CHAPTER IV
METHODS

4.1 Total RNA extraction

Total RNA was extracted from approximately 100 mg of tissues (brain, eyestalks, hepatopancreas, nerve cords, ovary, and thoracic ganglia) from wild female *P. monodon* broodstock by Tri reagent®. First, tissues were homogenized in 1 ml of Tri reagent® and incubated at room temperature for 5 min. Then, 200 µl of chloroform were added and mixed by vigorously shaking for 15 sec, followed by incubating at room temperature for 15 min. The mixture was centrifuged at 12,000 g for 15 min at 4˚C. An upper aqueous phase was transferred to a new tube, and 0.5 volume of isopropanol was added and incubated at room temperature for 15 min to precipitate the RNA followed by centrifugation at 12,000 g at 4˚C for 10 min. RNA pellet was washed with 1 ml of 75% ethanol and centrifuged at 7,500 g at 4˚C for 5 min. Finally, RNA pellet was air dried at room temperature and dissolved in DEPC-water. Total RNA was heated at 55˚C for 2 min and measured RNA concentration by NanoDrop™ 1000 Spectrophotometer.

4.2 RNA electrophoresis

Both the quantity and quality of the RNA was determined by agarorose gel electrophoresis. One gram of agarose powder was mixed with 100 ml of 1X TAE buffer (40 mM Tris-HCl, acetic acid, 2.5 mM EDTA, pH 8.0) and boiled until the agarose powder was completely dissolved. Then, the gel was poured into a tray and the comb was inserted, and then left at room temperature for 30 min. To prepare the sample for gel loading, 1µl of total RNA was mixed with 2 µl of RNA loading dye (50% glycerol, 0.4% bromophenol blue, 1 mM EDTA, 1mg/ml ethidium bromide) and 9 µl of RNA sample buffer (10 ml of formamide, 3.5 ml of 37% formaldehyde and 2

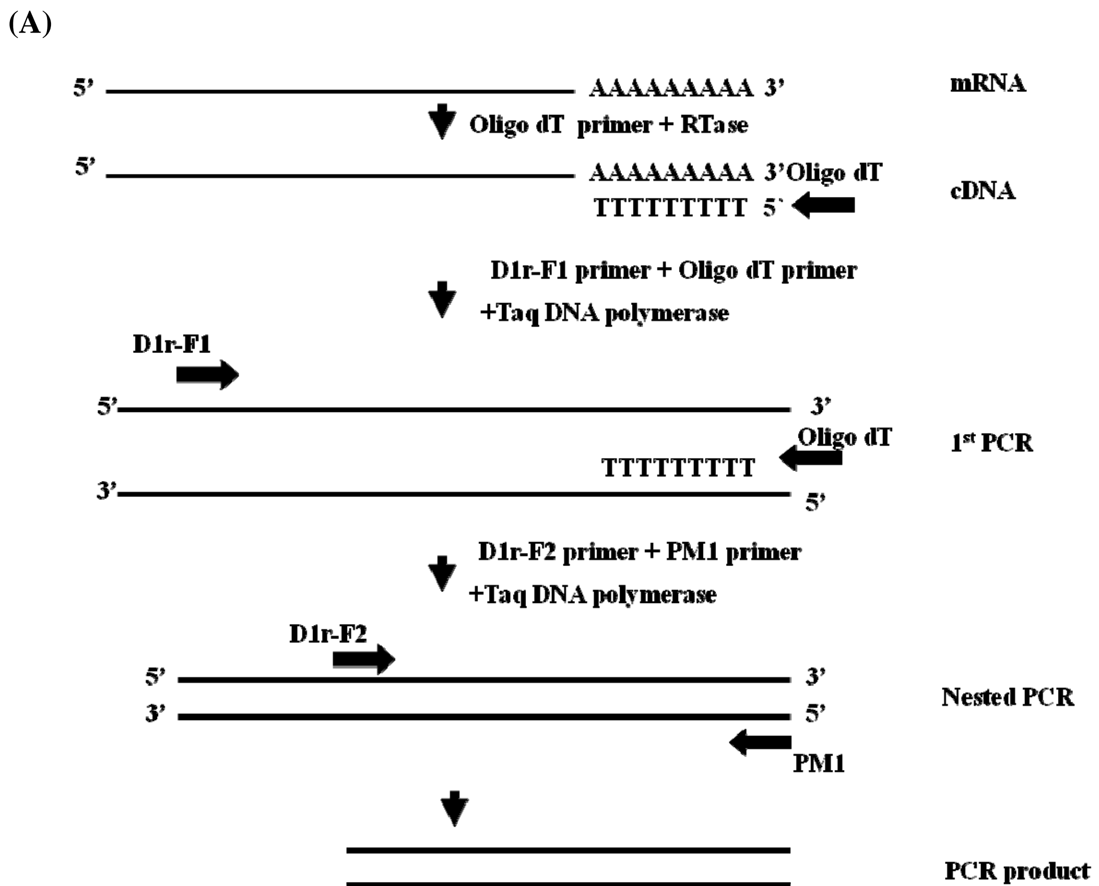
ml of 10X MOPS). The mixture was heated at 65˚C for 10 min and then incubated on ice for 10 min. The RNA samples were loaded on 1% agarose gel in 1X TAE buffer at electrical constant voltage at 90 volts for 1 hr. After gel electrophoresis, the RNA was visualized under UV light (BioDoc-It^{TM} System).

