CHAPTER III
MATERIALS AND METHODS

3.1 Materials

3.1.1 The black tiger shrimp culture

Penaeus monodon or the black tiger shrimp that have size about 1-10 g and viral pathogen free were obtained from shrimp farms in Nakhon Pathom and Cha-Cheung-Sao provinces, Thailand. Shrimp were maintained in tanks containing 30 liters of aerated artificial saltwater at 10 ppt salinity. Shrimp were acclimatized for 1 day before dsRNAs injection and fed with commercial feed twice a day. Half of the saltwater was exchanged every 2 days.

3.1.2 Chemicals

In these experiments, molecular and analytical grade of the chemicals were used in all experiments. They were purchased from several reliable manufacturers such as Promega, Invitrogen, Fermentas, BIO-RAD, Sigma, Merck, and Molecular Research Center.

3.1.3 Enzymes and Accessory buffers

Improm-II^{TM} reverse transcriptase	Promega
RNasin® ribonuclease inhibitor	Promega
Taq DNA polymerase	Home made
Taq DNA polymerase buffer + $(NH_4)_2SO_4$	Fermentas
RQ1 RNase free DNase	Promega
Ribonuclease A (RNase A)	USBiological
Short cut ribonuclease III (RNase III)	New England Biolab
Deoxyribonucleotide triphosphates (dNTPs)	Promega
Vent DNA polymerase	New England Biolab

3.1.4 Kits and miscellaneous

λ/*Hin*d III DNA ladder	(Invitrogen)
1 kb plus DNA ladder	(Invitrogen)
100 bp DNA ladder	(Invitrogen)
TRI® Reagent, TRIzol® Reagent	(Molecular Research Center)
Agarose	(SeaKem LE)
Ribomax™ Large scale RNA production systems	(Promega)
Ampicillin and tetracyclin	(GIBCO)
Syringes and needles (29G)	(BD Ultra-Fine™)
QIAquick® PCR Purification Kit	(QIAGEN)
QIAprep Spin Miniprep Kit	(QIAGEN)

3.1.5 Bacteria strains

***Escherichia coli* strain HT115** (RNase III mutant *E.coli* strain) was used in this study. The RNase III gene was disrupted by a Tn10 transposon which contains a tetracycline-resistance marker. The genotype of HT115 is as follows: F-, mcrA, mcrB, IN (rrnD-rrnE)1, lambda-, rnc14::Tn10(DE3) lysogen: lacUV5 promoter-T7 polymerase.

***Escherichia coli* strain DH5α** [*supE*44, Δ*lac*U169 (ϕ80 *lacZ* 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.

3.1.6 Culture media

E. coli was cultured in low salt Luria-Bertani (LB) medium and 2XYT medium. LB medium containing 1% (w/v) tryptone, 0.5% (w/v) yeast extract and 1% (w/v) NaCl. 2XYT medium contains 1.6 % (w/v) of tryptone, 1% (w/v) of yeast extract, and 0.5% (w/v) of NaCl. Ampicillin (100 µg/ml) and tetracyclin (12.5 µg/ml) were used for selection of the recombinant cells.

Media were sterilized by autoclaving at 15 p.s.i for 15 min. For agar plates, 15% European bacteriological agar was added before autoclaving.

3.1.7 Vectors and recombinant plasmids

pGEM®-T Easy vector was obtained from Promega (Figure 3-1). This vector is suitable for cloning of PCR product. This linearized plasmid was added with a 3' terminal thymidine to both ends. These single 3'-T overhang at the insertion site is compatible with a single deoxyadenosine overhang at 3' end of PCR-amplified fragments that were generated by certain thermostable polymerases.

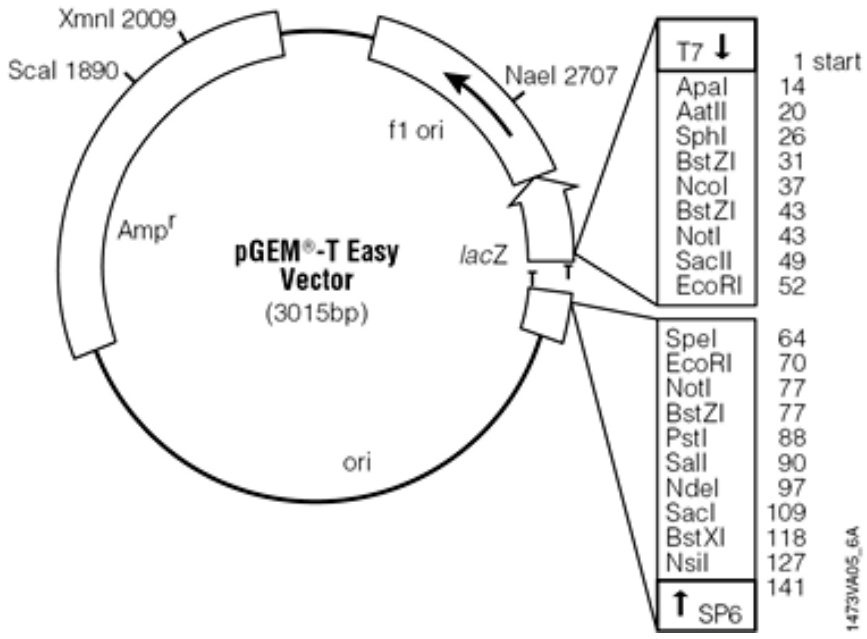


Figure 3-1 A schematic diagram of the pGEM®-T Easy vector (Promega) (Taken from www.promega.com/products/pcr/pcr-cloning/pgem_t-easy-vector-systems).

The figure illustrates the physical map of pGEM®-T Easy vector containing T7 and SP6 RNA polymerase promoters flanking a multiple cloning region surrounded by α-peptide coding region of the enzyme β-galactosidase (lac Z), amplicillin resistance gene (Amp^r), origin of replication (Ori) and single 3' terminal thymidine at both ends that is suitable for cloning of the PCR product.

pET-17b This vector has many cloning sites for insertion of an inverted repeat fragment of a template for dsRNA expression in *E. coli*. A variety of expression level can be controlled by T7 promoter under the induction of IPTG (Figure 3-2).

