CHAPTER II

MATERIALS AND METHODS

2.1 Experimental animals

Wild female *P. monodon* broodstock collected from Satun (Andaman Sea, west) was used for RACE-PCR. In addition, broodstock-sized male and female *P. monodon* were purchased from Angsila, Chonburi (Gulf of Thailand, east). Juvenile *P. monodon* male and female (approximately 20 g body weight, 4-month-old) were purchased from local farms in Chachengsao, eastern Thailand. These samples were used for RT-PCR and 20-hydroxyecdysone injection.

For quantitative RT-PCR analysis, progesterone and serotonin injection, cultured juveniles (4 months old, $N = 6$) and domesticated broodstock of *P. monodon* (18 months old, $N = 6$) were collected from the Broodstock Management Center, Burapha University (Chanthaburi, Thailand). Female broodstock were wild-caught from the Andaman Sea and acclimated under the farm conditions for 2-3 days. The post-spawning group was immediately collected after shrimp were ovulated ($N = 6$). Ovaries were dissected out from each juvenile and intact domesticated and wild broodstock and weighed. For the eyestalk ablation group, shrimp were acclimated for 7 days prior to unilateral eyestalk ablation. Ovaries of eyestalk-ablated shrimp were collected at 2-7 days after ablation. The gonadosomatic index (GSI, ovarian weight/body weight x 100) of each shrimp was calculated. Ovarian developmental stages were classified by conventional histology (Qiu et al., 2005) and divided to previtellogenic (I, $N = 10$ and 4 for intact and eyestalk-ablated broodstock, respectively), vitellogenic (II, $N = 7$ and 6), early cortical rod (III, $N = 7$ and 9) and mature (IV, $N = 9$ and 11) stages, respectively.

For tissue distribution analysis, various tissues of a female juvenile and broodstock were collected, immediately placed in liquid N_2 and kept at -80°C until required. Hemolymph was collected using 10% sodium citrate as an anticoagulant and

centrifuged at 1000 *g* for 10 minutes. Hemocytes were then subjected to RNA extraction.

For *in situ* hybridization and immunochemistry, the latter portion was excised to small pieces and fixed with the 4% paraformaldehyde prepared in 0.1 M phosphate buffer pH 7.2, washed 3 times with 0.1 M phosphate buffer pH 7.2 and 3 times with 50% ethanol for ovaries fixed in 4% paraformaldehyde. Tissue was subsequently stored in 70% ethanol before subjected to the standard paraffin section. Ovarian developmental stages were classified by conventional histology slightly modified from Qiu *et al.* (2005)

2.2 Nucleic acid extraction

2.2.1 Genomic DNA extraction

Genomic DNA was extracted from a piece of pleopod of each shrimp using a phenol-chloroform-proteinase K method (Klinbunga et al., 1999). A piece of pleopod tissue was dissected out from a frozen pleopod and placed in a prechilled microcentrifuge tube containing 500 µl of the extraction buffer (100 mM Tris-HCl, 100 mM EDTA, 250 mM NaCl; pH 8.0) and briefly homogenized with a micropestle. SDS (10%) and RNase A (10 mg/ml) solutions were added to a final concentration of 1.0 % (w/v) and 100 µg/ml, respectively. The resulting mixture was then incubated at 37°C for 1 hour. At the end of the incubation period, a proteinase K solution (10 mg.ml) was added to the final concentration of 300 µg/ml and further incubated at 55°C for 3–4 hours. An equal volume of buffer-equilibrated phenol: chloroform: isoamylalcohol (25:24:1) was added and gently mix for 10 minutes. The solution was centrifuged at 10,000 rpm for 10 minutes at room temperature. The upper aqueous phase was transferred to a newly sterile microcentrifuge tube. This extraction process was then repeated once with phenol:chloroform:isoamylalcohol (25:24:1) and once with chloroform:isoamylalcohol (24:1). The aqueous phase was transferred into a sterile microcentrifuge. One-tenth volume of 3 M sodium acetate, pH 5.2 was added. DNA was precipitated by an addition of two volume of prechilled absolute ethanol and mixed thoroughly. The mixture was incubated at -80°C for 30 minutes. The precipitated DNA was recovered by centrifugation at 12,000 rpm for 10 minutes at room temperature and washed twice with 1 ml of 70% ethanol (5 minutes and 2 – 3

minutes, respectively). After centrifugation, the supernatant was removed. The DNA pellet was air-dried and resuspended in 50 – 80 µl of TE buffer (10 mM Tris-HCl, pH 8.0 and 0.1 mM EDTA). The DNA solution was incubated at 37°C for 1 – 2 hours and kept at 4 °C until further needed.

2.2.2 RNA extraction

Total RNA was extracted from ovaries (or other tissue) of *P. monodon* using TRI Reagent®. A piece of tissue was immediately placed in a mortar containing liquid nitrogen and ground to fine powder. The tissue powder was transferred to a microcentrifuge tube containing 500 µl of TRI Reagent (50-100 mg tissue per 1 ml) and homogenized. Additional 500 µl of TRI Reagent was then added. The homogenate and left for 5 minutes, before adding 0.2 ml of chloroform. The homogenate was vortexed for 15 seconds and left at room temperature for 2-15 minutes and centrifuged at 12000g for 15 minutes at 4 °C. The mixture was separated into the lower red, phenol-chloroform phase, the interphase, and the colorless upper aqueous phase. The aqueous phase (inclusively containing RNA) was transferred to a new 1.5 ml microcentrifuge tube. Total RNA was precipitated by an addition of 0.5 ml pf isopropanol and mixed thoroughly. The mixture were left at room temperature for 10-15 minute and centrifuged at 12000g for 10 minutes at 4-25 °C. The supernatant was removed. The RNA pellet was washed with 1 ml of 75 % ethanol centrifuged at 7500g for 5 minutes. Total RNA was dissolved in appropriate volume of DEPC-treated H_2O for immediately used. Alternatively, the total RNA pellet was kept under absolute ethanol in a -80 °C freezer for long storage.

2.2.3 Preparation of DNase I-free total RNA

Fifteen micrograms of total RNA were treated with DNase I (0.5 U/1 µg of RNA, Promega) at 37°C for 30 minutes. After the incubation, the sample was gently mixed with a sample volume of phenol:chloroform:isoamylalcohol (25:24:1) for 10 minutes. The mixture was centrifuged at 12,000 g for 10 minutes at 4°C, and the upper aqueous phase was collected. The extraction process was then repeated once with chloroform:isoamylalcohol (24:1) and one with chloroform. The final aqueous phase was mixed with one-tenth final sample volume of 3 M sodium acetate (pH 5.2). After that, RNA was precipitated by adding two point five volume of -20°C-cold

absolute ethanol. The mixture was incubated at -80°C for 30 minutes, and the precipitated RNA was recovered by centrifugation at 12,000 g for 10 minutes at room temperature. The RNA pellet was then washed twice with 1 ml of -20°C cold 75% ethanol. Alternatively, the RNA pellet was kept in absolute ethanol at -80°C until required.

2.3 Measuring concentrations of nucleic acids by spectrophotometry and electrophoresis

The concentration of extracted DNA or RNA was estimated by measuring the optical density at 260 nanometre (OD_{260}). An OD_{260} of 1.0 corresponds to a concentration of 50 μg/ml double stranded DNA, 40 μg/ml single stranded RNA and 33 μg/ml oligonucleotide (Sambrook et al., 2001). Therefore, the concentration of DNA/RNA samples (μg/ml) were estimated by multiplying an OD_{260} value with a dilution factor and 50, 40, 33 for DNA, RNA and oligonucleotides, respectively. The purity of DNA samples can be guided by a ratio of OD_{260} /OD_{280}. The ratio much lower than 1.8 indicated contamination of residual proteins or organic solvents whereas the ratio greater than this value indicate contamination of RNA in the DNA solution (Kirby, 1992).

The amount of high molecular weight DNA can be roughly estimated on the basis of the direct relationship between the amount of DNA and the level of fluorescence after ethidium bromide staining after agarose gel electrophoresis. Genomic DNA was run in a 0.8 - 1.0% agarose gel prepared in 1x TBE buffer (89 mM Tris-HCl, 89 mM boric acid and 2.0 mM EDTA, pH 8.3) at 4 V/cm. After electrophoresis, the gel was stained with ethidium bromide (0.5 μg/ml). DNA concentration was estimated from the intensity of the fluorescent band by comparing with that of undigested λDNA.

