CHAPTER III

RESULTS

3.1 Isolation and characterization of the full length cDNA of *cathecol-O-methyalation (PmCOMT), farnesoic acid–O-methyltransferase (PmFAMeT)* and *broad complexes (PmBr-c)* genes in *P. monodon*

3.1.1 Total RNA extraction and first strand synthesis

The quantity and quality of total RNA were estimated by spectrophotometry. The ratio of OD_{260}/OD_{280} of extracted RNA ranged from 1.8-2.0 indicating that RNA samples were relatively pure. Agarose gel electrophoresis indicated smear total RNA with a few discrete bands implying the accepted quality of extracted total RNA (Fig. 3.1). The ovarian mRNA was purified and large amount of mRNA was obtained (typically 30-50 μg from each specimen). The purified mRNA was subjected to the synthesis of 5′ and 3′ RACE-PCR template.

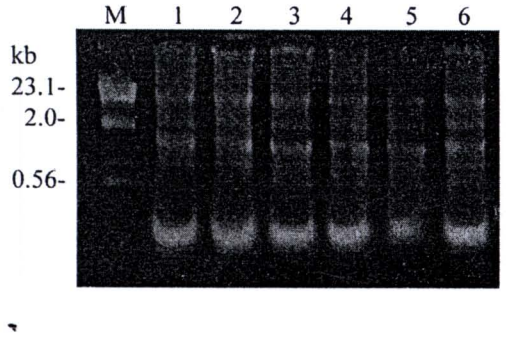

Figure 3.1 A 0.8% ethidium bromide-stained agarose gel showing the quality of total RNA from ovaries of *P. monodon* broodstock (Lanes 1 - 6). Lanes M is λ/*Hin*d III marker.

3.1.2 Characterization of the full length cDNA of *PmCOMT*

The partial nucleotide sequence of *PmCOMT* was initially obtained from EST analysis of the hemocyte cDNA library. This EST significantly match *O-methyltransferase* of *F. chinensis* (*E*-value = 1e-110, Fig 3.2). The primary 3′ RACE-PCR of *PmCOMT* was further carried out for isolation of the full length cDNA of this gene. The positive amplification product of 900 bp in size was obtained (Fig. 3.3). The RACE-PCR fragment was cloned and sequenced for both directions (Fig. 3.4). Nucleotide sequences of 3′ RACE-PCR and the original EST were assembled (Fig. 3.5A).

The full length cDNA of *PmCOMT* was 1176 bp in length containing an ORF of 666 bp corresponding to a polypeptide of 221 amino acids. The 5′ and 3′ UTRs of *PmCOMT* were 17 and 465 bp (excluding the poly A tail), respectively. The poly A additional signals (AATAAA) were located at 150 and 468 nucleotides upstream from the poly A tail (Fig. 3.5B). The closest similarity to *PmCOMT* was *O-methyltransferase* (*OMT*) of *F. chinensis* (*E*-value = 0.0). The calculated p*I* and molecular weight of the deduced PmCOMT protein was 5.73 and 24.1 kDa, respectively. A predicted *O*-methyltransferase domain was found at positions 18-221 (*E*-value = 1.7e-73) of the deduced PmCOMT. Two putative glycosylation sites were found at positions 19-21 (NTS) and 205-207 (NVT). Nine predicted phosphorylation sites (positions 6, 7, 21, 76, 125, 140, 150, 154, and 185) were found in the deduced PmCOMT (Fig. 3.5C).

A.

TTGACAGGTTCCTGAAG**ATG**TCTTCTCTGAAAAGTTACCATAATCCCGATCCTTTGGTGCAGTATTGTG
TAAATCATTCATTGAGATTAACCGACGCGCAAAAACGACTGAATGATGTAACTCTGCAGCACCGTAGAG
CGGCGATGTTGGGGGCACCTGAGGTTCTGCAGCTCAATGCCAACATAATGCAGGCTATCGGGGCAAAGA
AAGTACTAGACATTGGGGTGTTCACAGGCGCCAGTTCACTCTCTGCTGCTCTGGCACTGCCTCCGAATG
GCAAGGTCCACGCCCTTGACATAAGTGAAGAGTTTGCCAACATAGGAAAACCGTTCTGGGAGGAAGCTG
GAGTTATCAACAAGATAAGTCTGCACATCGCTCCAGCTGCTGAGACTCTCCAGAAGTTCATTGACGGCG
GAGAAGCTGGCACCTTCGACTATGCTTTCATTGATGCCGACAAAGGGAATTATGAGCTGTACTATGAAC
TTTGCCTCACTCTCTTGC**GCTCTGGTGGAGTCATCGCCTTCG**ACAACACACTTTGGGATGGAGCTGTGA
TTGACCCCACTGATCAAACCCCTGGCACAGTGGCTATTAGGAAAATTAACGAAAAGCTGAGAGATGACC
AGAGAATCAATATTTCCTTCCTGAAGATTGGTGATGGCGTGACTCTATGTTTTAAAAAATGAACATTTT
TTCACCCGATAGGCACACATCTTCCGCAGTAGTAAATTCCTGTTTCAGGAGAATGCTGATGAC

B.

O-methyltransferase [Fenneropenaeus chinensis] Length=221 Score = 402 bits (1032), Expect = 1e-110 Identities = 199/221 (90%), Positives = 209/221 (94%), Gaps = 0/221 (0%) Frame = +3

```
Query  18   MSSLKSYHNPDPLVQYCVNHSLRLTDAQKRLNDVTLQHRRAAMLGAPEVLQLNANIMQAI  197
            MSSLKSY N DPLVQYCVNHSLRLTD QKRLND TLQHRRAAMLGAPEVLQLNANIMQAI
Sbjct  1    MSSLKSYDNTDPLVQYCVNHSLRLTDVQKRLNDATLQHRRAAMLGAPEVLQLNANIMQAI  60

Query  198  GAKKVLDIGVFTGASSLSAALALPPNGKVHALDISEEFANIGKPFWEEAGVINKISLHIA  377
            GAKKVLDIGVFTGASSLSAALALPPNGKV+ALDISEEF NIGKP+WEEAGV NKISLHIA
Sbjct  61   GAKKVLDIGVFTGASSLSAALALPPNGKVYALDISEEFTNIGKPYWEEAGVSNKISLHIA  120

Query  378  PAAETLQKFIDGGEAGTFDYAFIDADKGNYELYYELCLTLLRSGGVIAFDNTLWDGAVID  557
            PAAETLQKFID GEAGTFDYAFIDADK +Y+ YYELCL LLR GGVIAFDNTLWDGAVID
Sbjct  121  PAAETLQKFIDAGEAGTFDYAFIDADKESYDRYYELCLILLRPGGVIAFDNTLWDGAVID  180

Query  558  PTDQTPGTVAIRKINEKLRDDQRINISFLKIGDGVTLCFKK  680
            PTDQ PGT+AIRK+NEKL+DDQRINISFL+IGDG++LCFKK
Sbjct  181  PTDQKPGTLAIRKMNEKLKDDQRINISFLRIGDGLSLCFKK  221
```

Figure 3.2 Nucleotide sequence (A) and BlastX results (B) of an EST from hemocyte cDNA library of *P. monodon* that significantly matched *F. chinensis OMT*. The positions of sequences primers were illustrated in boldface and underlined. The putative start codon was illustrated in boldface.

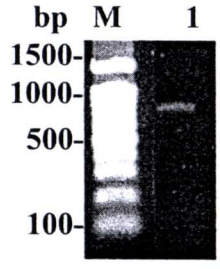

Figure 3.3 Agarose gel electrophoresis showing 3′ RACE-PCR of *PmCOMT* (lanes 1). A 100 bp DNA ladder (lane M) was used as the DNA marker.

A.

GCTCTGGTGGAGTCATCGCCTTCGACAACACACTTTGGGATGGAGCTGTGATTGACCCCACTGATCAAA
CCCCTGGCACAGTGGCTATTAGGAAAATTAACGAAAAACTGAGAGATGACCAGAGAATCAATATTTCCT
TCCTGAAAATTGGTGACGGCGTGACTCTATGTTTTAAAAAATGAATATTTTTTCCCCCCGAAAAGGACC
CCTCCTCCCCAATAATAAATTCCTGGTTCCAGAAAAAGGTTAAGAACTTTAACAAGGATGGAACAATTG
ACCCCCCATACCATACACCTATGAAAAGGTTTTAAAACAATTGGCCGGCCTTTACCGGCCCCTCCTGGC
ACGGGGGGCCAAAAACATCCTCCATTGGCCCCGAATTTACCGAAAAATCTTATTAAAACCCCTTTTAAA
ACCAGGGGCCTGTAACTGGGAATGGCTGAATATGGATTTCCTTTCCCCCCAAAGGTCCCTGGCCAGAAT
TGATTCTAAATAAAAATTGGCAACAACTTTAAATGGAAGTTTCCTCCGGTCCATTGCCACTTGGCCAAA
CTACCGCCAAATACTAACTTTTAATGGACCAAATGGAAATGGTAAAACCACCCCCCCCCCCCTATATTA
TCCCCGAATTAAAATCCTACGTCTCGAANAAAAAAANNAAAAAAAAAAAAAAAA**GGTCTCCGGCGTGAG**
ACCACCGGCTGGCCCATAGAGAGAGCCAATATAAACCAAAT

Figure 3.4 Nucleotide sequence of the 3′UTR of *PmCOMT* generated by the primary 3′ RACE-PCR. The positions of sequencing primers are illustrated in boldface and underlined.

A.

TTGACAGGTTCCTGAAG**ATG**TCTTCTCTGAAAAGTTACCATAATCCCGATCCTTTGGTGCAGTATTGTG
TAAATCATTCATTGAGATTAACCGACGCGCAAAAACGACTGAATGATGTAACTCTGCAGCACCGTAGAG
CGGCGATGTTGGGGGCACCTGAGGTTCTGCAGCTCAATGCCAACATAATGCAGGCTATCGGGGCAAAGA
AAGTACTAGACATTGGGGTGTTCACAGGCGCCAGTTCACTCTCTGCTGCTCTGGCACTGCCTCCGAATG
GCAAGGTCCACGCCCTTGACATAAGTGAAGAGTTTGCCAACATAGGAAAACCGTTCTGGGAGGAAGCTG
GAGTTATCAACAAGATAAGTCTGCACATCGCTCCAGCTGCTGAGACTCTCCAGAAGTTCATTGACGGCG
GAGAAGCTGGCACCTTCGACTATGCTTTCATTGATGCCGACAAAGGGAATTATGAGCTGTACTATGAAC
TTTGCCTCACTCTCTTGCGCTCTGGTGGAGTCATCGCCTTCGACAACACACTTTGGGATGGAGCTGTGA
TTGACCCCACTGATCAAACCCCTGGCACAGTGGCTATTAGGAAAATTAACGAAAAACTGAGAGATGACC
AGAGAATCAATATTTCCTTCCTGAAAATTGGTGACGGCGTGACTCTATGTTTTAAAAAA**TGA**ATATTTT
TTCCCCCCGAAAAGGACCCCTCCTCCCCAATAATAAATTCCTGGTTCCAGAAAAAGGTTAAGAACTTTA
ACAAGGATGGAACAATTGACCCCCCATACCATACACCTATGAAAAGGTTTTAAAACAATTGGCCGGCCT
TTACCGGCCCCTCCTGGCACGGGGGGCCAAAAACATCCTCCATTGGCCCCGAATTTACCGAAAAATCTT
ATTAAAACCCCTTTTAAAACCAGGGGCCTGTAACTGGGAATGGCTGAATATGGATTTCCTTTCCCCCCA
AAGGTCCCTGGCCAGAATTGATTCTAAATAAAAATTGGCAACAACTTTAAATGGAAGTTTCCTCCGGTC
CATTGCCACTTGGCCAAACTACCGCCAAATACTAACTTTTAATGGACCAAATGGAAATGGTAAAACCAC
CCCCCCCCCCCTATATTATCCCCGAATTAAAATCCTACGTCTCGAAAAAAAAAAAAAAAAAAAAAAAA
AAA

B.

O-methyltransferase [Fenneropenaeus chinensis] Length=221 Score = 402 bits (1032), Expect = 2e-110 Identities = 199/221 (90%), Positives = 209/221 (94%), Gaps = 0/221 (0%) Frame = +3

```
Query  18   MSSLKSYHNPDPLVQYCVNHSLRLTDAQKRLNDVTLQHRRAAMLGAPEVLQLNANIMQAI  197
            MSSLKSY N DPLVQYCVNHSLRLTD QKRLND TLQHRRAAMLGAPEVLQLNANIMQAI
Sbjct  1    MSSLKSYDNTDPLVQYCVNHSLRLTDVQKRLNDATLQHRRAAMLGAPEVLQLNANIMQAI  60

Query  198  GAKKVLDIGVFTGASSLSAALALPPNGKVHALDISEEFANIGKPFWEEAGVINKISLHIA  377
            GAKKVLDIGVFTGASSLSAALALPPNGKV+ALDISEEF NIGKP+WEEAGV NKISLHIA
Sbjct  61   GAKKVLDIGVFTGASSLSAALALPPNGKVYALDISEEFTNIGKPYWEEAGVSNKISLHIA  120

Query  378  PAAETLQKFIDGGEAGTFDYAFIDADKGNYELYYELCLTLLRSGGVIAFDNTLWDGAVID  557
            PAAETLQKFID GEAGTFDYAFIDADK +Y+ YYELCL LLR GGVIAFDNTLWDGAVID
Sbjct  121  PAAETLQKFIDAGEAGTFDYAFIDADKESYDRYYELCLILLRPGGVIAFDNTLWDGAVID  180

Query  558  PTDQTPGTVAIRKINEKLRDDQRINISFLKIGDGVTLCFKK  680
            PTDQ PGT+AIRK+NEKL+DDQRINISFL+IGDG++LCFKK
Sbjct  181  PTDQKPGTLAIRKMNEKLKDDQRINISFLRIGDGLSLCFKK  221
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C.

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TTGACAGGTTCCTGAAGATGTCTTCTCTGAAAAGTTACCATAATCCCGATCCTTTGGTGC    60
                  M  S  S  L  K  S  Y  H  N  P  D  P  L  V  Q    15
AGTATTGTGTAAATCATTCATTGAGATTAACCGACGCGCAAAAACGACTGAATGATGTAA   120
  Y  C  V  N  H  S  L  R  L  T  D  A  Q  K  R  L  N  D  V  T    35
CTCTGCAGCACCGTAGAGCGGCGATGTTGGGGGCACCTGAGGTTCTGCAGCTCAATGCCA   180
  L  Q  H  R  R  A  A  M  L  G  A  P  E  V  L  Q  L  N  A  N    55
ACATAATGCAGGCTATCGGGGCAAAGAAAGTACTAGACATTGGGGTGTTCACAGGCGCCA   240
  I  M  Q  A  I  G  A  K  K  V  L  D  I  G  V  F  T  G  A  S    75
GTTCACTCTCTGCTGCTCTGGCACTGCCTCCGAATGGCAAGGTCCACGCCCTTGACATAA   300
  S  L  S  A  A  L  A  L  P  P  N  G  K  V  H  A  L  D  I  S    95
GTGAAGAGTTTGCCAACATAGGAAAACCGTTCTGGGAGGAAGCTGGAGTTATCAACAAGA   360
  E  E  F  A  N  I  G  K  P  F  W  E  E  A  G  V  I  N  K  I   115
TAAGTCTGCACATCGCTCCAGCTGCTGAGACTCTCCAGAAGTTCATTGACGGCGGAGAAG   420
  S  L  H  I  A  P  A  A  E  T  L  Q  K  F  I  D  G  G  E  A   135
CTGGCACCTTCGACTATGCTTTCATTGATGCCGACAAAGGGAATTATGAGCTGTACTATG   480
  G  T  F  D  Y  A  F  I  D  A  D  K  G  N  Y  E  L  Y  Y  E   155
AACTTTGCCTCACTCTCTTGCGCTCTGGTGGAGTCATCGCCTTCGACAACACACTTTGGG   540
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  L  C  L  T  L  L  R  S  G  G  V  I  A  F  D  N  T  L  W  D  175
ATGGAGCTGTGATTGACCCCACTGATCAAACCCCTGGCACAGTGGCTATTAGGAAAATTA  600
  G  A  V  I  D  P  T  D  Q  T  P  G  T  V  A  I  R  K  I  N  195
ACGAAAAACTGAGAGATGACCAGAGAATCAATATTTCCTTCCTGAAAATTGGTGACGGCG  660
  E  K  L  R  D  D  Q  R  I  N  I  S  F  L  K  I  G  D  G  V  215
TGACTCTATGTTTTAAAAAATGAATATTTTTTCCCCCCGAAAAGGACCCCTCCTCCCCAA  720
  T  L  C  F  K  K  *                                          221
TAATAAATTCCTGGTTCCAGAAAAAGGTTAAGAACTTTAACAAGGATGGAACAATTGACC  780
CCCCATACCATACACCTATGAAAAGGTTTTAAAACAATTGGCCGGCCTTTACCGGCCCCT  840
CCTGGCACGGGGGGCCAAAAACATCCTCCATTGGCCCCGAATTTACCGAAAAATCTTATT  900
AAAACCCCTTTTAAAACCAGGGGCCTGTAACTGGGAATGGCTGAATATGGATTTCCTTTC  960
CCCCCAAAGGTCCCTGGCCAGAATTGATTCTAAATAAAAATTGGCAACAACTTTAAATGG  1020
AAGTTTCCTCCGGTCCATTGCCACTTGGCCAAACTACCGCCAAATACTAACTTTTAATGG  1080
ACCAAATGGAAATGGTAAAACCACCCCCCCCCCCCTATATTATCCCCGAATTAAAATCCT  1140
ACGTCTCGAAAAAAAAAAAAAAAAAAAAAAAAAAAA                          1176
```

Figure 3.5 The full lenght cDNA of *PmCOMT* (A) and BlastX result (B) of *PmCOMT* against the previously deposited sequences in the GenBank. (C). Nucleotide sequences illustrating organization of *PmCOMT* gene. Coding nucleotides and deduced amino acids of each exon are capitalized. Start and stop codons are illustrated in boldface and underlined. The catechol-*O*-methyltransferase domain is highlighted. Polyadenylation additional signals (AATAAA) are underlined.

3.1.3 Characterization of full length cDNA of *PmFAMeT*

The partial nucleotide sequence of *PmFAMeT* in this thesis was obtained from RT-PCR using the primers designed from the partial *FAMeT* gene sequence of *P. monodon* previously deposited in the GenBank (Accession no. AAX24112). Nucleotide sequence of the amplification product significantly match *farnesoic acid O-methyltransferase* of *P. monodon* (*E*-value = 4e-147, Fig 3.6).

Both 5′ and 3′ RACE-PCR of *PmFAMeT* were carried out (Fig. 3.7A). The discrete amplification bands of 254 and 1333 bp from the respective reactions were cloned and sequenced (Fig. 3.7B and C). Nucleotide sequences of RACE-PCR fragments and the original EST were assembled (Fig. 3.8A). Sequence similarity analysis revealed that the full length cDNA of *PmFAMeT* was already obtained and it significantly matched that of *L. vannamei* (*E*-value = 2e-164)

A.

```
CCGCTTCAGGGACATCAAGGGCAAGACCCTCCGGTTCCAGGTGAAGGCTGCCCATGATGCCCACCTTGC
CCTGACCTCAGGGGAGGAGGAGACTGACCCTATGCTGGAGGTGTTCATTG
```
GCGGATGGGAAGGCGCTGC CTCTGCC
```
ATTAGGTTCAAGAAAGCTGATGACTTAACTAAAGTGGACACCCCTGACATCCTGAGTGAAGA
AGAATATCGTGAATTCTGGGTTGCCTTCGACCATGATGTTATCCGTGTTGGCAAGGGAGGCGAGTGGGA
GCCATTCATGAGTGCCACCATTCCAGAGCCTTTCGACATCACTCATTACGGCTACAGTACTGGCTGGGG
```

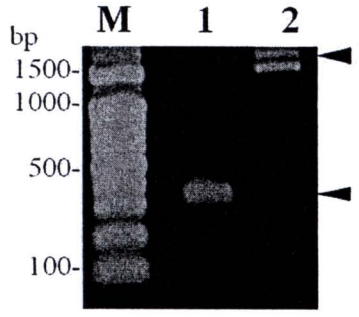
TGCTGTTGGCTGGTGGCAGTTCCATAGTGAGGTGCACTTCCAAACTGAGGACTGCCTCACGTACAACTT
CATTCCTGTGTACGGTGACACCTTTACCTTCAGTGTTGCCTGTAGCAATGATGCCCATCTGGCACTCAC
CTCTGGCCCTGAGGAGACCACACCCATGTATGAAGTGTTC***ATTGGTGGTTGGGAAAACCAGCACT***CTGC
CATTCGTCTCAGCAAGGAGGGAAGGGGATCTGGTGAGGACATGATCAAGGTCGACACCCCCGACGTTGT
CTGCTGCGAAGAGGAGAGGAAGTTCTACGTCAGCTTCAAGGACGGCCATATCAGGGTGGGATACCAGGA
CAGTGATCCCTTC

B.

```
Farnesoic acid O-methyltransferase [Penaeus monodon]
Length=280
 Score =  523 bits (1347),  Expect = 4e-147
 Identities = 245/250 (98%), Positives = 247/250 (98%), Gaps = 0/250 (0%)
 Frame = +2

Query  71   MGESWASYRTDENKQYRFKDIKDKTLRFQGKAAHDAHLALTSGEEETDPMLEVFIGGWEG  250
            MG+SWASY TDENKQYRF+DIK KTLRFQ KAAHDAHLALTSGEEETDPMLEVFIGGWEG
Sbjct  1    MGDSWASYGTDENKQYRFRDIKGKTLRFQVKAAHDAHLALTSGEEETDPMLEVFIGGWEG  60

Query  251  AASAIRFKKADDLTKVDTPDILSEEEYREFWVAFDHDVIRVGKGGEWEPFMSATIPEPFD  430
            AASAIRFKKADDLTKVDTPDILSEEEYREFWVAFDHDVIRVGKGGEWEPFMSATIPEPFD
Sbjct  61   AASAIRFKKADDLTKVDTPDILSEEEYREFWVAFDHDVIRVGKGGEWEPFMSATIPEPFD  120

Query  431  ITHYGYSTGWGAVGWWQFHSEVHFQTEDCLTYNFIPVYGDTFTFSVACSNDAHLALTSGP  610
            ITHYGYSTGWGAVGWWQFHSEVHFQTEDCLTYNFIPVYGDTFTFSVACSNDAHLALTSGP
Sbjct  121  ITHYGYSTGWGAVGWWQFHSEVHFQTEDCLTYNFIPVYGDTFTFSVACSNDAHLALTSGP  180

Query  611  EETTPMYEVFIGGWENQHSAIRLSKEGRGSGEDMIKVDTPDVVCCEEERKFYVSFKDGHI  790
            EETTPMYEVFIGGWENQHSAIRLSKEGRGSGEDMIKVDTPDVVCCEEERKFYVSFKDGHI
Sbjct  181  EETTPMYEVFIGGWENQHSAIRLSKEGRGSGEDMIKVDTPDVVCCEEERKFYVSFKDGHI  240

Query  791  RVGYQDSDPF  820
            RVGYQDSDPF
Sbjct  241  RVGYQDSDPF  250


 Score =  132 bits (331),  Expect = 3e-29
 Identities = 69/136 (50%), Positives = 86/136 (63%), Gaps = 6/136 (4%)
 Frame = +2

Query  92   YRTDENKQYRFKDIKDKTLRFQGKAAHDAHLALTSGEEETDPMLEVFIGGWEGAASAIRF  271
            ++T++   Y F  +   T  F    ++DAHLALTSG EET PM EVFIGGWE   SAIR
Sbjct  144  FQTEDCLTYNFIPVYGDTFTFSVACSNDAHLALTSGPEETTPMYEVFIGGWENQHSAIRL  203

Query  272  KK-----ADDLTKVDTPDILSEEEYREFWVAFDHDVIRVGKGGEWEPFMSATIPEPFDIT  436
             K      +D+ KVDTPD++  EE R+F+V+F    IRVG     +PFM  T PEP+ IT
Sbjct  204  SKEGRGSGEDMIKVDTPDVVCCEEERKFYVSFKDGHIRVGYQDS-DPFMEWTDPEPWKIT  262

Query  437  HYGYSTGWGAVGWWQF  484
            H GY TGWGA G W+F
Sbjct  263  HIGYCTGWGATGKWKF  278
```

Figure 3.6 (A) Partial nucleotide sequence of *FAMeT* amplified from the first strand cDNA template from ovaries of *P. monodon.* The positions of sequencing primers were illustrated in boldface and underlined. (B). BlastX results of nucleotide sequence of an EST exhibiting significant similarity with *FAMeT* of *F. chinensis.*

A.



B.

GAATGCTTGGGTGCTGCCGGTGTGCTGTGTCTGGATTGTGCTCTGCTCGCAAGTAACTCGGGATGGGCG
AGAGCTGGGCTTCCTACCGTACAGATGAGAACAAGCAGTACCGCTTCAAGGACATCAAGGACAAGACCC
TCCGGTTCCAGGGGAAGGCTGCCCATGATGCCCACCTTGCCCTGACCTCAGGAGAAGAG**GAGACTGACC**
CTATGCTGGAGGTGTTCA

C.

CGTCTCAGCAAGGAGTGAAGGGGATCTGGCGAGGACATGATCAAGGTCGACACCCCCGACGTTGTTTGC
TGCGAAGAGGAGAGGAAGTTTTACGTCAGCTTCAAGGACGGCCATATCAGGGTGGGATACCAGGACAGT
GATCCCTTCATGGAGTGGACTGACCCTGAGCCATGGAAGATCACCCACATTGGTTACTGCACAGGCTGG
GGAGCAACTGGAAAGTGGAAGTTCGAATTTTAAGTCCTGCTTTGTGGCTTTGTTCACGGAATGCACCAA
CCACTAATTTTTTTTTCTTTTCTTTTTTATTGTATTTTCTCATGGGACAAATGGTTTTACATGTTTTTG
GAGTATCTTTTTCCTCATGATGTATGTTTTTTCAGGTGAGGCAACATCAGTTTTGCATTTATTAACCTT
CAAAAGTAATATTAATTTTAAAAGTGCAAAGTGCATAGATTAATAAGGGCTACCAGAAGTATGCTCTTT
ATTTTTGCAAGCAGACATTGAGTTTATGATGTTATTGTAACAAGGTTATTCAGAGTAATAGTGAATGAA
AAAATGTATCATGACGATTCATTCAAATTATTAAACATTAAACAAATAAAAGGAATTTTACAGAAAAAA
AAAAAAAAAAAAAAAAAA

Figure 3.7 (A). 5′- and 3′RACE-PCR of *PmFAMeT* (lanes 1 and 2). A 100 bp DNA ladder (lanes m) was used as the DNA marker. Nucleotide sequence of 5′UTR (B) and 3′ UTR (C) of *PmFAMeT* generated by the secondary 5′ RACE PCR and the primary 3′ RACE-PCR, respectively. The positions of sequencing primers are illustrated in boldface and underlined.

A.

GAATGCTTGGGTGCTGCCGGTGTGCTGTGTCTGGATTGTGCTCTGCTCGCAAGTAACTCGGG**ATG**GGCG
AGAGCTGGGCTTCCTACCGTACAGATGAGAACAAGCAGTACCGCTTCAAGGACATCAAGGACAAGACCC
TCCGGTTCCAGGGGAAGGCTGCCCATGATGCCCACCTTGCCCTGACCTCAGGAGAAGAG**GAGACTGACC**
CTATGCTGGAGGTGTTCATTGGCGGATGGGAAGGCGCTGCCTCTGCCATTAGGTTCAAGAAAGCTGATG
ACTTAACTAAAGTGGACACCCCTGACATCCTGAGTGAAGAAGAATATCGTGAATTCTGGGTTGCCTTCG
ACCATGATGTTATCCGTGTTGGCAAGGGAGGCGAGTGGGAGCCATTCATGAGTGCCACCATTCCAGAGC
CTTTCGACATCACTCATTACGGCTACAGTACTGGCTGGGGTGCTGTTGGCTGGTGGCAGTTCCATAGTG
AGGTGCACTTCCAAACTGAGGACTGCCTCACGTACAACTTCATTCCTGTGTACGGTGACACCTTTACCT
TCAGTGTTGCCTGTAGCAATGATGCCCATCTGGCACTCACCTCTGGCCCTGAGGAGACCACACCCATGT
ATGAAGTGTTCATTGGTGGTTGGGAAAACCAGCACTCTGCCATT**CGTCTCAGCAAGGAGGGAAGGGGAT**
CTGGCGAGGACATGATCAAGGTCGACACCCCCGACGTTGTTTGCTGCGAAGAGGAGAGGAAGTTTTACG
TCAGCTTCAAGGACGGCCATATCAGGGTGGGATACCAGGACAGTGATCCCTTCATGGAGTGGACTGACC
CTGAGCCATGGAAGATCACCCACATTGGTTACTGCACAGGCTGGGGAGCAACTGGAAAGTGGAAGTTCG
AATTTTAAGTCCTGCTTTGTGGCTTTGTTCACGGAATGCACCAACCACTAATTTTTTTTTCTTTTCTTT

TTTATTGTATTTTCTCATGGGACAAATGGTTTTACATGTTTTTGGAGTATCTTTTTCCTCATGATGTAT
GTTTTTTCAGGTGAGGCAACATCAGTTTTGCATTTATTAACCTTCAAAAGTAATATTAATTTTAAAAGT
GCAAAGTGCATAGATTAATAAGGGCTACCAGAAGTATGCTCTTTATTTTTGCAAGCAGACATTGAGTTT
ATGATGTTATTGTAACAAGGTTATTCAGAGTAATAGTGAATGAAAAAATGTATCATGACGATTCATTCA
AATTATTAAACATTAAACAAATAAAAGGAATTTTACAGAAAAAAAAAAAAAAAAAAAAAAAA

B.

```
farnesoic acid O-methyltransferase [Litopenaeus vannamei]
Length=280

 Score =  584 bits (1505),  Expect = 1e-164
 Identities = 264/280 (94%), Positives = 274/280 (97%), Gaps = 0/280 (0%)
 Frame = +3

Query  63   MGESWASYRTDENKQYRFKDIKDKTLRFQGKAAHDAHLALTSGEEETDPMLEVFIGGWEG  242
            MG+SWASY TDENKQYRF+DIK KTLRFQ KAAHDAH+ALTSGEEETDPMLEVFIGGWEG
Sbjct  1    MGDSWASYGTDENKQYRFRDIKGKTLRFQVKAAHDAHIALTSGEEETDPMLEVFIGGWEG  60

Query  243  AASAIRFKKADDLTKVDTPDILSEEEYREFWVAFDHDVIRVGKGGEWEPFMSATIPEPFD  422
            AASAIRFKKADDL KVDTPDILSEEEYREFW+AFDHDV+RVGKG EWEPFMSATIPEPFD
Sbjct  61   AASAIRFKKADDLAKVDTPDILSEEEYREFWIAFDHDVVRVGKGAEWEPFMSATIPEPFD  120

Query  423  ITHYGYSTGWGAVGWWQFHSEVHFQTEDCLTYNFIPVYGDTFTFSVACSNDAHLALTSGP  602
            ITHYGYSTGWGA GWWQFHSE+HFQTEDCLTYNFIPVYGDTF+FSVACSNDAHLALTSGP
Sbjct  121  ITHYGYSTGWGATGWWQFHSEIHFQTEDCLTYNFIPVYGDTFSFSVACSNDAHLALTSGP  180

Query  603  EETTPMYEVFIGGWENQHSAIRLSKEGRGSGEDMIKVDTPDVVCCEEERKFYVSFKDGHI  782
            EET+PMYEVFIGGWENQHSAIRLSKEGRGSGEDMIKVDTPDVVCCEEERKFY+SFKDGHI
Sbjct  181  EETSPMYEVFIGGWENQHSAIRLSKEGRGSGEDMIKVDTPDVVCCEEERKFYISFKDGHI  240

Query  783  RVGYQDSDPFMEWTDPEPWKITHIGYCTGWGATGKWKFEF  902
            RVGYQDSDPFMEWTDPEPWKITHIGYCTGWGA+GKWKFEF
Sbjct  241  RVGYQDSDPFMEWTDPEPWKITHIGYCTGWGASGKWKFEF  280
```

Figure 3.8 The full length cDNA of *PmFAMeT* combined from 5′RACE-PCR, 3′RACE-PCR and an original EST (A). The positions of sequencing primers are illustrated in boldface and underlined. (B). Results from BlastX indicating significant similarity between *P. monodon FAMeT* and that of *L. vannamei.*

Kuballa et al. (2006) reported the existent of multiple isoforms of putative *FaMeT* from six crustacean species including *P. monodon.* Accordingly, a pair of primers covering the region that exhibited sequence polymorphism was designed. The amplification product was cloned and sequences (Fig. 3.9) and two different sequences with the presence or absence of a 15 bp indel (AGGGAAGGGGATCTG) were found (Fig. 3.10). This resulted in the identification of two different isoforms of *PmFAMeT.*

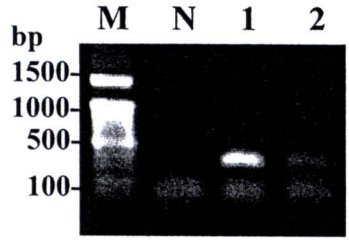


Figure 3.9 RT-PCR of *PmFAMeT* (lanes 1 and 2). Lanes M and N are a 100 bp DNA ladder and the negative control (without the cDNA template), respectively.

A.

```
CCACCATTCCAGAGCCTTTCGACATCACTCATTACGGCTACAGTACTGGCTGGGGTGCTGTTGGCTGGT
GGCAGTTCCATAGTGAGGTACGCTTCCAAACTGAGGACTGCCTCACGTACAACTTCATTCCTGTGTACG
GTGACACCTTTACCTTCAGTGTTGCCTGTAGCAATGATGCCCATCTGGCACTCACCTCTGGCCCTGAGG
AGACCACACCCATGTATGAAGTGTTCATTGGTGGTTGGGAAAACCAGCACTCTGCCATTCGTCTCAGCA
AGGAGGGAAGGGGATCTGGCGAGGACATGATCAAGGTCGACACCCCCGACGTTGTTTGCTGCGAAGAGG
```

B.

```
CCACCATTCCAGAGCCTTTCGACATCACTCATTACGGCTACAGTACTGGCTGGGGTGCTGTTGGCTGGT
GGCAGCTCCATAGTGAGGTGCACTTCCAAACTGAGGACTGCCTCACGTACAACTTCATTCCTGTGTACG
GTGACACCTTTACCTTCAGTGTTGCCTGTAGCAATGATGCCCATCTGGCACTCACCTCTGGCCCTGAGG
AGACCACACCCATGTATGAAGTGTTCATTGGTGGTTGGGAAAACCAGCACTCTGCCGTTCGTCTCAGCA
AGGGCGAGGACATGATCAAGGTCGACACCCCCGACGTTGTTTGCTGCGAAGAGG
```

C.

```
PmFAMeT-l CCACCATTCCAGAGCCTTTCGACATCACTCATTACGGCTACAGTACTGGCTGGGGTGCTG
PmFAMeT-s CCACCATTCCAGAGCCTTTCGACATCACTCATTACGGCTACAGTACTGGCTGGGGTGCTG
          ************************************************************
PmFAMeT-l TTGGCTGGTGGCAGTTCCATAGTGAGGTACGCTTCCAAACTGAGGACTGCCTCACGTACA
PmFAMeT-s TTGGCTGGTGGCAGCTCCATAGTGAGGTGCACTTCCAAACTGAGGACTGCCTCACGTACA
            ************** ************* * ****************************
PmFAMeT-l ACTTCATTCCTGTGTACGGTGACACCTTTACCTTCAGTGTTGCCTGTAGCAATGATGCCC
PmFAMeT-s ACTTCATTCCTGTGTACGGTGACACCTTTACCTTCAGTGTTGCCTGTAGCAATGATGCCC
          ************************************************************
PmFAMeT-l ATCTGGCACTCACCTCTGGCCCTGAGGAGACCACACCCATGTATGAAGTGTTCATTGGTG
PmFAMeT-s ATCTGGCACTCACCTCTGGCCCTGAGGAGACCACACCCATGTATGAAGTGTTCATTGGTG
          ************************************************************
PmFAMeT-l GTTGGGAAAACCAGCACTCTGCCATTCGTCTCAGCAAGGAGGGAAGGGGATCTGGCGAGG
PmFAMeT-s GTTGGGAAAACCAGCACTCTGCCGTTCGTCTCAGCAAGG---------------GCGAGG
            ********************** ***************              ******
PmFAMeT-l ACATGATCAAGGTCGACACCCCCGACGTTGTTTGCTGCGAAGAGG
PmFAMeT-s ACATGATCAAGGTCGACACCCCCGACGTTGTTTGCTGCGAAGAGG
          *********************************************
```

Figure 3.10 Nucleotide sequences of *PmFAMeT-l* (A) and *PmFAMeT-s* (B) obtained by sequencing of the RT-PCR product using primers flanking the expected indel. (C) Pairwise alignment indicating an indel of 15 nucleotide corresponding to a presence/absence of a pentapeptide in different isoforms of *PmFAMeT*.

The full length cDNA of *PmFAMeT* combined from 5′ and 3′ RACE PCR and EST sequences were found and they were 1296 and 1311 bp in length for the short (*PmFAMeT-s*) and the long form (*PmFAMeT-l*), respectively (Fig. 3.11). These transcripts were composed of an open reading frame (ORF) of 828 and 843 bp corresponding to the deduced proteins of 275 and 280 amino acids with the identical 5′ and 3′UTR of 69 and 315 bp (excluding the poly A tail), respectively. A pentapeptide (EGRGS) was found in *PmFAMeT-l* but not in *PmFAMeT-s*. The calculated p*I* and molecular weight of the deduced PmFAMeT-l and PmFAMeT-s protein was 4.69 and 32.1 kDa and 4.67 and 31.6, respectively. The deduced PmFAMeT-l and PmFAMeT-s proteins contained 2 crustacean FAMeT domains at positions 8 and 138 (*E*-value = 2.33e-32) and 144 and 278 (*E*-value = 2.33e-32) and 8 and 138 (*E*-value = 2.33e-32) and 144 and 273 (*E*-value = 2.33e-32), respectively (Fig. 3.12). *PmFAMeT* is significantly matched *FAMeT* of *L. vannamei* (*E*-value = 2e-157, GenBank accession no. AAX24111).

A.

ATGGGCGAGAGCTGGGCTTCCTACCGTACAGATGAGAACAAGCAGTACCGCTTCAAGGACATCAAGGAC
AAGACCCTCCGGTTCCAGGGGAAGGCTGCCCATGATGCCCACCTTGCCCTGACCTCAGGAGAAGAGGAG
ACTGACCCTATGCTGGAGGTGTTCATTGGCGGATGGGAAGGCGCTGCCTCTGCCATTAGGTTCAAGAAA
GCTGATGACTTAACTAAAGTGGACACCCCTGACATCCTGAGTGAAGAAGAATATCGTGAATTCTGGGTT
GCCTTCGACCATGATGTTATCCGTGTTGGCAAGGGAGGCGAGTGGGAGCCATTCATGAGTGCCACCATT
CCAGAGCCTTTCGACATCACTCATTACGGCTACAGTACTGGCTGGGGTGCTGTTGGCTGGTGGCAGTTC
CATAGTGAGGTGCACTTCCAAACTGAGGACTGCCTCACGTACAACTTCATTCCTGTGTACGGTGACACC
TTTACCTTCAGTGTTGCCTGTAGCAATGATGCCCATCTGGCACTCACCTCTGGCCCTGAGGAGACCACA
CCCATGTATGAAGTGTTCATTGGTGGTTGGGAAAACCAGCACTCTGCCATTCGTCTCAGCAAGGAGGGA
AGGGGATCTGGCGAGGACATGATCAAGGTCGACACCCCCGACGTTGTTTGCTGCGAAGAGGAGAGGAAG
TTTTACGTCAGCTTCAAGGACGGCCATATCAGGGTGGGATACCAGGACAGTGATCCCTTCATGGAGTGG
ACTGACCCTGAGCCATGGAAGATCACCCACATTGGTTACTGCACAGGCTGGGGAGCAACTGGAAAGTGG
AAGTTCGAATTT**TAA**

B.

ATGGGCGAGAGCTGGGCTTCCTACCGTACAGATGAGAACAAGCAGTACCGCTTCAAGGACATCAAGGAC
AAGACCCTCCGGTTCCAGGGGAAGGCTGCCCATGATGCCCACCTTGCCCTGACCTCAGGAGAAGAGGAG
ACTGACCCTATGCTGGAGGTGTTCATTGGCGGATGGGAAGGCGCTGCCTCTGCCATTAGGTTCAAGAAA
GCTGATGACTTAACTAAAGTGGACACCCCTGACATCCTGAGTGAAGAAGAATATCGTGAATTCTGGGTT
GCCTTCGACCATGATGTTATCCGTGTTGGCAAGGGAGGCGAGTGGGAGCCATTCATGAGTGCCACCATT
CCAGAGCCTTTCGACATCACTCATTACGGCTACAGTACTGGCTGGGGTGCTGTTGGCTGGTGGCAGTTC
CATAGTGAGGTGCACTTCCAAACTGAGGACTGCCTCACGTACAACTTCATTCCTGTGTACGGTGACACC
TTTACCTTCAGTGTTGCCTGTAGCAATGATGCCCATCTGGCACTCACCTCTGGCCCTGAGGAGACCACA
CCCATGTATGAAGTGTTCATTGGTGGTTGGGAAAACCAGCACTCTGCCATTCGTCTCAGCAAGGGCGAG
GACATGATCAAGGTCGACACCCCCGACGTTGTTTGCTGCGAAGAGGAGAGGAAGTTTTACGTCAGCTTC
AAGGACGGCCATATCAGGGTGGGATACCAGGACAGTGATCCCTTCATGGAGTGGACTGACCCTGAGCCA
TGGAAGATCACCCACATTGGTTACTGCACAGGCTGGGGAGCAACTGGAAAGTGGAAGTTCGAATTT**TAA**

Figure 3.11 The full length ORF of *PmFAMeT-l* (A), *PmFAMeT-s* (B).