4.3 Rapid Amplification of cDNA ends (RACE)

4.3.1 Amplification of 3'end of cDNA by 3'RACE

Approximately 1 µg of total RNA from brain tissues of wild female broodstock *P. monodon* at ovarian stage I was used for first stranded cDNA synthesis by Improm II Reverse transcriptase. The RNA sample was mixed with 10 pmole of oligo dT primer (table 3.2) and the volume was adjusted to 5 µl with DEPC-water. The mixture was heated at 70˚C for 5 min, followed by incubated on ice for 5 min. Then, the components containing 4 µl of 5X ImpromII™ reaction buffer, 2.4 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTPs, 1 µl of ImpromII Reverse transcriptase, adjusted with DEPC-water into 15 µl final volume were added to the RNA mixture reaction. The reaction was performed in a PCR machine (MJ Mini^{Tm}, BIO-RAD) with the following temperature profile; 25˚C for 5 min, 42˚C for 60 min and 70˚C for 15 min. After cDNA synthesis, the first strand cDNA was used as a template for PCR with D1r-F1 (table 3.2) and oligo dT primers in a 25 µl PCR reaction containing 2.5 µl of 10X taq DNA polymerase buffer (750 mM Tris HCl pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20) supplement with $(NH_4)_2SO_4$. 1.5 µl of 25 mM $MgCl_2$, 0.5 mM dNTPs, 1.25 µM of each primer, 1 µl of cDNA template and 0.2 µl of taq DNA polymerase (5U/µl). The reaction mixture was subjected to the following cycles; 94°C for 3 min followed by 30 cycles of denaturation at 94°C for 30 sec, decrease annealing temperature at 1˚C per cycle from 62˚C-55˚C for 30 sec per cycle and then constant at 58 °C for 25 cycles, and extension at 72°C for 1 min 30 sec. The first PCR product was used in nested amplification with D1r-F2 (table 3.3) and PM1 (table 3.2) primers that have the same sequence as the linker of the Oligo-dT primer. The PCR condition of nested amplification was the same as the first amplification. A diagram of 3'RACE method and PCR profiles were shown in figure 4.1.

(A)



(B)

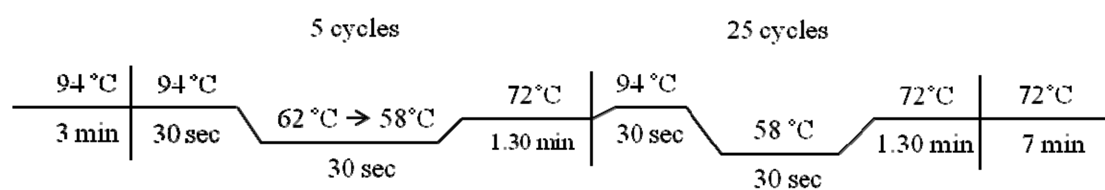


Figure 4.1 Amplification of the 3'end of putative DAR cDNA (A) A schematic diagram of 3' end amplification of DAR cDNA by 3'RACE. D1r-F1 and oligo-dT primers were used in the first amplification and PM1 primer containing the linker sequence of Oligo-dT and D1r-F2 were used in the nested amplification. (B) PCR profile of 3'RACE of DAR cDNA. First and nested amplifications were performed in the same condition.

4.3.2 Amplification of 5’end of cDNA by 5’RACE

Approximately 1 μg of total RNA from thoracic ganglia tissues of wild female brood stock shrimp at ovarian stage I was used for 5’RACE. The specific primer D1r-R4 was used for cDNA synthesis following the same protocol as that in 3’RACE. Subsequently, the first strand cDNA was tailed at the 3’end with 0.6 μl of 10 mM dATP by terminal deoxynucleotidyl transferase in a reaction containing 2.5 μl of 10X buffer, 1.5 μl of 25 mM $MgCl_2$, 0.2 μl of taq DNA polymerase and then final volume was adjusted to 25 μl with distilled water. Then, the tailed cDNA was amplified with D1r-R4 and oligo dT primers followed by nested amplification with D1r-R3 and PM1 primers with taq DNA polymerase to obtain specific product using the same condition as described in 3’RACE. A diagram of 5’RACE method was shown in figure 4.2.

4.3.3 Amplification of the coding sequence of *P. monodon*’s dopamine receptor (DAR_Pem) cDNA

To amplify the coding sequence of DAR_Pem cDNA, two gene specific primers, EX-DP-F and EX-DP-R were designed from the nucleotide of the sequences 3’ and 5’ cDNA fragments. Total RNA from previous 5’RACE was synthesized the first strand cDNA with oligo dT primer. One microliter of cDNA was amplified with EX-DP- and EX-DP-R primers in a 25 μl reaction containing 2.5 μl of 10X *Pfu* DNA polymerase buffer (200 mM Tris HCl pH 8.8, 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$, 1% Triton X-100 and 1 mg/ml nuclease-free BSA), 0.5 mM dNTP, 0.25 μM of primers each, and 0.25 μl of *Pfu* DNA polymerase (3U/μl) with the following temperature profile; denaturation at 95˚C for 30 sec, annealing at 62˚C for 30 sec and extension at 74˚C for 3 min for 35 cycles.