2.4 Agarose gel electrophoresis (Sambrook and Rassell, 2001)

Appropriate amount of agarose was weighed out and mixed with 1x TBE buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.0 mM EDTA, pH 8.3). The gel slurry was heated until complete solubilization in the microwave. The gel solution was left at room temperature to approximately 55°C before poured into a gel mould. The comb was inserted. The gel was allowed to solidify at room temperature for

approximately 45 minutes. When needed, the gel mould was placed in the gel chamber and sufficient 1x TBE buffer was added to cover the gel for approximately 0.5 cm. The comb was carefully withdrawn. To carry out agarose gel electrophoresis, one-fourth volume of the gel-loading dye (0.25% bromphenol blue and 25% ficoll) was added to each sample, mixed and loaded into the well. A 100-bp DNA ladder or λ-*Hind* III was used as the standard DNA markers. Electrophoresis was carried out at 4 - 5 V/cm until the tracking dye migrated about three-quartered of the gel. After electrophoresis, the gel was stained with ethidium bromide (0.5 µg/ml) for 5 minutes and destained to remove unbound EtBr by submerged in H_2O for 15 minutes. The DNA fragments were visualized under the UV light using a UV transilluminator.

2.5 Isolation and characterization of the full length cDNA and genomic DNA using rapid amplification of cDNA ends-polymerase chain reaction (RACE-PCR) and genome walking technique

2.5.1 RACE-PCR

2.5.1.1 Preparation of the 5' and 3' RACE-PCR template

Total RNA was extracted from ovaries of *P. monodon* using TRI Reagent. Messenger (m) RNA was purified using a QuickPrep micro mRNA Purification Kit (GE Healthcare). The RACE-Ready cDNA was synthesized using a BD SMART™ RACE cDNA Amplification Kit (BD Clontech) by combining 1.5 µg of ovarian mRNA with 1 µl of 5'CDS primer and 1 µl of 10 µM SMART II A oligonucleotide for 5'RACE-PCR and 1 µg of ovarian mRNA with 1 µl of 3'CDS primer A oligonucleotide for 3'RACE-PCR. The components were mixed and briefly centrifuged. The reaction was incubated at 70 °C for 2 minutes and snap-cooled on ice for 2 minutes. The reaction tube was briefly centrifuged. After that, 2 µl of 5X First-strand buffer, 1 µl of 20 mM DTT, 1 µl of dNTP Mix (10 mM each) and 1 µl of PowerScript Reverse Transcriptase were added. The reactions were mixed by gently pipetting and briefly centrifuged. The tubes were incubated at 42 °C for 1.5 hours in an air incubator. The first strand reaction products were diluted with 125 µl of Tricine-EDTA Buffer and heated at 72 °C for 7 minutes. The first strand cDNA template was stored at -20°C.

Table 2.1 Primer sequences for the first strand cDNA synthesis and RACE-PCR

Primer	Sequence
SMART II A Oligonucleotide	5′-AAGCAGTGGTATCAACGCAGAGTACGC GGG-3′
3'-RACE CDS Primer A	5′-AAGCAGTGGTATCAACGCAGAGTAC$(T)_{30}$ N-1N-3′ (N=A, C, G or T; N-1=A, G or C)
5'-RACE CDS Primer	5′-(T)25N-1N-3′ (N=A, C, G or T; N-1=A, G or C)
10X Universal Primer A Mix (UPM)	Long: 5′-CTAATACGACTCACTATAGGGCAA GCAGTGGTATCAACGCAGAGT-3′ (0.4 μm) Short: 5′-CTAATACGACTCACTATAGGGC-3′ (2 μm)
Nested Universal Primer A (NUP)	5′-AAGCAGTGGTATCAACGCAGAGT-3′ (10 μm)

2.5.1.2 Primer design

A gene-specific primer (GSPs) was designed from ESTs significantly matched *COMT* (hemocyte cDNA library; clone no. HC-H-S01-0684-LF), *Br-cZ1*(ovarian cDNA library, clone no. OV-N-S01-1207-W) and *Br-cZ4* (hemocyte cDNA library, clone no. HC-H-S01-0767-LF) and previously deposited *FAMeT* sequence (GenBank accession no. AAX24112.1) (Table 2.2).

2.5.1.3 RACE-PCR and cloning of amplification products

The same master mix for 5′- and 3′-RACE-PCR and control reactions was prepared. For each amplification reaction, 35.75 μl of deionized H_2O, 5 μl of 10X Advantage 2 PCR buffer, 1 μl of dNTP mix (10 μM each) and 1 μl of 50X Advantage 2 polymerase mix were combined. 5′-RACE-PCR and 3′-RACE-PCR were set up according to Table 2.4 and 2.5, respectively.

Table 2.2 The gene specific primer (GSP1), their sequences and Tm of *COMT*, *FAMeT* and *Br-C* gene.

Gene	Sequence	Tm
3'PmCOMT	F:5'-GCTCTGGTGGAGTCATCGCCTTC-3'	**66**
5'PmFAMeT	R:5'-GGCAGAGGCAGCGCCTTGGGAT CCGC -3'	**70**
3'PmFAMeT	F:5'-CTGCTCAGCAAGGAGGGAAGGGGAT -3'	**70**
5'PmBr-cZ1	R:5'-TGATCGGACCACGTGCGAACCAG-3'	**68**
3'PmBr-cZ1	F:5'-GCCACCAACCGCTCACGCATG-3'	**70**
3'PmBr-cZ4	R: 5'-TTGACCTCCTTGATCACACC- 3'	**60**

The reaction was carried out for 20 cycles composing of a 94 °C for 30 second, 68 °C for 30 seconds and 72 °C for 3 minutes. The primary 5′ and 3′ RACE-PCR products were electrophoretically analyzed.

After characterization of primary RACE product, if the discrete expected product (s) is not obtained. The primary PCR product was 50-fold diluted (1 µl of the product + 49 µl of TE) and amplified with nested GSP and NUP primer (5'-AAG CAGTGGTATCAACGCAGAGT-3'). The amplification reaction was performed using 5 µl of the diluted PCR product as a template using the same condition for the first PCR for 15 cycles.

The resulting products are size-fractionated through agarose gels. The expected fragment is eluted from the gel, cloned into pGEM-T Easy and further characterized by DNA sequencing.

Nucleotide sequences of EST and 5' and 3' RACE-PCR are assembled and blasted against data previously deposited in the GenBank using Blast*N* and Blast*X* (Altschul et al., 1990; available at http://ncbi.nlm.nih.gov). The protein domain of deduced amino acids of each gene is searched using SMART (http://smart.embl-heidelberg.de/). The p*I* and molecular weight of the deduced protein are estimated using Protparam (http://www.expasy.org/tools/protparam.html).

Table 2.3 Composition of 5′-RACE-PCR

Component	5′-RACE Sample	UPM only (Control)	GSP1 only (Control)
5′-RACE-Ready cDNA	1.25 µl	1.25 µl	1.25 µl
UPM(10X)	5.0 µl	5.0 µl	-
5′ GSP(GSP1, 10µM)	1.0 µl	-	1.0 µl
H_2O	-	1.0 µl	5.0 µl
Master Mix	42.75 µl	42.75 µl	42.75 µl
Final volume	50 µl	50 µl	50 µl

Table 2.4 Composition of 3′-RACE-PCR

Component	3′-RACE Sample	UPM only (Control)	GSP1 only (Control)
3′-RACE-Ready cDNA	1.25 µl	1.25 µl	1.25 µl
UPM(10X)	5.0 µl	5.0 µl	-
3′ GSP(GSP2, 10µM)	1.0 µl	-	1.0 µl
H_2O	-	1.0µl	5.0 µl
Master Mix	42.75 µl	42.75 µl	42.75 µl
Final volume	50 µl	50 µl	50 µl

2.5.2 Genome walking analysis

2.5.2.1 Digestion of genomic DNA

After the full length cDNA of *PmCOMT* was obtained from RACE-PCR,. Genomic organization of this gene was further characterized using genome walking analysis.

Two and a half micrograms of genomic DNA of an individual of *P. monodon* were singly digested in a reaction volume of 100 µl containing 40 units of a blunt end generating restriction enzyme (*Dra* I, *Eco RV*, *Pvu II* and *Ssp* I, respectively), 1X appropriate restriction enzyme buffer and deionized H_2O. The reaction was incubated at 37°C for 4 hours. Five microlitres of the digest was run on a 0.8% agarose gel to determine whether the digestion was complete.

