CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipments

Autoclave Model # LS-2D (Rex all industries Co. Ltd., Taiwan)

Automatic micropipettes P10, P100, P200, and P1000 (Gilson Medical Electrical S.A.)

Balance: Satorius 1702 (Scientific Promotion Co.)

CX31 Biological Microscope (Olympus)

Cytospin 4 (Shandon)

–20 °C Freezer (Whirlpool)

–80 °C Freezer (Revco)

Gene Pulser (Bio-RAD)

GenePix 4000B Microarrays Scanner (Amersham biosciences)

GS Gene Linker™: UV chamber (Bio-RAD Laboratories)

Hybridization oven (Hybrid, USA)

iCycler IQ Real-time Detection System (Bio-Rad)

Incubator 30 °C (Heraeus)

Incubator 37 °C (Memmert)

Larminar flow: Dwyer Mark II Model # 25 (Dwyer instruments)

MegaBACE DNA Analysis Systems (Amersham Biosciences)

Microcentrifuge tubes 0.5 ml and 1.5 ml (Bio-RAD Laboratories)

Minicentrifuge (Costar)

Nipro disposable syringes (Nissho)

Orbital Shaker (Gallenkamp)

PCR thermal cycler: Gene Amp PCR System 2400 (Perkin Elmer)

PCR workstation Model # P-036 (Scientific Co)

pH meter Model # SA720 (Orion)

Pipette tips 10, 20, 200, and 1000 µl (Bio-RAD Laboratories)

Poly-L-lysine coated slide (O.Kindler GmbH&/Co)

Power supply: Power PAC 3000 (Bio-RAD Laboratories)

Refrigerated microcentrifuge Kubota 1300 (Kubota)

Spectrophotometer: Spectronic 2000 (Bausch & Lomb)

Spectrophotometer DU 650 (Beckman, USA)

Sterile disposable plastic pipettes 1, 5 and 10 ml (Sterilin)

Sterring hot plate (Fisher Scientific)

Touch mixer Model # 232 (Fisher Scientific)

Trans-Blot® SD (Bio-RAD Laboratories)

Vacuum blotter Model # 785 (Bio-RAD Laboratories)

Vacuum pump (Bio-RAD Laboratories, USA)

Whatman® 3 MM Chromatography paper (Whatman International Ltd., England)

White/UV transilluminator: UVP ImageStore 7500 (Mitsubishi Electric Corporation, Japan)

96-well plate (Bio-RAD)

2.1.2 Software

Gene Cluster 3 Programme

2.1.3 Chemicals and reagents

Anti-Digoxigenin-AP Fab fragment (Roche, Germany)

Absolute ethanol, C_2H_5OH (BDH)

Acetic acid glacial, CH_3COOH (BDH)

Acetic anhydride (Sigma)

Acrylamide, C_3H_5NO (Merck)

Agarose (Sekem)

Bacto agar (Difco)

Bacto tryptone (Merck)

Bacto yeast extract (Scharlau)

BCIP (5-bromo-4-chloro-indolyl phosphate) (Roche, Germany)

Boric acid, BH_3O_3 (Merck)

Bromophenol blue (BDH)

Chloroform, $CHCl_3$ (Merck)

100 mM dATP, dCTP, dGTP, and dTTP (Promega)

Diethyl pyrocarbonate (DEPC), $C_6H_{10}O_5$ (Sigma)

Dimethyl sulfoxide (DMSO), C_6H_6SO (Amresco)

Ethidium bromide (Sigma)

Ethylene diamine tetraacetic acid (EDTA), disodium salt dihydrate (Fluka)

Ficoll™ 400 (Amersham)

Foetal bovine serum (Gibco BRL)

Formaldehyde, CH_2O (BDH)

Formamide deionized (Sigma)

GeneRuler™ 100bp DNA ladder

Glucose (Merck)

Glycerol, $C_3H_8O_3$ (BDH)

Glycine NH_2CH_2COOH (Scharlau)

Hydrochloric acid, HCl (Merck)

IQ SYBR Green Supermix (Bio-Rad)

Lamda DNA/*Eco*RI+*Hin*dIII marker (Promega)

Levamisol (Sigma)

Lobster haemolymph medium (LHM) (Merck)

Isoamylalcohol, $C_5H_{12}O$ (Merck)

Isopropanol, C_3H_7OH (Merck)

2-Mercaptoethanol, C_2H_6OS (Fluka)

Ethatimate (Toyama, Japan)

3-(N-morpholino)propanesulfonic acid (MOPS), $HO_3S(CH_2)_3(C_4H_8NO)$ (USB)

Nitroblue tetrazolium (NBT) (Roche, Germany)

Nytrans® super charge nylon membrane (Schleicher&Schuell)

Phenol crystals, C_6H_5OH (Carlo Erba)

Phenol, saturated (Merck)

RNA markers (Promega)

Sodium acetate, CH_3COONa (Merck)

Sodium chloride, NaCl (BDH)

Sodium citrate, $Na_3C_6H_5O_7$ (Carlo Erba)

Sodium dodecyl sulfate (Sigma Chemical Co., USA)

Sodium hydroxide, NaOH (Eka Nobel)

Tris-(hydroxy methyl)-aminomethane, $NH_2C(CH_2OH)_3$ (USB)

Triton X-100 (Merck)

Trizol reagent (Gibco BRL)

Tryptic soy broth (Difco)

Tween™-20 (Flula)

Xylene cyanol FF, $C_{25}H_{27}N_2O_6S_2Na$ (Sigma)

2.1.4 Enzymes

Ampli*Taq* DNA polymerase (Perkin-Elmer Cetus, USA)

*Bam*HI (Biolabs)

DNaseI (Promega)

*Eco*RI (Biolabs)

Proteinase K (Sigma)

RNase A (Sigma)

RQ1 RNase-free DNase (Promega)

T3 RNA polymerase (Roche, Germany)

T7 RNA polymerase (Roche, Germany)

*Xho*I (Biolabs)

2.1.5 Bacterial strains

Escherichia coli strain JM 109

E. coli strain XL-1 Blue MRF'

E. coli strain SOLR

E. coli strain DH10B

Vibrio harveyi 1526

2.1.6 Virus

White spot syndrome virus

2.1.7 Kits

DIG RNA Labeling Kit (Roche, Germany)

DYEnamic ET Dye Terminator Cycle Sequencing Kit (Amersham Biosciences)

LabelStar Array Kit (QIAGEN, Germany)

ImProm-II Reverse Transcription System Kit (Promega)

Gigapack® III Gold Cloning Kit (Stratagene)

Mini Quick Spin™ RNA column (Roche, Germany)

PCR cleanup kit (Millipore, USA)

QuickPrep® Micro mRNA Purification kit (Amersham Pharmacia Biotech)

QIAqiuck™ Gel Extraction Kit (Qiagen, Germany)

QIAqiuck™ PCR purification Kit (Qiagen, Germany)

ZAP-cDNA® Synthesis Kit (Stratagene)

2.2 Samples

Broodstock (approximately 800 g of body weight) and sub-adult *P. monodon* (approximately 3 month-old, 20 g of body weight) were purchased from the local farms, and separated into 5 groups. The first group was the unchallenged or normal broodstock shrimps. The rest of the shrimps were the sub-adult with the following treatments. The second group was experimentally injected with 0.85% (w/v) NaCl, the third group infected with *V. harveyi* 1526, the fourth group injected with LHM medium, and the last group infected with white spot syndrome virus. All groups were acclimatized in aquaria at the ambient temperature (28 ± 4 °C) and at the salinity of 15 ppt for at least 1 day before used in the experiments.