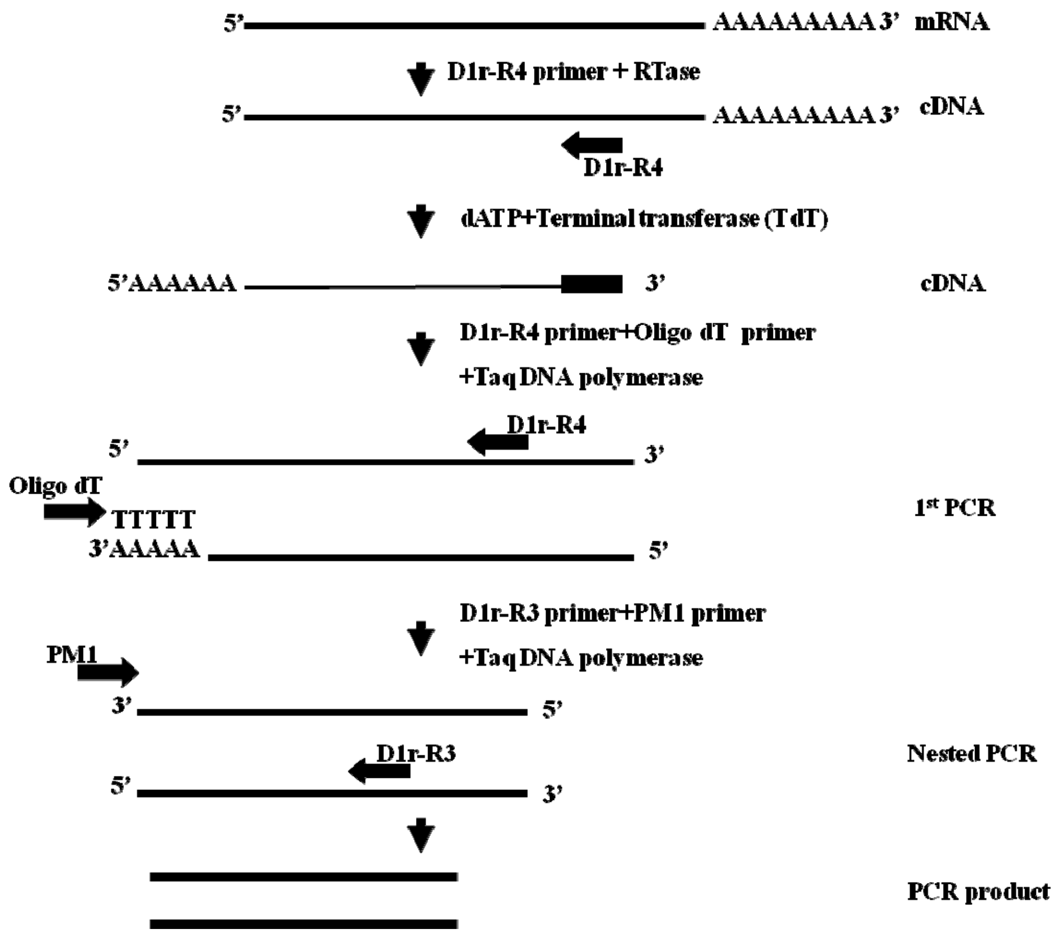


Figure 4.2 A schematic diagram of 5' end amplification of putative DAR cDNA by 5'RACE. D1r-R4 and oligo-dT primers were used for first amplification and D1r-R3 and PM1 were used for nested amplification.

4.4 Cloning of cDNA fragments

4.4.1 Purification of PCR product fragments and plasmid DNA by QIAquick Gel Extraction Kit (QIAGEN)

PCR products or plasmid DNA were run on 1% agarose gel in 1X TAE buffer [40 mM Tris-HCl, 40 mM acetic acid, 10 mM EDTA, pH 8.0] with constant voltage at 90 volts until the dye migrated to 2/3 of the length of agarose gel. DNA fragments in the gel were stained in ethydium bromide for about 2 min followed by destaining with distilled water for 10 min. The gel was exposed to UV light and the DNA fragments were sliced out of the gel with razor blade and transferred to 1.5 ml tube. The gel slices were weighed and melted at 55˚C for 10 min or until completely dissolved in three volumes of buffer QG assuming that 100 mg of the gel is equal to 100 µl. After that the mixture was transferred into a QIAquick column inserted in collecting tube and centrifuged at 13,000 rpm for 1 min. The DNA fragments were washed with 500 µl of PE buffer, centrifuged at 13,000 rpm for 1 min, and then the column was inserted into a new 1.5 ml tube. Finally, DNA fragments were eluted from the column with 30 µl of EB buffer, and the concentration of the DNA was estimated by agarose gel electrophoresis.

4.4.2 Agarose gel electrophoresis

To estimate the concentration and the size of the DNA, DNA samples were subjected to agarose gel electrophoresis that separate DNA fragments depending on their sizes. One gram of agarose powder was mixed in 1X TBE (89 mM Tris-HCl, 89 mM Boric acid, 2.5 mM EDTA, pH 8.0) and melted in a microwave oven about 2-3 min or until completely dissolved. The gel was poured into a tray with a comb inserted and left the gel at room temperature for 30 min for setting. Next, nucleic acid samples were mixed with loading dye [25% (v/v) Glycerol, 60 mM EDTA, 0.25% (w/v) Bromophenol Blue] and loaded into the wells of the agarose gel. Nucleic acid samples were run with constant voltage at 100 volts. The λ/*Hin*d III DNA or 100 bp DNA ladder were used as a marker for estimation of the size and concentration of the DNA samples. After agarose gel electrophoresis, nucleic acid samples were stained with ethydium bromide for about 2 min and followed by destaining in distilled water for 15 min. Nucleic acid samples were visualized under UV light (BioDoc-It^{TM} System)

4.4.3 DNA Ligation

After purification, DNA fragment was ligated to a plasmid vector (pGEM®-T easy, pET-17b or pcDNA3.1). The molecular ratio of the ligation was optimized at 3:1 molar ratio of insert to vector. The insert : vector ratio was calculated as follows;

$$\text{Amount of insert (ng)} = \frac{\text{Amount of vector (ng)} \times \text{size of insert (kb)} \times 3}{\text{Size of vector (kb)}}$$

Fifty nanogram of vector were used for ligation and the amount of insert fragments were calculated from the above formula. Components of the ligation reaction contained 2 µl of 5X T4 ligase buffer, 1 µl of 10 mM ATP, 1 µl of T4 DNA ligase (3U/µl), vector, insert fragment and the volume was adjusted to 10 µl with distilled water. Ligation reactions were incubated at 16˚C overnight.