A.

```
GAATGCTTGGGTGCTGCCGGTGTGCTGTGTCTGGATTGTGCTCTGCTCGCAAGTAACTCG    60
GGATGGGCGAGAGCTGGGCTTCCTACCGTACAGATGAGAACAAGCAGTACCGCTTCAAGG   120
   M  G  E  S  W  A  S  Y  R  T  D  E  N  K  Q  Y  R  F  K      19
ACATCAAGGACAAGACCCTCCGGTTCCAGGGGAAGGCTGCCCATGATGCCCACCTTGCCC   180
D  I  K  D  K  T  L  R  F  Q  G  K  A  A  H  D  A  H  L  A      39
TGACCTCAGGAGAAGAGGAGACTGACCCTATGCTGGAGGTGTTCATTGGCGGATGGGAAG   240
L  T  S  G  E  E  E  T  D  P  M  L  E  V  F  I  G  G  W  E      59
GCGCTGCCTCTGCCATTAGGTTCAAGAAAGCTGATGACTTAACTAAAGTGGACACCCCTG   300
G  A  A  S  A  I  R  F  K  K  A  D  D  L  T  K  V  D  T  P      79
ACATCCTGAGTGAAGAAGAATATCGTGAATTCTGGGTTGCCTTCGACCATGATGTTATCC   360
D  I  L  S  E  E  E  Y  R  E  F  W  V  A  F  D  H  D  V  I      99
GTGTTGGCAAGGGAGGCGAGTGGGAGCCATTCATGAGTGCCACCATTCCAGAGCCTTTCG   420
R  V  G  K  G  G  E  W  E  P  F  M  S  A  T  I  P  E  P  F     119
ACATCACTCATTACGGCTACAGTACTGGCTGGGGTGCTGTTGGCTGGTGGCAGTTCCATA   480
D  I  T  H  Y  G  Y  S  T  G  W  G  A  V  G  W  W  Q  F  H     139
GTGAGGTGCACTTCCAAACTGAGGACTGCCTCACGTACAACTTCATTCCTGTGTACGGTG   540
S  E  V  H  F  Q  T  E  D  C  L  T  Y  N  F  I  P  V  Y  G     159
ACACCTTTACCTTCAGTGTTGCCTGTAGCAATGATGCCCATCTGGCACTCACCTCTGGCC   600
D  T  F  T  F  S  V  A  C  S  N  D  A  H  L  A  L  T  S  G     179
CTGAGGAGACCACACCCATGTATGAAGTGTTCATTGGTGGTTGGGAAAACCAGCACTCTG   660
P  E  E  T  T  P  M  Y  E  V  F  I  G  G  W  E  N  Q  H  S     199
CCATTCGTCTCAGCAAGGAGGGAAGGGGATCTGGCGAGGACATGATCAAGGTCGACACCC   720
A  I  R  L  S  K  E  G  R  G  S  G  E  D  M  I  K  V  D  T     219
CCGACGTTGTTTGCTGCGAAGAGGAGAGGAAGTTTTACGTCAGCTTCAAGGACGGCCATA   780
P  D  V  V  C  C  E  E  E  R  K  F  Y  V  S  F  K  D  G  H     239
TCAGGGTGGGATACCAGGACAGTGATCCCTTCATGGAGTGGACTGACCCTGAGCCATGGA   840
I  R  V  G  Y  Q  D  S  D  P  F  M  E  W  T  D  P  E  P  W     259
AGATCACCCACATTGGTTACTGCACAGGCTGGGGAGCAACTGGAAAGTGGAAGTTCGAAT   900
K  I  T  H  I  G  Y  C  T  G  W  G  A  T  G  K  W  K  F  E     279
TTTAAGTCCTGCTTTGTGGCTTTGTTCACGGAATGCACCAACCACTAATTTTTTTTTCTT   960
F  *                                                           280
TTCTTTTTTATTGTATTTTCTCATGGGACAAATGGTTTTACATGTTTTTGGAGTATCTTT  1020
TTCCTCATGATGTATGTTTTTTCAGGTGAGGCAACATCAGTTTTGCATTTATTAACCTTC  1080
AAAAGTAATATTAATTTTAAAAGTGCAAAGTGCATAGATTAATAAGGGCTACCAGAAGTA  1140
TGCTCTTTATTTTTGCAAGCAGACATTGAGTTTATGATGTTATTGTAACAAGGTTATTCA  1200
GAGTAATAGTGAATGAAAAAATGTATCATGACGATTCATTCAAATTATTAAACATTAAAC  1260
AAATAAAAGGAATTTTACAGAAAAAAAAAAAAAAAAAAAAAAA                   1310
```

B.

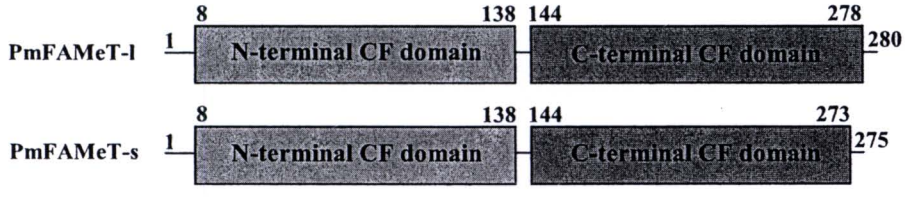


Figure 3.12 The full length cDNA and deduced amino acid sequences of *PmFAMeT-l* and *PmFAMeT-s* (A) which are different according to a pentapeptide (EGRGS, underlined). The start and stop codons are boldfaced and underlined. The poly A additional signal (AATAAA) is boldfaced, italicized and underlined. Crustacean FAMeT domains (positions 8 - 138 and 144 – 278) in the deduced FAMeT are highlighted. The diagram representing PmFAMeT is also illustrated (B).

Further analysis of the protein sequence, with NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos/), for potential posttranslational modifications shows multiple high scoring (score > 0.8) sites for possible phosphorylation at 6 threonine, 3 tyrosine and 6 or 5 serine side chains within the deduced PmFAMeT-l and PmFAMeT-s proteins, respectively.

3.1.4 Characterization of the full length cDNA of *PmBr-c* genes

3.1.4.1 *PmBr-cZ1*

The partial nucleotide sequence of *PmBr-cZ1* was initially obtained from EST analysis of the ovarian cDNA library. This EST significantly match *Broad complex core protein isoform 6* of *A. mellifera* (*E*-value = 1e-110, Fig 3.13).

A.

```
AAGCAGTGGTATCAACGCAGAGTACGCGGGGACAGAAAACGGGTACCTCGATCACCACAGAAAACTGGT
TCGCACGTGGTCCGATCATTTTTGAAGAGAAGGTGGTTCTGCAGACAGCATGGAGGAGGGCTACCTAGC
ACTGCGATGGAACAACCACAACACCATCTTCACCAAGATCCTCACCCTCCTTAGGGAGCAGGAGGCTTA
TGTGGATGTTTCCTTAGCTTGTGCGGGAAGATTATATCCTGCACACAAATTTGTACTTTCTACATGTAG
TGAGTATTTCAAGGAAATGTTTTCCAAGAACCCATGTAAGCATCCCATTGTTTTCATGAAGGATGTCTC
AACTAAGGACATGGAGGCCTTGCTGGACTTCATGTACAAGGGTGAGGTCCATGTACCACAAAGCGAGTT
GGGTTCATTGCTGCGTACAGCTGAAGGGCTTCAGGTAAAAGGCCTTGCTGTACCTGATGACTCTCCTCG
TGGTTCCTCTACCACCCCTATTGTGCCTTCTGCCTCGTCCGTCCCACGCTCACCTCCGCCCAGTCTTAT
GGCTCCAATGCATATGCGGGGTAAACGCAAAAGGCCACCAGAATCGGCTAAAAAGGATGACCCTCCAAA
GTTGACTTTACGTCCTGACCTTGGACCCCCGGCCACCAACCGCTCACGCATGAGGAACAGACCATCAGG
CATGCCAGAACTGAAGAGAATTAAGAGAGAGGAACATAGTGCGGCAAATGTGGGCACTATTGAGCCTGG
AGAGGTTCCAGGATCTCCAAGTCCAACACCAAGTAGTCACAACAGCGATGAACAGTCACAGAACCTTGC
CCATAAGATCAAGACCGAAAGATCAGAATACTCTAAAGAAGAAGCAGAAGACCTGGATGAGGATGAAGG
GGTGGCAGGAGAAGTAATGTCTGGGGAAGAAGAGCAGGAACAAGAACCAGAAGAGGAAGAAGAGGAAGA
GGAAGAAGAAGAGGAAGGAGCATTAGGGGAAGGGGAAGGCCTCTCCCACGGTGTTCTCTCAGATGTTGA
GGATGGCTATGAACAATCTAACTCTTCCCTTCCCAATTCTGACATATCCACAACGGAACTGCTACAGGT
TGATTTGAGTGAGGACGGGACACAGTTCATAATCGGTCCTGGTGGCTTTGGAGATATGATGTCAAGAAC
TTCGTCACTAGCAGGAGATGATGAGAGAAATGGTGACAAAGAGCAGAAGAAGCCATTTGTTTGTCCTCT
CTGTGGGCAGTCATTTACACGTCGTGACAACCTTGCCAACCATATCAAGACCCACACCGGTGACCGTCC
GTTTATGTGCCAGTACTGCCACAAGTGCTTCTCAAGGAAGGACTACTTGAAGCAGCATGAACGCATCCA
CACTGGAGAGAAGCCCTACCCCTGTGACATCTGTGGTCGTGCATTTACCAGGAAA
```

B.

```
REDICTED: similar to Broad-complex core-protein isoform 6 [Apis mellifera]
Length=454
 Score =  136 bits (342),  Expect = 4e-30
 Identities = 81/206 (39%), Positives = 114/206 (55%), Gaps = 14/206 (6%)
 Frame = +3

Query  165  DSMEEGYLALRWNNHNTIFTKILTLLREQEAYVDVSLACAGRLYPAHKFVLSTCSEYFKE  344
            DS ++    LRWNN     T     LR+ E +VDV+LAC GR   AHK VLS CS YFKE
Sbjct  3    DSQQQ--FCLRWNNFQANITSQFEALRDDEDFVDVTLACDGRRLQAHKVVLSACSPYFKE  60

Query  345  MFSKNPCKHPIVFMKDVSTKDMEALLDFMYKGEVHVPQSELGSLLRTAEGLQVKGLAVPD  524
            +F  NPCKHPI+FM+DV  + +++LL+FMY GEV++ Q+EL + LRTAE LQ++GL
Sbjct  61   LFKTNPCKHPIIFMRDVEFEHLQSLLEFMYAGEVNISQAELPTFLRTAESLQIRGLT---  117

Query  525  DSPRGSSTTPIVpsassvprspppsLMAPMHMRGKRKRPPESA--------KKDDPPKLT  680
```

```
            DS              +++  S    L++P     + K PP S+        K+ D P+++
Sbjct  118  DSQNNQHNNEKHLKTNNIHASNGRGLISPNLEEERSKTPPTSSPPPLKRLCKRSDSPQIS  177

Query  681  LRPDLGPPATNRSRMRNRPSGMPELK  758
               P      A       R RP    P+++
Sbjct  178  -SPVPAAAACASGTPRTRPLIEPQVQ  202

 Score = 41.2 bits (95),  Expect = 0.17
 Identities = 25/86 (29%), Positives = 39/86 (45%), Gaps = 9/86 (10%)
 Frame = +3

Query  1191  GPGGFGDMMSRTS--SLAGDDERNGDKEQK-------KPFVCPLCGQSFTRRDNLANHIK  1343
              G GG G +        S AG+ E N +++           P  CP+C + ++   NL  H++
Sbjct  269   GGGGGGSLEGGMPGPSHAGNTELNQEQQADLRKLHSLDPRPCPVCNRMYSNLSNLRQHMR  328

Query  1344  THTGDRPFMCQYCHKCFSRKDYLKQH  1421
                   +   C  C+K F  K YLK+H
Sbjct  329   LIHNPQSVTCPLCNKPFKTKLYLKRH  354
```

Figure 3.13 Nucleotide sequence (A) and BlastX result (B) of an EST showing significant similarity with *BrC-Z1* of *Apis mellifera*. The positions of sequences primers were illustrated in boldface and underlined.

The 5′ and 2 fragments of 3′ RACE-PCR of this gene homologue was further carried out. The positive amplification product of 158 and 1800 (fragment 1) and 1437 (fragment 2) bp in size were obtained, respectively (Fig. 3.14). The RACE-PCR fragments were cloned and sequenced for both directions (Fig. 3.15). Nucleotide sequences of RACE-PCR fragments and the original EST were assembled.

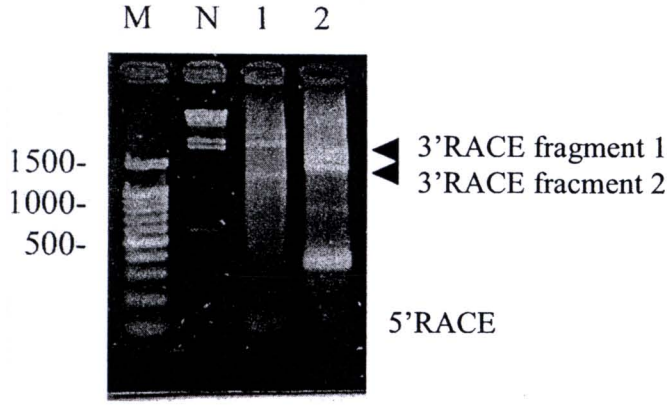


Figure 3.14 Agarose gel electrophoresis showing 5′-and 3′RACE-PCR of *PmBr-cZ1* (lanes 1 and 2, respectively). Lane N = the negative control (without cDNA template). A 100 bp DNA ladder (lanes M) was used as the DNA marker.

A.

GCCACCAACCGCTCACGCATGAGGAACAGACCATCAGGCATGCCAGAACTGAAGAGA
ATTAAGAGAGAGGAACATAGTGCGGCAAATGTGGGCACTATTGAGCCTGGAGAGGTT
CCAGGATCTCCAAGTCCAACACCAAGTAGTCACAACAGCGATGAACAGTCACAGAAC
CTTGCCCATAAGATCAAGACCGAAAGATCAGAATACTCTAAAGAAGAAGCAGAAGAC
CTGGATGAGGATGAAGGGGTGGCAGGAGAAGTAATGTCTGGGGAAGAAGAGCAGGAA
CAAGAACCAGAAGAGGAAGAAGAGGAAGAGGAAGAAGAAGAGGAAGGAGCATTAGGG
GAAGGGGAAGGCCTCTCCCACGGTGTTCTCTCAGATGTTGAGGATGGCTATGAACAA
TCTAACTCTTCCCTTCCCAATTCTGACATATCCACAACGGAACTGCTACAGGTTGAT
TTGAGTGAGGACGGGACACAGTTCATAATCGGTCCTGGTGGCTTTGGAGATATGATG
TCAAGAACTTCGTCACTAGCAGGAGATGATGAGAGAAATGGTGACAAAGAGCAGAAG
AAGCCATTTGTTTGTCCTCTCTGTGGGCAGTCATTTACACGTCGTGACAACCTTGCC
AACCATATCAAGACCCACACCGGTGACCGTCCGTTTATGTGCCAGTACTGCCACAAG
TGCTTCTCAAGGAAGGACTACTTGAAGCAGCATGAACGCATCCACACTGGAGAGAAG
CCCTACCCCTGTGACATCTGTGGTCGTGCATTTACCAGGAAAGGAGGATTGACAGAC
CACATGCGCTGTCATTCTGACTTCCGAGCCTTTTCCTGTGAAACATGTGGCAAGAGC
TTCAAGCAAAAATGTGGTTTGCGCTTCCATAAGAGGAATTATAAACAT**TAA**CCTGCC
ATAAAGATCTCCAACACTTAATTCACTGTTCCATTTATGAGGCGGATATGGATAGCC
TGTCTCTCAGTTAGTTGGATGTGGTCCCTTTAAAAATATGCCAGATAAAGGGCTAAT
TTATGCCCCTGTACTAAGTTAAGTCGTGCCATTTCCTTTATGTTGAGCACCTAATTT
CTGTGTTCAGATAGTCAATTTTACCCTGAGCTTTGTCTATGGCCCTGGCCAGATGTA
TATTTTGGGTATATGATTATGGTTAGTGAAATATCACAACTTGTATATTGCTGCTCA
CATACAGGGAACTTGTGTAAATTTGATTTATGTTTTGACTTCAACAGGCTTAATGTA
TGGCAAAGGTTTTGCAAAATGGAAATTGACTTTTAAATGTTAGACTAATCTCAGGTA
AGCTGTATTAATAACAGTGCTTACCTTGGTGGAGTCATTACCTAAAATTATGCAATC
ATAATATCAGTTTTAAAATATACTTTATATTCTTACTACTTTTGATATTTTCTTCAT
CTTTTATATATATATATATAATGTTTAAAAATTCAAATTTATGACATATTGCAGACC
TCCTAAACTTGGTCGTATGTTTCTTGTGTATTTATGACTCGCACAGAAAGAATTTGT
ATGACACCTTTTTTTTTCATGTTTGATACTCTCTCTCATCTGTAACTACACTTTAGG
TGCTCTTTGTGGTATGTTGCACTCCTTGAGTGCTGTTTTCTTCTGCCTTTTATAAGT
TAAGATTAGATGCACCAGTATTTATTTAATTATGAAAATAACCTTTTGTAAAAATAA
AAAAAAACAATAAGATTATGTTATAAATAATATATAATTAAGAAATAAAGAAATATG
ACACCTAAAAAAAAAAAAAAAAAAAAAAAAAA

B

GCCACCAACCGCTCACGCATGAGGAACAGACCACCAGGCATGCCAGAACTGAAGAGA
ATTAAGAGAGAGGAACGTAATGCGGCAAATGTGGGCACTATTGAGCCTGGAGAGGTT
CCAGGATCTCCAAGTCCAACACCAAGTAGTCACAACAGCGATGAACAGTCACAGAAC
CTTGCCCATAAGATCAAGACCGAAAGATCAGAATACTCTAAAGAAGAAGCAGAAGAC
CTGGATGAGGATGAAGGGGTGGCAGGAGAAGTAATGTCTGGGGAAGAAGAGCAGGAA
CAAGAACCAGAAGAGGAAGAAGAGGAAGAGGAAGAAGAAGAGGAAGGAGCATTA
GGGGAAGGGGAAGGCCTCTCCCACGGTGTTCTCTCAGATGTTGAGGATGGCTATGAA
CAATCTAACTCTTCCCTTCCCAATTCTGACATATCCACAACGGAACTGCTACAGGTT
GATTTGAGTGAGGACGGGACACAGTTCATAATCGGTCCTGGTGGCTTTGGAGATATG
ATGTCAAGAACTTCGTCACTAGCAGGAGATGATGAGAGAAATGGTGACAAAGAGCAG
AAGAAGCCATTTGTTTGTCCTCTCTGTGGGCAGTCATTTACACGTCGTGACAACCTT
GCCAACCATATCAAGACCCACACCGGTGACCGTCCGTTTATGTGCCAGTACTGCCAC
AAGTGCTTCTCAAGGAAGGACTACTTGAAGCAGCATGAACGCATCCACACTGGAGAG
AAGCCCTATCCCTGTGACATCTGTGGTCGTGCATTTACCAGGAAAGAAGGATTGACA

GACCACATGCGCTGTCATTCTGACTTCCGAGCCTTTTCCTGTGAAACATGTGGCAAG
AGCTTCAAGCAAAAATGTGGTCCGCGCTTCCATAAGAGGAATTATAAACATTAACCT
GCCATAAAGATCTCCAACACTTAATTCACTGTTCCATTTATGAGGCGGATATGGATA
GCCTGTCTCTCAGTTAGTTGGATGTGGTCCCTTTAAAAATATGCCAGATAAAGGGCT
AATTTATGCCCCTGTACTAAGTTAAGTCGTGCCATTTCCTTTATGTTGAGCACCTAA
TTTCTGTGTTCAGATAGTCAATTTTACCCTGAGCTTTGTCTATGGCCCTGGCCAGAT
GTATATTTTGGGTATATGATTATGGTTAGTGAAATATCACAACTTGTATATTGCTGC
TCACATACAGGGAACTTGTGTAAATTTGATTTATGTTTTGACTTCAACAGGCTTAAT
GTATGGCAAAGGTTTTGCAAAATGGAAATTGACTTTTAAATGTTAGATTAATCTCAG
GTAAGCTGTATTAATAACAGTGCTTACCTTGGTGGAGTCATTACCTAAAATTATGCA
ATCATAATATCAGTTTTAAAATATACTTTATATTCTTACTTAAAAAAAAAAAAAAAA
AAAAAAAAAAAA

C

AAGCAGTGGTATCAACGCAGAGTACGCGGGGACAGAAAACGGGTACCTCGATCACCA
CAGAAAACTGGTTCGCACGTGGTCCGATCATTTTTGAAGAGAAGGTGGTTCTGCAGA
CAGC***ATG***GAGGAGGGCTACCTAGCACTGCGATGGAACAACCACA

Figure 3.15 Nucleotide sequence of 3′UTR, fragment 1 (A), fragment 2 (B) and 5′UTR (C) of *PmBr-cZ1* generated by the secondary RACE-PCR. Nucleotide sequencing was carried out. The positions of sequencing primers are illustrated in boldface and underlined.

Two different forms of the full length cDNA of *PmBR-cZ1* (the log and short form; *PmBR-cZ1-s* and *PmBR-cZ1-l*) were found. The *PmBR-cZ1-s*and *PmBR-cZ1-l* were 2422 and 2060 bp in length containing ORFs of 1440 and 1443 bp corresponding to the polypeptides of 480 and 481 amino acids, respectively (Figs. 16 and 17). The calculated p*I* and molecular weight of the deduced *PmBR-cZ1-s* and *PmBR-cZ1-l* protein were 5.46 and 53.88 kDa and 5.54 and 53.64, respectively. Both deduced proteins contained BTB domains at positions 31 and 126 (*E*-value = 4.14e-22) and 4 ZnF C2H2 domains.

```
GACAGAAAACGGGTACCTCGATCACCACAGAAAACTGGTTCGCACGTGGTCCGATCATTT   60
TTGAAGAGAAGGTGGTTCTGCAGACAGCATGGAGGAGGGCTACCTAGCACTGCGATGGAA  120
                           M  E  E  G  Y  L  A  L  R  W  N    11
CAACCACAACACCATCTTCACCAAGATCCTCACCCTCCTTAGGGAGCAGGAGGCTTATGT  180
  N  H  N  T  I  F  T  K  I  L  T  L  L  R  E  Q  E  A  Y  V   31
GGATGTTTCCTTAGCTTGTGCGGGAAGATTATATCCTGCACACAAATTTGTACTTTCTAC  240
  D  V  S  L  A  C  A  G  R  L  Y  P  A  H  K  F  V  L  S  T   51
ATGTAGTGAGTATTTCAAGGAAATGTTTTCCAAGAACCCATGTAAGCATCCCATTGTTTT  300
  C  S  E  Y  F  K  E  M  F  S  K  N  P  C  K  H  P  I  V  F   71
CATGAAGGATGTCTCAACTAAGGACATGGAGGCCTTGCTGGACTTCATGTACAAGGGTGA  360
  M  K  D  V  S  T  K  D  M  E  A  L  L  D  F  M  Y  K  G  E   91
GGTCCATGTACCACAAAGCGAGTTGGGTTCATTGCTGCGTACAGCTGAAGGGCTTCAGGT  420
  V  H  V  P  Q  S  E  L  G  S  L  L  R  T  A  E  G  L  Q  V  111
AAAAGGCCTTGCTGTACCTGATGACTCTCCTCGTGGTTCCTCTACCACCCCTATTGTGCC  480
```

```
  K  G  L  A  V  P  D  D  S  P  R  G  S  S  T  T  P  I  V  P  131
TTCTGCCTCGTCCGTCCCACGCTCACCTCCGCCCAGTCTTATGGCTCCAATGCATATGCG  540
  S  A  S  S  V  P  R  S  P  P  P  S  L  M  A  P  M  H  M  R  151
GGGTAAACGCAAAAGGCCACCAGAATCGGCTAAAAAGGATGACCCTCCAAAGTTGACTTT  600
  G  K  R  K  R  P  P  E  S  A  K  K  D  D  P  P  K  L  T  L  171
ACGTCCTGACCTTGGACCCCCGGCCACCAACCGCTCACGCATGAGGAACAGACCATCAGG  660
  R  P  D  L  G  P  P  A  T  N  R  S  R  M  R  N  R  P  S  G  191
CATGCCAGAACTGAAGAGAATTAAGAGAGAGGAACATAGTGCGGCAAATGTGGGCACTAT  720
  M  P  E  L  K  R  I  K  R  E  E  H  S  A  A  N  V  G  T  I  211
TGAGCCTGGAGAGGTTCCAGGATCTCCAAGTCCAACACCAAGTAGTCACAACAGCGATGA  780
  E  P  G  E  V  P  G  S  P  S  P  T  P  S  S  H  N  S  D  E  231
ACAGTCACAGAACCTTGCCCATAAGATCAAGACCGAAAGATCAGAATACTCTAAAGAAGA  840
  Q  S  Q  N  L  A  H  K  I  K  T  E  R  S  E  Y  S  K  E  E  251
AGCAGAAGACCTGGATGAGGATGAAGGGGTGGCAGGAGAAGTAATGTCTGGGGAAGAAGA  900
  A  E  D  L  D  E  D  E  G  V  A  G  E  V  M  S  G  E  E  E  271
GCAGGAACAAGAACCAGAAGAGGAAGAAGAGGAAGAGGAAGAAGAAGAGGAAGGAGCATT  960
  Q  E  Q  E  P  E  E  E  E  E  E  E  E  E  E  E  G  A  L  291
AGGGGAAGGGGAAGGCCTCTCCCACGGTGTTCTCTCAGATGTTGAGGATGGCTATGAACA 1020
  G  E  G  E  G  L  S  H  G  V  L  S  D  V  E  D  G  Y  E  Q  311
ATCTAACTCTTCCCTTCCCAATTCTGACATATCCACAACGGAACTGCTACAGGTTGATTT 1080
  S  N  S  S  L  P  N  S  D  I  S  T  T  E  L  L  Q  V  D  L  331
GAGTGAGGACGGGACACAGTTCATAATCGGTCCTGGTGGCTTTGGAGATATGATGTCAAG 1140
  S  E  D  G  T  Q  F  I  I  G  P  G  G  F  G  D  M  M  S  R  351
AACTTCGTCACTAGCAGGAGATGATGAGAGAAATGGTGACAAAGAGCAGAAGAAGCCATT 1200
  T  S  S  L  A  G  D  D  E  R  N  G  D  K  E  Q  K  K  P  F  371
TGTTTGTCCTCTCTGTGGGCAGTCATTTACACGTCGTGACAACCTTGCCAACCATATCAA 1260
  V  C  P  L  C  G  Q  S  F  T  R  R  D  N  L  A  N  H  I  K  391
GACCCACACCGGTGACCGTCCGTTTATGTGCCAGTACTGCCACAAGTGCTTCTCAAGGAA 1320
  T  H  T  G  D  R  P  F  M  C  Q  Y  C  H  K  C  F  S  R  K  411
GGACTACTTGAAGCAGCATGAACGCATCCACACTGGAGAGAAGCCCTACCCCTGTGACAT 1380
  D  Y  L  K  Q  H  E  R  I  H  T  G  E  K  P  Y  P  C  D  I  431
CTGTGGTCGTGCATTTACCAGGAAAGGAGGATTGACAGACCACATGCGCTGTCATTCTGA 1440
  C  G  R  A  F  T  R  K  G  G  L  T  D  H  M  R  C  H  S  D  451
CTTCCGAGCCTTTTCCTGTGAAACATGTGGCAAGAGCTTCAAGCAAAAATGTGGTTTGCG 1500
  F  R  A  F  S  C  E  T  C  G  K  S  F  K  Q  K  C  G  L  R  471
CTTCCATAAGAGGAATTATAAACATTAACCTGCCATAAAGATCTCCAACACTTAATTCAC 1560
  F  H  K  R  N  Y  K  H  *                                    480
TGTTCCATTTATGAGGCGGATATGGATAGCCTGTCTCTCAGTTAGTTGGATGTGGTCCCT 1620
TTAAAAATATGCCAGATAAAGGGCTAATTTATGCCCCTGTACTAAGTTAAGTCGTGCCAT 1680
TTCCTTTATGTTGAGCACCTAATTTCTGTGTTCAGATAGTCAATTTTACCCTGAGCTTTG 1740
TCTATGGCCCTGGCCAGATGTATATTTTGGGTATATGATTATGGTTAGTGAAATATCACA 1800
ACTTGTATATTGCTGCTCACATACAGGGAACTTGTGTAAATTTGATTTATGTTTTGACTT 1860
CAACAGGCTTAATGTATGGCAAAGGTTTTGCAAAATGGAAATTGACTTTTAAATGTTAGA 1920
CTAATCTCAGGTAAGCTGTATTAATAACAGTGCTTACCTTGGTGGAGTCATTACCTAAAA 1980
TTATGCAATCATAATATCAGTTTTAAAATATACTTTATATTCTTACTACTTTTGATATTT 2040
TCTTCATCTTTTATATATATATATATAATGTTTAAAAATTCAAATTTATGACATATTGCA 2100
GACCTCCTAAACTTGGTCGTATGTTTCTTGTGTATTTATGACTCGCACAGAAAGAATTTG 2160
TATGACACCTTTTTTTTTCATGTTTGATACTCTCTCTCATCTGTAACTACACTTTAGGTG 2220
CTCTTTGTGGTATGTTGCACTCCTTGAGTGCTGTTTTCTTCTGCCTTTTATAAGTTAAGA 2280
TTAGATGCACCAGTATTTATTTAATTATGAAAATAACCTTTTGTAAA***AATAAA***AAAAAAC 2340
AATAAGATTATGTTATAAATAATATATAATTAAGAAATAAAGAAATATGACACCTAAAAA 2400
AAAAAAAAAAAAAAAAAAAAAA                                       2422
```

Figure 3.16 The full lenght cDNA and deduced amino acid sequences of the shrot form of *P. monodon Br-cZ1* (*PmBr-cZ1-s*). The start and stop codons are illustrated in boldface. The poly A additional signal site is underlined. The BTB domain is highlighted.

```
GACAGAAAACGGGTACCTCGATCACCACAGAAAACTGGTTCGCACGTGGTCCGATCATTT    60
TTGAAGAGAAGGTGGTTCTGCAGACAGCATGGAGGAGGGCTACCTAGCACTGCGATGGAA   120
                            M  E  E  G  Y  L  A  L  R  W  N     11
CAACCACAACACCATCTTCACCAAGATCCTCACCCTCCTTAGGGAGCAGGAGGCTTATGT   180
  N  H  N  T  I  F  T  K  I  L  T  L  L  R  E  Q  E  A  Y  V    31
GGATGTTTCCTTAGCTTGTGCGGGAAGATTATATCCTGCACACAAATTTGTACTTTCTAC   240
  D  V  S  L  A  C  A  G  R  L  Y  P  A  H  K  F  V  L  S  T    51
ATGTAGTGAGTATTTCAAGGAAATGTTTTCCAAGAACCCATGTAAGCATCCCATTGTTTT   300
  C  S  E  Y  F  K  E  M  F  S  K  N  P  C  K  H  P  I  V  F    71
CATGAAGGATGTCTCAACTAAGGACATGGAGGCCTTGCTGGACTTCATGTACAAGGGTGA   360
  M  K  D  V  S  T  K  D  M  E  A  L  L  D  F  M  Y  K  G  E    91
GGTCCATGTACCACAAAGCGAGTTGGGTTCATTGCTGCGTACAGCTGAAGGGCTTCAGGT   420
  V  H  V  P  Q  S  E  L  G  S  L  L  R  T  A  E  G  L  Q  V   111
AAAAGGCCTTGCTGTACCTGATGACTCTCCTCGTGGTTCCTCTACCACCCCTATTGTGCC   480
  K  G  L  A  V  P  D  D  S  P  R  G  S  S  T  T  P  I  V  P   131
TTCTGCCTCGTCCGTCCCACGCTCACCTCCGCCCAGTCTTATGGCTCCAATGCATATGCG   540
  S  A  S  S  V  P  R  S  P  P  P  S  L  M  A  P  M  H  M  R   151
GGGTAAACGCAAAAGGCCACCAGAATCGGCTAAAAAGGATGACCCTCCAAAGTTGACTTT   600
  G  K  R  K  R  P  P  E  S  A  K  K  D  D  P  P  K  L  T  L   171
ACGTCCTGACCTTGGACCCCCGGCCACCAACCGCTCACGCATGAGGAACAGACCACCAGG   660
  R  P  D  L  G  P  P  A  T  N  R  S  R  M  R  N  R  P  P  G   191
CATGCCAGAACTGAAGAGAATTAAGAGAGAGGAACGTAATGCGGCAAATGTGGGCACTAT   720
  M  P  E  L  K  R  I  K  R  E  E  R  N  A  A  N  V  G  T  I   211
TGAGCCTGGAGAGGTTCCAGGATCTCCAAGTCCAACACCAAGTAGTCACAACAGCGATGA   780
  E  P  G  E  V  P  G  S  P  S  P  T  P  S  S  H  N  S  D  E   231
ACAGTCACAGAACCTTGCCCATAAGATCAAGACCGAAAGATCAGAATACTCTAAAGAAGA   840
  Q  S  Q  N  L  A  H  K  I  K  T  E  R  S  E  Y  S  K  E  E   251
AGCAGAAGACCTGGATGAGGATGAAGGGGTGGCAGGAGAAGTAATGTCTGGGGAAGAAGA   900
  A  E  D  L  D  E  D  E  G  V  A  G  E  V  M  S  G  E  E  E   271
GCAGGAACAAGAACCAGAAGAGGAAGAAGAGGAAGAGGAAGAAGAAGAAGAGGAAGGAGC   960
  Q  E  Q  E  P  E  E  E  E  E  E  E  E  E  E  E  E  E  G  A   291
ATTAGGGGAAGGGGAAGGCCTCTCCCACGGTGTTCTCTCAGATGTTGAGGATGGCTATGA  1020
  L  G  E  G  E  G  L  S  H  G  V  L  S  D  V  E  D  G  Y  E   311
ACAATCTAACTCTTCCCTTCCCAATTCTGACATATCCACAACGGAACTGCTACAGGTTGA  1080
  Q  S  N  S  S  L  P  N  S  D  I  S  T  T  E  L  L  Q  V  D   331
TTTGAGTGAGGACGGGACACAGTTCATAATCGGTCCTGGTGGCTTTGGAGATATGATGTC  1140
  L  S  E  D  G  T  Q  F  I  I  G  P  G  G  F  G  D  M  M  S   351
AAGAACTTCGTCACTAGCAGGAGATGATGAGAGAAATGGTGACAAAGAGCAGAAGAAGCC  1200
  R  T  S  S  L  A  G  D  D  E  R  N  G  D  K  E  Q  K  K  P   371
ATTTGTTTGTCCTCTCTGTGGGCAGTCATTTACACGTCGTGACAACCTTGCCAACCATAT  1260
  F  V  C  P  L  C  G  Q  S  F  T  R  R  D  N  L  A  N  H  I   391
CAAGACCCACACCGGTGACCGTCCGTTTATGTGCCAGTACTGCCACAAGTGCTTCTCAAG  1320
  K  T  H  T  G  D  R  P  F  M  C  Q  Y  C  H  K  C  F  S  R   411
GAAGGACTACTTGAAGCAGCATGAACGCATCCACACTGGAGAGAAGCCCTATCCCTGTGA  1380
  K  D  Y  L  K  Q  H  E  R  I  H  T  G  E  K  P  Y  P  C  D   431
CATCTGTGGTCGTGCATTTACCAGGAAAGAAGGATTGACAGACCACATGCGCTGTCATTC  1440
  I  C  G  R  A  F  T  R  K  E  G  L  T  D  H  M  R  C  H  S   451
TGACTTCCGAGCCTTTTCCTGTGAAACATGTGGCAAGAGCTTCAAGCAAAAATGTGGTCC  1500
  D  F  R  A  F  S  C  E  T  C  G  K  S  F  K  Q  K  C  G  P   471
GCGCTTCCATAAGAGGAATTATAAACATTAACCTGCCATAAAGATCTCCAACACTTAATT  1560
  R  F  H  K  R  N  Y  K  H  *                                 481
CACTGTTCCATTTATGAGGCGGATATGGATAGCCTGTCTCTCAGTTAGTTGGATGTGGTC  1620
CCTTTAAAAATATGCCAGATAAAGGGCTAATTTATGCCCCTGTACTAAGTTAAGTCGTGC  1680
CATTTCCTTTATGTTGAGCACCTAATTTCTGTGTTCAGATAGTCAATTTTACCCTGAGCT  1740
TTGTCTATGGCCCTGGCCAGATGTATATTTTGGGTATATGATTATGGTTAGTGAAATATC  1800
ACAACTTGTATATTGCTGCTCACATACAGGGAACTTGTGTAAATTTGATTTATGTTTTGA  1860
CTTCAACAGGCTTAATGTATGGCAAAGGTTTTGCAAAATGGAAATTGACTTTTAAATGTT  1920
AGATTAATCTCAGGTAAGCTGTATTAATAACAGTGCTTACCTTGGTGGAGTCATTACCTA  1980
AAATTATGCAATCATAATATCAGTTTTAAAATATACTTTATATTCTTACTTAAAAAAAAA  2040
AAAAAAAAAAAAAAAAAAA                                            2060
```

Figure 3.17 The full lenght cDNA and deduced amino acid sequences of the long form of *P. monodon Br-cZ1* (*PmBr-cZ1-l*). The start and stop codons are illustrated in boldface. The poly A additional signal site is underlined. The BTB domain is highlighted.

Further analysis of the protein sequence, with NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos/) shows multiple high scoring (score > 0.8) sites for possible phosphorylation at 6 threonine, 3 tyrosine and 29 or 28 serine side chains within the respective deduced proteins.

Unlike *PmFAMeT*, the indel within the coding region was not observed in different isoforms of the *PmBr-cZ1*gene but sequence polymorphism was found suggesting allelic variation of this gene (Fig. 3.18).

```
Br C Z1-l       MEEGYLALRWNNHNTIFTKILTLLREQEAYVDVSLACAGRLYPAHKFVLSTCSEYFKEMF
Br C Z1-s       MEEGYLALRWNNHNTIFTKILTLLREQEAYVDVSLACAGRLYPAHKFVLSTCSEYFKEMF
                ************************************************************
Br C Z1-l       SKNPCKHPIVFMKDVSTKDMEALLDFMYKGEVHVPQSELGSLLRTAEGLQVKGLAVPDDS
Br C Z1-s       SKNPCKHPIVFMKDVSTKDMEALLDFMYKGEVHVPQSELGSLLRTAEGLQVKGLAVPDDS
                ************************************************************
Br C Z1-l       PRGSSTTPIVPSASSVPRSPPPSLMAPMHMRGKRKRPPESAKKDDPPKLTLRPDLGPPAT
Br C Z1-s       PRGSSTTPIVPSASSVPRSPPPSLMAPMHMRGKRKRPPESAKKDDPPKLTLRPDLGPPAT
                ************************************************************
Br C Z1-l       NRSRMRNRPPGMPELKRIKREERNAANVGTIEPGEVPGSPSPTPSSHNSDEQSQNLAHKI
Br C Z1-s       NRSRMRNRPSGMPELKRIKREEHSAANVGTIEPGEVPGSPSPTPSSHNSDEQSQNLAHKI
                *********.************:.**************************************
Br C Z1-l       KTERSEYSKEEAEDLDEDEGVAGEVMSGEEEQEQEPEEEEEEEEEEEEEGALGEGEGLSH
Br C Z1-s       KTERSEYSKEEAEDLDEDEGVAGEVMSGEEEQEQEPEEEEEEEEEEEE-GALGEGEGLSH
                ********************************************** ***********
Br C Z1-l       GVLSDVEDGYEQSNSSLPNSDISTTELLQVDLSEDGTQFIIGPGGFGDMMSRTSSLAGDD
Br C Z1-s       GVLSDVEDGYEQSNSSLPNSDISTTELLQVDLSEDGTQFIIGPGGFGDMMSRTSSLAGDD
                ************************************************************
Br C Z1-l       ERNGDKEQKKPFVCPLCGQSFTRRDNLANHIKTHTGDRPFMCQYCHKCFSRKDYLKQHER
Br C Z1-s       ERNGDKEQKKPFVCPLCGQSFTRRDNLANHIKTHTGDRPFMCQYCHKCFSRKDYLKQHER
                ************************************************************
Br C Z1-l       IHTGEKPYPCDICGRAFTRKEGLTDHMRCHSDFRAFSCETCGKSFKQKCGPRFHKRNYKH
Br C Z1-s       IHTGEKPYPCDICGRAFTRKGGLTDHMRCHSDFRAFSCETCGKSFKQKCGLRFHKRNYKH
                ******************** ****************************** *********
```

Figure 3.18 Pairwise alignment showing sequence polymorphism between the short and long form of *PmBr-cZ1*.

3.1.4.2 *PmBr-cZ4* genes

The partial sequences of *PmBr-cZ4* were initially obtained from EST analysis of the hemocyte cDNA library of *P. monodon*. It significantly matched *PmBr-cZ4* (*Lola-like protein*) of *Drosophila hydei* (*E*-value = 1e-26; Fig 3.19).

A.

GGCGGCGTGGTGATGGACGCTCATTAATGTGTCATAATGGAGGACGGACTACTAAGCTTGAAGTGGAAC
AACCACAAAACCACATTCTTTGAAATCCTCAGGGTATTAAGAGAAAAGGCAAATTATACAGATGCCACT
ATTGCAGTGGATGGAAAGTTTTATCCAGTTCACAAACTGGTAATGAGCACATGCAGTGAGTATTTTAGT
GAAATTTTTGAAAAAACACCATGCAAATCACCAGTGATAGTGCTAAAAGATGTGCGCAGTCAGGACATG
GAAGCGCTGTTGGACTATATGTACTTAGGTGAAGTTAACGTAAACCAAAATGACTTAGCCTCGTTATTG
AAGACAGCCGAATGCCTCAGAATTAAGGGCTTGGCAGTACCTGATGAAGACACTACAAAGGTAAGGAAA
GCACCTCCGGATGATAGACAAGAAAGTCCGCCACCAAAGAGAAGACGAAATGAAGACAACCCTTCCTCA
GCACCTAGGCCAGTTTCCCCATCAGTCAATGCACCCTCTAAAACCACGACGCCATCGGTAACACCTCCA
GTCCAGTCTGCATCGTTGCCCGGGTCCCAGTCTCAGGATGGGATTCAGGACTCCTCATTAGATGTCCCG
CCCATGGTGAAGGTAGAAATGCAAGAAGCTGACGACCCAGACGACTACAGAAAGGACAACAGTTATGAA
GGTGGATCAGTCAACGAAGGCGACATGGGATCAGACTTTGGCGCGGAATTATCTAAAGCGGAACA

B.

```
Lola-like protein [Drosophila hydei]
Length=1010

 Score =  123 bits (308),  Expect = 1e-26
 Identities = 56/137 (40%), Positives = 85/137 (62%), Gaps = 0/137 (0%)
 Frame = +1

Query  40   EDGLLSLKWNNHKTTFFEILRVLREKANYTDATIAVDGKFYPVHKLVMSTCSEYFSEIFE  219
            +D    L+WNNH++T   +   L E     D T+A +GKF   HK+V+S CS YF+ + +
Sbjct  3    DDQQFCLRWNNHQSTLISVFDTLLENETLVDCTLAAEGKFLKAHKVVLSACSPYFATLLQ  62

Query  220  KTPCKSPVIVLKDVRSQDMEALLDYMYLGEVNVNQNDLASLLKTAECLRIKGLAVPDEDT  399
            +   K P+ +LKDV+ Q++ A++DYMY GEVN++Q+ L +LLK AE L+IKGL+     T
Sbjct  63   EQYDKHPIFILKDVKYQELRAMMDYMYRGEVNISQDQLTALLKAAESLQIKGLSDNRSGT  122

Query  400  TKVRKAPPDDRQESPPP  450
                 A    +Q++P P
Sbjct  123  GPAAAAAAQQQQQAPKP  139
```

Figure 3.19 Nucleotide sequence (A) and the BlastX result (B) of an EST significantly similar to *Br-cZ4* of *Drosophila hydei*. The positions of sequences primers were illustrated in boldface and underlined.

For isolation of the full length cDNA, 3′ RACE-PCR of *PmBr-cZ4* was carried out and discrete amplification bands were obtained from RACE-PCR of this gene. Two fragments were found in the agarose gel. The correct fragment (long fragment) was chosen, cloned and sequenced (Fig. 3.20. The positive amplification product of approximately 1247 bp was obtained (Fig. 3.21). The amplification fragment was cloned and sequenced. Nucleotide sequences of 3′RACE-PCR fragments and the original EST were assembled.