An equal volume (95 µl) of buffer-equilibrated phenol was added. The mixture was vortexed at the low speed for 5-10 seconds and centrifuged for 5 minutes at room temperature to separate the aqueous and organic phases. The upper layer was transferred into a fresh 1.5 ml microcentrifuge tube. An equal volume (95 µl) of chloroform:isoamylalcohol was added, vortexed and centrifuged. The upper layer was transferred into a fresh 1.5 ml tube. One-tenth volume of 3 M NaOAc (pH 4.5) was added and mixed followed by 2.5 volume of ice cold absolute ethanol and thoroughly mixed. The mixture was kept at -80 °C for 30 minutes. The digested DNA was recovered by centrifugation at 12,000 rpm for 10 minutes at room temperature. The supernatant was discarded. The DNA pellet was briefly washed with ice-cols 70% ethanol and centrifuged at 12000 rpm for 5 minutes at room temperature. The supernatant was discarded. The pellet was air-dried. DNA was dissolved in 10 µl of TE (10 mM Tris, pH 8.0, 0.1 mM EDTA).

2.5.2.2 Ligation of genomic DNA to GenomeWalker adaptors

The ligation reaction was set up in a 10 µl reaction volume containing 4 µl of digested DNA, 1.9 µl of: 25 µM of GenomeWalker Adaptor (GenomeWalker Adaptor Forward: 5′-GTA ATA CGA CTC ACT ATA GGG CAC GCG TGG TCG ACG GCC CGG GCA GGT-3′ and GenomeWalker Adaptor Reverse: 5′-PO_4-ACC TGC CC-NH_2-3′), 1.6 µl of 10X ligation buffer and 3 units of T4 DNA ligase. The reaction mixture was incubated at 16 °C overnight. The reaction was terminated by incubation at 70 °C for 5 minutes. The ligated product was ten fold diluted by an addition of 72 µl of TE (10 mM Tris, pH 8.0, 0.1 mM EDTA).

2.5.2.3 PCR-based genomic DNA walking

PCR-based genomic DNA walking was carried out in a 25 µl reaction containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100, 200 µM each of dATP, dCTP, dGTP and dTTP, 2 mM $MgCl_2$, 0.2 µM each of Adaptor primer1 (AP1: 5′-GTAATACGACTCACTATAGGGC-3′) and gene specific primer (*PmCOMT* GWF:5'-CGCTCTGGTGGAGTCATCG-3'), 1 µl of each DNA library of *P. monodon* and 1.0 unit of DyNAzyme™ II DNA Polymerase (Finnzymes). The amplification reactions were carried out using the two-step cycle parameters including 7 cycles of a denaturing step at 94 °C for 25 seconds and an annealing/extension step at 70 °C for 3 minutes followed by 35 cycles of 94 °C for 25 second, annealing at 65 °C for 3 minutes and the final extension at 67 °C for an additional 7 minutes. Five microlitres of the primary PCR product was electrophoretically analyzed by a 1.2% agarose gel. A 100 bp ladder and λ–*Hin*d III was included as the DNA markers.

The primary PCR product was 50 fold-diluted (1 µl of each primary PCR and 49 µl of deionized H_2O) and 1 µl of this was used as the DNA template for the semi-nested PCR. The PCR components of the secondary PCR were similar as those of the primary PCR with the exception that AP1 was replaced with 0.2 µM of AP2 primer (5′-ACTATAGGGCACGCGTGGT-3′) and 0.2 µM of GSP.

PCR was carried out composing of 5 cycles of a denaturing step at 94 °C for 25 seconds and an annealing/extension step at 70 °C for 3 minutes followed by 20 cycles of 94 °C for 25 second and 65 °C for 3 minutes. The final extension at 67 °C was carried out for an additional 7 minutes. Five microlitres of the secondary PCR product was electrophoretically analyzed by a 1.2% agarose gel. A 100 bp ladder and λ–*Hin*d III was included as the DNA markers.

2.5.2.4 Overlapping PCR of genomic *PmCOMT*

For amplification of the remaining PmCOMT genomic sequence, two fragments were amplified using primers ORFPmCOMT-F (5′-ATGTCTTCTCTGAA AAGTTACCA -3′) and 5′COMT-R (5′-ACATCGCCGCTCTACGGTGCT-3′) and COMTRT-F (5′- AGCACCGTAGAGCGGCGATGTT-3′) and COMTRT-R (5′-CGAAGGCGATGACTCCACCAGA-3′). The amplification profiles were PCR was performed by predenaturation at 94°C for 3 min followed by 35 cycles of a 94°C denaturation for 30 s, a 53 (ORFPmCOMT-F + 5′COMT-R) or 55°C (COMTRT-

F/R), annealing for 1 min and a 72°C extension for 2 or 1 min, respectively. The final extension was carried out at 72°C for 7 min. The PCR products were cloned and sequenced. Nucleotide sequences of these genes were assembled and compared with their cDNAs.

2.6 Cloning of PCR-amplified DNA

2.6.1 Elution of DNA from agarose gels

The DNA fragment was fractionated through agarose gels in duplication. One was run side-by-side with a 100 bp DNA markers and the other was loaded into the distal well of the gel. After electrophoresis, lanes representing the DNA standard and its proximal DNA sample were cut and stained with ethidium bromide (0.5 μg/ml) for 5 minutes. Positions of the DNA markers and the EtBr-stained fragment were used to align the position of the non-stained target DNA fragment. The DNA fragment was excised from the gel with a sterile razor blade. DNA was eluted out from the agarose gels using a QIAquick gel Extraction kit (QIAGEN) according to the protocol recommended by the manufacture. The excised gel was transferred into a microcentrifuge tube and weighed. Three gel volumes of the QG buffer were added. The mixture was incubated at 50°C for 10 minutes with briefly vortexing every 2 – 3 minutes. After the gel was completely dissolved, 1 gel volume of isopropanol was added and gently mixed. The mixture was applied to the QIAquick spin column placed on a 2 ml collection tube and centrifuged at 13,000 rpm for 1 minute at room temperature. The flow-through was discarded and 0.75 ml of the PE buffer was added. The QIAquick spin column was centrifuged at 13,000 rpm for 1 minute at room temperature. The flow-through was discarded. The column was further centrifuged at room temperature for an additional 1 minute at 13,000 rpm to remove trance amount of the washing buffer. The column was then placed in a new microcentrifuge tube and 30 μl of the EB buffer (10 mM Tris-Cl, pH 8.5) was added to the center of the QIAquick membrane. The column was incubated at room temperature for 1 minute before centrifuged at 13,000 rpm for 1 minute. The eluted sample was stored at -20°C until further required.

2.6.2 Ligation of PCR product to pGEM-T easy vector

The ligation reaction was set up in the total volume of 10 µl containing 3 ul of the gel-eluted PCR product, 25 ng of pGEM-T easy vector, 5 µl of 2X rapid ligation buffer (60 mM Tris-HCl pH 7.8, 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP and 10 % PEG 8000) and 3 Weiss units of T4 DNA ligase. The ligation mixture was gently mixed by pipetting and incubated at 4°C overnight.

2.6.3 Preparation of competent cells

A single colony of *E. coli* JM109 was inoculated in 10 ml of LB broth (1% Bacto tryptone, 0.5% Bacto yeast extract and 0.5% NaCl) with vigorous shaking at 37°C overnight. The starting culture was inoculated into 50 ml of LB broth and continued culture at 37°C with vigorous shaking to the OD_{600} of 0.5 – 0.8. The cells were chilled on ice for 10 minutes before centrifuged at 3,000 g for 10 minutes at 4°C. The pellets were resuspended in 30 ml of ice-cold $MgCl_2$-$CaCl_2$ solution (80 mM $MgCl_2$ and 20 mM $CaCl_2$) and centrifuged as above. The supernatant was discarded and the pellet was resuspended in 2 ml of ice-cold 0.1 M $CaCl_2$ and divided into 200 µl aliquots. These competent cells could be used immediately or stored at – 70°C for subsequent used.

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

The competent cells were thawed on ice for 5 minutes and divided to aliquots of 100 µl. Two microlitres of the ligation mixture was added and gently mixed by pipetting. The mixture was incubated on ice for 30 minutes. The reaction tube was then placed in a 42°C water bath for 45 seconds without shaking. The tube was then immediately snapped on ice for 2-3 minutes. One microlitre of SOC medium (2% Bacto tryptone, 0.5% 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 tube. The cell suspension was incubated with shaking at 37°C for 1.5 hours. At the end on 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 and spread on a LB agar plate containing 50 µg/ml of ampicillin, 25 µg/ml of IPTG and 20 µg/ml of X-gal. The plate was left until the cell suspension was absorbed and further incubated at

37°C overnight. The recombinant clones containing inserted DNA are white whereas those without inserted DNA are blue.

