CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipments

Autoclave Model # LS-2D (Rexall Industries Co. Ltd., Taiwan)

Automatic micropipettes P10, P100, P200, and P1000 (Gilson®, France)

Balance: Satorius 1702 (Scientific Promotion Co.)

Bind Silane (Pharmacia, USA)

-20°C Freezer (Whirlpool)

-80°C Freezer (ThermoForma)

Gel Documention System (GeneCam FLEX1, SynGene)

Gene Pulser (Bio-RAD)

Hot plate (CERAMAG Midi, IKA® WORKS, USA)

Incubator 37°C (Memmert)

Laminar Airflow Biological Safety Cabinets Class II Model NU-440-400E (NuAire, Inc., USA)

Microcentrifuge tubes 0.6 ml and 1.5 ml (Bio-RAD Laboratories, USA)

Minicentrifuge (Costar, USA)

Orbital shaker SO3 (Stuart Scientific, Great Britain)

PCR Mastercycler (Eppendorf AG, Germany)

PCR thin wall microcentrifuge tubes 0.2 ml (Axygen® Scientific, USA)

PCR workstation Model # P-036 (Scientific Co., USA)

pH meter Model # SA720 (Orion)

Pipette tips 10, 20, 100 and 1000 µl (Axygen® Scientific, USA)

Power supply, Power PAC 3000 (Bio-RAD Laboratories, USA)

Refrigerated microcentrifuge MIKRO 22R (Hettich Zentrifugen, Germany)

Repel Silane (2 % dimethyldichlorosilane in octamethylcyclotetrasitoxone; Pharmacia, USA)

Spectrophotometer DU650 (Beckman, USA)

Sterring hot plate (Fisher Scientific)

Vacuum pump (Bio-RAD Laboratories, USA)

2.1.2 Chemicals and Reagents

Absolute ethanol, C_2H_5OH (BDH)

Acetic acid glacial, CH_3COOH (BDH)

Acrylamide, C_3H_5NO (Merck)

Agarose (Sekem)

Ammonium persulfate, $(NH_4)_2S_2O_8$ (USB)

Bacto agar (Difco)

Bacto tryptone (Scharlau)

Bacto yeast extract (Scharlau)

Boric acid, BH_3O_3 (Merck)

Bromophenol blue (Merck, Germany)

Chloroform, $CHCl_3$

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

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

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

Ethidium bromide (Sigma)

Ficoll^{TM} 400 (Amersham)

Formaldehyde (BDH)

Formamide (Gibco BRL, technologies, Co., USA)

Glucose (Merck)

Glycerol, $C_3H_8O_3$ (BDH)

Isopropanol (Merck)

Kodak Tri-Xpan400 film

N, N'-methylene-bisacrylamide, $C_7H_{10}N_2O_2$ (USB)

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

Phenol:chloroform:isoamyl alcohol (Sigma)

RNA markers (Promega)

Silver nitrate (Merck)

Sodium carbonate (Merck)

Sodium chloride (Carlo Erba)

Sodium hydrogen carbonate, $NaHCO_3$ (BDH)

Sodium hydroxide, NaOH (Eka Nobel)

Sodium thiosulfate (Merck)

N, N, N', N'-tetramethylethylenediamine (TEMED) (BDH)

Trizol reagent (Gibco BRL)

Urea (Fluka, Switzerland)

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

2.1.3 Bacterial strains

E. coli XL-I blue strain

2.1.4 Kits

Delta™ Differentia Display Kit (Clontech, USA)

HiYield™ Gel/PCR Mini Kit (RBC).

ImProm-IITM Reverse Transcription system kit (Promega)

pGem®-T Easy vector system I (Promega)

QIAprep spin miniprep kit (QIAGEN)

2.1.5 Enzymes

Advantage cDNA polymerase mix (Clontech)

DyNazyme II DNATM polymerase (Finnzymes, Finland)

Eco RI (Biolabs, UK)

RQ1 RNase – free DNase (Promega)

Taq DNA polymerase (Fermentus)

2.2 Shrimp samples

Sub-adult *P.monodon* (approximately 3 month old, about 20 g of body weight) was perchased from local farms. The shrimp were acclimated to 25 ppt salinity for 2 weeks in tank covered with a black plastic sheet to reduce light intensity and to minimize disturbance. They were fed with commercial food at a rate of 5% body weight per day. Water temperature was maintained at 26 to 28°C. Two airstones were provided on the bottom of tank. After 2 weeks, 4 individual shrimp assigned as control group were killed and tissues including, antennal gland, epipodite and gill were collected. The remaining shrimp were random by divided into two groups. The first group was abruptly transferred to 3 ppt salinity and another group was transferred to 40 ppt salinity. Antennal gland, epipodite and gill were taken from an individual shrimp at different time points (6, 24 h and 2 weeks) after the transfer, respectively. At each time point, the samples were taken from 4 individual shrimp. Two independent biological samples were prepared. One group was prepared for identification of functionally relevant genes controlling osmoregulation and another was prepared for conformation of DD-PCR.

