CHAPTER II

MATERIALS AND METHODS

The experiments were separated into 2 parts:

Part I : Examination of royal jelly composition

Part II : Cloning, screening and sequencing of cDNA of mandibular gland

2.1 Part I : Examinanation of royal jelly composition

2.1.1 Instruments

-Burning oven (Thermolyne Sybron Corporation, USA.)

-(-20 °C) Freezer (Krungthai Ltd., Thailand)

-Gas chromatography (Shimadzu 7-AG)

-Heater (Fisher Scientific, USA.)

-Magnetic Stirrer M21/1 (Franz Morat KG GambH, Germany)

-pH meter (Metar Lab®, Denmark)

-Precision calibrator model 1302 (Sartorius, Germany)

2.1.2 Inventory supplies

-Buret

-Crucible

-Desiccator

-Forceps

-Grafting frame with 3 bars

-Kjeldahl apparatus

-Larvae removing spoon

-Nitrogen gas tank

-Queen cages

-Queen cell cups(made of wax) size 9 x 10 mm (Inner diameter x Inner length)

-Separatory funnel

-Soxhlet apparatus

-Weighing dish

2.1.3 Chemicals

-Boric acid (A.R. grade) (Merck, Germany)

-Bromcresol green (A.R. grade) (Fluka, Swithzerland)

-BSA: N, O-bis (trimethylsilyl) acetamide (A.R. grade)(Sigma Chemical Company, USA.)

-Chloroform (A.R. grade) (Merck, Germany)

-Copper (II) sulfate (A.R. grade) (Carlo Erba Reagent, Italy)

-Diethyl ether (A.R. grade) (BDH, England)

-Hydrochloric acid (A.R. grade) (Lab-Scan, Thailand)

-10-Hydroxy-δ-2-decenoic acid (10-HDA) (A.R. grade) (Nippon Shoji, Japan)

-Margaric acid (GC grade) (Sigma Chemical Company, USA.)

-Methyl red (A.R. grade) (Merck, Germany)

-Nitrogen gas (Industrial grade) (TIG, Thailand)

-Potassium sulfate (A.R. grade) (Univar, Australia)

-Sodium hydroxide (A.R. grade) (Carlo Erba Reagent, Italy)

-Sulfuric acid (A.R. grade) (Merck, Germany)

-Trimethylchlorosilane (A.R. grade) (Fluka, Switzerland)

2.1.4 Identification of sample population

For producing royal jelly, the selected honeybee (*Apis cerana*) hives were firstly confirmed their population using PCR-RFLP of *Dra*I digested 3 mitochondrial (small subunit (srRNA), large subunit RNA (lrRNA) and intergenic COI-COII (inter COI-COII) genes of mitochondrial DNA) (Sihanuntavong, 1999).

The DNA extraction was extracted from individual bee thorax using modified method of Hall and Smith (1991). A thorax of each individual was transferred into 1.5-ml microcentrifuge tube containing 500 µl of STE buffer (100 mM NaCl, 50 mM Tris-HCl, pH 7.5 and 1 mM EDTA) and briefly honogenized with a plastic pestle. The cells were lysed by adding 20% SDS to final concentratin of 1% SDS. Then, 10 mg/ml proteinase K solution was added to final concentration of 500 µg/ml and incubated at 65 °C for 2 hours. The DNA was extracted using a standard phenol-chloroform method. The extraction was carried out twice with an addition of an equal volume of phenol-chloroform-isoamyl alcohol (25: 24: 1 v/v), mixed gently for 15 minutes and

centrifuged at 7,000 x g for 10 minutes at room temperature. The upper aqueous phase was transferred to a new microcentrifuge tube, carefully and further extracted once with an equal volume of chloroform-isoamyl alcohol (24: 1 v/v). After each extraction, the mixture was centrifuged at 7,000 x g for 10 minutes at room temperature. The upper aqueous phase was carefully transferred. The upper aqueous phase was carefully transferred to a new microcentrifuge tube.

The extracted DNA was used as the template for specific amplification of the srRNA, lrRNA genes and inter COI-COII region by PCR using the specific primers and condition of Sihanuntavong (1999). The reaction was performed in 25 µl containing 50 ng template DNA, 200 µM each of dNTPs (dATP, dTTP, dCTP and dGTP), 1x PCR buffer (10 mM Tris-HCl, pH 8.3 and 50 mM KCl), 2.5-3.2 mM $MgCl_2$, 0.1 µM each of primers and 0.6 unit of Ampli*Taq* DNA polymerase (Perkin Elmer, USA). The reaction was predenaturated at 94 °C for 1 minute following by 35 cycles of denaturating step at 94 °C for 1 minute, annealing step for 1 minute and extension step at 72 °C for 2 minutes, the last extension at 72 °C for 10 minutes. Appropriate $MgCl_2$ concentrations and annealing temperature for amplification of each region were illustrated in Table 2.1. Then, the amplification products were singly digested with *Dra*I restriction endonuclease according to the manufacture's recommendations. The reaction mixture was carried out in 20 µl containing 500 ng of amplified products, 1x the recommended buffer, 5 units of enzyme and sterile deinonized water. The reaction was incubated at 37 °C for

2-3 hours. At the end period, one-fifth volume of a blue/orange loading dye (0.4% orange G, 0.03% bromphenol blue, 0.03% xylene cyanol FF, 15% Ficoll® 400, 10 mM Tris-HCl, pH 7.5 and 50 mM EDTA, pH 8.0) was added to stop the reaction. The resulting mixture was electrophoretically analyzed as soon as possible.