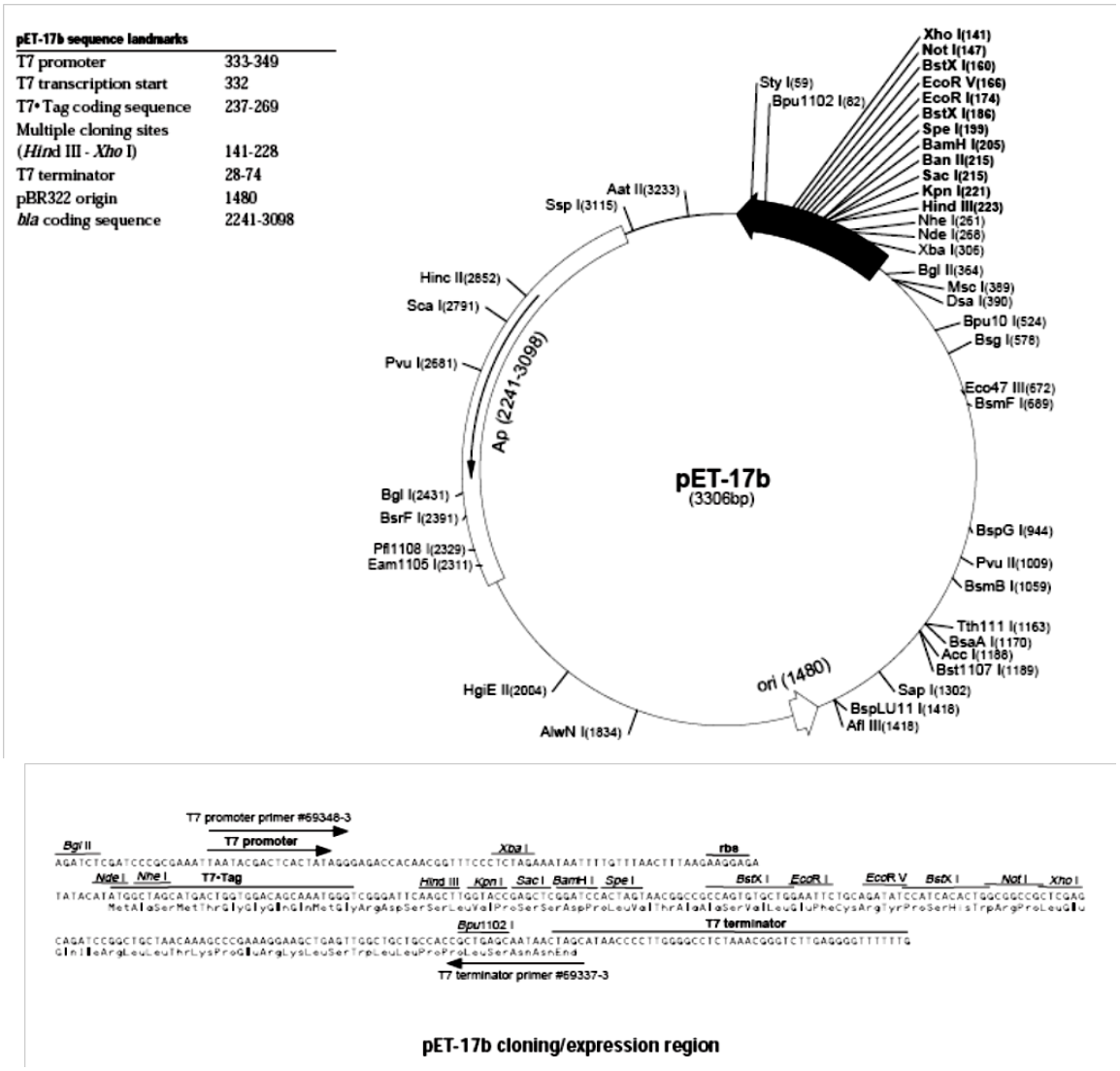


Figure 3-2 A schematic diagram of the plasmid pET-17b (Taken from Novagen).

The figure represents the physical map of pET-17b vector. This plasmid contains T7 promoter, ribosome binding site (rbs), T7 transcription start sites, multiple cloning site, T7 terminator and amplicillin resistance gene (Ap).

pET17b-stRab7 The recombinant plasmid that contains an inverted repeat of the stem loop of PmRab7 gene to produce dsRNA of PmRab7 (Figure 3-3) (kindly constructed by Ms. Mayuree Chanasakulniyom).

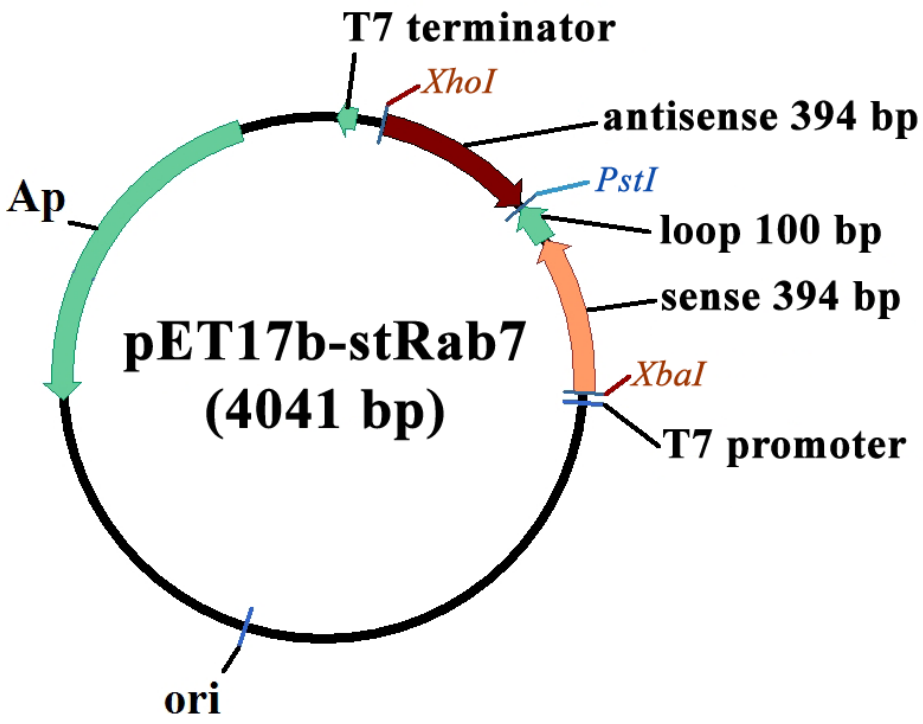


Figure 3-3 A schematic diagram of the recombinant plasmid, pET17b-stRab7.

The figure represents the physical map of pET17b-stRab7 vector. This recombinant plasmid contains 888 bp of fragment harboring the cassette for producing 394 bp of dsRNA-PmRab7 in the form of stem-loop structure. The plasmid was consisted of two fragments of PmRab7 (GenBank accession number DQ231062), a sense-loop strand (494 bp, nt 246-739) and an inverted anti-sense strand (394 bp, nt 246-639) cloned into pET-17b plasmid at *Xba*I / *Pst*I and *Pst*I / *Xho*I site, respectively.

pET3a-YHV (pro) A recombinant plasmid that contains an inverted repeat of the stem loop of YHV protease gene to produce dsRNA of YHV (pro) (Figure 3-4) (kindly provided by Asst. Prof. Dr. Witoon Tirasophon).

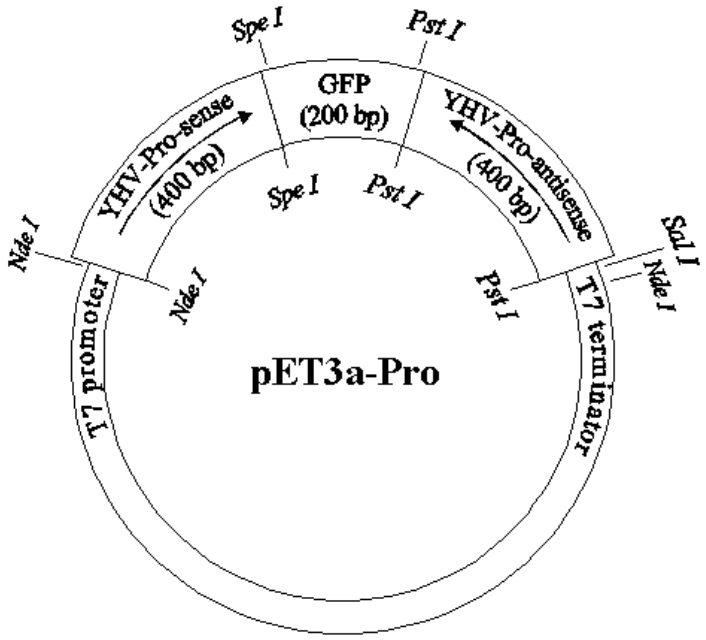


Figure 3-4. A schematic diagram of the recombinant plasmid, pET3a-YHV (pro) (Taken from Ongvarrasopone et al., 2007).

pET17b-stPmLoqs. A recombinant plasmid that contains an inverted repeat of the stem loop of PmLoqs gene to produce dsRNA of PmLoqs (Figure 3-5).

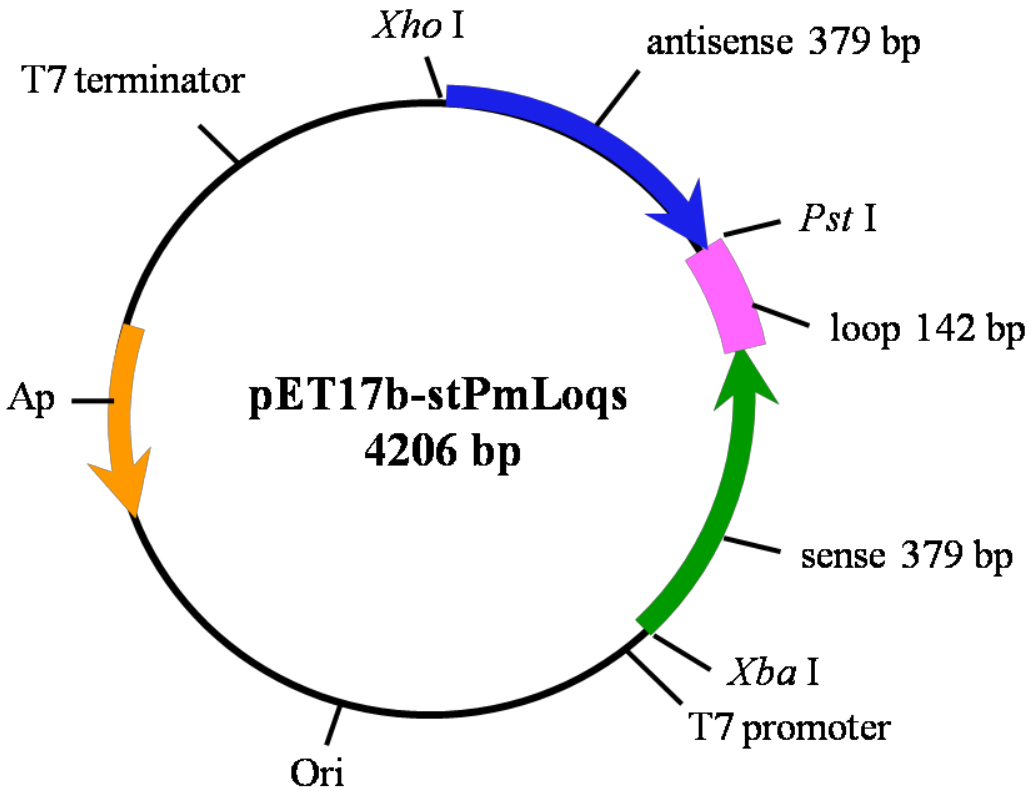


Figure 3-5 A schematic diagram of the recombinant plasmids, pET17b-stPmLoqs.