2.3 Sample collection and total RNA preparation

Antennal gland, epipodite and gill were isolated separately from an individual shrimp and immediately frozen in liquid nitrogen (-176°C) in order to preserve the intact tissue. The samples were briefly homogenized by a pestle in 1 ml of ice-cold Trizol reagent (Gibco BRL). The homogenate was stored at room temperature for 5 to 10 minutes to permit complete dissociation of nucleoprotein complexes. After that, 200 µl of chloroform were added and vigorously shaken for 15 seconds and incubated at room temperature for 3 minutes. The sample was centrifuged at 12,000xg for 15

minutes at 4°C. The colorless upper aqueous phase containing total RNA was transferred to a new 1.5 ml microcentrifuged tube. Total RNA was precipitated by the addition of 500 µl of isopropanol. Then, the mixture was incubated at room temperature for 10 minutes and centrifuged at 12,000xg for 10 minutes at 4°C. The supernatant was removed. The RNA pellet was washed with 1 ml of 75% ethanol. The RNA pellet was kept under 75% ethanol until used. When required, the sample was centrifuged at 12,000xg for 15 minutes at 4°C. The supernatant was removed. The RNA pellet was air dried about 30 minutes. The total RNA was dissolved with an appropriate amount of diethyl pyrocarbonate (DEPC) – treated water.

The total RNA concentration was determined by UV spectrophotometer at 260 nm and estimated in µg/ml using the following equation,

$$[RNA] = OD_{260} \times \text{dilution factor} \times 40^*$$

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

Protein had a maximum absorption at 280 nm. Determining the ratio of A_{260}/A_{280}, the relative purity of the sample could be estimated. RNA sample should not have an A_{260}/A_{280} ratio below 1.6. Then RNA was dissolved in RNase – free water and stored at -70°C until used.

2.4 Formaldehyde – agarose gel electrophoresis

After total RNA was extraction, RNA quality should be estimated by formaldehyde – agarose gel electrophoresis.

One % (w/v) formaldehyde – agarose gel was prepared using 1x MOPS buffer (diluted from a 10x MOPS buffer to 0.2 mM MOPS, 50 mM NaOAc, 10 mM EDTA, pH 7.0 final concentration) The gel slurry was boiled until complete solubilization and allowed to cool to about 60°C. Formaldehyde (0.66 M final concentration) and ethidium bromide (0.2 µg final concentration) were added to the gel and poured into a chamber set. The comb was inserted. Ten to twenty micrograms of total RNA in 3.5

µl of DEPC – treated water, 5 µl of formamide, 1.5 µl of 10x MOPS and 2 µl of formaldehyde were combined, mixed well and incubated for 15 minutes at 65°C. After that, the mixture was immediately placed on ice. One – forth volume of the gel – loading buffer (50% (v/v) glycerol, 1 mM EDTA, pH 8.0 and 0.5% (w/v) bromophenol blue) was added to each sample. The samples were loaded to the 1.0% formaldehyde – agarose gel. The standard RNA marker was used as a size marker. Electrophoresis was carried out in 1x MOPS buffer at 50 volts, until the bromophenol blue dye migrated approximately ¾ of the gel length. The ethidium bromide stained gel was visualized total RNA as fluorescent bands by a UV transilluminator (UVP Inc.)

2.5 DNase treatment of the total RNA samples

Chromosomal DNA contamination in the total RNA sample was removed by treating 25 µg of total RNA with 5 units of RQ1 RNase – free DNase (Promega) at 37°C for 1 h. Then, the reaction volume was adjusted to 40 µl with DEPC – treated water, 250 µl of Trizol reagent was added and mixed well for 5 seconds. Two hundred microliters of chloroform was added and vigorously shaken for 15 seconds. The mixture was added at room temperature for 2 to 5 minutes and centrifuged at 12,000xg for 15 minutes at 4°C. Then, the top layer was added to 1 volume of isopopanol and incubated for 10 minutes at room temperature. The resulting mixture was centrifuged at 12,000xg for 15 minutes at 4°C. The supernatant was removed. The RNA pellet was stored with 500 µl of 75% ethanol with used. When required, the sample was centrifuged at 12,000xg for 10 minutes at 4°C. The supernatant was removed. The RNA pellet was briefly air dried for 5 to 10 minutes. The total RNA was dissolved with an appropriate amount of DEPC – treated water. The concentration of DNA – free total RNA was determined as described in sample collection and total RNA preparation section.