2.6.5 Detection of recombinant clone by colony PCR

Colony PCR was performed in a 25 µl reaction volume containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100, 200 µM each of dATP, dCTP, dGTP and dTTP, 1.5 mM $MgCl_2$, 0.2 µM of pUC1 (5′-TCC GGC TCG TAT GTT GTG TGG A-3′) and pUC2 (5′-GTG GTG CAA GGC GAT TAA GTT GG-3′) primers and 0.5 unit of DyNAzyme™ II DNA Polymerase. A recombinant colony was picked up by the micropipette tip and mixed well in the amplification reaction. The PCR profiles was predenaturing at 94°C for 3 minutes, followed by 30 cycles of 94°C for 30 seconds, 55°C for 60 seconds and 72 °C for 90 seconds. The final extension was carried out at 72°C for 7 minutes. The resulting PCR products were analyzed by agarose gel electrophoresis.

2.6.6 Isolation and digestion of recombinant plasmid DNA

A recombinant clone was inoculated into 3 ml of LB broth (1% Bacto-tryptone, 0.5% Bacto-yeast extract and 1.0 % NaCl) containing 50 µg/ml of amphicillin and incubated at 37°C with constant shaking at 250 rpm overnight. The culture was transferred into 1.5 ml microcentrifuge tube and centrifuged at 12,000 g for 1 min. The cell pellet was collected and resuspended with 250 µl of the buffer P1. The mixture was completely dispersed by vortexing. The mixture was then treated with 250 µl of the buffer P2, gently mixed and placed on ice for 10 min. Additionally, 350 µl of the buffer N3 was added and gently mixed. To separate the cell debris, the mixture was centrifuged at 12,000 g for 10 minutes. The supernatant was transferred into the QIAprep column and centrifuged at 12,000 g for 30 - 60 seconds. The flow-through was discarded. The QIAprep spin column was washed by adding 0.75 ml of the buffer PE and centrifuged for 30-60 seconds. The flow-through was discarded. The spin tube was centrifuge for an additional 1 minute to remove the residual wash buffer. The QIAprep column was placed in a new 1.5 ml microcentrifuge tube and 40 µl of the EB buffer (10 mM Tris-Cl, pH 8.5) was added to elute the extracted plasmid

DNA. The column was left at room temperature for 1 minute and centrifuge at 12,000 g for 1 minute.

The insert size of each recombinant plasmid was examined by digestion of the plasmid with *Eco* RI. The digest was carried out in a 15 µl containing 1X restriction buffer (90 mM Tris-HCl; pH 7.5, 10 mM NaCl and 50 mM $MgCl_2$), 1 µg of recombinant plasmid and 2-3 units of *Eco* RI and incubated at 37°C for 3 hours before analyzed by agarose gel elctrophoresis.

In addition, clones showing corresponded DNA insert size were separately digested with *Hin*d III and *Rsa* I to verify whether a single insert possibly contained only one type of sequence. Typically, the digestion reaction was set up in the total volume of 15 µl containing appropriate restriction enzyme buffer (buffer E; 6 mM Tris-HCl; pH 7.5, 6 mM $MgCl_2$, 100 mM NaCl and 1 mM DTT for *Hin*d III and buffer C; 10 mM Tris-HCl; pH 7.9, 10 mM $MgCl_2$, 50 mM NaCl and 1 mM DTT for *Rsa* I), 5 µl of the amplified product and 2 units of either *Hin*d III or *Rsa* I. The reaction mixture was at incubated at 37°C for 3-4 hours. Digestion patterns were analyzed by agarose gel electrophoresis.

2.6.7 DNA sequencing

Cloned DNA fragments from typical PCR, RT-PCR, RACE-PCR and genome walking analysis were sequenced by automated DNA sequencer using M13 forward and/or M13 reverse primer as the sequencing primer by MACROGEN (Korea).

2.7 Phylogenetic analysis

The amino acid sequence of *PmCOMT* and *PmFAMeT* was phylogenetically compared with that from other species found in GenBank. Multiple alignments were carried out with ClustalW (Thompson et al., 1994). A bootstrapped neighbor-joining tree (Saitou and Nei 1987) was constructed with the Seqboot, Prodist, Neighbor, and Consense routines in Phylip (Felsenstein 1993) and illustrated with Treeview (http://taxonomy.zoology.gla.ac.uk/rod/ treeview.html).

2.8 RT-PCT and tissue distribution analysis of *PmCOMT, PmFAMeT, PmBr-cZ1* and *PmBr-cZ4*

2.8.1 Primer design

Forward and reverse primers of each gene were designed from nucleotide sequence obtained from ESTs library (*PmCOMT, PmBr-cZ1* and *PmBr-cZ4*) and partial sequence of *PmFAMeT* from *P. monodon* (*PmFAMeT*) (Table 2.5) using Primer Premier 5.0. Generally, the PCR primers is 20-24 bp in length with melting temperatures of 60-70°C and the GC content of 40-50% (Table 2.5).

2.8.2 First strand cDNA synthesis

The first strand cDNA was synthesized from 1.5 µg of DNase-treated total RNA were reverse-transcribed to the first strand cDNA using an ImPromp-II™ Reverse Transcription System Kit (Promega, U.S.A.). Total RNA was combined with 0.5 µg of oligo dT_{12-18} and appropriate DEPC-treated H_2O in final volume of 5 µl. The reaction was incubated at 70°C for 5 minutes and immediately placed on ice for 5 minutes. Then 5X reaction buffer, $MgCl_2$, dNTP Mix, RNasin were added to final concentrations of 1X, 2.25 mM, 0.5 mM and 20 units, respectively. Finally, 1 µl of ImProm-II™ Reverse transcriptase was add and gently mixed by pipetting. The reaction mixture was incubated at 25°C for 5 minutes and at 42°C for 90 minutes. The reaction mixture was incubated at 70°C for 15 minutes to terminate the reverse transcriptase activity. Concentration and rough quality of the newly synthesized first strand cDNA was spectrophotometeically examined (OD_{260}/OD_{280}) and electrophoretically analyzed by 1.0% agarose gels, respectively. The first stranded cDNA was 10 fold-diluted and kept at -20°C until required.

2.8.3 RT-PCR analysis

Generally, PCR was carried out in a 25 µl reaction mixture containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100, 200 µM each of dATP, dCTP, dGTP and dTTP, 2 mM $MgCl_2$, 0.2 µM of a primer, 200 ng of first strand cDNA of *P. monodon* and 1.0 unit of DyNAzyme™ II DNA Polymerase (Finnzymes, Finland). The PCR profiles was predenaturing at 94°C for 3 minutes, followed by 30 cycles of 94°C for 30 seconds, 55°C for 45 seconds and 72 °C for 1 minute. The final extension was carried out at 72°C for 7 minutes. The resulting PCR products were

electrophoretically analyzed through 1.0-2.0% agarose gels and visualized under a UV transilluminator after ethidium bromide staining.

2.8.4 Tissue distribution analysis by RT-PCR

Expression of each genes in various tissue of female juvenile and broodstock (eyestalk, gills, heart, hemocytes, hepatopancrease, lymphoid organ, intestine, pleopods, stomach, thoracic ganglion and ovaries) and testes of male juvenile and broodstock was analyzed by RT-PCR. *EF-1α*$_{500}$ (F: 5'-ATGGTTGTCAACTTTGCCCC-3' and R: 5'-TTGACCTCCTTGATCACACC-3') was included as the positive control. The thermal profiles were 94°C for 3 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45 s and extension at 72°C for 1 min. The final extension was carried out at 72°C for 7 min. The amplicon was electrophoretically analyzed through 1.5% agarose gels and visualized with a UV transilluminator after ethidium bromide staining (Sambrook and Russell, 2001).

2.9 Semi-quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Expression levels of *PmCOMT* and *PmFAMeT* were semiquatitatively determined. *Elongation factor 1 alpha (EF1-α)* was used as an internal control. The amplification conditions need to be optimized.

2.9.1 Primers

Primer pairs used for semiquantitative RT-PCR of *PmCOMT* and *PmFAMeT* were illustrated in Table 2.5. Primer for *EF1-α* were designed (F: 5′-ATGGTTGTCAACTTTGCCCC-3′ and R: 5′-TTGACCTCCTTGATCACACC-3′).

2.9.2 Total RNA extraction and the first strand synthesis

Total RNA was extracted from hemocyte, testes and ovaries of broodstock-sized and juvenile *P. monodon* using TRI Reagent as previously described.

2.9.3 Determination of PCR conditions

Amplification was performed in a 25 µl reaction volume containing 0.1 µg of the first strand cDNA template, 1X PCR buffer (10mM Tris-HCl pH 8.8, 50 mM KCl and 0.1% Triton X-100), 200 µM each of dNTP and 1 unit of Dynazyme™ DNA Polymerase (FINZYMES, Finland). PCR was carried out using the conditions described in Table 2.5.

2.9.4 Primer concentration

The optimal primer concentration for each primer pair (between 0 - 0.3 µM) was examined using the standard PCR conditions. The resulting product was electrophoretically analyzed. The primer concentration that gave specificity of the amplification product and clear results were selected for further optimization of PCR condition.

