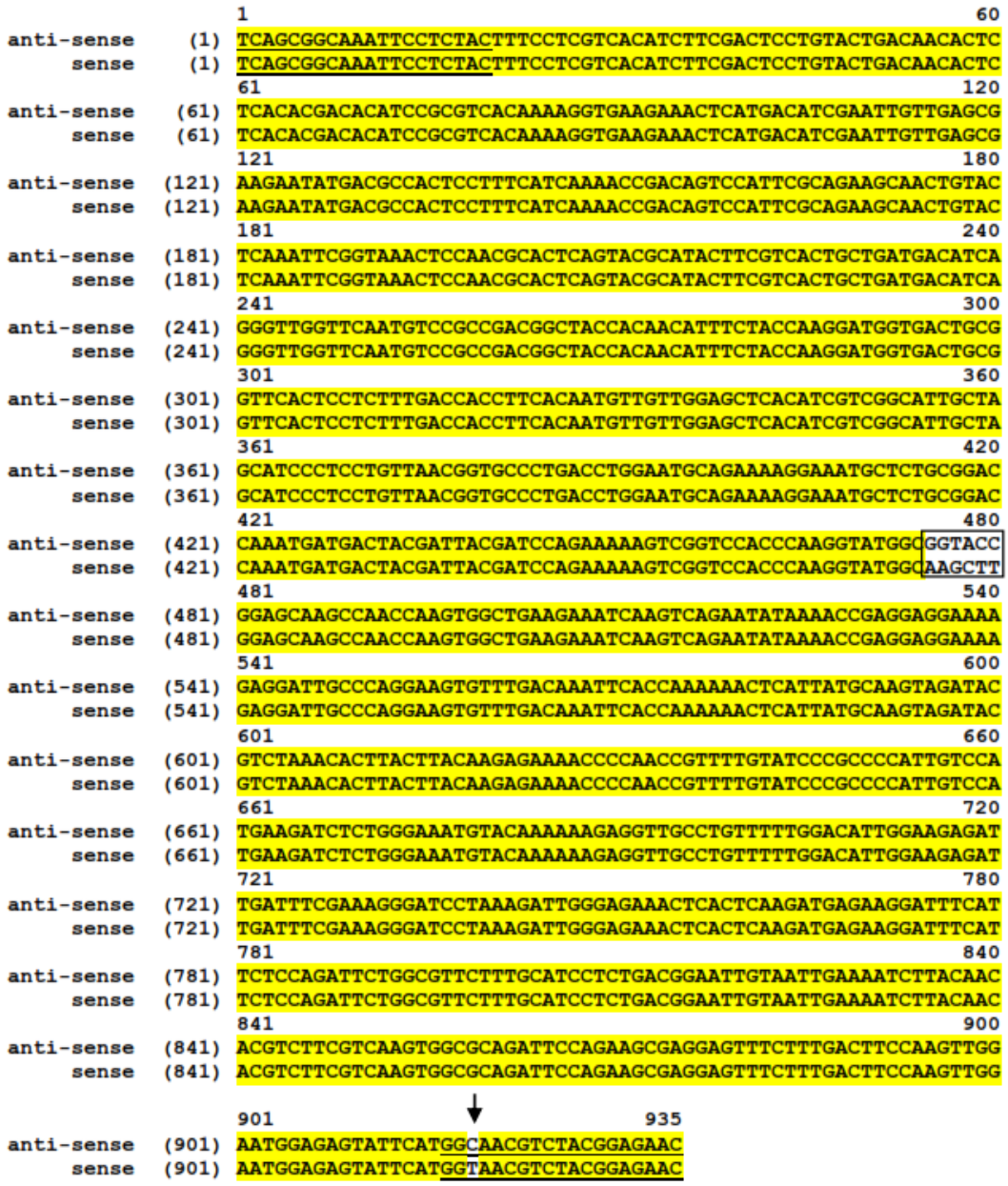


Figure 14 Nucleotide sequence alignment of the sense and anti-sense fragment of pET17b-dspro-rr2

Nucleotide sequences of the sense and anti-sense of stem region were compared together by Vector NTI Advance 10 (Align X) program. Nucleotides at position 1 - 474 and 481- 935 represent the protease region and rr2 region, respectively. Specific primer sequences are underlined. Box indicates restriction enzyme site (*Hin*d III and *Kpn* I) and arrow indicates nucleotide mismatch position.

4.1.2 Construction of plasmid pET17b-dspro-dsrr2 (two-stem)

4.1.2.1 Primer design

The specific primers were designed and synthesized (Table 3). Restriction enzyme recognition sequences were added into 5' end of each primer to provide directional cloning (Figure 15).

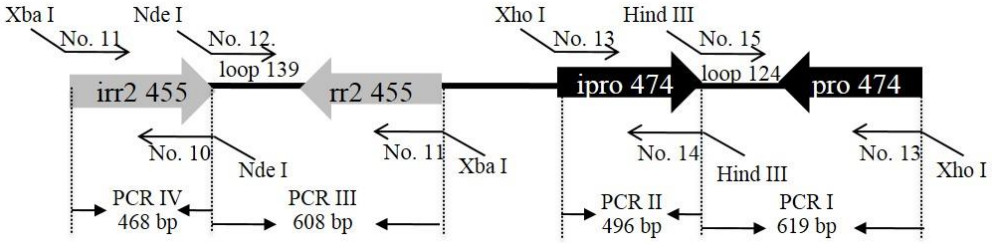


Figure 15 Diagram of an inverted repeat for two-stem construction and primers location

Primer No.13 + No.15 and No.13 + No.14 were used for the synthesis of PCR I (protease sense with loop fragment) and PCR II (protease anti-sense fragment (ipro)), respectively. In case of PCR III (rr2 sense with loop fragment) and PCR IV (rr2 anti-sense fragment (irr2)) were amplified by primer No.11 + No.12 and No.10 + No. 11, respectively. The restriction enzyme sites providing the directional cloning are indicated at 5' end of each primer. The black arrow and gray arrow represent the inverted repeats of protease and rr2 DNA fragments, respectively, while the solid line indicates the spacer between the inverted repeat.

4.1.2.2 Cloning strategy and recombinant clone screening

The cloning steps of the pET17b-dspro-dsrr2 were shown in Figure 16. The sense fragment of protease including loop (pro-loop) (619 bp) was amplified by using specific primers No.13 and 15 and anti-sense fragment of protease (ipor) (496 bp) was amplified by using specific primers No.13 and 14 (Figure 17). These two fragments were digested with *Hin*d III and ligated together. Then, the ligated fragment was digested with *Xho* I before cloning into the *Xho* I digested pET17b vector. After transformation of the ligated product into *E. coli* strain DH5α the recombinant clones were screened by rapid size screening, colony PCR and restriction enzyme digestion analysis. Only one clone was positive by rapid size screening. Then, it was verified by colony PCR using specific primer (No.13 and 15). It presented the 619 bp amplified PCR product indicating that it contained the protease insert fragment. To confirm by restriction enzyme analysis, the plasmid DNA was extracted and double digested with *Hin*d III and *Xho* I enzymes. This plasmid can generate three expected DNA fragments at 3,306 bp, 598 bp and 474 bp (Figure 18). Moreover, the DNA insert was confirmed by DNA sequencing. The sequence of the sense strand of protease region showed 100% homology with its anti-sense sequence and with the reference sequence (Figure 19). Thus, the size of dsRNA-protease was 480 bp. So, this clone was chosen for further cloning with the dsrr2 expression cassette. The sense fragment of rr2 including loop (rr2-loop) (608 bp) was amplified by using specific primers No.11 and 12. The anti-sense fragment of rr2 (irr2) (478 bp) was amplified by using specific primers No. 10 and 11 (Figure 20). These two PCR fragments were digested with *Nde* I and ligated together. Next, the ligated fragment was digested with *Xba* I before cloning into the pET17b-dspro vector to create the pET17b-dspro-dsrr2. Then, the ligated product was transformed into the competent cell DH5α. After that, the recombinant clones were verified by rapid size screening, colony PCR and restriction enzyme digestion. The result of size screening showed 5 positive clones. Then colony PCR by using specific primer to T7 promoter and primer No.10 showed 2 positive clones. They presented the 499 bp amplified PCR product indicating that they contained the rr2 insert fragment. To confirm by restriction enzyme analysis, the plasmid DNAs of clone No.14 and 29 were extracted and double digested with *Hin*d III and *Nde* I enzymes. The plasmid from two recombinant clones can generate two expected 474 bp and 594 bp DNA fragments.

After digestion with *Xba* I, the plasmids can generate the expected 1,049 bp DNA fragment (Figure 21). Finally, two positive clones (No.14 and 29) were confirmed by DNA sequencing. When comparing with the reference sequence, the result from both clones showed one mismatch at 3' end of the sense strand of rr2. Therefore, the size of dsRNA-rr2 was reduced from 455 bp to 438 bp while the size of dsRNA-pro was similar as expected (480 bp) (Figure 22). At this step, the correct plasmid pET17b-dspro-dsrr2 (No.14) was chosen but it still contained the ribosome binding site. Hence, the ribosome binding site has to be removed to create pET17b-dspro-dsrr2 (without the ribosome binding site) (two-stem). The plasmid two-stem was then used to construct the plasmid pET17b-dspro and pET17b-dsrr2 for producing an individual dsRNA; dsRNA-protease and dsRNA-rr2, respectively. These two dsRNAs were used as the control in this study.

The plasmid pET17b-dspro-dsrr2 (No.14) were double digested with *Xba* I and *EcoR* V to remove ribosome binding site then re-ligated with blunt end ligation for construction of pET17b-dspro-dsrr2. After screening and confirmation (Figure 23), we got the plasmid pET17b-dspro-dsrr2 (No.62). Then the stem of protease was removed to generate the pET17b-dsrr2 by digestion with *Xho* I or removed the stem of rr2 to generate the pET17b-dspro by digestion with *Xba* I. The clones of pET17b-dspro and pET17b-dsrr2 were verified by size screening and colony PCR as shown in Figure 24 and 25, respectively.

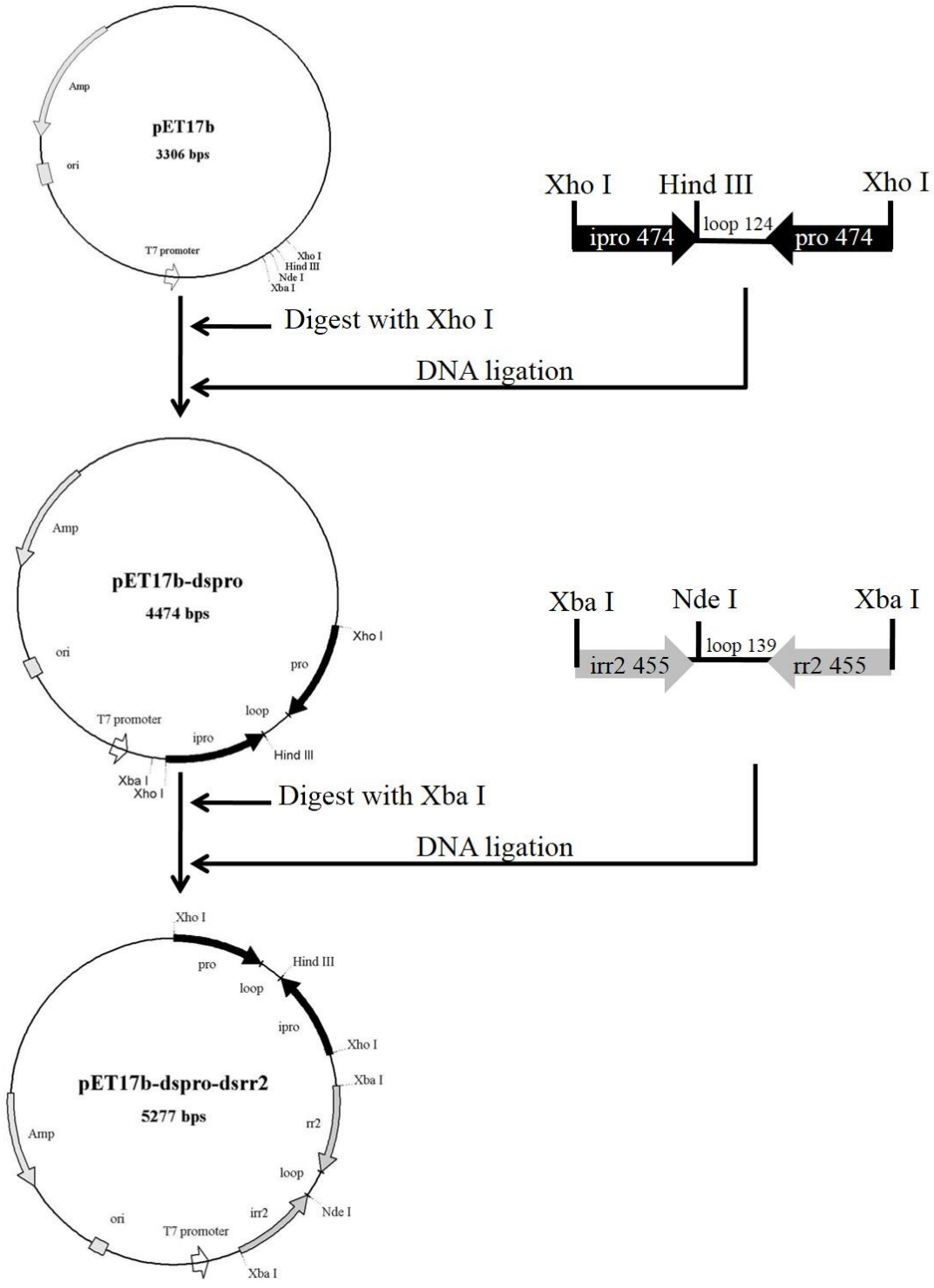


Figure 16 Schematic diagrams for construction of pET17b-dspro-dsrr2

The pET17b vector was digested with *Xho* I before ligating with the inverted repeat of dspro cassette. The resulting pET17b-dspro was then digested with *Xba* I before ligation with the inverted repeat of dsrr2 cassette.