The figure represents the physical map of pET17b-stPmLoqs vector. This recombinant plasmid contains 900 bp of fragment harboring the cassette for producing 379 bp of dsRNA-PmLoqs in the form of stem-loop structure. The plasmid was consisted of two fragments, a sense-loop strand (521 bp, nt 426-947) and an inverted anti-sense strand (379 bp, nt 426-805) into pET-17b plasmid at *Xba*I / *Pst*I and *Pst*I / *Xho*I site, respectively.

3.1.8 Synthetic oligonucleotide primers

Synthetic oligonucleotides that used in the experiments were purchased from Bio Basic Inc. The sequence of primers are shown in the Table 3-1

Table 3-1 List of the synthetic oligonucleotide primers.

Experiments	**Name**	**Sequences (5' → 3')**	**Tm (°C)**
Reverse transcription	PRT	CCGGAATTCAAGCTTCTAGAG GATCCTTTTTTTTTTTTTTT	70
Amplification of PmActin cDNA	PmActin-F	GACTCGTACGTCGGGCGACGA	62
	PmActin-R1	GACTCGTACGTCGGGCGACGA	65
	PmActin-R2	CGTAGATGGGCACGGTGTGGG	60
Amplification of PmRab7 cDNA	GTP-met	ATGGCATCTCGCAAGAAGATT	53
	GTP-stop	TTAGCAAGAGCATGCATCCTG	53
Amplification of PmLoqs cDNA	Loqs_F1	CTTCTGCAGATAGAGGGAGCA G	58
	Loqs_R1	GACATTGACTTGAGGTGCCTC	57
	Loqs_R2	CTCCACCAGTTACCACAGTCA	59
Amplification of dsPmLoqs (*in vitro*)	dsLoqsA_F1	GCAGCAAATGGATCTGGACA	57
	dsLoqsA_R1	GCTGTGTCATCTTGTAGGCT	56
	dsLoqsA_F1T7	*TAATACGACTCACTATAG*GCAGC AAATGGATCTGGACA T_7	66
	dsLoqsA_R1T7	*TAATACGACTCACTATAG*GCTGT GTCATCTTGTAGGCT T_7	65
	dsLoqsL_F1	CTGCAAGACATTGCCCAAGAA G	58
	dsLoqsL_F1T7	*TAATACGACTCACTATAG*CTGCA AGACATTGCCCAAGAAG T_7	66
	Loqs_R2T7	*TAATACGACTCACTATAG*CTCCA CCAGTTACCACAGTCA T_7	66
Amplification of dsPmLoqs (*in vivo*)	dsLoqsA_SF1	TCTAGAGCAGCAAATGGATCT GGACA *Xba*I	61
	dsLoqsA_AF1	CTGCAGGCTGTGTCATCTTGTA GGCT *Pst*I	64

	dsLoqsA_SR1	CTGCAGCTTCCGCGAGTCACT GGTGG *Pst*I	69
	dsLoqsA_AR1	AAGCTTCTCGAGGCAGCAAAT GGATCTGGACA *Hin*dIII, *Xho*I	68
Amplification of the full length PmLoqs	Loqs_5'	GGGCACTGAGGCAGGAGCACA CAA	67
	3_LoqsAB	CCGGAATTCAAGCTTCTAGAG	54
Amplification of YHV (helicase) cDNA	YHV (hel)-F	CAAGGACCACCTGGTACCGGT AAGAC	62
	YHV (hel)-R	GCGGAAACGACTGACGGCTAC ATTCAC	66
Real-time PCR	qRab7-F2	CTGGAGAATAGGGCGGTATCA ACG	60
	qRab7-R2	CGAGCAATGGTCTGGAAGGCT AAC	61
	EF1a-F	GAACTGCTGACCAAGATCGAC AGG	61
	EF1a-R	GAGCATACTGTTGGAAGGTCT CCA	60

* Underline indicates the restrictions site. Italic characters with underline represent T7 promoter sequences.

3.1.9 Virus stock

YHV stock was kindly provided by Ms. Pratsaneeyaporn Posiri. The virus was prepared from hemolymph of *Penaeus monodon* which was injected with YHV. The YHV was purified *via* Urografin® (Schering) gradient ultracentrifugation and stored at -80 °C until used.

3.2 Methods

3.2.1 Total RNA extraction by Trizol® Reagent

The tissues of *P. monodon* were collected from individual shrimp. After that, approximately 50 mg of each tissue was homogenized in 500 µl of Trizol® Reagent (Molecular Research Center) and incubated at room temperature for 5 minutes. Then, the mixture solution was added with 100 µl of chloroform (20% from volume of TRIzol® Reagent) following by vigorous shaking for 15 sec and incubated at room temperature for 15 min. The solution was centrifuged at 12,000 g for 15 min at 4 °C. Aqueous phase was transferred into a new 1.5 ml tube. Precipitation of RNA from the aqueous phase was performed by adding 500 µl of isopropanol and incubated at -20 °C for 1 hour. The solution was subsequently centrifuged at 12,000 g for 15 min at 4 °C. The supernatant was discarded and 1 ml of 75% ethanol was added for washing RNA pellet. Following centrifugation at 12,000 g for 5 min at 4 °C, the 75% ethanol was removed and the RNA pellet was air-dried for 5 min. Finally, the RNA pellet was resuspended with RNase-free sterile distilled water. For completely dissolved, the solution was incubated at 55-65 °C for 5 min and stored at -80 °C until used.

3.2.2 Determination of RNA concentration

The RNA concentration was determined by UV-spectrophotometry (Nano Drop 1000 UV-Visible). The absorbances were read at wavelength of 260 nm and 280 nm. One unit of the absorbance of 260 nm (A_{260}) equals approximately 40 µg/ml of RNA, so the concentration was calculated from the A_{260} by the formula:

$$\text{RNA concentration (µg/µl)} = \frac{A_{260} \times \text{dilution factor} \times 40}{1{,}000}$$

The purity of RNA was determined by absorbance ration of A_{260} / A_{280}. The ratio should be 1.8 - 2.0 which represents high purity of RNA.

3.2.3 Generation of first strand cDNA by reverse transcription (RT)

Total RNA that extracted from shrimp tissue was used as a template to generate first stranded cDNA by oligo(dT)-containing primer (PRT) (Table 3-1) and ImProm II™ reverse transcriptase (Promega). According to the manufacturer's

protocol, 1 μg of total RNA was mixed with 250 ng of oligo(dT)- containing primer (PRT) and adjusted the volume up to 5.5 μl with RNase-free sterile distilled water (DEPC-water). The reaction mixture was allowed to anneal at 70 °C for 5 min and immediately cooled on ice for 5 min. Subsequently, RT reaction mixture composing of 1X ImProm-II buffer, 3 mM $MgCl_2$, 0.5 mM dNTPs, 0.3 μl of sterile distilled water and 100 unit of ImProm-II™ Reverse transcriptase was added into the reaction mixture to the final volume of 10 μl. The combined mixture was gently mixed and incubated at the following profile; 25 °C for 5 min, 42 °C for 60 min and 70 °C for 15 min for synthesized first strand cDNA. The synthesized first strand cDNA was stored at -20 °C until used.