Table 2.1 Primer sequence, annealing temperatures, concentrations of primers and $MgCl_2$ used in PCR amplifiction of lrRNA and inter COI-COII genes of *A. cerana* mitochondrial.

Gene	Sequence	Annealing Temperature (°C)	Primer conc. (μM)	$MgCl_2$ conc. (mM)
srRNA	5' AAA AAG CTT CAA ACT GGG ATT AGA TAC CCC 3' 5' TGA CTG CAG AGG GTG ACG GGC GGT GTG T 3'	53	0.10	3.0
lrRNA	5' CTA TAG GGT CTT ATC GTC CC 3' 5' TTT TGT ACC TTT TGT ATC AGG GTT 3'	53	0.10	3.0
Inter COI-COII	5' TTG ATT TTT TGG TCA TCC AGA AGT 3' 5' CCA CAA ATT TCT GAA CAT TGA CC 3'	53	0.10	2.5

Finally, each of digested PCR products were electrophoretically analyzed in 2.5% (w/v) metaphore agarose in TBE buffer, pH 8.3 (89 mM Tris-Hydroxy, 89 mM boric acid and 2.5 mM EDTA).

2.1.5 Production of fresh royal jelly

The honeybee colonies were fed continuously with nectar and pollen to stimulate the production of royal jelly and the secretion of wax. Then, the healthy colony (large population of young bees and 1-3 days larvae, free of disease and well supplied with honey stores and pollen frames) was selected to be used as a cell builder. The honeybee queen was moved out from the hive into a queen cage about 10-12 hours before the producing step. To produce the royal jelly, 40-60 of the cell cups (made of wax) were stuck to the grafting bar and incubated in the queenless honeybee colony for 1 hour. After incubation, the grafting frame was carried out and a very small amount of royal jelly was poured onto the bottom of each cell cup. The 1 day larvae were then gently put into each royal jelly-poured cell cup (a larva per a cup). Then, the grafting frame was put between the larvae frame and the chrysalis frame in the honeybee colony. After 3 days of incubation, the grafting frame was carried out. The larvae were removed and the royal jelly was collected and immediately stored at –70 °C.

2.1.6 Determination of moisture content (followed by National Royal jelly Fair Trade Conference, 1980)

Accurate 2 to 3 g of homogeneous raw royal jelly was weight out into the constant-weight weighing dish. Then, the weighing dish containing royal jelly was heated on a water bath until most of moisture was evaporated off. The weighing dish was placed in vacuum oven adjusted at 70 ± 2 °C and

25 ± 5 mmHg and dried for 4 hours. After drying, the weighing dish was cooled in desiccator for about 30 minutes. Then, the weighing dish was weighed and calculated as follow:

$$\text{Moisture content (\%)} = \frac{B - C}{B - A} \times 100$$

where, A : weight of weighing dish (g)

B : initial weight of weighing dish containing raw royal jelly (g)

C : weight of weighing dish containing the royal jelly, after drying (g)

2.1.7 Determination of crude protein content (followed by National Royal jelly Fair Trade Conference, 1980)

A gram of homogeneous raw royal jelly was weighed out and transferred into a Kjeldahl digestion flask. Five grams catalyst (1:9 mixture of copper sulfate and potassium sulfate) and 15 ml of sulfuric acid were then added intimately. After that, the Kjeldahl flask containing royal jelly was slowly heated in a draft chamber at the beginning and the contents were digested gradually intensely. After the content had turned transparent, the digestion was finished by heating for 1 hour. Then, the contents were left cooling, sufficiently. About 100 ml of water were gradually poured into flask, mixed and the flask was fit to a distillation apparatus. Forty milliliters of 40%

sodium hydroxide were added into the flask and the distillation step was started.

In the distillation step, the flask containing 40 ml of 4% boric acid solution was used for collecting the distilled ammonia and the distillation was continued until about 100 ml of distillate were collected (about 20 minutes). Then, a few drop of the indicator mixture (0.3 g bromocresol green and 0.2 g methyl red dissolved in 400 ml ethyl alcohol) was added into the distillate and the distillate was titrated with the standard 0.1 N sulfuric acid solution. The crude protein content could be calculated as follow:

$$\text{Crude protein content (\%)} = \frac{A \times B \times 6.25 \times 1.4}{C}$$

where, A : concentration of H_2SO_4 (N)

B : amount of H_2SO_4 consumed (ml)

C : amount of raw royal jelly taken (g)