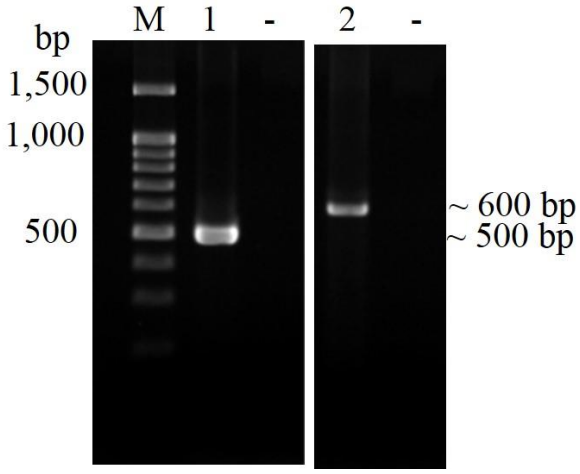


Figure 17 PCR amplification of protease sense and anti-sense fragments

The YHV specific primers (No.13 and 15) were used for amplification of the protease-loop sense fragment (619 bp) and the specific primers (No.13 and 14) were used to amplify the protease anti-sense fragment (496 bp). The PCR products were detected by agarose gel electrophoresis stained with ethidium bromide.

Lane M: 100bp ladder

Lane 1: The amplified protease anti-sense fragment at 496 bp

Lane 2: The amplified protease-loop sense fragment at 619 bp

Lane -: PCR negative control (each pair of primers)

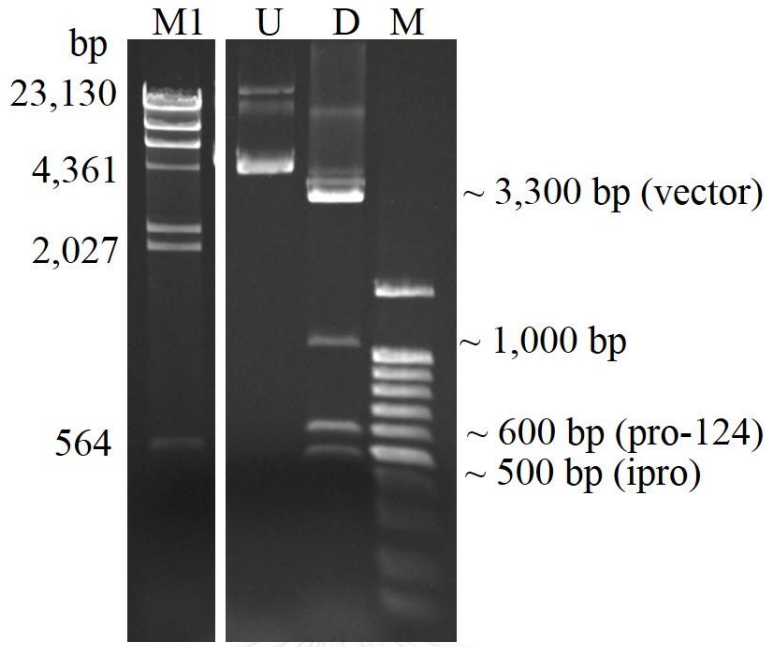


Figure 18 Verification of pET17b-dspro by restriction endonuclease analysis

Only one positive recombinant clone from rapid size screening and colony PCR analysis was verified by restriction enzyme digestion. The recombinant plasmid DNA was double digested with *Hin*d III and *Xho* I to verify the cloning. The expected DNA fragments at 598 bp of protease-loop region, 474 bp of protease region and 3,306 bp of linearized pET17b vector were observed.

Lane M1: λ/*Hin*d III marker

Lane M: 100bp DNA marker

Lane U: Undigested plasmid DNA

Lane D: Digested plasmid DNA with *Hin*d III and *Xho* I

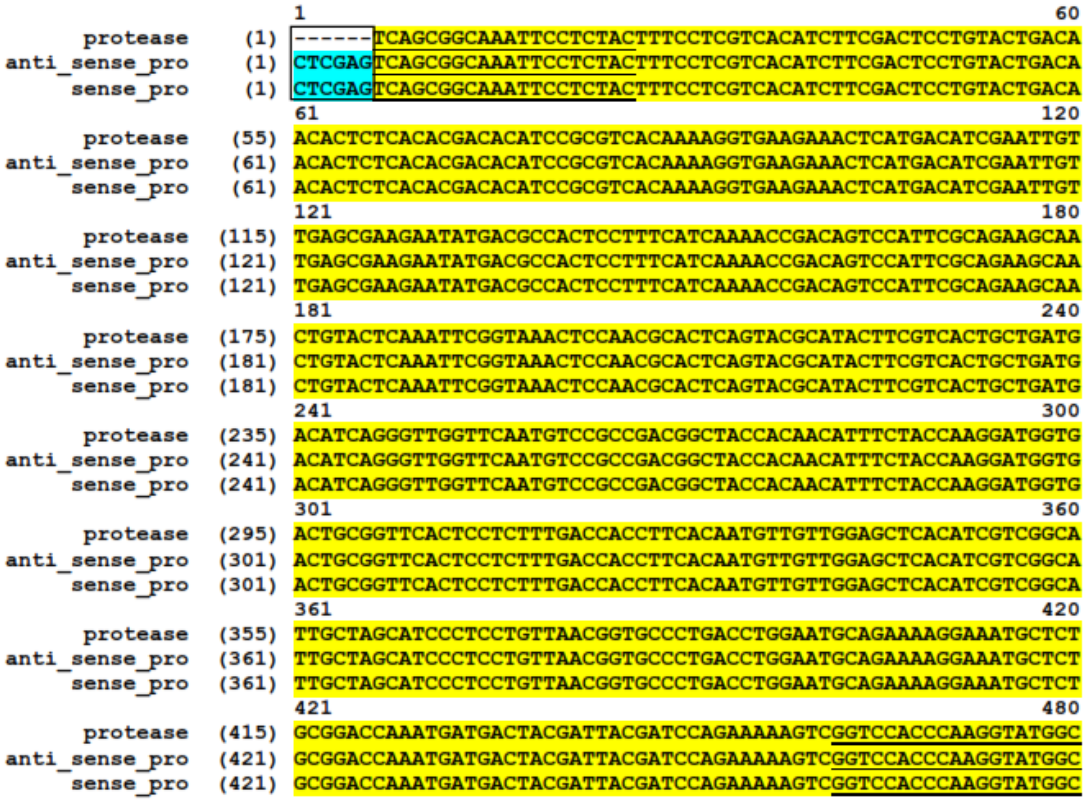


Figure 19 Nucleotide sequence alignment of the stem protease of pET17b-dspro

Nucleotide sequences of the sense and anti-sense in the stem region of protease were compared together and with the reference sequence (protease) by Vector NTI Advance 10 (Align X) program. Specific primer sequences are underlined. Box indicates restriction enzyme site (*Xho* I).

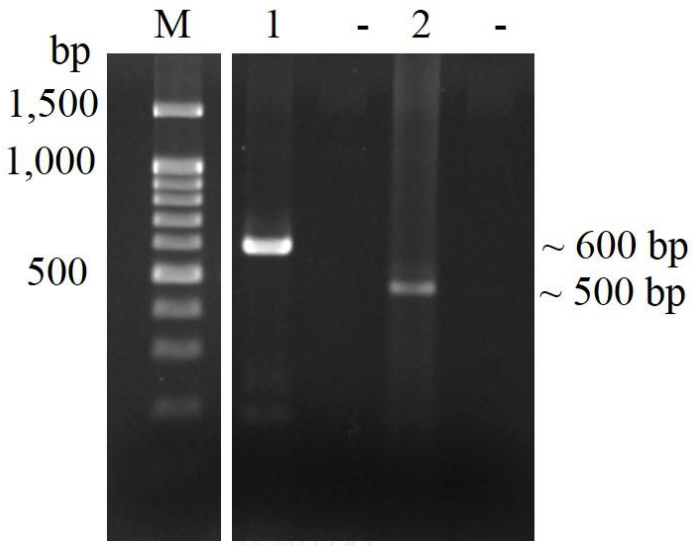


Figure 20 PCR amplification of rr2 sense and anti-sense fragments

The WSSV specific primers (No.11 and 12) were used for amplification of the rr2-loop sense fragment (608 bp) and the specific primers (No.10 and 11) were used to amplify the rr2 anti-sense fragment (478 bp). The PCR products were detected by agarose gel electrophoresis stained with ethidium bromide.

Lane M: 100bp ladder

Lane 1: The amplified rr2 loop sense fragment at 608 bp

Lane 2: The amplified rr2 anti-sense fragment at 478 bp

Lane -: PCR negative control (each pair of primers)

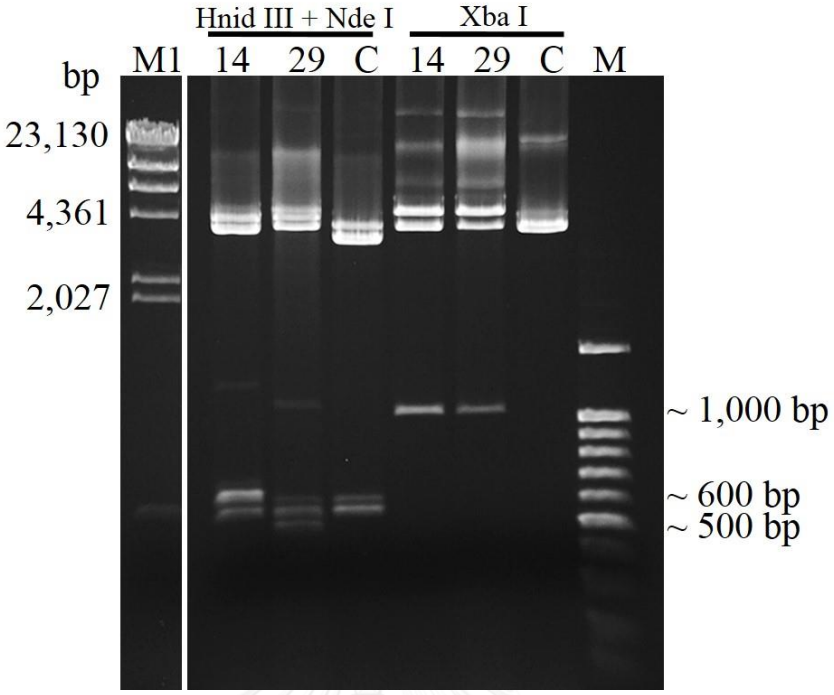


Figure 21 Verification of pET17b-dspro-dsrr2 by restriction endonuclease analysis

The positive recombinant clones from rapid size screening and colony PCR analysis were verified by restriction enzyme digestion. The plasmids were isolated from clone No.14 and 29 and double digested with *Hin*d III and *Nde* I to verify the cloning. The expected DNA fragments at 594 bp of rr2-stem including loop region and 474 bp of protease region were observed. In addition the plasmids were digested with *Xba* I and the expected DNA fragment at 1,049 bp of cassette of dsrr2 was observed.