2.3 Preparation of *Vibrio harveyi* challenged shrimps

The preparation of *Vibrio harveyi* challenged shrimps was performed according to Supungul et al. (2002). A single colony of *V. harveyi* 1526 (kindly provided by Charoenpokphand (CP) Group of Companies) was inoculated in tryptic soy broth (TSB) supplemented with 2% (w/v) NaCl at 30 °C for 12-16 hours. The overnight culture was diluted (1:100) in the same medium and grown at 30 °C for 2 hours. The culture was then diluted 1:100 with a sterile 0.85% (w/v) NaCl. The titer of this dilution was monitored by a plate count method in tryptic soy agar (TSA) supplemented with 2% (w/v) NaCl (modified from Austin, 1988). The 10^5 CFU diluted culture was intramuscularly injected into the forth abdominal segment, whereas the control group was injected with 100 µl of 0.85% (w/v) NaCl solution.

At 0, 6, 24, 48 hours post injection (hpi), haemolymph were collected from the ventral sinus in the first abdominal segment using a needle on a 1-ml syringe. Each syringe was pre-filled with 200 µl of 10% sodium citrate as an anticoagulant. Then shrimps were tested whether the infection was successful by culturing the suspensions of hepatopancreas on TSA plates supplemented with 2% (w/v) NaCl and incubating at 30 °C overnight. Colonies of *V. harveyi* 1526 from infected shrimps were identified as the strong luminescent spots in the dark.

2.4 Preparation of white spot syndrome virus challenged shrimp

The protocol used was modified from that of the CP laboratory. The initial white spot syndrome virus stock (kindly provided by Charoenpokphand Group of Companies) contained 8.8×10^6 copies. The viral stock was diluted 100-fold with lobster haemolymp medium (LHM). One hundred microliters of the diluted virus were intramuscularly injected into the forth abdominal segment, while the control group was injected with 100 µl of LHM.

At 0, 6, 24, 48, 72 hpi, haemolymph was collected using the above procedure, and shrimps were tested whether the viral infection was successful using polymerase

chain reaction (PCR). The templates for the PCR reactions were the DNAs of the virus extracted from the gills of the sacrificed shrimps.

2.5 Lymphoid organ collection and total RNA preparation

Lymphoid organs were isolated separately from the internal thorax of each sacrificed shrimp, normal and *V. harveyi* injection at 6, 12, 24 and 48 hours, and immediately frozen in liquid nitrogen (-176 °C) in order to preserve the intact tissue. The lymphoid organs were briefly homogenized in 1 ml of Trizol reagent (Gibco BRL). The lymphoid organ homogenate was stored at room temperature for 5 minutes to permit complete dissociation of nucleoprotein complexes. After that, 200 µl of chloroform were added and vigorously shaken for 15 seconds. The resulting mixture was stored at room temperature for 2 - 5 minutes and centrifuged at 12,000*g* for 15 minutes at 4 °C. The colorless upper aqueous phase was transferred to a new 1.5 ml microcentrifuge tube. Total RNA was precipitated by the addition of 500 µl of isopropanol. The mixture was left at room temperature for 5 - 10 minutes and centrifuged at 12,000*g* for 10 minutes at 4 °C. The supernatant was removed. The RNA pellet was washed with 500 µl of 75% ethanol. The RNA pellet was kept under 75% ethanol until used. When required, the samples were centrifuged at 12,000*g* for 15 minutes at 4°C. The supernatant was removed. The RNA pellet was briefly air dried for 5 - 10 minutes. The total RNA was dissolved with an appropriate amount of diethyl pyrocarbonate (DEPC)-treated water.

The concentration of total RNA was determined by measuring the OD at 260 nm, and estimated in µg/ml using the following equation:

$$[\text{RNA}] = \text{OD}_{260} \times \text{dilution factor} \times 40$$

An OD unit at 260 nm corresponds to approximately 40 µg/ml of RNA (Sambrook et al., 1989)

2.6 Formaldehyde-agarose gel electrophoresis

A 1% (w/v) formaldehyde agarose gel was prepared using 1× MOPS buffer (diluted from a 10× MOPS buffer to 0.2 mM MOPS, 50 mM NaOAc, 10 mM EDTA,

pH 7 final concentration). The gel slurry was boiled till complete solubilization, and allowed to cool to 60 °C. Formaldehyde (0.66 M final concentration) and ethidium bromide (0.2 μg) were added, and the gel solution was poured into a gel setting chamber. The comb was then inserted.

Ten to twenty micrograms of total RNA in 3.5 μl of DECP-treated H_2O, 5 μl of formamide, 1.5 μl of 10× MOPS and 2 μl of formaldehyde were combined, mixed well, and incubated at 65 °C for 15 minutes. Then, the mixture was immediately placed on ice. One-forth volume of the gel-loading buffer (50% (v/v) glycerol, 1 mM EDTA, pH 8, and 0.5% (w/v) bromphenol blue) was added to each sample. The samples were loaded to the 1% formaldehyde agarose gel. The standard RNA marker was used as a size marker. Electrophoresis was carried out in 1× MOPS buffer at 50 volts, until the bromphenol blue dye migrated approximately ¾ of the gel length. The gel was washed 4-5 times of water before it was stained with ethidium bromide solution (0.5 μg/ml) for 1 hour. The total RNA in the stained gel was visualized as fluorescent bands using an UV transilluminator.

2.7 DNase treatment of the total RNA samples

Chromosomal DNA contaminated in the total RNA samples was removed by treating 25 μg of total RNA with 5 units of RQ1 RNase-free DNase (Promega) at 37 °C for 1 hour. Then, the reaction volume was adjusted to 40 μl with DEPC-treated water, 250 μl of Trizol reagent was added, and the mixture was vortexed for 5 seconds. Two hundred microliters of chloroform was then added and vigorously vortexed for 15 seconds. The resulting mixture was stored at room temperature for 2-5 minutes and centrifuged at 12,000g for 15 minutes at 4 °C. The top layer was added to 1 volume of isopropanol, and incubated for 10 minutes at room temperature. The resulting mixture was centrifuged at 12,000g for 15 minutes at 4 °C. The supernatant was removed. The RNA pellet was washed with 500 μl of 75% (v/v) ethanol. The supernatant was removed. The RNA pellet was briefly air dried for 5-10 minutes. The DNase-treated total RNA was dissolved with an appropriate amount of DEPC-treated water. The concentration of DNA-free total RNA was determined as described in section 2.5.

2.8 Preparation of mRNA

The mRNA was purified from the total RNA using a QuickPrep® Micro mRNA Purification kit (Amersham Pharmacia Biotech). The oligo(dT)-cellulose was gently swirled, and 1 ml of the cellulose suspension was immediately pipetted into a 1.5 ml microcentrifuge tube. This tube was centrifuged for 1 minute, and the buffer was removed. The solution containing 50 µl of the total RNA, 400 µl of the extraction buffer containing quanidinium thiocyanate and *N*-lauroyl sarcosine and 750 µl of elution buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) was added onto the oligo(dT)-cellulose. The tube was inverted gently for 3 minutes to resuspend the oligo(dT)-cellulose. The sample was centrifuged at the top speed for 1 minute. The supernatant was removed. The sample was washed five times with the high-salt buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 M NaCl) followed by three times with the low-salt buffer (10 mM Tris-HCl; pH 7.5, 1 mM EDTA, 0.1 M NaCl). After the last washing step, the resin was resuspended with 300 µl of the low salt buffer and transferred to a microspin column placed in a microcentrifuge tube. The column was added with 500 µl of the low-salt buffer and was centrifuged at the full speed for 1 minute. The effluent in the collection tube was discarded. This step was repeated twice. After that, the column was placed in a sterile 1.5 ml microcentrifuge tube. Two hundred microlitres of pre-warmed (65 °C) elution buffer was added to the top of the resin bed. The eluted mRNA was collected by centrifugation at the full speed for 1 minute. The eluted mRNA was kept at -80 °C until used.