The purity of RNA sample was evaluated from a ratio of A_{260}/A_{280}. The ratio of the purified RNA was approximately about 1.8 to 2.0 respectively (Sambrook et al., 1989)

2.6 First-stranded cDNA synthesis

DNA - free total RNA isolated from each tissue was pooled of 4 individuals for each experiment and used as template for the first-stranded cDNA synthesis by using ImPromp-II™ Reverse Transcription System (Promega). One microgram of DNA-free total RNA was combined with 1 µl of oligo (dT) primer (0.5 µg/µl) and appropriate nuclease-free water in a final volume of 5 µl. The mixture was incubated for 5 minutes at 70°C. Then, the mixture was immediately chilled on ice-water for at least 5 minutes and spinned for 10 seconds in a microcentrifuge to collect the condensate and maintain the original volume. After that, 4 µl of 5x reaction buffer, 2.6 µl of 25 mM $MgCl_2$, 1 µl of dNTP mix (10 mM each dNTP), 20 units of Ribonuclease inhibitor and 1 µl of Improm-II™ Reverse Transcriptase were added and gently mixed. The reaction mixture was incubated for 5 minutes at 25°C and for 60 minutes at 42°C. To inactivate reverse transcriptase activity, the reaction mixture was incubated for 15 minutes at 70°C. All cDNA samples were stored at -20°C until ready for use.

2.7 Differential Display PCR amplification

DD-PCR primers from Delta® Differential Display Kit (Clontech) consisting of 10 arbitary or "P" primers and 9 oligo (dT) or "T" primers are listed in Table 2.1.

The cDNA samples obtained from the previous cDNA synthesis of gill, epipodite and antennal gland were diluted to 1:2 and then subjected to DD-PCR reaction. For each experiment display, 1 µl of each diluted cDNA sample was mixed with a master mix containing 0.25 µl of 20 µM P primer, 0.25 µl of 20 µM T primer, 1 µl of 10X cDNA PCR reaction buffer, 0.3 µl of 10 mM dNTP mix (10 mM each dNTP), 0.4 µl of 50X Advantage cDNA polymerase mix (Clontech) and 6.8 µl of sterile water to 10 µl final volume. The PCR amplification was carried out in a Mastercycler® PCR thermal cycler (Eppendorf, Germany).

The thermal cycles were one cycle at 94°C for 5 minutes, 40°C for 5 minutes, 72°C for 5 minutes, two cycles at 94°C for 30 seconds, 40°C for 30 seconds , 72°C for 5 minutes and then amplified using 40 cycles. Each cycle composed of DNA denaturation at 94°C for 30 seconds, primer annealing at 60°C for 30 seconds, and primer elongation at 72°C for 2 minutes. After 40 cycles, the samples were incubated at 72 °C for 7 minutes for final elongation to fully extend the amplified products. The DD-PCR products were stored at –20°C.

DD-PCR products were analyzed by using 4% denaturing polyacrylamide gel electrophoresis and visualized by silver staining.

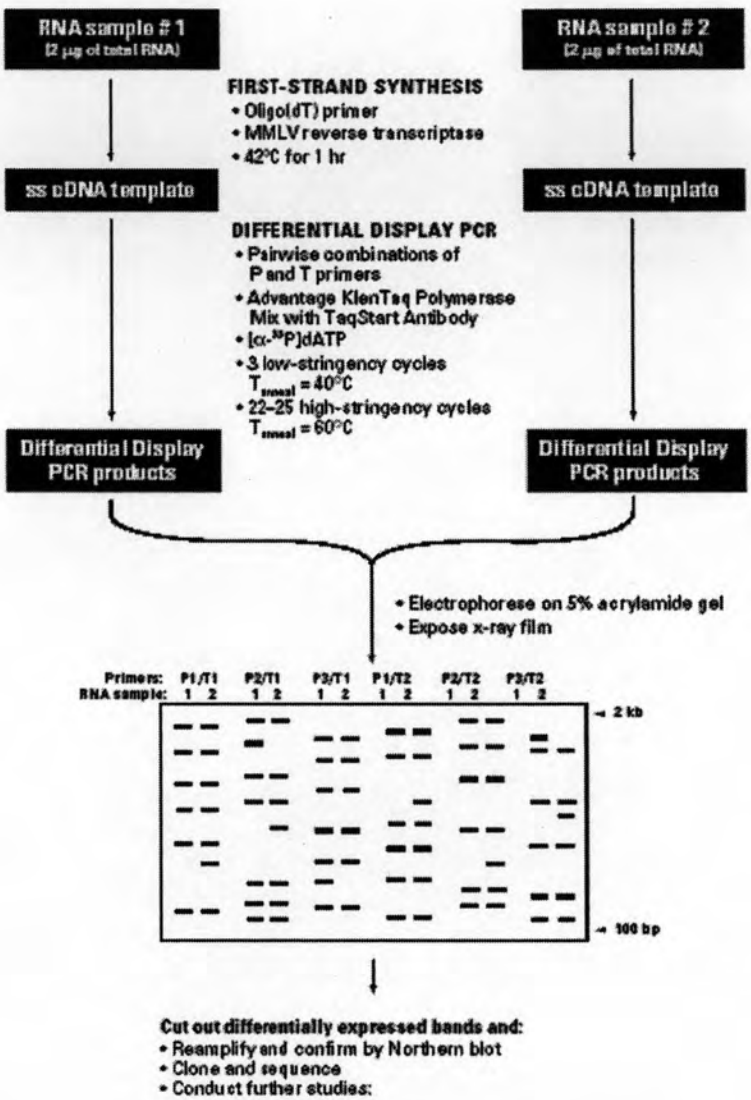


Figure 2.1 Overview of the Delta Differential Display protocol

First-strand cDNA were synthesized from total RNA and subjected to differential display PCR combinations of P and T primers. DD-PCR products were analyzed by polyacrylamide gel electrophoresis and autoradiography.