Lane M1: λ/*Hin*d III marker

Lane M: 100bp DNA marker

Lane C: pET17b-dspro (No.17)

Lane 14, 29: plasmid DNA of clone No.14 and 29

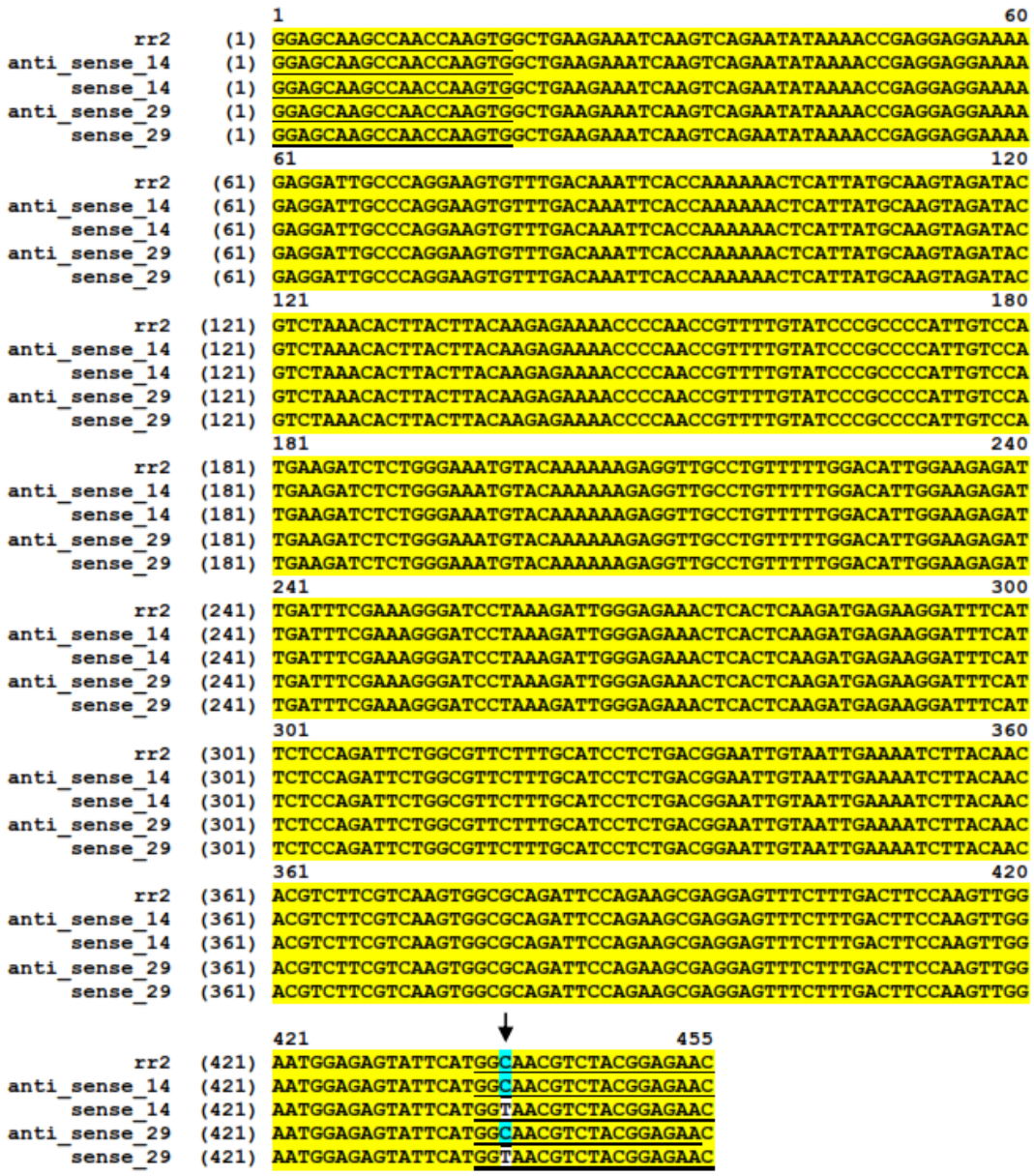


Figure 22 Nucleotide sequence alignment of stem rr2 of pET17b-dsrr2

Nucleotide sequences of the sense and anti-sense in the stem region of rr2 (clone No.14 and 29) were compared together and with the reference sequence (rr2) by Vector NTI Advance 10 (Align X) program. Specific primer sequences are underlined. Arrow indicates the nucleotide mismatch position.

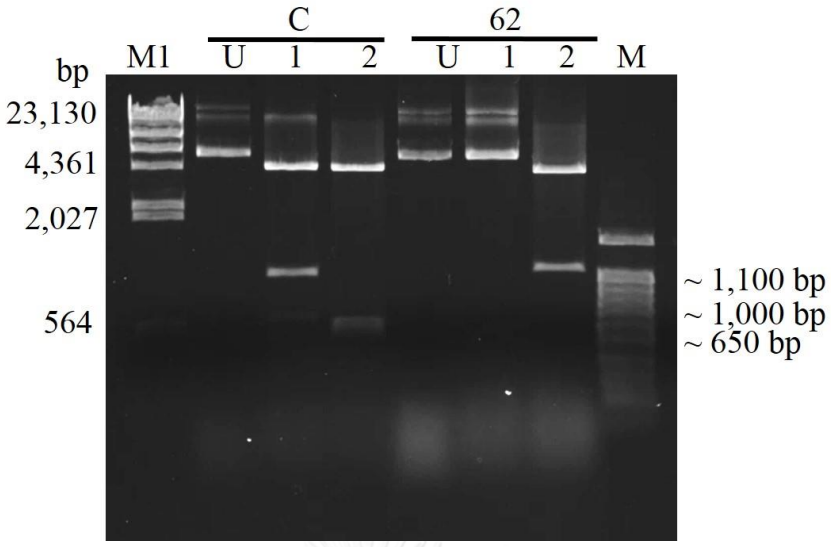


Figure 23 Verification of pET17b-dspro-dsrr2 by restriction endonuclease analysis

The recombinant plasmids isolated from clone No.14 (before) and 62 (after removing the ribosome binding site) of pET17b-dspro-dsrr2 were digested with *Xba* I and double-digested with *Hin*d III and *Nde* I to verify the removing. The plasmid of clone No.62 showed the correct pattern that it could not be digested with *Xba* I while the expected DNA fragments at 1,075 bp of rr2-loop joined with the anti-sense of protease region was observed after digested with *Hin*d III and *Nde* I.

Lane M1: λ/*Hin*d III marker

Lane M: 100bp DNA marker

Lane C: pET17b-dspro-dsrr2 (No.14)

Lane 62: pET17b-dspro-dsrr2 (No.62)

Lane U: Undigested plasmid DNA

Lane 1: Digested plasmid DNA with *Xba* I

Lane 2: Digested plasmid DNA *Hin*d III and *Nde* I

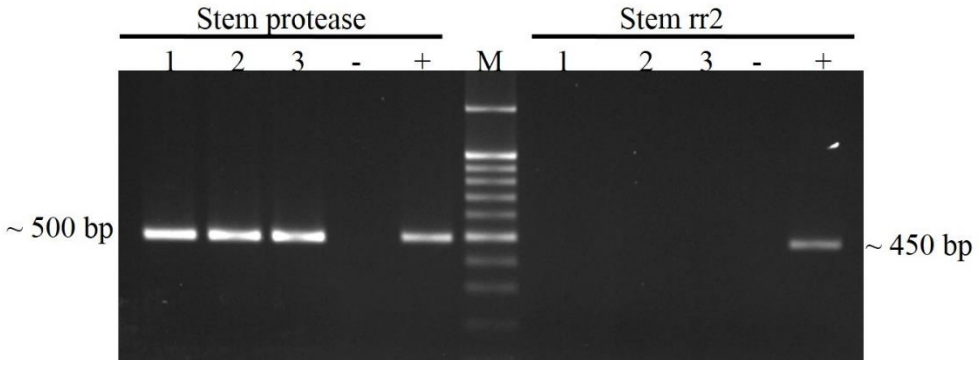


Figure 24 Verification of pET17b-dspro by PCR analysis

The plasmid of pET17b-dspro (clone No. 1, 2 and 3) were verified by amplification with the specific primers for protease (left) and rr2 (right) regions to confirm the present of protease but not the rr2. The expected size 496 bp of protease was detected only from the reaction with the protease primers.

Lane M: 100bp DNA marker

Lane 1-3: PCR product of pET17b-dspro (clone No. 1, 2, 3)

Lane -: PCR negative control (each pair of primers)

Lane +: PCR positive control (pET17b-dspro-dsrr2)

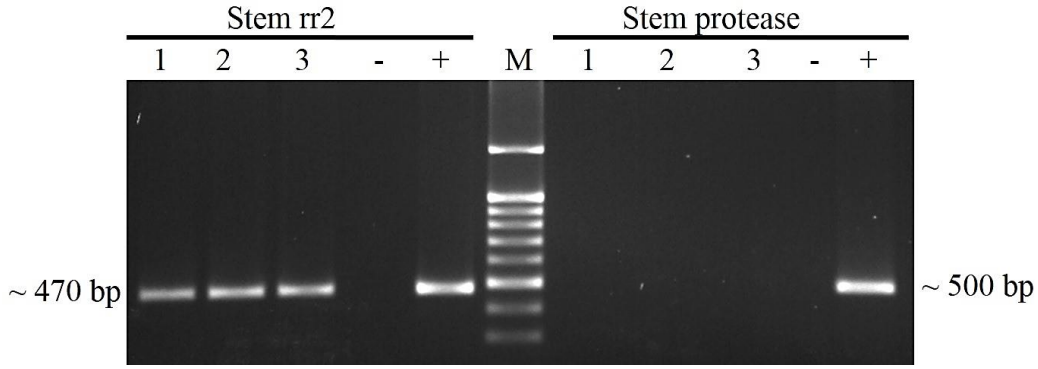


Figure 25 Verification of pET17b-dsrr2 by PCR analysis

The plasmid of pET17b-dsrr2 (clone No. 1, 2 and 3) were verified by amplification with the specific primers for rr2 (left) and protease (right) regions to confirm the present of rr2 but not the protease. The expected size 478 bp of rr2 was detected only from the reaction with the rr2 primers.

Lane M: 100bp DNA marker

Lane 1-3: PCR product of pET17b-dsrr2 (clone No. 1, 2, 3)

Lane -: PCR negative control (each pair of primers)

Lane +: PCR positive control (pET17b-dspro-dsrr2)

4.2 Production and characterization of dsRNAs

The recombinant plasmid containing dsRNA expression cassette was re-transformed into *E. coli* HT115 bacterial host cells. Since dsRNA is sensitive to RNase III, HT115, which lacks RNase III activity, was use as a bacterial host cell for dsRNA production. The *E. coli* strain HT115 was modified to express T7 RNA polymerase from an isopropyl-β-D-thiogalactopyranoside (IPTG) inducible promoter. After IPTG induction, the transcribed RNA can form stem-loop structured dsRNA automatically by complementary hybridization of the sequences in the inverted repeat of stem region.

4.2.1 Production and extraction of dsRNAs

In this study, Ribozol was used to extract total RNA from the bacterial cells that were either boiled or not boiled. The extraction steps were followed the manufacturer's protocol. Patterns of total RNA isolated from the boiled and normal cells were not different. One-stem showed the expected dsRNA-pro-rr2 (935 bp), which included the region of dsRNA-protease (474 bp), the dsRNA-rr2 (455 bp) and the restriction enzyme site (6 bp) (Figure 26 A and B). Two-stem showed the bands pattern similar to the pattern of dsRNA-protease and dsRNA-rr2 (Figure 26 A and B). To identify the presence of dsRNA-protease and dsRNA-rr2 in the extracted RNA, one microgram of each total RNA was digested with RNase A to remove the host ssRNA, the ssRNA in the loop and junction regions before loading into an agarose gel. We can detect the expected bands of dsRNA-protease at 474 bp (from one-stem), at 480 bp (from two-stem and pET17b-dspro) and dsRNA-rr2 at 438 bp (from one-stem, two-stem and pET17b-dsrr2) (Figure 26 C and D). Nevertheless, the dsRNA band patterns of one-stem were different among these two methods, in which the normal cells showed two dsRNA bands (dsRNA-protease and dsRNA-rr2) more clearly than the boiled cells. Therefore, the HT115 cells expressing dsRNA without boiling were used for the RNA extraction in the next experiment.

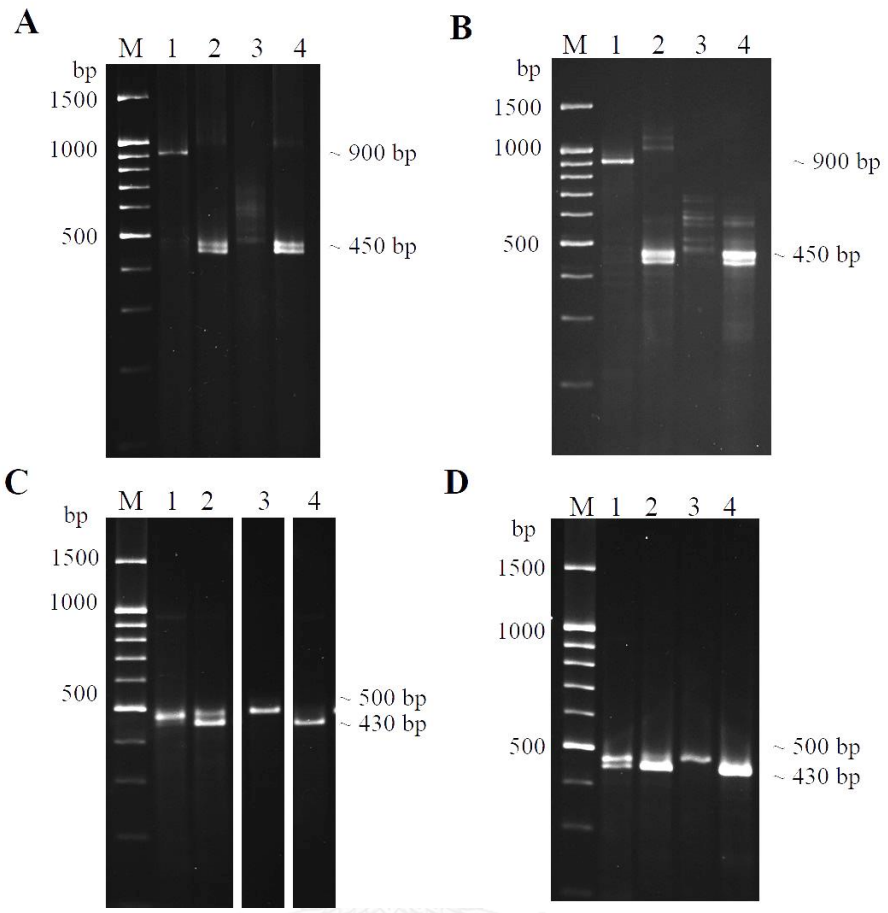


Figure 26 Extraction of total RNA by Ribozol method

The bacterial cells expressing dsRNA with (A) and without (B) boiling treatment were used in this study. After adding the Ribozol to the cells, total RNA was extracted following the manufacturer's protocol. Then, one microgram of total RNA extracted from the boiled cells (C) and the normal cells (D) were digested with RNase A to determine the presence of dsRNA-protease and dsRNA-rr2. Approximately 100 ng of the treated RNA was loaded on 2% agarose gel.