2.9.5 $MgCl_2$ concentration

The optimal $MgCl_2$ concentration of each primer pair (between 0 - 4 mM $MgCl_2$) was examined using the standard PCR conditions and the optimal primer concentration in 2.10.4. The concentration that gave the highest specificity was chosen.

2.9.6 Cycle number

The PCR amplifications were carried out at different cycles (e.g. 20, 25, 30 and 35 cycles) using the optimal concentration of primers $MgCl_2$ and analyzed by gel electrophoresis. Relationships between the number of cycles and the intensity of the PCR product were plotted. The number of cycles that still provided the PCR product in the exponential rage and did not reach a plateau level of amplification was chosen.

2.9.7 Gel electrophoresis and quantitative analysis

The amplification product of genes under investigation and EF-1α were electrophoretically analyzed by the same gel. The intensity of interesting genes and that of *EF-1α* was quantifiedly from glossy prints of the gels using the Quantity One programme (BioRad)

Table 2.5 The gene specific primer (GSP1), their sequences and Tm of *PmCOMT*, *PmFAMeT*, *PmBr-cZ1* and *PmBr-cZ4* gene for RT-PCR and Tissue distribution analysis

Gene	Sequence	Tm
PmCOMT-RT-F	F: 5′- TTGACATAAGTGAAGAGTTTGC -3′	60
PmCOMT-RT-R	F: 5′- GAAGGCGATGACTCCACCAG -3′	64
PmFAMeT-RT-F	F: 5′- CCACCATTCCAGAGCCTTTC -3′	62
PmFAMeT-RT-R	R: 5'- TTCCCTCCTTGCTGAGACGA - 3'	62
PmBr-cZ1-RT-F	F: 5'- ACGCTCACCTCCGCCCAGTC - 3'	68
PmBr-cZ1-RT-R	R: 5'- AGTGCCCACATTTGCCGCATTAT - 3'	68
PmBr-cZ4-RT-F	F: 5′- CTCAGAATTAAGGGCTTGGCAG -3′	66
PmBr-cZ4-RT-R	R: 5'- TGGAGGTGTTACCGATGGCTGC - 3'	70
EF-1α_{500}-F	R: 5'- ATGGTTGTCAACTTTGCCCC - 3'	60
EF-1α_{500}-R	R: 5'- TTGACCTCCTTGATCACACC - 3'	60

2.10 Effects of dopamine and serotonin on expression of genes in ovaries of juvenile *P. monodon*

Expression levels of *PmCOMT* and *PmFAMeT* related with dopamine and serotonin were examined using semi-quantitative RT-PCR analysis.

2.10.1 Dopamine administration

Female juvenile *P. monodon* (approximately 5 months old with the body weight of 20-25 g) were purchased from Chonburi (eastern Thailand) used in this experiments. They were acclimatized at the laboratory conditions (ambient temperature of 28-30 °C, salinity of 12 ppt) for 7 days in 150-liter fish tanks. The experimental animals were not fed approximately 24 hours before the treatment.

Shrimp were intramuscularly into the first abdominal segment injected individually with dopamine hydrochloride to obtain the doses of 10^{-6} mole/shrimp (N = 5 for each group). Shrimp injected with the 0.85% saline solution (at 0 hr) were included as the control. The samples were collected at 0, 3, 6, 12 and 24 hr post injection. Ovaries of each shrimp were collected and subjected to total RNA

extraction. The first strand cDNA was synthesized and used as the template for semi-quantitative RT-PCR of *PmCOMT* and *PmFAMeT*.

2.10.2 Serotonin administration

For serotonin (5-HT) injection, juvenile *P. monodon* (5-month-old) were purchased from a commercial farm in Chonburi (eastern Thailand) and acclimated for 7 days at the laboratory conditions (28–30 °C and 30 ppt seawater) in 150-liter fish tanks.

Four groups of female shrimp (approximately 35 g body weight) were injected intramuscularly into the first abdominal segment with 5-HT (50 μg/g body weight, N=5 for each group). Specimens were collected at 12, 24, 48 and 72 h post treatment (hpt). Shrimp injected with the 0.85% saline solution (at 0 hpi) were included as the control. Specimens were collected at 0, 3, 6, 12 and 24 hr post injection. Ovaries of each shrimp were collected and subjected to total RNA extraction. The first strand cDNA was synthesized and used as the template for semi-quantitative RT-PCR of *PmCOMT* and *PmFAMeT*.

2.10.3 Data analysis

The expression level of each gene was normalized by that of *EF-1α*. Expression levels between different groups of *P. monodon* were statistically tested using one way analysis of variance (ANOVA) followed by Duncan's new multiple range test. Significant comparisons were considered when the P-value was < 0.05.

2.11 Quantitative real-time PCR of *PmCOMT, PmFAMeT, PmBr-cZ1* and *PmBr-cZ4* in ovaries of *P. monodon*

2.11.1 Experimental animals

2.11.1.1 Intact wild and domesticated *P. monodon* used for expression analysis of various genes during ovarian development

Female juveniles of *P. monodon* (N = 4, average body weight 33 g, 6-month-old), domesticate broodstock: 10-month-old (N = 6, average body weight 46.68 ± 3.55 g and GSI <1), 14- month-old (N = 4, average body weight 64.06 ± 3.20 g and GSI =

<1), and 18-month-old (N = 8, average body weight 77.12 ± 3.10 g and GSI = 1-1.5), wild intact broodstock (N = 8, average body weight 135.29 ± 8.20 g) and eyesstalk ablated broodstock(N = 8, average body weight 135.29 ± 8.20 g) were used for real-time PCR analysis.

2.11.1.2 Serotonin administration

Domesticated female *P. monodon* (18-month-old with the average body weight of 107±16.24g) were sampled from the earth ponds and acclimated in the fish tank for 96 hours. Five group of shrimp are single injected intramuscularly with serotonin (50 μg/g of body weight)into the first abdominal segment of each shrimp and specimens are collected at 0 hr, 1, 12, 24 ,48 and 72 hr post injection. Shrimp injected with the normal saline (0.85% at 0 hr) is also included as the control. Ovaries of each shrimp were sampling and immediately placed in liquid N_2. Specimens were stored at -80°C prior to RNA extraction and first-strand cDNA synthesis.

2.11.1.3 Progesterone administration

Domesticated female *P. monodon* (18-month-old with the average body weight of 100.79±17.59 g) were sampled from the earth ponds and acclimated in the fish tank for 96 hours. Five group of shrimp are single injected intramuscularly with progesterone (0.1 μg/g of body weight) into the first abdominal segment of each shrimp and specimens are collected at 0 hr, 1, 12, 24 , 48 and 72 hr post injection (hpi). Shrimp injected with the absolute ethanol was used as the vehicle control (at 12 and 72 hpi). Ovaries of each sample were sampling and immediately placed in liquid N_2. Specimens were stored at -80°C prior to RNA extraction and first-strand cDNA synthesis.

2.11.1.4 20β-hydroxyecdysone administration

Commercially cultured female juveniles of *P. monodon* (the average body weight of 17.56±3.46) were acclimated in the fish tank for 2 weeks. Five group of juvenile shrimp are single injected intramuscularly with 20β-hydroxyecdysone (1 μg/g of body weight)into the first abdominal segment of each shrimp and specimens are collected at 0 hr, 6, 12, 24 ,48 72, 96 and 168 hr post injection. Shrimp injected with 10% ethanol was also included as a control (at 0 hr). Ovaries of each sample

were sampling and immediately placed in liquid N_2. The sample were stored at -80°C prior to RNA extraction and first-strand cDNA synthesis.

2.11.2 Primer design and construction of the standard curve

The intron/exon structure of the target gene was characterized. Several primer pairs were designed from cDNA sequence of each gene and used to PCR against genomic DNA as the template. The PCR fragment was cloned and sequenced. The forward or reverse primer covering intron/exon boundaries or alternatively, a primer pairs sandwiching the large intron was designed. Sizes of the expected PCR product size was usually less than 100-200 bp or less than 500 bp when the small amplification producy could not be designed.

For construction of the standard curve of each gene, the DNA segment covering the target PCR product and *EF*-1α were amplified from primers used for quantitative real-time PCR. The amplification products were cloned and sequenced. The recombinant plasmid was extracted and used as the template for estimation of the copy number of a particular gene. A 10 fold-serial dilution corresponding to 10^3-10^8 molecules (10^3-10^8 molecules for *PmFAMeT*) was prepared. The copy of standard DNA molecules can be calculated using the following formula:

X g/µl DNA/(plasmid length in bp x 660) x6.022x10^{23}= Y molecules/µl

Standard curves representing 10^3-10^8 copies (in triplicate) of each gene and the internal control, *EF-1*α_{214} (F: 5′-GTCTTCCCCTTCAGGACGTC-3′ and R: 5′-CTTTACAGAC ACGTTCTTCACG TTG-3′) were constructed using the conditions described below. The standard curves with correlation coefficient = 0.995-1.000 or efficiency higher than 95% were accepted to be used to deduce the copy number of examined genes.