2.1.8 Determination of acidity (followed by National Royal jelly Fair Trade Conference, 1980)

A gram of homogeneous raw royal jelly was weighed out in a beaker. About 80 ml of water were added and the mixture was stirred thoroughly. Then, the mixture was titrated with 0.1 N standard sodium hydroxide (NaOH) solution to a pH value of 8.3. The acidity was calculated by the formula below

$$\text{Acidity (ml of NaOH/100 g royal jelly)} = \frac{A \times B \times 100}{C}$$

where, A : amount of NaOH consumed (ml)

B : concentration of NaOH (N)

C : amount of raw royal jelly taken (g)

2.1.9 Determination of 10-hydroxy-δ-2-decenoic acid (10-HDA) content (followed by National Royal Jelly Fair Trade Conference, 1980)

A gram of the raw royal jelly (containing about 2 to 10 mg of the 10-HDA) was weighed out in a flask. A small amount of water and a few drop of 30% sodium hydroxide were added to the flask containing the royal jelly. Then, the mixture was stirred to dissolve the royal jelly and diluted with water to 100 ml. Five to twenty milliliters of the diluted solution were introduced into a 200-ml separatory funnel. The contents were then adjusted to 200 ml with the water and acidified with 1 N HCl solution (9 ml concentrated hydrochloric acid diluted with water to 100 ml) to a pH value not more than 3. After that, the acidified contents were once extracted with 40 ml of ethyl ether then three times with 20 ml each of ethyl ether by shaking the contents. The ethyl ether layer was washed four times with 20 ml each of water. The water layer was removed as far as possible by standing and then the ether layer was transferred into a 200-ml fig-shaped flask (ground joint to evaporator). The separatory

funnel was washed with a small amount of ethyl ether and the washing was combined into the same fig-shaped flask as the ether layer. The ether was then evaporated off in the flask with a rotary evaporator at about 40 °C. Two milliliters of internal standard solution (0.5 mg/ml margaric acid (high quality) in chloroform) were added in the flask. Then, the chloroform was removed in a similar manner to that above. The solvents and water were completely removed in a stream of dry nitrogen gas. The 0.5 ml of TMS reagent (mixture of two volumes of BSA (N,O-bis (trimethylsilyl) acetamide) and one volume of TMCS (Trimethyl chlorosilane)) was added and the contents were shaked thoroughly. Then, a milliliter of the contents was injected into a gas chromatograh attached with a hydrogen flame ioinzation detector (FID) to prepare a chromatogram. The injection condition was performed using 5% Silicone SE-30 column at 200 °C of column temperature and 240 °C of injection temperature. The nitrogen gas flow was 50 ml/min. The 10-HDA was calculated by the following:

$$\text{10-HDA (\%)} = \frac{A \times B \times 100}{C \times 1000} = \frac{A \times B \times 0.1}{C}$$

where, A : amount of 10-HDA (mg) obtained from the calibration curves prepared by similar procedures

B : dilution number (Time)

C : amount of royal jelly taken (g)

Preparation of calibration curve

The standard solution was prepared by dissolving the dried 10-HDA in chloroform precisely to about 0.2 mg/ml concentration. Then, the standard solution was taken 1.0, 2.0, 3.0, 4.0 and 5.0 ml into five 200 ml fig-shaped flask, respectively. Two milliliters of the internal standard solution (margaric acid) were added in the respective flasks. After the solvent having been evaporated off by nitrogen gas, 0.5 ml each of TMS reagent was added. The contents were shaked thoroughly and 2 µl of the contents were injected, separately, into the gas chromatograph to prepare five chromatograms.

The peak area was measured. Then, the calibration curves were plotted from the ratio of the peak areas for the standard product (10-hydroxy-δ-2-decenoic acid) to those for the internal standard substance.

2.1.10 Determination of ash (followed by A.O.A.C. Method, 1984)

A gram of raw royal jelly was accurately weighed out in a constant-weight crucible. Then, the crucible containing royal jelly was dried at 105 °C and followed by burning at 550 °C until the weight was constant. The crucible was cooled down in desiccator and then calculated as follow:

$$\text{Total ash (\%)} = \frac{B - C}{B - A} \times 100$$

where, A : weight of crucible (g)

B : initial weight of crucible containing raw royal jelly (g)

C : weight of crucible containing the royal jelly, after burning (g)

2.1.11 Determination of lipid content (followed by A.O.A.C. Method, 1984)

The raw royal jelly was dried at 70 °C for 4 hours and was then grinded. The dried-grinded royal jelly was redried for 2 hours. After that, two grams of the dried-grinded royal jelly were weighed out in a filter paper and brought into the thimble of Soxhlet apparatus. Then, 200 ml ether were added into a constant-weight round-bottom flask and the flask was refluxed on a heating mantle at 2-3 drops/sec of diethyl ether refluxing rate for 16 hours. The diethyl ether was evaporated out from the flask. Then, the flask was incubated at 100 °C for 30 minutes and cooled in desiccator. The flask was weighed and calculated the lipid content as follow:

$$\text{Lipid content (\%)} = \frac{(B - A)}{C} \times 100$$

where, A : weight of the round-bottom flask (g)

B : weight of the round-bottom flask containing lipids after refluxing (g)

C : weight of the raw royal jelly (g)

2.1.12 Statistical Analysis

The chemical compositions of commercial and harvested royal jelly from Northern and Southern honeybees were analyzed using t distribution analysis to compare the difference between royal jelly produced by .population and species.