4.4.4 Competent *Escherichia coli* cells preparation

E. coli strain DH5α was streaked on LB agar plate at 37˚C overnight. About 8-10 colonies were cultured in 5 ml of SOB broth at 18˚C with shaking 250 rpm overnight. The culture was used as a starter for culturing in 250 ml of SOB broth at 18˚C with 250 rpm shaking until OD_{600} reached 0.4-0.6. The culture was transferred to 50 ml tubes and incubated on ice for 10 min. Cells were centrifuged at 3,000 rpm for 10 min at 4˚C, then the pellet was resuspended in 10 ml of ice-cold TB buffer (10 mM PIPES, 55 mM $MnCl_2$, 15 mM $CaCl_2$, and 250 mM KCl) per 50 ml culture and incubated on ice for 10 min. Cells were collected by centrifugation at 3,000 rpm for 10 min at 4˚C and gently resuspended in 5 ml of TB buffer per 50 ml culture. Then, the cell mixture from each tube was combined and gently swirled in a new 50 ml tube followed by the addition of 7% DMSO. Competent cells were left on ice for 10 min and a 200 µl aliquot of the cells suspension was transferred to a 1.5 ml tube and stored at -80 ˚C.

4.4.5 Recombinant plasmid DNA transformation by heat-shock

Ten microliter of the ligation mixture was added into 200 µl of competent *E. coli* cells and incubated on ice for 30 min. Then, the cell mixture was incubated at 42˚C for 90 sec and immediately cooled on ice for 2 min. Heat-shocked cells were mixed with 800 µl of LB broth and incubated at 37˚C for 1 hr with shaking at 250 rpm to allow plasmid DNA replication. Then, the heat-shocked cells was collected by centrifugation at 3,000 rpm for 8 min and the cell pellets were resuspended with LB medium and spreaded on LB agar containing appropriate antibiotic for recombinant plasmid DNA selection.

4.5 Verification of recombinant plasmid DNA

4.5.1 Recombinant plasmid DNA determination by colony PCR

PCR technique was used to screen recombinant plasmids by using specific primers for amplification. A single colony of each recombinant clones was picked up with a toothpick and spotted on LB agar plate containing appropriate antibiotic, then incubated at 37˚C overnight. A single colony was lysed in 10 µl of 10X taq DNA polymerase buffer and centrifuged at 13,000 rpm for 1 min. Then, 1.25 µl of the buffer containing the lysed cells were used as a template for PCR reaction containing 0.75 µl of 25 mM $MgCl_2$, 0.25 mM dNTPs, 0.25 µM of each primer, and 0.1 µl of taq DNA polymerase in a final volume of 12.5 µl. The profile of PCR condition was set up according to the Tm of specific primers of each experiment.

4.5.2 Recombinant plasmid DNA determination by size screening

A single colony from a master plate was picked up with toothpick and the cells were lysed in 30 µl of lysis buffer [5 mM EDTA, 100 mM NaOH, 60 mM KCl, 10% (w/v) sucrose, 0.25% (w/v) SDS]. The lysed cell was incubated at 37˚C for 5 min and cooled at 4˚C for 5 min. Before loading, the lysed cell was centrifuged at 13,000 rpm for 1 min to remove cell debris. About 15 µl of the supernatant were loaded on 1% agarose gel and run at 120 volts for 45 min. After gel electrophoresis, the DNA was detected by ethidium bromide staining and visualized under UV light as

described earlier. The size of recombinant plasmids were compared with the size of plasmid vector alone.

4.5.3 Recombinant plasmid DNA determination by restriction enzyme digestion

4.5.3.1 Plasmid DNA extraction by CTAB mini-preparation

A Single colony of bacteria was cultured in 3 ml of LB broth containing appropriate antibiotic and grown at 37˚C for 12-16 hr or overnight. The cell culture was transferred to 1.5 ml tube and centrifuged at 13,000 rpm for 1 min. The cell pellet was completely resuspended in 200 µl of STET buffer [0.1% (v/v) Triton X-100, 8% (w/v) sucrose, 50 mM EDTA, 50 mM Tris-HCl (pH 8.0)] containing lysozyme (final concentration at 250 mg/ml) and incubated at room temperature for 10 min. After that, the mixture was boiled for 45 sec and immediately centrifuged for 15 min at room temperature. Cell debris was removed from the supernatant by a toothpick and 1/10 volume of 5% CTAB was added, mixed and centrifuged at 13,000 rpm for 5 min. The supernatant was discarded before the pellet was resuspended in 300 µl of 1.2 M NaCl and mixed by vortexing. Then, 5 µl of 50 mg/ml of RNase A was added to the mixture and incubated at 37˚C for 15 min. Next, equal volume of chloroform was added, thoroughly mix and centrifuged at 13,000 rpm for 5 min. An upper phase was transferred to a new 1.5 ml tube and the DNA was precipitated with 2 volumes of absolute ethanol, mixed and centrifuged at 13,000 rpm for 10 min at room temperature. The DNA pellet was washed with 70% ethanol, air dried and dissolved with appropriate volume of distilled water.