Lane M: 100bp ladder

Lane 1: one-stem

Lane 2: two-stem

Lane 3: pET17b-dspro

Lane 4: pET17b-dsrr2

4.2.2 Optimization of the dsRNAs expression condition

To find the optimum temperature for producing dsRNA, HT115 with four recombinant plasmids were grown at 37°C until OD_{600} of the bacterial cells reached 0.4 and then IPTG was added. Then the cells were divided into three parts to be incubated at 37°C, 30°C and 25°C (first experiment) or 37°C, 25°C and 18°C (second experiment) for 4 hours. After dsRNA production, total RNA was extracted by Ribozol method and the ssRNA was removed by RNase A digestion. The results (Figure 27) showed that the dsRNAs can be expressed at all four temperatures. But the yield of dsRNA of one-stem, two-stem and pET17-dsrr2 were decreased when growing the cells at low temperature. It suggested that the optimal temperature for producing dsRNA in HT115 was 37°C. Therefore, the yield of dsRNA at 37°C of one-stem was approximately 8 µg/OD and the weight ratio of dsRNA-protease per dsRNA-rr2 was 1:1, two-stem was approximately 23 µg/OD and the weight ratio of dsRNA-protease per dsRNArr2 was 1:4, dsRNA-protease was approximately 7 µg/OD and dsRNA-rr2 was approximately 38 µg/OD.

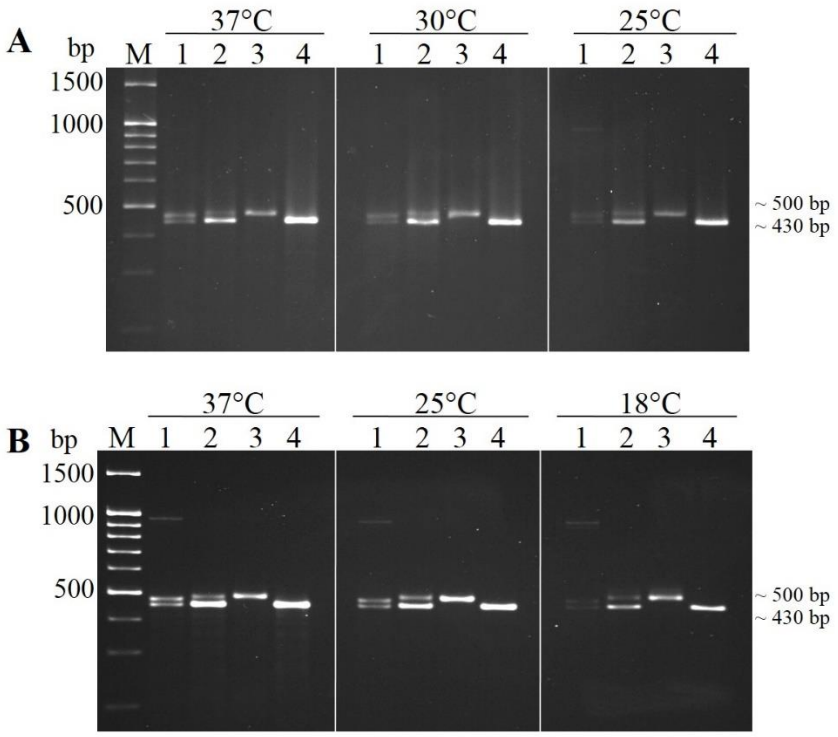


Figure 27 Production of dsRNA at various temperatures after IPTG induction

Each bacterial cell suspension after IPTG addition was separately cultured at 37°C, 30°C and 25°C (first experiment (A)) or 37°C, 25°C and 18°C (second experiment (B)) for 4 hours. Then, total RNA was extracted by Ribozol method and one microgram of total RNA was digested with RNase A before loading. Approximately 100 ng of total RNA were loaded on 2% agarose gel.

Lane M: 100bp ladder

Lane 1: dsRNA production from one-stem

Lane 2: dsRNA production from two-stem

Lane 3: dsRNA production from pET17b-dspro

Lane 4: dsRNA production from pET17b-dsrr2

4.2.3 Verification of dsRNAs formation

The integrity of dsRNAs were characterized by enzymatic digestion and analyzed by agarose gel electrophoresis. One microgram of total RNA was incubated with RNase A and RNase III to digest ssRNA and dsRNA, respectively. As expected, two fragments of dsRNA (dsRNA-protease and dsRNA-rr2) were obtained from one-stem and two-stem after digesting with RNase A (Figure 28) whereas only one expected RNA band was detected from pET17b-dspro and pET17b-dsrr2. In addition, these RNA bands were completely digested when incubating with RNase III. These results suggested that the RNA produced from each recombinant plasmids were in the form of dsRNA.

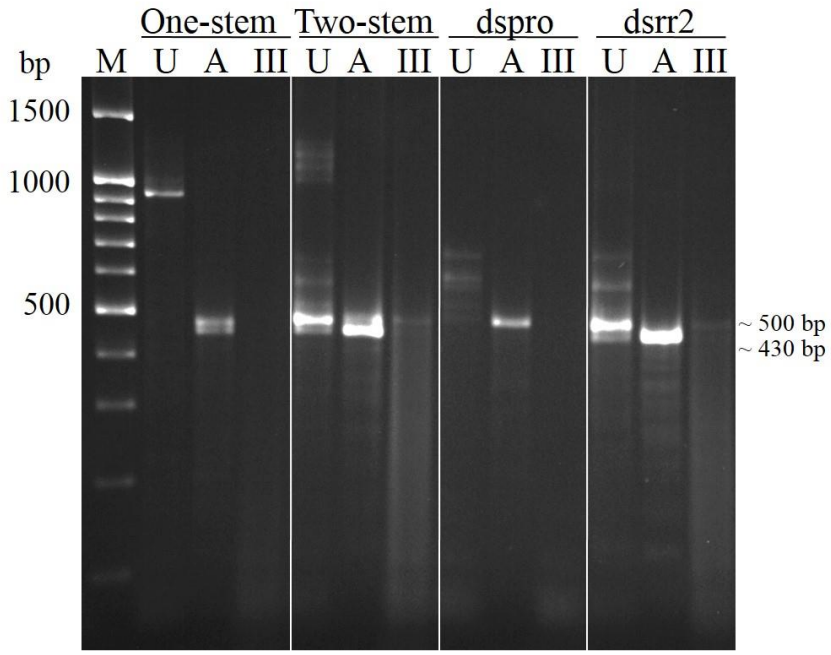


Figure 28 Verification of dsRNAs by enzymatic digestion

The integrity of dsRNA was verified by RNase digestion. One microgram of total RNA was treated with RNase A and RNase III digestion. After RNase digestion, approximately 100 ng of total RNA were loaded on 2% agarose gel.

Lane M: 100bp ladder

Lane U: Untreated total RNA

Lane A: Total RNA treated with RNase A

Lane III: Total RNA treated with RNase III

4.3 Investigation of the efficiency of dsRNAs on viral inhibition and shrimp mortality suppression

In this study, the dsRNAs (one-stem and two-stem) that were produced from the plasmids pET17b-dspro-rr2 and pET17b-dspro-dsrr2 contained different weight ratio of dsRNA-protease (specific to YHV) and dsRNA-rr2 (specific to WSSV). Therefore, their efficacies on YHV and WSSV inhibition have to be determined and compared in shrimp before being selected for further study.

4.3.1 Infectivity test of YHV and WSSV

To investigate the suitable amount of viruses (YHV and WSSV) for shrimp infection, the infectivity of YHV and WSSV in shrimp were monitored. Two viral stocks of YHV and WSSV were used in this study.

For the viral stock (preparation 1), the 400 mg shrimp was injected with YHV at dilution 10^{-6}, $5x10^{-7}$, 10^{-7}, $5x10^{-8}$ and 10^{-8} or WSSV at dilution $5x10^{-3}$, 10^{-3}, 10^{-4} and 10^{-5} and the shrimp mortality was then recorded every day for 7 days. The cumulative mortality of shrimp infected with the YHV and WSSV were plotted in the graphs as shown in Figure 29 A and B, respectively. From the results, the YHV at $5x10^{-7}$, 10^{-6} dilution and the WSSV at 10^{-3} dilution, which showed 100% of viral-infected shrimp were used to infect shrimp in the experiment of viral suppression test (short term) and the YHV at $5x10^{-7}$ and the WSSV at $5x10^{-4}$, which showed 80-90% death of viral-infected shrimp within 5-7 days were used in the shrimp mortality reduction experiment (long term).

For the viral stock (preparation 2), the 400 mg shrimp was injected with YHV at dilution $5x10^{-6}$, 10^{-6}, $5x10^{-7}$ and 10^{-7} or WSSV at dilution 10^{-2} $5x10^{-3}$ and 10^{-3} and the shrimp mortality was then recorded every day for 7 days. The infectivity of the YHV and WSSV (prep.2) were presented in Figure 30 A and B, respectively. From the results, the YHV at 10^{-6} dilution and the WSSV at $5x10^{-3}$ were used in the experiment of shrimp mortality suppression (long term) and the YHV at $5x10^{-7}$ and WSSV at $2.5x10^{-3}$ dilution were used for dual infection.

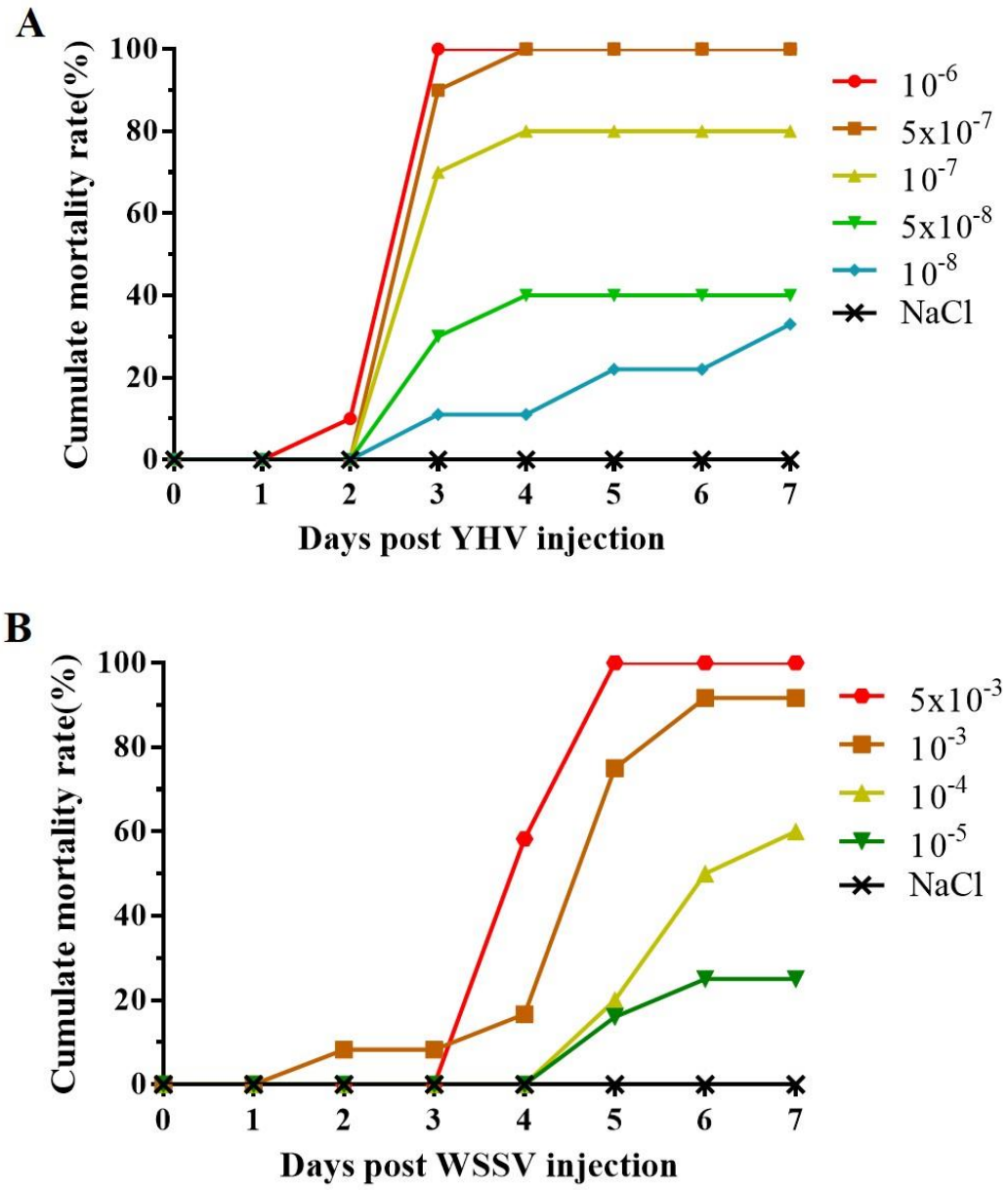


Figure 29 Infectivity of YHV and WSSV in shrimp (preparation 1)

Shrimp at 400 mg body weight (n = 10) were injected with YHV (A) or WSSV (B) at different dilutions. The cumulative mortality of the infected shrimp was recorded every day for 7 days and plotted by using GraphPad program.