3.2.4 Polymerase chain reaction (PCR)

3.2.4.1 Multiplex PCR amplification of PmLoqs and Actin

The first stranded cDNA was used as a template for determination of PmLoqs and PmActin expression by PCR. The PCR composed of 1 μl of the first stranded cDNA, 1X *Taq* DNA polymerase buffer + $(NH_4)_2SO_4$ (Fermentas), 2 mM $MgCl_2$, 0.4 mM dNTPs, 0.4 μM of Loqs_F1 and Loqs_R1 primers to detect all isoforms of PmLoqs (Table 3-1), 0.04 μM of PmActin-F and PmActin-R2 primers (Table 3-1), and 1 units of *Taq* DNA polymerase (home made) in a final volume of 25 μl. MJ-mini Thermal (PTC-1148) machine was used to perform PCR. The condition for multiplex PCR of all isoforms of PmLoqs and PmActin are shown below (Table 3-2). Multiplex PCR products of PmLoqs targeted to all isoform and PmActin have size 485 bp and 350 bp, respectively were analyzed by 1.5% agarose gel electrophoresis. In addition, to perform multiplex PCR of PmLoqs targeted to only long isoform and PmActin, 0.4 μM of Loqs_F1 and Loqs_R2 primers and 0.04 μM of PmActin-F and PmActin-R1 primers (Table 3-1) were used. The condition for multiplex PCR of long isoforms of PmLoqs and PmActin are shown below (Table 3-3). The PCR products of PmLoqs targeted to long isoforms and PmActin have size 1006 bp and 550 bp, respectively were analyzed by 1.5% agarose gel electrophoresis.

Table 3-2 PCR condition for multiplex PCR of PmLoqs (all isoforms) and PmActin.

Steps of PCR		**Temperature (°C)**	**Time**
Pre-denaturation		94 °C	5 min
Step cycles (5 cycles) Touchdown	Denaturation	94 °C	30 sec
	Annealing	57-53 °C	30 sec
	Extension	72 °C	30 sec
Step cycles (20 cycles)	Denaturation	94 °C	30 sec
	Annealing	52 °C	30 sec
	Extension	72 °C	30 sec
Extra-extension		72 °C	10 min
Hold		20 °C	∞

Table 3-3 PCR condition for multiplex PCR of PmLoqs (long isoform) and PmActin.

Steps of PCR		**Temperature (°C)**	**Time**
Pre-denaturation		94 °C	5 min
Step cycles (5 cycles) Touchdown	Denaturation	94 °C	30 sec
	Annealing	55-51 °C	30 sec
	Extension	72 °C	1 min
Step cycles (25 cycles)	Denaturation	94 °C	30 sec
	Annealing	50 °C	30 sec
	Extension	72 °C	1 min
Extra-extension		72 °C	10 min
Hold		20 °C	∞

3.2.4.2 Multiplex PCR amplification of PmRab7 and Actin

The first stranded cDNA was used as a template for determination of PmRab7 and PmActin expression by PCR. The PCR composed of 1 µl of the first stranded cDNA, 1X *Taq* DNA polymerase buffer + $(NH_4)_2SO_4$ (Fermentas), 2 mM $MgCl_2$, 0.4 mM dNTPs, 0.4 µM of GTP-met and GTP-stop

primers (Table 3-1), 0.06 µM of PmActin-F and PmActin-R2 primers (Table 3-1), and 1 units of *Taq* DNA polymerase (home made) in a final volume of 25 µl. MJ-mini Thermal (PTC-1148) machine was used to perform a PCR. The condition for multiplex PCR of PmRab7 and PmActin are shown below (Table 3-4). Multiplex PCR products of PmRab7 and PmActin which have size 617 bp and 350 bp, respectively were analyzed by 1.5% agarose gel electrophoresis.

Table 3-4 PCR condition for multiplex PCR of PmRab7 and PmActin.

Steps of PCR		**Temperature (°C)**	**Time**
Pre-denaturation		94 °C	1.30 min
Step cycles (5 cycles) Touchdown	Denaturation	94 °C	30 sec
	Annealing	58-54 °C	30 sec
	Extension	72 °C	45 sec
Step cycles (25 cycles)	Denaturation	94 °C	30 sec
	Annealing	53 °C	30 sec
	Extension	72 °C	45 sec
Extra-extension		72 °C	7 min
Hold		20 °C	∞

3.2.4.3 Multiplex PCR amplification of YHV (hel) and Actin

The first stranded cDNA was used as a template for determination of YHV (hel) and PmActin expression by PCR. The PCR composed of 1 µl of the first stranded cDNA, 1X *Taq* DNA polymerase buffer + $(NH_4)_2SO_4$ (Fermentas), 2 mM $MgCl_2$, 0.4 mM dNTPs, 0.4 µM of YHV (hel)-F and YHV (hel)-R primers (Table 3-1), 0.1 µM of PmActin-F and PmActin-R1 primers (Table 3-1), and 1 units of *Taq* DNA polymerase (home made) in a final volume of 25 µl. MJ-mini Thermal (PTC-1148) machine was used to perform a PCR. The condition for multiplex PCR of YHV (hel) and PmActin are shown below (Table 3-5). Multiplex PCR products of YHV (hel) and PmActin which have size 850 bp and 550 bp, respectively were analyzed by 1.5% agarose gel electrophoresis.

Table 3-5 PCR condition for multiplex PCR of YHV (hel) and PmActin.

Steps of PCR		**Temperature (°C)**	**Time**
Pre-denaturation		94 °C	1 min
Step cycles (25 cycles)	Denaturation	94 °C	30 sec
	Annealing	55 °C	30 sec
	Extension	72 °C	45 sec
Extra-extension		72 °C	7 min
Hold		20 °C	∞

3.2.5 Agarose gel electrophoresis

Agarose gel electrophoresis is used to separate, purify and analyze the nucleic acid. DNA fragments were separated according to their molecular size. The agarose gel was prepared by completely dissolving appropriate amount of agarose powder in 1X Tris-acetate EDTA (TAE) buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) and boiled until it completely melted. After gel solution cooled down about 55 °C, it was poured into the clean tray which sealed with tape and allowed gel to set completely. After the gel was set, solid gel was placed into the chamber and 1X TAE buffer was added just enough to cover the gel. The PCR product sample was mixed with 6x loading dye [15% (w/v) ficoll 400, 0.01% (w/v) Bromophenol blue] to the final concentration of 1X. The mixture was loaded into slots of submerged gel using pipette. The lid of gel chamber was closed. The electrical leads were attached, so that the DNA was migrated toward the positive anode. After gel electrophoresis was completed, gel was stained in ethidium bromide for 5 min and destained in water for 15 min. The ethidium-bromide-stained gel was visualized under UV light and then photographed.

3.2.6 Semi-quantitative analysis of mRNA expression levels

The multiplex PCR products of mRNA targets were analyzed on 1.5% agarose gel electrophoresis. The intensity of each band after subtracting the background was quantified by using Scion image analysis program. The signal of mRNA band was collected. The relative expression levels of the target gene

normalized with the internal control gene was calculated as the ratio of the intensity and expressed in arbitary unit.

3.2.7 Statistical analysis

The semi-quantitative data were expressed as mean ± standard error of mean (SEM). The statistical analysis of mean ± SEM was performed by using ANOVA test. The probability (*p*) value of < 0.05 was accepted as statistically significant.

3.2.8 Plasmid DNA extraction using QIAprep Spin Miniprep Kit (QIAGEN)

This kit was used for extraction of plasmid DNA from bacterial culture. Before extraction, a single colony of each clone from the master plate or flash streaked plate was picked and inoculated into 3 ml LB medium containing 100 μg/ml amplicillin and/or 12.5 μg/ml of tetracycline according to the characteristics of the recombinant plasmids. After incubated at 37 °C for 16 hours with shaking, the bacterial culture was harvested by centrifugation at 13,000 rpm for 60 sec at room temperature. After the supernatant was removed, the cell pellet was resuspended with 250 μl of Buffer P1 (50 mM Tris-Cl pH 8.0, 10 mM EDTA, and 100 μg/ml RNase A). After that, 250 μl of Buffer P2 [200 mM NaOH, 1% (w/v) SDS] was added and gently mixed by inverting the reaction tube for 4-6 times. The reaction was placed at room temperature for 5 min and then added with Buffer P3 (3 M potassium acetate, pH 5.5) following by gently inverting the tube for 4-6 times. Then, it was centrifuged at 13,000 rpm for 10 min and the supernatant was transferred to the QIAprep spin column prior to centrifugation at 13,000 rpm for 1 min. After that, the flow-through solution was discarded and 750 μl of Buffer PE was added into the column. Subsequently, the column was centrifuged at 13,000 rpm for 1 min and the filtrate was removed. After additional centrifugation for 1 min to dry the membrane, the QIAprep column was placed in a new 1.5 ml microcentrifuge tube and 20 μl of sterile distilled water was added into the middle of column and left at room temperature for 1-5 min. Then the microcentrifuge tube containing QIAprep column was centrifuged at 13,000 rpm for 1

min to collect plasmid DNA solution. The DNA plasmids were subsequently analyzed by 1% agarose gel electrophoresis.