Table 2.1 Differential Display primers* ; oligo(dT) (T) primers and arbitrary (P) primers

Primer name	Primer's sequence
P1	5'-ATTAACCCTCACTAAATGCTGGGGA-3'
P2	5'-ATTAACCCTCACTAAATCGGTCATAG-3'
P3	5'-ATTAACCCTCACTAAATGCTGGTGG-3'
P4	5'-ATTAACCCTCACTAAATGCTGGTAG-3'
P5	5'-ATTAACCCTCACTAAAGATCTGACTG-3'
P6	5'-ATTAACCCTCACTAAATGCTGGGTG-3'
P7	5'-ATTAACCCTCACTAAATGCTGTATG-3'
P8	5'-ATTAACCCTCACTAAATGGAGCTGG-3'
P9	5'-ATTAACCCTCACTAAATGTGGCAGG-3'
P10	5'-ATTAACCCTCACTAAAGCACCGTCC-3'
T1	5'-CATTATGCTGAGTGATATCTTTTTTTTTAA-3'
T2	5'-CATTATGCTGAGTGATATCTTTTTTTTTAC-3'
T3	5'-CATTATGCTGAGTGATATCTTTTTTTTTAG-3'
T4	5'-CATTATGCTGAGTGATATCTTTTTTTTTCA-3'
T5	5'-CATTATGCTGAGTGATATCTTTTTTTTTCC-3'
T6	5'-CATTATGCTGAGTGATATCTTTTTTTTTCG-3'
T7	5'-CATTATGCTGAGTGATATCTTTTTTTTTGA-3'
T8	5'-CATTATGCTGAGTGATATCTTTTTTTTTGC-3'
T9	5'-CATTATGCTGAGTGATATCTTTTTTTTTGG-3'

* obtained from Delta™ Differential Display kit (Clonetech).

2.8 Denaturing polyacrylamide gel electrophoresis

2.8.1 Preparation of glass plate

The long and short glass plates were throughly cleaned with 95% commercial grade ethanol for 3 times in one direction. The short glass plate was coated with 1 ml of Repel Silane (2% dimethyldichlorosilane in octamethylcyclotetrasitoxone; Pharmacia, USA). For the long glass plate, it was coated with 1,500 µl of freshly prepared Bind Silane (6 µl of Bind Silane; Pharmacia, USA, 1,500 µl of ethanol and 8 µl of glacial acetic acid). Both of glass plates were left for approximately 20 to 30 minutes. After that, they were assembled to each other with a pair of spacer in between. The bottom and both sides of assembled glass plates were sealed with the plastic tape.

2.8.2 Preparation of denaturing polyacrylamide gel electrophoresis

DD-PCR products were analyzed on the denaturing polyacrylamide gel. The gel was prepared by combining 60 ml of the acrylamide solution (57 g acrylamide, 5 g N'N' methylenebisacrylamide, 7.66 M urea per 1 liter in 1x TBE buffer) with 360 µl of freshly prepared 10% ammonium persulphate and 36 µl of TEMED. The denaturing polyacrylamide gel was poured onto preset plates. The filled plate sandwich was left in the horizontal position. The flat edge of the shark – tooth comb was then inserted. The gel was left at room temperature for 1 h. After that, the polymerized gel was covered by water – soaked tissue paper and left at room temperature for 4 h (or overnight) for complete polymerization. When all was done, the spring clips and the sealing tape were carefully removed. The shark – tooth comb was rinsed with water.

2.8.3 Electrophoresis

The gel sandwich was placed in the vertical sequencing apparatus with the short glass plate inward. The gel sandwich was clamped with the integral gel clamps along with the sides of the sequencing apparatus. The upper and lower buffer champers were filled with approximately 300 ml of 1x TBE buffer. The gel was pre – runned at 30 to 40 watts for at least 30 minutes.

For each amplification product (10 µl) was mixed with 2 µl of the stopping/loading dye (10 mM NaOH, 99% formamide, 0.1% bromophenol blue and 0.1% xylene cyanol) and then heated at 95°C for 5 minutes and immediately placed on ice at least 5 minutes. The samples were carefully loaded into the well and electrophoresed with 1x TBE buffer at 50 watts for approximately 5 to 7 hours.