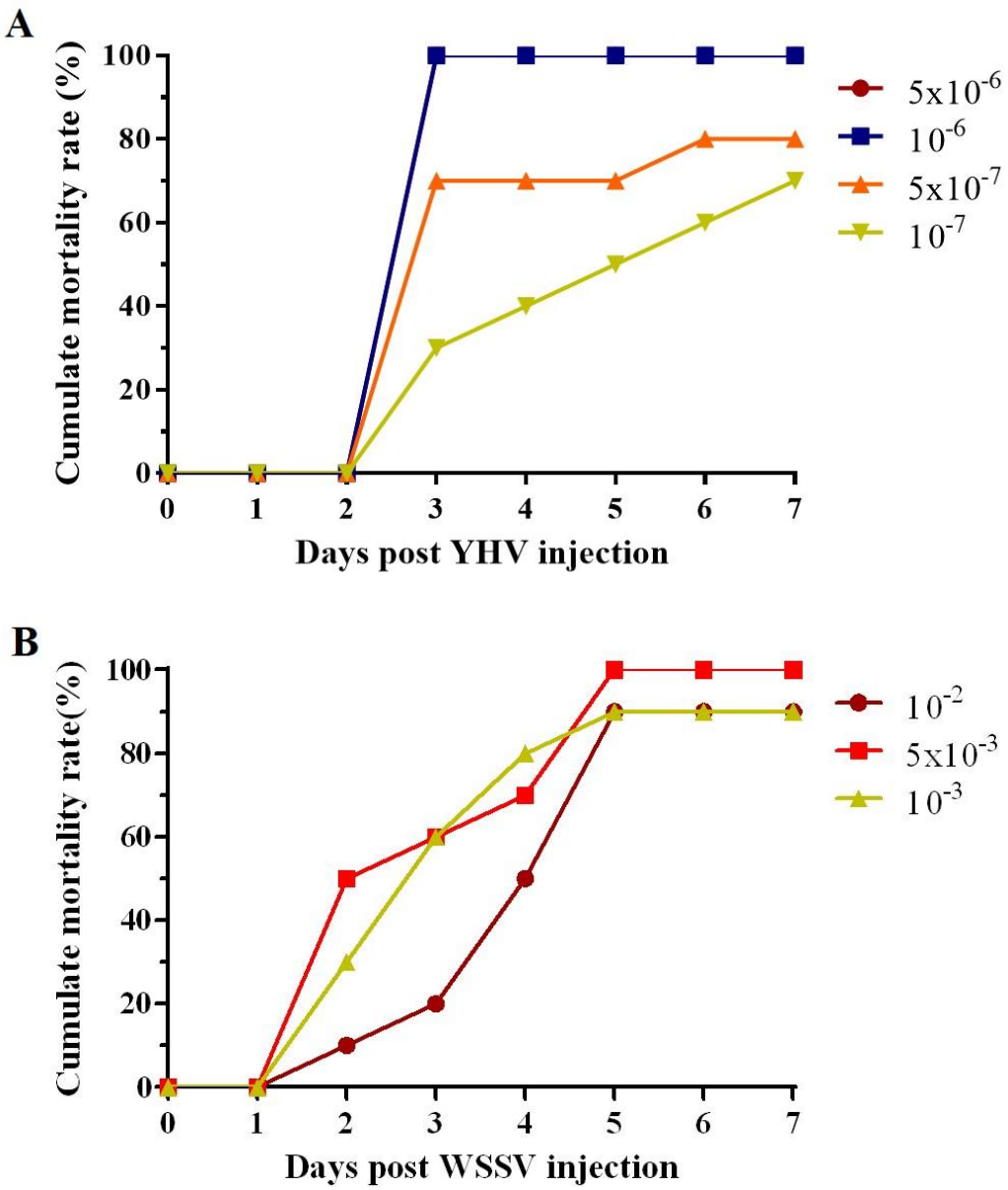


Figure 30 Infectivity of YHV and WSSV in shrimp (preparation 2)

Shrimp at 400 mg body weight (n = 10) were injected with YHV (A) or WSSV (B) at different dilutions. The cumulative mortality of the infected shrimp was recorded every day for 7 days and plotted by using GraphPad program.

4.3.2 Determination of the inhibitory effect of dsRNAs on YHV and WSSV infection in shrimp

4.3.2.1 Inhibition of YHV replication in shrimp by dsRNAs

To investigate the inhibitory effect of dsRNA, shrimps size about 400 mg were divided into seven groups. For the first and second group, shrimp were injected with 20 µl of 150 mM NaCl which were used as negative and positive control. The third to sixth groups were injected with either total RNA of HT115 containing 1 µg of dsRNA-one-stem, dsRNA-two-stem, dsRNA-protease or dsRNA-rr2, respectively. The last group was injected with total RNA of HT115 with empty vector. Then 24 hours after 150 mM NaCl or total RNA injection, only the first group was injected with 150 mM NaCl (negative control) and the rest was challenged with $5x10^{-7}$ dilution of YHV. The gill of an individual shrimp was collected 48 hours after YHV injection. RT-PCR analyses were performed to detect expression levels of Helicase and *Lv*-Actin, which indicated the amount of YHV in the infected shrimp.

The results showed that the Helicase mRNA could be detected in all samples of the positive control group, whereas it could not be detected in the negative control group. And no significant levels of Helicase in the other groups (Figure 31). The results demonstrated that shrimp were YHV-free before experimental infection and the YHV ($5x10^{-7}$ dilution) were inhibited with specific dsRNA and non-specific dsRNA when comparing with the positive control. Therefore, the second experiment was conducted in order to investigate the effect of specific dsRNA by using higher amount of YHV (10^{-6} dilution).

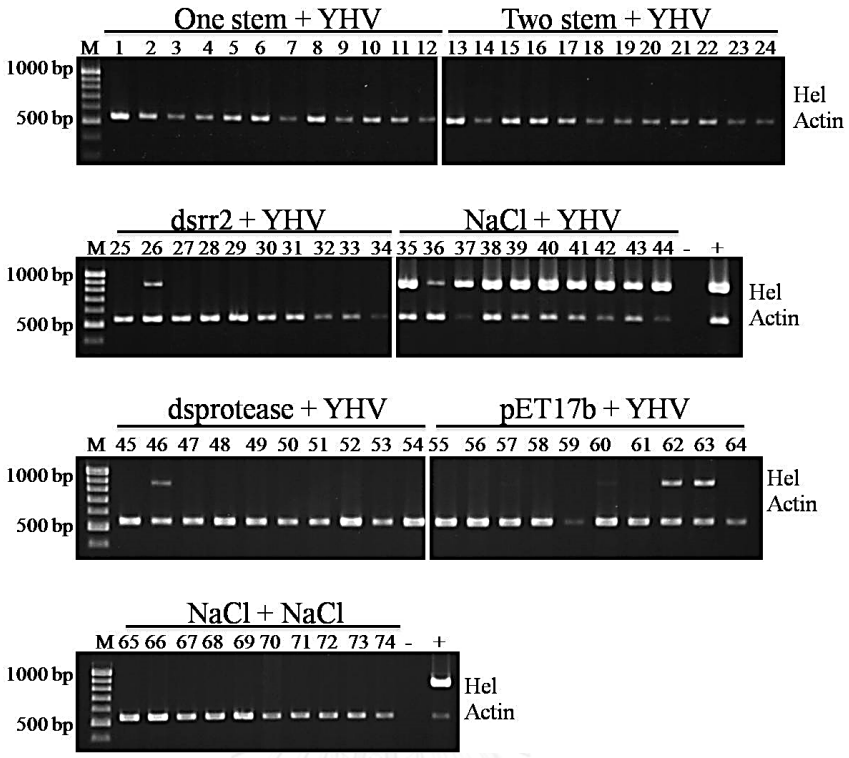


Figure 31 Suppression of YHV replication in shrimp by dsRNAs (experiment 1)

The 400 mg shrimp were separately injected with total RNA of HT115 containing 1 µg of dsRNA-one-stem (One stem), dsRNA-two-stem (Two stem), dsRNA-protease (dsprotease) (suppression control) or dsRNA-rr2 (dsrr2) (non-specific dsRNA), respectively. The shrimp were treated with NaCl (infection control), 1 µg of total RNA from HT115 with empty vector (pET17b). At 24 hour post injection, shrimp were challenged with YHV at dilution $5x10^{-7}$. After 48 hour post viral challenge, the gills were collected to extract RNA and subjected to RT-PCR analysis using the primers specific to YHV-helicase (Hel) and *Lv*-actin (Actin). Shrimp that double-injected with NaCl was the negative control of infection. The number represents an individual shrimp.

Lane M: 100bp ladder

Lane -: PCR negative control

Lane +: PCR positive control

In this second experiment, shrimp were also divided into seven groups which were separately injected with 150 mM NaCl, total RNA containing 1 µg dsRNA-one-stem, dsRNA-two-stem, dsRNA-protease, dsRNA-rr2, total RNA of HT115 with pET17b or total RNA from the host cell (*E. coli* HT115). Similarly, at 24 hours post injection, the samples were challenged with the same stock of YHV. At 48 hours after viral infection, gill was collected and the YHV replication was followed by RT-PCR. The result showed strong YHV inhibition of shrimp treated with dsRNA-one-stem, dsRNA-two-stem or dsRNA-protease when compared with the control (no dsRNA) (Figure 32). But partial inhibition of YHV was observed in shrimp injected with the non-specific dsRNA (dsRNA-rr2), the RNA of HT115 with pET17b or the RNA of HT115. The results suggested that the dsRNAs (one-stem and two-stem) gave high potency of YHV inhibition comparable to the dsRNA-protease

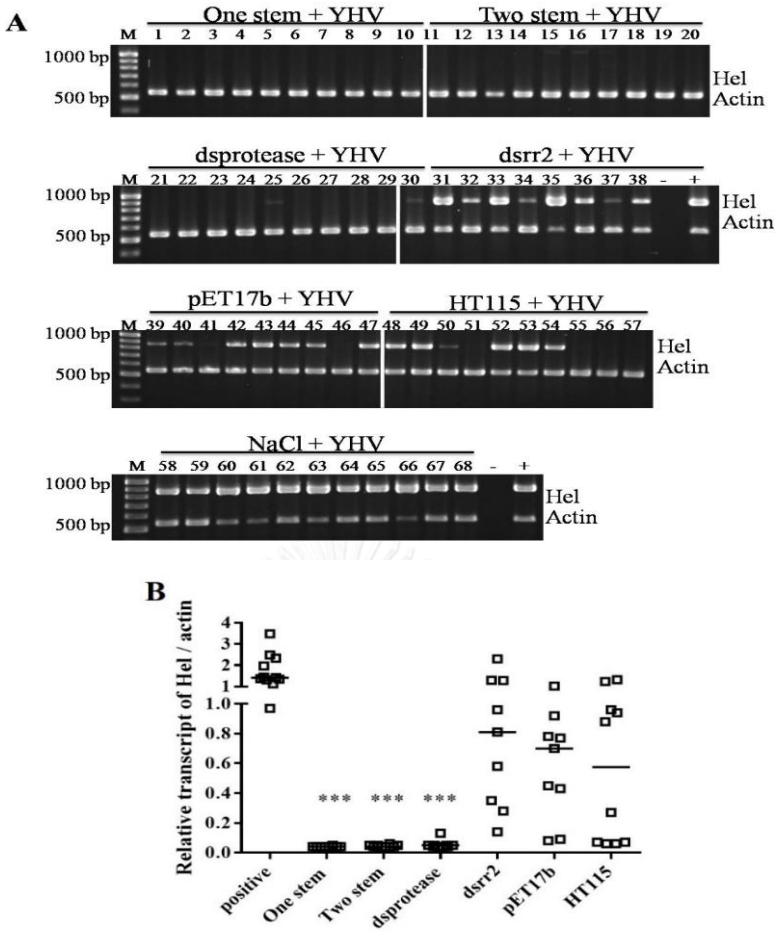


Figure 32 Suppression of YHV replication in shrimp by dsRNAs (experiment 2)

The 400 mg shrimp were separately injected with total RNA of HT115 containing 1 µg of dsRNA-one-stem (One stem), dsRNA-two-stem (Two stem), dsRNA-protease (dsprotease) (suppression control) or dsRNA-rr2 (dsrr2) (non-specific dsRNA), respectively. The shrimp were treated with NaCl (infection control), 1 µg of total RNA from HT115 with empty vector (pET17b) or without vector (HT115). At 24 hour post injection, shrimp were challenged with YHV at dilution 10^{-6}. After 48 hour post viral challenge, the gills were collected to extract RNA and subjected to RT-PCR analysis using the primers specific to YHV-helicase (Hel) and *Lv*-actin (Actin). The number represents an individual shrimp.