The null hypothesis was

$H_0 : \mu = \mu_0$

$H_1 : \mu \neq \mu_0$

Where, μ : Average value of royal jelly chemical composition of Northern honeybee population

μ_0 : Average value of royal jelly chemical composition of Southern honeybee population

2.2 Part II : Cloning, screening and sequencing of cDNA of mandibular gland

2.2.1 Instruments

-Autoclave HA-30 (Hirayama Manufacturing Co., Japan)

-Automated sequencer

: ABIprism DNA sequencer model 377 (Applied Biosystem, USA.)

: ALFexpress DNA sequencer

-Automatic micropipette P2, P20, P100, P200 and P1000 (Gilson Medical Electronics S.A., France)

-Camera Pentax super A (Asahi Opt. Co., Japan)

-Centrifuge model J2-21 (Beckman, USA.)

-Electrophoresis apparatus

: Horizontal gel electrophoresis apparatus (9 x 12 cm)

: Vertical gel electrophoresis apparatus for sequencing (Amersham Pharmacia Biotech, England)

-(-20 °C) Freezer (Krungthai Ltd.,Thailand)

-High speed microcentrifuge MC-15A (Tomy-Seiko, Japan)

-Heating Block 1761G-26 (Sybron Thermermolyne Co., USA)

-Incubator BM-600 (Memmert GambH, Germany)

-Incubator shaker

: Innova 4080 (New Brunswick Scientific Co., Inc. USA.)

: Kühner shaker (Kühner, Switzerland)

-Magnetic stirrer M21/1 (franz Morat KG GambH, Germany)

-Microcentrifuge model 1300 (Kubota, Japan)

-Microscope

-Microwave oven TRX1500 (Turbora International Co., Ltd., Korea)

-Power supply: Power Pac 300 (BIO-RAD Laboratories, USA.)

-Thermal cycler: Gene Amp PCR system 2400 (Perkin Elmer Cetus, USA.)

-UV transilluminator 2011 (San Gabriel California, USA.)

2.2.2 Inventory supplies

-Black and White print film TriX-pan 400 (Eastman Kodak Company, USA)

-Microcentrifuge tubes 0.5, 1.5 and 2.0 ml (Axygen Hayward, USA.)

-Pipette tips 10, 200 and 1000 µl (Axygen Hayward, USA.)

-Thin-wall microcentifuge tubes 0.2 ml (Axygen Hayward, USA.)

2.2.3 Chemicals

-Absolute ethanol (Merck, Germany)

-Acrylamide (Merck, Germany)

-Agarose gel (FMC Bioproducts, USA.)

: Seakem LE Agarose

: Metaphor Agarose

-Ammonium persulfate (Promega, USA.)

-100 bp DNA ladder (Promega, USA.)

-BigDye® Terminator v3.1 cycle Sequencing kit (Applied Biosysterms, USA.)

-Boric acid (Merck, Germany)

-Bromophenol blue (Merck, Germany)

-Chloroform (Merck, Germany)

-Delta® Differential Display Kit (Clonetech, USA.)

-DEPC (Sigma Chemical Company, USA.)

-Ethidium bromide (Sigma Chemical Company, USA.)

-Ethylene diamine tetraacetic acid, disodium salt dihydrate (Fluka, Switzerland)

-Ficoll 400 (Sigma Chemical Company, USA.)

-Formaldehyde (BDH, England)

-Gene Amp PCR core reagents (Perkin Elmer, USA.)

: 10x PCR buffer

: Magnesium chloride

: dNTPs (dATP, dGTP, dCTP, dTTP)

-Glacial acetic acid (BDH, England)

-Hydrochloric acid (Merck, Germany)

-Lamda/*Hin*dIII digest

-N,N-methylene-bis-acrylamide (Sigma Chemical Company, USA.)

-N,N,N',N'-tetramethylenediamine (Sigma Chemical Company, USA.)

- plasmid pBR322

- plasmid pGEM –3f(+) (Promega Co., USA.)

-pGEM®-T easy vector system (Promega, Co., USA.)

-Phenol crystal (Fluka, Switzerland)

- plasmid pUC18

-QIAprep® Miniprep plasmid DNA purification kit (QIAGEN, Germany)

-QIAquick® Gel Extraction kit (QIAGEN, Germany)

-QuickPrep® Micro mRNA Purification Kit (Amersham Pharmacia Biotech, England)

-Sodium acetate (Merck, Germany)

-Sodium chloride (Merck, Germany)

-Sodium carbonate anhydrous (AJAX, Australia)

-Sodium dodecyl sulfate: SDS (Sigma Chemical Company, USA.)

-Sodium thiosulfate (Sigma Chemical Company, USA.)

-TEMED (Sigma Chemical Company, USA.)

-TimeSaver™ cDNA Synthesis Kit (Amersham Pharmacia Biotech, England)

-Tris-(Hydroxy methyl)-aminomethane (Fluka, Switzerland)

-Urea (Fluka, Switzerland)

-Xylene cyanol (Sigma, USA.)