2.11.3 Quantitative real-time PCR analysis

The target transcripts and the internal control *EF*-1α of the synthesized cDNA were amplified in a reaction volume of 10 µl using 2X LightCycler® 480 SYBR Green I Master (Roche, Germany). The specific primer pairs were used at a final

Table 2.6 Nucleotide sequences and Tm of primers used for quantitative real-time PCR of *PmCOMT*, *PmFAMeT* , *PmBr-cZ1*, *PmBr-cZ4 and EF1α*

Gene	Sequence	Tm
PmCOMT-qRT-F	F: 5'- AACGACTGAATGATGTAACTCT -3'	60
PmCOMT-qRT-R	R: 5'- CTCCCAGAACGGTTTTCCTAT -3'	62
PmFAMeT-qRT-F	F: 5'-TTCGACATCACTCATTACGGC-3'	62
PmFAMeT-qRT-R	R: 5'-GAACACTTCATACATGGGTGTGG- 3'	68
PmBr-cZ1-qRT-F	F: 5'- CGCAAAAGGCCACCAGAATCG - 3'	66
PmBr-cZ1-qRT-R	R: 5'-TCTGTGACTGTTCATCGCTGTTG - 3'	68
PmBr-cZ4-qRT-F	F: 5'- ACGCAGGACCATCCATTCAACC - 3'	68
PmBr-cZ4-qRT-R	R: 5'- CGGACAGGCACCAGTCATTAGCT - 3'	72
EF-1α_{214}-F	F: 5'- GTCTTCCCCTTCAGGACGTC - 3'	64
EF-1α_{214}-R	R: 5'- CTTTACAGACACGTTCTTCACGTTG - 3'	72

concentration of 0.3μM and 50 ng of the first strand cDNA template. The thermal profile for SYBR Green real-time PCR was 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds, 58°C for 30 seconds and at 72°C for 30 seconds. The cycles for the melting curve analysis were 95°C for 15 seconds, 65°C for 1 minute and at 98°C for continuation and cooling was 40°C for 10 seconds. The real-time RT-PCR assay was carried out in a 96 well plate and each sample was run in duplicate using a LightCycler® 480 Instrument II system (Roche). A ratio of the absolute copy number of the targer gene and that of *EF*-1α was calculated. . The relative expression level (copy number of each gene /that of *EF-1α*) between shrimp possessing different stages of ovarian development (or treatment) were statistically tested ($P < 0.05$).

2.12 *In situ* hybridization (ISH)

2.12.1 Sample preparation

Ovaries of intact and eyestalk-ablated *P. monodon* broodstock were fixed in 4% paraformaldehyde prepared in 0.1% phosphate-buffered saline (PBS, pH 7.2) overnight at 4°C. The fixed ovarian tissue was washed four times with PBS at room temperature and stored in 70% ethanol at -20°C until used. Tissue was histrogically

prepared, embedded in paraffin and Conventional paraffin sections (5 μm) were carried out onto poly-L-lysine-coated slides.

2.12.2 Preparation of cRNA probes

For *PmFAMeT, PmBr-cZ1 and PmBr-cZ4* the template used for synthesis of the cRNA probes were PCR-amplified. The T7 (TAATACGACTCACTAT AGGG) and SP6 sequence (ATTTAGGTGACACTATAGAA) (Table 2.7) promoter sequences were added to the 5′ of forward and reverse primers, respectively. PCR was carried out in a 25 μl reaction volume containing 10 ng of recombinant plasmid containing the complete ORF of the target transcripts were used as the template. The PCR condition was initially performed by predenaturation at 94°C for 2 minutes followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds and at 72°C for 1 minute. The PCR product was purified using a MinElute PCR purification Kit (Qiagen). The concentration of purified PCR product was estimated by comparing with the DNA marker after electrophoresis and also spectrometrically estimated.

Table 2.7 Nucleotide sequences and Tm of primers for synthesis of the cRNA probes of *PmCOMT*, *PmFAMeT*, *PmBr-cZ1* and *PmBr-cZ4*

Gene	Sequence	Tm
PmCOMT-ISH-F	F:5′-**TAATACGACTCACTATAGGG**CATAATCCCGATCCT TTGGT3'-3′	**66**
PmCOMT-ISH-R	F:5′**ATTTAGGTGACACTATAGAA**TTCCTAATAGCCACTG TGCC3'-3′	**60**
PmFAMeT-ISH –F	F:5′-**TAATACGACTCACTATAGGG**ACACCCCTGACATCC TGAGT-3′	**60**
PmFAMeT-ISH –R	R: 5'- **ATTTAGGTGACACTATAGAA**TCCATGAAGGGATC ACTGTC3'- 3'	**60**
PmBr-cZ1-ISH –F	F: 5'- **TAATACGACTCACTATAGGG**GGAAGATTATA TCCTGCACACA - 3'	**62**
PmBr-cZ1-ISH –R	R: 5'- **ATTTAGGTGACACTATAGAA**CTGTGACTGTT CATCGCTGTTG -3'	**66**
PmBr-cZ4-ISH –F	F: 5'-**TAATACGACTCACTATAGGG**TTCACAAACTG GTAATGAGCAC - 3'	**62**
PmBr-cZ4-ISH R	R:5'-**ATTTAGGTGACACTATAGAA**TGTAGTCGTCT GGGTCGTCA -3'	**70**

For *PmCOMT*, the sense and anti-sense cRNA probes were synthesized from a linearlized plasmid containing the full length ORF of *PmCOMT* using primers (ORFPmCOMT-F (5′-ATGTCTTCTCTGAAAAGTTACCA-3′) and ORFPmCOMT-R (5′-TTTATTATTGGGGAGGAGGG -3′). Briefly, 2.5 μg of recombinant *PmCOMT* plasmid was digested with of *Nde* I (sense, NEB) or *Nco* I (anti-sense, promega) in a 50 μl containing 1X appropriate buffer and 20 units of each restriction enzyme. The reaction was incubated at 37°C overnight. The digestion product was purified using a MinElute PCR purification Kit (Qiagen). The concentration of purified PCR product were estimated by comparing with the DNA marker after electrophoresis and also spectrophotometrically estimated.

2.12.3 Synthesis of the cRNA probes

For synthesis of the cRNA probe, 0.4 μg of the gel-eluted product (for linearlized plasmid) or 1 μg of the gel-eluted PCR product was used as the template using the protocol recommended by the manufacturer (Roche). The mixture was incubated at 40°C for 2 hours for the antisense probe and 37°C for 2 hours for the sense probe. The template DNA was eliminated by treating with DNase I at 37°C for 20 minutes. The reaction was terminated by adding 2 μl of 0.2 M EDTA (pH 8.0). The synthesized probe (1 μl) was determined by electrophoresis and the remaining reaction mixture was purified using an RNeasy® MinElute® Cleanup kit (Qiagen). The cRNA probe concentration was spectrophotometrically measured and stored at -80°C until needed.

2.12.4 Dot blot analysis

The quality of cRNA probes was determined before used for *in situ* hybridization using dot blot analysis. Serial dilutions of the pre-diluted probe and control cRNA were made. The diluted probe (1 μl) was spotted on a piece of the Hybond N^+membrane. The spotted probe was fixed to the membrane by cross-linking with UV-light for 1 minute. The membrane was washed with the washing buffer for 1 minute and incubated in the blocking solution for 1 minute. After that, the membrane was incubated in Anti-DIG-alkaline phosphatase (1:5,000 in the blocking solution) for 3 minute, washed with the washing buffer for 1 minute and incubates in the detection buffer. The positive hybridization signals was developed using NBT/BCIP solution.

The intensities of the control and the dilution of probe were compared to estimate the concentration of the cRNA probe.