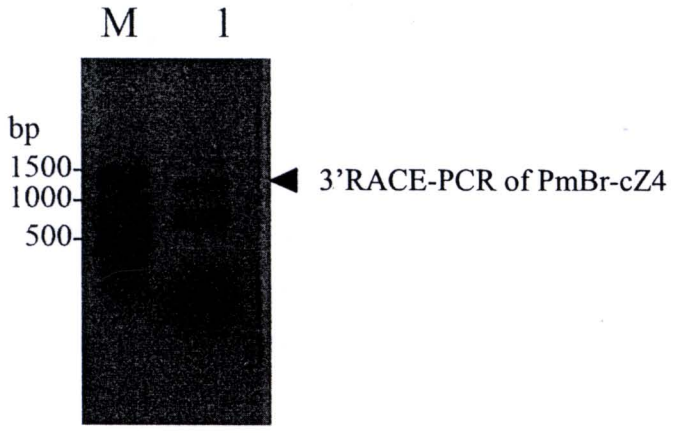


Figure 3.20 Agarose gel electrophoresis showing 3′ RACE-PCR of *PmBr-c Z4* (lanes 1 and 2, respectively). Lane N = the negative control (without cDNA template). A 100 bp DNA ladder (lanes M) was used as the DNA marker.

TGACGACCCAGACGACTACAGAAAGGACAACAGTTATGAAGGTGGATCAGTCAACGA
AGGCGAAATGGGATCAGACTTTGGCGCGGAATTATCTAAAGCGGAACACGACCCTGA
CAGTTACGGCAGTGGATCATACGCAGGACCATCCATTCAACCTGGGGGTGACCTCCC
TTGGGATGAGGGAGATTCAAGCAGTTTTCCACAAGAAGGCTTCTCAGGGGACTTACC
AGCAGGCCAGCAACCTCAAGGGGACTGGGATCATGTTCGTCCAGCTGCCCTCATTCC
TGTGGTGGAGATACGCCAACCTGTTGCTGCAAGCACACCCACAAGCATCGCTCAGCT
AATGACTGGTGCCTGTCCGAAGACTGCTGCTATACCTATGGGCCCAGCTAGTGGGGC
CTTCACTCCCTACACGGCCTCAACCTCCCCAAGGGGCAGTACCCCAGACTTGGCCCA
GGTGGCACCTGCCGACAAGCAGTCTTTTATGTGCCCCGTGTGTGGGAAACAGTTTGG
GCAGCCCTACAACCTCCGCCGCCATCTGACCACCCACACGGGAGAGAGACCTTACCG
TTGCCCCCACTGTAATTATGCCGCCTCTCAGAATGTCCACCTGGAGAAGCACATCCG
ACGCATACACTTGAACAATGGCCAGAATGAAACACCCACTGGGCCAGCGGTCACTTG
GGCCCCTGCAGCCACAGCTGTAACTCCT**TAA**TAAATTCTCATATCTGCACATCATGA
TAAGCCCTGTGATGAAAAAAGTGATAAAATACATATATCAATATTTCGTTATTACTC
AGATGGTCAGTGTTTTTTCTATCATATTTAGAGTTGTCTATGAATATTCAGAACAGA
ATACAATTTATAGTAAAACTTCCTTTTATATCATATCTATTTCATATACTACTTTTT
ATATACAATGATGCAGTATACAGAGGCCTTTGATTATTTTCTAAAAAGTATTTTGGT
ATAAGGATAGTGAAAGTTATTTTACAAACATTATTCACCCAACAGTCGGGTGGTCAT
CCGAGAGATTAGTTCAAATGGCGACAAAGCCAAGAACTATAATTATATCCGAGAGTC
TTCTTTAGGATACTAGCTCTTACACATGAAAACCATAGCAAAGCACTTACATGAATA
CATTTTATAAGAGGGCAATATCTCTTTTTTTTTCATAGGTATCTTACAGTATTTGCA
GAGTTGTGAAAATACAACCAGAAAAAAAAAAAAAAAAAAAAAAAAAAA

Figure 3.21 Nucleotide sequence of 3′UTR of *PmBr-cZ4* generated by the secondary 3′ RACE-PCR. The positions of sequencing primers are illustrated in boldface and underlined.

The full length cDNA of *PmBr-cZ4* was successfully characterized. The full length cDNA of *PmBr-cZ4* is 1896 bp long composing of an ORF of 1332 bp encoding a polypeptide of 443 amino acids (Fig. 3.22). The predict protein was a theoretical p*I* and Mw of *PmBr-cZ4* were 5.16 and 47.87 kDa, respectively. The predicted *PmBr-cZ4* protein composted of one BTB domain at positions 31 and 126 (*E*-value = 4.02e-20) and two ZnF C2H2 domains. Sequence alignment between the BTB domain of *PmBr-cZ1* and *PmBr-cZ4* suggested that this functional domain is conserved across the protein family (Fig. 3.23).

A.

```
GGCGGCGTGGTGATGGACGCTCATTAATGTGTCATAATGGAGGACGGACTACTAAGCTTG    60
                                      M  E  D  G  L  L  S  L     8
AAGTGGAACAACCACAAAACCACATTCTTTGAAATCCTCAGGGTATTAAGAGAAAAGGCA   120
 K  W  N  N  H  K  T  T  F  F  E  I  L  R  V  L  R  E  K  A    28
AATTATACAGATGCCACTATTGCAGTGGATGGAAAGTTTTATCCAGTTCACAAACTGGTA   180
 N  Y  T  D  A  T  I  A  V  D  G  K  F  Y  P  V  H  K  L  V    48
ATGAGCACATGCAGTGAGTATTTTAGTGAAATTTTTGAAAAAACACCATGCAAATCACCA   240
 M  S  T  C  S  E  Y  F  S  E  I  F  E  K  T  P  C  K  S  P    68
GTGATAGTGCTAAAAGATGTGCGCAGTCAGGACATGGAAGCGCTGTTGGACTATATGTAC   300
 V  I  V  L  K  D  V  R  S  Q  D  M  E  A  L  L  D  Y  M  Y    88
TTAGGTGAAGTTAACGTAAACCAAAATGACTTAGCCTCGTTATTGAAGACAGCCGAATGC   360
 L  G  E  V  N  V  N  Q  N  D  L  A  S  L  L  K  T  A  E  C   108
CTCAGAATTAAGGGCTTGGCAGTACCTGATGAAGACACTACAAAGGTAAGGAAAGCACCT   420
 L  R  I  K  G  L  A  V  P  D  E  D  T  T  K  V  R  K  A  P   128
CCGGATGATAGACAAGAAAGTCCGCCACCAAAGAGAAGACGAAATGAAGACAACCCTTCC   480
 P  D  D  R  Q  E  S  P  P  P  K  R  R  R  N  E  D  N  P  S   148
TCAGCACCTAGGCCAGTTTCCCCATCAGTCAATGCACCCTCTAAAACCACGACGCCATCG   540
 S  A  P  R  P  V  S  P  S  V  N  A  P  S  K  T  T  T  P  S   168
GTAACACCTCCAGTCCAGTCTGCATCGTTGCCCGGGTCCCAGTCTCAGGATGGGATTCAG   600
 V  T  P  P  V  Q  S  A  S  L  P  G  S  Q  S  Q  D  G  I  Q   188
GACTCCTCATTAGATGTCCCGCCCATGGTGAAGGTAGAAATGCAAGAAGCTGACGACCCA   660
 D  S  S  L  D  V  P  P  M  V  K  V  E  M  Q  E  A  D  D  P   208
GACGACTACAGAAAGGACAACAGTTATGAAGGTGGATCAGTCAACGAAGGCGAAATGGGA   720
 D  D  Y  R  K  D  N  S  Y  E  G  G  S  V  N  E  G  E  M  G   228
TCAGACTTTGGCGCGGAATTATCTAAAGCGGAACACGACCCTGACAGTTACGGCAGTGGA   780
 S  D  F  G  A  E  L  S  K  A  E  H  D  P  D  S  Y  G  S  G   248
TCATACGCAGGACCATCCATTCAACCTGGGGGTGACCTCCCTTGGGATGAGGGAGATTCA   840
 S  Y  A  G  P  S  I  Q  P  G  G  D  L  P  W  D  E  G  D  S   268
AGCAGTTTTCCACAAGAAGGCTTCTCAGGGGACTTACCAGCAGGCCAGCAACCTCAAGGG   900
 S  S  F  P  Q  E  G  F  S  G  D  L  P  A  G  Q  Q  P  Q  G   288
GACTGGGATCATGTTCGTCCAGCTGCCCTCATTCCTGTGGTGGAGATACGCCAACCTGTT   960
 D  W  D  H  V  R  P  A  A  L  I  P  V  V  E  I  R  Q  P  V   308
GCTGCAAGCACACCCACAAGCATCGCTCAGCTAATGACTGGTGCCTGTCCGAAGACTGCT  1020
 A  A  S  T  P  T  S  I  A  Q  L  M  T  G  A  C  P  K  T  A   328
GCTATACCTATGGGCCCAGCTAGTGGGGCCTTCACTCCCTACACGGCCTCAACCTCCCCA  1080
 A  I  P  M  G  P  A  S  G  A  F  T  P  Y  T  A  S  T  S  P   348
AGGGGCAGTACCCCAGACTTGGCCCAGGTGGCACCTGCCGACAAGCAGTCTTTTATGTGC  1140
 R  G  S  T  P  D  L  A  Q  V  A  P  A  D  K  Q  S  F  M  C   368
CCCGTGTGTGGGAAACAGTTTGGGCAGCCCTACAACCTCCGCCGCCATCTGACCACCCAC  1200
 P  V  C  G  K  Q  F  G  Q  P  Y  N  L  R  R  H  L  T  T  H   388
ACGGGAGAGAGACCTTACCGTTGCCCCCACTGTAATTATGCCGCCTCTCAGAATGTCCAC  1260
 T  G  E  R  P  Y  R  C  P  H  C  N  Y  A  A  S  Q  N  V  H   408
CTGGAGAAGCACATCCGACGCATACACTTGAACAATGGCCAGAATGAAACACCCACTGGG  1320
```

```
 L  E  K  H  I  R  R  I  H  L  N  N  G  Q  N  E  T  P  T  G   428
CCAGCGGTCACTTGGGCCCCTGCAGCCACAGCTGTAACTCCTTAATAAATTCTCATATCT  1380
 P  A  V  T  W  A  P  A  A  T  A  V  T  P  *                 443
GCACATCATGATAAGCCCTGTGATGAAAAAAGTGATAAAATACATATATCAATATTTCGT  1440
TATTACTCAGATGGTCAGTGTTTTTTCTATCATATTTAGAGTTGTCTATGAATATTCAGA  1500
ACAGAATACAATTTATAGTAAAACTTCCTTTTATATCATATCTATTTCATATACTACTTT  1560
TTATATACAATGATGCAGTATACAGAGGCCTTTGATTATTTTCTAAAAAGTATTTTGGTA  1620
TAAGGATAGTGAAAGTTATTTTACAAACATTATTCACCCAACAGTCGGGTGGTCATCCGA  1680
GAGATTAGTTCAAATGGCGACAAAGCCAAGAACTATAATTATATCCGAGAGTCTTCTTTA  1740
GGATACTAGCTCTTACACATGAAAACCATAGCAAAGCACTTACATGAATACATTTTATAA  1800
GAGGGCAATATCTCTTTTTTTTTCATAGGTATCTTACAGTATTTGCAGAGTTGTGAAAAT  1860
ACAACCAGAAAAAAAAAAAAAAAAAAAAAAAAAAAA                          1896
```

Figure 3.22 The full lenght cDNA and deduced amino acid sequences of *PmBr-cZ4* The start and stop codons are illustrated in boldface and underlined. The poly A additional signal site are underlined. The BTB domain is highlighted.

```
Br-CZ1l     VDVSLACAGRLYPAHKFVLSTCSEYFKEMFSKNPCKHPIVFMKDVSTKDMEALLDFMYKG
Br-CZ1s     VDVSLACAGRLYPAHKFVLSTCSEYFKEMFSKNPCKHPIVFMKDVSTKDMEALLDFMYKG
Br-CZ4      TDATIAVDGKFYPVHKLVMSTCSEYFSEIFEKTPCKSPVIVLKDVRSQDMEALLDYMYLG
            .*.::*  *::**.**:*:*******.*:*.*.*** *::.:*** ::********:** *

Br-CZ1l     EVHVPQSELGSLLRTAEGLQVKGLAVPDDSPRGSST
Br-CZ1s     EVHVPQSELGSLLRTAEGLQVKGLAVPDDSPRGSST
Br-CZ4      EVNVNQNDLASLLKTAECLRIKGLAVPDEDTTKVRK
           **:* *.:*.***:*** *::*******:..    .
```

Figure 3.23 Sequence alignments of the BTB domain of *PmBr-cZ1-s, PmBr-cZ1-l* and *PmBr-cZ4.*

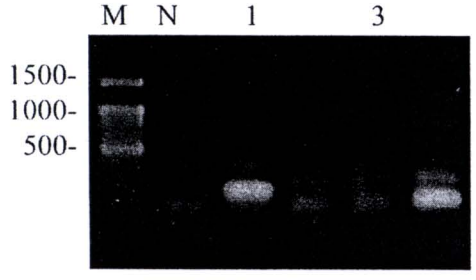
Further analysis of the protein sequence, with NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos/), for potential posttranslational modifications shows multiple high scoring (score > 0.8) sites for possible phosphorylation at 7 threonine, 4 tyrosine and 18 serine side chains within the deduced PmBr-cZ4 proteins, respectively.

3.2 Characterization of the genomic organization *PmCOMT* by using Genome Walking Technique

Genomic organization of *PmCOMT* has not been reported in any crustacean. Genome walking was carried out and the resulting fragment of approximately 200 bp obtained from the *Dra*-I mini-library was cloned and sequenced (Fig. 3.24). In addition, nucleotide sequences of the amplification product from overlapping PCR was also characterized (Fig. 3.25).

The genomic sequence of *PmCOMT* deduced from nucleotide sequences of the genome walking and overlapping amplification clones spanned 1470 bp in length and contained 3 exons (194, 111 and 361 bp) and 2 introns (143 and 147 bp). The GC content of exons (46-54%) was greater than that of introns (33%), reflecting a greater thermal stability of the coding regions than that of the noncoding regions.

A

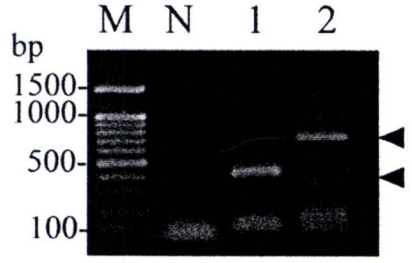


B

ACTATAGGGCACGCGTGGTCGACGGCCCGGGCAGGTAAAACATAGAGTCACGCCGTCACCAATTTTCAG
GAAGGAAATATTGATTCTCTGGTCATCTCTCAGTTTTTCGTTAATTTTCCTAATAGCCACTGTGCCAGG
GGTTTGATCAGTGGGGTCAATCACAGCTCCATCCCAAAGTGTGTTGTCGAAAGCGATGACTCCACCAGA
GCG

Figure 3.24 (A) Agarose gel electrophoresis showing the amplification products of the 5′UTR of *PmCOMT* generated by the secondary 5′ genome walking analysis against the template from *Alu* I, *Dra* I, *Hae* III *Stu* I, and *Rsa* I mini-libraries (lanes 1-4). Lanes M and N is a 100 bp DNA ladder marker and the negative control (without genomic DNA template), respectively. (B) The amplification product obtained from *Dra* I mini-library template was cloned and sequenced. The positions of sequencing primers are illustrated in boldface and underlined.

A.



B.

AGCACCGTAGAGCGGCGATGTTGGGGGCACCTGAGGTTCTGCAGCTCAATGCCAACATAATGCAGGCTA
TCGGGGCAAAGAAAGTGAGTTTAGTCATATGATGGTATTTAAAGACCAACTGGATATATGTGCTTGTTT
TAGAATATGGCGTTCTATGCATTTTTAAGCATTTTGCTATTTCCTCACAGTCATGTATTGAGGGTTGTT
CTGTATGATTATTTAACAGGTACTAGACATTGGGGTGTTCACAGGCGCCAGTTCACTCTCTGCTGCTCT
GGCACTGCCTCCGAATGGCAAGGTCTACGCCCTTGACATAAGTGAAGAGTCTGCCAACATAGGTATTAA
GACTATCAGAGCAGATCAGATGAATTATGCACATATTCATGTATAGATAATGAAATAGATAGTGACTCA
AAATGTCTCATAGGTACCTGTGGTACTATGAACATAACATGTTGACATTCCTAAGGAAACGAGTATTCC
AGGCAAACCGTTCTGGGAGGAAGCTGGAGTTATCAACAAGATAAGTCTGCACATCGCTCCAGCTGCTGA
GACTCTCCAGAAGTTCATTGACGGCGGAGAAGCTGGCACCTTCGACTATGCTTTCATTGATGCCGACAA
AGGGAATTATGAGCTGTACTATGAACTTTGCCTCACTCTCTTGCGCT**CTGGTGGAGTCATCGCCTTCG**

Figure 3.25 The PCR product of *PmCOMT* (lanes 1 and 2 using cDNA and genomic DNA as template, respectively). Lane N = the negative control (without cDNA template). A 100 bp DNA ladder (lanes M) was used as the DNA marker. (A) and nucleotide sequence (B) of *PmCOMT* using genomic DNA as the template. The positions of sequencing primers are illustrated in boldface and underlined.

The exon-intron boundaries of *PmCOMT* did not follow the GT/AG rule. Both introns interrupt the ORFs within the same codons (type 1 intron) (Fig. 3.26). Schematic diagram illustrating the genomic organization of *PmCOMT* is shown by Fig. 3.27.

```
TTGACAGGTTCCTGAAGATGTCTTCTCTGAAAAGTTACCATAATCCCGATCCTTTGGTGC   60
                    M  S  S  L  K  S  Y  H  N  P  D  P  L  V  Q   15
AGTATTGTGTAAATCATTCATTGAGATTAACCGACGCGCAAAAACGACTGAATGATGTAA  120
  Y  C  V  N  H  S  L  R  L  T  D  A  Q  K  R  L  N  D  V  T   35
CTCTGCAGCACCGTAGAGCGGCGATGTTGGGGGCACCTGAGGTTCTGCAGCTCAATGCCA  180
  L  Q  H  R  R  A  A  M  L  G  A  P  E  V  L  Q  L  N  A  N   55
ACATGATGCAGGCTATCGGGGCAAAGAAAGTgagtttagtcatatgatggtatttaaaga  240
  M  M  Q  A  I  G  A  K  K  V                                  65
ccaactgaatgtatgtgcttgttttagaatatggcgttctatgcattttaagcatttttg  300
caatttcctcacagtcatgtattgagggttgttctgtatgattatttaacaggtACTAGA  360
                                                  L  D          67
CATTGGGGTGTTCACAGGCGCCAGTTCACTCTCTGCTGCTCTGGCACTGCCTCCGAATGG  420
  I  G  V  F  T  G  A  S  S  L  S  A  A  L  A  L  P  P  N  G   87
CAAGGTCCACGCCCTTGACATAAGTGAAGAGTTTGCCAACATAGGtattaagactatcag  480
  K  V  H  A  L  D  I  S  E  E  F  A  N  I  G                  102
agcagatcagatgaattatgcacatattcatgtatagataatgaaatagatagtgactca  540
aaatgtctcataggtacctgtggtactatgaacataacatgttaacattcctaaggaaac  600
gagtattccaggAAAACCGTTCTGGGAGGAAGCTGGAGTTATCAACAAGATAAGTCTGCA  660
                K  P  F  W  E  E  A  G  V  I  N  K  I  S  L  H  118
CATCGCTCCAGCTGCTGAGACTCTCCAGAAGTTCATTGACGGCGGAGAAGCTGGCACCTT  720
  I  A  P  A  A  E  T  L  Q  K  F  I  D  G  G  E  A  G  T  F  138
CGACTATGCTTTCATTGATGCCGACAAAGGGAATTATGAGCTGTACTATGAACTTTGCCT  780
  D  Y  A  F  I  D  A  D  K  G  N  Y  E  L  Y  Y  E  L  C  L  158
CACTCTCTTGCGCTCTGGTGGAGTCATCGCCTTCGACAACACACTTTGGGATGGAGCTGT  840
  T  L  L  R  S  G  G  V  I  A  F  D  N  T  L  W  D  G  A  V  178
GATTGACCCCACTGATCAAACCCCTGGCACAGTGGCTATTAGGAAAATTAACGAAAAACT  900
  I  D  P  T  D  Q  T  P  G  T  V  A  I  R  K  I  N  E  K  L  198
GAGAGATGACCAGAGAATCAATATTTCCTTCCTGAAAATTGGTGACGGCGTGACTCTATG  960
  R  D  D  Q  R  I  N  I  S  F  L  K  I  G  D  G  V  T  L  C  218
TTTTAAAAAATGAATATTTTTTCCCCCCGAAAAGGACCCCTCCTCCCCAATAATAAATTC 1020
  F  K  K  *                                                    221
CTGGTTCCAGAAAAAGGTTAAGAACTTTAACAAGGATGGAACAATTGACCCCCCATACCA 1080
TACACCTATGAAAAGGTTTTAAAACAATTGGCCGGCCTTTACCGGCCCCTCCTGGCACGG 1140
GGGGCCAAAAACATCCTCCATTGGCCCCGAATTTACCGAAAAATCTTATTAAAACCCCTT 1200
TTAAAACCAGGGGCCTGTAACTGGGAATGGCTGAATATGGATTTCCTTTCCCCCCAAAGG 1260
TCCCTGGCCAGAATTGATTCTAAATAAAAATTGGCAACAACTTTAAATGGAAGTTTCCTC 1320
CGGTCCATTGCCACTTGGCCAAACTACCGCCAAATACTAACTTTTAATGGACCAAATGGA  1380
AATGGTAAAACCACCCCCCCCCCCCTATATTATCCCCGAATTAAAATCCTACGTCTCGAA  1440
AAAAAAAAAAAAAAAAAAAAAAAAA                                     1470
```

Fig 3.26 Nucleotide sequences illustrating organization of *PmCOMT* genes. Coding nucleotides and deduced amino acids of each exon are capitalized. Introns are italicized and illustrated with lower letters. Start and stop codons are illustrated in boldface and underlined. The catechol-*O*-methyltransferase domain is highlighted. Polyadenylation signals (AATAAA) are underlined.

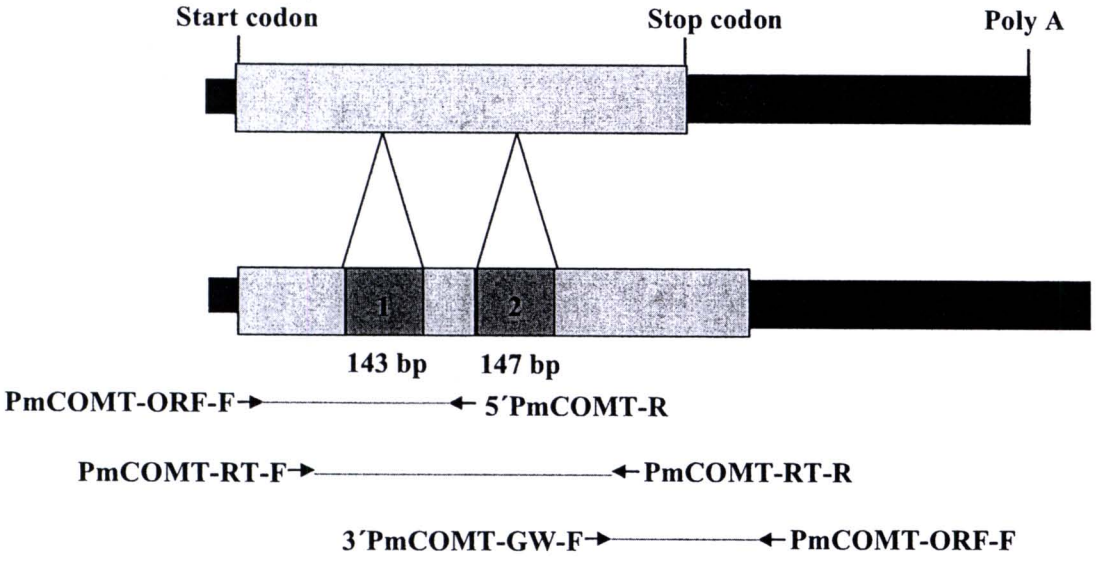


Fig. 3.27 Schematic diagrams of *PmCOMT* cDNA and gene. The complete *PmCOMT* cDNA were obtained by RACE–PCR. Genomic DNA fragments of *PmCOMT* were obtained from both genome walking analysis and overlapping PCR amplification. Noncoding regions are represented by solid bars. Introns (with numbers) are gray-shaded. Primers used for amplification of genomic *PmCOMT* and corresponding clones are illustrated.

3.3 Phylogenetic analysis

3.3.1 Phylogenetic analysis of *PmCOMT*

Phylogenetic relationships between *PmFAMeT* and *PmCOMT* and their orthologues were examined. A neighbor-joining tree clearly indicated that *COMT* and *FAMeT* from various species were allocated to be different groups of OMT. Both *PmFAMeT-l* and *PmFAMeT-s* are closely related to *FAMeT* of other decapod crustaceans and are regarded as members of crustacean *FAMeT* rather than *COMT* (Fig 3.28).

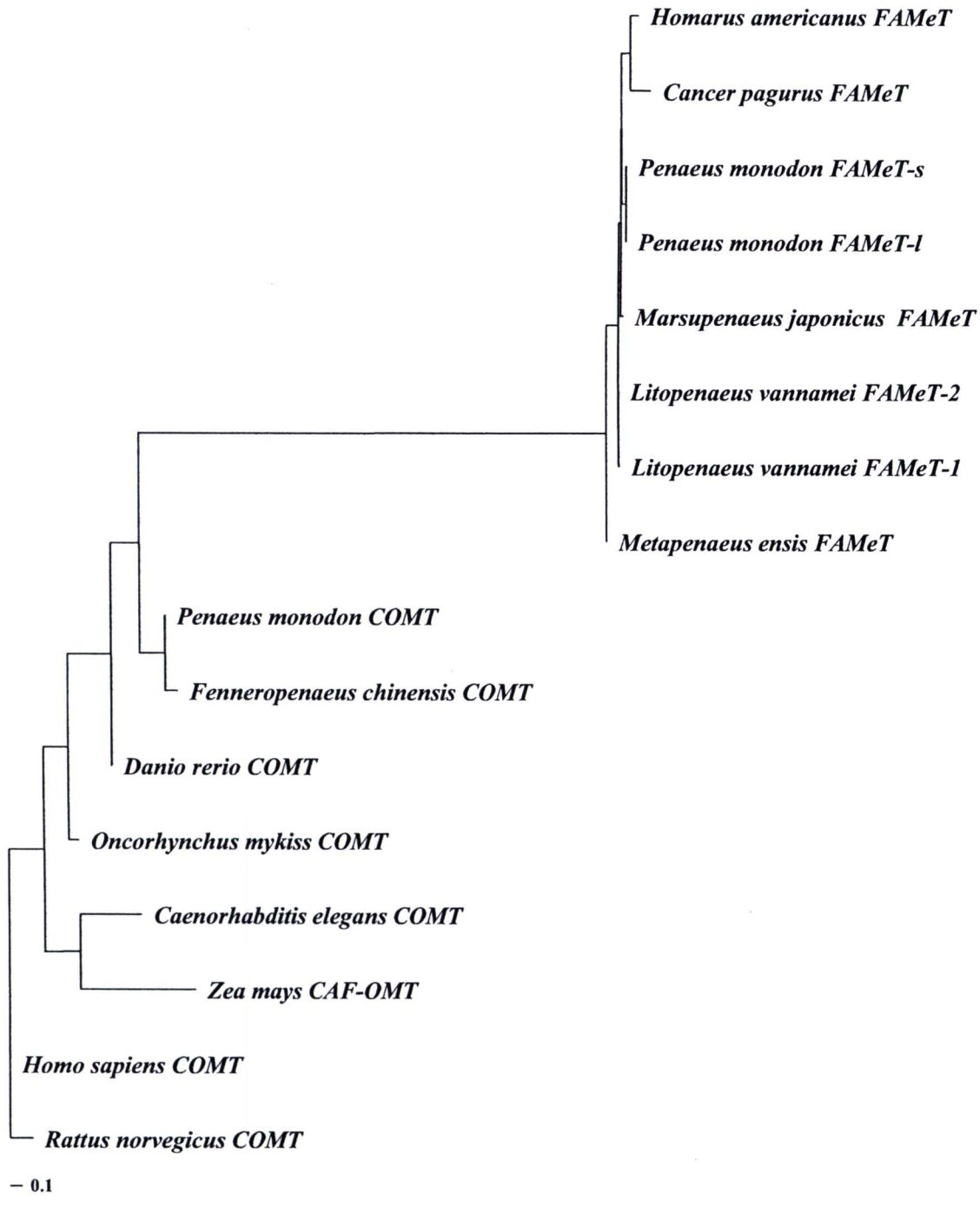


Figure 3.28 A neighbor-joining tree illustrating phylogenetic relationships of *catechol-O-methyltransferase* (*COMT*) and *farnesoic-O-methyltransferase* (*FAMeT*) of various taxa.

3.4. Determination of expression profile and tissue distribution of *PmCOMT, PmFAMeT, PmBr-cZ1* and *PmBr-cZ4* genes in *P. monodon* by RT-PCR

3.4.1 Determination of expression profile of *PmCOMT, PmFAMeT, PmBr-cZ1* and *PmBr-cZ4* genes in *P. monodon* by RT-PCR

Total RNA were extracts from ovaries or testes of 4-month-old juveniles and wild broodstock of male and female *P. monodon* (N = 4 for each group). DNase I-treated total RNA of each specimen was reverse-transcribed. RT-PCR was carried out.

The end-point PCR revealed greater expression levels of *PmCOMT* (Figs. 3.29-3.30 and Table 3.1) and *PmBr-cZ4* (Figs. 3.35-3.36 and Table 3.4) genes in ovaries (N = 4) than testes in both juvenile and broodstock of *P. monodon* ($P < 0.05$). Interestingly, the expression levels in ovaries of juvenile were greater than that in ovaries of broodstock ($P < 0.05$).

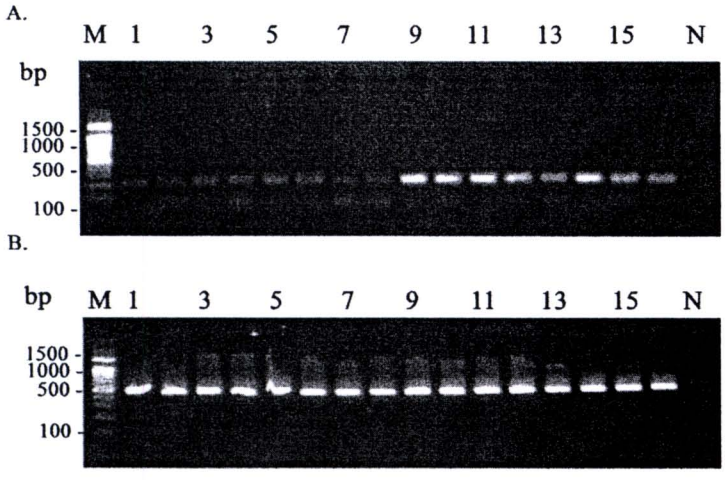


Figure 3.29 Agarose gel electrophoresis showing RT-PCR of *PmCOMT* using the first strand cDNA from ovaries of cultured juveniles (lanes 1-4, A) and wild broodstock (lanes 5-8, A) and testes of cultured juveniles (lanes 9-12, A) and wild broodstock (lanes 13-16, A) *P. monodon*. *EF-1α* was successfully amplified from the same template (B). Lanes M and N are a 100 bp DNA marker and the negative control (without cDNA template), respectively.

Table 3.1 Relative expression level of *PmCOMT* in ovaries and testes of *P. monodon*

Stage	Relative expression level
JNTT ($N = 4$)	0.3974 ± 0.1821^{a}
BSTT ($N = 4$)	0.5595 ± 0.2841^{a}
JNOV ($N = 4$)	3.2787 ± 0.2695^{b}
BSOV ($N = 4$)	1.6116 ± 0.4066^{c}

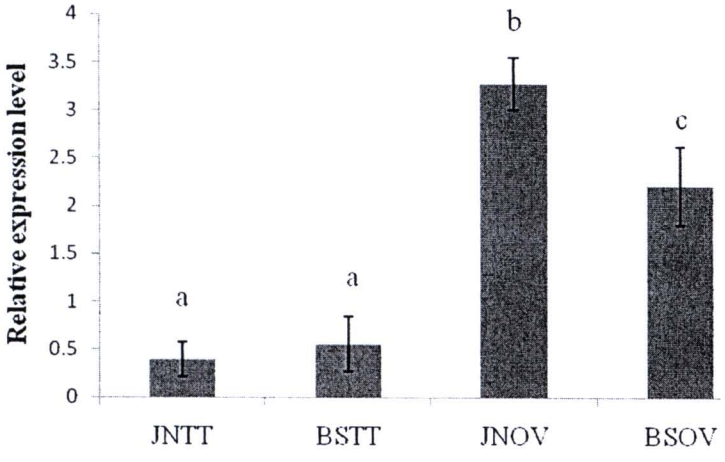


Figure 3.30 Histograms showing the relative expression profiles of *PmCOMT* in testes of cultured juveniles (JNTT) and wild broodstock (BSTT) and ovaries of cultured juveniles (JNOV) and wild broodstock (BSOV) of *P. monodon*.

In contrast, the expression of *PmFAMeT* was not significantly different between ovaries and testes in both juvenile and broodstock *(P* > 0.05*)* (Figs 3.31-3.32 and Table 3.2).

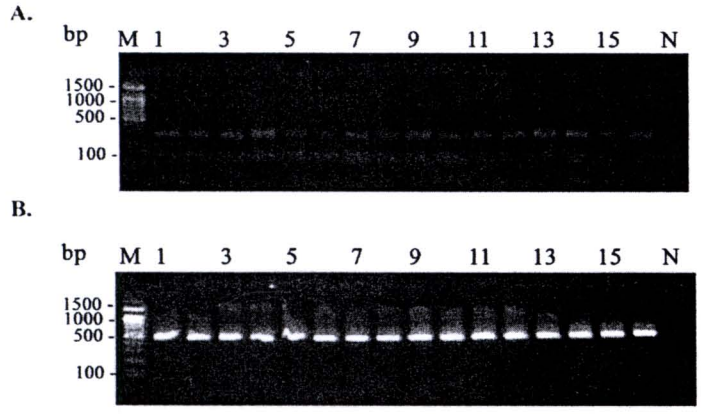


Figure 3.31 Agarose gel electrophoresis showing RT-PCR of *PmFAMeT* using the first strand cDNA from ovaries of cultured juveniles (lanes 1-4, A) and wild broodstock (lanes 5-8, A) and testes of cultured juveniles (lanes 9-12, A) and wild *P. monodon* broodstock (lanes 13-16, A). *EF-1α* was successfully amplified from the same template (lanes 1-16, B). Lanes M and N are a 100 bp DNA marker and the negative control (without cDNA template), respectively.

Table 3.2 Relative expression level of *PmFAMeT* ovaries and testes of *P. monodon*

Stage	Relative expression level
JNTT ($N = 4$)	0.2644 ± 0.0421^{a}
BSTT ($N = 4$)	0.2609 ± 0.0544^{a}
JNOV ($N = 4$)	0.2521 ± 0.0967^{a}
BSOV ($N = 4$)	0.2448 ± 0.0904^{a}

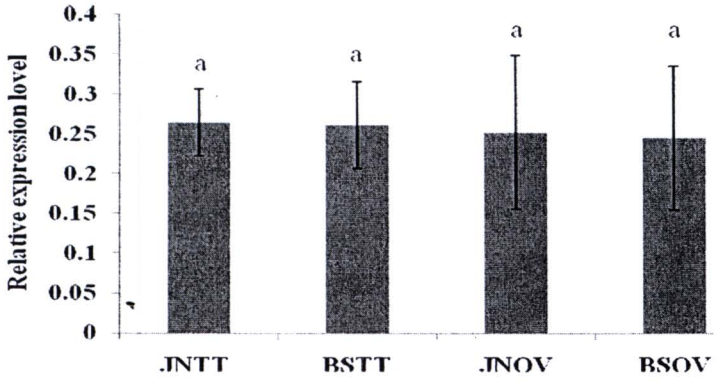


Figure 3.32 Histograms showing the relative expression profiles of *PmFAMeT* in testes of cultured juveniles (JNTT) and wild broodstock (BSTT) and ovaries of cultured juveniles (JNOV) and wild broodstock (BSOV) of *P. monodon*.

The expression profiles of *PmBr-cZ1* and *PmBr-cZ4* in ovaries of juvenile and broodstock were greater than those in testes of both stages of *P. monodon* ($P < 0.05$; Figs. 3.33-3.36 and Tables 3.3 and 3.4).

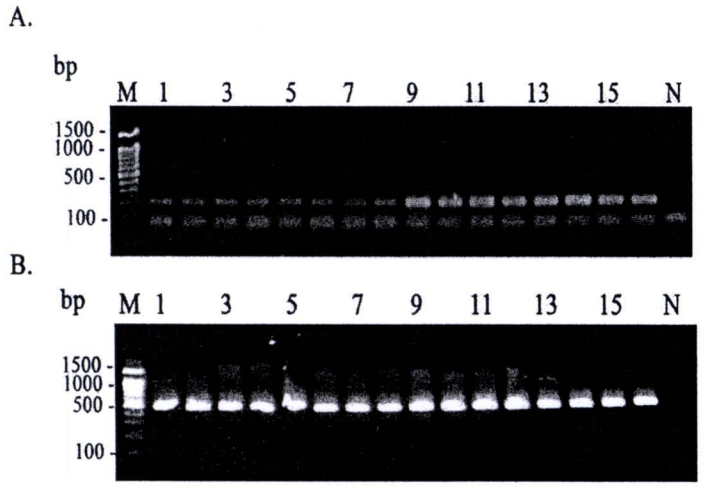


Figure 3.33 Agarose gel electrophoresis showing RT-PCR of *PmBR-cZ1* using the first strand cDNA from ovaries of cultured juveniles (lanes 1-4, A) and wild broodstock (lanes 5 - 8, A) and testes of cultured juveniles (lanes 9-12, A) and wild broodstock (lanes 13-16, A) of *P. monodon*. *EF-1α* was successfully amplified from the same template (lanes 1-16, B). Lanes M and N are a 100 bp DNA marker and the negative control (without cDNA template), respectively.

Table 3.3 Relative expression level of *PmBr-cZ1* ovaries and testes of *P. monodon*

Stage	Relative expression level
JNTT($N = 4$)	0.2876 ± 0.0711^{a}
BSTT($N = 4$)	0.2609 ± 0.0505^{a}
JNOV($N = 4$)	1.2458 ± 0.1067^{b}
BSOV($N = 4$)	1.0984 ± 0.1748^{b}

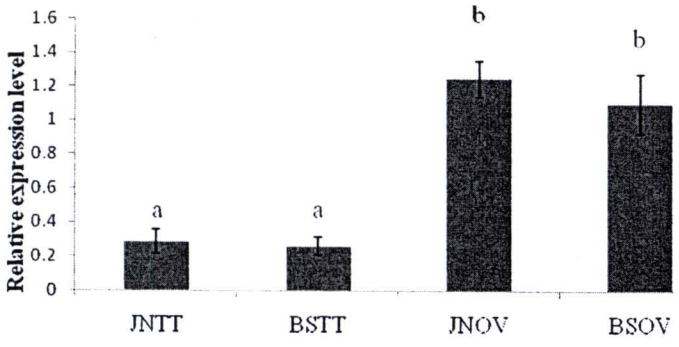


Figure 3.34 Histograms showing the relative expression profiles of *PmBr-cZ1* in testes of cultured juveniles (JNTT) and wild broodstock (BSTT) and ovaries of cultured juveniles (JNOV) and wild broodstock (BSOV) of *P. monodon*.

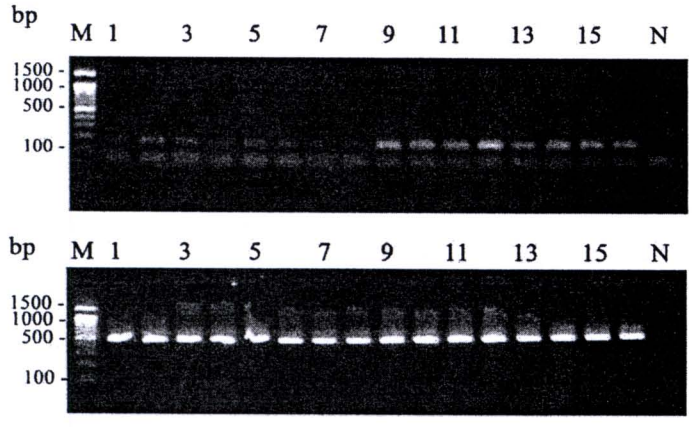


Figure 3.35 Agarose gel electrophoresis shwing RT-PCR of *PmBR-cZ4* using the first strand cDNA from testes of cultured juveniles (lanes 1 - 4, A) and wild broodstock (lanes 5 - 8, A) and ovaries cultured juveniles (lanes 9 - 12, A) and wild broodstock (lanes 13 - 16, A) of *P. monodon*. *EF-1α* was successfully amplified from the same template (lanes 1-16, B). Lanes M and N are a 100 bp DNA marker and the negative control (without cDNA template), respectively.

Table 3.4 Relative expression level of *PmBr-cZ4* in ovaries and testes of *P. monodon*

Stage	Relative expression level
JNTT($N = 4$)	0.1404 ± 0.0795^{a}
BSTT($N = 4$)	0.1012 ± 0.0445^{a}
JNOV($N = 4$)	0.8544 ± 0.3060^{c}
BSOV($N = 4$)	0.4509 ± 0.0536^{b}

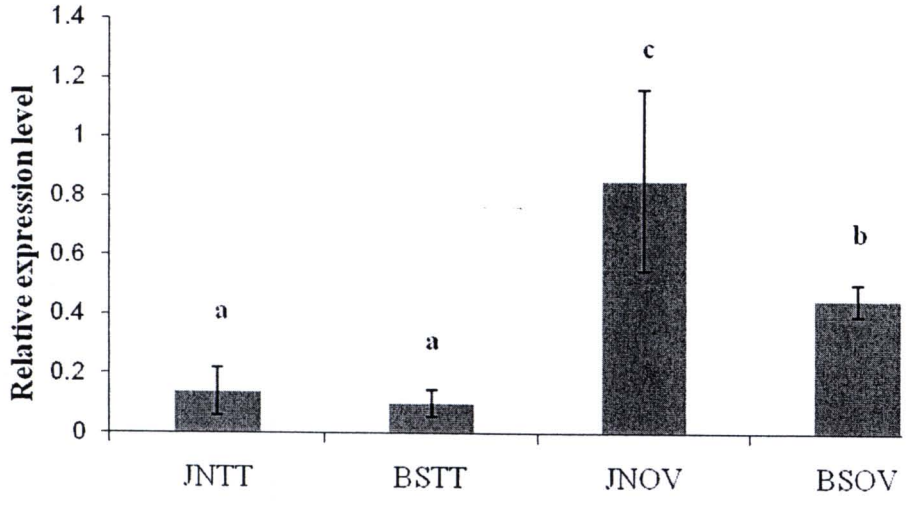


Figure 3.36 Histograms showing the relative expression profiles of *PmBr-cZ4* in testes of cultured juveniles (JNTT) and wild broodstock (BSTT) and ovaries of cultured juveniles (JNOV) and wild broodstock (BSOV) of *P. monodon*.

3.4.2 Tissue distribution analysis of *PmCOMT, PmFAMeT, PmBr-cZ1* and *PmBr-cZ4* genes in *P. monodon* examined by RT-PCR

The expression of *PmCOMT, PmFAMeT, PmBr-cZ1* and *PmBr-cZ4* in ovaries and testes of 6-month-old juveniles, domesticated male and female broodstock, various tissues (heart, hemocytes, lymphoid organs, intestine, gill, pleopods, thoracic ganglion, stomach, eyestalk, hepatopancreas, ovaries and testes) of female juvenile and broodstock and testes of male juvenile and broodstock were examined using RT-PCR analysis.