4.5.3.2 Restriction enzyme digestion of plasmid DNA

Recombinant plasmids harboring interested fragment were verified by restriction enzyme digestion. After plasmid DNA extraction, about 100-200 ng of plasmid DNA was added into the digestion reaction composing of 1 µl of 1x reaction buffer, 1 µl of 10 U of restriction enzyme and the volume was adjusted to 10 µl. The digestion reaction was incubated at 37˚C for 2 hr then the digested recombinant plasmid DNA was analyzed by gel electrophoresis.

4.5.4 Determination of recombinant plasmid by sequencing

Two microgram of plasmid DNA (100 ng/µl) was sent for automated DNA sequencing at Macrogen Inc., Korea. The DNA sequences were searched against NCBI database using Blastn program sequence homology. The sequences were edited by Chromas® program and were compared with homologous genes of other species by Vector NTI, GenDoc and Clustal W programs.

4.5.5 Analysis of DAR structure by computer programs

The full-length of DAR cDNA was determined for translational start site by ATGpr program available at http://atgpr.dbcls.jp/. The putative seven hydrophobic regions were predicted by TMpred program available at http://www.ch.embnet.org/ software/TMPRED_form.html. Phosphorylation sites were investigated in the coding DAR cDNA sequence by PhosphoBase program available at http://kinasephos.mbc. nctu.edu.tw/. The potential N-linked glycosylation sites were predicted by NetNGlyc program available at http://www.cbs.dtu.dk/.

4.6 Determination of DAR_Pem mRNA expression level in the black tiger shrimp

4.6.1 Shrimp sample preparation

Shrimp tissues (brain, thoracic ganglia, abdominal nerve cords, ovary and hepatopancrease) were dissected from wild female brood stock *P. monodon* that were in different ovarian stages (stage I-stage IV) by scissors and forceps. One hundred milligrams of tissues were sliced into small pieces and total RNA was extracted by Trizol reagent® as described earlier. The RNA was kept at -30˚C until used.

4.6.2 Determination of DAR_Pem mRNA expression by RT-PCR

One microgram of total RNA was used to synthesize first strand cDNA by Improm II Reverse transcriptase. The cDNA synthesis was primed with oligo dT as described in 4.3.1. After cDNA synthesis, 1 µl of cDNA was used to amplify the region from the second extracellular loop to the third intracellular loop of DAR_Pem

with the primer D1r-F1 and i3-DAR-R. The PCR reaction contained 2.5 µl of 10X taq DNA polymerase buffer (750 mM Tris HCl pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20) supplement with $(NH_4)_2SO_4$. 1.5 µl of 25 mM $MgCl_2$, 0.5 mM dNTPs, 0.25 µM of each primers, 1 µl of cDNA template and 0.2 µl of taq DNA polymerase in a final volume of 25 µl adjusted with milli Q water. The PCR reaction mixture was denatured at 94˚C for 30 sec, followed by annealing at 55˚C for 30 sec and extension at 72˚C for 30 sec for 30 cycles. Shrimp actin transcript was used as an internal control by amplification with Actin-F and Actin-R primers using the following temperature profile; denaturation at 94˚C for 30 sec, annealing at 55˚C for 30 sec and extension at 72˚C for 45 sec for 21 cycles.

4.6.3 DAR_Pem mRNA expression analysis

To analyze DAR_Pem mRNA expression level in each tissue at different ovarian stages, PCR products of DAR_Pem and actin amplification were run on 1.5% agarose gel. After gel electrophoresis, mRNA expression levels of DAR_Pem and actin were quantitated by scion image program. Relative mRNA expression of DAR_Pem to actin was calculated and compared between each different ovarian stage.

4.7 Expression of recombinant C-terminal tail of *P. monodon*'s dopamine receptor (C-tail DAR)

4.7.1 Construction of recombinant plasmid for expression of C-tail DAR

A DNA fragment encoding the C-tail DAR was amplified with specific primers (C-ter-DAR-F and C-ter-DAR-R) that link with *Nde* I and *Bam*H I restriction sites. The PCR reaction was performed in a 25 µl reaction containing 2.5 µl of 10X *Pfu* DNA polymerase buffer (200 mM Tris HCl pH 8.8, 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$, 1% Triton X-100 and 1 mg/ml nuclease-free BSA), 0.5 mM dNTP, 0.25 µM of each primer, 1 µl (100 ng) of plasmid DNA template harboring the coding region of DAR and 0.5 µl of taq DNA polymerase. The temperature profile of the PCR was composted of 30 cycles of denaturation at 95˚C for

30 sec, annealing at 60˚C for 30 sec and extension at 74˚C for 1 min. The PCR fragment was purified by QIAquick Gel Extraction Kit (Qiagen). After that, the fragment was cloned into pGEM-T easy vector, and subsequently released from the vector with *Nde* I and *Eco*R I digestion. The digested fragment was then cloned into pET-17b vector at the same restriction sites for protein expression. Finally, the recombinant plasmid was verified with restriction enzyme digestion and sequencing analysis.