2.12.5 Hybridization and detection

Tissue sections were dewaxed with xylene and dehydrated in absolute ethanol. The sections were prehybridized with 2x SSC containing 50% deionized formamide, 1 μg/μl yeast tRNA, 1 μg/μl salmon sperm DNA, 1 μg/μl BSA and 10% (w/v) dextran sulfate at 50°C for 30 min and hybridized with either the sense or antisense probe in the prehybridization solution overnight at 50°C. After hybridization, the tissue sections were washed twice with 4x SSC for 5 min each and once with 2x SSC containing 50% (v/v) formamide for 20 min at 50°C. The sections were immersed in prewarmed RNase A buffer (0.5 M NaCl, 10 mM Tris–HCl, pH 8.0, 1 mM EDTA) at 37 °C for 30 min and treated with RNase A (10 μg/ml) at 37 °C for 30 min. Tissue sections were washed four times with the RNase A buffer (37°C, 10 min each) and 2x SSC (50°C, 15 min each). Then tissue sections was wash twice with 2x SSC for 15 min at 50°C. And high stringent washing was carried out twice in 0.2x SSC at 50°C for 20 min each. Detection of the positive hybridization signals was carried out with a DIG Wash and Block Buffer kit (Roche) (Qui and Yamano, 2005).

2.13 *In vitro* expression of recombinant protein using the bacterial expression system

2.13.1 Primer design

A pair of primer was designed to amplify the full length cDNA of each gene. The forward and reverse primers contained a *Nde* I site and a *Bam* HI site and six His encoded nucleotides, respectively (Table 2.8).

2.13.2 Construction of recombinant plasmid in cloning and expression vectors

The full length cDNA of each genes were amplified by PCR, ligated to pGEM®-T Easy vector and transformed in to *E. coli* JM109. Plasmid was extracted from a positive clone and used as the template for PCR amplification using 0.2 μM of each primer, 0.75 unit *Pfu* DNA polymerase (Promega) and 0.2 mM of each dNTP

Table 2.8 Nucleotide sequences and Tm of primers for amplification the ORF of *PmCOMT*, *PmFAMeT*, *PmBr-cZ1* and *PmBr-cZ4*

Gene	Sequence	Tm
PmCOMT-ORF-F	F: 5′-ATGTCTTCTCTGAAAAGTTACCA -3′	62
PmCOMT-ORF-R	R:5′- TTTATTATTGGGGAGGAGGG -3′	58
PmFAMeT-ORF-F	F: 5′-TGCTCGCAAGTAACTCGGGATG-3′	68
PmFAMeT-ORF-R	R:5′ -GTGAACAAAGCCACAAAGCAGGA-3′	88
PmBr-cZ1-ORF-F	F: 5′ -AAAACT GGTTCGCACGTGG -3′	58
PmBr-cZ1-ORF-R	R:5′-GGAGATCTTTATGGCAGGTTAAT -3′	64
PmBr-cZ4-ORF-F	F: 5′-GCGGCGTGGTGATGGACGCTC-3′	76
PmBr-cZ4-ORF-R	R:5′- TTTTCATCACAGGGCTTATC -3′	56

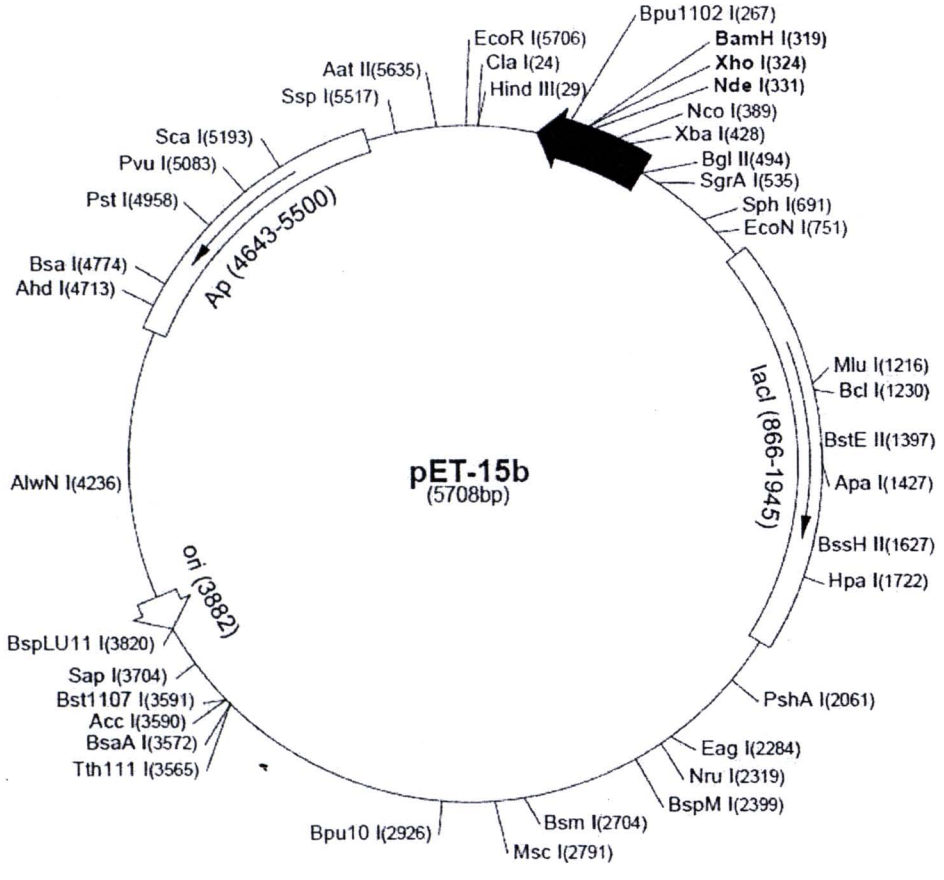

Figure 2.1 The pET-15b vector map (Novogen).

The thermal profiles were predenaturation at 95°C for 2 minutes followed by 25 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds and at 72°C for 4 minutes and the final extension at 72°C for 7 minutes. The amplification product was digested with *Nde* I and *Bam* RI and analyzed by agarose gel electrophoresis. The gel-eluted product was ligated into pET15b and transformed into *E. coli* JM109. The recombinant plasmid was subsequently transformed into *E. coli* BL21-CodonPlus(DE3)-RIPL.

Table 2.9 The gene specific overhang primer, their sequences and Tm of *COMT*, *FAMeT* and *Br-C* gene for *in vitro* expression.

Gene	Sequence	Tm
PmCOMT-ORF/ *Nde* I-F*	F: 5'-CCGCATATGTCTTCTCTGAAAAGTTACCA -3'	70
PmCOMT-ORF/*Bam HI*-R*	R:5'-CGG*GGATCC*TCA**ATGATGATGATGATGATG** TTTTTTAAAACATAGAGTCA-3'	76
PmFAMeT-ORF/ *Nde* I-F*	F:5'-CCGCATATGGGCGAGAGCTGGGCTTCCTA -3'	70
PmFAMeT-ORF/*Bam HI*-R*	R:5'-CGG*GGATCC*TTA**ATGATGATGATGATGATG** AAATTCGAACTTCCACT -3'	74
PmBr-cZ1-ORF/*Xba I*-F	F:5'-CGGTCTAGAATGGAGGAGGGCTACCTAGCA C-3'	68
PmBr-cZ1-ORF/ *Bam HI*-R*	R:5'-CGGGGATCCTTA**ATGATGATGATGATGATG** TTAATGTTTATAATTCCTCTTATGG-3'	76
PmBr-cZ4-ORF/*Xba I*-F	F: 5'-CGGTCTAGAATGGAGGACGGACTACTAAGCT-3'	64
PmBr-cZ4-ORF/ *Nde I*-R*	R5'-CGGCATATGTTA**ATGATGATGATGATGATG** TTAAGGAGTTACAGCTGTGGCT-3'	72

**Nde* I site (underlined) and an *Bam* HI site (italicized) and six His encoded nucleotides (boldfaced),

2.13.3 Expression of recombinant proteins\

A bacterial colony carrying recombinant plasmid of each gene was inoculated into 3 ml of LB medium containing 50 µg/ml ampicillin and 50 µg/ml chloramphenicol at 37°C and 50 µl of the overnight culture was transferred to 50 ml of LB medium containing ampicillin and chloramphenicol and further incubated to an OD_{600} of 0.4-0.6. One OD_{600} milliliter was time-interval taken at 1, 2, 3, 6, 12 and 24 hr after IPTG induction (0.4 mM final concentration). The culture was centrifuged at 12000 *g* for 1 min, resuspended with 1x PBS and analyzed by 15% SDS-PAGE

(Laemmli, 1970). In addition, 20 ml of the IPTG induced-culture (6 hr) were centrifuged, resuspended in the lysis buffer (0.05 M Tris-HCl; pH 7.5, 0.5 M Urea, 0.05 M NaCl, 0.05 M EDTA; pH 8.0 and 1 mg/ml lysozyme) and sonicated 2-3 times at 15-30% amplitude, pulsed on for 10 seconds and pulsed off for 10 seconds in a period of 2-5 min. The protein concentration of both soluble and insoluble fractions was measured (Bradford, 1976). Overexpression of recombinant protein was analyzed by 15% SDS-PAGE. For western blot analysis, the electrophoresed proteins were transferred to a PVDF membrane (Towbin et al., 1979) and analyzed as previously described in Imjongjairak et al. (2005).