3.4.2.1 *PmCOMT*

PmCOMT was constitutively expressed in all examined tissues and abundantly expressed in ovaries and intestine of female broodstock and testes of a male broodstock. Lower expression levels of *PmCOMT* were observed in, for example, hepatopancreas, stomach, thoracic ganglion, eyestalk, pleopods and epicuticle of female broodstock (Fig. 3.37).

In juveniles, *PmCOMT* was constitutively expressed in all examined tissues. It was abundantly expressed in hemocytes, hepatopancreas, stomach and thoracic ganglion of a female juvenile and testes of a male juvenile. Lower expression levels of *PmCOMT* were observed in other tissues for example, gill, heart, intestine, lymphoid organs, eyestalk and pleopods of a female juvenile (Fig. 3.38).

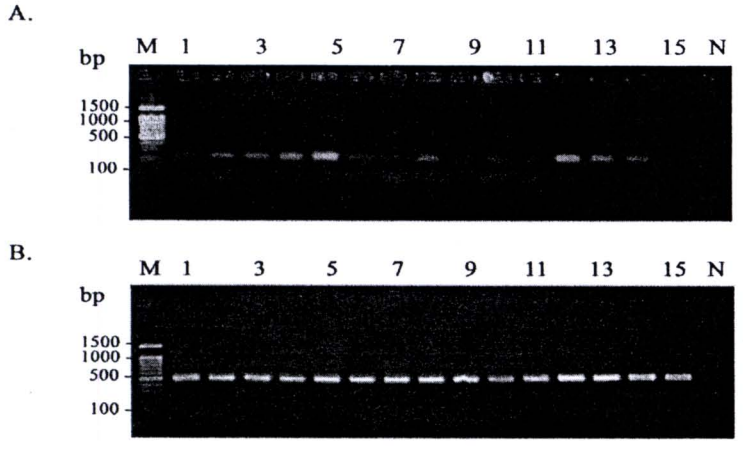


Figure 3.37 1.5% ethidium bromide-stained agarose gels showing results from tissue distribution analysis of *PmCOMT* (A) using the first strand cDNA of various tissues of females: HE = heart, HC = hemocytes, LO = lymphoid organs, IT = intestine, GL = gill, PL = pleopods, TG = thoracic ganglion, ST = stomach, ES = eyestalk, HP = hepatopancreas, OJ = juvenile ovaries, OJ = broodstock ovaries and males: TJ = juvenile testes, TB = broodsotck testes, of *P. monodon* corresponding to lanes 1-15, respectively. *EF-1α* was successfully amplified from the same template (lanes 1-15, B). Lanes M and N are a 100 bp DNA marker and the negative control (without cDNA template), respectively.

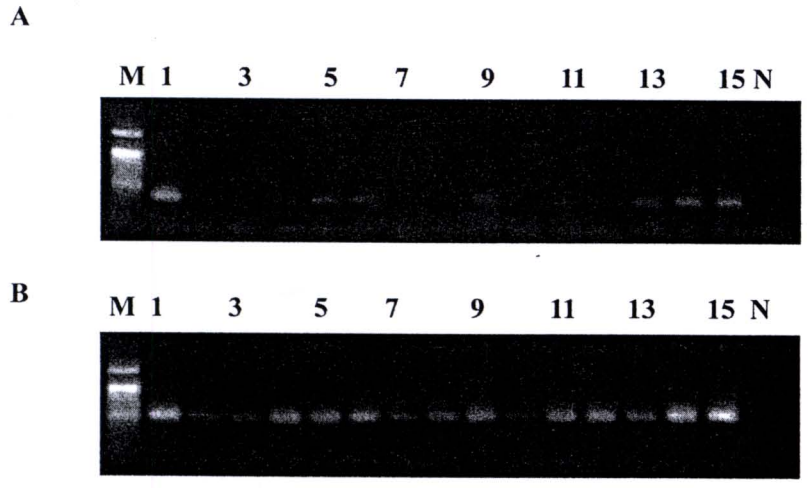


Figure 3.38 1.5% ethidium bromide-stained agarose gels showing results from tissue distribution analysis of *PmCOMT* (A) using the first strand cDNA of various tissues of female juvenile (HC = hemocytes, GL = gill, HE = heart, OJ = juvenile ovaries, HP = hepatopancreas, ST = stomach, IT = intestine, LO = lymphoid organs, TG = thoracic ganglion, ES = eyestalk, PL = pleopods) and male juvenile (TJ = juvenile testes), female broodstock (OB = broodsotck ovaries) and male broodstock (TB = broodsotck testes) of *P. monodon* corresponding to lanes 1-15 (A). *EF-1α* was successfully amplified from the same template (lanes 1-15, B). Lanes M and N are a 100 bp DNA marker and the negative control (without cDNA template), respectively.

3.4.2.2 *PmFAMeT*

PmFAMeT was constitutively expressed in all examined tissues of broodstock. Slightly lower expression patterns of *PmFAMET* were observed in stomach and hemocyte of a female broodstock and testes of a male broodstock (Fig. 3.39).

In juvenile, *PmFAMeT* was expressed in all examined tissues. It was abundantly expressed in hemocytes and thoracic ganglion of a female juvenile. Lower expression levels of *PmFAMeT* were observed in other tissues for example, gill, heart, intestine, lymphoid organs and pleopods of a female broodstock and testes of male juvenile (Fig. 3.40).

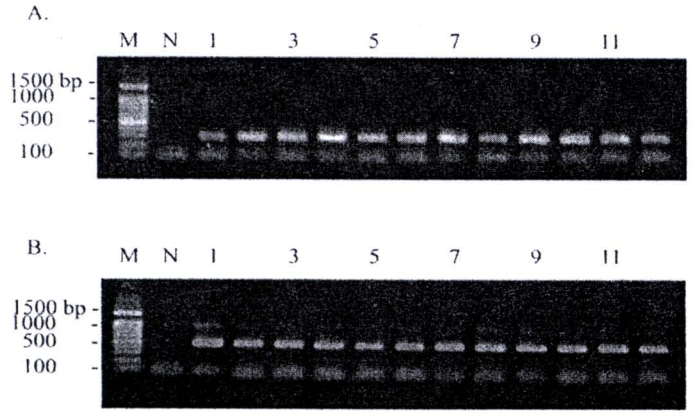


Figure 3.39 Tissue expression analysis of *PmFAMeT* (A) and *EF-1α* (B) in various tissues of female (HE; hemocytes, gills, GL; heart, HC; ovaries, OV; lymphoid organs, LO; intestine, IT; hepatopancreas, HP; stomach, ST; thoracic ganglion, TG; eyestalk, ES, pleopods, PL; lanes 1-11) and testes (testes, TT; lane 12) of male *P. monodon* broodstock. *EF-1α* was successfully amplified from the same template. Lanes M are a 100 bp DNA marker.

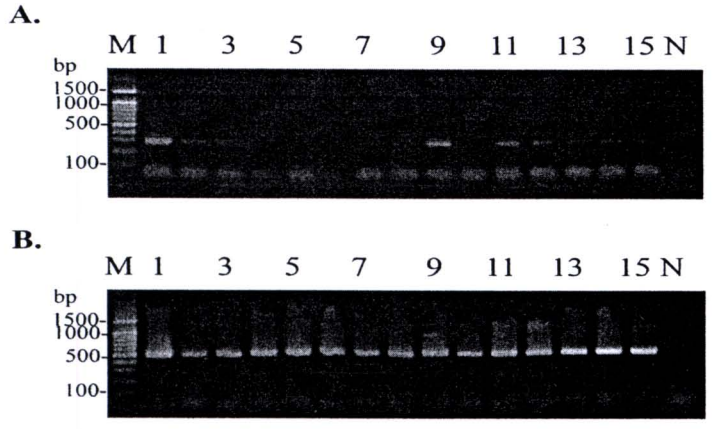


Figure 3.40 1.5% ethidium bromide-stained agarose gels showing results from tissue distribution analysis of *PmFAMeT* using the first strand cDNA of various tissues of a female juvenile (HC = hemocytes, GL = gill, HE = heart, OJ = juvenile ovaries, HP = hepatopancreas, ST = stomach, IT = intestine, LO = lymphoid organs, TG = thoracic ganglion, ES = eyestalk, PL = pleopods, male juvenile (TJ = juvenile testes), male broodstock (TB = broodsotck testes) and female broodstock (OB = broodsotck ovaries; lanes 1-15) of *P. monodon*. *EF-1α* was successfully amplified from the same template (B). Lanes M and N are a 100 bp DNA marker and the negative control (without cDNA template), respectively.

3.4.2.3 *PmBr-cZ1*

PmBr-cZ1 was abundantly expressed in ovaries of *P. monodon* broodstock. Lower expression was observed in heart, lymphoid organs, intestine, hepatopancreas, stomach, thoracic and testes of both juvenile and broodstock (Fig. 3.41). It was abundantly expressed in hemocyte of a female juvenile. A lower expression was found in ovaries but not in other tissues of a juvenile shrimp (Fig. 3.42).

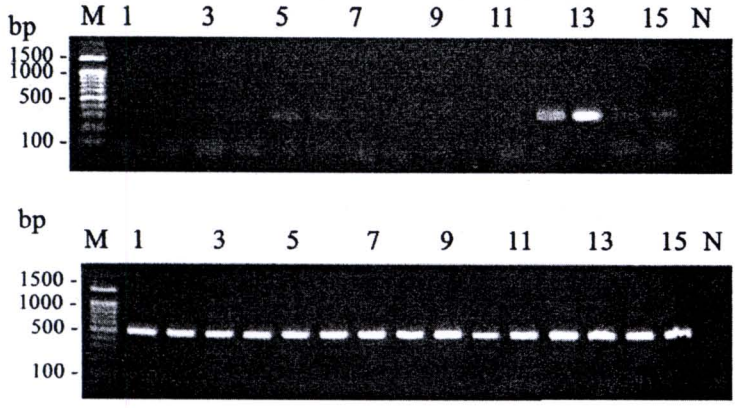


Figure 3.41 Tissue distribution analysis of *PmBr-cZ1* using the first strand cDNA of hemocytes, gills, heart, lymphoid organs, intestine, hepatopancreas, stomach, thoracic ganglia, Eyestalk, pleopods, epidermis, ovaries of juvenile, ovaries of broodstock, testes of juvenile and testes of broodstock (lanes 1 - 15) and *EF-1α* (B). Lanes M and N are a 100 bp DNA marker and the negative control (without cDNA template), respectively.

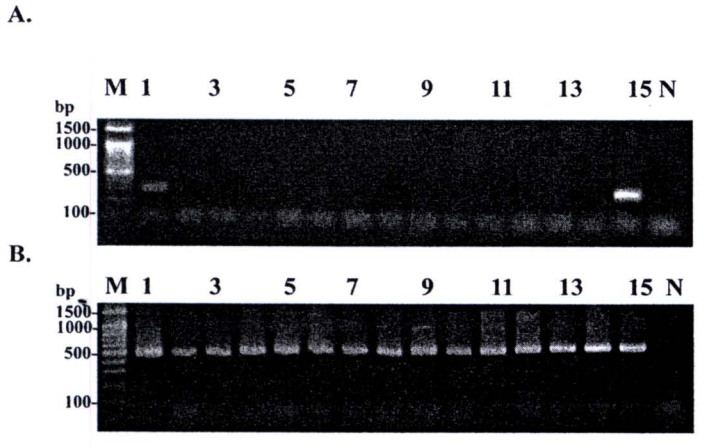


Figure 3.42 1.5% ethidium bromide-stained agarose gels showing results from tissue distribution analysis of *PmBr-cZ1* (A) using the first strand cDNA of various tissues

of female juvenile (HC = hemocytes, GL = gill, HE = heart, OJ = juvenile ovaries, HP = hepatopancreas, ST = stomach, IT = intestine, LO = lymphoid organs, TG = thoracic ganglion, ES = eyestalk, PL = pleopods, male juvenile (TJ = juvenile testes), male broodstock (TB = broodsotck testes) and female broodstock (OB = broodsotck ovaries) of *P. monodon*. *EF-1α* was successfully amplified from the same template (B). Lanes M and N are a 100 bp DNA marker and the negative control (without cDNA template), respectively.

3.4.2.4 *PmBr-cZ4*

PmBr-cZ4 was abundantly expressed in ovaries of broodstock of *P. monodon* with a lower express pattern in hemocytes, gills, heart,, lymphoid organs, intestine, stomach, thoracic ganglion, pleopods, antennal gland and testes of broodstock. This transcript was not expressed in hepatopancreas and eyestalk of a shrimp broodstock (Fig. 3.43). *PmBr-cZ4* was only expressed in hemocyte and ovaries of a female juvenile (Fig. 3.44)

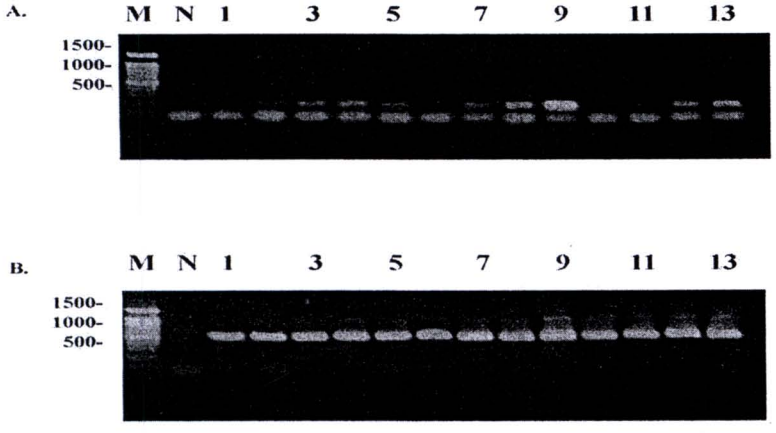


Figure 3.43 Tissue distribution analysis of *PmBr-cZ4* (A) using the first strand cDNA of hemocytes, gills, heart, lymphoid organs, intestine, hepatopancreas, stomach, thoracic ganglion, ovaries of broodstock, eyestalk, pleopods, antennal gland and testes of broodstock (lanes 1-13) and *EF-1α* (B). Lanes M and N are a 100 bp DNA marker and the negative control (without cDNA template), respectively.

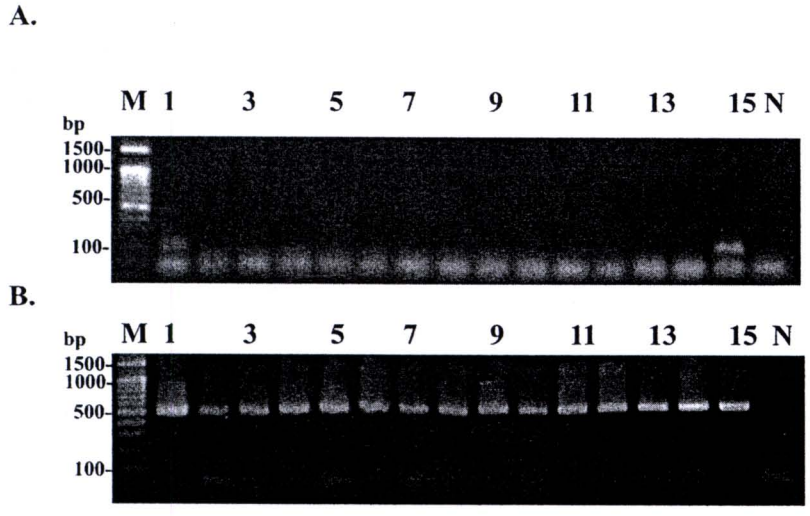


Figure 3.44 1.5% ethidium bromide-stained agarose gels showing results from tissue distribution analysis of *PmBr-cZ4*(A) using the first strand cDNA of various tissues of female juvenile (HC = hemocytes, GL = gill, HE = heart, OJ = juvenile ovaries, HP = hepatopancreas, ST = stomach, IT = intestine, LO = lymphoid organs, TG = thoracic ganglion, ES = eyestalk, PL = pleopods), male juvenile (TJ = juvenile testes), male broodstock (TB = broodsotck testes) and female broodstock (OB = broodsotck ovaries) of *P. monodon*. *EF-1α* was successfully amplified from the same template (B). Lanes M and N are a 100 bp DNA marker and the negative control (without cDNA template), respectively.

3.4.3 Expression levels of *PmCOMT* and *PmFAMeT* during ovarian development of wild *P. monodon* examined by semi-quantitative RT-PCR

3.4.3.1 Optimization of PCR conditions for semi-quantitative RT-PCR

Semi-quantitative RT-PCR was carried out to determine whether the expression levels of *PmCOMT*, and *PmFAMeT* were significantly different during ovarian development of *P. monodon*. In addition, this technique was also applied to evaluate the effects of dopamine (at 10^{-6} M/shrimp) and serotonin (50 ug/g body weight) administration.

To carry out semi-quantitative RT-PCR, several parameters of the amplification and PCR components required further optimization. As a result, primer and $MgCl_2$ concentrations and the number of amplification cycles were carefully

optimized. *EF-1α* was used as the internal control. The chosen parameter for each factor was that generating the highest specificity with the relatively intense product.

The standard RT-PCR was carried out by using 100 ng of the first strand cDNA template from ovaries of juvenile *P. monodon* (approximately 15-20 g body weight) at the annealing temperature of 55 °C, 1 U of Dynazyme DNA polymerase and 0.2 μM of each primer and various $MgCl_2$ concentrations for 28 cycles. After the most suitable $MgCl_2$ concentration was chosen. RT-PCR was carried out using an optimized $MgCl_2$ concentration for further optimization of primer concentration. Finally, selected primer and $MgCl_2$ concentrations were included for optimization of the suitable number of the amplification cycles. 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. The most suitable condition for amplification of *PmCOMT*, *PmFAMeT* and *EF-1α* were showed in Table 3.5.

3.4.3.2 Differential expression of *PmCOMT* and *PmFAMeT* during ovarian development of wild *P. Monodon*

Total RNA were extracted from different stages of ovaries of wild intact and eyestalk-ablated females of *P. monodon*. After reverse transcription, RT-PCR was carried out.

Table 3.5 Optimized $MgCl_2$ and primer concentrations, number of amplification cycles and thermal profiles for semi-quantitative RT-PCR of *EF1-α, PmCOMT* and *PmFAMeT* in ovaries of *P. monodon*

Gene	$MgCl_2$ (mM)	Primer con. (μM)	No. of cycles	PCR condition
1. *EF-1α*	1.5	0.15	23	94°C for 3 min followed by optimized cycles of 94°C for 30 sec, 55°C for 45 sec and 72°C for 45 sec and 72°C for 7 min
2. *PmCOMT*	1.5	0.15	28	As described in 1.
3. *PmFAMeT*	1.5	0.15	30	As described in 1.

Results showed that the expression levels of *PmCOMT* and *PmFAMeT* were significantly increased at stages II and stages III and IV ovaries of intact broodstock, respectively ($P < 0.05$) (Fig. 3.45 and Table 3.6). In contrast, levels of both transcripts were comparable throughout the ovarian developmental stages (stages I-IV) in eyestalk ablated female broodstock ($P > 0.05$) (Figs. 3.46-3.47 and Table 3.7).

3.4.3.3 The expression profiles of *PmCOMT* and *PmFAMeT* in ovaries of *P. monodon* following dopamine and serotonin administration

Semi-quantitative RT-PCR was then applied for determining effects of neurotransmitters (serotonin and dopamine) on expression levels of *PmCOMT* and *PmFAMeT* in ovaries of juvenile *P. monodon*.

3.4.3.3.1 Dopamine administration

Results from semi-quantitative RT-PCR indicated that dopamine (10^{-6} M/shrimp) resulted in significant lower expression of *PmCOMT* in ovaries of juvenile shrimp at 24 hour post injection (hpi, $P < 0.05$; Fig. 3.48 and Table 3.7) but significant higher expression of *PmFAMeT* in ovaries of juvenile shrimp was observed at 12 and 24 hpi ($P < 0.05$) (Fig. 3.48 and Table 3.8).

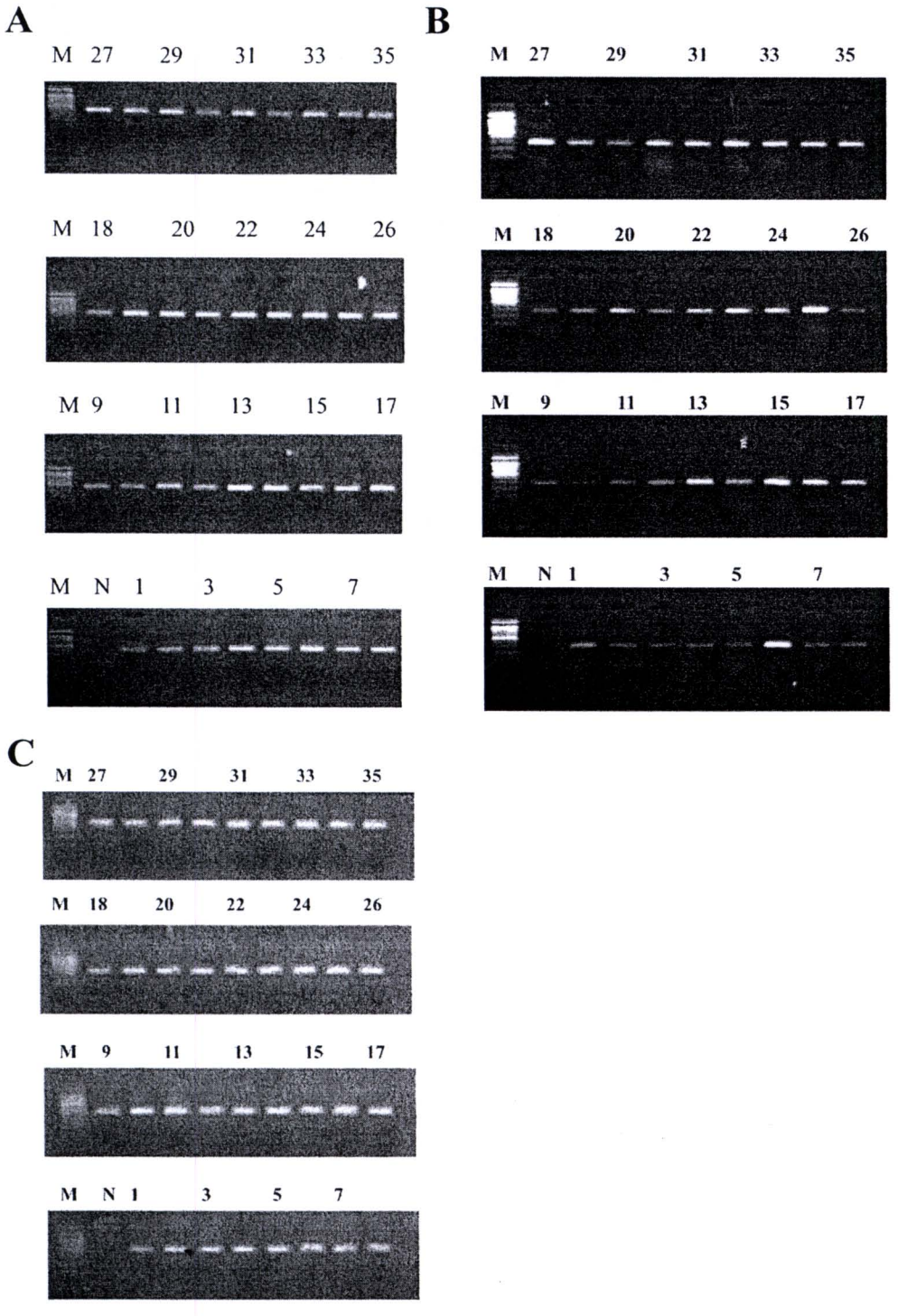


Figure 3.45 Semi-quantitative RT-PCR of *PmCOMT* (A) and *PmFAMeT* (B) and *EF-1α* (C) in different stages of ovaries of intact *P. monodon*.

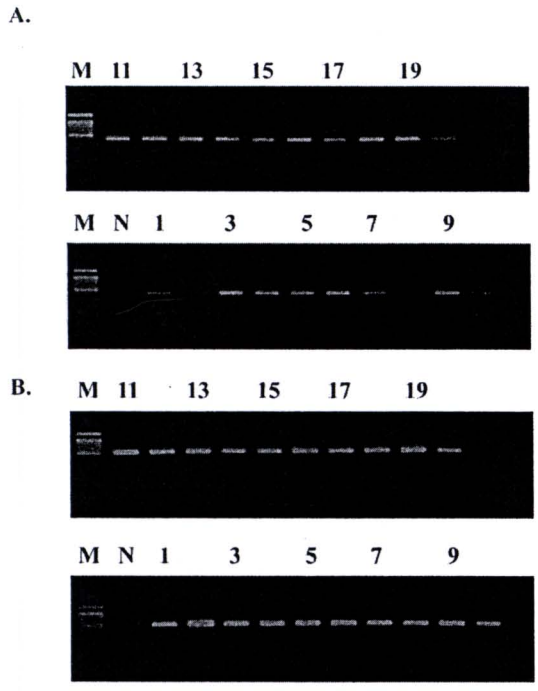


Figure 3.46 Semi-quantitative RT-PCR of *PmCOMT* (A) and *PmEF-1α* (B) in at different ovarian stages of eyestalk-ablated *P. monodon*

Table 3.6 Time course relative expression levels of *PmCOMT* and *PmFAMeT* in different ovarian stages of intact *P. monodon*

Stage	Relative expression level	
	PmFAMeT	***PmCOMT***
I Previtellogenetic ovaries ($N = 8$)	0.7534 ± 0.0855^{a}	1.3187 ± 0.0958^{a}
II Vitellogenetic ovaries ($N = 5$)	0.7421 ± 0.1580^{a}	1.1948 ± 0.0970^{b}
III Early cortical rod ($N = 9$)	0.8825 ± 0.0731^{b}	1.4069 ± 0.1830^{a}
IV Mature ($N = 13$)	0.9299 ± 0.0745^{b}	1.3712 ± 0.1248^{a}

Table 3.7 Time course relative expression levels of *PmCOMT* and *PmFAMeT* in different ovarian stages of eyestalk-ablated *P. monodon*

Stage	Relative expression level	
	PmFAMeT	***PmCOMT***
I Previtellogenetic ovaries ($N = 8$)	0.7170 ± 0.0970^{a}	0.7025 ± 0.1626^{a}
II Vitellogenetic ovaries ($N = 5$)	0.8880 ± 0.1212^{a}	0.7624 ± 0.0589^{a}
III Early cortical rod ($N = 9$)	0.7535 ± 0.1212^{a}	0.7501 ± 0.0972^{a}
IV mature ($N = 13$)	0.8541 ± 0.1772^{a}	0.7658 ± 0.1024^{a}

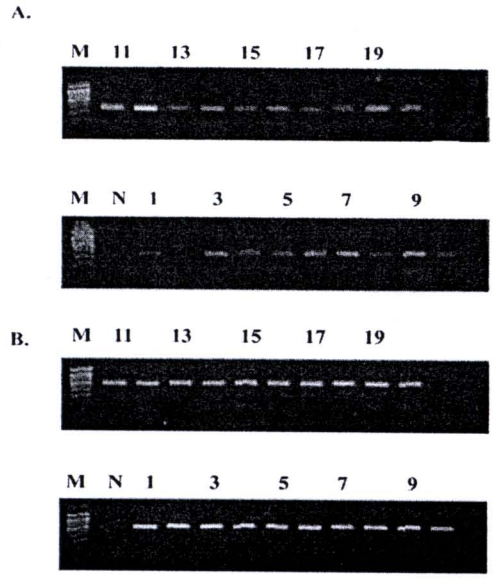


Figure 3.47 Semi-quantitative RT-PCR of *PmFAMeT* (A) and *PmEF-1α* (B) in different ovarian stages of eyestalk-broodstock *P. monodon*.

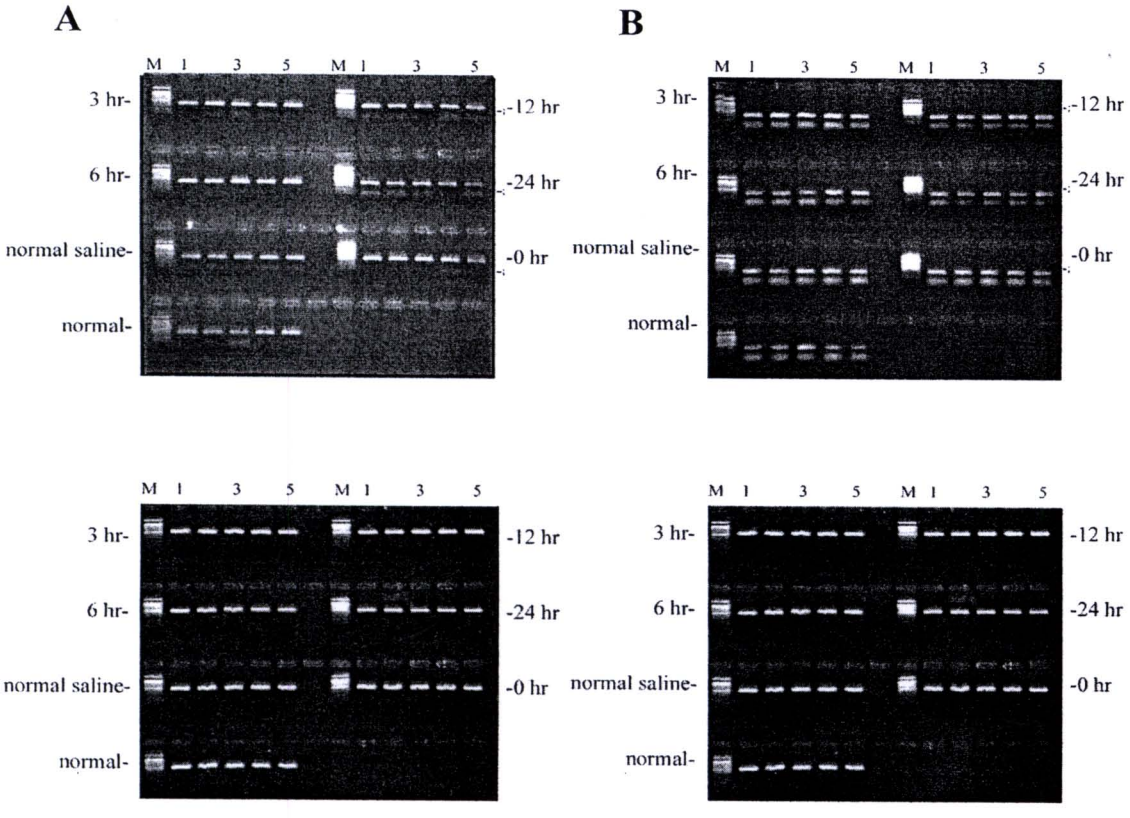


Figure 3.48 Semi-quantitative RT-PCR of *PmCOMT* (A) and *PmFAMeT* (B) of juvenile *P. monodon* treated with dopamine at 10^{-6} mole/shrimp. *EF1-α* was successfully amplified from the same template (bottom, A and B)

Table 3.8 Time course relative expression levels of *PmCOMT* and *PmFAMeT* in ovaries of *P. monodon* treated with dopamine at 10^{-6} M/shrimp

Gene	Relative expression level						
	Normal	**Vehicle control**	**0 hpt**	**3 hpt**	**6 hpt**	**12 hpt**	**24 hpt**
PmCOMT	0.31 ± 0.06^{ab}	0.36 ± 0.06^{abc}	0.38 ± 0.04^{c}	0.38 ± 0.04^{c}	$0.37 \pm 0.04b^{c}$	0.39 ± 0.04^{c}	0.30 ± 0.04^{a}
PmFAMeT	0.54 ± 0.20^{a}	0.88 ± 0.13^{cd}	0.71 ± 0.13^{abc}	0.66 ± 0.17^{ab}	0.53 ± 0.14^{a}	0.96 ± 0.10^{d}	0.75 ± 0.10^{bc}

3.4.3.3.2 Serotonin administration

Results indicated that the expression of *PmFAMeT* in ovaries of juvenile *P. monodon* upon serotonin administration (50 μg/g body weight) was significantly down-regulated at 48 and 72 hpi ($P < 0.005$) (Fig. 3.49 and Table 3.9). In contrast, serotonin had no significant effects on expression of *PmCOMT* in ovaries of juvenile *P. monodon* ($P > 0.005$, Fig. 3.50 and Table 3.9).

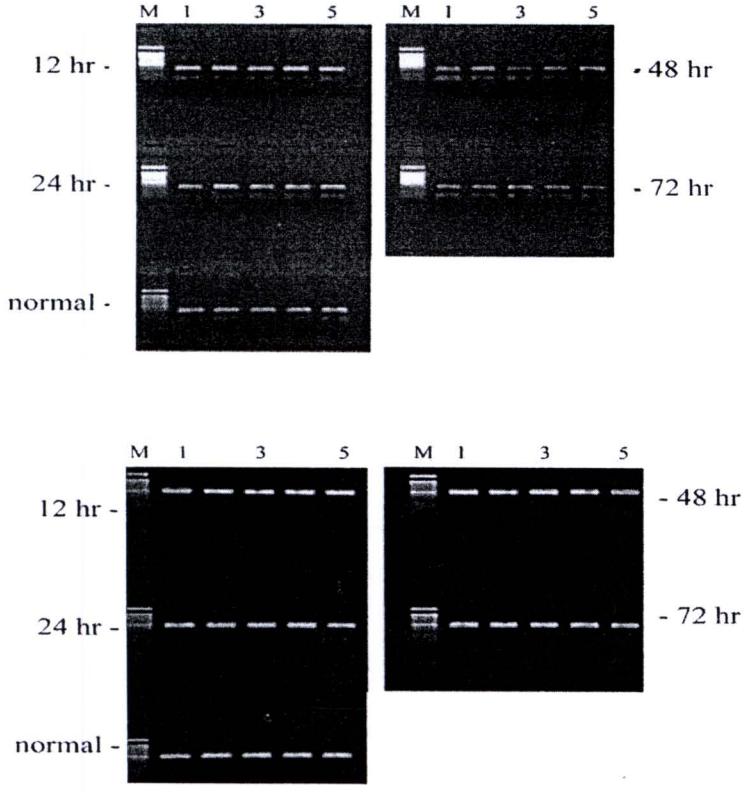


Figure 3.49 Semi-quantitative RT-PCR of *PmCOMT* (top) in ovaries of juvenile *P. monodon* treated with serotonin (50 ug/g body weight). *EF*1-α (bottom) was successfully amplified from the same template.

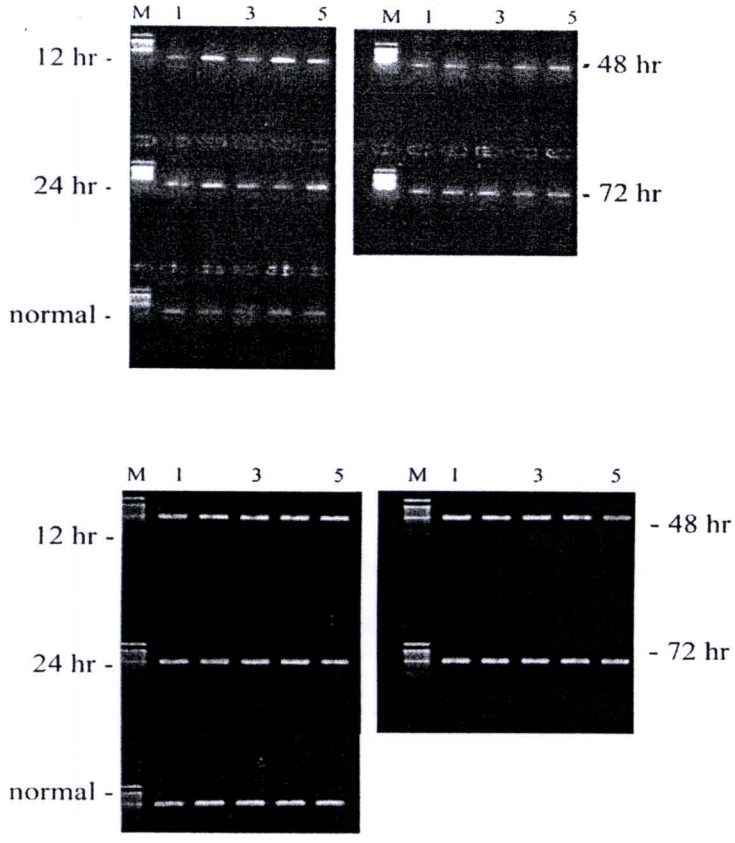


Figure 3.50 Semi-quantitative RT-PCR of *PmCOMT* (top) in ovaries of juvenile *P. monodon* treated with serotonin (50 ug/g body weight). *EF*1-α (bottom) was successfully amplified from the same template.

Table 3.9 Time-course relative expression levels of *PmCOMT* and *PmFAMeT* in ovaries of *P. monodon* juveniles treated serotonin (50 ug/g body weight)

Gene	Average of intensity of band				
	Normal saline (*N*=5)	**12 hpt (*N*=5)**	**24 hpt (*N*=5)**	**48 hpt (*N*=5)**	**72 hpt (*N*=5)**
PmCOMT	1.00 ± 0.18^{a}	0.90 ± 0.10^{a}	0.90 ± 0.07^{a}	0.83 ± 0.16^{a}	0.83 ± 0.13^{a}
PmFAMeT	0.17 ± 0.03^{a}	0.21 ± 0.02^{a}	0.21 ± 0.03^{a}	0.09 ± 0.05^{b}	0.09 ± 0.02^{b}

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

The expression levels of *PmCOMT, PmFAMeT, PmBr-cZ1* and *PmBr-cZ4* genes in ovaries of *P. monodon* were examined by quantitative real-time PCR analysis.

The standard curve of each target gene and the control (*EF-1α*) was constructed from the 10-fold dilutions covering 10^3-10^8 copy numbers of all genes except *PmFAMeT* where 10^2–10^8 copy numbers was used. The amplification efficiency of the target genes and the internal control are shown by Fig. 3.51

Quantitative real-time PCR was carried out in duplicate using 100 ng of the first strand cDNA template for the target genes and 1 ng of the first strand cDNA template for *EF-1α*.

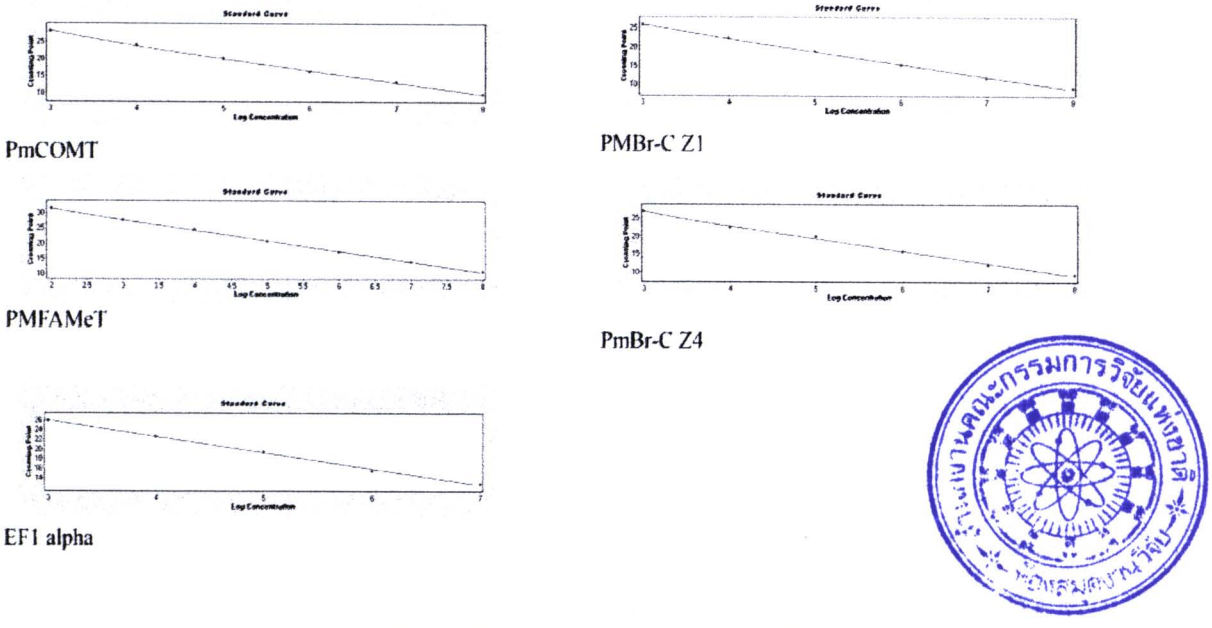
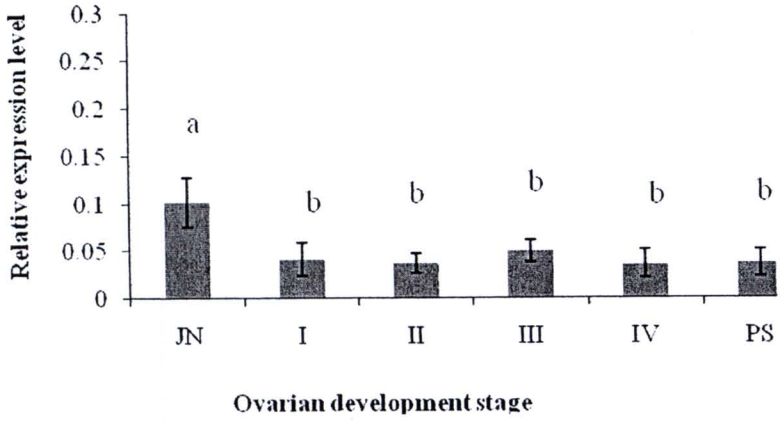


Figure 3.51 Standard curves of *PmCOMT* (Error: 0.0285, efficiency = 1.951 and the equation Y = -3.445*log(X) + 38.62), *PmFAMeT* (Error:0.0208, efficiency = 1.975 and the equation Y = -3.384 *log(X) + 37.93), *PmBr-cZ1*(Error:0.0251, efficiency = 2.005 and the equation Y = -3.309*log(X) + 35.89), *PmBr-cZ4* (Error:0.0385, efficiency = 1.953 and the equation Y = -3.440*log(X) + 37.10),*EF1-α* (Error:0.0285, efficiency = 1.994 and the equation Y = -3.335*log(X) + 35.87).

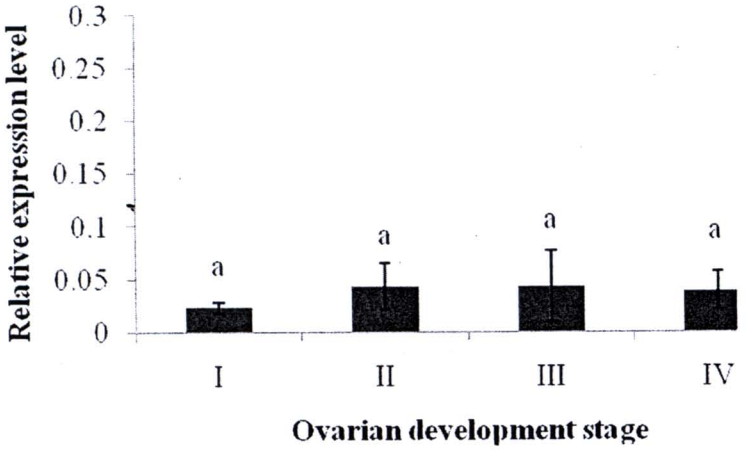
3.5.1 Expression profiles of *PmCOMT* during ovarian development of *P. monodon*

Quantitative real-time PCR revealed that the expression level of *PmCOMT* in ovaries of juveniles (4 months old) was greater than that of intact broodstock ($P < 0.05$). Interestingly, *PmCOMT* was comparably expressed during ovarian development in intact broodstock ($P > 0.05$). The expression level of *PmCOMT* in ovaries of eyestalk-ablated broodstock was not significantly different ($P>0.05$). At these stages, the *PmCOMT* mRNA was not significantly different from that in ovaries of juveniles ($P > 0.05$) but greater than that in ovaries of intact broodstock ($P > 0.05$) (Fig. 3.52 and Table 3.10).

