# CHAPTER 2

# Material and Methods

## 2.1 Insects

The *O. fuscidentalis* larvae were obtained from bamboo forest in Maewang District, Chiang Mai Province, Thailand. The diapausing fifth larval instars were collected from October to March, and pupae were collected in April. Larvae were reared in containers (12 x 14 x 8 cm) lined with sheets of wet paper towel and were maintained continuously in the dark at 25 ºC and 95% humidity (Singtripop *et al*., 1999).

## 2.2 Hormones

The JHA (S-methoprene, >95% stereochemically pure; SDS Biotech, Japan) was dissolved in acetone (final concentration 5 mg /ml) and stored at -35 °C as a stock solution. The stock solution was diluted to 0.5 µg/5 µl with acetone, and a 5 µl aliquot was applied to the dorsal abdomen of individual diapausing larvae using a 50 µl microsyringe. Individuals in the control were treated with 5 µl acetone. After JHA treatment, the larvae (designated as G0) became motionless and their epidermis turned brown and formed a hard, pigmented cuticle. The physiological age of these larvae was divided into six stages, from G0 to G5, based on body color and deposition of pigmented pupal cuticle (Fig. 1) (Singtripop *et al*., 2000). The 20-hydroxyecdysone (20E, Sigma, St. Louis, Missouri) was dissolved in distilled water at a concentration of 1 mg/ ml and stored at -35 °C until use. The stock solution was diluted to 0.5 µg/5 µl in distilled water (DW), and diapausing larvae were injected with a 5 µl aliquot through the second proleg (Singtripop *et al*., 2002b). Control larvae were injected with 5 µl DW. The amounts of JHA and 20E applied were based on Singtripop *et al*. (2000).

## 2.3 Total RNA extraction, cDNA synthesis, and PCR amplification

Total RNA was isolated from SG, other neural tissues (e.g., brain, thoracic ganglia and abdominal ganglia) and non-neural tissues (e.g., fat body, integument and Malpighian tubules) with the single-step method of acid guanidinium thiocyanate phenol chloroform (AGPC) extraction according to the manufacturer's instructions (Chomczynski & Sacchi, 1987). Prior to cDNA synthesis, the RNA was treated with RNase-free DNaseI (Fermentas, Vilnius, Lithuania) to eliminate contaminating DNA. A quantity of 1 µg of total RNA was used to generate first-strand cDNA with an oligo-dT primer and M-MuLV-Reverse Transcriptase (Fermentas). The degenerate primers for PCR amplification are listed in Table 2.1.

## 2.4 Rapid amplification of cDNA ends (5' and 3' RACE)

Two specific primers, OfDP-SPF (5'-CTT AGA CTA TTG GAG GCT GCA-3') and OfDP-SPR (5'-GAT CCT TAG GGA GCG TTT GCC-3'), were synthesized for RACE based on the cDNA sequences obtained from internal amplification. 3' and 5' RACE were conducted using a SMART$^{TM}$ RACE cDNA amplification kit (Clontech, Palo Alto, California) according to the manufacturer's instructions. The 5'-ready-cDNA had a nested universal primer (5'-AAG CAG TGG TAT CAA CGC AGA GT-3') and OfDP-SPR (5'-GAT CCT TAG GGA GCG TTT GCC-3'). The 3'-ready-cDNA had a nested universal primer (Clontech) and OfDP-SPF (5'-CTT AGA CTA TTG GAG GCT GCA-3'). The reaction mixture was subjected to 1 cycle for 5 min at 94 ºC, 45 cycles that consisted of 94 ºC for 30 sec, 68 ºC for 30 sec, 72 ºC for 30 sec and then a final extension step for 2 min at 72 ºC.

Table 2.1 Gene-degenerate primer used in PCR amplification

| Gene name | Forward primer | Reverse primer | Reaction conditions |
|---|---|---|---|
| DH-PBAN | TGGTTCGGHCCYAGRHTNGGS | GAAGATBACYTTYTTBGTHAC | 94 ºC, 3 min<br>94 ºC, 30 sec<br>56 ºC, 45 sec<br>72 ºC, 30 sec } 40 cycles<br>72 ºC, 2 min |
| Met | TKGACAARACNRSHRYBCT | ADRWARATVAANYSNCCVKT | 94 ºC, 5 min<br>94 ºC, 30 sec<br>48 ºC, 45 sec<br>72 ºC, 3 min } 45 cycles<br>72 ºC, 2 min |
| HR3 | ACAGTGGTGAACTACCARTGYCC | ARYGTRTCBARHACKGTGAC | 94 ºC, 5 min<br>94 ºC, 30 sec<br>50 ºC, 45 sec<br>72 ºC, 3 min } 45 cycles<br>72 ºC, 2 min |
| E75C | ACTCSGTSATYCARTGCATG | ATCATCTAGCTCTGCGGTGG | 94 ºC, 5 min<br>94 ºC, 30 sec<br>56 ºC, 45 sec<br>72 ºC, 30 sec } 45 cycles<br>72 ºC, 5 min |

## 2.5 Cloning and sequencing

The PCR products were separated on 1.5% agarose gel and purified using a GEL/PCR Purification Mini Kit (Favorgen Biotech Corp., Ping-Tung, Taiwan). Purified DNA fragments were subcloned into pTZ57R/T vectors using an InsTAclone™ PCR cloning Kit (Fermentas) and amplified in *Escherichia coli* DH5α competent cells (One Shot, Invitrogen, Carlsbad, California) after transformation. Recombinant plasmids were isolated using a GeneJET™ Plasmid Miniprep Kit (Fermentas), and DNA sequencing was performed by an ABI PRISM® Bigdye® Terminator (version 3.1) cycle Sequencing Kit (Applied Biosystems, Foster city, California) and an automated DNA sequencer (ABI PRISM® 3100 Genetic Analyzer; Applied Biosystems). The cDNA sequences of DH-PBAN were searched against the GenBank database using FASTA (European Molecular Biology Laboratory, European Bioinformatics Institute).