2.9 Preparation of host bacteria XL 1 blue MRF' and SOLR

A single colony of a particular bacterial strain was inoculated into 2 ml of LB broth (1% bactotryptone, 1% NaCl and 0.5% bactoyeast extract) and incubated at 37 °C with shaking overnight. Fifty microlitters of the starter were added into 5 ml of LB broth containing 0.2% (w/v) maltose and 10 mM $MgSO_4$ and incubated at 37 °C for 4 hours. The culture was then transferred into the microcentrifuge tubes and centrifuged 800g for 3 minutes at room temperature. The supernatant was removed and the

bacterial pellets were resuspended in 500 µl of 10 mM $MgSO_4$. All bacterial suspension could be kept at 4 °C for 1 week.

2.10 Construction of the cDNA libraries

2.10.1 Construction of a lymphoid organ cDNA library from unchallenged shrimps

2.10.1.1 cDNA synthesis

Five micrograms of mRNA from the lymphoid organs of unchallenged shrimps were used to synthesize the cDNA using a ZAP-cDNA® Synthesis Kit (Stratagene). The oligo(dT)-*Xho*I linker-primer was used to prime at the 3'-end of the polyadenylated mRNA for the synthesis of the first-standed cDNA, which was catalyzed by StrataScript™ reverse transcriptase. The reaction mixture was incubated at 42 °C for 1 hour. RNA:cDNA hybrid from the first step was used to synthesized the second-stranded cDNA. The RNA strand was nicked by Rnase H. Then, the DNA polymerase I replaced RNA with DNA by the nick translation reaction at 16 °C for 2.5 hour. To blunt the cDNA termini, cloned *Pfu* DNA polymerase was added and the reaction was incubated at 72 °C for 30 minutes. The cDNA product was purified with phenol/chloroform and precipitated by adding sodium acetate and absolute ethanol. After precipitating overnight at -20 °C, the precipitated cDNA solution was centrifuged at 12,000g for 60 minutes at 4 °C. The supernatant was removed. The cDNA pellet was washed with 500 µl of 75% (v/v) ethanol. The supernatant was removed. The cDNA pellet was briefly air dried for 5-10 minutes. The cDNA was dissolved with *Eco*RI adaptor solution. After that, T4 DNA ligase was added to ligate the *Eco*RI adaptor to the termini of the blunt-ended cDNA at 8 °C for 14-16 hours. Then the *Eco*RI-ended cDNA was phosphorylated by T4 polynucleotide kinase at 37 °C for 30 minutes. Subsequently, the phosphorylated cDNA was digested with *Xho*I and then precipitated overnight at -20 °C. The cDNA pellet was then resuspended with 1× STE buffer (100 mM NaCl, 20 mM Tris-HCl, pH 7.5, 10 mM EDTA). The *Eco*RI-*Xho*I-ended cDNAs were size-fractionated on Sepharose CL-2B to remove the adapters and the low molecular weight cDNAs.

Aliquots of fractions (about 200 µl per fraction) were collected. Eight microliters of each fraction were analyzed by electrophoresis on a 5% polyacrylamide gel. Fractions, having cDNAs with size between 200-2000 bp were combined and extracted with 1 volume phenol:chloroform (1:1 v/v). After vortexing, centrifuging and collecting the supernatant, the aqueous solution was extracted again with equal volume of chloroform, then precipitated with 2 volume of pre-chilled absolute ethanol, and incubated at -20 °C overnight. Then the cDNA solution was centrifuged at 4 °C for 60 minutes at 12,000 rpm. The supernatant was removed. The pellet was washed with pre-chilled 70% ethanol, and centrifuged for 2 minutes at 12,000 rpm. The supernatant was removed. The cDNA pellet was dried at 37 °C for 10 minutes and then resuspended with 5 µl of sterile water.

2.10.1.2 Cloning and packaging the λZAP

The *Eco*RI/*Xho*I-ended cDNA was directionally ligated with the Uni-ZAP XR vector arms using T4 DNA ligase at 12 °C for an overnight. Then, the mixture was incubated at 65 °C for 15 minutes to inactivate the ligase. Four microliters of the cloning vector were assembled into the phage particles *in vitro* using Gigapack® III Gold Cloning Kit (Stratagene). The reaction was incubated at room temperature for 2 hours. Five hundred microliters of SM buffer (0.1 M NaCl, 0.8 mM $MgSO_4.7H_2O$, 0.05 Tris-HCl, pH 7.5) and 20 µl of chloroform were added to the reaction and mixed by pipetting. This reaction was briefly spun to sediment the debris, and collected the supernatant. The phage packaging supernatant was kept at 4 °C until used.

2.10.1.3 Titering of the phage library

For titering the phage from the normal library, XL1-Blue MRF' was prepared as described in section 2.9, and diluted to an OD_{600} of 0.5 with sterile 10 mM $MgSO_4$. The phage supernatant was serially diluted with the SM buffer. Each phage dilution was mixed with the prepared bacteria and incubated at 37 °C for 15 minutes. The NZY top agar (0.5% NaCl, 0.2% $MgSO_4$ $7H_2O$, 0.5% yeast extract, 1% NZ amine (casein hydrolysate), 0.7% agarose) was added into each mixture, and

immediately poured onto the NZY agar plates (0.5% NaCl, 0.2% $MgSO_4$ $7H_2O$, 0.5% yeast extract,1% NZ amine (casein hydrolysate) and 1.5% agar). The top agar was allowed to solidify at room temperature for 10 minutes, and the plates were incubated at 37 °C for 6-8 hours. The number of plaques was counted, and the plaque forming units (pfu) per milliliter for each concentration were calculated.

2.10.1.4 *In vivo* excision

Approximately 10^7 plaque forming units (pfu) of the lambda phage (packaging phage stock), 10^8 cfu/ml of XL1-Blue MRF' cell and 10^9 pfu/ml of ExAssist helper phage were combined in a conical tube. The mixture was incubated at 37 °C for 15 minutes to allow the attachment of the phage to the cells. Then, 20 ml of LB broth (1% bactotryptone, 1% NaCl and 0.5% bactoyeast extract) were added. The reaction was incubated with shaking at 37 °C for 2.5–3 hours. The reaction was then heated at 65-70 °C for 20 minutes and spun down at 1000*g* for 10 minutes. The supernatant was decanted into a sterile conical tube. This stock contained the excised pBluescript phagemid packaged as filamentous phage particles. This stock was kept at 4 °C before the excised phagemids were plated.

To plate the excised phagemids, 200 µl of the freshly grown SOLR cells and 1 µl of the excised phage supernatant were combined, and incubated at 37 °C for 15 minutes. After that, 100 µl of the cell mixture were plated on LB-ampicillin agar plates (50 mg/ml) and incubated overnight at 37 °C.

2.10.1.5 Amplification of the cDNA library

The phage packaging supernatant containing about 5×10^4 pfu of bacteriophage and 600 µl of XL1-Blue MRF' cell at OD_{600} of 0.5 were combined in a conical tube, and incubated for 15 minutes at 37 °C. The NZY top agar (6.5 ml) was added and spreaded evenly onto a freshly poured 150-mm NZY agar plate. The plate was incubated at 37 °C for 6-8 hours, then overlaid with 8-10 ml of the SM buffer and stored at 4 °C overnight. The SM buffer was collected into a sterile polypropylene container. The plate was rinsed with 2 ml of the SM buffer and pooled. The pooled SM buffer was added with chloroform to a final concentration of 5% (v/v),

mixed well, and incubated at room temperature for 15 minutes. The cell debris was removed by centrifugation at 500*g* for 10 minutes at room temperature. The supernatant was transferred into a sterile conical tube and chloroform was added to a final concentration of 0.3% (v/v). Then, DMSO were added to a final concentration of 7% (v/v), and the amplified library was kept at –80 °C.