4.7.2 Protein expression of C-tail DAR by *Escherichia coli* expression system

The recombinant plasmid containing C-tail DAR fragment in pET-17b vector was used to transformed into *E. coli* BL21(DE3)pLysS. After screening for recombinant clone, the *E. coli* BL21(DE3)pLysS containing C-tail DAR plasmid DNA was streaked on LB agar supplemented with antibiotics (100 µg/ml of ampicillin and 34 µg/ml of chloramphenicol). A single colony was cultured in 2 ml of LB broth containing the same antibiotics and grown at 37˚C with 250 rpm shaking for overnight. Culture cells were used as a starter at dilution 1:5 in 10 ml of culture medium. Cells were grown at 37˚C until OD_{600} reached 0.4-0.6 or about 2 hr. After that, the cells were induced with 0.4 mM IPTG at 30˚C for 4 hr with shaking at 250 rpm. Finally, the cells density by spectrophotometry and 1 OD_{600} cells were harvested by centrifugation at 13,000 rpm for 1 min. Protein expression from 0.1 OD_{600} was analyzed by SDS-PAGE.

4.7.3 Protein expression analysis by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

4.7.3.1 Separation of protein fractions

One OD_{600} of protein samples was separated into inclusion and soluble fractions by sonicator. The bacterial cells were first lysed with lysis buffer [25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 10% Triton X-100 (v/v)], mixed by vortexing and incubated on ice for 15 min. Then, the mixture was frozen in liquid nitrogen followed by thawing at 42˚C for 3 times and subsequently sonicated at amplitude 20% for 5 min for 3 times to disrupt the cell. Protein pellet or inclusion

proteins were separated from soluble proteins with centrifugation at 13,000 rpm for 10 min at room temperature.

4.7.3.2 Soluble fraction preparation

Proteins in the soluble fraction were precipitated with 100% TCA and incubated at 4˚C for 30 min or kept at -30˚C overnight. The proteins were centrifuged at 13,000 rpm for 10 min at room temperature. The supernatant was discarded and the pellet was washed with acetone followed by air dry. Finally, the pellet of the soluble proteins was dissolved with 0.1 N NaOH.

4.7.3.3 Preparation of protein sample for gel loading

Protein samples were prepared for analysis by SDS-PAGE as followed. 1 OD_{600} of total protein or crude cell, inclusion protein and soluble protein were mixed with 1X SDS sample buffer (25 mM Tris-HCl pH 6.8 (w/v), 1% glycerol (w/v), 1% (w/v) SDS, 0.1% (w/v) bromphenol blue containing 2.5% β-mercaptoethanol) and boiled for 5 min. Next, the protein samples were centrifuged at 13,000 rpm for 2 min and placed on ice until loaded on SDS-PAGE.

4.7.3.4 SDS-PAGE preparation

SDS-PAGE was performed according to the manufacture's Protocol for Mini Protein II electrophoresis (BIO-RAD). A component of SDS polyacrylamide gel was prepared as shown in table 4.1 (the percent of the gel depends on molecular weight of the protein samples to be analyzed). The C-tail DAR protein (approximately 15 kDa) was run on 15% gel whereas a 49 kDa DAR_Pem was run on 10% gel. To assemble the gel system, two glass plates at a thickness of 0.75 mm were attached together with a spacer. Separating gel was poured into the space between the two glass plates to the height about one inch from the top. Subsequently, stacking gel mixture was poured to fill up to the edge of the glass plates and the comb was inserted. After the gel polymerized, it was fixed in an electrophoresis chamber containing 1X Tris-glycine buffer [25 mM Tris-HCl pH 8.3, 192 mM glycine, 0.1% (w/v) SDS]. The protein samples were loaded on SDS-PAGE gel using bromphenol blue as a tracking dye and broad range SDS PAGE standard as a marker. The protein samples were run

in electrophoresis chamber with constant voltage at 100 volts until the dye run out of the gel. The protein bands were visualized with staining solution (0.1% Coomassie Brilliant Blue R250, 50% methanol, 10% glacial acetic acid) with shaking at room temperature overnight. The background was removed with destaining solution (10% methanol and 10% glacial acetic acid) with shaking at room temperature until the protein bands were clearly visible.

Table 4.1 SDS-PAGE components

Solution	10% Separating gel	15% Separating gel	5.2% Stacking gel
H_2O	1.65 ml	1.05 ml	1.8375 ml
40% Acrylamide[a]	1.2 ml	1.8 ml	0.325 ml
1.0 M Tris pH6.8	-	-	0.3125
1.5 M Tris pH8.8	1.875 ml	1.875 ml	-
10% SDS	50 µl	50 µl	25 µl
10% APS	25 µl	25 µl	12.5 µl
TEMED	3.75 µl	3.75 µl	2 µl

[a] Acrylamide : N,N'- methylene-bis-acrylamide 37.5:1 (w/w)

APS = Ammonium persulphate

TEMED = N,N,N,N'- tetramethyl-ethylenediamide

4.8 Polyclonal antibody production

4.8.1 Protein purification by Electro-Eluter (Model 422, BIO-RAD)

An inclusion protein was run on 15% SDS-PAGE gel at a constant 100 volts until the dye run out of the gel and stained by coomassie brilliant blue R-250. The protein band was sliced with a razor blade and loaded into a glass tube containing 1X protein elution buffer [25 mM Tris-HCl, 192 mM Glycine, 0.04% SDS (w/v)] in a buffer tank. The protein was eluted through the membrane at 10 mM/glass tube for 3-5 hr or completely eluted. Finally, the purified protein was collected in a 1.5 ml tube and stored at -30˚C until used for polyclonal antibody production.

4.8.2 Polyclonal antibody production in rabbit

The concentration of the purified protein was estimated by SDS-PAGE compared to the broad range SDS-PAGE standard marker. Approximately 4.5 mg of the purified protein were used to raise antibody in rabbit at Biomedical Technology Research Unit, Faculty of Associated Medical Sciences, Chiangmai University.