A



B



C

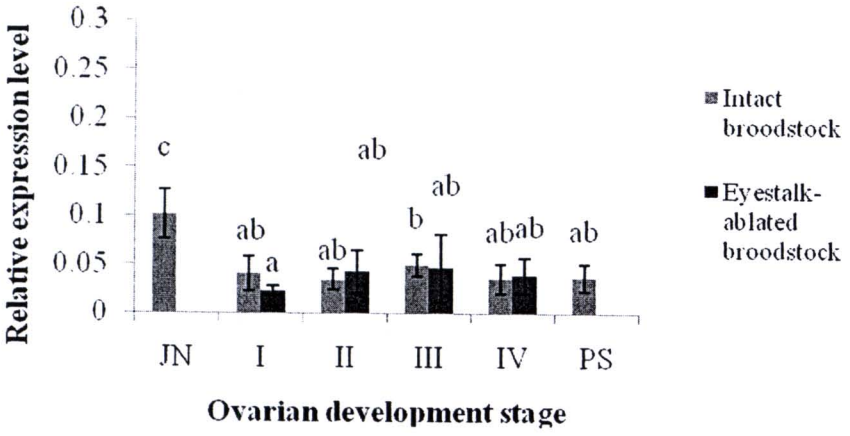


Figure 3.52 Histograms showing the relative expression profiles of *PmCOMT* in ovaries of cultured 4-month-old juveniles (JN, A) and different stages of ovarian development (stages I, previtellogenic; II, vitellogenic; III, early cortical rod; and IV, mature ovaries) of intact (A) and unilateral eyestalk-ablated (B) and intact post-spawning broodstock (PS; A). Data of intact and eyestalk-ablated broodstock were also analyzed together (C). Each bar corresponds to a particular ovarian stage. The same letters indicate that the expression levels were not significantly different ($P > 0.05$).

In domesticated broodstock, *PmCOMT* in ovaries of cultured 6-month-old juvenile and domesticated 14-month-old and 18-month-old broodstock were comparable ($P > 0.05$) (Fig. 3.53).

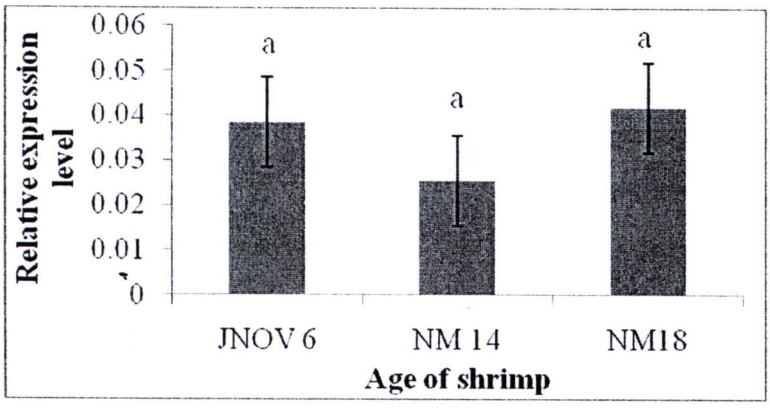


Figure 3.53 Histograms showing the relative expression profiles of *PmCOMT* in ovaries of domesticated juveniles (6 months old) and broodstock (14, and 18 months

old) *P. monodon*. Each bar corresponds to a particular ovarian stage. The same letters indicate that the expression levels were not significantly different ($P > 0.05$).

Table 3.10 Relative expression levels of *PmCOMT* in different ovarian stages of wild (A) and domesticated (B) *P. monodon*. females

A

Ovarian stage	Relative expression level			
	Intact shrimp	*N*	Eyestalk ablated shrimp	*N*
Juvenile	0.101483±0.025784^{c}	6	-	-
Stage I (GSI<1.5)	0.041000±0.017623^{ab}	10	0.022850±0.004649^{a}	4
Stage II (GSI 2-<4)	0.034825±0.010918^{ab}	8	0.042929±0.021993^{ab}	7
Stage III (GSI 4- <6)	0.049457±0.011968^{b}	7	0.046614±0.035120^{ab}	7
Stage IV (GS I>6)	0.035933±0.015196^{ab}	9	0.038838±0.017976^{ab}	8
Post-spawning	0.036917±0.013846^{ab}	6	-	-

B

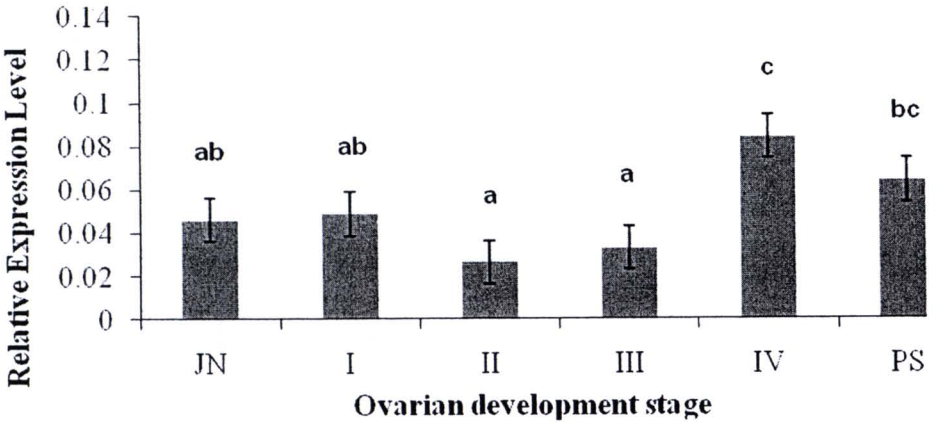
Group	Relative expression level
6 months old ($N = 4$)	0.0385±0.0085^{a}
14 months old ($N = 4$)	0.0254±0.0032^{a}
18 months old ($N = 3$)	0.0417±0.0158^{a}

3.5.2 Expression profiles of *PmFAMeT* during ovarian development of *P. monodon*

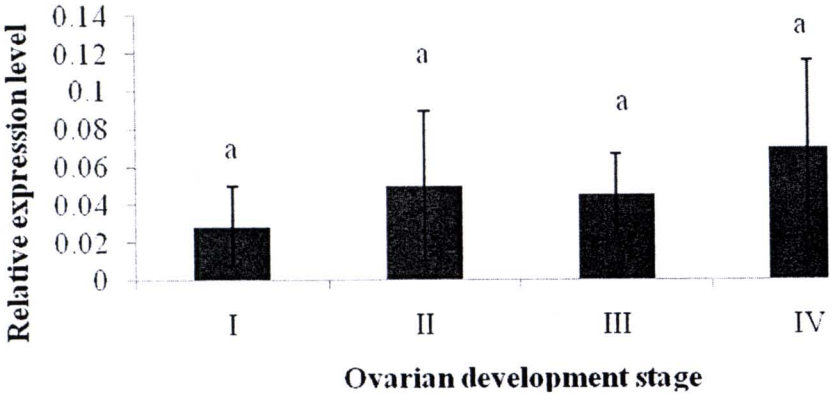
The *PmFAMeT* mRNA in ovaries of 4-month-old juveniles was comparable with that in stage I, II and III ovaries of intact broodstock. *PmFAMeT* was significantly up-regulated at stage IV (mature) ovaries in intact wild broodstock ($P < 0.05$) and returned to the basal level after spawning.

In eyestalk-ablated broodstock, its expression level seemed to be increased in stages II (vitellogenic), III (early cortical rod) and IV (mature ovaries) greater than that in stage I (previtellogenic) ovaries ($P > 0.05$). Results clearly indicated that eyestalk ablation did not have direct effects on expression of this gene (Fig. 3.54).

A


Relative Expression Level
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
ab
ab
a
a
c
bc
JN
I
II
III
IV
PS
Ovarian development stage

B.


Relative expression level
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
a
a
a
a
I
II
III
IV
Ovarian development stage

C.

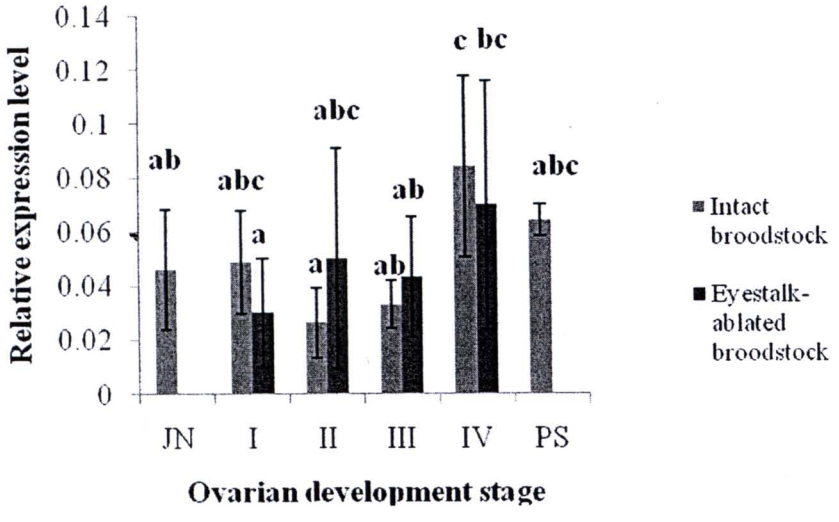

Relative expression level
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
ab
abc
a
a
abc
ab
ab
c bc
abc
JN
I
II
III
IV
PS
Ovarian development stage
Intact broodstock
Eyestalk-ablated broodstock

Figure 3.54 Histograms showing the relative expression profiles of *PmFAMeT* in ovaries of cultured 4-month-old juveniles (JN, A) and different stages of ovarian development (stages I, previtellogenic; II, vitellogenic; III, early cortical rod; and IV, mature ovaries) of intact (A) and unilateral eyestalk-ablated (B) and intact post-spawning broodstock (PS; A). Data of intact and eyestalk-ablated broodstock were also analyzed together (C). Each bar corresponds to a particular ovarian stage. The same letters indicate that the expression levels were not significantly different ($P > 0.05$).

The expression levels of *PmFAMeT* in ovaries of cultured 6-month-old juvenile and domesticated 14-month-old was greater than that of 18-month-old broodstock ($P > 0.05$). Although *PmFAMeT* in ovaries of 6-month-old juveniles was gradually decreased in domesticated broodstock (14 and 18 months old), results were not statistically significant when compared with 14-month-old shrimp owing to large standard deviation ($P > 0.05$) (Fig. 3.55).

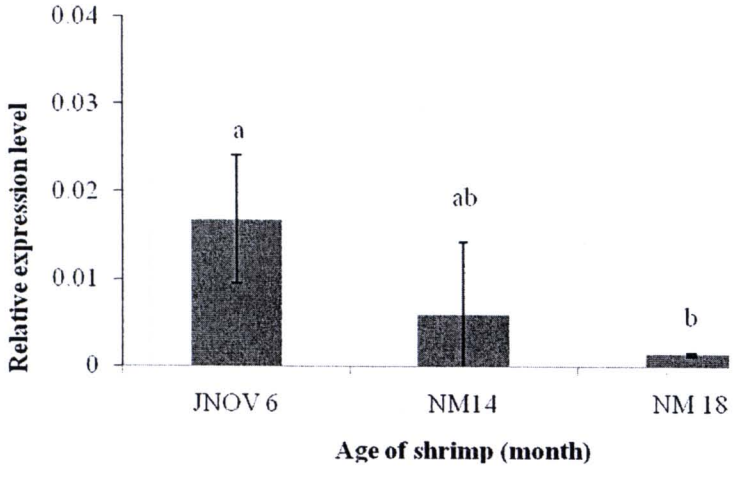


Figure 3.55 Histograms showing the relative expression profiles of *PmFAMeT* in ovaries of domesticated juveniles (6 months old) and broodstock (14, and 18 months old) *P. monodon*. Each bar corresponds to a particular ovarian stage. The same letters indicate that the expression levels were not significantly different ($P > 0.05$).

Table 3.11 Relative expression levels of *PmFAMeT* in different ovarian stages of wild (A) and domesticated (B) *P. monodon* females

A

Ovarian Stage	Relative expression level			
	Intact shrimp	*N*	Eyestalk ablated shrimp	*N*
Juvenile	0.046168±0.022243^{ab}	5	-	-
Stage I (GSI<1.5)	0.046657±0.019139^{abc}	7	0.030000±0.020000^{c}	4
Stage II (GSI 2-<4)	0.026416±0.013025^{a}	6	0.050000±0.040825^{abc}	7
Stage III (GSI 4- <6)	0.033000±0.008819^{ab}	6	0.043333±0.022361^{ab}	9
Stage IV (GS I>6)	0.084242±0.033545^{a}	7	0.070000±0.045947^{bc}	10
Post-spawning	0.064200±0.006082^{abc}	5	-	-

B

Group	Relative expression level
6 months old ($N = 3$)	0,0168±0.0073^{a}
14 months old ($N = 3$)	0.0059±0.0083^{ab}
18 months old ($N = 3$)	0.0013±0.0001^{b}

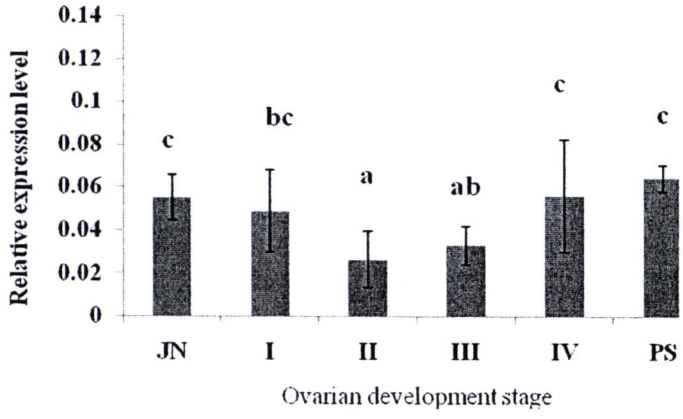
3.5.3 Expression profiles of *PmBr-cZ1* during ovarian development of *P. monodon*

Quantitative real-time PCR revealed that the expression levels of *PmBr-cZ1* in ovaries of juveniles and stage I ovaries of broodstock was comparable. *PmBr-cZ1* was significantly down-regulated at stage II and III ovaries in intact wild broodstock ($P < 0.05$) and returned to the basal level at stage IV ovaries and after spawning.

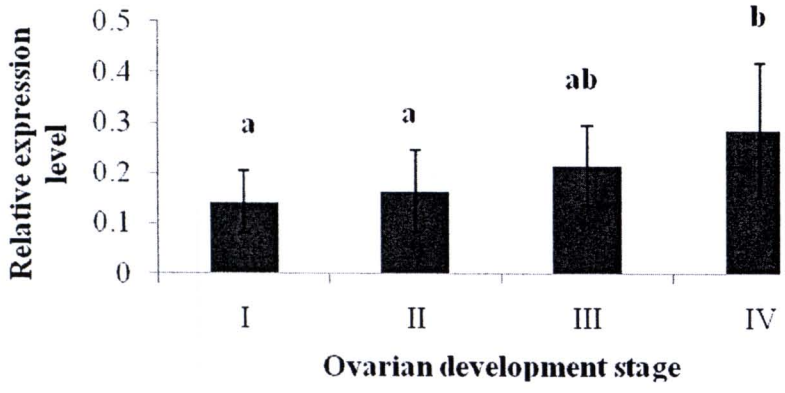
In eyestalk-ablated broodstock, its expression level in stages IV (mature ovaries) was significantly greater than that in stage I (previtellogenic) ovaries and II (vitellogenic) ($P > 0.05$). Eyestalk ablation clearly promoted the expression of *PmFAMeT* during vitellogenesis and final maturation of ovaries compared to intact broodstock (Fig. 3.56). Nevertheless, eyestalk ablation potentially reduced the expression level of for *PmBr-cZ1* approximately 3.5-7 times. Therefore, the

expression profiles of *PmBr-cZ1* can be used as the biomarker to indicate degrees of ovarian maturation of *P. monodon*.

A



B.



C.

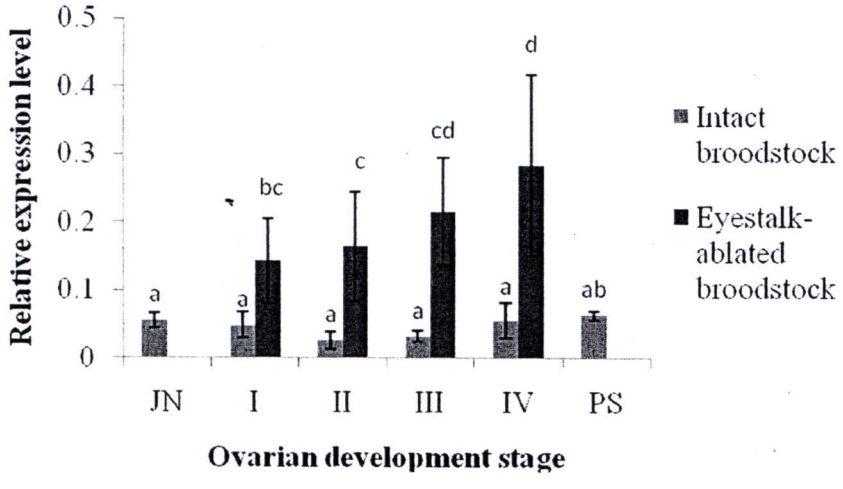


Figure 3.56 Histograms showing the relative expression profiles of *PmBr-cZ1* in ovaries of cultured 4-month-old juveniles (JN, A) and different stages of ovarian development (stages I, previtellogenic; II, vitellogenic; III, early cortical rod; and IV, mature ovaries) of intact (A) and unilateral eyestalk-ablated (B) and intact post-spawning broodstock (PS; A). Data of intact and eyestalk-ablated broodstock were also analyzed together (C). Each bar corresponds to a particular ovarian stage. The same letters indicate that the expression levels were not significantly different ($P > 0.05$).

PmBr-c Z1 in ovaries of cultured 6-month-old juvenile and domesticated, 14-month-old and 18-month-old broodstock were not significantly different ($P > 0.05$). The expression levels of *PmBr-cZ1* in ovaries of domesticated 14-month-old broodstock seem to be increased compared to other ages of domesticated stocks but results were not statistically significant owing to large standard deviation ($P > 0.05$) (Fig. 3.57).

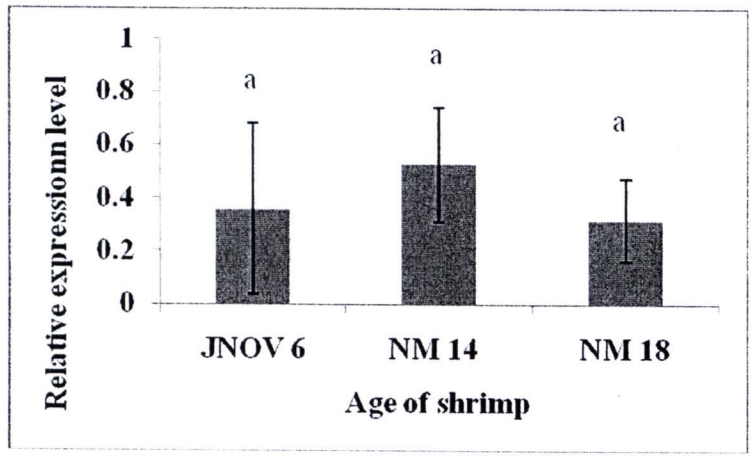


Figure 3.57 Histograms showing the relative expression profiles of *PmBr-cZ1* in ovaries of domesticated juveniles (6 months old) and broodstock (14, and 18 months old) *P. monodon*. Each bar corresponds to a particular ovarian stage. The same letters indicate that the expression levels were not significantly different ($P > 0.05$).

Table 3.12 Relative expression levels of *PmBr c Z1* in different ovarian stages of wild (A) and domesticated (B) *P. monodon* females

A

Ovarian Stage	Relative expression level			
	Intact shrimp	*N*	Eyestalk-ablated shrimp	*N*
Juvenile	0.055250±0.01047[a]	4	-	-
Stage I (GSI<1.5)	0.048857±0.01913[a]	7	0.143257±0.06200[bc]	4
Stage II (GSI 2-<4)	0.026417±0.01302[a]	6	0.164939±0.80753[c]	7
Stage III (GSI 4- <6)	0.033000±0.00819[a]	6	0.216274±0.07822[c]	10
Stage IV (GS I>6)	0.056057±0.02627[a]	7	0.285269±0.13323[d]	10
Post-spawning	0.064200±0.00608[ab]	5	-	-

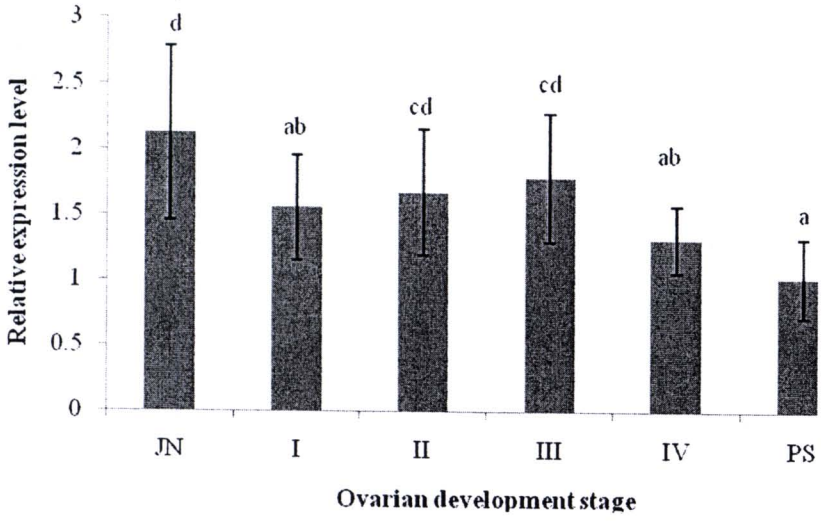
B

Group	Relative expression level
6 months old (*N* = 5)	0.3587 ±0.3226[a]
14 months old (*N* = 14)	0.5258±0.2145[a]
18 months old (*N* = 4)	0.3169±0.1560[a]

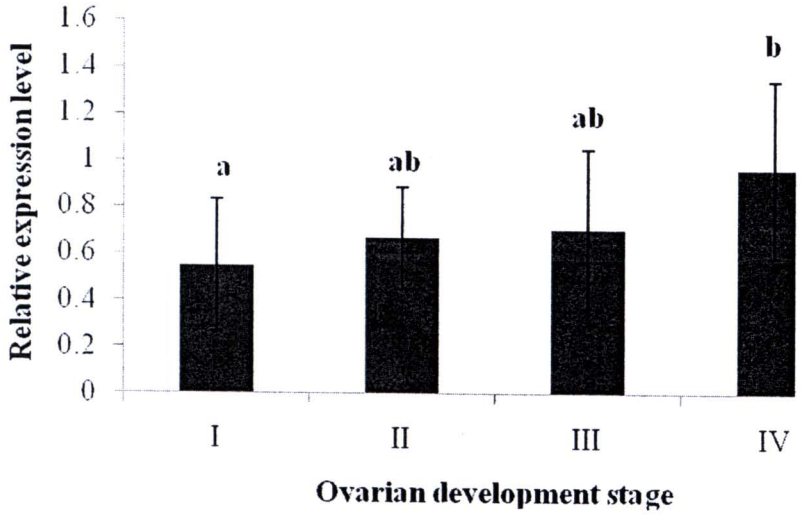
3.5.4 Expression profiles of *PmBr-cZ4* during ovarian development of *P. monodon*

The level of *PmBr-cZ4* mRNA was significantly decreased during ovarian development of intact wild *P. monodon* ($P > 0.05$). In eyestalk-ablated broodstock, its expression level in stages IV ovaries was significantly greater than that in other stage of ovaries ($P < 0.05$) (Fig. 3.58).

A


Relative expression level
3
2.5
2
1.5
1
0.5
0
d
ab
cd
cd
ab
a
JN
I
II
III
IV
PS
Ovarian development stage

B.


Relative expression level
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
ab
ab
b
I
II
III
IV
Ovarian development stage

C.

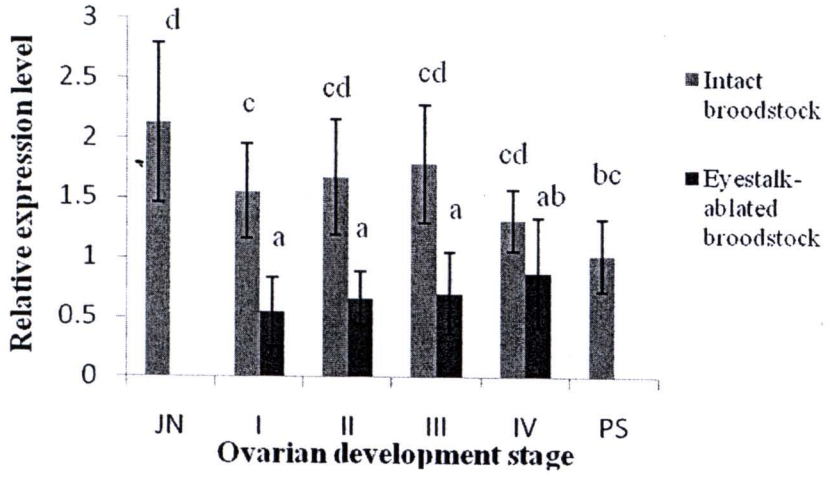

Relative expression level
3
2.5
2
1.5
1
0.5
0
d
c
a
cd
a
cd
a
cd
ab
bc
JN
I
II
III
IV
PS
Ovarian development stage
Intact broodstock
Eyestalk-ablated broodstock

Figure 3.58 Histograms showing the relative expression profiles of *PmBr-cZ4* in ovaries of cultured 4-month-old juveniles (JN, A) and different stages of ovarian development (stages I, previtellogenic; II, vitellogenic; III, early cortical rod; and IV, mature ovaries) of intact (A) and unilateral eyestalk-ablated (B) and intact post-spawning broodstock (PS; A). Data of intact and eyestalk-ablated broodstock were also analyzed together (C). Each bar corresponds to a particular ovarian stage. The same letters indicate that the expression levels were not significantly different ($P > 0.05$).

Quantitative real-time PCR revealed that the expression levels of *PmBr-cZ4* in ovaries was reduced in 18-month-old broodstock compared to domesticated 6-month-old juveniles and 18-month-old broodstock ($P > 0.05$; Fig. 3.59). This indicated that 18-month-old shrimp should possess greater degrees of ovarian maturation than 14-month-old broodstock.

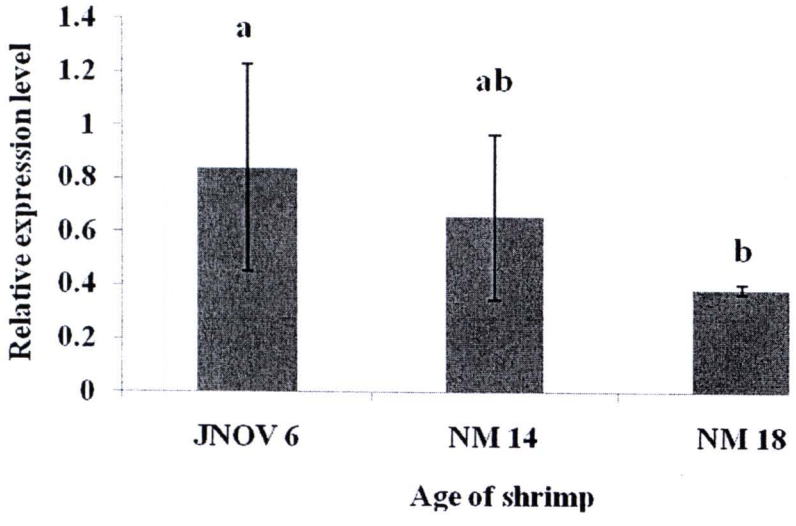


Figure 3.59 Histograms showing the relative expression profiles of *PmBr-cZ4* in ovaries of domesticated juveniles (6 months old) and broodstock (14, and 18 months old) *P. monodon*. Each bar corresponds to a particular ovarian stage. The same letters indicate that the expression levels were not significantly different ($P > 0.05$).

Table 3.13 Relative expression levels of *PmBr-cZ4* in different ovarian stages of wild (A) and domesticated (B) *P. monodon* females

A

Ovarian Stage	Relative Expression Level			
	Intact shrimp	***N***	**Eyestalk-ablated shrimp**	***N***
Juvenile	2.12200±0.66292^{d}	8	-	-
Stage I (GSI<1.5)	1.55550±0.39587^{c}	5	0.55250±0.28335^{a}	4
Stage II (GSI 2-<4)	1.67400±0.47914^{c}	5	0.66857±0.219502^{a}	7
Stage III (GSI 4- <6)	1.78440±0.49191^{c}	9	0.70700±0.34098^{a}	10
Stage IV (GS I > 6)	1.31580±0.25519^{bc}	5	0.87636±0.46007^{ab}	10
Post-spawning	1.02400±0.30369^{ab}	5	-	-

B

Group	Relative expression level
6 months old (N = 5)	0.8402±0.3886^{a}
14 months old (N = 17)	0.6580±0.3093^{ab}
18 months old (N = 4)	0.3883±0.0170^{b}

3.6 Effects of 5-HT, progesterone and 20β-hydroxyecdysone administration on transcription of reproduction-related genes in ovaries of *P. monodon*

PmCOMT, *PmFAMeT*, *PmBr-cZ1* and *PmBr-cZ4* are involved in ovarian development and/or molting of shrimp. To verify the regulatory effects of neurotransmitters, steroid hormones and ecdysteriods on expression of these genes, various groups of domesticated shrimp samples were treated with 5-HT, progesterone or 20β-hydroxyecdysone (20E).

3.6.1 Effects of 5-HT administration on transcription of *PmCOMT*, *PmFAMeT*, *PmBr-cZ1* and *PmBr-cZ4* in ovaries of domesticated 18-month-old broodstock

The effects of 5-HT on expression of *PmCOMT*, *PmFAMeT*, *PmBr-cZ1* and *PmBr-cZ4* in ovaries of 18-month-old *P. monodon* were examined. For *O-methyltransferase* genes, the injection of 5-HT did not promote the expression level of *PmCOMT* in ovaries of domesticated *P. monodon* broodstock (Fig. 3.60). In contrast, 5-HT administration resulted in increasing of ovarian *PmFAMeT* expression for approximately 50-fold at 1 hpt ($P < 0.05$; Fig. 3. 61).

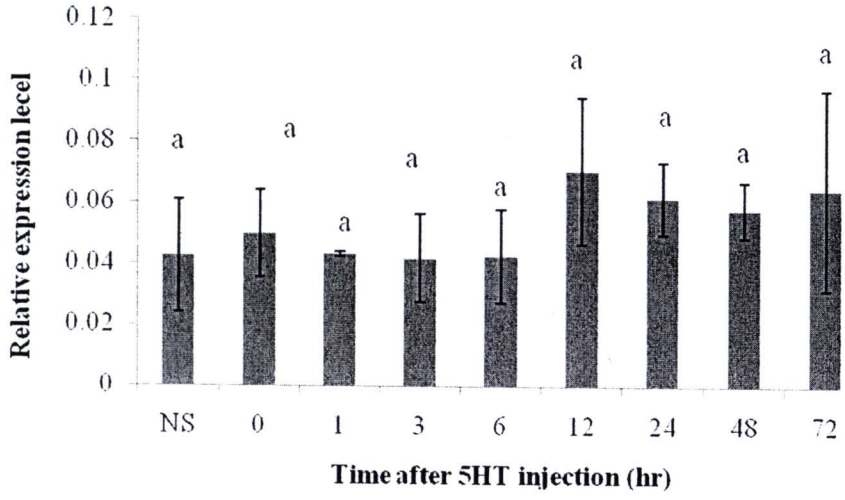


Figure 3.60 Time-course relative expression levels of *PmCOMT* in ovaries of 18 months old after serotonin injection (50 µg/g body weight) at 1, 2, 3, 6, 12, 24, 48 and 72 hours post injection (hpt; $N = 4$ for each stage). Shrimp injected with 0.85% saline solution at 0 hpi were included as the vehicle control.

Table 3.14 Time course relative expression levels of *PmCOMT* in ovaries of juvenile *P. monodon* treated with serotonin (50 µg/g body weight)

Group (N=3)	Relative expression level
NS (control)	0.042 ± 0.018^{a}
0 hpi (control)	0.049 ± 0.014^{a}
1 hpi	0.043 ± 0.001^{b}
3 hpi	0.042 ± 0.014^{a}
6 hpi	0.042 ± 0.015^{a}
12 hpi	0.070 ± 0.024^{a}
24 hpi	0.061 ± 0.011^{a}
48 hpi	0.058 ± 0.009^{a}
72 hpi	0.064 ± 0.032^{a}

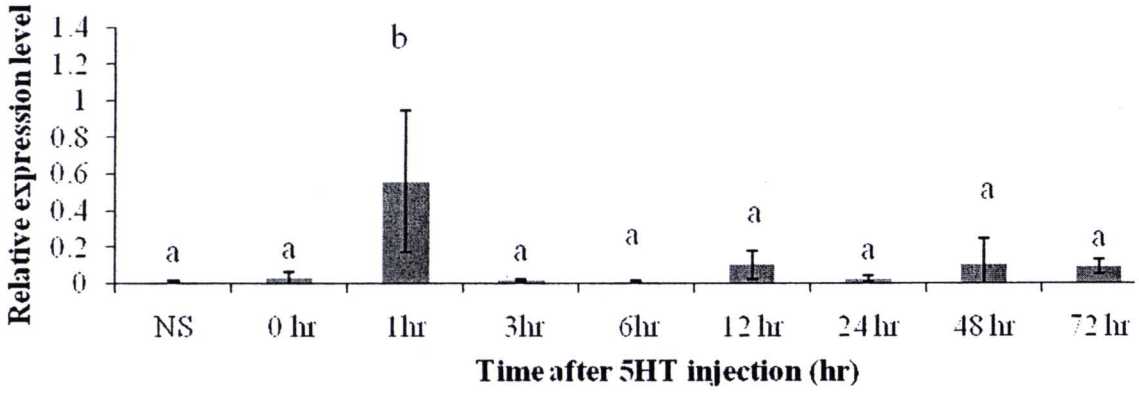


Figure 3.61 Time-course relative expression levels of *PmFAMeT* in ovaries of 18 months old after serotonin injection (50 μg/g body weight) at 1, 2, 3, 6, 12, 24, 48 and 72 hpt ($N = 4$ for each stage). Shrimp injected with absolute ethanol and 0.85% saline solution at 0 hpi were included as the vehicle control.

Table 3.15 Time course relative expression levels of *PmFAMeT* in ovaries of 18-month-old *P. monodon* treated with serotonin (50 μg/g body weight)

Group (N=3)	Relative expression level
NS (control)	0.009 ± 0.004^{a}
0 hpi (control)	0.065 ± 0.063^{a}
1 hpi	1.356 ± 0.936^{b}
3 hpi	0.035 ± 0.009^{a}
6 hpi	0.032 ± 0.009^{a}
12 hpi	0.176 ± 0.030^{a}
24 hpi	0.032 ± 0.006^{a}
48 hpi	0.396 ± 0.583^{a}
72 hpi	0.194 ± 0.028^{a}

For ecdysteroid responsive genes, exogenous administration of 5-HT did not affect the expression of *PmBr-cZ1* (Fig. 3.62) but promote the expression level of *PmBr-cZ4* at 12 hpt ($P < 0.05$) (Fig. 3.63).

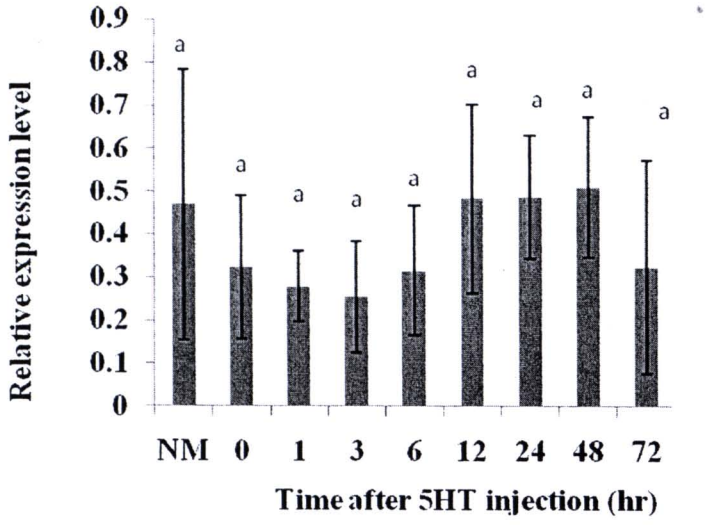


Figure 3.62 Time-course relative expression levels of *PmBr-cZ1* in ovaries of 18 months old after serotonin injection (50 µg/g body weight) at 1, 2, 3, 6, 12, 24, 48 and 72 hpt ($N = 4$ for each stage). Shrimp injected with absolute ethanol and 0.85% saline solution at 0 hpi were included as the vehicle control.

Table 3.16 Time course relative expression levels of *Br-cZ1* in ovaries of juvenile *P. monodon* treated with serotonin (50 µg/g body weight)

Group (N=3)	Relative expression level
NS (control)	0.4688±0.3136[a]
0 hpi (control) *	0.3234±0.1669[a]
1 hpi	0.2792±0.0830[a]
3 hpi	0.2546±0.1299[a]
6 hpi*	0.3165±0.1515[a]
12 hpi*	0.4835±0.2192[a]
24 hpi*	0.4873±0.1438[a]
48 hpi **	0.5105±0.1626[a]
72 hpi	0.3250±0.2477[a]

*$N = 4$; **$N = 5$

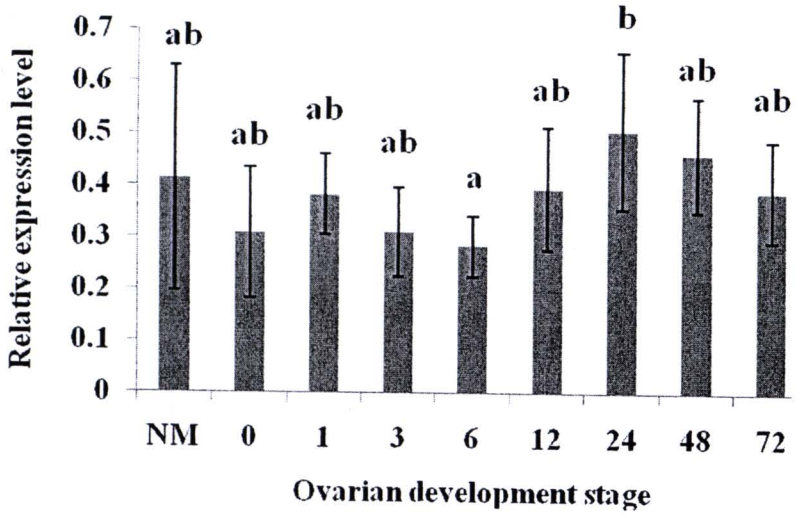


Figure 3.63 Time-course relative expression levels of *PmBr-cZ4* in ovaries of 18 months old after serotonin injection (50 μg/g body weight) at 1, 2, 3, 6, 12, 24, 48 and 72 hpt ($N = 4$ for each stage). Shrimp injected with absolute ethanol and 0.85% saline solution at 0 hpi were included as the vehicle control.

Table 3.17 Time course relative expression levels of *Br c Z4* in ovaries of juvenile *P. monodon* treated with serotonin (50 μg/g body weight)

Group (N=3)	Relative expression level
NS (control)	0.4141 ± 0.2166^{ab}
0 hpi (control)*	0.3093 ± 0.1269^{ab}
1 hpi	0.3838 ± 0.0773^{ab}
3 hpi	0.3119 ± 0.0860^{ab}
6 hpi*	0.2840 ± 0.0574^{a}
12 hpi*	0.3951 ± 0.1181^{ab}
24 hpi*	0.5070 ± 0.1512^{b}
48 hpi**	0.4613 ± 0.1085^{ab}
72 hpi	0.3910 ± 0.0966^{ab}

*$N = 4$; **$N = 5$

3.6.2 Effects of progesterone administration on transcription of *PmCOMT*, *PmFAMeT*, *PmBr-cZ1* and *PmBr-cZ4* in ovaries of domesticated 18- and 14-month-old broodstock

3.6.2.1 18-month-old shrimp

The effects of progesterone administration on expression of *PmCOMT* and *PmFAMeT* in ovaries of domesticated broodstock of *P. monodon* were examined at 12, 24 and 72 after administration. Expression of these genes in ovaries of shrimp injected with 40% ethanol were dissected out at 0 hpi and used as the control group. The result showed that the expression levels of *PmCOMT* (Fig. 3.64) and *PmFAMeT* (Fig. 3.65) in ovaries of domesticated 18-month-old broodstock were not significantly affected by progesterone injection ($P > 0.05$).

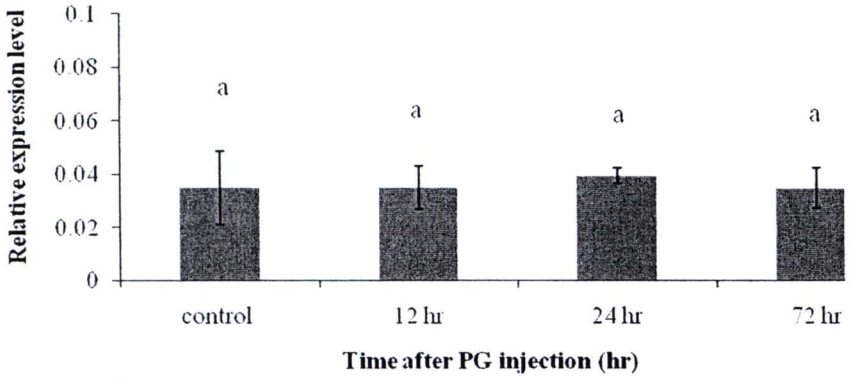


Figure 3.64 Time-course relative expression levels of *PmCOMT* in ovaries of 18 months old at 12, 24 and 48 hpi of progesterone (1 μg/g body weight; $N = 4$ for each stage). Shrimp injected with absolute ethanol and 0.85% saline solution at 0 hpi were included as the vehicle control and the positive control, respectively.

Table 3.18 Time course relative expression levels of *PmCOMT* in ovaries of 18 months old *P. monodon* treated with progesterone (0.1 μg/g body weight)

Group (N=3)	Relative expression level
0 hpi (control)	0.034±0.014[a]
12 hpi	0.034±0.007[a]
24 hpi	0.040±0.002[a]
48 hpi	0.034±0.007[a]

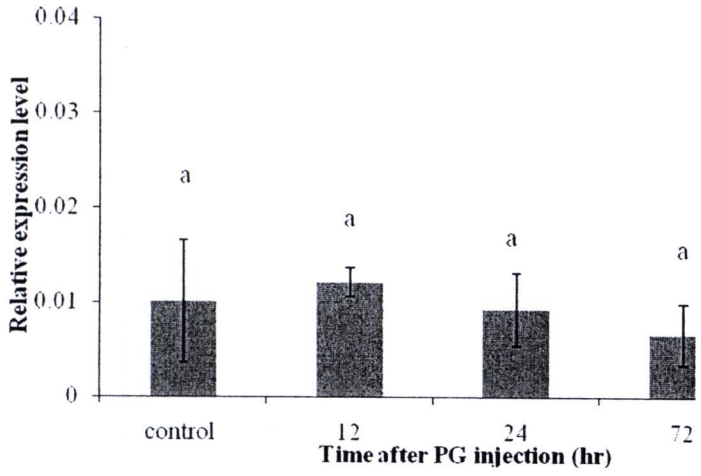


Figure 3.65 Time-course relative expression levels of *PmFAMeT* in ovaries of 18 months old at 12, 24 and 48 hpi of progesterone (1 μg/g body weight; $N = 4$ for each stage). Shrimp injected with absolute ethanol and 0.85% saline solution at 0 hpi were included as the vehicle control and the positive control, respectively.