2.9 Silver staining

A silver staining protocol used to stain PCR products in polyacrylamide gel was that described by Soot-anan (1999). After electrophoresis, the long glass plate with the gel was submerged for 30 minutes in the fix/stop solution (10% acetic acid). The gel was then briefly rinsed 3 times with the deionized water and was shaken well for 2 minutes each. Then, the gel was transferred to the staining solution (0.1% silver nitrate containing 1.5 ml/liter of 37% formaldehyde) and incubated with agitation at room temperature for 30 minutes. The excess silver ions were eliminated by brief rinse with the deionized water and immediately placed in the cold (8 to 10°C) developing solution (3% sodium carbonate containing 1.5 ml/liter of 37% formaldehyde and 200 µl/liter of 10 mg/ml sodium thiosulfate). The gel was well agitated until the first band could be seen (usually 1.5 to 2 minutes). The gel was then transferred to another tray containing the fresh developing solution. The gel was continued development and shaken until optimal image intensity was observed (usually 2 to 3 minutes). The developing reaction was stopped by using 10% acetic acid with agitating for 5 minutes. The stained gel was soaked in deionized water twice for 3 minutes each. The gel was dried by air dry at room temperature.

2.10. Isolation of differentially expressed bands

DD-PCR patterns with visually different signal intensities were compared between control group (25 ppt salinity) and stress groups (3 and 40 ppt salinities) for each pair of primers. To isolate differential expressed cDNA fragments, the strong intensity cDNA band of each primer combination was excised using a clean, sharp razor and washed 3 times of sterile deionized water. Each cDNA fragment was placed in a 0.5 ml microcentrifuged tube. Twenty microliters of TE buffer was then added to each tube and incubated overnight at 37°C for extraction of cDNA fragment from the gel. The extracted cDNA fragments were further reamplified using polymerase chain reaction.

2.11 Reamplification of the cDNA bands

The eluted cDNAs were reamplified in a total volume of 50 µl. Each amplification reaction contained 5 µl of eluted DD-PCR product, 1.25 µl of 20 µM of the corresponding P and T primers used in DD-PCR reaction, 5 µl of 10X cDNA PCR reaction buffer, 1.5 µl of 10 mM dNTP mix (10 mM each dNTP), 1 µl of 50X Advantage cDNA polymerase mix (Clontech) and 35 µl of sterile water.

The thermal cycling was performed on Mastercycler® PCR thermal cycler (Eppendorf, Germany). DNA was denatured at 94°C for 5 minutes. Forty five PCR cycles were performed using 94°C for 30 seconds to denature, 60°C for 30 seconds to anneal primers, and 72°C for 2 minutes to synthesize DNA and a final extension step of the product at 72°C for 7 minutes.

2.12 Elution of DNA from agarose gel

Amplified cDNA was electrophoresed on 1.5% agarose gel in 1x TBE buffer. The gel was stained with ethidium bromide and determined the size of product by visualization under UV light. The expected product was purified using HiYield™ Gel/PCR Mini Kit (RBC). The expected products were excised from the gels using a

clean sharp scalpel and then determined the weight of the gel slice. Five hundred microliter of DF buffer was added to 300 mg of gel weight and incubated at 55°C for 10 to15 minutes or until the gel slice was completely dissolved. During incubation, the tube was inverted every 2 to 3 minutes. To bind DNA, the DF column was placed in a collection tube and applied 800 µl of the sample mixture from previous step into the DF column. After that, DF column was centrifuged at 8,000 rpm for 30 seconds. The flow-through was discarded and placed the DF column back in the collection tube. After binding step, 500 µl of wash buffer was added into the DF column and centrifuged at 8,000 rpm for 30 seconds. The flow-through was discarded and placed the DF column back in the collection tube. DF column was centrifuged again at 14,000 rpm for 2 minutes to completely dry the column matrix. Dried column was transferred in a new microcentrifuge tube and added 15 µl of elution buffer or sterile water in the center of the column matrix. The column matrix was left for 2 minutes until elution buffer or sterile water completely absorbed by the matrix. Finally the tube was centrifuged at 14,000 rpm for 2 minutes to elute purified DNA.