4.9 Sensitivity and specificity of polyclonal antibody

4.9.1 Dot blot analysis

Sensitivity and specificity of the polyclonal antibody was primarily determined by dot blot analysis. Various amounts of the purified recombinant C-tail DAR protein (1-200 ng), total protein from 0.025 OD_{600} of *E. coli* BL21(DE3)pLysS and *E. coli* BL21(DE3)pLysS carrying pET-17b vector were dotted onto PVDF membranes. The membrane was subsequently soaked with blocking solution [5% skim milk in 1X PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4)] for 2 hr at room temperature. Then, the membrane was incubated with different dilution of C-tail DAR antibody (anti C-tail DAR) for 1.5 hr following by washing in washing solution (PBS containing 0.2% Tween 20) 3 times, 10 min each. The membrane was then incubated with secondary antibody (HRP conjugated anti-rabbit IgG) at 1:20,000 dilution for 1.5 hr at room temperature and subsequently washed with washing solution 3 times, 10 min each. The signals were detected with Immobilon^{TM} Western Chemiluminescent HRP Substrate (Millipore) and exposed to X-ray film.

4.9.2 Western blot analysis

The specificity of the antibody was further determined by western blot analysis. After the protein were separated by SDS-PAGE, the gel was soaked in 1X transfer buffer for 10 min (48 mM Tris-HCl, 390 mM Glycine, 0.1% (w/v) SDS containing 20% (v/v) methanol. At the same time, filter paper and PVDF membrane were cut to the same size as the gel. The membrane was immersed in methanol followed by soaking in 1X transfer buffer for 10 min. Then, the gel, the PVDF membrane and the filter paper were assembled and placed in an electrophoresis

transfer tank containing 1X transfer buffer. The protein was transferred to membrane by wet blot at 4˚C for 12-14 hr with constant voltage at 50 volts. After protein was transferred, the membrane was washed with PBS for 10 min then immersed in blocking solution for 2 hr. The proteins into membrane were incubated with 1:15,000 dilution of anti C-tail DAR antibody in blocking solution for 2 hr and then washed with PBS for 3 times, 10 min each before incubated with secondary antibody (HRP conjugated anti-rabbit IgG) for 1.5 hr. After that the membrane was washed with PBS for 10 min, 3 times. Signals were detected with Immobilon^{TM} Western Chemiluminescent HRP Substrate (Millipore) and exposed to X-ray film.

4.10 Determination of DAR expression protein in black tiger shrimp

4.10.1 Total protein extraction

Approximately 100 mg of tissues dissected from broodstock *P. monodon* at developmental stage I (brain, thoracic ganglia, abdominal nerve cord, ovary and hepatopancreas) were washed twice in PBS before chopped into small pieces and homogenized in buffer M (100 mM NaCl, 20 mM Tris-Cl, 2 mM $MgCl_2$, 1 mM EDTA, 0.2% Triton X-100, 1 mM PMSF, pH 7.4). The cell debris was removed by centrifugation at 600 g for 3 min. The supernatant was kept as total proteins, and about 50 µg were used to analyze DAR_Pem expression by western blot.

4.10.2 Membrane protein extraction

About 100 mg of the tissues from adult female *P. monodon* were homogenized in buffer M as described above. The cell lysate was sonicated with sonicator for 15 min and centrifuged at 600 g for 3 min to remove the cell debris. Subsequently, other organelles such as mitochondria, lysozomes, peroxizomes were removed by further centrifugation at 6,000 g for 8 min. Finally, the supernatant was centrifuged at 40,000 g for 1 hr for membrane protein separation. The membrane protein pellet was dissolved in buffer M, and 50 µg of membrane protein were analyzed by western blot analysis.

4.10.3 Measurement of protein concentration by Bradford method

The concentration of protein samples was measured by Bio-Rad protein assay based on Bradford method. A bovine serum albumin (BSA) was used as a protein standard for setting up a calibration curve in the range of 0, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1 mg/ml. The protein samples were diluted in a diluent solution at 1:20 dilution and 20 µl of the diluted samples were mixed with 200 µl of protein assay dye reagent concentrate and then the mixture was added to a well of a microplate. Protein standard and samples were assayed in triplicate. Protein samples were measured in triplicate by ELISA microplate reader (spectra max 190) at absorbance at 595 nm. The concentration of protein was calculated from the standard curve.

4.11 Expression of DAR_Pem in COS-1 cells

4.11.1 Construction of DAR_Pem expression plasmid

A cDNA for a coding region of DAR_Pem was amplified with specific primers (DAR-F and DAR-R) that linked with *Eco*R I and *Hin*d III sites. The Kozak sequence (ACCATGG) was added the forward primer for protein expression in mammalian cell. The PCR reaction contained 2.5 µl of 10X *Pfu* DNA polymerase buffer, 0.5 mM dNTP, 0.25 µM of primers each, 1 µl (100 ng) of plasmid DNA template and 0.5 µl of taq DNA polymerase in a final volume of 25 µl adjusted with distilled water. The PCR profile was as follows; 95˚C for 30 sec, 60˚C for 30 sec and 74˚C for 2 min for 30 cycles. The DNA fragment was purified by QIAquick Gel Extraction Kit (Qiagen) and cloned into pcDNA 3.1 (+) vector at *Eco*R I and *Hin*d III sites. The recombinant plasmid was verified with restriction enzyme digestion and sequencing analysis.

4.11.2 COS-1 cell culture

COS-1 cells were cultured in completed DMEM medium consisting of DMEM medium supplemented with 10% heat inactivated fetal bovine serum (FBS), 5 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were maintained in 100 mm culture plate at 37˚C with 10% CO_2 in humidity condition and the cells were split twice a week.