Table 3.19 Time course relative expression levels of *PmFAMeT* in ovaries of 18 months old *P. monodon* treated with progesterone (0.1 μg/g body weight)

Group (*N*=3)	Relative expression level
0 hpi (control)	0.024±0.019[a]
12 hpi	0.037±0.005[a]
24 hpi	0.030±0.021[a]
72 hpi	0.020±0.008[a]

3.6.2.2 14-month-old domesticated broodstock

The effects of progesterone administration on expression of *PmCOMT*, *PmFAMeT*, *PmBr-cZ1* and *PmBr-cZ4* in ovaries of domesticated 14-month-old broodstock were examined at 12, 24 and 72 after administration. Expression of these genes in ovaries of shrimp infected with 10% ethanol were dissected out at 12 hpi and used as the vehicle control.

Results from quantitative real-time PCR revealed that progesterone did not affect the expression level of *PmCOMT* in ovaries of 14-month-old shrimp (Fig. 3.66). Although a trend of inducing effects of progesterone on the expression of *PmFAMeT* was observed at 72 hpi, results were not statistically significant due to a large standard deviation within each treatment group (Fig. 3.67).

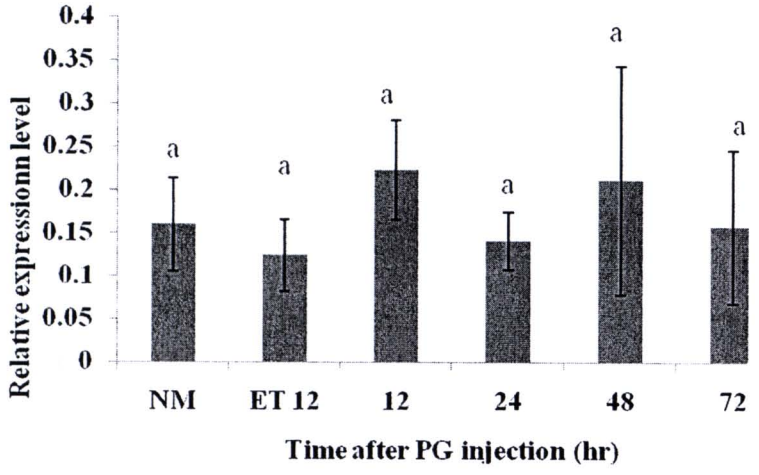


Figure 3.66 Time-course relative expression levels of *PmCOMT* in ovaries of 14 months old at 12, 24, 48 and 72 hpi of progesterone (0.1 μg/g body weight; $N = 4$ for each stage). Shrimp injected with absolute ethanol and 0.85% saline solution at 0 hpi were included as the vehicle control and the positive control, respectively.

Table 3.20 Time course relative expression levels of *PmCOMT* in ovaries of 14 months old *P. monodon* treated with progesterone (0.1μg/g body weight)

Group	Relative expression level
Normal (control; $N = 5$)	0.1596±0.0538[a]
10% ethanol at 12 hpi (control; $N = 5$)	0.1240±0.0415[a]
12 hpi ($N = 5$)	0.2233±0.0581[a]
24 hpi ($N = 5$)	0.1412±0.0337[a]
48 hpi ($N = 5$)	0.2117±0.1328[a]
72 hpi (N=3)	0.1576±0.0892[a]

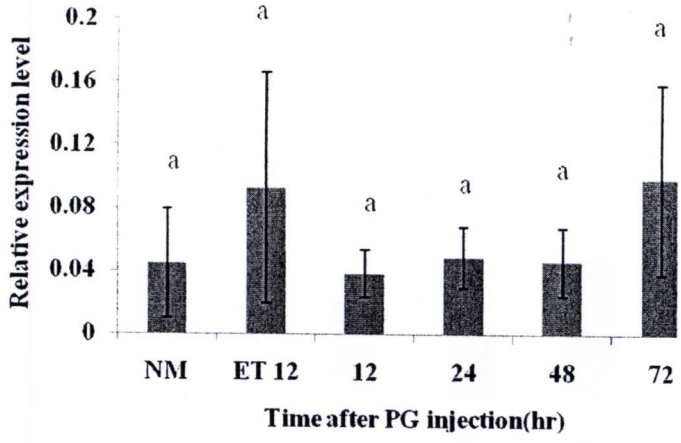


Figure 3.67 Time-course relative expression levels of *PmFAMeT* in ovaries of 14 months old at 12, 24, 48 and 72 hpi of progesterone (0.1 μg/g body weight; $N = 4$ for each stage). Shrimp injected with absolute ethanol and 0.85% saline solution at 0 hpi were included as the vehicle control and the positive control, respectively.

Table 3.21 Time course relative expression levels of *PmFAMeT* in ovaries of 14 months old *P. monodon* treated with progesterone (0.1 μg/g body weight)

Group	Relative expression level
Normal ($N = 4$)	0.0447 ± 0.0348^a
10% Ethanol at 12 hpi (control, $N = 6$)	0.0925 ± 0.0731^a
12 hpi ($N = 5$)	0.0383 ± 0.0150^a
24 hpi ($N = 5$)	0.0489 ± 0.0192^a
48 hpi ($N = 5$)	0.0457 ± 0.0219^a
72 hpi ($N = 4$)	0.0983 ± 0.0600^a

Like *PmCOMT* and *PmFAMeT*, the expression level of *PmBr-cZ1* was not affected by exogenous progesterone administration ($P > 0.05$) (Fig. 3.68). Nevertheless, *PmBr-cZ4* was up-regulated at 48 and 72 hpi after progesterone administration ($P > 0.05$) (Fig. 3.69).

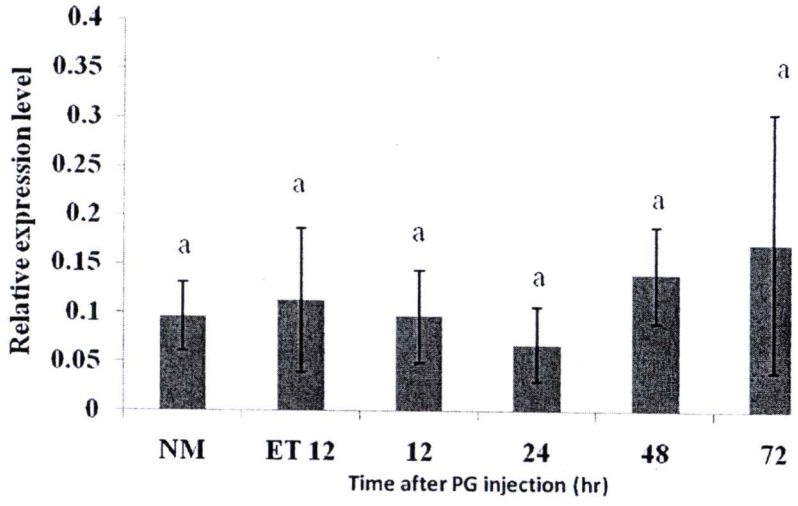


Figure 3.68 Time-course relative expression levels of *PmBr-cZ1* in ovaries of 14 months old at 12, 24, 48 and 72 hpi of progesterone (0.1 µg/g body weight; $N = 4$ for each stage). Shrimp injected with absolute ethanol and 0.85% saline solution at 0 hpi were included as the vehicle control and the positive control, respectively.

Table 3.22 Time course relative expression levels of *PmBr-cZ1* in ovaries of 14 months old *P. monodon* treated with progesterone (0.1 µg/g body weight)

Group	Relative expression level
Normal ($N = 3$)	0.0961±0.0356[a]
10%Ethanol at 12 hpi (control, $N = 6$)	0.1133±0.0738[a]
12 hpi ($N = 5$)	0.0969±0.0470[a]
24 hpi ($N = 5$)	0.0681±0.0384[a]
48 hpi ($N = 5$)	0.1398±0.0493[a]
72 hpi ($N = 4$)	0.1726±0.1326[a]

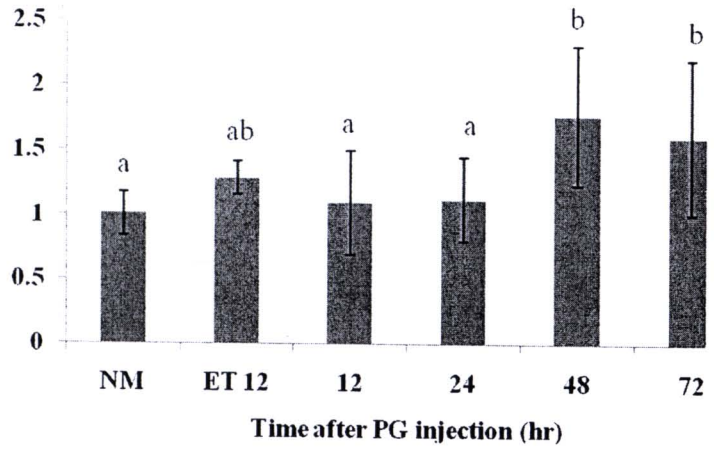


Figure 3.69 Time-course relative expression levels of *PmBr-cZ4* in ovaries of 14 months old at 12, 24, 48 and 72 hpi of progesterone (0.1 μg/g body weight; $N = 4$ for each stage). Shrimp injected with absolute ethanol and 0.85% saline solution at 0 hpi were included as the vehicle control and the positive control, respectively.

Table 3.23 Time course relative expression levels of *PmBr-cZ4* in ovaries of 14 months old *P. monodon* treated with progesterone (0.1μg/g body weight)

Group	Relative expression level
Normal ($N = 4$)	1.0125 ± 0.1678^{a}
10% Ethanol at 12 hpi (control, $N = 5$)	1.2879 ± 0.1267^{ab}
12 hpi ($N = 5$)	1.0971 ± 0.4010^{a}
24 hpi ($N = 5$)	1.1241 ± 0.3217^{a}
48 hpi ($N = 5$)	1.7720 ± 0.5343^{b}
72 hpi ($N = 3$)	1.6049 ± 0.5940^{b}

3.6.3 Effects of 20-hydroxyecdysone (20E) administration on transcription of *PmCOMT*, *PmFAMeT*, *PmBr-cZ1* and *PmBr-cZ4* in ovaries of commercially cultured *P. monodon* juveniles

The effects of 20E administration on expression of *PmCOMT*, *PmFAMeT*, *PmBr-cZ1* and *PmBr-cZ4* in ovaries of juvenile *P. monodon* females were examined at 6, 12, 24, 48, 72, 96 and 168 after administration.

The expression of *PmCOMT* in ovaries of juvenile *P. monodon* was significantly decreased following 20E administration ($P > 0.05$). However, the results should be interpreted with caution as the expression of *PmCOMT* was also significantly in the vehicle control group (Fig. 3.70).

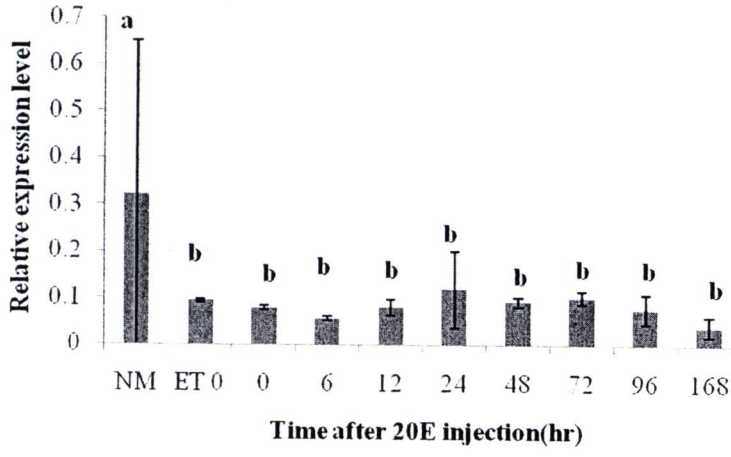


Figure 3.70 Time-course relative expression levels of *PmCOMT* in ovaries of 4 months-old at 6, 12, 24,48, 72, 96 and 168 hpi of 20E (1 μg/g body weight; $N = 3$ for each treatment). Shrimp injected with 10% absolute ethanol and 20E (1 μg/g body weight) at 0 hpi were included as the vehicle control and the positive control, respectively.

Table 3.24 Time course relative expression levels of *PmCOMT* in ovaries of 4 months- old *P. monodon* treated with 20E(1μg/g body weight)

Group (*N*=3)	Relative expression level
NM (normal shrimp)	0.3216 ± 0.3293^{a}
10% EtOH (control)	0.0940 ± 0.0025^{b}
0 hpi (control)	0.0792 ± 0.0035^{b}
6 hpi	0.0563 ± 0.0040^{b}
12 hpi	0.0808 ± 0.0163^{b}
24 hpi	0.1192 ± 0.0823^{b}
48 hpi	0.0920 ± 0.0105^{b}
72 hpi	0.1021 ± 0.0128^{b}
96 hpi	0.0768 ± 0.0317^{b}
168 hpi	0.0393 ± 0.0217^{b}

In contrast, the expression level of *PmFAMeT* in ovaries of cultured juveniles was obviously affected after administration with 20E for 6 hpt ($P < 0.05$). The induced effects remained for 24 hours ($P < 0.05$) before reduced to the basal level since 48 hpi ($P > 0.05$) (Fig. 3.71).

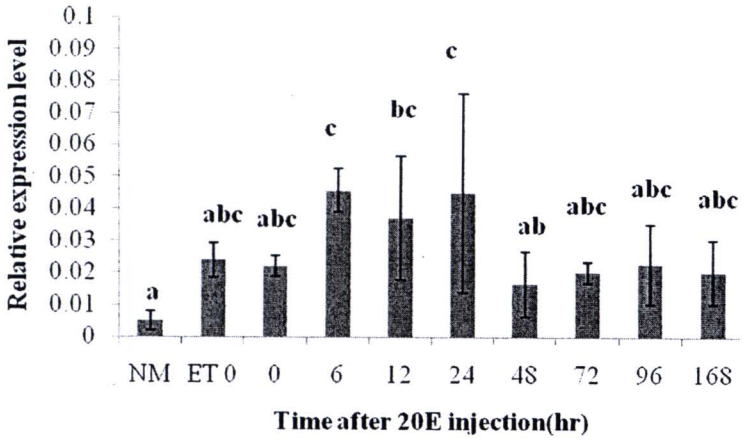


Figure 3.71 Time-course relative expression levels of *PmFAMeT* in ovaries of 4 months-old at 6, 12, 24,48, 72, 96 and 168 hpi of 20E (1 μg/g body weight; $N = 3$ for each treatment). Shrimp injected with 10% absolute ethanol and 20E (1 μg/g body weight) at 0 hpi were included as the vehicle control and the positive control, respectively.

Table 3.25 Time course relative expression levels of *PmFAMeT* in ovaries of 4 months- old *P. monodon* treated with 20E(1μg/g body weight)

Group (N=3)	Relative expression level
NM (normal shrimp)	0.0040 ± 0.0028^{a}
10% EtOH (control)	0.0239 ± 0.0054^{abc}
0 hpi (control)	0.0219 ± 0.0032^{abc}
6 hpi	0.0457 ± 0.0067^{c}
12 hpi	0.0371 ± 0.0194^{bc}
24 hpi	0.0449 ± 0.0312^{c}
48 hpi	0.0165 ± 0.0101^{ab}
72 hpi	0.0200 ± 0.0032^{abc}
96 hpi	0.0226 ± 0.0124^{abc}
168 hpi	0.0201 ± 0.0100^{abc}

Interestingly, late response effects of 20E on expression of *PmBr-cZ1* (Fig. 3.72) and *PmBr-cZ4* (Fig. 3.73) were observed at 168 hpi. Notably, juvenile shrimp treated with 20E showed immediate response to exogenous 20E administration

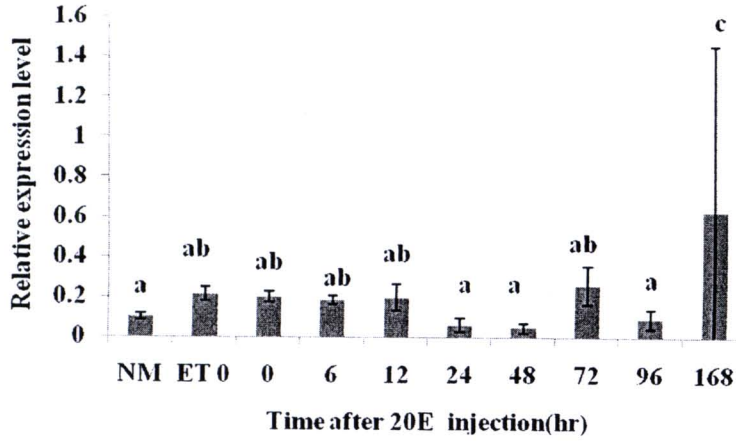


Figure 3.72 Time-course relative expression levels of *PmBr-cZ1* in ovaries of 4 months-old at 6, 12, 24,48, 72, 96 and 168 hpi of 20E (1 μg/g body weight; $N = 3$ for each treatment). Shrimp injected with 10% absolute ethanol and 20E (1 μg/g body weight) at 0 hpi were included as the vehicle control and the positive control, respectively.

Table 3.26 Time course relative expression levels of *PmBr-cZ1* in ovaries of 4 months old *P. monodon* treated with 20E (1μg/g body weight)

Group (N=3)	Relative expression level
NM (Intact shrimp)	0.0992 ± 0.0147^{a}
10%EtOH (Control)	0.2133 ± 0.0317^{ab}
0 hpi (Control)	0.1983 ± 0.0265^{ab}
6 hpi	0.1843 ± 0.0223^{ab}
12 hpi	0.1966 ± 0.0656^{ab}
24 hpi	0.0610 ± 0.0321^{a}
48 hpi	0.0467 ± 0.0245^{a}
72 hpi	0.2590 ± 0.0955^{ab}
96 hpi	0.0888 ± 0.0469^{a}
168 hpi	0.6232 ± 0.8340^{c}

reflected by molting within 48 hours following the treatment in most individuals of all treatment. Accordingly, differential expression of both *PmBr-cZ1* and *PmBr-cZ4* should have reflected long duration effects of the ecdysteroid on expression of these genes.

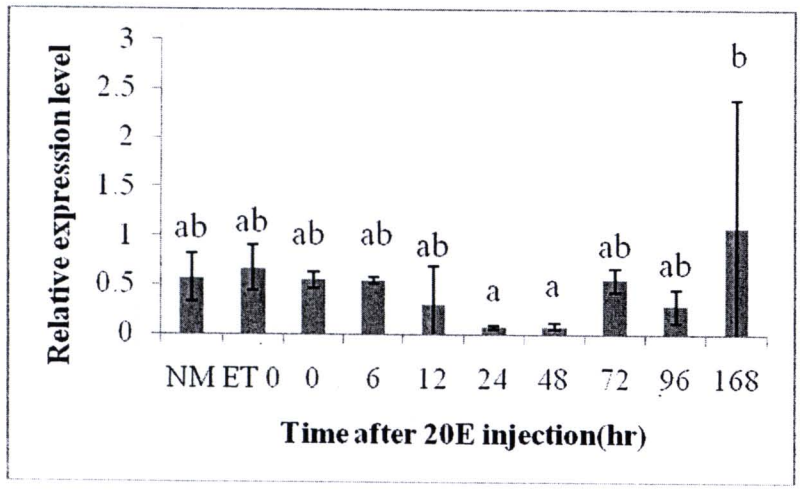


Figure 3.73 Time-course relative expression levels of *PmBr-cZ4* in ovaries of 4 months-old at 6, 12, 24,48, 72, 96 and 168 hpi of 20E (1 µg/g body weight; $N = 3$ for each treatment). Shrimp injected with 10% absolute ethanol and 20E (1 µg/g body weight) at 0 hpi were included as the vehicle control and the positive control, respectively.

Table 3.27 Time course relative expression levels of *PmBr-cZ4* in ovaries of 4 months- old *P. monodon* treated with 20E (1 µg/g body weight)

Group (N=3)	Relative expression level
NM (normal shrimp)	0.5690 ± 0.2452^{ab}
10% EtOH (control)	0.6733 ± 0.2356^{ab}
0 hpi (control)	0.5496 ± 0.0784^{ab}
6 hpi	0.5453 ± 0.0289^{ab}
12 hpi	0.3023 ± 0.3947^{ab}
24 hpi	0.0718 ± 0.0145^{a}
48 hpi	0.0793 ± 0.0303^{a}
72 hpi	0.5493 ± 0.1252^{ab}
96 hpi	0.2886 ± 0.1711^{ab}
168 hpi	1.0900 ± 1.3084^{b}

3.7 Localization of all genes in ovaries of *P. monodon* broodstock

3.7.1. Quantification of the cRNA probe

The sense and antisense cRNA probes were synthesized from the recombinant insert (700 bp) for *PmCOMT* (Fig. 3.73) or the PCR product for those of *PmFAMeT* (Fig. 3.74), *PmBr-cZ1* (Fig. 3.75) and *PmBr-cZ4* (Fig. 3.76). The amount of cRNA probes was roughly estimated by dot blot analysis. The control RNA was used as the positive control and gave the positive signal between 10 pg to 10ng.

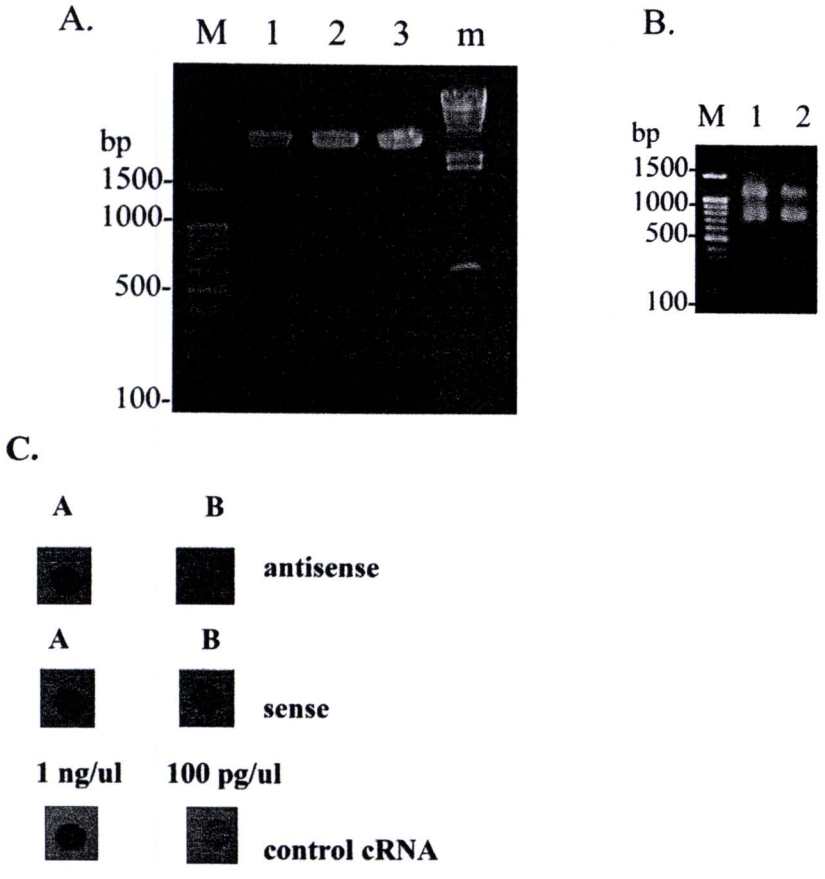


Figure 3.73 (A) The digested plasmid used as te template for synthesis of the cRNA probe of *PmCOMT* (lane 1-3, A). (B) The antisense (lane 1, B) and sense (lane 2, B) were synthesized from the gel-eluted digested plasmid template. A 100 bp ladder (lanes 1, A and B) and λ-*Hin*d III was used as the DNA marker. (C) Dot blot hybridization for estimation of the concentration of the antisense, and sense *PmCOMT* and the control RNA probes.

The antisense and sense probes of these genes gave the positive signal at approximately 1 ng/µl. However, the sense cRNA probe of *PmFAMeT* which was lower than 1 ng/µl (Fig. 3.74C) and the antisense and sense probes of PmCOMT more than 1 ng/µl (Fig. 3.73C). An appropriate amount of the cRNA probe of each transcript was applied for examination of transcriptional localization using *in situ* hybridization.

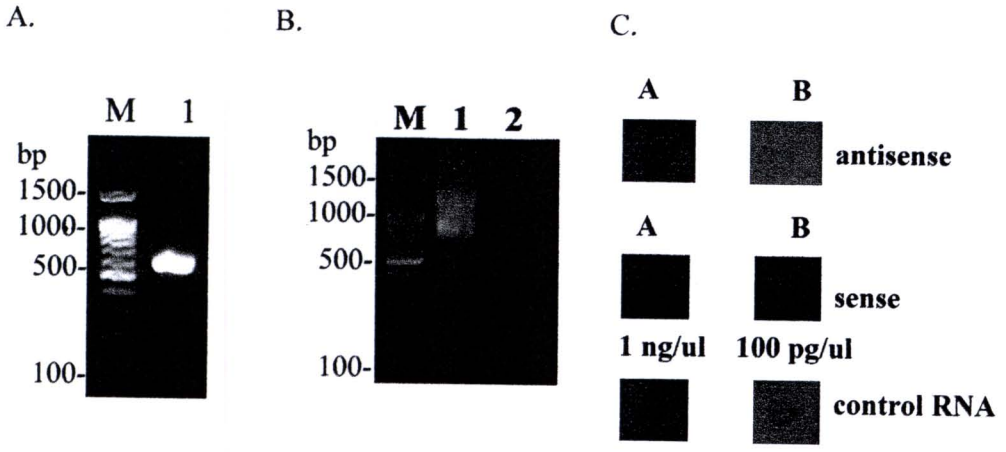


Figure 3.74 (A) The amplification product for synthesis of the cRNA probe of *PmFAMeT* (lane 1, A). (B) The antisense (lane 1, B) and sense (lane 2, B) were synthesized from the gel-eluted PCR template. A 100 bp ladder (lanes 1, A and B) was used as the DNA marker. (C) Dot blot hybridization for estimation of the concentration of the antisense, sense *PmFAMeT* and the control RNA probes.

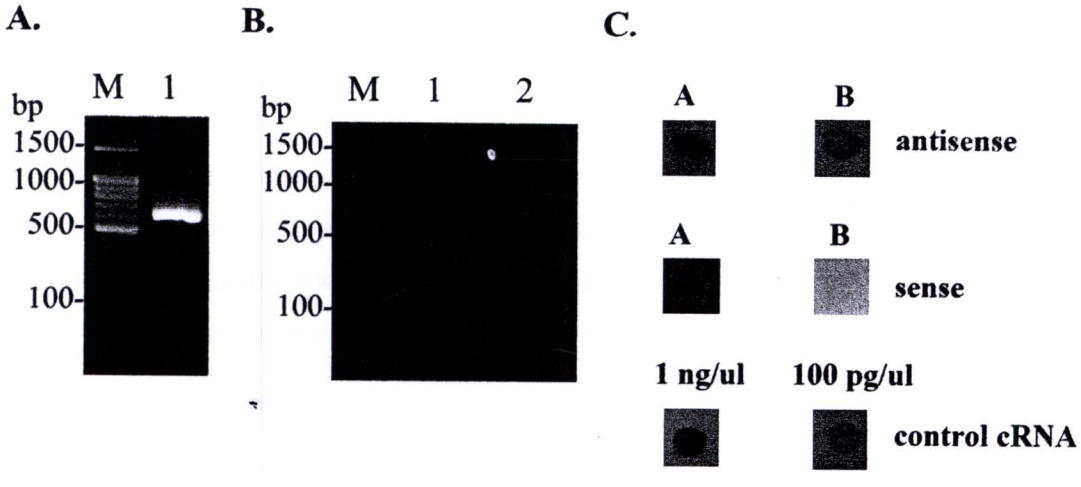


Figure 3.75 (A) The amplification product used as the template for synthesis of the cRNA probe of *PmBr-cZ1* (lane 1, A). (B) The antisense (lane 1, B) and sense (lane 2, B) were synthesized from the gel-eluted PCR template. A 100 bp ladder (lanes 1, A and B) was used

as the DNA marker. (C) Dot blot hybridization for estimation of the concentration of the antisense, sense *PmBr-cZ1* and the control RNA probes.

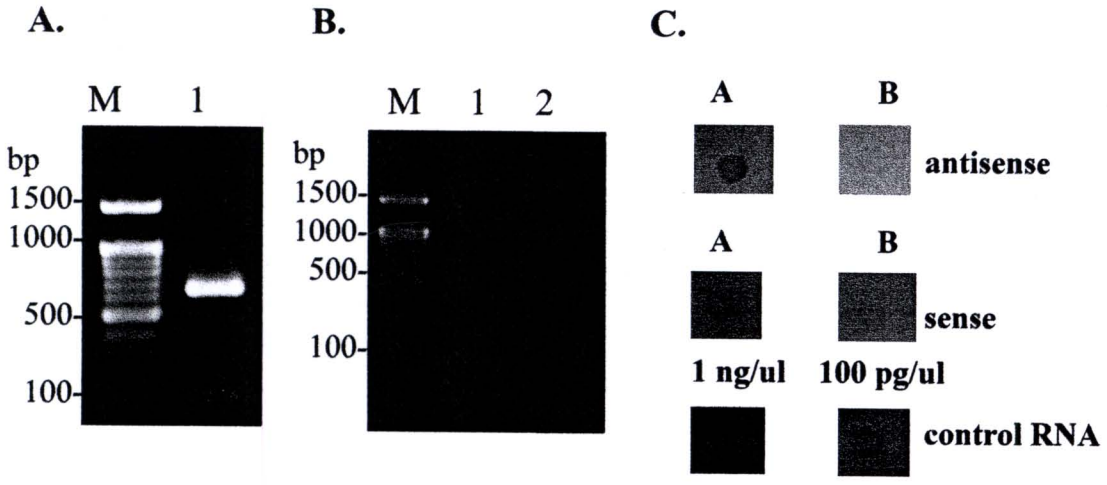


Figure. 3.76 (A) The amplification product used as the template for synthesis of the cRNA probe of *PmBr-cZ4* (lane 1, A). (B) The antisense (lane 1, B) and sense (lane 2, B) were synthesized from the gel-eluted PCR template. A 100 bp ladder (lanes 1, A and B) was used as the DNA marker. (C) Dot blot hybridization for estimation of the concentration of the antisense and sense *PmBr-cZ4* and the control RNA probes.

3.7.2 *In situ* hybridization (ISH)

The cellular localization of *PmCOMT, PmFAMeT, PmBr-cZ1* and *PmBr-cZ4* transcripts in ovaries of *P. monodon* broodstock was determined by *in situ* hybridization. No signal was observed with the sense probe for all transcripts (Figs 3.77-3.84). The positive signal was observed when the tissue sections were hybridized with the antisense probe of *PmCOMT, PmFAMeT, PmBr-cZ1* and *PmBr-cZ4.* Only the antisense *PmCOMT* probe gave the positive signal in cytoplasm of oogonia, previtellogenic oocytes and follicular cells. The remaining transcripts gave the clear signals in the cytoplasm of oogonia and previllogenic oocytes in different stages of ovaries in both intact and eyestalk-ablated broodstock (Figs. 3.77-3.84, Table 3.28)

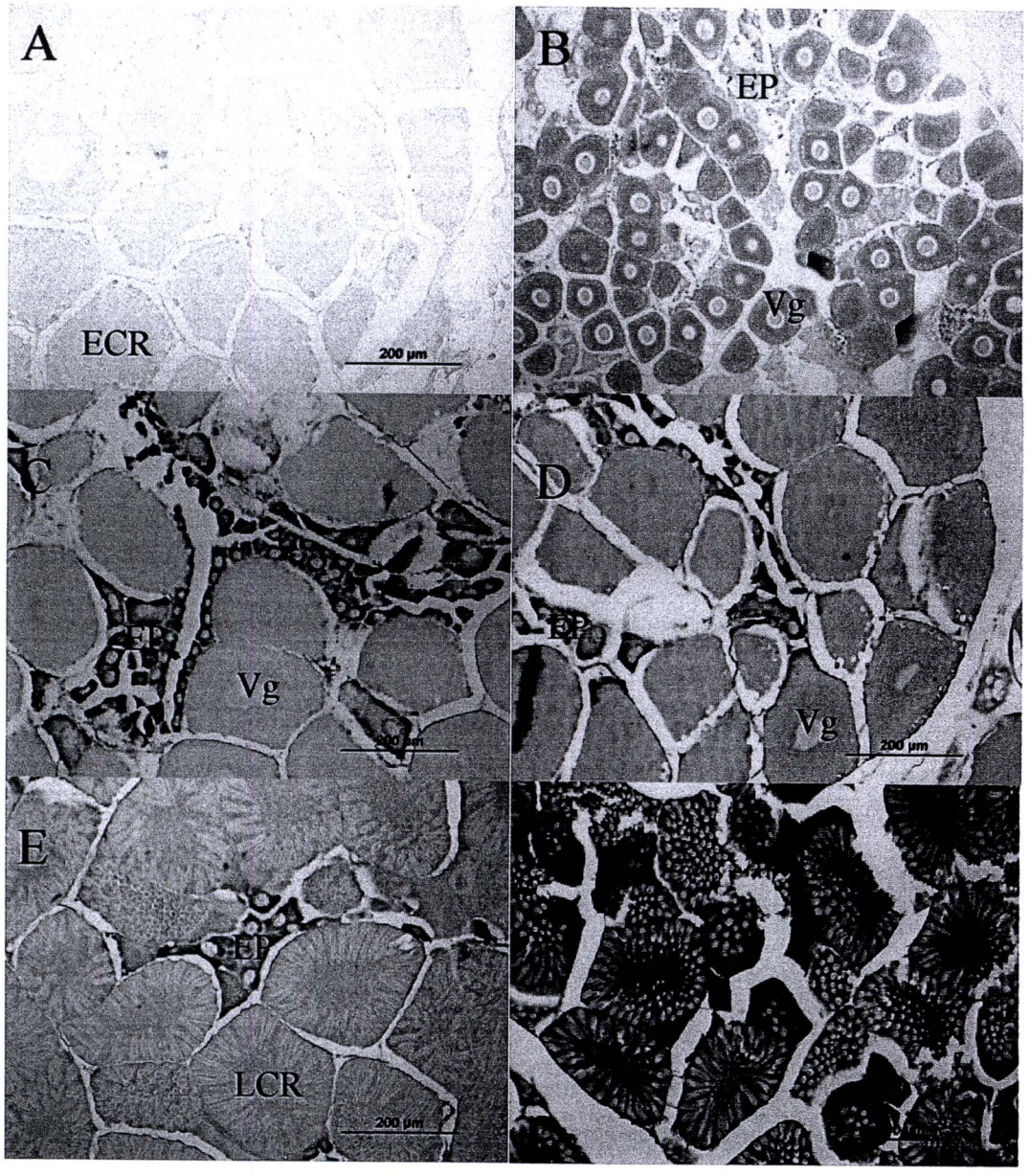


Figure 3.77 Localization of *PmCOMT* transcript during ovarian development of intact *P. monodon* broodstock visualized by *in situ* hybridization using the antisense(B-E), sense (A) cRNA probes. The conventional hematoxylin/eosin staining was carried out for identification of oocyte stages (F). EP = early previtellogenic oocytes; ECR = early cortical rod oocytes; LCR=late cortical rod oocytes; Vg = vitellogenic oocyte

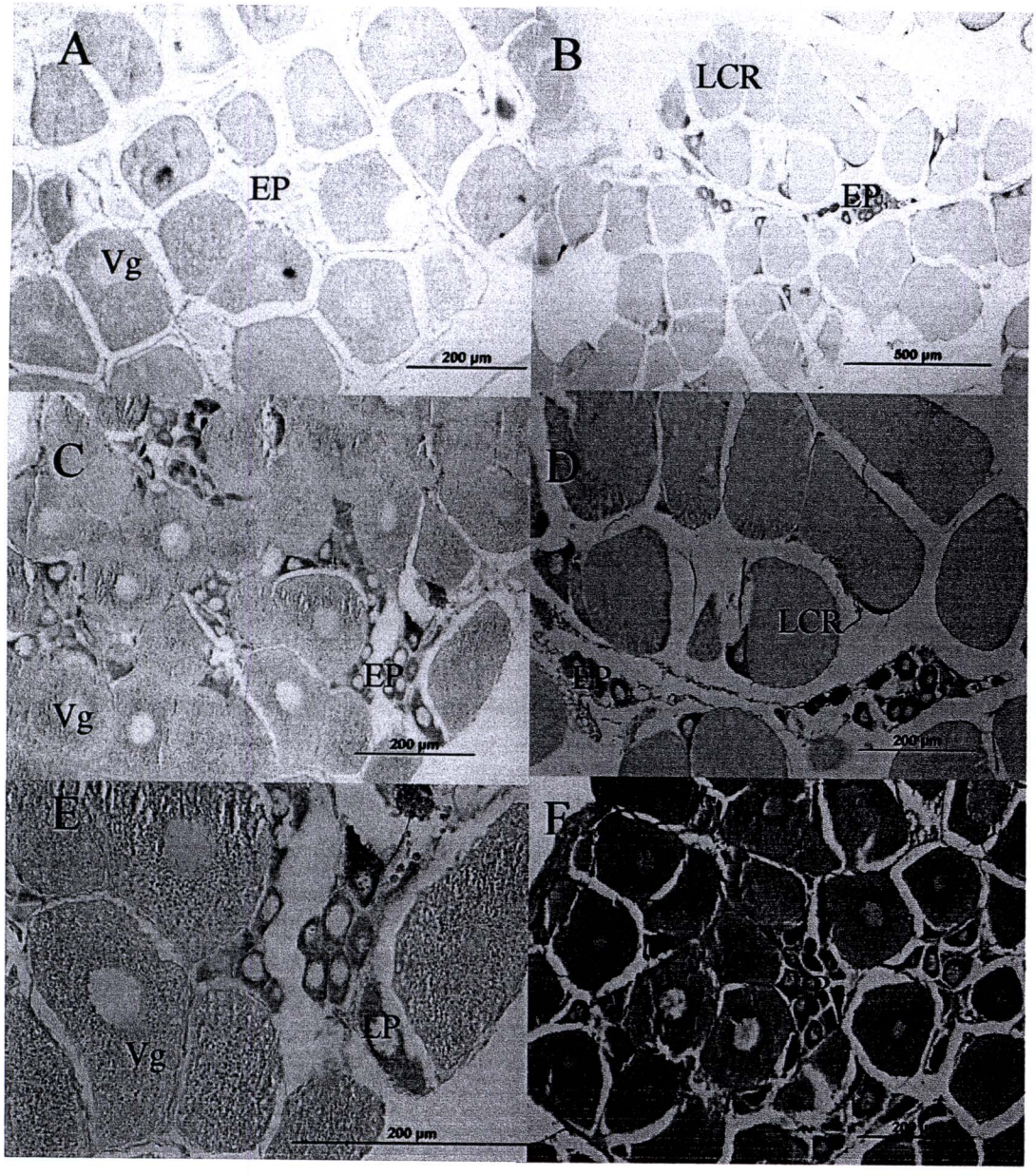


Figure 3.78 Localization of *PmCOMT* transcript during ovarian development of eyestalk-ablated *P. monodon* broodstock visualized by *in situ* hybridization using the antisense (B-E), sense (A) cRNA probes. The conventional hematoxylin/eosin staining was carried out for identification of oocyte stages (F). EP = early previtellogenic oocytes; ECR = early cortical rod oocytes; LCR=late cortical rod oocytes; Vg = vitellogenic oocyte

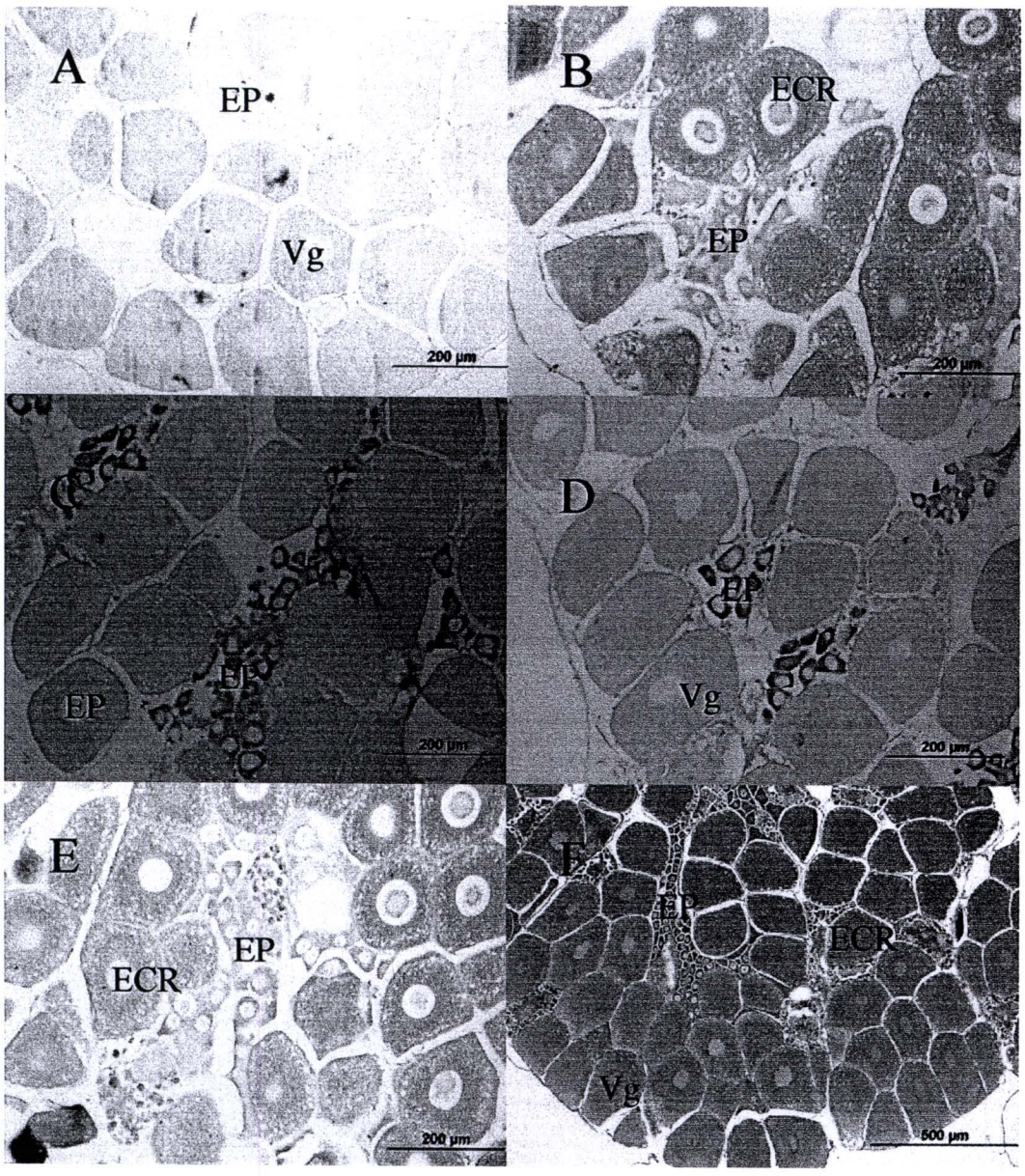


Figure 3.79 Localization of *PmFAMeT* transcript during ovarian development of intact *P. monodon* broodstock visualized by *in situ* hybridization using the antisense (B-E), sense (A) cRNA probes. The conventional hematoxylin/eosin staining was carried out for identification of oocyte stages (F). EP = early previtellogenic oocytes; ECR = early cortical rod oocytes; LCR = late cortical rod oocytes; Vg = vitellogenic oocyte