2.10.2 Construction of a lymphoid organ cDNA library from the *V. harveyi* infected shrimps

Five micrograms of the pooled mRNA from lymphoid organ of *V. harveyi* infected shrimps at 6, 12, 24 and 48 hours post injection were used to construct a *V. harveyi* infected shrimp cDNA library using the same procedures as descript in section 2.10.1.

2.10.3 Plasmid DNA preparation

A recombinant plasmid was inoculated into 2 ml of LB broth containing 50 mg/ml of ampicillin and cultured overnight at 37 °C. The culture was centrifuged at 800*g* for 3 minutes. The supernatant was removed. The pellets were completely resuspended in 100 µl of solution I (25 mM Tris–HCl, pH 8, 10 mM EDTA, pH 8, 50 mM glucose). For cell lysis and DNA denaturation, 200 µl of a freshly prepared solution II (0.2 N NaOH and 1% (w/v) SDS) were added and mixed gently. This was followed by adding 150 µl of solution III (3 M sodium acetate, pH 4.8), mixed gently, and placed on ice for 10 minutes. After centrifugation at 10,000g for 5 minutes, the supernatant was transferred to a new microcentrifuge tube. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added, mixed and spun at 10,000*g* for 10 minutes. The upper aqueous phase was transferred to a new microcentrifuge tube. Plasmid DNA was precipitated by adding 2 volumes of absolute ethanol. The mixture was kept at –80 °C for 15 minutes and centrifuged at 10,000*g* for 10 minutes. The pellet was washed with cold 70% ethanol. The pellet was air dried and dissolved in 50 µl of TE buffer (10 mM Tris–HCl, pH 8, 1 mM EDTA, pH 8). Three microlitres of 5 mg/ml of RNase A were added and incubated at 37 °C for 30

minutes. After that, 32 µl of 20% polyethyleneglycol (MW 6,000, PEG 6,000) in 2.5 M NaCl were added. The mixture was mixed gently by pipetting and placed on ice for 1 hour. The mixture was centrifuged at 10,000*g* for 10 minutes at room temperature and washed with cold 70% ethanol. The pellet was air-dried and resuspended in 50 µl TE buffer.

2.10.4 Determination of the DNA insert sizes by colony PCR

After *in vivo* excision, λ-phages containing cDNAs from both libraries were converted to the recombinant pBluescripSK phagemid clones. The sizes of the cDNA inserts were determined by using colony PCR. The pBluescript SK phagemid was 3000 bp in length (Figure 2.1). This phagemid has the multiple cloning sites flanked by T3 and T7 RNA promoter and a choice of 6 different primer-annealing sites for DNA sequencing. Two of these sites are M13 forward and M13 reverse primer-annealing sites. Therefore, the M13 forward and reverse primers can be used to determine the insert sizes of cDNA. Colony PCR was performed in a 25 µl reaction volume containing 1.25 mM of each dNTP (dATP, dCTP, dGTP and dTTP), 1× PCR buffer, 1.2 mM $MgCl_2$, 2 pmole of each primer and 1 unit of *Taq* DNA polymerase (5 unit/µl). A recombinant colony was scraped by a micropipette tip and mixed well in the amplification reaction. The PCR profile were one cycle of 96 °C denaturing step for 2 minutes, followed by 30 cycles of 96 °C for 30 seconds, 50 °C for 30 seconds and 70 °C for 2 minutes. The reaction was final extended of at 72 °C for 5 minutes. The PCR products were electrophoretically analyzed in 1× TBE buffer at 100 volts. A 100 bp DNA ladder was used as DNA markers. The cDNA inserts whose sizes over 200 bp were selected for DNA sequencing

2.10.5 Agarose gel electrophoresis

Agarose gel electrophoresis was done according to Sambrook et al. (1989). One percent (w/v) of agarose gel was prepared using 1× TBE buffer (89 mM Tris-HCl, 8.9 mM boric acid, 2.5 mM EDTA, pH 8). The gel slurry was heated until complete solubilization, and then the gel was poured into a setting chamber. A comb was inserted. After the gel was solidified, the comb was carefully withdrawn and

sufficient 1× TBE buffer was added to cover the gel. Each sample was mixed with ¼ volume of the gel-loading dye (0.25% bromphenol blue, 0.25% xylene cyanol FF, 15% ficoll) and loaded into the well. The λ/*Hin*dIII fragments or a 100 bp DNA ladder were used as standard DNA markers. Electrophoresis was carried out in 1× TBE buffer at 100 volts until the bromophenol blue dye marker migrated about ¾ of the gel length. After electrophoresis, the gel was stained in a 2.5 μg/ml ethidium bromide solution for 5 minutes and destained in distilled water for 15 minutes to remove excess ethidium bromide. The DNA fragment was visualized under a UV transilluminator.

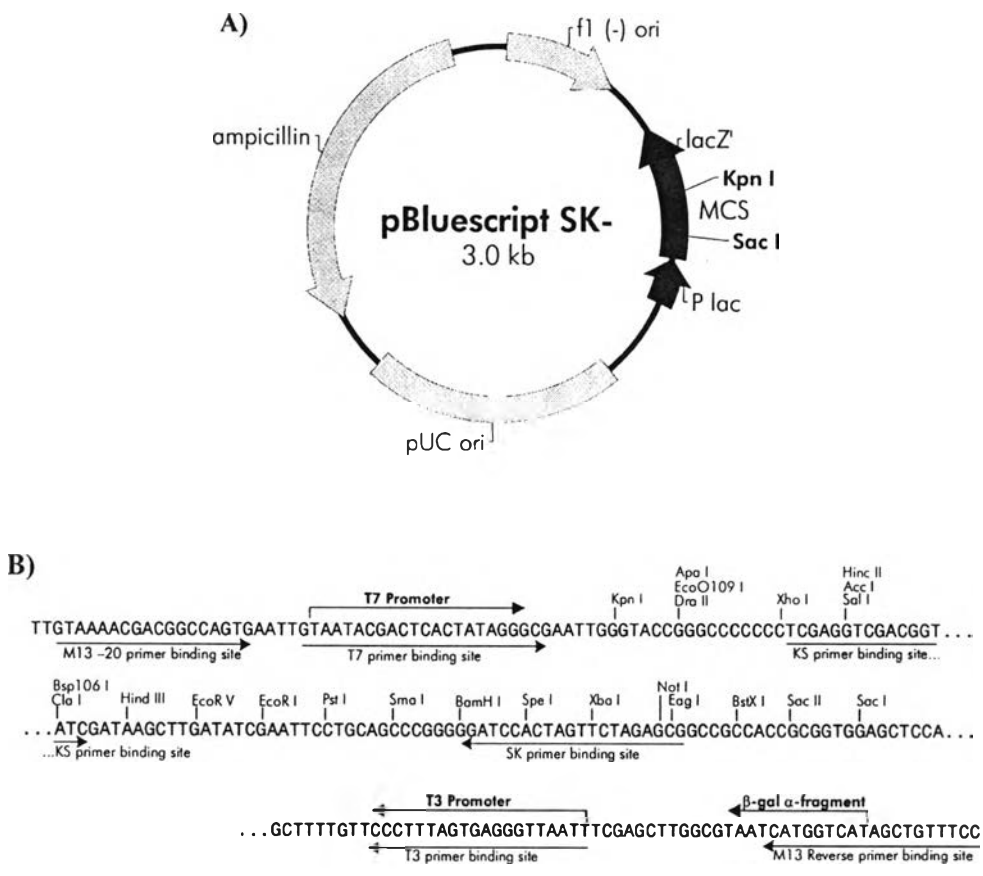

Figure 2.1 The circular map (A) and the multiple cloning sites (B) of the pBluescript II SK (+/-) vector.