2.2.4 Enzymes

-MMLV Reverse transcriptase (Clontech, USA.)

-*Taq* DNA polymerase (Amersham Pharmacia Biotech, England)

-*Taq* DNA polymerase (Perkin Elmer, USA.)

-The advantage polymerase mix (Clontech, USA.)

-T_4 DNA ligase (Amersham Pharmacia Biotech, England)

-T_4 DNA ligase (Promega, USA.)

-T_4 polynucleotide kinase (Amersham Pharmacia Biotech, England)

2.2.5 Primers

-Arbitrary primers (Clontech, USA.)

-Oilgo (dT) primers (Clontech, USA.)

-pUC/M13 Forward primer (Amersham Pharmacia Biotech, England)

-pUC/M13 Reward primer (Amersham Pharmacia Biotech, England)

- pUC/M13 Forward primer (Applied Biosysterm, USA.)

-T7 primer (QIAGEN, Germany)

2.2.6 Sample collections

For honeybees samples, the 0-day adult honeybees (*Apis cerana indica*) were tagged by different colors each day. After 5-15 days, those of adult honeybees (nurse bees) were collected and kept immediately in the liquid nitrogen until used.

For mandibular gland samples, under the RNase-free condition, the nurse bees in liquid nitrogen were taken out and their heads were separated from their bodies. Then, each head was dissected under a microscope and the mandibular glands near the mandibles (two glands per one honeybee) were removed. All mandibular glands were kept in the extraction buffer (a buffer

aqueous solution containing the guanidinium thiocyanate and N-lauroyl sarcosine for RNA extraction).

2.2.7 Preparation of RNase-free solution, glassware and plasticware (Sambrook and Russell, 2001)

This step is a most important consideration for RNA research. The 0.1% diethyl pyrocarbonate (DEPC) in water was used for ribonuclease (RNase) inactivation. All aqueous solutions were prepared under RNase-free condition by autoclaving the stand-overnight 0.1% DEPC-treated water for 15 minutes at 15 psi (1.05 kg/cm^3). RNase-free glassware and plasticware were prepared by washing them with detergent and dried in a hot air oven at 240 °C for 4 hours or overnight. Then, they were filled overnight with 0.1% diethyl pyrocarbonate (DEPC) in water at 37 °C for 1 hour or overnight at room temperature. After that, they were rinsed several times with DEPC-treated water and were then autoclaved for 15 minutes at 15 psi. The new microcentrifuge tubes and tips were used and autoclaved at the same condition above for 2 times.

2.2.8 Extraction of mRNA of mandibular gland (Amersham Pharmacia Biotech, England)

The oligo (dT)-cellulose slurry was gently swirled to obtained a uniform suspension. Then, 1 ml of the oligo (dT)-cellulose was immediately pipetted into a microcentrifuge tube and centrifuged for 1 minute at top speed

(16,000xg). After that, the solution was removed from the oligo (dT)-cellulose pellet by pipeting.

The six hundreds of the mandibular glands were homogenized in 0.4 ml of extraction buffer until they were a uniform suspension. Then, 0.8 ml of elution buffer (10 mM Tris-HCl, pH 7.5 and 1 mM EDTA) was added and mixed thoroughly. The cellular suspension was then centrifuged for 1 minute at top speed (16,000xg) and the supernatant was placed on top of the oligo (dT)-cellulose pellet. The suspension was gently mixed by inverting the tube for 3 minutes and then centrifuged at a maximum speed (16,000xg) for 10 seconds. The supernatant was discarded and the oligo (dT)-cellulose was washed by adding 1 ml of high-salt buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA and 0.5 M NaCl) and centrifuged for 10 seconds, alternatively for 5 times. After that, 1 ml of low-salt buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA and 0.1 M NaCl) was added to the oligo (dT)-cellulose pellet. The tube was resuspended by inversion and centrifuged for 10 seconds. This step of washing with low-salt buffer was repeated one more time. Then, the pellet was resuspended in 0.3 ml of low-salt buffer and transferred to a Microspin column (placed in a 2-ml microcentrifuge tube). The column in the microcentrifuge tube was then centrifuged at full speed for 5 seconds. The effluent was discarded. The 0.5 ml of low-salt buffer was added to the column and centrifuged for 5 seconds at full speed. This step was repeated two more times.

After the effluent was discarded, the column was placed in a new sterile 1.5-ml microcentrifuge tube. The 0.2 ml of 65 °C prewarmed elution

buffer was added to the top of resin bed and then centrifuged at top speed for 5 seconds. The obtained eluate was not discarded. Then, the elution was repeated by adding 0.2 ml of prewarmed elution buffer and centrifuged at top speed for 5 seconds. The eluted mRNA was kept at –80 °C for further use (0.4 ml total volume).

2.2.9 Construction of mandibular gland cDNA

a) Preparation of cDNA by TimeSaver™ cDNA synthesis kit (Amersham Pharmacia Biotech, England)

The 1-5 µg of mRNA was placed in a steriled microcentrifuge tube and the volume was adjusted to 20 µl by RNase-free water (from kit). In order to denature mRNA, the tube containing mRNA was heated at 65 °C for 10 minutes and chilled on ice.