A: Multiplex RT-PCR analysis

Lane M: 100bp ladder

Lane -, +: negative and positive PCR control, respectively

B: Relative transcription level of helicase normalized by the shrimp's actin

The relative level of viral genome normalized by the shrimp's actin was measured by scion image and plotted by Graphpad program. *** represents statistically difference ($p < 0.01$) between infection control group with the group injected with dsRNAs (one-stem, two-stem and protease), respectively.

4.3.2.2 Inhibition of WSSV transcription in shrimp by dsRNAs

To investigate the inhibitory effect of dsRNAs, shrimp at size about 400 mg were divided into seven groups similar to 4.3.2.1. After 24 hours post injection, the samples were challenged with WSSV lysate at dilution 10^{-3} into hemolymph of shrimp. At 48 hours after viral infection, the gill was collected and the WSSV genome was detected by PCR with specific primers to viral vp28 and *Lv*-Actin (internal control). The results showed WSSV inhibition in shrimp treated with the RNA containing the specific dsRNA (dsRNA-rr2), in which the dsrr2 and dsRNA-one-stem groups showed comparable inhibition while the dsRNA-two-stem showed the lowest one (Figure 33). This result was un-expected, therefore, the experiment was repeated again and the similar results were obtained as one-stem and dsRNA-rr2 gave higher inhibition of WSSV than two-stem (data not shown). It was not correlated with the amount of WSSV specific dsRNA-rr2 of the two-stem, which was higher than the one-stem. This might be the result of their differences in the structure. Then the third experiment was performed to test this assumption. Shrimp were divided into seven groups. The first group was injected with 20 µl of 150 mM NaCl, which was used as positive control. The second to seventh were injected with total RNA containing 1 µg of un-treated dsRNAs (one-stem or two-stem), 1 µg of RNase A-treated dsRNAs (one-stem or two-stem), mixed dsRNA-protease and dsRNA-rr2 at ratio 1:1 and 1:4, respectively. Then 24 hours after 150 mM NaCl or dsRNA injection, shrimp were challenged with WSSV lysate at dilution 10^{-3} into hemolymph. The gill of an individual shrimp was collected 48 hours after infection to total DNA extraction. PCR analyses were performed to detect viral genome (vp28) and shrimp internal control (*Lv*-Actin). The result showed that the one-stem gave the WSSV inhibition effect equivalent to the mixed dsRNAs (1:4) but higher than the two-stem and the mixed dsRNAs (1:1) (Figure 34). In contrast to the untreated dsRNAs, the two-stem can inhibit WSSV better than the one-stem when they were pre-treated with RNase A (to destroy the secondary structure) before injection into shrimp. It indicated that not only the amount of specific dsRNA but the secondly structure of that dsRNA also affect the efficacy on the WSSV inhibition in shrimp.

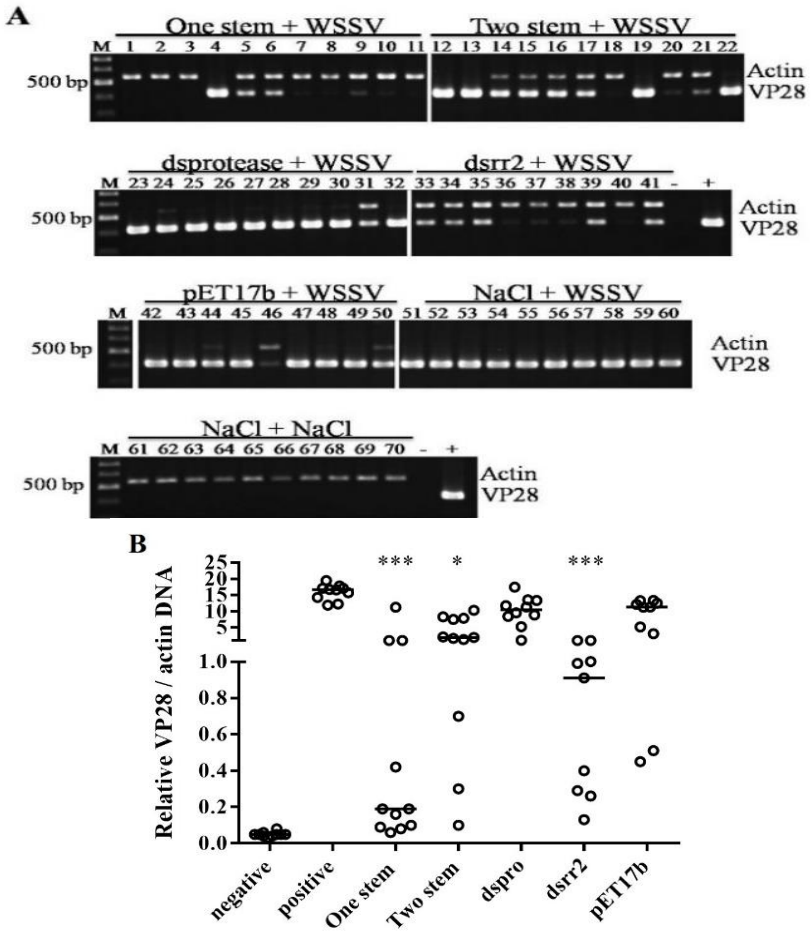


Figure 33 Suppression of WSSV replication in shrimp by dsRNAs

The 400 mg shrimp were separately injected with total RNA of HT115 containing 1 µg of dsRNA-one-stem (One stem), dsRNA-two-stem (Two stem), dsRNA-protease (dsprotease) (non-specific dsRNA) or dsRNA-rr2 (dsrr2) (a suppression control), respectively. The shrimp were treated with NaCl (infection control), or 1 µg of total RNA from HT115 with empty vector (pET17b). At 24 hour post injection, shrimp were challenged with WSSV at dilution 10^{-3}. After 48 hour post viral challenge, the gills were collected to DNA extraction and subjected to PCR analysis using the primers specific to WSSV-vp28 (vp28) and *Lv*-actin (Actin). Shrimp that double-injected with NaCl was the negative control of infection. The number represents an individual shrimp.

A: Multiplex PCR analysis

Lane M: 100bp ladder

Lane -, +: PCR negative and positive control, respectively

B: Relative level of VP28 normalized by the shrimp's actin

The relative level of viral genome normalized by the shrimp's actin was measured by scion image and plotted by Graphpad program. * and *** represent statistically difference ($p < 0.05$ and $p < 0.01$) between infection control group with the group injected with dsRNAs (one-stem, two-stem and rr2), respectively.

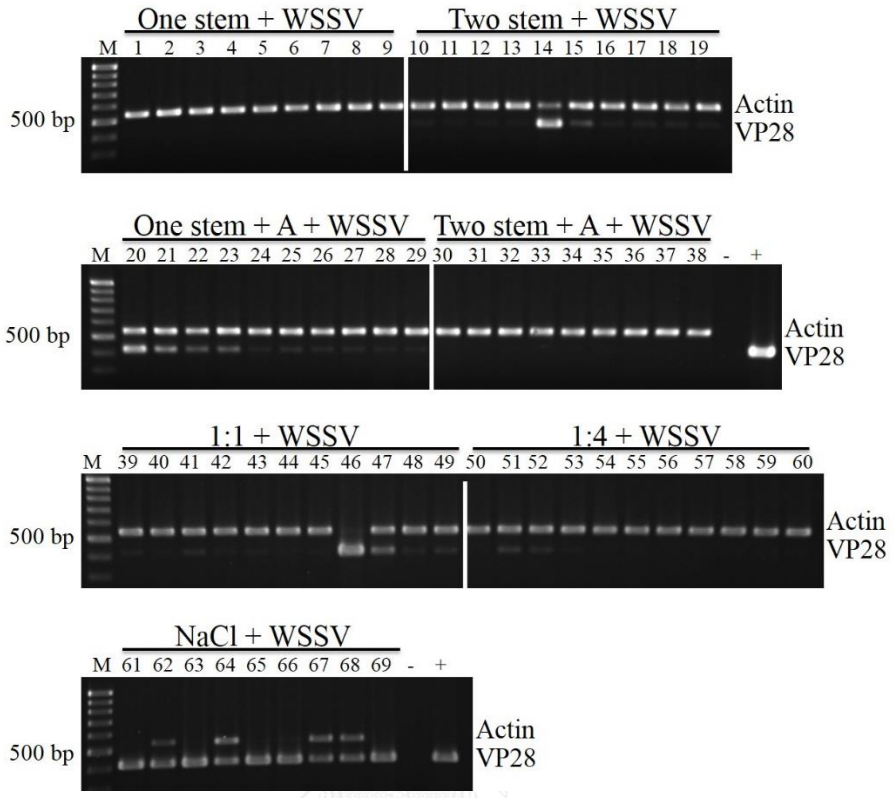


Figure 34 Suppression of WSSV replication in shrimp by dsRNAs (secondary structure test)

The 400 mg shrimp were separately injected with total RNA of HT115 containing 1 µg of dsRNA-one-stem (One-stem), dsRNA-two-stem (Two-stem), the RNase A pre-treated dsRNA-one-stem (One-stem+A), the RNase A pre-treated dsRNA-two-stem (Two-stem+A), the mixed dsRNA-protease and dsRNA-rr2 at ratio 1:1 (1:1) and 1:4 (1:4), respectively. The control shrimp were treated with NaCl. At 24 hour post injection, shrimp were challenged with WSSV at dilution 10^{-3}. After 48 hour post viral challenge, the gills were collected to DNA extraction and subjected to PCR analysis using the primers specific to WSSV-vp28 (vp28) and *Lv*-actin (Actin). The number represents an individual shrimp.

Lane M: 100bp ladder

Lane -: PCR negative control

Lane +: PCR positive control

4.3.3 Suppression of shrimp mortality

4.3.3.1 Suppression of shrimp mortality by dsRNAs (short term)

As the previous experiment demonstrated that total RNA from one-stem could inhibit YHV and WSSV replication at 48 hour after infection. Therefore, the inhibitory effect of one-stem on the shrimp mortality from YHV and WSSV infection was investigated. In this experiment, shrimp (400 mg) were divided into four groups. The first was injected with 20 µl of 150 mM NaCl, which was used as a positive control. The second to fourth was injected with total RNA containing 1 µg of dsRNA (one-stem), dsRNA-protease and dsRNA-rr2, respectively. After 24 hours post injection, the samples were challenged with either YHV or WSSV lysate at dilution $5x10^{-7}$ (YHV) or $5x10^{-4}$ (WSSV) into hemolymph of shrimp and their mortality was monitored every day for 7 days. In the case of YHV, the result showed 90-100% cumulative mortality of shrimp injected with NaCl and non-specific dsRNA (dsRNA-rr2), respectively (Figure 35 A). The significant reduction of shrimp death (from 100% to 10%) was observed when shrimp received the specific dsRNA-protease and dsRNA-one-stem. For WSSV inhibition, the 100% cumulative mortality was observed in the infection control shrimp (injected with 150 mM NaCl) and 70% cumulative mortality was observed in the nonspecific dsRNA (dsRNA-protease). The cumulative mortality was significantly reduced to 0-10% when shrimp were injected with the specific dsRNA-rr2 and dsRNA-one-stem, respectively (Figure 35 B).