2.10.6 DNA sequencing and data analysis

The cDNA clones were identified by DNA sequencing. The DNA sequencing was carried out using DYEnamic ET Dye Terminator Cycle Sequencing Kit (Amersham Biosciences) with the M13 forward primer and the MegaBACE DNA Analysis Systems (Amersham Biosciences). Two microlitres (0.2 μg) of plasmid DNA and 2 pmol of M13 forward primer were added into 4 μl of a sequencing reagent premix (supplied by the kit). After mixing with plasmid and primer, 1.5 μl of distilled water were added into the mixture to adjust the total volume of the reaction to 10 μl.

The reaction was amplified in a thermal cycling using a denaturation step at 95 °C, 20 seconds, an annealing step at 45 °C, 30 second, and an extension step at 60 °C, 2 minutes for 30 cycles. After amplification, the reaction was added 1 μl of 7.5 M ammonium acetate and 27.5 μl of absolute ethanol to precipitate the amplified DNA product. The reaction was centrifuged at room temperature for 15 minutes at 12,000 rpm. The supernatant was removed and the pellet was washed with 100 μl of 70% ethanol. After re-centrifugation for 10 minutes at 12,000 rpm, the supernatant was removed, and the pellet was air dried for 30 minutes. Subsequently, the pellet was dissolved in 10 μl of MegaBACE loading solution. Then, the sample was vigorously vortexed for 10-20 seconds and briefly centrifuged to collect the sample at the bottom of the tube. The sample was then loaded into the MegaBACE sequencing instrument according to the manufacturer instruction.

Sequences of the cDNA clones were edited and compared with the DNA sequence in the nucleotide sequence database of the GenBank (the National Center for Biotechnology Information; NCBI) using the BLASTX program (Altschul et al., 1997). The significant probabilities and the numbers of matched nucleotide/proteins were considered when the E-value was less than 10^{-4} and a match was more than 10 amino acid residues for the BLASTX analysis.

After homology search, matched ESTs were categorized into 12 broad functional categories: 1) gene expression, regulation and protein synthesis; 2) internal/external structure and motility; 3) metabolism; 4) defense and homeostasis; 5) signaling and communication; 6) cell division/DNA synthesis, repair and replication;

7) ribosomal protein and rRNA; 8) mitochondrial protein; 9) transport; 10) miscellaneous function; 11) unidentified (hypothetical)-similar to other cDNA/DNA; and 12) unknown based on significant sequence homology according to the criteria proposed by the Genomic Researches for Increasing Culture Efficiency of the Black Tiger Shrimp (*Penaeus monodon*). EST nucleotide sequences were submitted to the nucleotide sequence database (dbEST) of the GenBank (Altschul et al., 1997).

2.11 Microarray analysis

2.11.1 Preparation of cDNA microarray

The cDNA microarray slide, containing 1026 cDNA clones, was prepared from the cDNA clones of the species of penaeid shrimps, 718 clones from *P. monodon* and 308 clones from *Marsopenaeus japonicus*, cloned and identified by Dr. Premreuthai Supunkul and Dr. Jiraporn Rojtinnakorn, respectively. Each cDNA clone was amplified by PCR amplification. The primers were the M13 forward primer, 5'-GTGCTGCAAGGCGATTAAGTTGG-3' and the M13 reverse primer 5'-TCCGGCTCGTATGTTGTGTGGA-3', which annealed to the vector regions. The PCR reaction was performed in a 150 µl final volume containing 200 ng of the plasmid, 0.12 mM of each dNTP (dATP, dCTP, dGTP and dTTP), 1× PCR buffer, 30 pmole of each reaction primers and 2.5 unit of *Taq* DNA polymerase (5unit/µl). The PCR profile was 95 °C for 2 minutes, followed by 35 cycles of 95 °C for 30 seconds, 58 °C for 30 seconds and 72 °C for 1 minute. The reaction was finally extended at 72 °C for 5 minutes. The PCR product was purified by adding 100 µl of 20% PEG 600, vortexing and leaving on ice for 1 hour. Then, the mixture was centrifuged for 20 minutes at 13,500 rpm at 4 °C. The supernatant liquid was removed, and the pellet was washed with 500 µl of 75% ethanol. The wash was then centrifuged for 5 minutes at 13,500 rpm at 4 °C. The supernatant liquid was removed, and the amplified pellet was air dried. The pellet was dissolved in 10-30 µl of distilled water such that a final concentration of DNA was at least 500 µg/ml. The success of amplification was confirmed by electrophoretically analyzing the PCR products in 1× TBE buffer at 100 volts. A 100 bp DNA ladder was used as DNA size markers. The amplified PCR

products were then sent to the DNA Chip Research Inc. (Tokyo, Japan) for printing onto a slide in the process of making the DNA microarray chips. There are duplicated spot for each gene on the cDNA chips.

2.11.2 Shrimp sample preparation

WSSV- and *V. harveyi*-free shrimps were obtained from Charoenpokphand Group, Nakhonsrithamarat province and challenged with the pathogens of interest as consecutively described in section 2.2, 2.3 and 2.4. Haemolymph was collected from the shrimps at 4 time points after WSSV-infection (6, 24, 48 and 72 hpi) and at 3 time points after *V. harveyi* infection (6, 24, and 48 hpi). In the latter case, most of the animal died after 48 hours. Haemolymph of shrimps injected with either 0.85% NaCl or lobster haemolymph medium (LHM) was also collected to use as controls.

2.11.3 Haemocyte collection and total RNA preparation

Haemolymph was collected from the ventral sinus of the shrimp using a 24G×1 inch needle fitted onto a 1.0 ml syringe pre-loaded with 200 µl of anticoagulant (10% sodium citrate (w/v)). Haemolymph from individual shrimp was immediately centrifuged at 800g for 10 minutes at 4 °C to separate haemocytes from the plasma. The haemocyte pellet was resuspended in 1 ml of TRIreagent (Molecular biology) and briefly homogenized. The homogenate was stored at room temperature for 5 minutes to permit complete dissociation of nucleoprotein complexes. After that 200 µl of chloroform was added and vigorously vortexed for 15 seconds. The resulting mixture was stored at room temperature for 2-5 minutes and centrifuged at 12,000 rpm for 15 minutes at 4 °C. The colorless upper aqueous phase was transferred to a new 1.5 ml microcentrifuge tube. RNA was precipitated by the addition of 500 µl of isopropanol and mixed. The mixture was left at room temperature for 5-10 minutes and centrifuged at 12,000 rpm for 10 minutes at 4 °C. The supernatant was removed. The RNA pellet was washed with 500 µl of 75% (v/v) ethanol and centrifuged at 12,000 rpm for 15 minutes at 4 °C. The supernatant was removed. The RNA pellet was briefly air dried for 5-10 minutes. The total RNA was dissolved with an

appropriate amount of diethy pyrocarbonate (DEPC)-treated water, and then the total RNA concentration was measured as described in section 2.5.