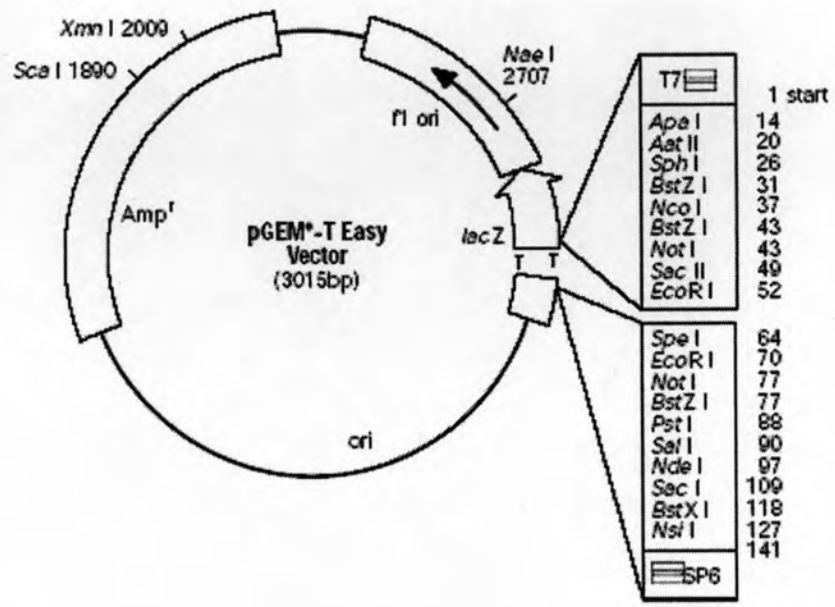
2.13 Ligation of PCR product to vector

The purified differential display cDNA fragments were cloned into pGEM-T easy vector (Promega). The map of pGEM-T easy vector was shown in Figure 2.2. The ligation reaction was set up in the total volume of 10 µl. The reaction component contained 5 µl of 2x Rapid ligation buffer (60 mM Tris-HCl pH 7.8, 2.0 mM DTT, 2 mM ATP and 10% PEG 8000), 1 µl of pGEM-T easy vector (50 ng), proper amount of PCR product to achieve 1:3 insert : vector molar ratio, 1 µl of T4 DNA ligase (3 Weiss unit/µl) and sterile deionized water to a final volumn of 10 µl.

The ligation mixture was carefully mixed by pipetting and incubated overnight at 4°C. To calculate the appropriate amount of insert to include in ligation reaction, use the following equation:

$$\frac{\text{ng of vector X kb size of insert X insert}}{\text{kb size of vector}} : \text{vector molar ratio} = \text{ng of insert}$$

a)



b)

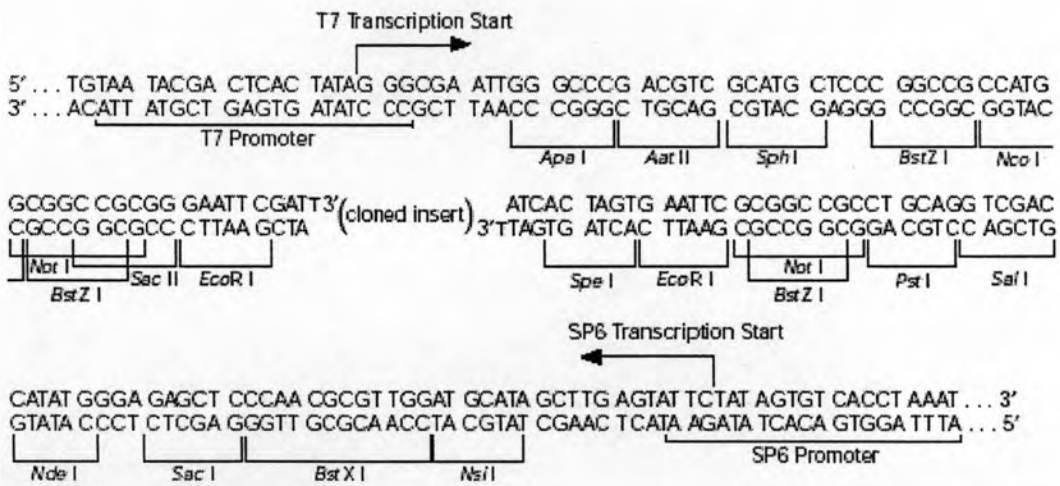


Figure 2.2 The circular map of the pGEM®-T Easy vector showing sequence reference points (a) and the linear map showing promotor and multiple cloning sites (b)

2.14 Transformation of the ligation product to *E.coli* host cells

The portion of the ligation mixture was transformed into host cell *E.coli* strain XL1 blue by electroporation procedure (Dower et al., 1988)8). The procedure to prepare *E.coli* competent cell was described in Section 2.15. The apparatus was set as followed; 25 μF of the Gene pulser, 200 Ω of the Pulse controller unit and 2.50 kV of the Gene pulser apparatus (BIO – RAD). Forty microliters of the cell suspension were gently set on ice. Then, 2 μl of each ligation reaction was added and mixed well. The reaction was placed on ice for 1 minute. After that, the reaction mixture was transferred to a cold electroporation cuvette. After one pulse was applied at the above setting, one milliliter of SOC medium (2% (w/v) bacto tryptone, 0.5% (w/v) bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose) was added to the cuvette and quickly resuspended the cells with a pasture pipette. The cell suspension was transferred to a sterile test tube and incubated at 37°C with shaking at 250 rpm for 1 h. At the end of the incubation period, the cultured cell suspension was centrifuged at 12,000 rpm for 20 seconds at room temperature. The pellet was gently resuspended in 100 μl of SOC medium and spread on the LB agar plate, which contained 50 μg/ml amplicillin, 25 μg/ml of IPTG and 20 μg/ml of X-gal. After that, it was incubated at 37°C overnight. As a result, the recombinant clones, containing inserted DNA, were white, wherease those without inserted DNA were blue.