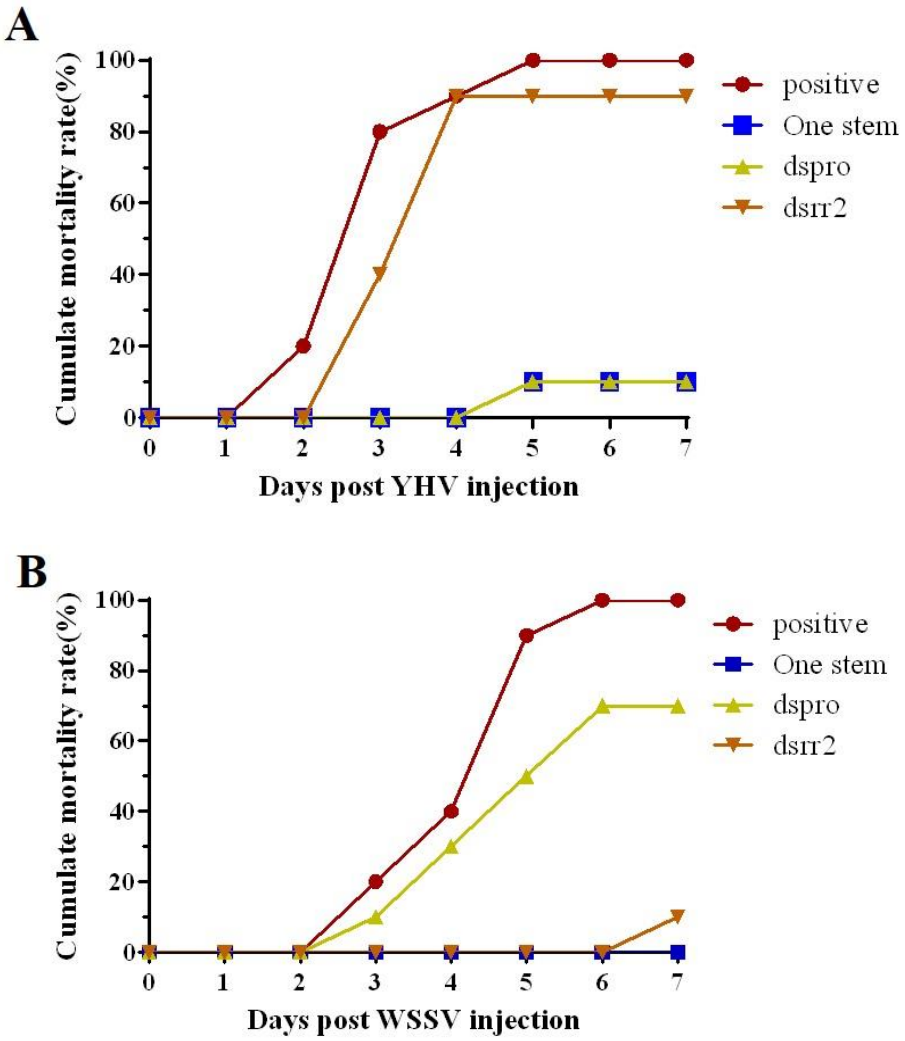


Figure 35 Suppression of shrimp mortality by dsRNAs (short term)

Shrimp (400 mg) (n = 10) were injected with 1 µg of total RNA from one-stem, dsRNA-protease, dsRNA-rr2, and 150 mM NaCl (infection control), respectively. At 24 hour post injection, shrimp were challenged with either YHV lysate at dilution $5x10^{-7}$ (A) or with WSSV lysate at dilution $5x10^{-4}$ (B). The cumulative percent mortality was recorded every day for 7 days.

4.3.3.2 Suppression of shrimp mortality by dsRNAs (long term)

To examine the stability of dsRNA in shrimp, shrimp (400 mg) were divided into five groups. The first was injected with NaCl, which was used as positive control. The second to fifth were injected with total RNA containing 1 µg dsRNA (one-stem), dsRNA (two-stem), the mixed dsRNA-protease and dsRNA-rr2 at weight ratio 1:1 and 1:4, respectively. After 1 day or 5 days post injection, the samples were challenged either with YHV lysate at dilution 10^{-6} or WSSV at dilution $5x10^{-3}$ by injection into hemolymph of shrimp and their mortality was monitored for 14 days. For YHV infection, 1 day after dsRNA injection (Figure 36 A), one-stem showed 100% shrimp survival as similar to the group of the mixed dsRNAs (1:1 and 1:4) and two-stem showed 80% survival whereas there was no shrimp left in the control. When shrimp was infected with YHV 5 days after dsRNA injection (Figure 36 B), 100% cumulative mortality that observed in the control shrimp (NaCl) was reduced to 50%, 40% and 0% cumulative mortality when shrimp injected with the mixed dsRNA 1:1, 1:4, two-stem and one-stem, respectively. It indicated that the dsRNA (one-stem) was stable in shrimp at least 5 days post injection, which was similar to the mixed dsRNAs (1:1). In contrast, the dsRNA (two-stem) and the mixed dsRNAs (1:4) lost their inhibitory effect when they were injected into shrimp for 5 days before YHV infection. In case of inhibitory effect on WSSV infection (both 1 and 5 days post dsRNA injection), 90-100% cumulative mortality was observed in the infection control shrimp (injected with NaCl) whereas 0-10% was found in the others (Figure 36 C and D). There was no significant change of inhibitory effect of the dsRNAs on WSSV infection when they were injected into shrimp for 5 days prior WSSV infection.

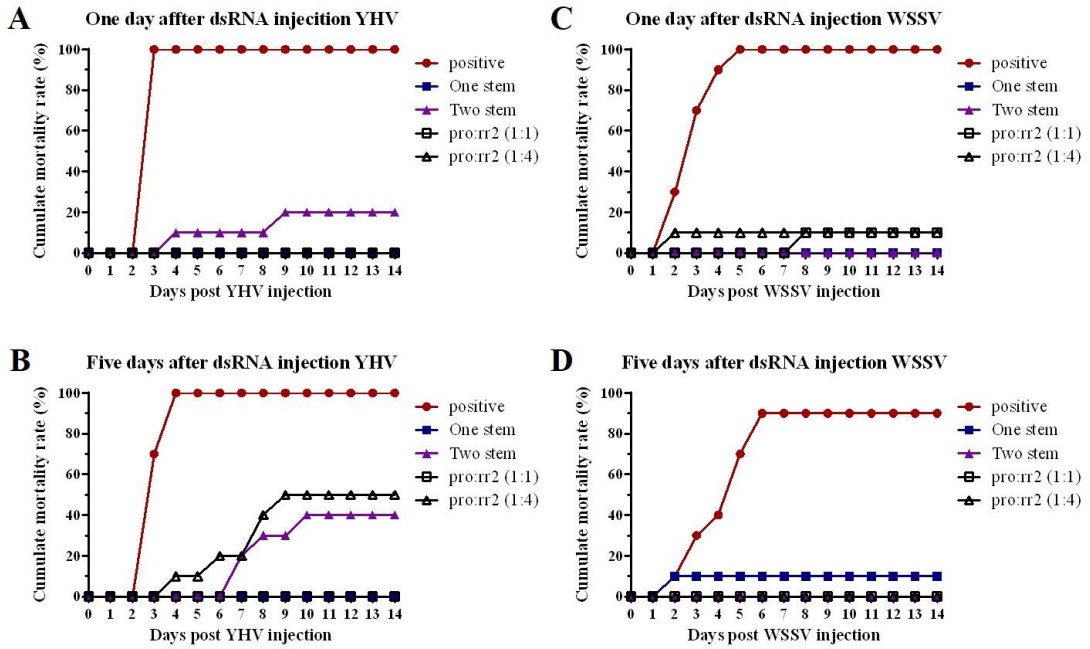


Figure 36 Suppression of shrimp mortality by dsRNAs (long term)

Shrimp (400 mg) were divided into five groups that injected with 1 µg of total RNA from one-stem, two-stem, the mixed dsRNA-protease and dsRNA-rr2 at ratio 1:1 and 1:4 and NaCl (infection control), respectively. At 1 day or 5 days post injection, shrimp were challenged with either YHV lysate at dilution 10^{-6} (A, B) or WSSV lysate at dilution $5x10^{-3}$ (C, D) into hemolymph and mortality was monitored for 14 days. The cumulative percent mortality was recorded every day for 14 day

4.3.3.3 Suppression of shrimp mortality by dsRNAs from dual infection (YHV and WSSV)

To examine protective effect of dsRNA against dual infection of YHV and WSSV, the experimental design was similar to the previous experiment except shrimp were infected with the mixture of YHV at dilution $5x10^{-7}$ and WSSV at dilution $2.5x10^{-3}$. A duplicate experiment was performed in this study. All dsRNA of clone one-stem, two-stem, mixed dsRNA-protease and dsRNA-rr2 (weight ratio = 1:1 and 1:4) showed no significant reduction of shrimp mortality from dual infection when compared with positive control (Figure 37). To identify the causative pathogen of shrimp death, gills of the dead shrimp were collected to DNA and RNA extraction and perform PCR and RT-PCR analysis, respectively. Multiplex PCR and RT-PCR analysis showed almost dead shrimp were resulted from WSSV infection.

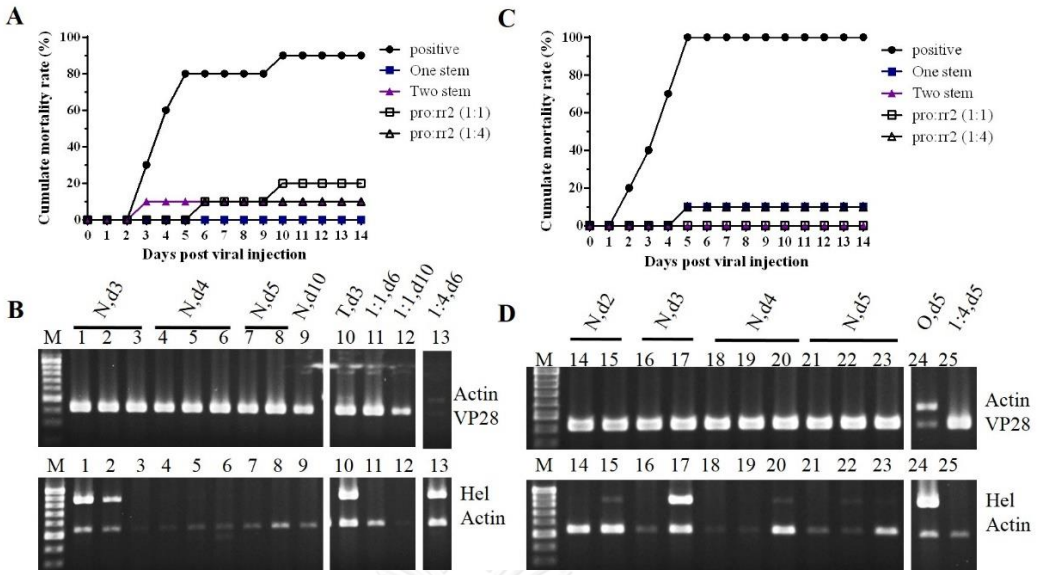


Figure 37 Suppression of shrimp mortality after shrimp infected with both viruses (YHV and WSSV)

Shrimp 400 mg divided into five groups that injected with 1 µg of total RNA from one-stem, two-stem, mixed dsRNA-protease and dsRNA-rr2 ratio 1:1 and 1:4 and 150 mM NaCl, respectively. After 24 hour post injection, shrimps were challenged with both virus (mixed YHV lysate at dilution $5x10^{-7}$ and WSSV lysate at dilution $2.5x10^{-3}$) by injection into hemolymph (A-B and C-D were the first and second experiment, respectively). The cumulative percent mortality (A, C) was recorded every day for 14 days and presented from n = 10. The gills of dead shrimp was collected to DNA and RNA extraction and subjection to PCR and RT-PCR analysis (B, D). Multiplex PCR analysis with VP28 and shrimp's Actin primers was used for WSSV detection while RT-PCR with Helicase and shrimp's Actin primers was used for detection of YHV. The number represents individual shrimp.

Lane M, 1-25: 100bp ladder, individual shrimp

N, O, and T: Shrimp was injected with NaCl, one-stem, two-stem respectively

1:1 Shrimp was injected with mixed dsRNA-protease and dsRNA-rr2 at ratio 1:1

1:4 Shrimp was injected with mixed dsRNA-protease and dsRNA-rr2 at ratio 1:4

d: Time of death

CHAPTER V
DISCUSSION AND CONCLUSIONS

DsRNA mediated RNAi approach is a powerful tool for silencing gene expression [3, 64] as well as viral inhibition [4]. One limitation is that one molecule of dsRNA is specific to a single target gene. Therefore, several strategies such as combined shRNAs/dsRNAs [48-61] or multi-target shRNA/dsRNA [60, 65] have been developed for simultaneous suppression of multiple targets.

In this study, long dsRNAs that have capacity to simultaneously knock down the protease and rr2 genes of YHV and WSSV, respectively were produced from two different recombinant plasmids. The pET17b-dspro-rr2 (one-stem) was constructed by cloning inverted repeat of DNA fragments containing YHV protease sequence linked with WSSV rr2 sequence intervening with one spacer. While pET17b-dspro-dsrr2 (two-stem) was derived from insertion of inverted repeat of protease DNA fragments and inverted repeat of rr2 DNA fragments. Two different structures of dsRNA molecule were expected to produce from these plasmids. The dsRNA one-stem contained one long stem (935 bp) with a shared loop. The dsRNA two-stem contained two short stems; 474 bp of protease and 455 bp of rr2 with their own loops. RNase A treatment was used to dissociate dsRNA-protease from dsRNA-rr2 that presented in two dsRNA bands (Figure 28). Unlike dsRNA one-stem, yield of dsRNA-protease and dsRNA-rr2 of dsRNA two-stem were not equal. Yield of the dsRNA-rr2 was 4 times higher than dsRNA-protease. Two possibilities for explaining this event were premature-termination of T7 RNA polymerase before entering into the protease region or instability of dsRNA-protease folding at 37°C. Since the position of inverted repeat of rr2 in pET17b-dspro-dsrr2 (two-stem) was close to the T7 promoter then the transcribed RNA automatically formed stem-loop structure that might interfere forming of the stem-loop of protease or the stem-loop of rr2 might represent the transcriptional termination signal [66] leading to no transcription of protease transcript. Moreover, the dsRNA-protease was found that it may not be stable at high temperature as higher yield of dsRNA-protease was obtained when bacteria were grown at 25°C (Figure 27).