3.2.9 DNA ligation

The appropriate amount of vector and DNA insert fragment in the ligation reaction was calculated from the molar ratio of vector to DNA insert. Normally, the molar ratio could be varied from 1:1 to 1:7 depended on the size of the vector compared to the insert DNA. The optimal ratio (1:3 of vector:insert) could be applied when the size of DNA vector and DNA insert were not different. Molar of DNA can be calculated by the following equation:

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

The plasmid vector and DNA fragment were digested with the same or compatible restriction enzymes and purified by QIAquick Purification Kit. Both of them were mixed and added with the final concentration of 1X ligation buffer (50 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 25 µg/ml BSA and 1 mM ATP) and 2 units T4 DNA ligase (Promega) in the total volume of 20 µl. The reaction mixture was incubated overnight at 16ºC.

3.2.10 Transformation of recombinant plasmid DNA into competent cells by heat-shock method

The ligation mixture (4 µl) was added into an aliquot of 100 µl *E. coli* competent cells and incubated on ice for 30 min. Subsequently, the mixture was subjected to heat-shock at 42 ºC for 50 seconds and immediately placed on ice for an additional 5 min. After incubation, 900 µl of LB medium were added into the transformed cells and incubated at 37ºC for 1 hr with shaking at 250 rpm. After centrifugation at 13,000 g for 60 sec, the supernatant was discarded. Then, the cell pellets were resuspended in the remaining medium. The suspended cells (~200 µl) were spread on the LB agar plate containing ampicillin (100 µg/ml) and cells were allowed to grow by incubation overnight at 37ºC.

3.2.11 Rapid size screening for recombinant clone

The recombinant clone containing an insert fragment was identified by comparing the size with the plasmid without the insert. The single colony from the transformed agar plate was picked and lysed in 30 µl of pre-warmed lysis buffer (100 mM NaOH, 5 mM EDTA, 10% (w/v) sucrose, 0.25% (w/v) SDS, 100 mM KCl , 0.1% (w/v) Bromophenol Blue). After vigorous vortex, the mixture was incubated at 37ºC for 5 min and then placed on ice for an additional 5 min. Subsequently, the mixture was centrifuged at 13,000 rpm for 5 min. Then, 25 µl of supernatant were analyzed by agarose gel electrophoresis. After electrophoresis, the recombinant clone that contained the insert DNA fragment would present as the bands of the recombinant plasmid DNA that migrated slower than the bands of the plasmid without the insert DNA.

3.2.12 Production of dsRNA

In vitro transcription method was used to produce dsRNAs targeting all isoforms (dsRNA-PmLoqs) and long isoform (dsRNA-PmLoqsL) of PmLoqs. DsRNA-PmLoqs was designed to cover between domain 1 and 2 (nt 426-805) whereas dsRNA-PmLoqsL was designed in the region of domain 3 (nt 360-1366). In addition, dsRNA-PmLoqs that produced by *in vivo* bacterial expression was designed to target all isoforms in the region of domain 1 and 2 of PmLoqs (nt 426-805). Moreover, the specific primers; Loqs_F1/Loqs_R1 (Table 3-1) were used to detect all isoforms (nt 360-846) of PmLoqs expression whereas the primers; Loqs_F1/Loqs_R2 (Table 3-1) were designed to detect only the long isoforms (nt 360-1366) of PmLoqs (Figure 3-6).

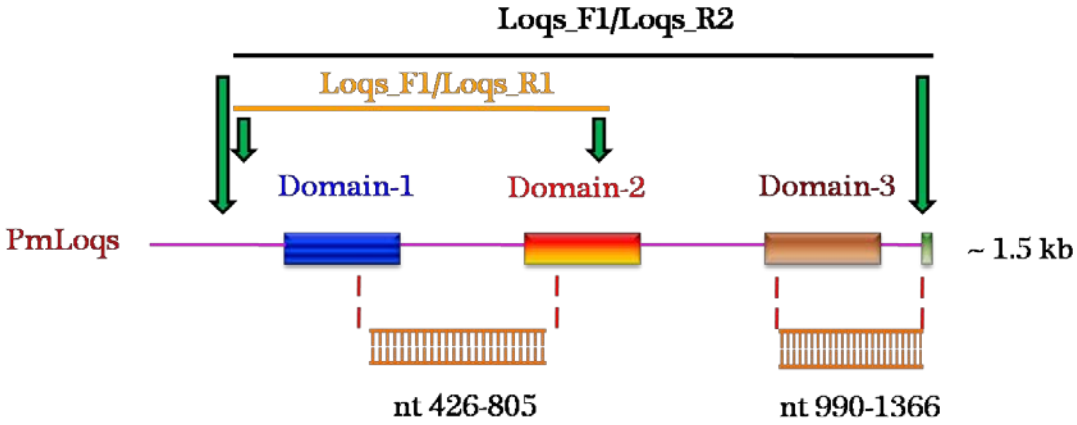


Figure 3-6 Schematic diagram of dsRNA-PmLoqs targeted on PmLoqs domain and the target regions for detection.

dsRNA-PmLoqs (*in vitro* transcription and *in vivo* bacterial expression) were designed to correspond with domain 1 and 2 of PmLoqs (nt 426-805) and have size about 379 bp whereas dsRNA-PmLoqsL (*in vitro* transcription) were designed to correspond with domain 3 (nt 990-1366) and have size about 379 bp. The primer Loqs_F1/Loqs_R1 was designed to detect PmLoqs in all isoforms (nt 360-846) and Loqs_F1/Loqs_R2 was used to detect PmLoqs only long isoforms (nt 360-1366).

3.2.12.1 *In vitro* dsRNA-PmLoqs production

3.2.12.1.1 Amplification of dsRNA-PmLoqs templates

PCR reaction of sense and antisense DNA templates were amplified from a plasmid containing the partial nucleotide sequences of PmLoqs cDNA (pGEM®-T Easy-PmLoqs). Each PCR contained 20 ng of plasmid DNA template, 1X Thermopol buffer , 0.4 mM dNTPs, 0.4 µM of each primer (for dsLoqs targeting all isoforms; sense reaction: dsLoqsA_F1 and dsLoqsA_R1T7, antisense reaction: dsLoqsA_R1 and dsLoqsA_F1T7; for dsLoqsL targeting long isoforms; sense reaction: dsLoqsL_F1 and Loqs_R2T7, antisense reaction: Loqs_R2 and dsLoqsL_F1T7) (Table 3-1) and 1.0 unit of Vent DNA polymerase in 25 µl reaction. The PCR was performed by incubation at 94 ºC for 5 min. Then the touchdown PCR was performed for 5 cycles by decreasing the annealing temperature 1 ºC per cycle from 55-51 ºC for 30 sec. After that the PCR was performed for 25 cycles under these following conditions: a denaturation at 94 ºC for 30 sec; an annealing at 50 ºC for 30 sec; and an extension at 72 ºC for 30 sec. The PCR product was run on 1% agarose gel electrophoresis. An expected size of the PCR products of 379 bp was excised and the DNA fragments were purified by using QIAquick® Gel Extraction Kit (QIAGEN).

3.2.12.1.2 Purification of sense and antisense DNA template

QIAquick® Gel Extraction Kit (QIAGEN) was used to purify the PCR products of dsLoqs DNA templates. To purify DNA fragment from agarose gel according to the manufacturer's protocol, the expected DNA fragment was excised from the gel and transferred into a 1.5 ml microcentrifuge tube and weighed. Three volume of buffer QG were added to one volume of the gel (100 mg equals approximately 100 µl). The mixture was incubated at 50 °C for 10 min or until the gel was completely dissolved. One volume of the isopropanol was added to the sample and mixed thoroughly. Then, the sample mixture was transferred into the QIAquick column that placed into 2 ml collection tube and centrifugation at 13,000 rpm for 1 min. After the flow-through solution was discarded, column was washed by

adding 750 µl of buffer PE prior to centrifugation at 13,000 rpm for 1 min. Residual ethanol was removed by an additional centrifugation at 13,000 rpm for 1 min. The column was air dried and placed into a new 1.5 ml microcentrifuge. The DNA was eluted from the column by adding 50 µl EB buffer (10 mM Tris·Cl, pH 8.5) or water (pH 7.0-8.5) at the center of column. After standing at room temperature for 1 min, the column was centrifuged at 13,000 rpm for 1 min. Finally, the concentration of DNA was determined by analyzing on agarose gel electrophoresis

3.2.12.1.3 Production of dsRNA-PmLoqs by *in vitro* transcription

The dsRNA-PmLoqs was produced by *in vitro* transcription using Ribomax™ Large scale RNA production systems (Promaga). The 100 µl reaction was set up at room temperature by adding the reaction components in the order as followed: 20 µl of 5X transcription buffer, 30 µl of rNTPs mix (25 mM each rATP, rGTP, rCTP, rUTP), 5 µg of each sense and antisense DNA templates, 10 µl of T7 RNA polymerase enzyme mix and nuclease-free water was added up to 100 µl. The reaction was then incubated at 37 °C for 4 hrs. After that the reaction containing dsRNA was reannealed by incubated at 70 °C for 15 min. and then left at room temperature to cool down until the temperature reached about 25 °C.

