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CHARACTERIZATION AND EXPRESSION OF O-METHYLTRANSFERASE
AND BROAD COMPLEX GENES AND PROTEINS IN THE GIANT TIGER
SHRIMP *Penaeus monodon*

Mr. Arun Buaklin

A Dissertation Submitted in Partial Fulfillment of the Requirements
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Thesis Title	Characterization and expression of o-methyltransferase and broad complex genes and proteins in the giant tiger shrimp <i>Penaeus monodon</i>
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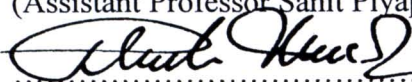
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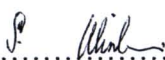
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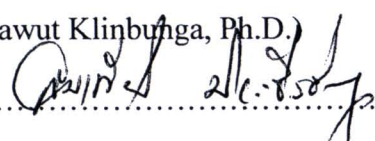
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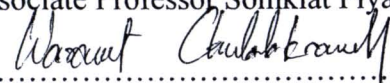
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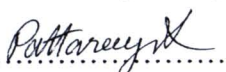
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นายอรุณ บัวกลิ่น: ลักษณะสมบัติและการแสดงออกของยีนโอ-เมทิลทรานส์เฟอเรส และบรอดคอมเพล็กซ์และโปรตีนในกุ้งกุลาดำ *Penaeus monodon* (CHARACTERIZATION AND EXPRESSION OF O-METHYLTRANSFERASE AND BROAD COMPLEX GENES AND PROTEINS IN THE GIANT TIGER SHRIMP *Penaeus monodon*.) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ศ.ดร.เปี่ยมศักดิ์ เมนะเศวต, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร.ศิริวรุณ กลิ่นบุหงา, 221 หน้า.

การหาลำดับนิวคลีโอไทด์และลักษณะสมบัติของยีนและโปรตีนที่เกี่ยวข้องกับการพัฒนารังไข่ เป็นจุดเริ่มต้นของความเข้าใจกลไกระดับโมเลกุลของการสมบูรณ์พันธุ์ของกุ้งกุลาดำ จึงหาลำดับนิวคลีโอไทด์ที่สมบูรณ์ของยีน catechol-O-methyltransferase (*PmCOMT*), farnesic-O-methyltransferase (*PmFAMEt*), broad complex Z1 (*PmBr-cZ1*) และ broad complex Z4 (*PmBr-cZ4*) โดยรูปแบบของยีน *PmCOMT* และ *PmBr-cZ4* เพียงรูปแบบเดียว และพบว่า *PmCOMT* และ *PmBr-cZ1* มี 2 รูปแบบของยีน นอกจากนี้ยังพบว่ายีน *PmCOMT* ประกอบด้วย 3 intron (ขนาด 194, 111 และ 361 คู่เบส) 2 exon (ขนาด 143 และ 147 คู่เบส)

เมื่อตรวจสอบการแสดงออกของยีนดังกล่าวด้วยวิธี quantitative real-time PCR พบว่ายีน *PmCOMT* มีระดับการแสดงออกที่ไม่แตกต่างกันในรังไข่ของกุ้งกุลาดำแม่พันธุ์ธรรมชาติปกติและกุ้งกุลาดำที่ตัดก้านตา ($P > 0.05$) และพบว่ายีน *PmFAMEt* มีระดับการแสดงออกที่สูงขึ้นในรังไข่ระยะที่สี่ของกุ้งกุลาดำแม่พันธุ์ปกติ ($P < 0.05$) แต่มีระดับการแสดงออกที่ไม่แตกต่างกันในรังไข่ของกุ้งกุลาดำที่ตัดก้านตา ($P > 0.05$) ส่วนระดับการแสดงออกของยีน *PmBr-cZ1* นั้นลดลงในรังไข่ระยะที่สองและสามของกุ้งกุลาดำปกติ ($P < 0.05$) โดยการตัดก้านตามีผลให้การแสดงออกของยีนนี้เพิ่มขึ้น ($P < 0.05$) ในขณะที่ยีน *PmBr-cZ4* มีระดับการแสดงที่ลดลงในระยะที่สี่ในกุ้งกุลาดำแม่พันธุ์ปกติ ($P < 0.05$) และการตัดก้านตามีผลให้การแสดงออกของยีนนี้ลดลง ($P < 0.05$)