Unexpected result, WSSV was inhibited by dsRNA one-stem more efficient than dsRNA two-stem, which had higher amount of viral specific dsRNA-rr2 than one-stem (Figure 33 and 34). The predicted secondary structure of dsRNA two-stem (see Appendix A) might interfere the entry of dsRNA into shrimp cells or hinder the binding of dsRNA with enzyme Dicer leading to loss activity of RNAi. As dicer requires an open ends for proper substrate (dsRNA) recognition and processing [67, 68], according to the predicted dsRNA structure, dsRNA two-stem containing one open end may not represent a proper substrate for dicer. This speculation was confirmed by RNase A treatment. After ssRNA removal by RNase A digestion, the structure of dsRNA two-stem was destroyed, the inhibitory effect of dsRNA two-stem was then recovered as shown in Figure 34. As a result, the inhibitory effect of treated dsRNA two-stem was correlated with the amount of specific dsRNA-rr2 that dsRNA two-stem showed higher strength of WSSV inhibition than dsRNA one-stem.

In this study, dsRNA one-stem showed high level of suppression against YHV and WSSV infection even though it contained only half of each specific dsRNA (dsRNA-protaese for YHV and dsRNA-rr2 for WSSV) (Figure 32 and 33). These results demonstrated an ability of longer hairpin RNA structure in improving gene silencing efficiency. It was consistent with the previous reports that the dsRNA mediated RNAi efficiency was length dependent [69, 70]. It has also been demonstrated in *Caenorhabditis elegans*, *Diabrotica virgifera* and *Tribolium castaneum* that the efficiency of cellular uptake and/or spreading of longer dsRNA was higher than the shorter one [71-73]. Recently, the Electrophoretic Mobility Shift Assay (EMSAs) results revealed that recombinant extracellular domain of *C. elegans* SID-1, a membrane protein required for systemic RNA, bound longer dsRNA more robustly but less effectively to shorter dsRNAs [74]. Moreover, when using more than one dsRNA, competition between dsRNAs might occur at several levels such as cellular uptake, transport of the dsRNA, processing of dsRNA or mRNA silencing [65, 69, 70, 72, 75]. Hence, two dsRNAs bound to each other was created to solve this problem and showed higher efficacy on gene knockdown than by a mixture of two dsRNAs. Therefore, the multi-target dsRNAs (one-stem and two-stem) produced in this study would be beneficial to effectively inhibit YHV and WSSV in shrimp rather than mixture of dsRNA-protease and dsRNA-rr2.

Longevity of dsRNA from one-stem and two-stem to reduce the mortality of shrimp after YHV and WSSV challenge at 1 day and 5 days post injection with dsRNA was evaluated. The data showed that stability of dsRNA from one-stem in shrimp hemolymph to effectively inhibit both viruses (YHV and WSSV) was at least 5 days after injection (Figure 36 B and 36 D) whereas that of two-stem was reduced at 5 days of injection. This was in agreement with the previous result of Yodmaung [7] that inhibition of YHV was reduced when injection of YHV was performed after 5 days of dsRNA-protease introduction. Nevertheless, there was no different effect among dsRNAs against WSSV (Figure 36 C and 36 D), which was not correlated with the suppression result (Figure 31 and 32). Therefore, the longevity of dsRNAs in shrimp for WSSV inhibition is needed to be repeated.

Shrimp mortality from dual viruses (mixed YHV and WSSV) infection was also reduced when receiving dsRNAs either one-stem or two-stem. It indicated that these dsRNAs were effective to simultaneously suppression of YHV and WSSV. However, most shrimp were dead from WSSV infection (90%) rather than YHV (10%).

The dsRNA one-stem and two-stem in this study are the first long dsRNA showing efficient wide range inhibition of both YHV and WSSV in shrimp. This study provides an important information essential to design and achieve an effective multi-target dsRNAs for multiple genes silencing in shrimp. This also revealed the potential anti-YHV and WSSV strategy for application in the shrimp farm in the future.

CONCLUSIONS

1. Two recombinant plasmids, pET17b-dspro-rr2 (one-stem) and pET17b-dspro-dsrr2 (two-stem), for producing long dsRNA, which has sequence corresponding to the YHV protease gene as well as to the WSSV rr2 gene, were successfully constructed.
2. The dsRNAs generated from pET17b-dspro-rr2 and pET17b-dspro-dsrr2 contained different ratio of dsRNA-pro and dsRNA-rr2, which were 1:1 for dsRNA one-stem and 1:4 for dsRNA two-stem.
3. Both dsRNA one-stem and dsRNA two-stem could effectively inhibit YHV and WSSV in shrimp, however; but the dsRNA one-stem had more potent on YHV and WSSV suppression and mortality reduction of YHV infected shrimp than the dsRNA two-stem.
4. The inhibitory effect of dsRNA one-stem could maintain in shrimp hemolymh for at least 5 days whereas dsRNA two-stem against YHV was reduced after injection into shrimp for 5 days.
5. Both dsRNA one-stem and dsRNA two-stem could simultaneously suppress YHV and WSSV in shrimp from dual infection.

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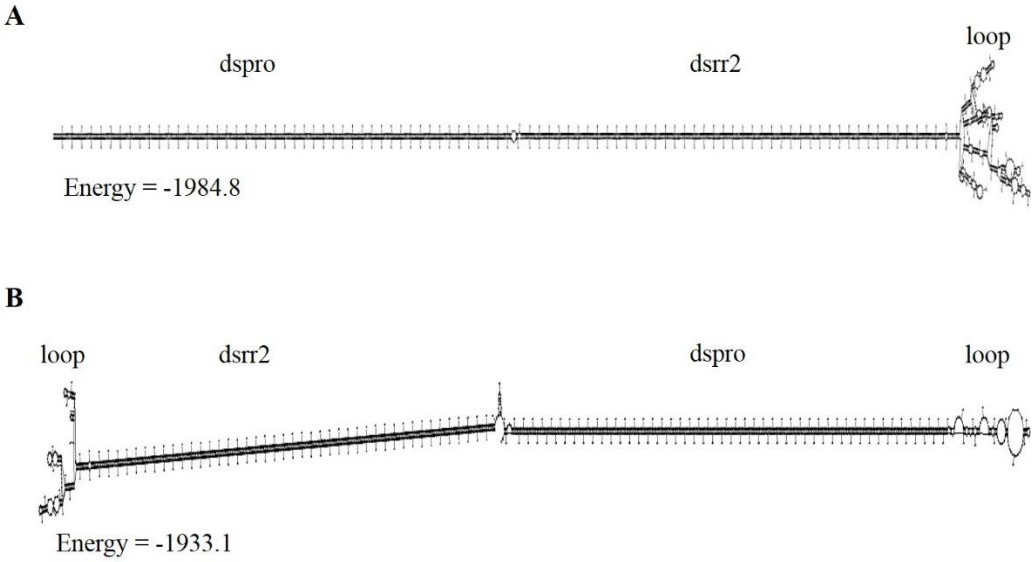
APPENDIX

Appendix A: The predicted secondary structure of dsRNA one-stem and dsRNA two-stem

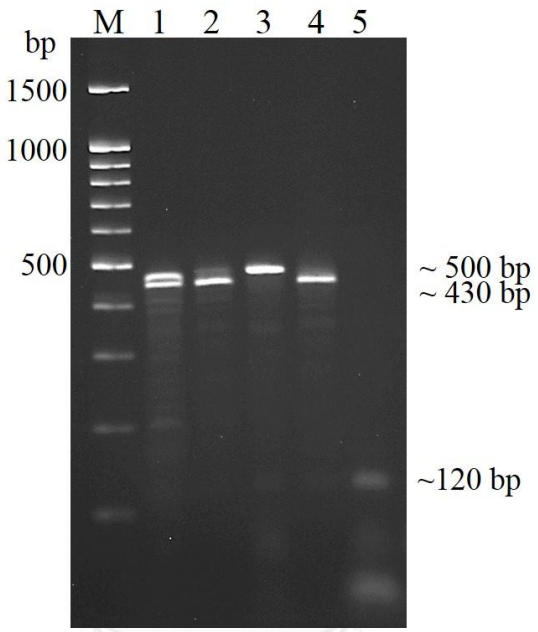
The predicted secondary structure of RNAs; one-stem (A) and two-stem (B) under thermodynamics at 37°C condition are shown. The possibility of stem-loop structures of dsRNA-protease and dsRNA-rr2 are indicated. The RNA secondary structure prediction was performed using free software RNA structure version 5.7 on the webservers [76].

(http://rna.urmc.rochester.edu/RNAstructureWeb/Servers/Fold/Fold.html)



Appendix B: Concentration of dsRNAs prior injection

Concentration of each RNase A treated-dsRNA was quantified by band intensity estimation comparing with a standard DNA marker. One microgram of total RNA was treated with RNase A digestion. Approximately 50 ng of the treated RNA were loaded on 2% agarose gel.



Lane M: 100bp DNA ladder

Lane 1: dsRNA one-stem

Lane 2: dsRNA two-stem

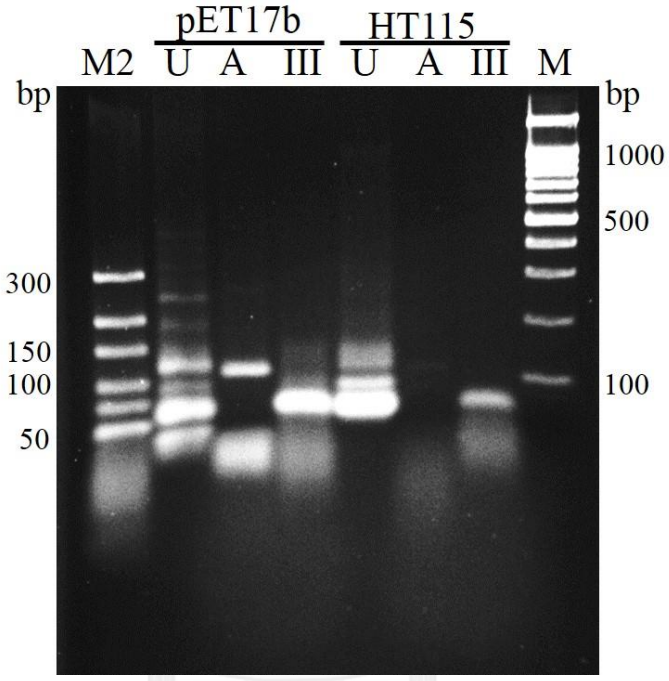
Lane 3: dsRNA-protease

Lane 4: dsRNA-rr2

Lane 5: dsRNA from pET17b

Appendix C: Verification of dsRNA from empty vector by enzymatic digestion

Production of dsRNA from empty vector (pET17b) or HT115 was verified by RNase digestion. One microgram of total RNA was treated with RNase A (A) and RNase III (III) digestion. Approximately 100 ng of the treated RNA were loaded on 2.5% agrarose gel.



Lane M2: Ultra low range DNA marker

Lane M: 100bp DNA ladder

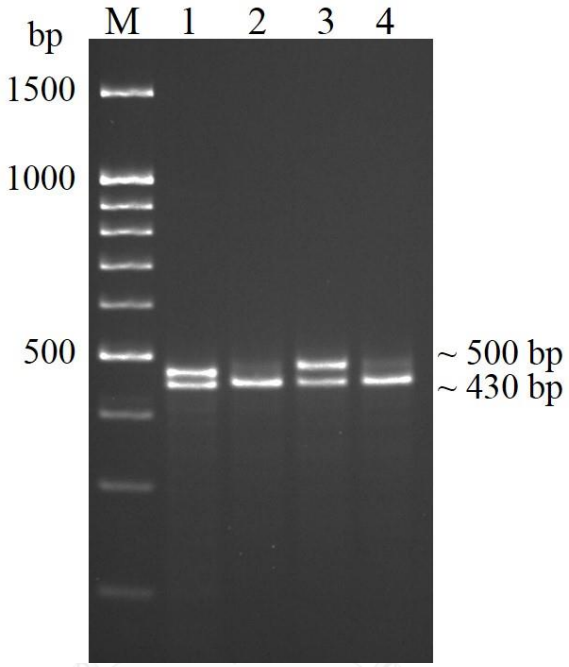
Lane U: Untreated total RNA

Lane A: Total RNA treated with RNase A

Lane III: Total RNA treated with RNase III

Appendix D: Concentration of dsRNA prior injection

Concentration of each dsRNA was verified by agarose gel electrophoresis. One microgram of total RNA was treated with RNase A digestion. Approximately 50 ng of treated RNA were loaded on 2% agrarose gel.



Lane M: 100bp DNA ladder

Lane 1: dsRNA one-stem

Lane 2: dsRNA two-stem

Lane 3: Mixed dsRNA-protease and dsRNA-rr2 at 1:1 ratio

Lane 4: Mixed dsRNA-protease and dsRNA-rr2 at 1:4 ratio

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