2.11.4 Preparation of cDNA probe

The total RNA from pathogen challenged and unchallenged *P. monodon* at indicated time points was extracted as described above. At each time point, the total RNA from 10 individuals was pooled. The first-stranded cDNA probe labeled with aminoallyl-dUTP was generated from the total RNA sample from each time point and the oligo(dT) primers using a LabelStar Array Kit (QIAGEN). The labeling reactions (1× buffer RT, 0.5 mM dATP, 0.5 mM dGTP, 0.5 mM dCTP, 0.5 mM aminoallyl-dUTP, 20 units RNase inhibitor (40 units/μl), 2.5 μl LabelStar reverse transcriptase, 25 μg of the total RNA and 2 μM of the oligo(dT) primers) were incubated at 37 °C for 2 hours and subsequently stopped by adding 2 μl of the LS stop solution. The aminoallyl-labeled cDNA probes were purified using a QIAquick PCR Purification Kit (QIAGEN). The reactions were added with 260 μl of PB buffer and mixed by vortexing. Then, the aminoallyl-labeled cDNA probe samples were applied onto the MinElute spin columns and centrifuged at 13,500 rpm for 1 minute. The flow-through fractions were discarded. The aminoallyl-labeled cDNA probes were washed with 750 μl of PE buffer and centrifuged at 13,500 rpm for 1 minute. After discarding the flow-through fractions, the aminoallyl-labeled cDNA probes were eluted with 100 μl of buffer EB, 1 minute incubation and centrifugation at 13,500 rpm for 1 minute. The aminoallyl-labeled cDNA probes were precipitated by adding 10 μl of 3 M sodium acetate, pH 5.2, 0.5 μl of ethachinmate and 250 μl absolute ethanol. The mixtures were incubated at -20 °C for 20 minutes and centrifuged at 13,500 rpm for 30 minutes. The aminoallyl cDNA probes were washed with 500 μl of 75% (v/v) ethanol and centrifuged at 13,500 rpm for 10 minutes. The supernatant was removed. The aminoallyl cDNA probes were air-dried and subsequently resuspended with 9 μl of sodium bicarbonate buffer, pH 8.5-9. The aminoallyl cDNA probes were further coupled with Cy3 (control shrimps injected with LHM or saline solution) and Cy5 (WSSV- or *V. harveyi*-challenged shrimps). The dye-labeled cDNA probes were then

purified using the QIAquick PCR Purification Kit. The purified cDNA probes were kept protected from light.

2.11.5 Microarray hybridization and scanning

The cDNA probes were dried in a speedvac concentrator and dissolved in 3 µl of nuclease-free water. The Cy3 and Cy5 labeled cDNAs at each time point were combined and incubated at 95 °C for 2 minutes. Then the combined cDNA at each time point was immediately chilled on ice for 30 seconds. After that, 1.5 ng of oligonucleotide (A_{280}) were added into each combined cDNA probe. The cDNA probes were incubated at 75 °C for 45 minutes and then added with 7.5 µl of microarray hybridization buffer and 15 µl of 100% (v/v) formamide. The probes were mixed and dropped onto the microarray slide. A glass slip was used to cover the hybridization reaction of the cDNA array. Hybridization was carried out for 16 hours at 42 °C in adequately humid chamber. The slides were washed at room temperature for 20 minutes with 2× SSC and 0.1% SDS with shaking in a platform shaker and 0.2× SSC and 0.1% SDS without shaking. The slides were then consecutively washed with 0.2× SSC and 0.1% SDS twice for 20 minutes at 55 °C, 0.2× SSC and 0.1% SDS for 20 minutes at room temperature, 0.2× SSC for 5 minutes at room temperature, and 0.05× SSC for 5 minutes at room temperature. The slides were dried by centrifugation at 1,500 rpm and scanned immediately using a GenePix 4000B Microarrays Scanner (Amersham Biosciences). The scanned images were analyzed with the GenePix Pro 3.0 program (Axon Instrument). The signal intensities of the Cy5 and Cy3 were measured and calculated from the intensity means of the duplet spots. After subtracting the background fluorescence, differences in the Cy5 and Cy3 incorporation efficiencies were corrected by global normalization. The results were expressed as the gene expression ratio, i.e., the ratio of the intensities of Cy5/Cy3. Genes with the spot (expression) ratios over 2.0 and under 0.5 were considered as up-regulated genes and down-regulated genes, respectively.

2.11.6 Microarray data mining

The processed data were subjected to cluster analysis using the Gene Cluster 3 developed by Michael Eisen at the Stanford site (http://rana.stanford.edu/software). Tree View software, also available at the Stanford site, was used to generate visual representations of the classification. Hierarchical clustering classifies the samples according to their overall gene expression profiles, and groups genes on the basis of correlation of their expression level pattern in all samples.

2.12 Quantitative real-time RT-PCR

The mRNA expression of some differentially expressed genes identified by microarray analysis, i.e., calmodulin (CaM), tubulin, and asialoglycoprotein receptor (ASGPR), was confirmed by real-time RT-PCR. In addition, the expression of calcinuerin, CDC like kinase 2 and protein phosphatase 1 were also studied.

2.12.1 Total RNA isolation and DNase treatment

The total RNA samples were extracted individually from the haemocytes of *V. harveyi*- and saline-injected *P. mondon* at time 0, 6, 24, and 48 hpi as described in section 2.3 and from the haemocytes of white spot syndrome virus- and LHM-injected *P. mondon* at time 0, 6, 24, 48 and 72 hpi as described in section 2.4. At each time point, the total RNA from 10 individuals was pooled and subsequently treated with RQ1 RNase-free DNase (Promega) to remove genomic DNA contaminant as mentioned above. After phenol-chloroform extraction, DNA-free total RNA from each time point was subjected to first strand cDNA synthesis reaction.

2.12.2 First strand cDNA synthesis

The first strand cDNAs were generated from 1 μg of DNA-free total RNA sample and 0.5 μg of oligo(dT_{18}) primer using the ImProm-II™ Reverse

Transcriptase System kit (Promega). The reaction was incubated at 70 °C for 5 minutes and immediately placed on ice for 5 minutes. After that, 4 µl of 5× reaction buffer, 3.8 µl of 25 mM $MgCl_2$, 1 µl of dNTP mix (10 mM each), 20 units of ribonuclease inhibitor and 1 µl of ImProm-II™ reverse transcriptase were added and gently mixed. The total volume of reactions was adjusted to 20 µl with DNase free water. The reaction was incubated at 25 °C for 5 minutes and at 42 °C for 60 minutes. Then, the reaction was incubated at 70 °C for 15 minutes to terminate the reverse transcriptase activity. The cDNAs were stored at –20 °C until use.

2.12.3 Real-time RT-PCR

The SYBR Green I real-time RT-PCR assay was carried out using the iCycler iQ™ Real-Time Detection System (Bio-Rad). The amplifications were carried out in a 96-well plate in a 20 µl reaction volume containing 10 µl of 2× SYBR Green supermix (Bio-rad), the appropriate amounts of forward and reverse primers, 5 µl of 10× diluted cDNA from each reverse transcription reaction as template and adjusted the reaction volume with sterile water. The required final concentrations of primers are shown in Table 2.1. The thermal profile for SYBR Green real-time RT-PCR was 95 °C for 3 minutes followed by 40 cycles of denaturation, annealing, and extension as indicated in Table 2.1. Fluorescent data were collected at the end of extension step. In a 96-well plate, each sample was conducted in triplicate. Sterile-water was used in place of the DNA templates as the negative control. To determine the specificity of the PCR amplification of each primer pair, after the amplification, a melting curve cycling was done immediately. The reactions were incubated at 95 °C for 1 minute, and 50 °C for 1 minute, and followed by 80 repeats of heating for 10 seconds starting at 50 °C with 0.5 °C increment.