ตรวจสอบผลของซีโรโทนิน (5-HT) โปรเจสเตอโรน และ 20-hydroxysteriod (20-E) ต่อการแสดงออกของยีนดังกล่าวในกุ้งแม่พันธุ์ตัดพันธุ์และกุ้งเลี้ยงขนาดวัยรุ่น พบว่าระดับการแสดงออกของยีน *PmFAMEt* จะเพิ่มขึ้นประมาณ 50 เท่า หลังถูกฉีดด้วย 5-HT เป็นเวลา 1 ชั่วโมง ($P < 0.05$) แต่ไม่พบระดับการแสดงออกที่แตกต่างกันของยีนต่างๆในกุ้งกุลาดำแม่พันธุ์ที่ถูกกระตุ้นด้วยโปรเจสเตอโรน แต่พบว่าการฉีด 20E ส่งผลให้ระดับการแสดงออกของยีน *PmCOMT* ในรังไข่ของกุ้งวัยรุ่นลดลง ($P < 0.05$) แต่ส่งผลให้ยีน *PmFAMEt*, *PmBr-cZ1* และ *PmBr-cZ4* มีการแสดงออกที่สูงขึ้น ($P < 0.05$)

เมื่อตรวจสอบตำแหน่งการแสดงออกของยีนที่สนใจในรังไข่ของกุ้งกุลาดำด้วยวิธี *in situ* hybridization พบว่า *PmCOMT*, *PmFAMEt*, *PmBr-cZ1* และ *PmBr-cZ4* มีตำแหน่งการแสดงออกของ mRNA ในส่วนของไฮโดพลาสซึมของเซลล์ไข่ระยะ previtellogenesis โดยพบการแสดงออกของยีน *PmCOMT* ในส่วนของไฮโดพลาสซึม ของ follicular cell และ โอลิโกเนียอีกด้วย

สร้างโปรตีนลูกผสมของ *PmCOMT*, *PmFAMEt-I* และ *PmFAMEt-s* ในแบคทีเรีย และผลิตโพลีโคลอนแอนติบอดีของโปรตีนดังกล่าวในกระต่าย เมื่อตรวจสอบระดับการแสดงของโปรตีนในรังไข่ของกุ้งกุลาดำ พบว่าโปรตีน *PmCOMT* มีระดับการแสดงออกในรังไข่ระยะที่หนึ่งและสองสูงกว่าในรังไข่ระยะที่สามและสี่ ในขณะที่พบระดับการแสดงออกของโปรตีน *PmFAMEt* ในรังไข่ระยะที่หนึ่งและสอง แต่ไม่พบการแสดงออกในรังไข่ระยะที่สามและสี่ของกุ้งแม่พันธุ์ธรรมชาติ และพบว่าในกุ้งวัยรุ่นมีแถบโปรตีน *PmFAMEt* ขนาด 32 และ/หรือ 37 kDa โดยพบเฉพาะแถบโปรตีนขนาด 37 kDa ในรังไข่ของกุ้งแม่พันธุ์ธรรมชาติ

ผลจาก immunohistochemistry พบโปรตีน *PmCOMT* ในส่วนไฮโดพลาสซึมของไข่ระยะที่หนึ่งและสอง แต่พบ *PmCOMT* ในคอติคูลลอร์ดของไข่ระยะที่สามและสี่ของกุ้งกุลาดำแม่พันธุ์ปกติและกุ้งกุลาดำที่ตัดก้านตา ส่วนโปรตีน *PmFAMEt* นั้นพบว่ายูอยู่ในส่วนคอติคูลลอร์ดของไข่ระยะที่สามและสี่ในทั้งกุ้งกุลาดำแม่พันธุ์ปกติและกุ้งกุลาดำตัดตา โดยไม่พบโปรตีนนี้ในรังไข่ระยะที่หนึ่งและสอง ผลการทดลองบ่งชี้ว่ายีนและโปรตีน *PmCOMT*, *PmFAMEt*, *PmBr-cZ1* และ *PmBr-cZ4* มีหน้าที่สำคัญเกี่ยวกับการพัฒนารังไข่ของกุ้งกุลาดำ นอกจากนี้ยังพบว่าการแสดงออกของยีน *PmFAMEt*, *PmBr-cZ1* และ *PmBr-cZ4* สามารถใช้เป็นชีวเครื่องหมายสำหรับติดตามการพัฒนาไข่และรังไข่ของกุ้งกุลาดำ

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ARUN BUAKLIN : CHARACTERIZATION AND EXPRESSION OF O-METHYLTRANSFERASE AND BROAD COMPLEX GENES AND PROTEINS IN THE GIANT TIGER SHRIMP *Penaeus monodon*. THESIS ADVISOR : PROF.PIAMSAK MENASVETA, Ph.D., THESIS CO-ADVISOR : SIRAWUT KLINBUNGA, Ph.D., 221 pp.