A first-strand reaction mix (FPLC*pure*™ Cloned Murine Reverse Transcriptase, RNAguard™ (porcine), RNase/DNase-free BSA, dATP,dCTP, dGTP and dTTP, in aqueous buffer) was briefly spun down to collect the solution at the bottom of the tube. The 1 µl of DTT solution and 1 µl of oligo $(dT)_{12-18}$ primer (0.5 mg/ml, aqueous solution) were added. Then, the heated-denatured mRNA was mixed into the reaction mix. The mixture was mixed by pipeting up and down and incubated at 37 °C for 1 hour. After 1 hour, the mixture in first-strand reaction mix including DTT and oligo$(dT)_{12-18}$ primer (about 33 µl) was transferred to the second-strand reaction mix (*E. coli* RNase-H and *E. coli* DNA polymerase I in aqueous buffer containing dNTPs) and

mixed, gently. The reaction was incubated at 12 °C for 30 minutes and then 22 °C for 1 hour.

During the incubation, the spun column was prepared as follow. The spun column was inverted several times to resuspend the sepharose CL-4B gel. Then, the top and bottom caps of the column were removed for draining the solution in column and the caps was recapped again after the solution surface was near the gel bed. The column was equilibrated with 2 ml of ligation buffer (66 mM Tris-HCl, pH 7.6, 0.1 mM spermidine, 6.6 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 150 mM NaCl) by inverting the column and the buffer was drained. The equilibration was performed twice more times. After that, the column was transferred into the 15-ml tube and centrifuged at 400xg for 2 minutes in a swinging bucket rotor. The column was transferred to a new sterile 15-ml tube with the 1.5-ml tube inside.

After the incubation of second-strand reaction was finished, the reaction was heated at 65 °C for 10 minutes and cooled down to room temperature. The 100 µl of phenol/chloroform was added, mixed and centrifuged for 1 minute to collect the upper aqueous layer (≈ 100 µl). This aqueous solution was then loaded onto the top of the gel bed of the spun column, carefully and centrifuged at 400xg for 2 minutes in the swinging bucket rotor. The cDNA was eluted in the solution into the 1.5-ml microcentrifuge tube within the 15-ml tube of the spun column.

To prepare the cDNA for cloning, the *Eco*RI/*Not* I adaptors were added to the ends of cDNA fragments by ligation. The ligated reaction was

performed in X µl of *Eco*RI/*Not* I adaptor solution (where X = µg of mRNA used for cDNA synthesis), 30 µl of PEG buffer, 1µl of diluted ATP solution (1:4 volume of ATP solution to RNase-free water) and 1 µl of T4 DNA ligase. Then, the reaction mixture was mixed, gently, spun briefly and incubated at 16 °C for 1 hour (the ligation was not proceeded for more than 4 hours). After incubation, the reaction was heated at 65 °C for 10 minutes to denature the DNA ligase and then chilled on ice. The procedure was followed by adding 1.5 µl of undiluted ATP solution and 1 µl of T4 polymucleotide kinase. The mixture was gently mixed and incubated at 37 °C for 30 minutes and then heated at 65 °C for 10 minutes. At the room temperature, 140 µl of phenol/chloroform (1:1 v/v) solution was added, mixed and centrifuged for 1 minute. The upper aqueous phase (≈140-150 µl) was applied to a equilibrated spun column for purification in a similar manner to that above.

b) Preparation of cDNA by modifying Delta® Differential Display Kit (Clontech, USA.)

The RNA prepared from the previous experiment was treated with DNase I. For 25 µl of RNA sample, the DNase mixture was prepared by combining of 1 µl of 0.5 M Tris-HCl, pH 7.5, 1 µl of 0.5 M $MgCl_2$, 22 µl of sterile water and 1 µl of RNase-free DNase (1 unit/µl). Then, the DNase mixture was added to the RNA sample in a 0.5-ml microcentrifuge tube and mixed, thoroughly. The mixture containing DNase and RNA sample was then

incubated at 37 °C for 30 minutes. After incubation, 2.5 µl of 0.2 M EDTA and 2 µl of 3M NaOAc were added to the tube. Then, an equal volume of phenol-chloroform-isoamyl alcohol (25: 24: 1 v/v) was added to the reaction and mixed. The reaction was centrifuged at 14,000 rpm for 10 minutes to separate phases. The upper aqueous phase was transferred to new 0.5-ml microcentrifuge tube and a volume of chloroform-isoamyl alcohol (24: 1 v/v) was added. The reaction was centrifuged at the 14,000 rpm for 10 minutes again and the top aqueous phase was transferred to new 0.5-ml microcentrifuge tube. After that, 1/10 volume of 3 M NaOAc and 2.5 volumes of 95% ethanol were added and then centrifuged at 14,000 rpm for 20 minutes. The supernatant was discarded. The RNA pellet was dried and dissolved for 2 µg of starting RNA in RNase-free water. The RNA solution was kept at –80 °C until further used.