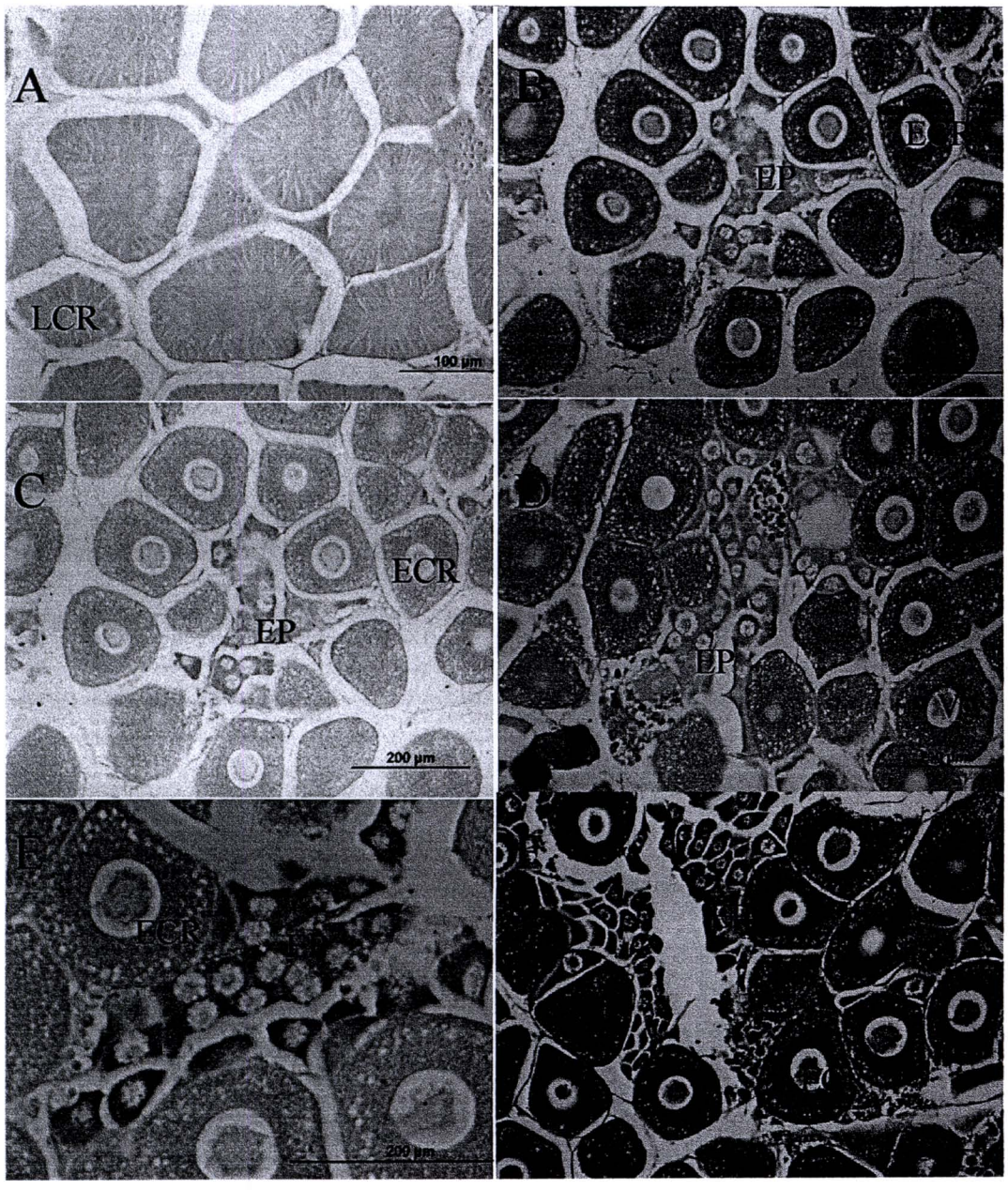


Figure 3.80 Localization of *PmFAMeT* transcript during ovarian development of eyestalk-ablated *P. monodon* broodstock visualized by *in situ* hybridization using the antisense (B-E), sense (A) cRNA probes. The conventional hematoxylin/eosin staining was carried out for identification of oocyte stages (F). EP = early previtellogenic oocytes; ECR = early cortical rod oocytes; LCR=late cortical rod oocytes; Vg = vitellogenic oocyte

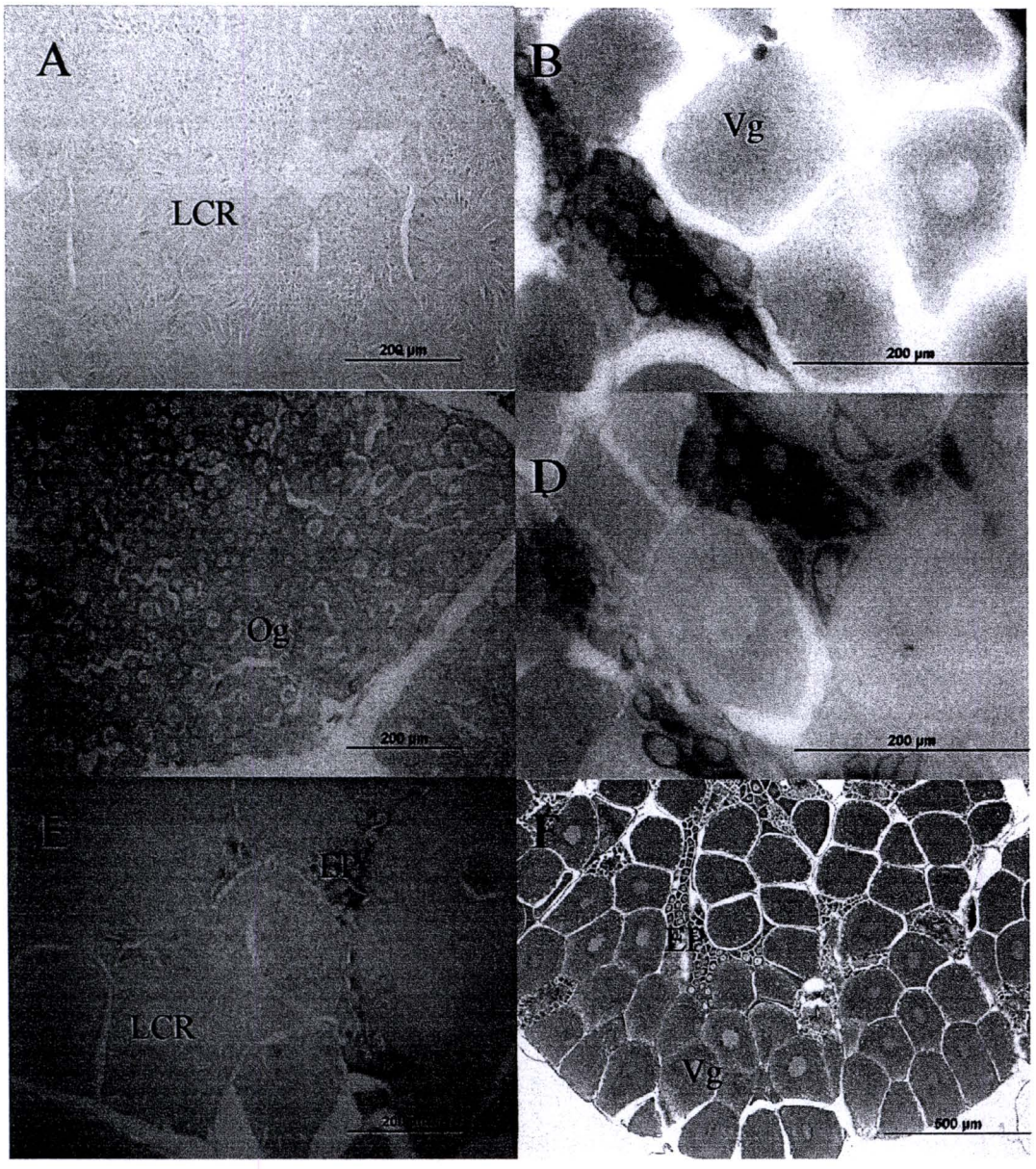


Figure 3.81 Localization of *PmBr-cZ1* transcript during ovarian development of intact *P. monodon* broodstock visualized by *in situ* hybridization using the antisense(B-E), sense (A) cRNA probes. The conventional hematoxylin/eosin staining was carried out for identification of oocyte stages (F). EP = early previtellogenic oocytes; ECR = early cortical rod oocytes; LCR=late cortical rod oocytes; Vg = vitellogenic oocyte

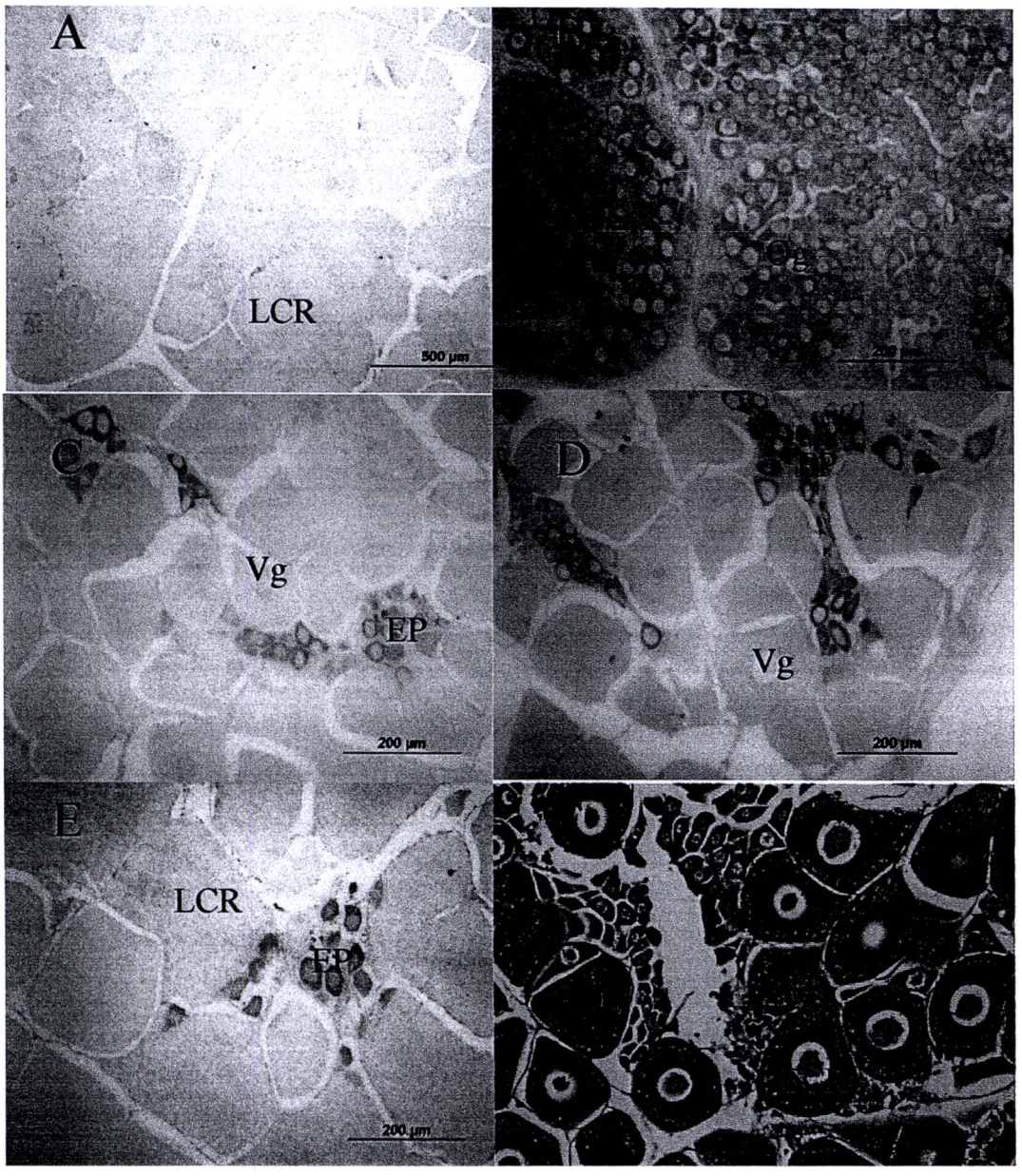


Figure 3.82 Localization of *PmBr-cZ1* transcript during ovarian development of eyestalk-ablated *P. monodon* broodstock visualized by *in situ* hybridization using the antisense(B-EC), sense (A) cRNA probes. The conventional hematoxylin/eosin staining was carried out for identification of oocyte stages (F). EP = early previtellogenic oocytes; ECR = early cortical rod oocytes; LCR=late cortical rod oocytes; Vg = vitellogenic oocyte

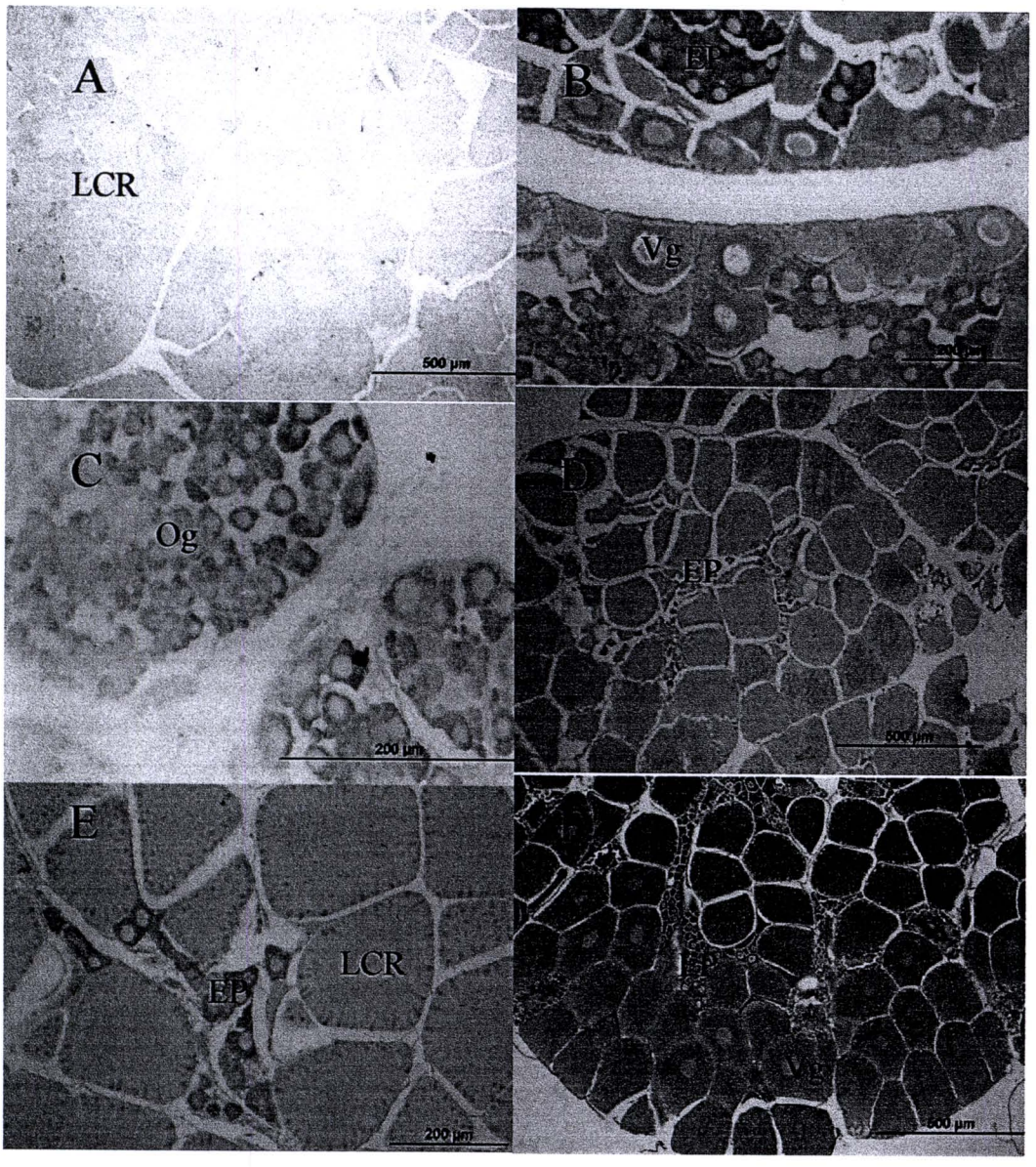


Figure 3.83 Localization of *PmBr-cZ4* transcript during ovarian development of intact *P. monodon* broodstock visualized by *in situ* hybridization using the antisense(B-E), sense (A) cRNA probes. The conventional hematoxylin/eosin staining was carried out for identification of oocyte stages (F). EP = early previtellogenic oocytes; ECR = early cortical rod oocytes; LCR=late cortical rod oocytes; Vg = vitellogenic oocyte

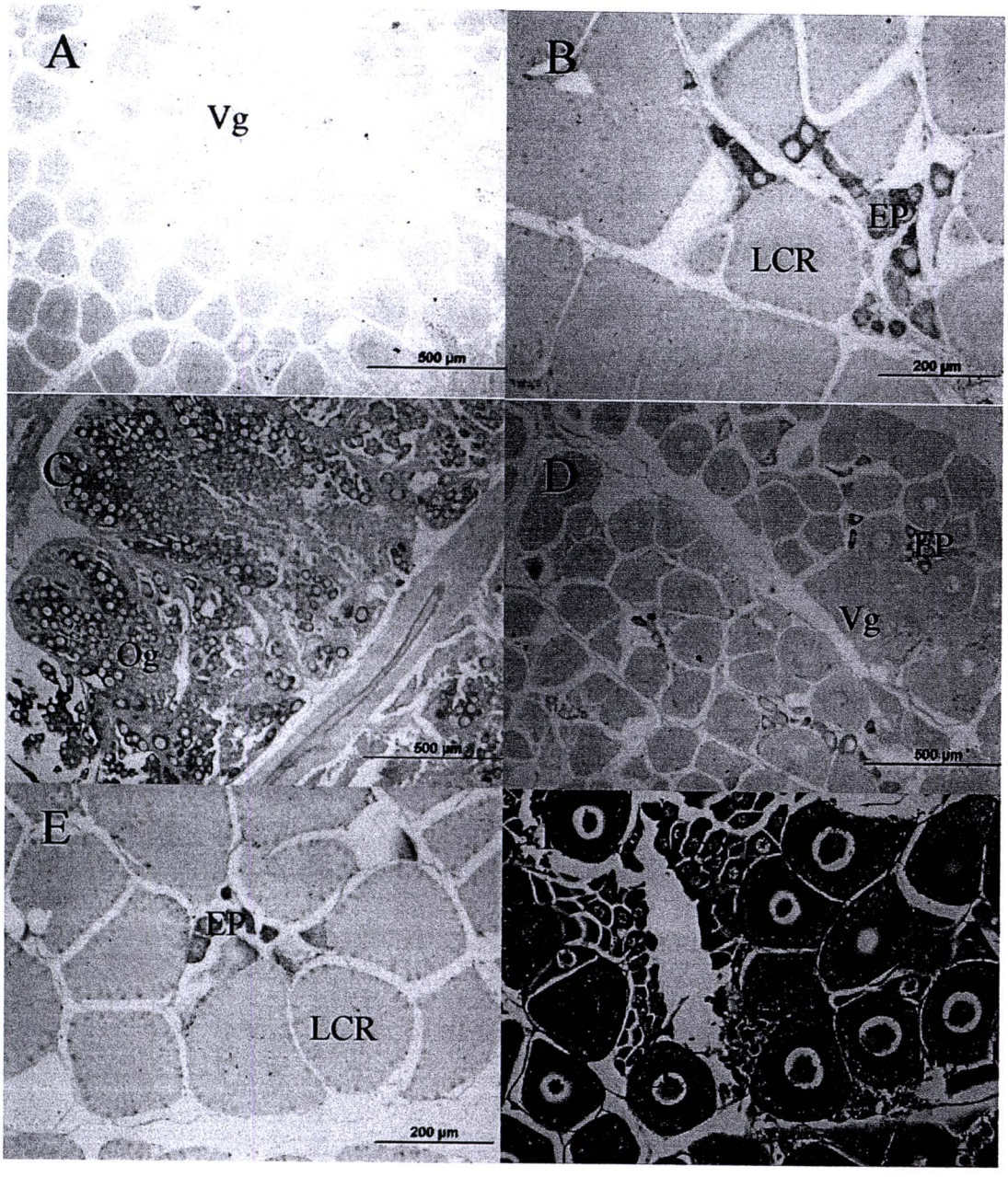


Figure 3.84 Localization of *PmBr-cZ4* transcript during ovarian development of ey *P. monodon* eyestalk-ablated broodstock visualized by *in situ* hybridization using the antisense(B-E), sense(A) cRNA probes. The conventional hematoxylin/eosin staining was carried out for identification of oocyte stages (F). EP = early previtellogenic oocytes; ECR = early cortical rod oocytes.

Table 3.28 A summary for localization of *PmCOMT, PmFAMeT, PmBr-cZ1* and *PmBr-cZ4* transcripts in ovaries of intact and eyestalk-ablated *P. monodon* broodstock determined by *in situ* hybridization.

Sample	**Positions of signals**			
	PmCOMT	***PmFAMeT***	***PmBr-cZ1***	***PmBr-cZ4***
Intact shrimp	Oogonia and previltellogenic	Previltellogenic oocytes	Oogonia and previltellogenic oocytes	Oogonia and previltellogenic oocytes
Eyestalk ablated shrimp	Oogonia, pre-viltellogenic and follicular cell surrounding stage III and IV oovytes	Oogonia and previltellogenic oocytes	Previltellogenic oocytes	Oogonia and previltellogenic oocytes

3.8 *In vitro* expression of recombinant *PmCOMT*, *PmFAMeT*, *PmBr-C Z1* and *PmBr-C Z4* using the bacterial expression system

3.8.1 Construction of recombinant plasmids in cloning and expression vector

Three recombinant plasmids carrying the full length cDNA (5′UTR + ORF + 3′UTR) of *PmCOMT*, *PmFAMeT-l* and *PmFAMeT-l* were successfully constructed for *in vitro* expression of the corresponding recombinant protein. A recombinant clone of each construct was sequenced for both directions to identify any misincorporation of nucleotides during the PCR amplification (Figs. 3.85-3.86). BlastX analysis indicated that the target genes were successfully cloned and no stop codon was misplaced to the ORF of each recombinant clone (Figs 3.87-3.89).

Figure 3.85 Agarose gel electrophoresis showing RT-PCR for amplification of the full length ORF of *PmCOMT* using the first strand cDNA of ovaries (lane 1) and hemocyte (lane 2) as the template (A) and the ORF of *PmCOMT* overhang with *Nde* I and *Bam* HI-6His tag using the first strand cDNA of ovaries as the template(lane 1, B). A 100 bp DNA ladder (lanes 1, A and B) was used as the DNA marker.

A.

ATGTCTTCTCTGAAAAGTTACCATAATCCCGATCCTTTGGTGCAGTATTGTGTAAATCATTCATT
GAGATTAACCGACGCGCAAAAACGACTGAATGATGTAACTCTGCAGCACCGTAGAGCGGCGATGT
TGGGGGCACCTGAGGTTCTGCAGTTCAATGCCAACATAATGCAGGCTATCGGGGCAAAGAAAGTA
CTAGACATTGGGGTGTTCACAGGCGCCAGTTCACTCTCTGCTGCTCTGGCACTGCCTCCGAATGG
CAAGGTCCACGCCCTTGACATAAGTGAAGAGTTTGCCAACATAGGCAAACCGTTCTGGGAGGAAG
CTGGAGTTATCAACAAGATAAGTCTGCACATCGCTCCAGCTGCTGAGACTCTCCAGAAGTTCATT
GACGGCGGAGAAGGTGGCACCTTCGACTATGCTTTCATTGATGCCGACAAGGGGAATTATGAGCT
GTACTATGAACTTTGCCTCACTCTCTTGCGCTCTGGTGGAGTCATCGCTTTCGACAACACACTTT
GGGATGGAGCTGTGATTGACCCCACTGATCAAACCCCTGGCACAGTGGCTATTAGGAAAATTAAC

GAAAAACTGAGAGATGACCAGAGAATCAATATTTCCTTCCTGAAAATTGGTGATGGCGTGACTCT
ATGTTTTAAAAAATGA

B.

O-methyltransferase [Fenneropenaeus chinensis] Length=221

Score = 355 bits (912), Expect = 8e-97
Identities = 197/221 (89%), Positives = 207/221 (93%), Gaps = 0/221 (0%)
Frame = +1

```
Query  1    MSSLKSYHNPDPLVQYCVNHSLRLTDAQKRLNDVTLQHRRAAMLGAPEVLQFNANIMQAI  180
            MSSLKSY N DPLVQYCVNHSLRLTD QKRLND TLQHRRAAMLGAPEVLQ NANIMQAI
Sbjct  1    MSSLKSYDNTDPLVQYCVNHSLRLTDVQKRLNDATLQHRRAAMLGAPEVLQLNANIMQAI  60

Query  181  GAKKVLDIGVFTGasslsaalalPPNGKVHALDISEEFANIGKPFWEEAGVINKISLHIA  360
            GAKKVLDIGVFTGASSLSAALALPPNGKV+ALDISEEF NIGKP+WEEAGV NKISLHIA
Sbjct  61   GAKKVLDIGVFTGASSLSAALALPPNGKVYALDISEEFTNIGKPYWEEAGVSNKISLHIA  120

Query  361  PAAETLQKFIDGGEGGTFDYAFIDADKGNyelyyelcltllRSGGVIAFDNTLWDGAVID  540
            PAAETLQKFID GE GTFDYAFIDADK +Y+ YYELCL LLR GGVIAFDNTLWDGAVID
Sbjct  121  PAAETLQKFIDAGEAGTFDYAFIDADKESYDRYYELCLILLRPGGVIAFDNTLWDGAVID  180

Query  541  PTDQTPGTVAIRKINEKLRDDQRINISFLKIGDGVTLCFKK  663
            PTDQ PGT+AIRK+NEKL+DDQRINISFL+IGDG++LCFKK
Sbjct  181  PTDQKPGTLAIRKMNEKLKDDQRINISFLRIGDGLSLCFKK  221
```

Figure 3.86 (A) Nucleotide sequence of the amplified full length *PmCOMT* generated by the start-to-stop codon primers. (B) BlastX analysis of nucleotide sequence of *PmCOMT*. Primer sequences are underlined.

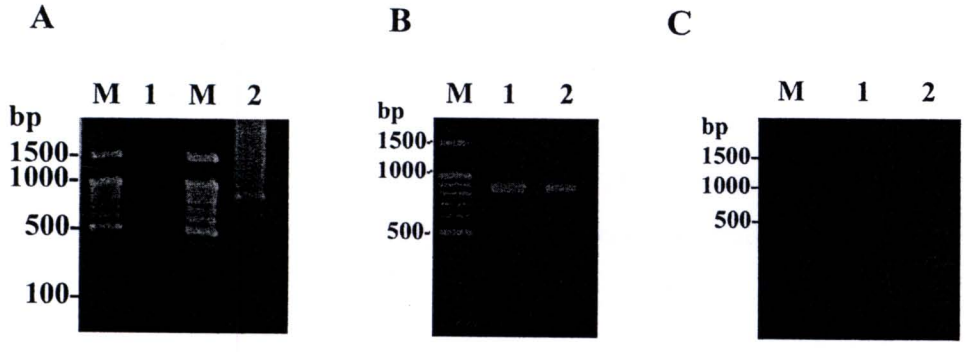


Figure 3.87 Agarose gel electrophoresis showing RT-PCR for amplification of the full length of *PmFAMeT- l* (lane 1, A) and *PmFAMeT-s* (lane 2, A), *PmFAMeT-l* (lane 1, B) and *PmFAMeT-s* (lane 2, B) overhang with *Nde* I and *Bam* HI-6XHis using thee first strand cDNA from ovaries as a template. The overhang products of *PmFAMeT- l* and *PmFAMeT-s* were digested with the corresponding restriction enzymes (lanes 1 and 2, C). Lanes M = a 100 bp DNA marker.

A

TGCTCGCAAGTAACTCGGGATGGGCGATAGCTGGGCTCCCTACGGTGCCGATGAGAACAAGCAGTACCGCT
TCAGGGACATCAAGGGCAAGACCCTCCGGTTCCAGGTGAAGGCTGCCCATGATGCCCACCTTGCCCTGACC
TCAGGGGAGGAGGAGACTGACCCTATGCTGGAGGTGTTCATTGGCGGATGGGAAGGCGCTGCCTCTGCCAT
TAGGTTCAAGAAAGCTGATGACTTAACTAAAGTGGACACCCCTGACATCCTGAGTGAAGAAGAATATCGTG

AATTCTGGGTTGCCTTCGACCATGATGTTATCCGTGTTGGCAAGGGAGGCGAGTGGGAGCCATTCATGAGT
GCCACCATTCCAGAGCCTTTCGACATCACTCATTACGGCTACAGTACTGGCTGGGGTGCTGTTGGCTGGTG
GCAGTTCCATAGTGAGGTACACTTCCAAACTGAGGACTGCCTCACGTACAACTTCATTCCTGTGTACGGTG
ACACCTTTACCTTCAGTGTTGCCTGTAGCAATGATGCCCATCTGGCACTCACCTCTGGCCCTGAGGAGACC
ACACCCATGTATGAAGTGTTCATTGGTGGTTGGGAAAACCAGCACTCTGCCATTCGTCTCAGCAAGGAGGG
AAGGGGATCTGGCGAGGACATGATCAAGGTCGACACCCCCGACGTTGTCTGCTGCGAAGAGGAGAGGAAGT
TCTACGTCAGCTTCAAGGACGGCCATATCANGGTGGGATACCAGGACAGTGATCCCTTCATGGAGTGGACT
GACCCTGAGCCATGGAAGATCACCCACATTGGTTACTGCACAGGCTGGGGAGCAACTGGAAAGTGGAAGTT
CGAATTTTAAGTCCTGCTTTGTGGCTTTGTTCAC

B

```
farnesoic acid O-methyltransferase [Penaeus monodon]
Length=280
 Score =  602 bits (1551),  Expect = 2e-170
 Identities = 277/280 (98%), Positives = 277/280 (98%), Gaps = 0/280 (0%)
 Frame = +2

Query  20   MGDSWAPYGADENKQYRFRDIKGKTLRFQVKAAHDAHLALTSGEEETDPMLEVFIGGWEG  199
            MGDSWA YG DENKQYRFRDIKGKTLRFQVKAAHDAHLALTSGEEETDPMLEVFIGGWEG
Sbjct  1    MGDSWASYGTDENKQYRFRDIKGKTLRFQVKAAHDAHLALTSGEEETDPMLEVFIGGWEG  60

Query  200  AASAIRFKKADDLTKVDTPDILSEEEYREFWVAFDHDVIRVGKGGEWEPFMSATIPEPFD  379
            AASAIRFKKADDLTKVDTPDILSEEEYREFWVAFDHDVIRVGKGGEWEPFMSATIPEPFD
Sbjct  61   AASAIRFKKADDLTKVDTPDILSEEEYREFWVAFDHDVIRVGKGGEWEPFMSATIPEPFD  120

Query  380  ITHYGYSTGWGAVGWWQFHSEVHFQTEDCLTYNFIPVYGDTFTFSVACSNDAHLALTSGP  559
            ITHYGYSTGWGAVGWWQFHSEVHFQTEDCLTYNFIPVYGDTFTFSVACSNDAHLALTSGP
Sbjct  121  ITHYGYSTGWGAVGWWQFHSEVHFQTEDCLTYNFIPVYGDTFTFSVACSNDAHLALTSGP  180

Query  560  EETTPMYEVFIGGWENQHSAIRLSKEGRGSGEDMIKVDTPDVVCCEEERKFYVSFKDGHI  739
            EETTPMYEVFIGGWENQHSAIRLSKEGRGSGEDMIKVDTPDVVCCEEERKFYVSFKDGHI
Sbjct  181  EETTPMYEVFIGGWENQHSAIRLSKEGRGSGEDMIKVDTPDVVCCEEERKFYVSFKDGHI  240

Query  740  XVGYQDSDPFMEWTDPEPWKITHIGYCTGWGATGKWKFEF  859
             VGYQDSDPFMEWTDPEPWKITHIGYCTGWGATGKWKFEF
Sbjct  241  RVGYQDSDPFMEWTDPEPWKITHIGYCTGWGATGKWKFEF  280
```

Figure 3.88 Nucleotide sequence of the full length of recombinant *PmFAMeT-l* (A) and its similarity analysis using BlastX (B). Primer sequences are underlined.

A.

TGCTCGCAAGTAACTCGGGATGGGCGATAGCTGGGCTTCCTTCGGTACCGATGAGAACAAGCAGTACCGCT
TCAGGGACATCAAGGGCAAGACCCTCCGGTTCCAGGTGAAGGCTGCCCATGATGCCCACCTTGCCCTGACC
TCAGGGGAAGAGGAGACTGACCCTATGCTGGAGGTGTTCATTGGCGGATGGGAAGGCGCTGCCTCTGCCAT
TAGGTTCAAGAAAGCTGATGACTTAACTAAAGTGGACACCCCTGACATCCTGAGTGAAGAAGAATATCGTG
AATTCTGGGTTGCCTTCGACCATGATGTTATCCGTGTTGGCAAGGGAGGCGAGTGGGAGCCATTCATGAGT
GCCACCATTCCAGAGCCTTTCGACATCACTCATTACGGCTACAGTACTGGCTGGGGTGCTGTTGGTTGGTG
GCAGTTCCATAGTGAGGTACACTTCCAAACTGAGGACTGCCTCACGTACAACTTCATTCCTGTGTACGGTG
ACACCTTTACCTTCAGTGTTGCCTGTAGCAATGATGCCCATCTGGCACTCACCTCTGGCCCTGAGGAGACC
ACACCCATGTATGAAGTGTTCATTGGTGGTTGGGAAAACCAGCACTCTGCCATTCGTCTCAGCAAGGGCGA
GGACATGATCAAGGTCGACACCCCCGACGTTGTCTGCTGCGAAGAGGAGAGGAAGTTCTACGTCAGCTTCA
AGGACGGCCATATCAGGGTGGGATACCAGGACAGTGATCCCTTCATGGAGTGGACTGACCCTGAGCCATGG
AAGATCACCCACATTGGTTACTGCACAGGCTGGGGAGCAACTGGAAAGTGGAAGTTCGAATTTTAAGTCCT
GCTTTGTGGCTTTGTTCAC

B

```
farnesoic acid O-methyltransferase [Penaeus monodon]
Length=280
```

```
 Score =  590 bits (1521),  Expect = 7e-167
 Identities = 274/280 (97%), Positives = 275/280 (98%), Gaps = 5/280 (1%)
 Frame = +2

Query  20   MGDSWASFGTDENKQYRFRDIKGKTLRFQVKAAHDAHLALTSGEEETDPMLEVFIGGWEG  199
            MGDSWAS+GTDENKQYRFRDIKGKTLRFQVKAAHDAHLALTSGEEETDPMLEVFIGGWEG
Sbjct  1    MGDSWASYGTDENKQYRFRDIKGKTLRFQVKAAHDAHLALTSGEEETDPMLEVFIGGWEG  60

Query  200  AASAIRFKKADDLTKVDTPDILSEEEYREFWVAFDHDVIRVGKGGEWEPFMSATIPEPFD  379
            AASAIRFKKADDLTKVDTPDILSEEEYREFWVAFDHDVIRVGKGGEWEPFMSATIPEPFD
Sbjct  61   AASAIRFKKADDLTKVDTPDILSEEEYREFWVAFDHDVIRVGKGGEWEPFMSATIPEPFD  120

Query  380  ITHYGYSTGWGAVGWWQFHSEVHFQTEDCLTYNFIPVYGDTFTFSVACSNDAHLALTSGP  559
            ITHYGYSTGWGAVGWWQFHSEVHFQTEDCLTYNFIPVYGDTFTFSVACSNDAHLALTSGP
Sbjct  121  ITHYGYSTGWGAVGWWQFHSEVHFQTEDCLTYNFIPVYGDTFTFSVACSNDAHLALTSGP  180

Query  560  EETTPMYEVFIGGWENQHSAIRLSK-----GEDMIKVDTPDVVCCEEERKFYVSFKDGHI  724
            EETTPMYEVFIGGWENQHSAIRLSK     GEDMIKVDTPDVVCCEEERKFYVSFKDGHI
Sbjct  181  EETTPMYEVFIGGWENQHSAIRLSKEGRGSGEDMIKVDTPDVVCCEEERKFYVSFKDGHI  240

Query  725  RVGYQDSDPFMEWTDPEPWKITHIGYCTGWGATGKWKFEF  844
            RVGYQDSDPFMEWTDPEPWKITHIGYCTGWGATGKWKFEF
Sbjct  241  RVGYQDSDPFMEWTDPEPWKITHIGYCTGWGATGKWKFEF  280
```

Figure 3.89 Nucleotide sequence of the full length of recombinant *PmFAMeT-s* (A) and its similarity analysis using BlastX (B). Primer sequences are underlined.

The forward primer containing the *Nde* I restriction site and the reverse primer containing the *Bam* HI restriction site and a 6XHis tag of each gene were designed. The cDNA representing each of the complete ORFs of *PmCOMT*, *PmFAMeT* and *PmFAMeT-s* using the recombinant plasmid of each gene as the template. The amplified full length cDNA was cloned into pGEM-T easy vector, transformed into *E. coli* JM109 and sequenced to confirm the orientation and nucleotide sequence of recombinant clone (Fig. 3.90). The amplification product was digested with *Nde I* and *Bam HI*, eluted from the gel and ligated into pET15 expression vector. The recombinant clone was transformed into *E. coli* JM109 and subsequently into *E. coli* BL21(DE3) codon+ RIPL (Figs. 3.90-3.92)

For the amplified full length ORF of *PmFAMeT-l* and *PmFAMeT-s*, colony PCR was carried out and the amplification products were digested with *Xho II* (which recognized the 15 bp insertion in *PmFAMeT-l*) to classify the short and long forms of the insert. The selected cloned were sequenced to confirm the orientation of the recombinant clones.

A.

ATATCCGATCTTTGGTGCAGTATTGTGTAATCATTCATTGAGATTAACCGACGCGCAAAAACGAC
TCAATGATGTAACTCTGCAGCACCGTAGAGCGGCGATGTTGGCCGCACCTGAGGTTCTGCAGTTC
AATGCCAACATAATGCAGGCTATCGGGGCAAAGAAAGTACTAGACATTGGGGTGTTCACAGGCGC
CAGTTCACTCTCTGCTGCTCTGGCACTGCCTCCGAATGGCAAGGTCCACGCCCTTGACATAAGTG
AAGAGTTTGCCAACATAGGCAAACCGTTCTGGGAGGAAGCTGGAGTTATCAACAAGATAAGTCTG
CACATCGCTCCAGCTGCTGAGACTCTCCAGAAGTTCATTGACGGCGGAGAAGGTGGCACCTTCGA
CTATGCTTTCATTGATGCCGACAAGGGGAATTATGAGCTGTACTATGAACTTTGCCTCACTCTCT
TGCGCTCTGGTGGAGTCATCGCTTTCGACAACACACTTTGGGATGGAGCTGTGATTGACCCCACT
GATCAAACCCCTGGCACAGTGGCTATTAGGAAAATTAACGAAAAACTGAGAGATGACCAGAGAAT
CAATATTTCCTTCCTGAAAATTGGTGATGGCGTGACTCTATGTTTTAAAAAACATCATCATCATC
ATCATTGAGGATCC

B.

```
O-methyltransferase [Fenneropenaeus chinensis]
Length=221
 Score =  321 bits (823),  Expect = 4e-86
 Identities = 180/203 (88%), Positives = 190/203 (93%), Gaps = 0/203 (0%)
 Frame = +2

Query  29   NHSLRLTDAQKRLNDVTLQHRRAAMLAAPEVLQFNANIMQAIGAKKVLDIGVFTGassls  208
            NHSLRLTD QKRLND TLQHRRAAML APEVLQ NANIMQAIGAKKVLDIGVFTGASSLS
Sbjct  19   NHSLRLTDVQKRLNDATLQHRRAAMLGAPEVLQLNANIMQAIGAKKVLDIGVFTGASSLS  78

Query  209  aalalPPNGKVHALDISEEFANIGKPFWEEAGVINKISLHIAPAAETLQKFIDGGEGGTF  388
            AALALPPNGKV+ALDISEEF NIGKP+WEEAGV NKISLHIAPAAETLQKFID GE GTF
Sbjct  79   AALALPPNGKVYALDISEEFTNIGKPYWEEAGVSNKISLHIAPAAETLQKFIDAGEAGTF  138

Query  389  DYAFIDADKGNyelyyelcltllRSGGVIAFDNTLWDGAVIDPTDQTPGTVAIRKINEKL  568
            DYAFIDADK +Y+ YYELCL LLR GGVIAFDNTLWDGAVIDPTDQ PGT+AIRK+NEKL
Sbjct  139  DYAFIDADKESYDRYYELCLILLRPGGVIAFDNTLWDGAVIDPTDQKPGTLAIRKMNEKL  198

Query  569  RDDQRINISFLKIGDGVTLCFKK  637
            +DDQRINISFL+IGDG++LCFKK
Sbjct  199  KDDQRINISFLRIGDGLSLCFKK  221
```

Figure 3.90 Nucleotide sequence of the amplified ORF of *PmCOMT* overhang with *Nde* I- *Bam* HI-6XHis sequenced with the PmCOMT-ORF/*Nde* I-F primer (A) and compared with sequences in the GenBank database using BlastX results of nucleotide sequence of the ORF of *PmCOMT* overhang with *Nde* I- *Bam* HI-6His tag (B). Primer sequences are underlined.

A.