After *in vitro* transcription, the DNA templates were removed by adding RQ1 RNase-free DNase (1 unit/ µl) to a concentration of 1 unit/µg of DNA template and incubated at 37 °C for 15 min. After the reaction was incubated for 10 min, RNaseA (1 µg) was added in 100 µl reaction in order to eliminate residual ssRNA. The reaction was continuously incubated for 15 min.

The dsRNA-PmLoqs was extracted with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1), pH 4.5. Then the reaction was vortexed for 1 min and centrifuged at 13,000 rpm for 2 min. The upper aqueous phase was transferred to a new microcentrifuge tube. The organic phase was performed back extraction by adding 100 µl nuclease free water and centrifuged at 13,000 rpm for 2 min. The upper aqueous phase from back extraction was transferred to the same tube as above. The aqueous phase was then added 1 volume of chloroform: isoamyl alcohol (24:1) and vortexed for 1 min. The reaction was

centrifuged at 13,000 rpm for 2 min. The aqueous phase was transferred to a new microcentrifuge tube and mixed with 0.1 volume of 3 M sodium acetate, pH 5.3 and 2.5 volume of absolute ethanol, respectively. The reaction was incubated at -20 °C for 1 hour. The precipitated dsRNA were centrifuged at 13,000 rpm for 20 min at 4 °C. Then the dsRNA pellet was washed with 1 ml of 70% ethanol and centrifuged at 7,500 rpm for 5 min at 4 °C. The pellet were air-dried, dissolved in 100 µl of 150 mM NaCl and stored at -80 °C.

3.2.12.2 *In vivo* dsRNA-PmLoqs production

3.2.12.2.1 Amplification of dsRNA-PmLoqs templates

The sense-loop and antisense DNA templates that corresponded to domain 1 and 2 of PmLoqs were amplified from a plasmid containing the partial nucleotides sequence of PmLoqs cDNA (pGEM®-T Easy-PmLoqs). Each PCR reaction contained 10 ng of plasmid DNA template, 1X Thermopol buffer, 0.4 mM dNTPs, 0.4 µM of each primer (sense reaction: dsLoqsA_SF1 containing recognition site for *Xba*I and dsLoqsA_SR1 containing recognition site for *Pst*I; antisense reaction: dsLoqsA_AF1 containing recognition site for *Pst*I and dsLoqsA_AR1 containing recognition site for *Xho*I and *Hin*dIII) (Table 3-1) and 1.0 unit of *Vent* DNA polymerase in 25 µl reaction. The PCR was performed by incubation at 94 ºC for 5 min. Then the PCR was performed for 25 cycles under these following conditions: a denaturation at 94 ºC for 30 sec; an annealing at 50 ºC for 30 sec; and an extension at 72 ºC for 30 sec and final extension step at 72 °C for 10 min. The PCR product was run on 1% agarose gel electrophoresis. Expected sizes of the PCR products are 521 bp for sense-loop strand and 379 bp for antisense strand. The DNA fragments were purified by using QIAquick® PCR Purification Kit (QIAGEN).

3.2.12.2.2 Purification of sense and antisense DNA template

QIAquick® PCR Purification Kit (QIAGEN) was used to purify the PCR product of dsRNA-PmLoqs DNA templates. According to the manufacturer's protocol, five volume of buffer PB were added to one volume of the

sample. The mixture was applied to a QIAquick spin column and followed by centrifugation at 13,000 rpm for 1 min. After the filtrate was discarded, column was washed by adding 750 µl of buffer PE prior to centrifugation at 13,000 rpm for 1 min. Residual ethanol was removed by an additional centrifugation at 13,000 rpm for 1 min. The column was placed into a new 1.5 ml microcentrifuge and the DNA was eluted from the column by adding 50 µl EB buffer (10 mM Tris·Cl, pH 8.5) or water (pH 7.0-8.5) at the center of column. After standing at room temperature for 1 min, The column was centrifuged at 13,000 rpm for 1 min. Finally, the concentration of DNA was determined by analyzing on 1.5 % agarose gel electrophoresis.

3.2.12.2.3 Construction of dsRNA-PmLoqs expression vector

PCR product of sense-loop and antisense DNA templates were added with A tail before cloning into pGEM®-T Easy vector. Each reaction containing DNA template, 1X Thermopol buffer, 0.2 mM dATP and 5 unit of *Taq* DNA polymerase (New England Biolab) in 10 µl reaction. This reaction was incubated at 70 ºC for 30 min. After that, the product of each fragment was used to ligate with pGEM®-T Easy vector at a raito 1:3 (vector:insert). The reaction composed of 1X rapid ligation buffer, 25 ng of pGEM®-T Easy vector, PCR product (A tail) in a ratio 1:3 and 2 unit of T4 DNA ligase (Promega). The reaction was incubated at 4 ºC overnight. The recombinant plasmid was transformed into *E.coli* DH5α before extracted by using QIAprep Spin Miniprep Kit (QIAGEN).

The recombinant plasmid was digested by restriction enzyme *Xba*I + *Pst*I and *Hin*dIII + *Pst*I for sense-loop and antisense fragment, respectively. The reaction containing 1 µg of the recombinant plasmid, 1X reaction buffer and 5 units of the corresponding restriction enzymes was incubated at 37 °C overnight. The digestion reaction was analyzed by agarose gel electrophoresis. Then, the *Xba*I-*Pst*I-sense-loop fragment was ligated with plasmid vector pGEM-3Zf(+) that had been previously digested with *Xba*I and *Pst*I at a ratio 1:5 (vector:insert). After ligation at 4 °C overnight, the recombinant plasmid was transformed into *E.coli* DH5α competent cell. The rapid size screening technique was used to screen the recombinant clones that contain sense-loop fragment. The positive

recombinant clones were digested with *Hind*III and *Pst*I before further ligated with the *Hin*dIII-*Pst*I-antisense fragment prior to transforming into *E. coli* DH5α competent cell. The rapid size screening technique was used again to screen the recombinant clones. The positive clones were digested with *Pst*I to generate linearized plasmid for automated DNA sequencing by T7 and Sp6 promoter to verify the nucleotide sequences of the stem-loop dsRNA-PmLoqs expression cassette.

After obtaining the recombinant plasmid containing inverted repeat fragments of the stem-loop dsRNA-PmLoqs (pGEM-3Zf-stPmLoqs), an inverted repeat of stem loop dsRNA-PmLoqs was digested by *Xba*I and *Xho*I and subcloned into the pET-17b plasmid (pET-17b-stPmLoqs) before transforming into a ribonuclease III mutant *E.coli* strain HT115 competent cell for *in vivo* expression of dsRNAs.

3.2.12.3 Production of dsRNAs by *in vivo* bacterial expression

A single colony of bacteria containing pET-17b-stPmLoqs was inoculated in LB media having 12.5 µg/ml of tetracycline and 100 µg/ml of ampicillin and incubated at 37 °C overnight with shaking (250 rpm). The culture was transferred to 2XYT media at a ratio 1:100 and incubated at 37 °C with shaking until the OD_{600} reached 0.4. Then, the Isopropyl-β-D thiogalactoside (IPTG) was added to a final concentration of 0.1 mM in order to induce an expression of stem loop dsRNA-PmLoqs. The bacterial cultures were further incubated at 37 ºC until OD_{600} per ml reached about 1. The 100 OD_{600} of bacteria culture was harvested by centrifugation at 6,000 g for 15 min. at 4 °C and extracted dsRNAs by using TRI-reagent.