Table 2.1 Primer pairs and conditions for the real-time RT-PCR

Gene	Primer	Final primer concentration (μM)	Denaturing (Temp/sec)	Annealing (Temp/sec)	Elongatio (Temp/sec
calmodulin (CaM)	Forward: CAGTTCCTTGGTGGTGAT	5	95/10	56/15	72/10
	Reverse: CGATTGGCTTGTGATACA				
asialoglycoprotein receptor (ASGPR)	Forward: GAACGAGAATGCTGACCTGA	5	95/10	56/15	72/20
	Reverse: AGTATTTGCGAGTATGGGAG				
tubulin	Forward: GAAAACACCGATGAAACTTACTG	5	95/10	58/15	72/10
	Reverse: GAGAGGAGCAAAACCAGGCAT				
EF-1alpha	Forward: GGTGCTGGACAAGCTGAAGGC	5	95/10	55/15	72/10
	Reverse: CGTTCCGGTGATCATGTTCTTGATG				
Calcineurin	Forward: CTGACTACGGCTTCCAAATG	5	95/10	55/15	72/10
	Reverse: CCATCTTCATCCTTGTCGGC				
CDC like-kinase 2	Forward: TGTGTTATGCTGTCAAGTTC	5	95/10	53/15	72/10
	Reverse: CCAGGTGTTCTCGGTTATC				
Protein phosphatase 1	Forward: CGCCAGATTATTAGAAGTTCG	5	95/10	55/15	72/10
	Reverse: CAGTTTGATGTTATACCGCC				

2.12.4 Data analysis of real-time RT-PCR

Fluorescence signal was analyzed by the data analysis software of the iCycler iQ™ Real-Time Detection System (Bio-Rad) using the PCR base line subtracted curve fit method. For each sample, the cycle number at which the fluorescence crosses the arbitrary line is called a threshold. The threshold should be in the line part of the reaction and higher than the background signal to ensure that the reactions cross the line due to the amplification rather than noise. This crossing point, CP, is also known as the threshold cycle or Ct value. The obtained Ct values were used to calculate a relative expression ratio (R).

The relative quantification analyses the amount of a target transcript relatively to an internal standard, i.e., elongation factor 1-alpha gene (EF 1α) in the same sample. Moreover, the Ct values of *V. harveyi*-injected sample at each time point were normalized with the saline-injected samples, and the Ct values of white spot syndrome virus-injected samples were also normalized with the LHM-injected

samples at each time point. A mathematical model described by Pfaffl (2001) was used to determine the relative expression ratio according to the equation:

$$\text{Relative expression ratio} = \frac{(E_{\text{target}})^{\Delta Ct}\text{target}^{(\text{control-sample})}}{(E_{\text{ref}})^{\Delta Ct}\text{ref}^{(\text{control-sample})}}$$

E_{target} is the real-time PCR efficiency of the target gene trancript; E_{ref} is the real-time PCR efficiency of reference gene trancript; ΔCt_{target} is the CP deviation of the control (saline- or LHM-injected) minus the sample (*V. harveyi*- or white spot syndrome virus-injected) of the target gene transcript; and ΔCt_{ref} is the CP deviation of the control (saline- or LHM-injected) minus the sample (*V. harveyi*- or white spot syndrome virus-injected) of the reference gene transcript.

2.12.5 Determination of the PCR efficiency

The different PCR efficiencies can be obtained for the target and the reference genes because they have different sequences and amplicon lengths. In order to compare the results from each run of the real-time RT-PCR, the PCR efficiencies are incorporated into the calculation of the relative expression ratio as shown above. Therefore, it is necessary to determine the PCR efficiency of each gene, amplified with a specific pair of primers. This was done by constructing a standard curve. The standard cDNA was prepared from the total RNA of the haemocytes from normal *P. monodon* using the same procedure as described above. This cDNA was used for creating all standard curves of both target and reference genes. The standard cDNA was diluted five steps from 5 or 10 to 5^5 or 10^5. The amplification was performed in triplicate. For each run, water was used in place of template as a negative control. At the end of each run, the amplification plots were automatically analyzed by the system. The standard curves were obtained. A calibration curve of the Ct values against the input template quantities in log scale was constructed for either reference (EF-1α) or each target gene. In each plot, a linear graph should give an excellent correlation coefficient (more than 0.990). The PCR efficiency was equal to $10^{-1/\text{slope}}$. These efficiencies were taken into account in relative quantification.

2.13 Gene expression analysis using *in situ* hybridization

2.13.1 Haemocyte preparation

The shrimp haemolymph was collected from the ventral sinus, located at the base of the first abdominal segment, using a 27G×1 inch needle fitted onto a 1.0 ml syringe containing 500 µl of the MAS anticoagulant (Modified Alsever Solution: 27 mM sodium citrate, 336 nM NaCl, 115 mM glucose, 9 mM EDTA, pH 7). The haemolymph was immediately centrifuged at 800g at 4 °C for 10 minutes to separate the haemocytes from plasma. The supernatant was removed, and the haemocytes were resuspended in freshly prepared ice-cold MAS containing 4% (w/v) paraformaldehyde. The resuspended haemocytes were incubated on ice for 10 minutes for the fixation. After centrifugation as above, the haemocytes were washed twice with MAS to eliminate the plasma proteins. The haemocytes were resuspended in MAS. The total number of haemocytes was determined using a haemocytometer. By using a cyto-centrifuge, 2×10^5 haemocytes were centrifuged onto a poly-L-lysine-coated slide at 1,000g for 5 minutes. The haemocytes were dried at room temperature for a few minutes. The slide was stored at –20 °C until use.

2.13.2 Riboprobe preparation

The pBluescriptSK plasmid, containing a calmodulin (CaM) cDNA, was linearized by restriction enzyme digestion. The plasmid was digested with *Bam*HI for the in vitro synthesis of the antisense probe for in situ hybridization, and *Xho*I for the sense probe. One hundred microliters of the linearized plasmid was diluted to 400 µl with sterile water. An equal volume of phenol:chloroform:isoamylalcohol (25:24:1) was added and mixed by vortexing. The mixture was centrifuged at 12,000 rpm for 10 minutes at 4 °C. The aqueous phase was transferred to a new tube. An equal volume of chloroform was added, mixed, and centrifuged as above. The linearized plasmid was precipitated by adding 50 µl of 3 M sodium acetate and 250 µl of cold absolute ethanol, and incubated at –80 °C for at least 1 hour. The mixture was centrifuged at 12,000 rpm for 20 minutes at 4 °C, air-dried the pellet, and suspended with 20 µl

DEPC-treated water. The linearized plasmid was quantified using agarose gel electrophoresis.

Dioxigenin (DIG)-labeled probes were prepared by in vitro transcription using T7 polymerase for CaM-antisense probe and T3 polymerase for CaM-sense probe. The reaction is composed of 1 μg of the purified, linearized plasmid DNA, 2 μl of 10× DIG RNA labeling mix (10 mM each ATP, CTP, and GTP, 6.5 mM UTP, 3.5 mM DIG-UTP, pH 7.5), 2 μl of 10× transcription buffer (400 mM Tris-HCl, pH 8, 60 mM $MgCl_2$, 100 mM dithiothreitol (DTT), and 20 mM spermidine), 40 units of T7 or T3 RNA polymerase. The DEPC-treated water was added to make a total reaction volume of 20 μl. The components were mixed and briefly centrifuged. The reaction was incubated at 37 °C for 2 hours. Two units of RNase-free DNaseI were added to the tube and incubated at 37 °C for 2 hours. The reaction was added with 1 μl of 0.5 M EDTA, pH 8, to stop the polymerase reaction.