The RNA solution prepared from previous was used for first-strand cDNA construction. Two micrograms of total RNA sample and 1 µl of 1 µM cDNA synthesis primer was pipetted to a new 0.5-ml microcentrifuge tube. The volume was then adjust to 5 µl. The mixture was mixed and spun briefly. Then, the mixture was incubated at 70 °C for 3 minutes and immediately cooled on ice for 2 minutes. Five micrometers of master mix containing 2 µl of 5x first-strand buffer (250 mM Tris, pH 8.3, 30 mM $MgCl_2$ and 375 mM KCl), 2 µl of 5 mM dNTP mix and 1 µl of 200 units/µl MMLV reverse transcriptase were added to the mixture. The content was mixed by gently pipeting. The mixture was incubated at 42 °C for 1 hour in an air incubator and the reaction

was terminated by incubating at 75 °C for 10 minutes and then was placed on ice. The reaction was diluted to 2 various conditions. The dilution A, 8 µl of first-strand cDNA sample were diluted with 72 µl of sterile water and the dilution B, 2 µl of first-strand cDNA sample were diluted with 78 µl of sterile water. Both of dilution conditions were used for constructing the second-strand cDNA.

To constructing of second-strand cDNA, the first-strand cDNA was amplified by PCR technique using arbitrary primer and oligo (dT) primer Table 2.2 and Table 2.3).

Table 2.2 Arbitrary primers and Oligo (dT) primers used in second-strand cDNA construction.

Primer name	Primer sequence
Arbitrary primer	
P1	5'-ATT AAC CCT CAC TAA ATG CTG GGG A-3'
P2	5'-ATT AAC CCT CAC TAA ATC GGT CAT AG-3'
P3	5'-ATT AAC CCT CAC TAA ATG CTG GTG G-3'
P4	5'-ATT AAC CCT CAC TAA AGC ACC GTC C-3'
P5	5'-ATT AAC CCT CAC TAA AGA TCT GAC TG-3'
P6	5'-ATT AAC CCT CAC TAA ATG CTG GGT G-3'
P7	5'-ATT AAC CCT CAC TAA ATG CTG TAT G-3'
P8	5'-ATT AAC CCT CAC TAA ATG GAG CTG G-3'
P9	5'-ATT AAC CCT CAC TAA ATG TGG CAG G-3'
Oligo (dT) primer	
T1	5'-CAT TAT GCT GAG TGA TAT CTT TTT TTT TAA-3'
T2	5'-CAT TAT GCT GAG TGA TAT CTT TTT TTT TAC-3'
T3	5'-CAT TAT GCT GAG TGA TAT CTT TTT TTT TGC-3'
T4	5'-CAT TAT GCT GAG TGA TAT CTT TTT TTT TCA-3'
T5	5'-CAT TAT GCT GAG TGA TAT CTT TTT TTT TCC-3'
T6	5'-CAT TAT GCT GAG TGA TAT CTT TTT TTT TCG-3'

Table 2.3 Pairs of primers used in the experiment

Pair name of primers	Arbitrary primer	Oligo (dT) primer
D1	P1	T4
D2	P1	T8
D3	P2	T1
D4	P2	T5
D5	P3	T2
D6	P3	T6
D7	P5	T4
D8	P5	T8
D9	P6	T1
D10	P6	T5
D11	P7	T2
D12	P7	T6
D13	P8	T2
D14	P8	T6
D15	P9	T1
D16	P9	T5
D17	P4	T4
D18	P4	T8

The reaction was performed in 20 µl total volume containing 1 µl of each first-strand cDNA template, 1 µM of each primer (arbitrary primer and oligo (dT) primer), 1x PCR reaction buffer containing 1.5 mM Mg^{2+} final concentration, 50 µM each of dNTPs (dATP, dCTP, dGTP and dTTP) and 0.4 µl of 50x advantage KlenTaq Polymerase mix. The sterile water was added to adjust volume to 20 µl. The reaction was separated to 3 steps of cycles. First, the reaction was respectively preheated at 94 °C, 40 °C and 68 °C for 5 minutes per each temperature. Next, two cycles of 94 °C for 2 minutes, 40 °C for 5 minutes and 68 °C for 5 minutes were performed. Then, 25 cycles of 94 °C for 1 minute, 60 °C for 1 minute and 68 °C for 2 minutes were performed. Last extension was performed at 68 °C for 7 minutes. After PCR, the PCR product was electrophoretically analyzed in 1% agarose gel. And the cDNA was then eluted from agarose gel using QIAGEN gel extraction kit to prepare pure cDNA for cDNA cloning.

2.2.10 cDNA cloning and screening

The cDNA obtained from 2.2.9 was ligated by pGEM® -T Easy vector system (Promega, USA.). The procedure was started by spinning the pGEM-T easy vector, briefly to collect the contents at the bottom of the tube. The 2x rapid ligation buffer was then mixed, vigorously before used. Then, the ligation reaction was performed in 5 µl of 2x rapid ligation buffer, 1 µl of pGEM-T easy vector (50 ng), X µl of cDNA (where X = optimal cDNA

amount) and 1 µl of T4 DNA ligase (3 weiss units/µl). The sterile deionized water was filled to the reaction to 10 µl. The reaction was mixed by pipeting and was then incubated at 4 °C overnight.