Recombinant plasmids containing an inverted repeat of stem loop GFP (pET3a-stGFP), YHV protease gene (pET3a-YHV (pro) (kindly provided by Asst. Prof. Dr.Witoon Tirasophon) and PmRab7 (pET17b-stRab7) (kindly constructed by Miss Mayuree Chanasukulniyom) were transformed into a RNaseIII mutant HT115 *E.coli* strain. A single colony of bacteria was inoculated in 5 ml LB media having 12.5 µg/ml of tetracycline and 100 µg/ml of ampicillin at 37 °C overnight (approximately 14 hours) with shaking (250 rpm). The culture (1 ml) was transferred to 100 ml of 2XYT media containing ampicillin (100 µg/ml) and

tetracycline (12.5 µg/ml) and cultured until OD_{600} reached 0.4. Then, isopropyl-β-D thiogalactoside (IPTG) was added to a final concentration 0.4 mM for pET3a-YHV (pro) and pET3a-GFP and 0.1 mM IPTG pET17b-stRab7 in order to induce an expression of dsRNAs. The bacterial cultures were further incubated at 37 ºC until OD_{600} per ml reached about 1. The 100 OD_{600} of bacteria culture was harvested by centrifugation at 6,000 g for 15 min. at 4 °C. The dsRNAs were extracted from the bacterial pellet by using TRI-reagent.

3.2.12.4 Extraction dsRNAs by using TRI® Reagent

bacterial cells (100 OD) were lysed by boiling with 0.1% SDS in 5 ml of phosphate buffer saline (PBS) for 2 min. The RNaseA was then added to a concentration 100 µg of RNaseA per 100 OD_{600} of bacterial culture in order to eliminate the bacterial ssRNA and the loop region of stem loop dsRNAs. The dsRNAs was extracted by TRI® Reagent. The TRI® Reagent was added at a ratio 10 ml of TRI® Reagent per 100 OD_{600} of bacterial culture. The reaction was vigorously shaken and incubated at room temperature for 5 min before adding 2 ml of chloroform (ratio 0.2 ml of chloroform per 1 ml TRI® Reagent). The reaction was vigorously shaken for 15 sec. and incubated at room temperature for 15 min. Then it was centrifuged at 9,000 g for 20 min. at 4 °C. The upper phase was transferred to a new tube and 5 ml of isopropanol was added (ratio 0.5 ml of isopropanol per 1 ml TRI® Reagent) and stored at -20 °C for at least 1 hour to precipitate the dsRNAs. The reaction was centrifuged at 9,000 g for 20 min at 4 °C and the supernatant was discarded. The pellet was washed with 10 ml of 75% ethanol (ratio 1 ml of 75% ethanol per 1 ml TRI® Reagent) and the mixture was subsequently centrifuged at 7,500 g for 5 min at 4 °C. The ethanol was removed and the dsRNA pellet was air dried. Finally, the pellet was dissolved in 1 ml of 150 mM NaCl. The dsRNAs were stored at -80 °C for subsequent analysis.

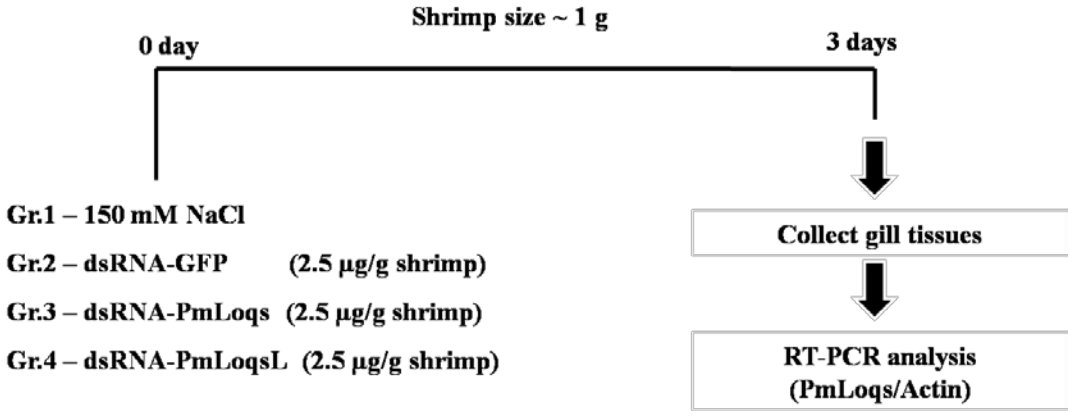
3.2.13 Double stranded RNA digestion assay

The dsRNAs qualities were determined by RNase digestion. First, control reaction was undigested dsRNA. Second, RNaseA digested reaction contained 3 µg of dsRNA, 1X RNaseA buffer (0.3 M Sodium Acetate, 10 mM Tris-HCl, 5 mM EDTA), 5 ng of RNaseA and nuclease-free water up to 10 µl. After that the reaction was

incubated at 37 °C for 5 min. Third, RNaseIII digested reaction contained 3 µg of dsRNA, 1X RNaseIII buffer (50 mM Tris-HCl, 1 mM Dithiothreitol, pH 7.5), 20 mM $MnCl_2$, 1.3 units of RNaseIII (New England Biolab) and nuclease-free water up to 10 µl. Then the reaction was incubated at 37 °C for 30 min. All of the reactions were run in 1.5% agarose gel to analyze dsRNA integrity. RNaseA digests only ssRNA whereas RNaseIII digests dsRNA. If a dsRNA can be digested by RNaseIII and remains intact in the same size as undigested dsRNA in RNaseA digested reaction, it means that the good quality of dsRNA was obtained and can be used in the experiment.

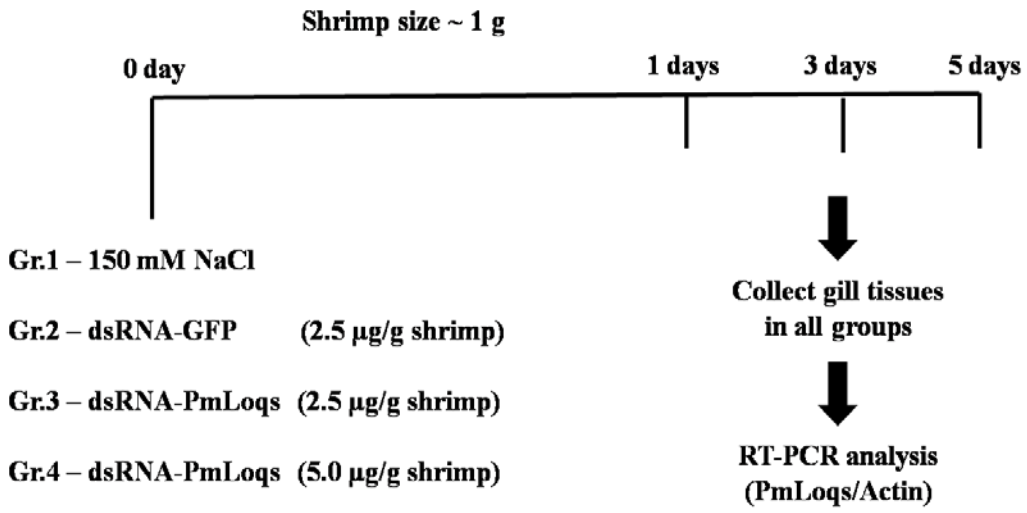
3.2.14 Inhibition of PmLoqs mRNA expression by dsRNA (*in vitro* transcription)

To investigate the knockdown effect of PmLoqs gene by using dsRNA-PmLoqs that were produced by *in vitro* transcription, shrimp size about 1 g were injected with 2.5 µg/g shrimp of dsRNA-PmLoqs into muscle by 0.5 ml U-100 Insulin syringe with 29 gauge needles. Shrimp injected with 150 mM NaCl and 2.5 µg/g shrimp of dsRNA-GFP were used as control groups. After 3 days dsRNA injection, gills from individual shrimp were collected to extract total RNA. The expression levels of PmLoqs gene were determined by RT-PCR analysis. The actin gene was used as an internal control to normalize the expression levels of PmLoqs mRNA. The injection plan was shown in the diagram below.