2.15 Preparation of competent cell

A colony of *E.coli* strain XL 1 blue was cultured and then it was used as the starter inoculums in 15 ml of LB broth medium (1% (w/v) bacto tryptone, 0.5% (w/v) bacto yeast extract and 1% (w/v) NaCl) at 37°C with shaking at 250 rpm overnight. Two and a half milliliters of the microbial starter was added in 250 ml of LB broth medium. The culture was incubated at 37°C with vigorous shaking for 3 to 4 h until the optical density at 600 nm (OD_{600}) of the cell reached 0.5 to 0.8. The cells were

then chilled on ice for 15 to 30 minutes and harvested by centrifugation at 5,000 rpm for 15 minutes at 4°C. The supernatant was removed as much as possible. The cell pellet was washed by resuspending in a total of 250 ml of cold sterile water, gently mixing and then it was centrifuged again. The pellet was further washed with 125 ml of cold sterile water and followed by 5 ml of ice cold sterile 10% (v/v) glycerol. Finally, the cells were resuspended to a final volume of 500 to 750 µl ice cold sterile 10% (v/v) glycerol. This cell suspension was aliquoted (40 µl) into 1.5 ml microcentrifuge tubes and stored at – 80 °C until required. The cells are good for at least 6 months under these conditions.

2.16 Detection of recombinant clone by colony PCR

Three to six of recombinant clones were randomly selected for each cDNA fragment and screened by colony PCR. The pGEM-T easy vector was 3,015 bp in length. This plasmid had unique restriction sites in the multiple cloning region flanked by T7 and SP6 RNA promoters, therefore T7 and SP6 primers can be used to analyze insert size. These primer sets anneal at T7 and SP6 RNA promoter and amplify the insert cDNA. Colony PCR was performed in a 25 µl reaction volume containing 2.5 µl of 2.5 mM of dNTP mix, 0.5 µl of each 50 µM T7 and SP6 primers, 2 units of DyNAzyme™ II DNA polymerase (Finnzymes, Finland) . For the DNA template, the single colony was picked by using a sterile toothpick and resuspended in the amplification reaction. The PCR profiles were one cycle at 96°C for 2 minutes and followed by 30 cycles of 96°C for 30 seconds, 50°C for 30 seconds and 72°C for 1 minute. The final extension was carried out at 72°C for 5 minutes. The PCR products were analyzed by 1.5% agarose gel electrophoresis. The clone containing an expected size of insert was selected and the recombinant plasmid was isolated.

2.17 Plasmid extraction

Plasmid extraction was performed according to the protocol provided with a QIAprep spin miniprep kit (QIAGEN). The colonies were inoculated into 1.5 to 2 ml of LB-broth medium (one colony per tube) and incubated at 37°C with constant

shaking at 250 rpm overnight. Then, the cell culture was transferred into 1.5 ml microcentrifuge tube and centrifuged at 12,000 rpm for 1 minute. After that, supernatant was removed by decanting or pipetting. The cell pellets were resuspended in 250 µl of the P1 buffer. A 250 µl of the P2 buffer was added and mixed gently by inverting the tube 4 to 6 times. After that, 350 µl of the N3 buffer was added and inverted the tube immediately after addition of N3 buffer but gently 4 to 6 times. The mixture was centrifuged at 12,000 rpm for 10 minutes. The supernatant was carefully transferred to the QIAprep column by decanting or pipetting. The QIAprep column was centrifuged at 12,000 rpm for 1 minute. Then, the flow-through solution was discarded. To wash the QIAprep column, 500 µl of the PB buffer was added and centrifuged at 12,000 rpm for 1 minute. The flow-through solution was discarded. A 750 µl of the PE buffer was added and centrifuged at 12,000 rpm for 1 minute. After the flow-through solution was discarded, the QIAprep column was centrifuged at 12,000 rpm for an additional 1 minute to remove residual wash buffer. Then, the QIAprep column was placed in a clean 1.5 ml microcentrifuge tube. To elute plasmid DNA, 50 µl of EB buffer (10 mM Tris-Cl, pH 8.5) was added to the center of each QIAprep column. The QIAprep column was left for at least 1 minute in room temperature before centrifuged at 12,000 rpm for 1 minute. The concentration of extracted plasmid DNA was calculated using spectrophotometer.

2.18 DNA sequencing and data analysis

The recombinant plasmids of each differentially expressed cDNA clones were identified by sequencing. DNA sequencing was carried out using Macrogen Sequencing System, Macrogen Inc. (Korea).

After cluster analysis of sequenced clones, all unique sequences were blasted with the GenBank database (the National Center for Biotechnology Information; NCBI) using BLASTN and BLASTX programs (NCBI Advanced Blast Search, http://www.ncbi.nlm.nih.gov) (Altschul et al., 1997). The identification of homologue sequence was based on the smallest probability (E) < 10^{-4}. Identified sequences generally shared a high sequence identity > 65% over a relatively long range > 150 bp

with the most similar sequence for BLASTN (Zeng and Gong, 2002) and a minimum amino acid sequence identity of 35% for BLASTX (Anderson and Brass, 1998).