4.11.3 Plasmid DNA purification by plasmid DNA extraction with mini prep QIAquick Kit (Qiagen)

A single colony of *E. coli* containing recombinant DAR_Pem expressing plasmid was grown in LB broth containing appropriate antibiotic for 14-16 hr. The cell culture was centrifuged at 13,000 rpm for 1 min, and the pellet was lysed with 250 µl of buffer P1 buffer, followed by 250 µl of buffer P2. The cell lysate was incubated for 5 min at room temperature. Then, 350 µl of buffer N3 was added to the mixture and centrifuged at 13,000 rpm for 10 min. The supernatant was transferred to a column and centrifuged at 13,000 rpm for 1 min. After that, 500 µl of buffer PB was added to the column and centrifuged at 13,000 rpm for 1 min. The column was then washed with 750 µl of PE and centrifuged at 13,000 rpm. The supernatant was completely discarded out of the column by repeating the centrifugation one more time. Finally, plasmid DNA was eluted with 30 µl of EB buffer.

4.11.4 Plasmid DNA transfection

One day before transfection, 2.3×10^5 cells of COS-1 were seeded in a 6-well plate at 37˚C with 10% CO_2 humidity until 90-95% confluent. Approximately 4 µg of plasmid DNA was diluted in 250 µl of Opti-MEM® I medium (medium free serum). At the same time, 10 µl of lipofectamine™ 2000 were also diluted in 250 µl of opti-MEM® I medium. Each mixture was mixed by pipette and incubated at room temperature for 5 min. The mixture was gently spun down in centrifugation and the mixture of plasmid DNA and lipofectamine 2000 were then combined together. The combined mixtures were gently mixed and incubated at room temperature for 20 min. Next, the combined mixture was dropped in each well plate (before adding, DMEM medium was aspirated out of the well), mixed well by rocking. After 6 hr, the medium were replaced with completed DMEM medium and grown until 48 hr at 37˚C in 10% CO_2 incubator.

4.12 Immunofluorescent

To determine protein expression and localization of DAR_Pem in COS-1 cells, immunofluorescent staining technique was employed. A cover slip was placed in

each well of the 6-well plate and 1x10^5 cells were grown on the cover slip until 70-80% confluent or about 20 hr. After that, cells were transfected with 4 µg of plasmid DNA and incubated at 37˚C, 10%CO_2 humidity for 22 hr. The medium was aspirated out, and the cells were washed with pre-warm PBS then fixed with 4% formaldehyde at room temperature for 10 min. Next, the cells were washed with PBS 3 times, 5 min each and incubated at room temperature for 10 min. Non-specific binding was blocked with blocking solution [3% (v/v) FBS in PBS] at room temperature for 1 hr, prior to incubation with anti C-tail DAR antibody (dilution 1:500 in blocking solution) at room temperature for 1 hr, then washed with PBS 3 times, 10 min each. The cells were subsequently incubated with secondary fluorescein isothiocyanate labeled anti-rabbit IgG (FITC) at dilution 1:100 in PBS for 1hr at room temperature in the dark. Finally, the cells were washed with PBS 3 times, 3 min each then the cover slip was put on a slide and mounted with glycerol before visualize under confocal laser scanning fluorescence microscopy (OLYMPUS FV1000).

4.13 cAMP assay

4.13.1 Activation of biogenic amine to DAR_Pem with biogenic amine

The cells that were grown in completed DMEM medium for 48 hr were washed twice with 1 ml of warm PBS and pre-incubated with 2.5 mM of 3-Isobutyl-1-methylxanthine solution at 37˚C for 10 min to prevent cAMP hydrolysis. After that, 2.5 µM forskolin solution were added for adenylyl cyclase activation followed by addition of 10^{-5} M monoamine neurotransmitter; dopamine (DA) or serotonin (5-HT), then incubated at 37˚C for 30 min. Subsequently, cells were washed with cold PBS 4 times before lysed with 300 µl of 1X cell lysis buffer (Cell Signaling Technology®) and kept on ice for 5-10 min. Finally, the supernatant was taken for cAMP level measurement by cyclic AMP XP™ assay kit.

4.13.2 Measurement of cAMP level

The cAMP XP™ Assay Kit is a competitive enzyme-linked immunoassay used to determine cAMP level in cells or tissues of interest. In this assay, cAMP samples and HRP-linked cAMP were competed to bind with an anti cAMP XP™

Rabbit mAb immobilized onto a 96-well plate assay. The cAMP standard was performed for setting up a calibration curve in the range of 3.75 nM, 7.5 nM, 15 nM, 30 nM, 60 nM, 120 nM and 240 nM. The diluent without cAMP was served as the 0 nM cAMP. The supernatant of samples from previous step was used for cAMP level measurement by using cAMP XP^{TM} Assay Kit. Fifty microliter of the sample or approximately five microgram of the sample was mixed with HRP-linked cAMP solution in 96 well cAMP assay plate at room temperature for 3 hr. Then, the mixture was discarded and washed with 200 µl of washing buffer for 4 times to remove both an excess cAMP samples and HRP-linked cAMP. Then, all liquid was discarded out of the wells and incubated with 100 µl of TMB substrate for 30 min at room temperature to develop color. The reaction was stopped with 100 µl of stop solution. cAMP levels were measured by absorbance at 450 nm by ELISA microplate reader (spectra max 190). The cAMP sample of interest was calculated from the standard curve. Because of the competition between cAMP samples and HRP-linked cAMP, the absorbance for this developed color is inversely proportion to the quantity of cAMP sample.