Identification and characterization of genes and proteins involved in ovarian development are the initial step necessary for understanding molecular mechanisms of reproductive maturation in the giant tiger shrimp (*Penaeus monodon*). The full length cDNAs of catechol-O-methyltransferase (PmCOMT), farnesoic-O-methyltransferase (PmFAMeT), broad complex Z1 (PmBr-cZ1) and broad complex Z4 (PmBr-cZ4) were successfully characterized. Only a single form of PmFAMeT and PmBr-cZ4 was found but two isoforms were observed in PmFAMeT (PmFAMeT-l and PmFAMeT-s) and PmBr-cZ1 (PmBr-cZ1-l and PmBr-cZ1-s). In addition, genomic organization of PmCOMT (3 exons of 194, 111 and 361 bp and 2 introns of 143 and 147 bp) was also isolated.

Quantitative real-time PCR indicated that the expression level PmCOMT was not significantly different during ovarian development in both intact and eyestalk-ablated *P. monodon* broodstock ($P > 0.05$). PmFAMeT mRNA was significantly up-regulated at stage IV ovaries in intact wild broodstock ($P < 0.05$). In contrast, its expression level was not significantly different during ovarian development of eyestalk-ablated broodstock ($P > 0.05$). In intact wild broodstock, PmBr-cZ1 was significantly down-regulated at stages II and III ovaries ($P < 0.05$) and returned to the basal level at stage IV ovaries and after spawning. Eyestalk ablation resulted in its up-regulation at stage IV ovaries of *P. monodon* broodstock. The level of PmBr-cZ4 mRNA was down-regulated at stage IV ovaries of intact *P. monodon* broodstock ($P < 0.05$). Nevertheless, this transcript was up-regulated at stage IV ovaries of eyestalk-ablated broodstock ($P > 0.05$).

Effects of serotonin (5-HT), progesterone (P4) and 20-hydroxysteroid (20E) on expression of these genes in domesticated shrimp were examined. Serotonin administration immediately elevated the expression level of FAMeT approximately 50 fold at 1 hpi ($P < 0.05$). In contrast, progesterone had no effects on expression of these genes ($P > 0.05$). The expression levels of PmCOMT (at 6 hpi), PmBr-cZ1 (at 168 hpi) and PmBr-cZ4 (at 168 hpi) in ovaries of juvenile *P. monodon* was significantly decreased following 20E treatment ($P < 0.05$).

In situ hybridization revealed that PmFAMeT, PmBr-cZ1 and PmBr-cZ4 transcripts were localized in cytoplasm of previtellogenic oocytes while PmCOMT mRNA was clearly observed in the cytoplasm of follicular cells, oogonia and previtellogenic oocytes.

Recombinant protein of PmCOMT, PmFAMeT-l and PmFAMeT-s were successfully expressed *in vitro*. The polyclonal antibody against each recombinant protein was produced. Western blot analysis indicated more preferentially expressed of PmCOMT in previtellogenic and vitellogenic ovaries than that in early cortical rod and mature ovaries of *P. monodon*. PmFAMeT was found in ovaries of juveniles and stages I and II ovaries of broodstock. Interestingly, juvenile shrimp possessed either 32 kDa, 37 kDa or both positive bands whereas only a 37 kDa band owing to posttranslational modifications of ovarian FAMeT was observed in stages I and II ovaries of shrimp broodstock.

Immunohistochemistry revealed the positive signals of the PmCOMT protein in cytoplasm of previtellogenic and vitellogenic oocytes. Subsequently, the positive signals were observed in cortical rods of stages III and IV oocytes in both intact and eyestalk-ablated broodstock. Interestingly, the PmFAMeT protein was detected in stages III and IV oocytes but not in stage I and II oocytes of *P. monodon* broodstock. Taken the information together PmCOMT, PmFAMeT, PmBr-cZ1 and PmBr-cZ4 gene products seem to play the important role on ovarian development and PmFAMeT, PmBr-cZ1 and PmBr-cZ4, in particular, may be used as the bioindicators for monitoring progression of oocyte/ovarian maturation in *P. monodon*.

Field of Study : Biotechnology

Student's Signature Arun Buaklin

Academic Year : 2010

Advisor's Signature Prof. Piamsak Menasveta

Co-Advisor's Signature Sirawut Klinbunga

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LIST OF ABBREVIATIONS

bp	base pair
°C	degree celcius
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
DNA	deoxyribonucleic acid
HCl	hydrochloric acid
IPTG	isopropyl-thiogalactoside
Kb	kilobase
M	Molar
MgCl ₂	magnesium chloride
mg	milligram
ml	milliliter
mM	millimolar
ng	Nanogram
OD	optical density
PCR	polymerase chain reaction
RNA	Ribonucleic acid
RNase A	Ribonuclease A
rpm	revolution per minute
RT	reverse transcription
SDS	sodium dodecyl sulfate
Tris	tris(hydroxyl methyl) aminomethane
µg	microgram
µl	microlitre
µM	micromolar
UV	ultraviolet