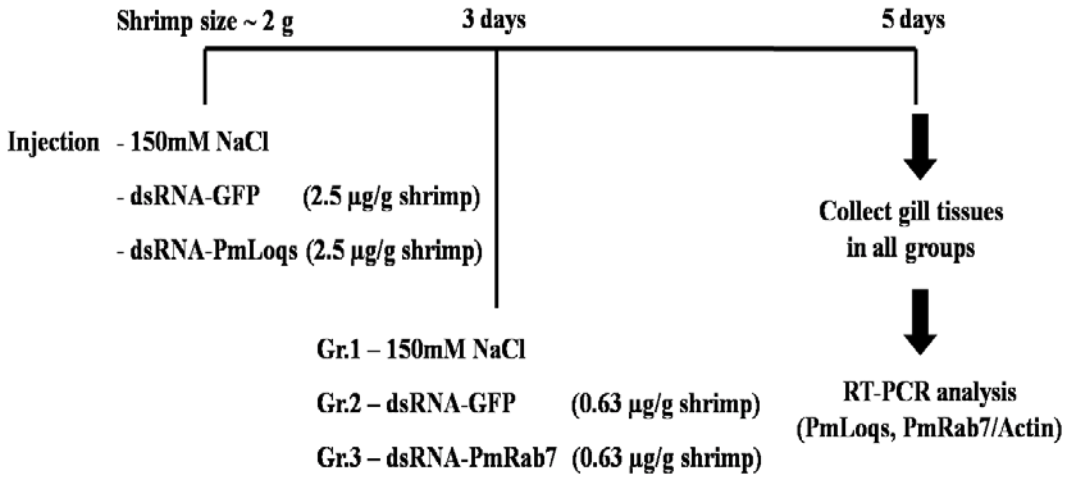
3.2.15 Inhibition of PmLoqs mRNA expression by dsRNA (*in vivo* bacterial expression)

To investigate the knockdown effect of PmLoqs by using dsRNA, shrimps size about 1 g were injected with 2.5 or 5.0 µg/g shrimp of dsRNA-PmLoqs. Shrimp injected with 150 mM NaCl or dsRNA-GFP (2.5 µg/g shrimp) was used as control groups. After 1, 3 and 5 days dsRNA injection, gill tissues from individual shrimp were collected and total RNA were extracted. RT-PCR analysis was performed to determine expression levels of PmLoqs. The actin gene was used as an internal control. The injection plan was shown in the diagram below.



3.2.16 Suppression effect of PmLoqs expression on the ability of dsRNA-PmRab7 to knockdown PmRab7 mRNA

In order to study the functions of PmLoqs in the RNAi pathway, shrimps size about 2 g were injected with 2.5 µg/g shrimp of dsRNA-PmLoqs. Shrimp injected with 150 mM NaCl or dsRNA-GFP (2.5 µg/g shrimp) was used as control groups. After 3 days dsRNA injection, all groups were injected with 150 mM NaCl, dsRNA-GFP (0.63 µg/g shrimp) and dsRNA-PmRab7 (0.63 µg/g shrimp). After 2 days, gill tissues were collected and total RNA was extracted to perform RT-PCR analysis to determine expression levels of PmLoqs and PmRab7. The actin gene was used as an internal control. The injection plan was shown in the diagram below.

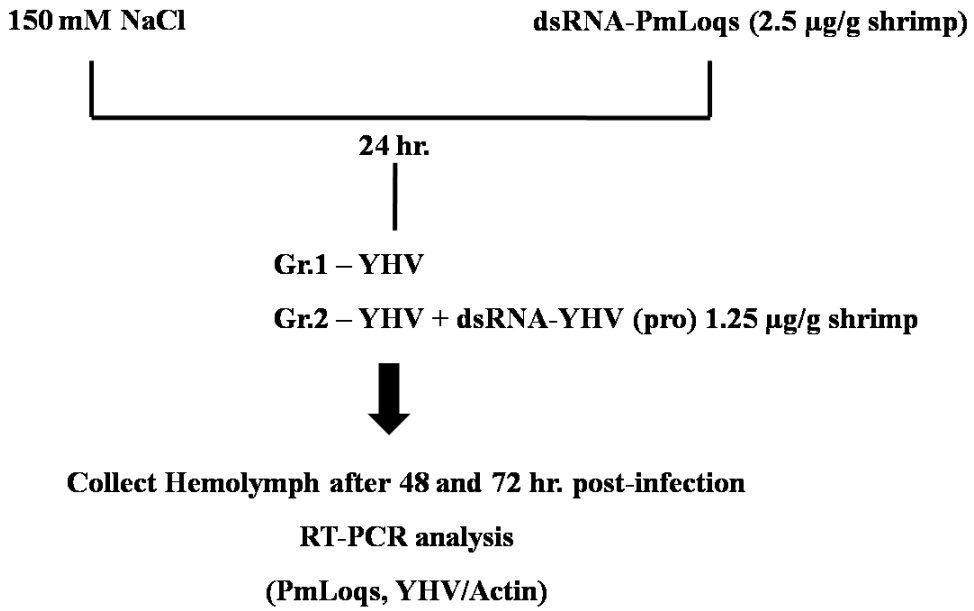


3.2.17 Suppression effect of PmLoqs expression on the ability of dsRNA-PmRab7 to knockdown PmRab7 mRNA by real-time PCR

Total RNA (1 µg) was used for cDNA synthesis. The expression of PmRab7 was determined by quantitative real-time PCR (qRT-PCR) analysis. qRT-PCR was performed in an ABI 7500 real-time detection system (Applied Biosystems) using KAPA_ SYBR_ FAST master mix (2X) ABI Prism_ (KAPA Biosystems). The primers for PmRab7 were qRab7- F2 and qRab7-R2 (Table 3-1). The primers for an internal control gene (EF1-α) were EF1a-F and EF1a-R (Table 3-1). The qRT-PCR was carried out in triplicates for each sample in a 20-µl reaction containing 10 µl of 2X KAPA-SYBR-FAST master mix, 5 µl of 1:50 diluted cDNA, 0.25 µl of 10 mM of each primer and 4.5 µl of sterile water. PCR amplification was performed under the following conditions: enzyme activation at 95°C for 3 min, followed by 40 cycles of 95°C for 3 sec and 60°C for 31 sec. The specificity of primers was determined by melting curve analysis from the ABI Prism 7500 detection system. The cycle threshold (Ct) value for the PmRab7 target genes and the internal control EF1-α gene were determined for each sample. The expression levels of PmRab7 in each treatment group relative to NaCl-injected group were determined by comparative Ct method ($2^{-\Delta\Delta Ct}$) (48).

3.2.18 Function of PmLoqs in RNAi pathway to virus infection

In order to study the functions of PmLoqs in the RNAi pathway that responded to viral infection, shrimps size about 10 g were injected with 2.5 µg/g shrimp of dsRNA-PmLoqs. Shrimp injected with 150 mM NaCl was used as a control group. After 24 hours dsRNA injection, each group was injected with YHV alone or co-injection of YHV and dsRNA-YHV (pro) 1.25 µg/g shrimp. After 48 and 72 hours, hemolymph was collected individually and total RNA was extracted to perform RT-PCR analysis to determine expression levels of PmLoqs and YHV replication. The actin gene was used as an internal control. The injection plan was shown in the diagram below.



3.2.19 Cloning of the full-length cDNA of PmLoqs

To identify the full-length cDNA encoding Loquacious, primers were designed from the sequence of TRBP (*Fenneropenaeus chinensis*) in the 5’ direction to amplify the sequence of PmLoq in the 5’ end. The PCR contained 1 µl cDNA from ovary tissue, 1X Thermopol buffer , 0.4 mM dNTPs, 0.4 µM of primer Loqs_5’ and 3_LoqsAB (Table3-1) and 1.0 unit of Vent DNA polymerase (New Englang Biolabs) in 25 µl reaction. The PCR was performed by incubation at 94 ºC for 5 min. Then the

touchdown PCR was performed for 5 cycles by decreasing the annealing temperature 1 ºC per cycle from 67-63 ºC for 30 sec after that the PCR was performed for 30 cycles under these following conditions: a denaturation at 94 ºC for 45 sec; an annealing at 62 ºC for 30 sec; and an extension at 72 ºC for 1.30 min. The final extension was carried out at 72 ºC for 10 min. PCR product was purified, cloned and subjected for sequencing.

3.2.20 DNA sequencing and data analysis

The nucleotide sequences of insert DNA fragment in recombinant plasmid were determined by DNA sequencing service at 1stBASE (Malaysia). After automated DNA sequencing, the sequence data was compared with sequence of the template by using BioEdit, Vecter NTI, GeneDoc and Clustal X programs. The phylogenetic analysis was performed by using MEGA 4 program.

3.2.21 Tissues distribution analysis

To study the expression levels of PmLoqs, the experiment was performed by extraction total RNAs from lymphoid organ, gill, testis, stomach, muscle, pleopod, hepatopancreas, thoracic ganglia, hemolymph, heart and ovary by using TRI® Reagent according to manufacturer's protocol. Then, total RNAs from various tissues were used as a template for multiplex RT-PCR with primers for PmLoqs and Actin in order to observe the expression levels of PmLoqs in different tissues of shrimp.