## 2.6 Effects of JHA and 20E on *OfMet, OfDH-PBAN, OfEcR-A, OfEcR-B1, OfBr-C, OfE75A, OfE75B, OfE75C* and *OfHR3* genes expression *in vitro*

A diluted stock solution of JHA (0.02 µg/µl) was prepared in ethanol. The solution was evaporated in air and resuspended in Grace's insect cell culture medium (Gibco Invitrogen Corporation, Grand Island, New York). All equipment used in the JHA experiments was coated with 1% solution of polyethylene glycol. For the 20E experiments, the stock solution (0.02 µg/µl) was prepared in distilled water. Both JHA and 20E stock solutions were serially diluted in culture medium to the appropriate working concentrations. In brief, the diapausing larvae were anesthetized on ice for 30 min. The SG and PG were dissected and rinsed 5 times in ice-cold Ringer's solution (130 mM NaCl, 4.7 mM KCl and 1.9 mM $CaCl_2$). The tissue was rinsed again in culture medium five times. The hormones were added to 50 µl medium containing 1 lobe of SG and PG in 96-well culture plates (Sero-Wel, Bibby Sterilin, U.K.) and were incubated at 25 °C with gentle shaking. For the controls, equal volumes of the medium without JHA or 20E were added. Total RNAs from SG and PG in both hormone treatments and control experiments were extracted at the same time points after incubation.

## 2.7 Quantitative real-time PCR (Q-RT-PCR)

The primers used for amplifying cDNA fragment are listed in Table 2.2. The *O. fuscidentalis* ribosomal protein L3 (*OfRpL3*) mRNA was used as an endogenous control. Quantitative real-time PCR was conducted using the SensiFAST™ SYBR® No-Rox Kit (Bioline, U.K.) and an iCycler iQ5™ Real-Time PCR Detection System (Bio-Rad, Hercules, California). One reaction contained 1 µl of template cDNA sample and 0.2 µM primers in a final reaction volume of 20 µl. The thermal cycling parameters were 95 ºC for 2 min, followed by 40 cycles at 95 ºC for 5 sec, 60 ºC for 10 sec, and 72 ºC for 20 sec. After Q-RT-PCR, the absence of undesired by-products was confirmed by an automated melting curve analysis and agarose gel electrophoresis of the PCR product. The amplification efficiency for both the reference and target genes was analyzed. The relative expression level of *OfMet*, *OfDH-PBAN*, *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA from samples with different treatments was estimated by a comparative $C_T$ method ($\Delta\Delta C_T$) for relative quantitation of gene expression. The $C_T$ (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the background level. The dynamic range of both the target (*OfMet*, *OfDH-PBAN*, *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* or *OfHR3*) and normalizer (*OfRpL3*) were determined. After normalization with *OfRpL3* [i.e., $\Delta C_T = C_{T(OfMet,\ OfDH\text{-}PBAN,\ OfEcR\text{-}A,\ OfEcR\text{-}B1,\ OfBr\text{-}C,\ OfE75A,\ OfE75B,\ OfE75C \text{ or } OfHR3)} - C_{T(OfRpL3)}$], the $\Delta C_T$ value of the treatment group was compared with that of the control group known as the calibrator (Livak and Schmittgen, 2001) (i.e., $\Delta\Delta C_T$). All results of Q-RT-PCR were analyzed using Pfaffl's mathematical model (Pfaffl, 2001). The relative expression was calculated using three samples from independent experiments. The data were subjected to one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons, where differences were considered significant at $P < 0.01$. Data are presented as the mean ± SEM.

Table 2.2 Forward and reverse primers used in Q-RT-PCR.

| **Gene name** | **Forward primer** | **Reverse primer** | **Accession no.** |
|---|---|---|---|
| *Met* | TTTGTAGGTGTCGTGCGTGT | CGCTCGTGTGTCATGTATCC | KT223005 |
| *DH-PBAN* | TATGAGAGTCGAGCTGATGAC | GTGACGGAAAGCTTCTCCGG | JX195641 |
| *EcR-A* | GTGAAATCGTGAGGCCGTAT | ACACTTCACTGGAGGCGTTT | EF667890 |
| *EcR-B1* | TGCGTGGATTGTGTTTTGTT | CACTTTTTCCCCGCACTAAA | EF667891 |
| *Br-C* | GCGCATAGAGTGGTCCTTTC | GGTTTTCAGGAACGAGGACA | KT223007 |
| *E75A* | CATTACGGTGTGCATTCCTG | GACAGCATCTCTGCTCATGC | KT224348 |
| *E75B* | CGGTGCTAGTGAGCATGTTG | AGGATGGAGCACTGCTGATT | KT224349 |
| *E75C* | CCGGATCTAGAGTTCGATGG | AGGATGGAGCACTGCTGATT | KT223004 |
| *HR3* | CCAACGCAGTCCGATGGATA | GCCCATCCCACTCAAGTTCA | KT223006 |
| *RpL3* | TCTACCCCAAGAAGAGGTCTCG | ACGACAGTCCTCAGACATGTGC | EF453378 |

## 2.8 Effect of photoperiod on the gene expression of *OfDH-PBAN*

Larvae were reared at 25 ºC combined with various photoperiods (LD 0:24 h, LD 2:22 h, LD 14:10 h, and LD 18:6 h). The number of larvae used in each tested photoperiod was at least 300. The *OfDH-PBAN* mRNA expression level was examined every 5 days until the larvae entered pupation.