Recombinant protein was analyzed in 15% SDS-PAGE. The electrophoresed proteins were transferred onto a PVDF membrane (Hybond P; GE Healthcare) (Towbin et al., 1979) in 25 mM Tris, 192 mM glycine (pH 8.3) buffer containing 10% methanol at 100 V for 90 min. The membrane was treated in the DIG blocking solution (Roche) for 1 hr and incubated with the Anti-His antibody IgG2a (GE Healthcare; 1:5000 in the blocking solution) for 1 h at room temperature. The membrane was washed 3 times with 1× Tris Buffer Saline-Tween20 (TBST; 50 mM Tris–HCl,0.15 M NaCl, pH 7.5, 0.1% Tween20) and incubated with goat anti mouse IgG (H+L) conjugated with alkaline phosphatase (Bio-Rad Laboratories) at 1:3000 for 1 h and washed 3 times with 1× TBST. Immunoreactive signals were visualized using NBT/BCIP (Roche) as the substrate.

2.13.4 Purification of recombinant proteins

Recombinant protein was purified using a His GraviTrap kit (GE Healthcare). Initially, 1 liter of IPTG-induced cultured overnight at 37°C was harvested by centrifugation at 5000 rpm for 15 min. The pellet was resuspended in the binding buffer (20 mM sodium phosphate, 500 mM NaCl, pH 7.4), sonicated and centrifuged at 14000 rpm for 30 min. The insoluble fraction was purified by using a His GraviTrap kit (GE Healthcare) under denaturing .The insoluble fraction was loaded into the column and washed with 10 ml of the binding buffer containing 20 mM imidazole(20 mM sodium phosphate, 500 mM NaCl, pH 7.4 and 8M urea), 5 ml of the binding buffer containing 50 mM imidazole (20 mM sodium phosphate, 500 mM NaCl, 50 mM imidazole, pH 7.4 and 8M urea) and 5 ml of the binding buffer containing 80 mM imidazole (20 mM sodium phosphate, 500 mM NaCl, 80 mM

imidazole, pH 7.4 and 8M urea), respectively. The recombinant protein was eluted with 6 ml of the elution buffer (20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4 and 8M urea). Fractions from the washing and eluting steps were analyzed by SDS-PAGE and western blotting. The purified protein was stored at 4°C or -20°C for long term storage.

2.13.5 Peptide sequencing of recombinant proteins

Peptide sequencing was applied to confirm that the expressed proteins were rPmCOMT, rPmFAMeT-l and rPmFAMeT-s by using NanoLC-MS/MS

2.13.5.1 In-gel digestion

The expected sizes (on SDS-PAGE or western blot) were excised, the gel pieces were subjected to in-gel digestion using a method developed by Proteomics Laboratory, Genome Institute, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Thailand (Jaresitthikunchai et al., 2009). Briefly, the excised band was washed 3 times with 3% hydrogen peroxide and water, respectively. The gel plugs were dehydrated with 100% acetonitrile (ACN), reduced with 10mM DTT in 10mM ammonium bicarbonate at room temperature for 1 hour and alkylated at room temperature for 1 hour (dark) in 100 mM iodoacetamide (IAA) supplemented with 10 mM ammonium bicarbonate. After alkylation, the gel pieces were dehydrated twice with 100% ACN for 5 minutes. To perform in-gel digestion of proteins, 10 µl of trypsin solution (10 ng/µl trypsin in 50% ACN/10mM ammonium bicarbonate) was added to the gels followed by incubation at room temperature for 20 minutes, and then 20 µl of 30% ACN was added to keep the gels immersed throughout digestion. The gels were incubated at 37°C for a few hours or overnight. To extract peptide digestion products, 30 µl of 50% ACN in 0.1% formic acid (FA) was added into the gels, and then the gels were incubated at room temperature for 10 minutes in a shaker for three times. Peptides extracted were collected and pooled together in the new tube. The pool extracted peptides were dried by incubated at 40°C for 3-4 hours and kept at -80°C for further mass spectrometric analysis.

2.13.5.2 NanoLC-MS/MS

Nano-electrospay liquid chromatography ionization tandem mass spectrometry (nanoLC-MS/MS) was performed as followed. Selected protein spots were submitted to the HCTultra ETD II system™ (Bruker Daltonics). This system was controlled by the Chromeleon Chromatography Management system and comprised a two-pump Micromass/Loading Iontrap system with an autosampler. Injected samples were first trapped and desalted on an AccLaim PepMap C18 μ Precolumn Cartridge (5 μm, 300-μm inside diameter by 5 mm) for 3 minutes with 0.1% formic acid delivered by a loading pump at 20 μl/minutes, after which the peptides were eluted from the pre-column and separated on a nano column, AccLaim PepMap 100 C18 (15 cm x 3 μm) connected in-line to the mass spectrometer, at 300 nl/minutes using a 30 minutes fast gradient of 4 to 96% solvent B (80% acetronitrile in 0.1% formic acid).

2.13.5.3 Database searches

After data acquisition, MS/MS ion from nanoLC-MS/MS were identified using MASCOT (http://www. matrixscience.com) searched against data of the local shrimp database. In addition, data from nanoLC-MS/MS were searched against data of the National Central for Biotechnology Information (NCBI, nr). For MS/MS ion search, the peptide charge was 1+, 2+ and 3+, MS/MS ion mass tolerance was ±1.2 Da, fragment mass tolerance ± 0.6 Da, and allowance for 1 miss cleavage. Variable modification was methionine oxidation and cysteine carbamidomethylation. Proteins with the highest score or higher significant scores were selected. The significant hit proteins were selected according to Mascot probability analysis and regarded as positive identification after additional conformation with molecular weight (MW)/ isoelectric point (p*I*) values

2.13.6 Polyclonal antibody production and western blot analysis

Polyclonal antibody against rPmCOMT, rPmFAMeT-s and rPmFAMeT-l was immunologically produced in rabbit by Faculty of Associated Medical Sciences, Chiangmai University. Western blot analysis was carried out to examine specificity and sensitivity of the antibody.

For western blot analysis, ovarian tissues of *P. monodon* were homogenized in the sample buffer (50 mM Tris-HCl; pH 7.5, 0.15 M NaCl) supplemented with the proteinase inhibitors cocktail (EDTA free; Roche). The homogenate was centrifuged

at 12000g for 30 min at 4°C and the supernatant was collected. Protein concentrations of the tissues extract were determined by the dye binding method (Bradford, 1976). Thirty micrograms of ovarian proteins were heated at 100°C for 5 min, immediately cooled on ice and size-fractionated by 15% SDS-PAGE (Laemmli, 1970). Electrophoretically separated proteins were transferred onto a PVDF membrane (Hybond P; GE Healthcare) (Towbin et al., 1979) in the 25 mM Tris, 192 mM glycine (pH 8.3) buffer containing 10% methanol at a constant current of 350 mA for 1 h. The membrane was treated with the DIG blocking solution (Roche) for 1 hr and incubated with the primary antibody (1:300 in the blocking solution) for 1 hr at room temperature. The membrane was washed 3 times with 1x Tris Buffer Saline-Tween-20 (TBST; 50 mM Tris–HCl, 0.15 M NaCl, pH 7.5, 0.1% Tween-20) and incubated with goat anti-rabbit IgG (H+L) conjugated with alkaline phosphatase (Bio-Rad Laboratories) at 1:3000 for 1 h and washed 3 times with 1x TBST. Immunological signals were visualized using NBT/BCIP (Roche) as the substrate.

2.14 Immunohistochemistry

The paraffin sections were prepared from pieces of ovaries fixed with 4%paraformaldehyde. Deparafinized sections were autoclaved in 0.01 sodium citrate(pH 6.0) containing 0.1% Tween-20 at 120°C for 5 minutes. Then incubated in the blocking solution I(3% H_2O_2 in methanol) for 15 minutes. After treatment in the blocking solution II (Roche) for 4 hours, section were incubated with purified anti-COMT, anti-FAMeT-s, anti-FAMeT-l, preimmunise and blocking solution(control) for 1 hour in the humid chamber. The sections were rinsed three times for 5 minutes with 1XPBS (pH 7.2) and incubate with goat anti-rabbit IgG conjugated with horse radish peroxidase for 1 hour. The section were again rinse three times for 5 minutes with 1XPBS (pH 7.2). Localization of antigen was visualized using diaminobenzidine (Wako) as the substrate. Tissue section were dehydrated and mounted for long term storage.