The labeled RNA transcript was purified using the RNA mini Quick Spin column (Roche). The column was prepared by resuspending the Sephadex matrix in the column by mixing. The top cap was removed, and then the bottom tip was snapped off. The excess buffer was removed by centrifugation at 3,000 rpm for 1 minute. The column was placed in a clean, sterile 1.5 ml microcentrifuge tube and then slowly and carefully applied the sample to the center of the column bed. The column was centrifuged at 3,000 rpm for 4 minutes at room temperature. The eluate was stored at –20 °C until use. Each labeled RNA run-off transcript was quantified using the agarose gel electrophoresis.

2.13.3 Prehybridization treatment

The cytospin haemocyte slides were incubated in 0.2 M Tris-HCl, pH 7.4, twice for 5 minutes each and in 0.1 M glycine in 0.2 M Tris-HCl, pH 7.4, for 10 minutes. The cells were rinsed with PBS for 5 minutes.

The slides were incubated in 4% paraformaldehyde in phosphate buffer containing 5 mM $MgCl_2$ for 15 minutes to fix the haemocytes and rinsed with PBS for 5 minutes. The cells were incubated in 0.1 M triethanolamine, pH 8 containing 0.25% acetic anhydride for 10 minutes. The cells were then dehydrated in 30%, 70%, and

100% graded ethanol for 5 minutes each. The cells were dried at room temperature for at least 2 hours.

2.13.4 Riboprobe hybridization

The DIG-labeled riboprobe (40-100 ng per slide) was mixed with 2× SSC containing 50% formamide and 10% dextran sulfate, 10× Denhart's solution, 0.5 mg/ml tRNA from *E. coli*, 100 mM dithiothreitol, and 0.5 mg/ml salmon sperm DNA. The mixture was denatured at 55 °C for 10 minutes and immediately placed on ice for 5 minutes. The riboprobe was dropped onto the slides. The hybridization solution was covered by a glass slip in order for the solution to spread over the cells. The slides were incubated overnight at 55 °C in an adequately humid chamber with 1× SSC and 30% formamide. The cells were washed twice for 15 minutes with 2× SSC with shaking in a platform shaker. The cells were treated with 20 μg/ml RNase A in 2× SSC at 37 °C for 30 minutes, and then consecutively rinsed for 10 minutes at room temperature with 1× SSC supplemented with 0.07% 2-mercaptoethanol twice, 0.5× SSC supplemented with 0.07% 2-mercaptoethanol, and 0.1× SSC supplemented with 0.07% 2-mercaptoethanol. The slides were then rinsed twice with 0.1× SSC supplemented with 0.07% 2-mercaptoethanol for 30 minutes at 55 °C. The last wash solution with the slides was finally left on ice for 1 hour.

2.13.5 Riboprobe detection

The slides were washed in 0.1× SSC for 5 minutes and rinsed twice in TBS (0.1 M Tris-HCl, 0.9% NaCl, pH 7.4) containing 0.05% Triton X-100 for 5 minutes. The slides were pre-incubated in blocking buffer (TBS supplemented with 1% normal sheep serum and 0.05% Triton X-100) for 20 minutes. Five hundred microliters of antibody solution (1/1000 diluted alkaline phosphatase-conjugated sheep anti-DIG antibody in blocking buffer) were added onto each slide and incubated overnight at room temperature in a humid chamber. After incubation, the slides were washed 3 times in TBS for 10 minutes each and 2 times in buffer B (0.1 M Tris-HCl, pH 9.5, 50 mM $MgCl_2$, and 0.9% NaCl) for 5 minutes each. Five hundred microliters of detection solution (375 μg/ml NBT (nitroblue tetrazolium in dimethyl formamide),

188 μg/ml of BCIP, 1 mM levamisole in buffer B) were overlaid onto the slides and incubated in the dark at room temperature. The reaction should be monitored until the optimal development was obtained. For CaM detection, the optimal development was observed within 2 hours. The slides were then washed by TBS for 10 minutes to stop the reaction, and rinsed briefly with distilled water. The slides were mounted with a mixture of TBS:glycerol (1:9) and stored at 4 °C.

2.13.6 Control

Sense riboprobe and RNase-treatment antisense riboprobe were used as negative controls. For RNase-treatment antisense riboprobe, 10 μg/ml of RNase A was added onto the slide and incubated at 37 °C for 30 minutes. The slide was then washed 3 times with TBS for 5 minutes each before proceeding to the hybridization step.

2.14 Immunohistochemistry

2.14.1 Tissue preparation

The tissue from the juvenile shrimps was prepared by dissecting the animal to get the cepharothorax. The dissected tissue was immediately fixed in a freshly prepared RNA friendly fixative solution (220 ml 37% formaldehyde, 315 ml absolute ethanol, 115 ml glacial acetic acid, 350 ml water) for 24 hours at 4 °C. Use an adequate supply of fixative; a minimum of approximately 10 volume should be used for each specimen, e.g., a tissue of 10 ml would require 100 ml of fixative. Following the fixation, the specimens were transferred to 50% ethanol, where it can be stored indefinitely. The cuticle is slit before transferring to 50% ethanol, paying particular attention not to cut deeply into the underlying tissue. A history of the specimen is recorded by assigning the number of specimen and the collection time post-infection. The tissues were dehydrated by treating at room temperature 3 times in 70% ethanol for 20 minutes, 3 times in 90% ethanol for 20 minutes, 3 times in 100% ethanol for 20 minutes, and 3 times in 70% ethanol overnight. The dehydratedly fixed tissues were embedded in the paraplast by incubating 3 times in xylene for 20 minutes

at room temperature, in xylene for a minimum of 12 hours at room temperature, in a solution of xylene/paraplast (v/v) for 12 hours at 60 °C, and 3 times in paraplast for 1 hour at 60 °C. The paraplast blocks were prepared for sections. The 6 μm sections were prepared, mounted on the poly-L-lysine coated slides, and stored at –20 °C until use.

2.14.2 Immunodetection

To detect the protein of interest, the six μm-thick paraffin sections were eliminated of paraffin and hydrated by consecutively bathing twice in xylene for 15 minutes, twice in 96% ethanol for 5 minutes, twice in 70% ethanol for 5 minutes, twice in 30% ethanol for 5 minutes. Then, the slides were equilibrated for 1 hour at room temperature in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.7H_2O$, and 1.4 mM KH_2PO_4, pH 7.4). The slides were then incubated for 10 minutes in a new PBS solution, and pre-incubated with PBS containing 1% normal rabbit serum (NRS), 1% BSA, and 0.1% Triton X-100 (PBS/NRS/BSA/Triton X-100) for 1 hour at room temperature. Five hundred microliters of the purified mouse anti-CaM polyclonal antibody (1 μg/ml in PBS/NRS/BSA/Triton X-100) were overlaid onto the shrimp tissue and incubated overnight at 37 °C. After incubation, the slides were washed 3 times for 5 minutes with TBS. The secondary antibody, alkaline phosphatase-conjugated rabbit anti-mouse IgG (diluted 1:500 in PBS/NRS/BSA/Triton X-100) was added and incubated for 2 hours at room temperature. After washing 2 times for 5 minutes each with TBS, they were equilibrated in 0.1 M Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5 for 10 minutes. Immunodetection was performed by incubating the slides in the dark box at room temperature with 500 μl of detection solution (375 μg/ml of NBT, 188 μg/ml of BCIP, and 1 mM of levamisole in 0.1 M Tris-HCl, pH 9.5, 50 mM $MgCl_2$, and 0.9% NaCl). The slides were washed by TBS for 10 minutes to stop the reaction and rinsed briefly with distilled water. The slides were mounted with a mixture of TBS:glycerol (1:9) and stored at 4 °C. The slides were examined using a light microscope (Olympus).