After the ligation was finished, 1 µl of ligation mixture was transformed to the JM109 host by electroporation (Sambrook and Russell, 2001). The blue/white screening was used for selected the cDNA clones on the LB agar plates containing 50 µg/µl ampicillin. The white colonies were collected and the plasmid were extracted by miniprep alkaline lysis method (Sambrook and Russell, 2001).

2.2.11 cDNA sequencing

In this experiment, the sequencing reactions were prepared for 2 models of automated sequencer: ABIprism model 377; and ALFexpress DNA sequencer by using BigDye® Terminator v3.1 Cycle Sequencing kit and ALFexpress™ AutoCycle™ Sequencing kit, respectively.

The bacteria ,*E.coli*, strain JM109 containing plasmid was innoculated to 2 ml of LB broth (per liter: 10 g of tryptone, 5 g of yeast extract and 10 g of NaCl) containing 50 µg/ml of ampicillin and shaked at 37 °C, overnight. At log phase, the cell was collected by centrifuged at 5,000 rpm for 5 minutes. The plasmid was extracted from cell pellet by QIAprep® Miniprep plasmid purification kit (QIAGEN, Germany). Then, plasmid was applied to sequencing reaction.

a) Preparation of sequncing reaction by BigDye® Terminator V.3.1 Cycle Sequencing kit (Applied Biosystem, USA.)

The reaction was performed in 20 µl total volume. Four microliters ready reaction premix, 4 µl of 5x BigDye sequencing buffer, 3.2 pmol of M13 or T7 primers and the 500 ng of plasmid DNA were added in a new sterile 0.2-ml thin-wall PCR tube. The reaction was adjusted to 20 µl with water. Then, the tube was placed in a thermal cycler and set to the correct volume. The initial denaturation was started at 96 °C for 1 minute. The 25 cycles of 96 °C for 10 second, 50 °C for 5 second and 60 °C for 4 minutes were repeated. The reaction was hold at 4 °C until ready to purify.

To purify the reaction, 5 µl of 125 mM EDTA and 60 µl of absolute ethanol were added to the reaction tube. The content was mixed by inverting 4 times. The reaction was incubated at room temperature for 15 minutes and then centrifuged at 2,000 x g for 45 minutes. The supernatant was discarded and the pellet was washed with 70% ethanol and the centrifuged was applied again at 1,650 x g for 15 minutes. The supernatant was discarded and the pellet was air-dried in a dark place and the reaction was covered with aluminum foil and stored at 4 °C until injected to the sequencer.

b) Preparation of sequencing reaction by ALFexpress™ AutoCycle™ Sequencing kit (Amersham Pharmacia Biotech, England)

For each template, four reaction of each ddNTPs (ddATP, ddGTP, ddCTP and ddTTP) were required. Two microliters of each ddNTP

were pipetted to 0.2-ml thin-wall PCR tube and placed on ice. Then, four microliters of master mix containing 1x reaction buffer, 1 μg DNA template (DNA sample), 2 μl of DMSO, 5 μl of dNTPs solution (dATP, dCTP, dGTP and dTTP), cyanine-labelled M13 forward primer and 2.5 units of *Taq* DNA polymerase were added to the tube. The reaction mixture tube was transferred to the thermocycle model 2400 (Perkin Elmer, USA.) and programmed to predenaturate at 94 °C for 2 minutes. Then, the program was followed by 36 cycles of denaturating step at 94 °C for 15 seconds, annealing step at 54°C for 15 seconds and extension at 72 °C for 40 seconds. The reaction was ended by post extension at 72 °C for 5 minutes and 4 μl of stop solution was applied to the tube. The sequencing reaction was stored at 4 °C until loading.

Sequencing gel preparation, 8% polyacrylamide was prepared. Seventy-six grams of polyacrylamide, 4 g of bis-acrylamide and 480 g of urea were weighed out in a brown bottle and dissolved with 100 ml of 10x TBE buffer. After the contents were dissolved, the mixture was adjusted to 1 liter with water and then degassed using vacuum pump. The polyacrylamide solution was kept at 4°C until the sequencing gel was set. To set the sequencing gel, the sequencing mirror was washed with detergent and clean water. Then, the glass spacers and the inner side of mirror were treated with 95% ethanol and the sequencing apparatus was set . Fifty milliliters of 8% polyacrylamide gel, 242 μl of 10% (g/ml) ammonium persulfate and 49 μl of TEMED were mixed in a 250-ml beaker. And the mixed gel was poured to the space inside the mirror set. After the gel was set, the sequencing gel was applied to the

ALFexpress DNA sequencer. Before loading, each reaction was heated at 95 ° C for 2-3 minutes and immediately placed on ice until loading. Ten microliters of each reaction was removed into an appropriate well on the sequencing gel. The 1x TBE buffer was used as the electrolyte.

The nucleotide sequences obtained from automated sequencing were compared with GenBank database by blastn and blastx programs.