Significant probabilities between cDNAs sequences and GenBank database were considered when E-values was less than 10^{-4}.

2.19 Expression confirmation of cDNA fragments of interest by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

Sequences of interested cDNA fragments from DD-PCR analysis were used to design specific primers for-PCR analysis using β-actin as the internal standard. The first stranded cDNA of the confirmation experiment was synthesized from total RNA of different group of shrimp that used in DD-PCR experiment. RT PCR experiments were replicated for at least thrice with the same template to confirm the intensity of bands and then run on the same 1.5% agarose gel and stained by ethidium bromide for quantitation.

2.19.1 RT-PCR amplification

PCR primers were designed from nucleotide sequencing using the SECentral program. Each pair of forward and reverse primers had closely similar Tm values minimal self-priming and upper/lower dimer formation. A housekeeping gene, β-actin gene in present experiment was used as an internal control.

Amplification reactions were performed in a 25 µl total volume containing 5 µl of appropriate diluted cDNA sample, 2.5 µl of 10X buffer, 2.1 µl of 1.2 mM dNTP mix, 0.15 µl of 5 units *Taq* DNA polymerase (Fermentus), optimum concentration of F, R primers and $MgCl_2$. Sterile water was used to adjust total volume. The optimum concentration of F, R primers and $MgCl_2$ were determined to avoid non specific products. The PCR amplification was carried out in a Mastercycler® PCR thermal cycler (Eppendorf, Germany).

Cycle profile was a denaturation step of 94°C for 2 minutes, followed by varying numbers of cycles of 94°C for 30 seconds, optimum annealing temperature for 30 seconds, 72°C for 1 minute and a final extension of the product at 72°C for 5 minutes. The optimum annealing temperatures were determined for each primer pairs to avoid non-specific amplification. The reactions were performed in triplicated.

2.19.2 Gel electrophoresis and quantitative analysis

PCR products were determined by electrophoresis on 1.5% agarose gels in TBE buffer (89 mM Tris-base pH 7.6, 89 mM boric acid, 2 mM EDTA). The slurry of agarose in TBE buffer was melted in microwave oven until completely dissolved. The solution was allowed to cool at 55 to 60 °C before pouring into a casting tray with a well comb. After hardening, the gel was submerged in a chamber containing an enough amount of 1x TBE buffer covering the gel for approximately 0.5 centimeters.

Ten microliters of PCR product were combined with 1 µl of 10x loading dye (0.25% bromphenol blue, 0.25% xylene cyanol FF, 15% ficoll) and loaded to the agarose gel. A DNA ladder (100 bp marker) was used as a standard DNA marker.

After electrophoresis at 100 volts, the gel was stained with ethidium bromide (10 µg/ml) for 30 seconds and destained in sterile water for 30 minutes to remove excess ethidium bromide. The gel was photographed on top of a UV light box. The intensity of the amplified bands was measured by using Gel Documention System (GeneCam FLEX1, SynGene). Prior to the statistical analysis, the intensity of bands generated by primers of interesting cDNA fragments was normalized by that of β-actin using the Gene Tools analysis program.

Significantly different expression levels were treated using One Way Analysis of Variance (ANOVA) following by a post hoc test (Duncan's new multiple range test). Significant differences were indicated at $p < 0.05$.

2.20 Identification of domain proteins from unknown gene sequences

Differentially expressed cDNA fragments of unknown genes which regulated by salinity from RT-PCR analysis were performed domain prediction using SMART program (Ponting et al., 1999; Schultz et al., 1998; Schultz et al., 2000). Knowing the domain composition of a protein is essential for a detailed understanding of its function. SMART (http://smart.embl-heidelberg.de) is a web-based resource used for the annotation of protein domains and the analysis of domain architecture. Over 600 domain families that are widely represented among nuclear, signaling and extracellular proteins are included in the SMART database. The first step of domain analysis, sequences of cDNA fragments were translated to six frames of amino acid sequences using GENETYX program (Software Development, Tokyo, Japan). The amino acid sequences of each frame of cDNA fragment were performed for domain prediction. In addition, possible to be transmembrane segments were also determined by one rather popular method for detecting hydrophobic regions, J. Kyte and R. F. Doolittle program (1982).

The Kyte and Doolittle hydrophobicity/hydrophilicity plot analyses were performed on http://gcat.davidson.edu/DGPB/kd/kyte-doolittle.htm. Kyte-Doolittle hydropathy plots give you information about the possible structure of a protein. A hydropathy plot can indicate potential transmembrane or surface regions in proteins. By varying the parameters of Kyte-Doolittle tests done on proteins whose structure was known, Kyte and Doolittle (1982) found the parameters that predicted protein structure the best. Window size refers to the number of amino acids examined at a time to determine a point of hydrophobic character. Window size can be varied from 5 to 25. When looking for a transmembrane region in a protein, a window size of 19 is needed. Transmembrane regions are identified by peaks with scores greater than 1.8 using a window size of 19 (Kyte and Doolittle, 1982).