GGACGGGCATAAAGAGTACGCTTCGGGACATCAAGGGCAAGACCCTCCGGTTCCAGGTGAAGGCCCCTCAT
GATGCCCACCTTGCCCTGACCTCAGGGGAGGAGGACCCTGACCCTATGCTGGAGGTGTTCATTGGCGGATG
GGAAGGCGCTGCCTCTGCCATTAGGTTCAAGAAAGCTGATGACTTAACTAAAGTGGACACCCCTGACATCC
TGAGTGAAGAAGAATATCGTGAATTCTGGGTTGCCTTCGACCATGATGTTATCCGTGTTGGCAAGGGAGGC
GAGTGGGAGCCATTCATGAGTGCCACCATTCCAGAGCCTTTCGACATCACTCATTACGGCTACAGTACTGG
CTGGGGTGCTGTTGGCTGGTGGCAGTTCCATAGTGAGGTACACTTCCAAACTGAGGACTGCCTCACGTACA
ACTTCATTCCTGTGTACGGTGACACCTTTACCTTCAGTGTTGCCTGTAGCAATGATGCCCATCTGGCACTC
ACCTCTGGCCCTGAGGAGACCACACCCATGTATGAAGTGTTCATTGGTGGTTGGGAAAACCAGCACTCTGC
CATTCGTCTCAGCAAGGAGGGAAGGGGATCTGGCGAGGACATGATCAAGGTCGACACCCCCGACGTTGTCT

GCTGCGAAGAGGAGAGGAAGTTCTACGTCAGCTTCAAGGACGGCCATATCAGGGTGGGATACCAGGACAGT
GATCCCTTCATGGAGTGGACTGACCCTGAGCCATGGAAGATCACCCACATTGGTTACTGCACAGGCTGGGG
AGCAAACTGGAAGTGGAAGTTCGAATTCATCATCATCATCATTAAGGATCC

B.

```
farnesoic acid O-methyltransferase [Penaeus monodon]
Length=280

Score =  558 bits (1437),  Expect = 4e-157
 Identities = 257/262 (98%), Positives = 258/262 (98%), Gaps = 0/262 (0%)
 Frame = +3

Query  24   RDIKGKTLRFQVKAPHDAHLALTSGEEDPDPMLEVFIGGWEGAASAIRFKKADDLTKVDT  203
            RDIKGKTLRFQVKA HDAHLALTSGEE+ DPMLEVFIGGWEGAASAIRFKKADDLTKVDT
Sbjct  19   RDIKGKTLRFQVKAAHDAHLALTSGEEETDPMLEVFIGGWEGAASAIRFKKADDLTKVDT  78

Query  204  PDILSEEEYREFWVAFDHDVIRVGKGGEWEPFMSATIPEPFDITHYGYSTGWGAVGWWQF  383
            PDILSEEEYREFWVAFDHDVIRVGKGGEWEPFMSATIPEPFDITHYGYSTGWGAVGWWQF
Sbjct  79   PDILSEEEYREFWVAFDHDVIRVGKGGEWEPFMSATIPEPFDITHYGYSTGWGAVGWWQF  138

Query  384  HSEVHFQTEDCLTYNFIPVYGDTFTFSVACSNDAHLALTSGPEETTPMYEVFIGGWENQH  563
            HSEVHFQTEDCLTYNFIPVYGDTFTFSVACSNDAHLALTSGPEETTPMYEVFIGGWENQH
Sbjct  139  HSEVHFQTEDCLTYNFIPVYGDTFTFSVACSNDAHLALTSGPEETTPMYEVFIGGWENQH  198

Query  564  SAIRLSKEGRGSGEDMIKVDTPDVVCCEEERKFYVSFKDGHIRVGYQDSDPFMEWTDPEP  743
            SAIRLSKEGRGSGEDMIKVDTPDVVCCEEERKFYVSFKDGHIRVGYQDSDPFMEWTDPEP
Sbjct  199  SAIRLSKEGRGSGEDMIKVDTPDVVCCEEERKFYVSFKDGHIRVGYQDSDPFMEWTDPEP  258

Query  744  WKITHIGYCTGWGANWKWKFEF  809
            WKITHIGYCTGWGA  KWKFEF
Sbjct  259  WKITHIGYCTGWGATGKWKFEF  280

 Score =  132 bits (331),  Expect = 6e-29
 Identities = 70/136 (51%), Positives = 87/136 (63%), Gaps = 6/136 (4%)
 Frame = +3

Query  399  FQTEDCLTYNFIPVYGDTFTFSVACSNDAHLALTSGPEETTPMYEVFIGGWENQHSAIRL  578
            + T++   Y F  + G T  F V  ++DAHLALTSG EET PM EVFIGGWE   SAIR
Sbjct  8    YGTDENKQYRFRDIKGKTLRFQVKAAHDAHLALTSGEEETDPMLEVFIGGWEGAASAIRF  67

Query  579  SKEGRGSGEDMIKVDTPDVVCCEEERKFYVSFKDGHIRVGY-QDSDPFMEWTDPEPWKIT  755
             K      +D+ KVDTPD++  EE R+F+V+F    IRVG   + +PFM  T PEP+ IT
Sbjct  68   KK-----ADDLTKVDTPDILSEEEYREFWVAFDHDVIRVGKGGEWEPFMSATIPEPFDIT  122

Query  756  HIGYCTGWGANWKWKF  803
            H GY TGWGA   W+F
Sbjct  123  HYGYSTGWGAVGWWQF  138
```

Figure 3.91 Nucleotide sequence of the complete ORF of *PmFAMeT-l* overhang with *Nde* I- *Bam* HI-6XHis (A) and its similarity analysis using BlastX (B). Primer sequences are underlined.

A.

GGAAGCCTGAGAATTCCNTCTGAATATTTTGTTAACTTTAAGAAGGAGATATACCATGGGCAGCAGCCATC
ATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGGGCGAGAGCTGGGCTTCCTTCGGT
ACCGATGAGAACAAGCAGTACCGCTTCAGGGACATCAAGGGCAAGACCCTCCGGTTCCAGGTGAAGGCTGC
CCATGATGCCCACCTTGCCCTGACCTCAGGGGAAGAGGAGACTGACCCTATGCTGGAGGTGTTCATTGGCG
GATGGGAAGGCGCTGCCTCTGCCATTAGGTTCAAGAAAGCTGATGACTTAACTAAAGTGGACACCCCTGAC

```
ATCCTGAGTGAAGAAGAATATCGTGAATTCTGGGTTGCCTTCGACCATGATGTTATCCGTGTTGGCAAGGG
AGGCGAGTGGGAGCCATTCATGAGTGCCACCATTCCAGAGCCTTTCGACATCACTCATTACGGCTACAGTA
CTGGCTGGGGTGCTGTTGGTTGGTGGCAGTTCCATAGTGAGGTACACTTCCAAACTGAGGACTGCCTCACG
TACAACTTCATTCCTGTGTACGGTGACACCTTTACCTTCAGTGTTGCCTGTAGCAATGATGCCCATCTGGC
ACTCACCTCTGGCCCTGAGGAGACCACACCCATGTATGAAGTGTTCATTGGTGGTTGGGAAAACCAGCACT
CTGCCATTCGTCTCAGCAAGGGCGAGGACATGATCAAGGTCGACACCCCCGACGTTGTCTGCTGCGAAGAG
GAGAGGAAGTTCTACGTCAGCTTCAAGGACGGCCATATCAGGTTGGGATACCAGGACAGTGATCCCTTCAT
GGAG
```

B.

```
farnesoic acid O-methyltransferase [Penaeus monodon]
Length=280
 Score =  518 bits (1333),  Expect = 3e-145
 Identities = 244/252 (96%), Positives = 247/252 (98%), Gaps = 5/252 (1%)
 Frame = +2
Query  119  MGESWASFGTDENKQYRFRDIKGKTLRFQVKAAHDAHLALTSGEEETDPMLEVFIGGWEG  298
            MG+SWAS+GTDENKQYRFRDIKGKTLRFQVKAAHDAHLALTSGEEETDPMLEVFIGGWEG
Sbjct  1    MGDSWASYGTDENKQYRFRDIKGKTLRFQVKAAHDAHLALTSGEEETDPMLEVFIGGWEG  60

Query  299  AASAIRFKKADDLTKVDTPDILSEEEYREFWVAFDHDVIRVGKGGEWEPFMSATIPEPFD  478
            AASAIRFKKADDLTKVDTPDILSEEEYREFWVAFDHDVIRVGKGGEWEPFMSATIPEPFD
Sbjct  61   AASAIRFKKADDLTKVDTPDILSEEEYREFWVAFDHDVIRVGKGGEWEPFMSATIPEPFD  120

Query  479  ITHYGYSTGWGAVGWWQFHSEVHFQTEDCLTYNFIPVYGDTFTFSVACSNDAHLALTSGP  658
            ITHYGYSTGWGAVGWWQFHSEVHFQTEDCLTYNFIPVYGDTFTFSVACSNDAHLALTSGP
Sbjct  121  ITHYGYSTGWGAVGWWQFHSEVHFQTEDCLTYNFIPVYGDTFTFSVACSNDAHLALTSGP  180

Query  659  EETTPMYEVFIGGWENQHSAIRLSK-----GEDMIKVDTPDVVCCEEERKFYVSFKDGHI  823
            EETTPMYEVFIGGWENQHSAIRLSK     GEDMIKVDTPDVVCCEEERKFYVSFKDGHI
Sbjct  181  EETTPMYEVFIGGWENQHSAIRLSKEGRGSGEDMIKVDTPDVVCCEEERKFYVSFKDGHI  240

Query  824  RLGYQDSDPFME  859
            R+GYQDSDPFME
Sbjct  241  RVGYQDSDPFME  252
 Score =  137 bits (345),  Expect = 1e-30
 Identities = 72/136 (52%), Positives = 87/136 (63%), Gaps = 6/136 (4%)
 Frame = +2
Query  140  FGTDENKQYRFRDIKGKTLRFQVKAAHDAHLALTSGEEETDPMLEVFIGGWEGAASAIRF  319
            F T++   Y F  + G T  F V  ++DAHLALTSG EET PM EVFIGGWE   SAIR
Sbjct  144  FQTEDCLTYNFIPVYGDTFTFSVACSNDAHLALTSGPEETTPMYEVFIGGWENQHSAIRL  203

Query  320  KK-----ADDLTKVDTPDILSEEEYREFWVAFDHDVIRVGKGGEWEPFMSATIPEPFDIT  484
             K      +D+ KVDTPD++  EE R+F+V+F    IRVG     +PFM  T PEP+ IT
Sbjct  204  SKEGRGSGEDMIKVDTPDVVCCEEERKFYVSFKDGHIRVGYQDS-DPFMEWTDPEPWKIT  262

Query  485  HYGYSTGWGAVGWWQF  532
            H GY TGWGA G W+F
Sbjct  263  HIGYCTGWGATGKWKF  278
```

Figure 3.92 Nucleotide sequence of the complete ORF of *PmFAMeT-s* overhang with *Nde* I- *Bam* HI-6XHis (A) and its similarity analysis using BlastX (B). Primer sequences are underlined.

3.8.2 Optimization of conditions for an *in vitro* expression of rPmCOMT, rPmFAMeT-1, and rPmFAMeT-s protein

Expression of 3 recombinant clones of PmCOMT, PmFAMeT-1 and PmFAMeT-s cultured at 37°C and induced with 0.4 mM IPTG for 1 and 6 hours were examined. No obvious difference on the expression level was observed when the recombinant clones of a particular protein was induced with IPTG for different period of time (Figs.3.93-3.95).

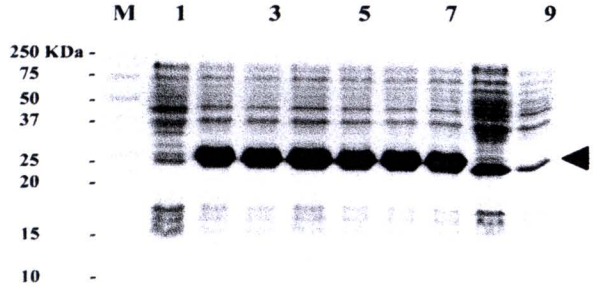


Figure 3.93 SDS-PAGE showing *in vitro* expression of rPmCOMT from the recombinant clones at 3 and 6 hours after induction with 0.4 mM IPTG (lanes 2-3, 4-5 and 6-7), respectively. A pET15b vector in *E. coli* BL21-CodonPlus (DE3)-RIPL (lane 8) and *E. coli* BL21-CodonPlus (DE3)-RIPL (lane 9) were included as the control. Lane M = the protein standard marker.

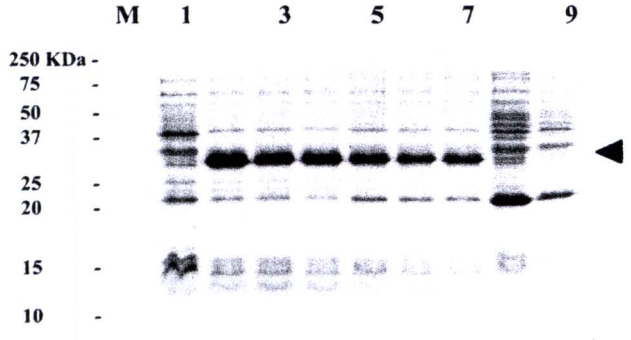


Figure 3.94 SDS-PAGE showing *in vitro* expression of rPmFAMeT-1 from the recombinant clones at 3 and 6 hours after induction with 0.4 mM IPTG (lanes 2-3, 4-5 and 6-7), respectively. A pET15b vector in *E. coli* BL21-CodonPlus (DE3)-RIPL (lane 8) and *E. coli* BL21-CodonPlus (DE3)-RIPL (lane 9) were included as the control. Lane M = the protein standard marker.

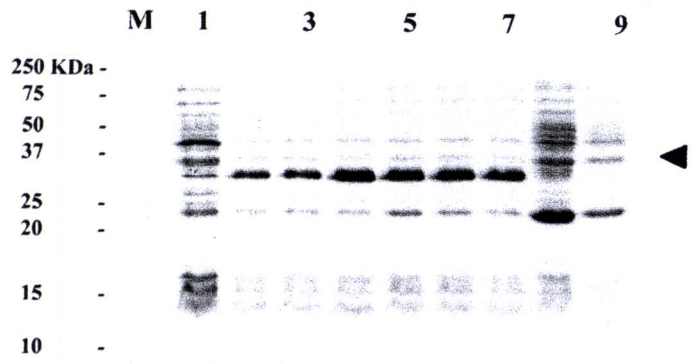


Figure 3.95 SDS-PAGE showing *in vitro* expression of rPmFAMeT-s from the recombinant clones at 3 and 6 hours after induction with 0.4 mM IPTG (lanes 2-3, 4-5 and 6-7), respectively. A pET15b vector in *E. coli* BL21-CodonPlus (DE3)-RIPL (lane 8) and *E. coli* BL21-CodonPlus (DE3)-RIPL (lane 9) were included as the control. Lane M = the protein standard marker.

Only one recombinant clone of each recombinant protein was selected and cultured at 37°C. The optimal concentration of IPTG (0.4, 0.6, 0.8 and 1.0 mM) for the production of each recombinant protein after IPTG induction for 3 and 6 hours was examined (Figs 3.96-3.98). No different effect of IPTG concentration on expression of rPmCOMT, rPmFAMeT-l and rPmFAMeT-s was observed. Therefore, the IPTG concentration at 0.4 mM was used to induce the overexpression of these recombinant clones.

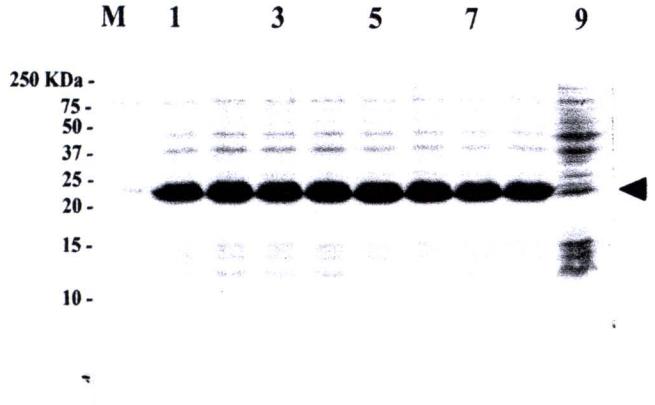


Figure 3.96 SDS-PAGE showing *in vitro* expression of rPmCOMT after induced by 0.4, 0.6, 0.8 and 1 mM IPTG for 3 (lanes 1-4) and 6 hr (lane 5-8), respectively. A pET15b vector in *E. coli* BL21-CodonPlus (DE3)-RIPL (lane 9) was included as the control.

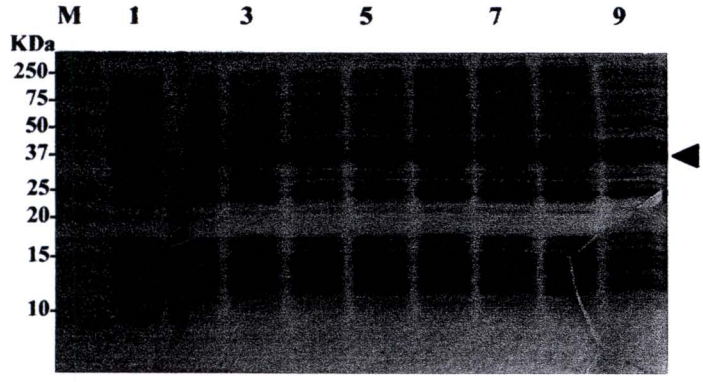


Figure 3.97 SDS-PAGE showing *in vitro* expression of rPmFAMeT-l after induced by 0.4, 0.6, 0.8 and 1 mM IPTG for 3 (lanes 1-4) and 6 hr (lane 5-8), respectively. A pET15b vector in *E. coli* BL21-CodonPlus (DE3)-RIPL (lane 9) was included as the control.

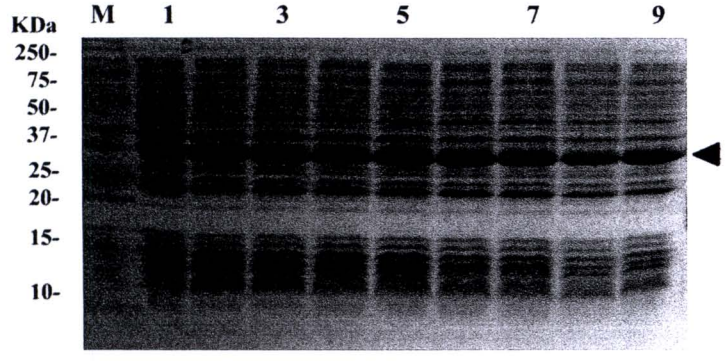
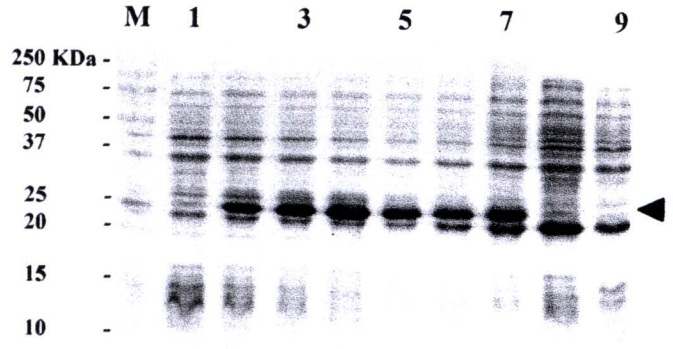


Figure 3.98 SDS-PAGE showing *in vitro* expression of rPmFAMeT-s after induced by 0.4, 0.6, 0.8 and 1 mM IPTG for 3 (lanes 1-4) and 6 hr (lane 5-8), respectively. A pET15b vector in *E. coli* BL21-CodonPlus (DE3)-RIPL (lane 9) was included as the control.

Afterwards, the expression of rPmCOMT, rPmFAMeT-l and rPmFAMeT-s cultured at 37°C and induced with 0.4 mM IPTG for 0, 1, 2, 6, 12 and 24 hours was examined. These proteins seems to be stably expressed during the induction period therefore, the induction period of 3 hours was used for cell localization of these recombinant proteins (Figs. 3.99-3.101).

A.



B.

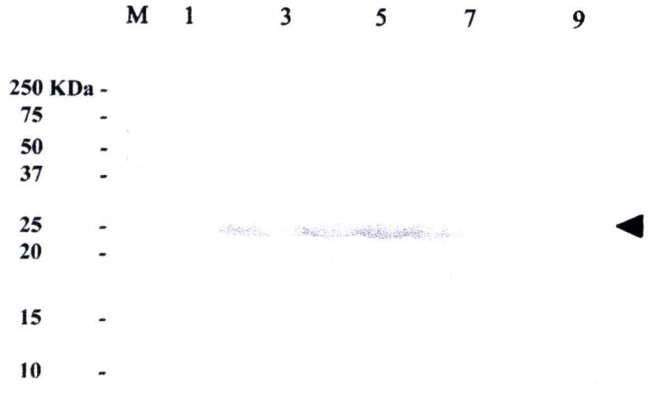
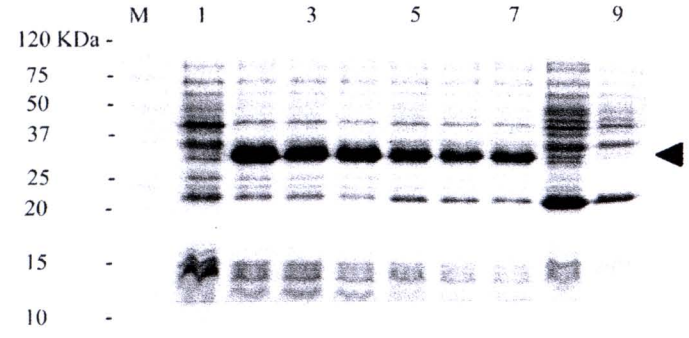


Figure 3.99 SDS-PAGE (A) and Western blot analysis (B) showing *in vitro* expression of rPmCOMT at 0, 1, 2, 6, 12 and 24 hours after induction with 0.4 mM IPTG (lanes 1-7), respectively. A pET15b vector in *E. coli* BL21-CodonPlus (DE3)-RIPL (lane 8) and *E. coli* BL21-CodonPlus (DE3)-RIPL (lane 9) were included as the control.

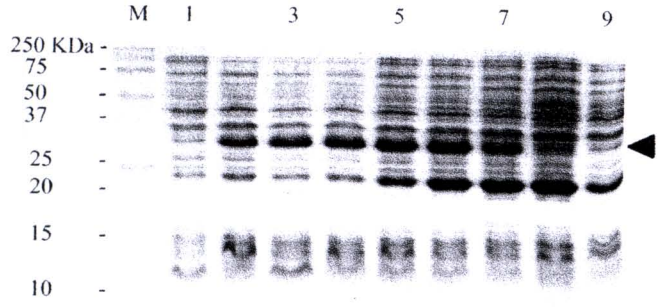
A.



B.

Figure 3.100 SDS-PAGE (A) and Western blot analysis (B) showing *in vitro* expression of rPmFAMeT-1 at 0, 1, 2, 6, 12 and 24 hours after induction with 0.4 mM IPTG (lanes 1-7), respectively. A pET15b vector in *E. coli* BL21-CodonPlus (DE3)-RIPL (lane 8) and *E. coli* BL21-CodonPlus (DE3)-RIPL (lane 9) were included as the control.

A.



B.

Figure 3.101 SDS-PAGE (A) and Western blot analysis (B) showing *in vitro* expression of rPmFAMeT-s at 0, 1, 2, 6, 12 and 24 hours after induction with 0.4 mM IPTG (lanes 1-7), respectively. A pET15b vector in *E. coli* BL21-CodonPlus (DE3)-RIPL (lane 8) and *E. coli* BL21-CodonPlus (DE3)-RIPL (lane 9) were included as the control.

3.8.3 Cell localization of rPmCOMT, rPmFAMeT-l and rPMFAMeT-s proteins

Cell localization of rPmCOMT, rPmFAMeT-l and rPMFAMeT-s proteins was examined. Proteins from the while cells, soluble and insoluble fractions of cultured recombinant cloned were electrophoretically analyzed by 15% SDS-PAGE and Western blot.

All recombinant proteins were greater expressed as the insoluble form than the soluble form when cultured at 37°C (Figs. 3.102, 3.104 and 3.106, respectively). The cultured temperature was then decreased from at 37°C to 25°C. Although these recombinant proteins were more abundantly expressed in the soluble form, the major products were still in the insoluble form (Figs. 3.103, 3.105 and 3.107). Accordingly, the rPmCOMT, PmFAMeT-l and PmFAMet-s were purified as the insoluble proteins under the denaturing conditions.

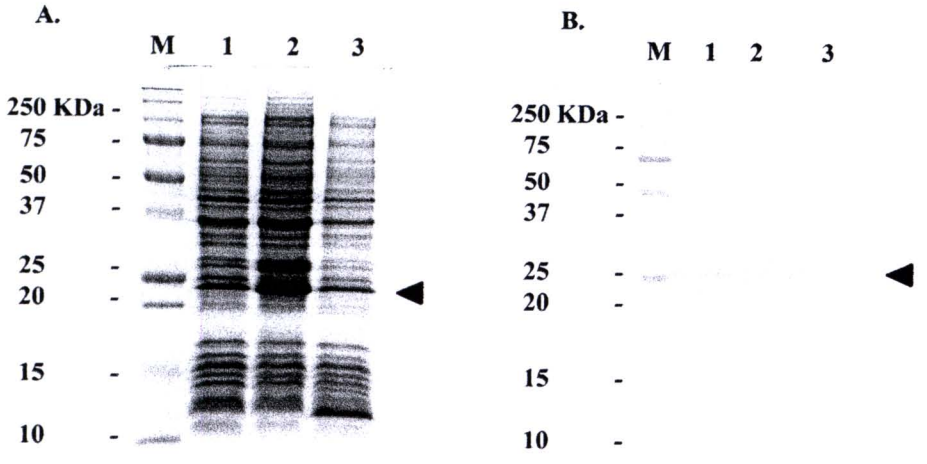


Figure 3.102 SDS-PAGE (A) and Western blot analysis (B) showing *in vitro* expression of a recombinant clone of rPmCOMT cultured at 37°C after induction with 0.4 mM IPTG. Lane 1 = whole cell, lane 2 = insoluble fraction and lane 3 = soluble fraction.

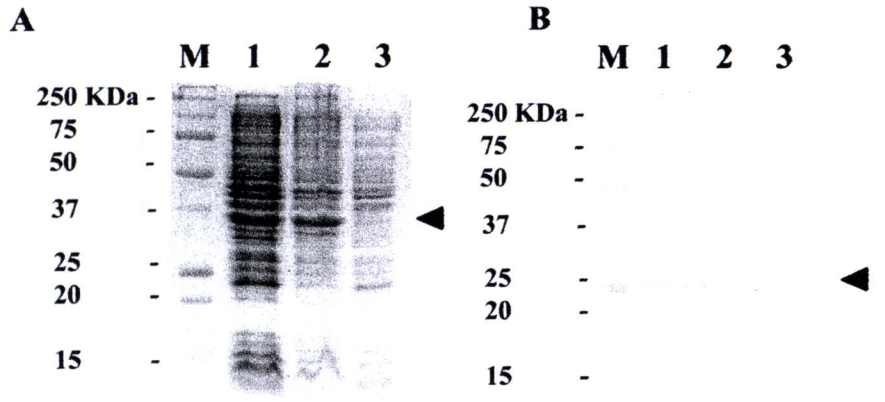


Figure 3.103 SDS-PAGE (A) and Western blot analysis (B) showing *in vitro* expression of a recombinant clone of rPmCOMT cultured at 25°C after induction with 0.4 mM IPTG. Lane 1 = whole cells, lane 2 = insoluble fraction and lane 3 = soluble fraction.

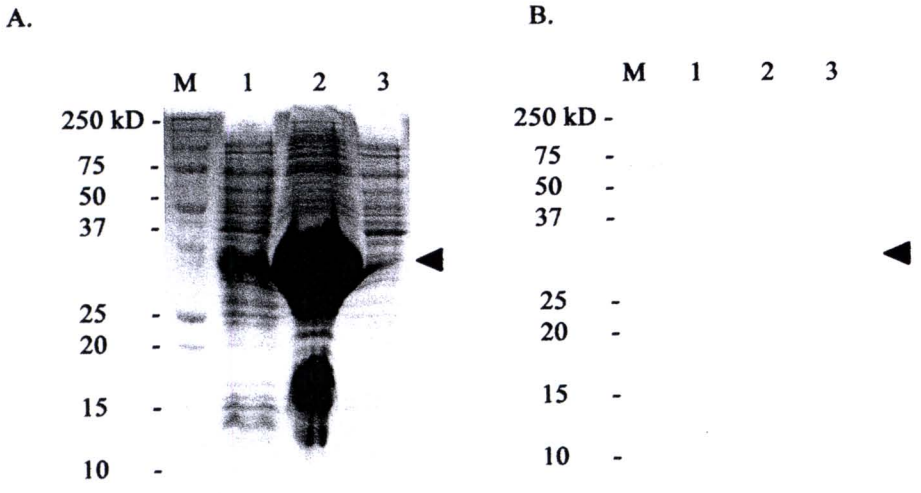


Figure 3.104 SDS-PAGE (A) and Western blot analysis (B) showing *in vitro* expression of a recombinant clone of rPmFAMeT-1 cultured at 37°C after induction with 0.4 mM IPTG. Lane 1 = whole cells, lane 2 = insoluble fraction and lane 3 = soluble fraction.

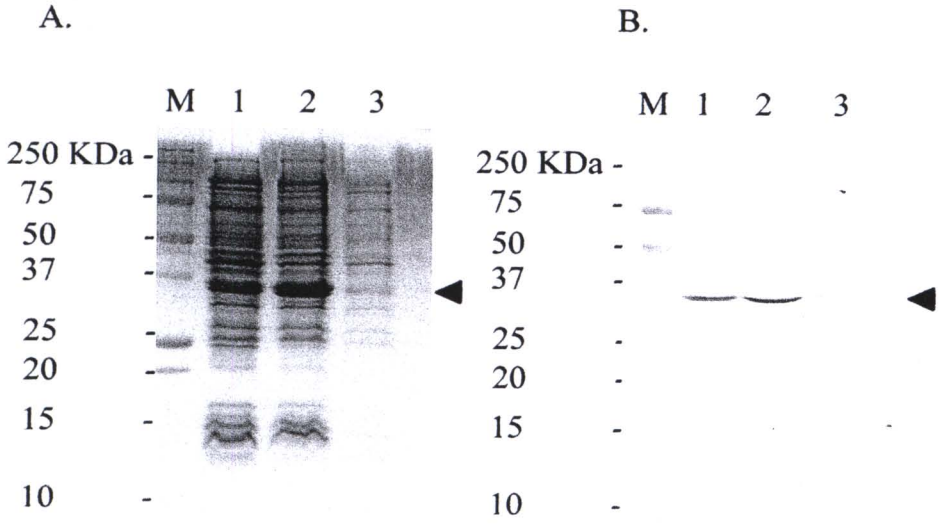


Figure 3.105 SDS-PAGE (A) and Western blot analysis (B) showing *in vitro* expression of a recombinant clone of rPmFAMeT-1 cultured at 25°C after induction with 0.4 mM IPTG. Lane 1 = whole cells, lane 2 = insoluble fraction and lane 3 = soluble fraction.

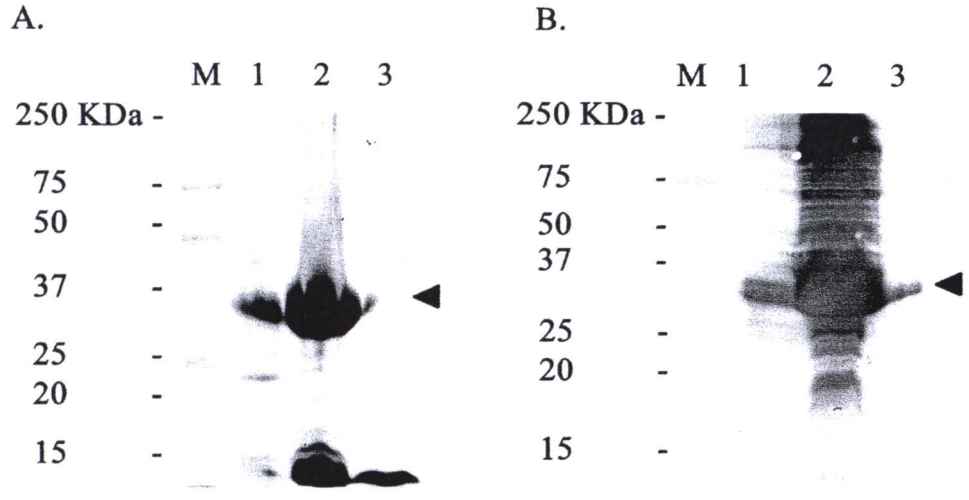


Figure 3.106 SDS-PAGE (A) and Western blot analysis (B) showing *in vitro* expression of a recombinant clone of rPmFAMeT-s cultured at 37°C after induction with 0.4 mM IPTG. Lane 1 = whole cells, lane 2 = insoluble fraction and lane 3 = soluble fraction.

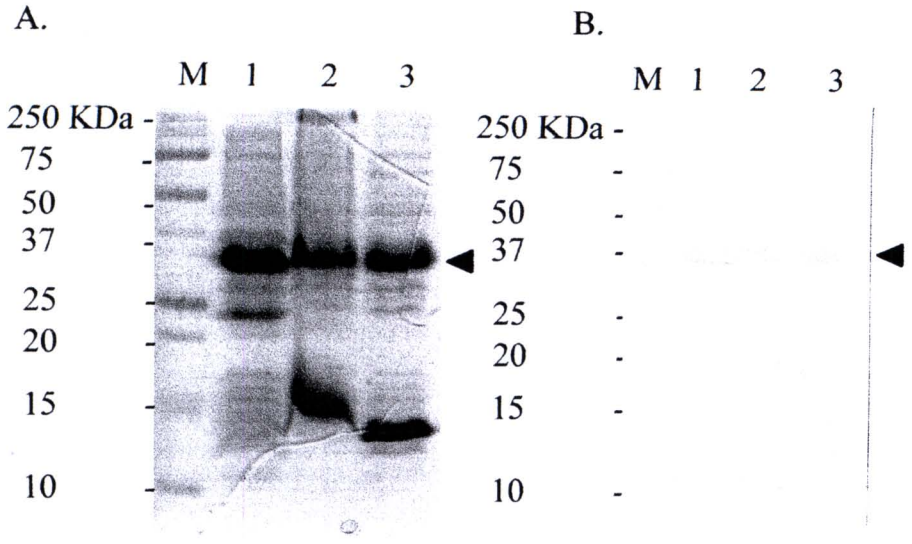


Figure 3.107 SDS-PAGE (A) and Western blot analysis (B) showing *in vitro* expression of a recombinant clone of rPmFAMeT-s cultured at 25°C after induction with 0.4 mM IPTG. Lane 1 = whole cells, lane 2 = insoluble fraction and lane 3 = soluble fraction.

3.8.4 Purification of recombinant protein

The insoluble proteins of all recombinant clones were purified in the denaturing conditions. The cells were washed using 10 ml of 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 loaded into the column and washed with the binding buffer. 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 15% SDS-PAGE and western blot analysis. The purified fractions (Figs. 3.108-3.110) were concentrated by ultrafiltration and kept at 4°C (Fig. 3.111). The obtained protein was subjected to polyclonal antibody production in rabbit.

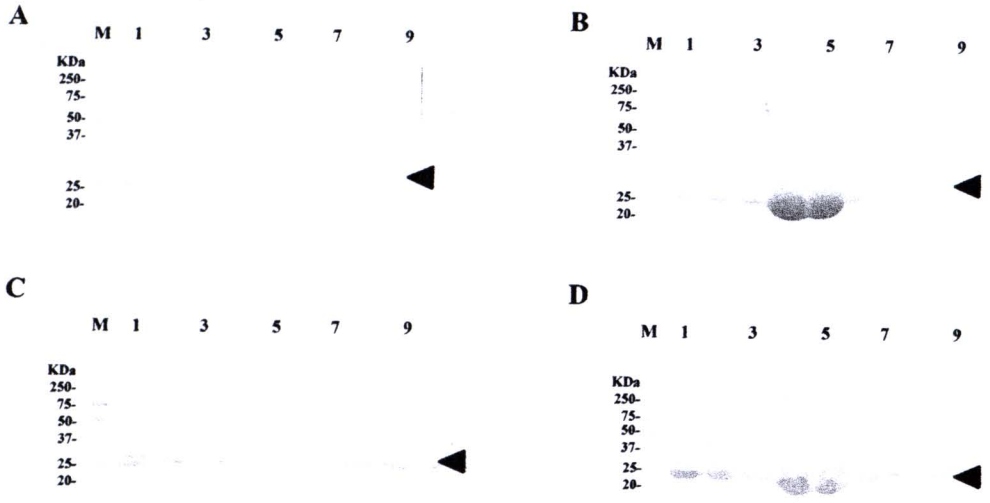


Figure 3.108 SDS-PAGE (A) and Western blot analysis (B) of purified rPmCOMT in the denaturing conditions. The culture was carried at 37 °C and induced with 0.4 mM IPTG for 3 hours. A and C: lane 1 = whole cells, 2 = the insoluble fraction after pass through the column, 2-7 = the first wash fractions, and 8-9 = the second wash fractions, respectively. B and D: lane 1 = the third wash fraction, 2 = the elution fraction, respectively.

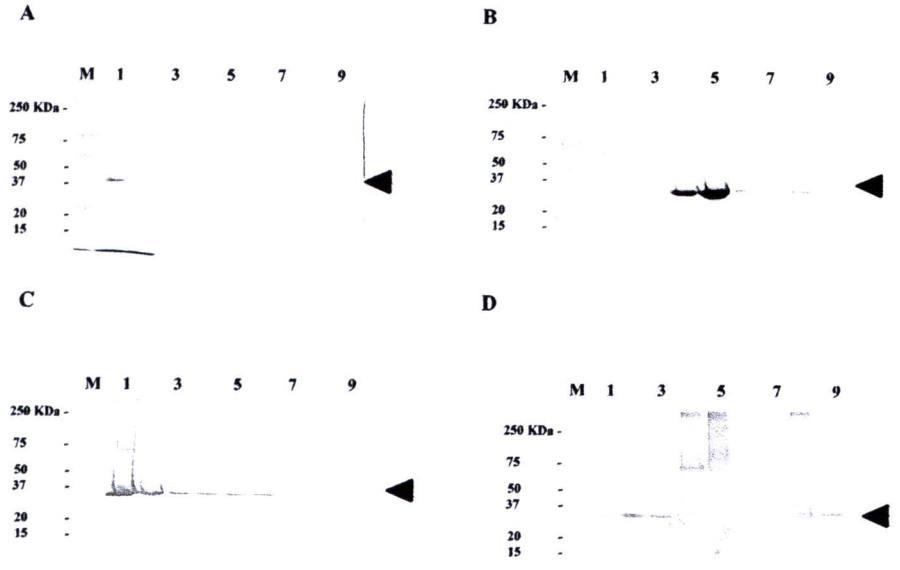


Figure 3.109 SDS-PAGE (A) and Western blot analysis (B) of purified rPmFAMeT-1 in the denaturing conditions. The culture was carried at 37 °C and induced with 0.4 mM IPTG for 3 hours. A and C: lane 1 = whole cells, 2 = the insoluble fraction after pass through the column, 2-7 = the first wash fractions, and 8-9 = the second wash fractions, respectively. B and D: lane 1 = the third wash fraction, 2 = the elution fraction, respectively.

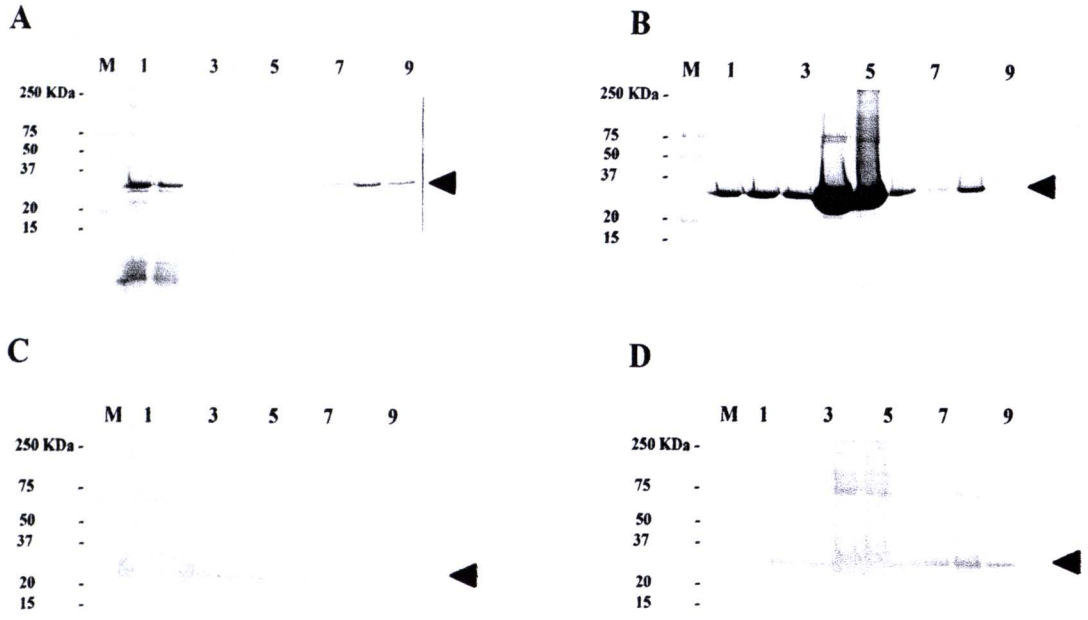


Figure 3.110 SDS-PAGE (A) and Western blot analysis (B) of purified rPmFAMeT-s in the denaturing conditions. The culture was carried at 37 °C and induced with 0.4 mM IPTG for 3 hours. A and C: lane 1 = whole cells, 2 = the insoluble fraction after pass through the column, 2-7 = the first wash fractions, and 8-9 = the second wash fractions, respectively. B and D: lane 1 = the third wash fraction, 2 = the elution fraction, respectively.

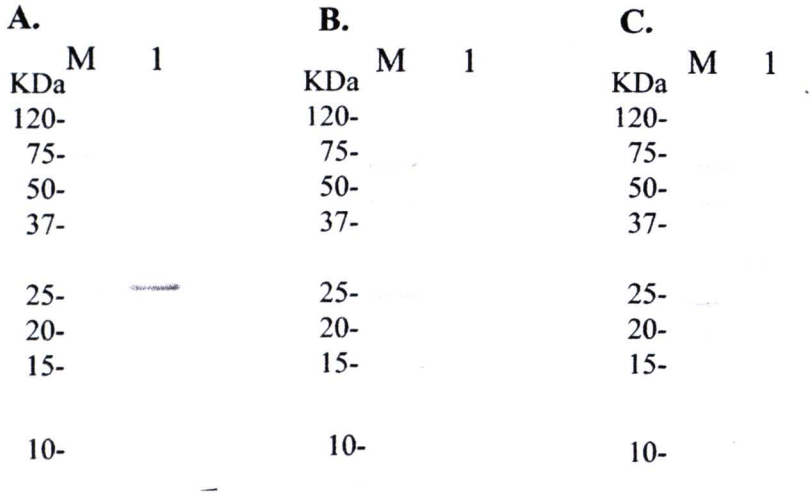


Figure 3.111 SDS-PAGE (A) and Western blot analysis (B) of the insoluble protein fractions of rPmCOMT, rPmFAMeT-s and rPmFAMeT-1 purified in denaturing conditions after culture at 37 °C and induced with 0.4 mM IPTG for 3 hours.

3.8.5 Peptide sequencing of purified rPmCOMT, rPmFAMeT-l and rPmFAMeT-s

The peptide sequencing was applied to confirm whether the purified proteins were rPmCOMT, rPmFAMeT-l and rPmFAMeT-s. After size-fractionated, the expected recombinant protein was further analyzed by NanoLC-MS/MS.

Internal peptide sequences of rPmCOMT was SYHNPDPLVQ YCVNHSLR and IGDGVTLCFKK which significantly matched *O*-methyltransferase of *Fenneropenaeus chinensis* (clone no. HC-H-S01-0684-LF; http://pmonodon.biotec.or.th) at 11% of sequence coverage while those of rPmFAMeT-l were VDTPDVVCCEEER and VGYQDSDPFMEWTDPEPWK which significantly matched farnesoic acid *O*-methyltransferase of *P. monodon* (clone no. IN-N-S01-1195-LF) at 13% of sequence coverage. Those of rPmFAMeT-s were VGYQDSDPFMEWTDPEPWK which significantly matched farnesoic acid *O*-methyltransferase of *P. monodon* (clone no. IN-N-S01-1195-LF) at 7% of sequence coverage, respectively. Results illustrated that the purified recombinant proteins were the target proteins of this study.

3.8.6 The production of polyclonal antibodies against rPmCOMT, rPmFAMeT-l and PmFAMeT-s

Anti-PmCOMT, anti-PmFAMeT-l and anti-PmFAMeT-s polyclonal antibodies were successfully produced in rabbits. The titer of PmFAMeT-s polyclonal antibody (PAb) was high after the third immunization but that of PmFAMeT-l PAb and PmFAMeT PAb was quite low, thus further antigen administration was required (Tables 3.29-3.31). Rabbits were sacrificed and their serum was collect, filtrated through 0.22 μM membrane and kept at -20 °C.

Table 3.29 Titers of anti-PmCOMT after the rabbit was immunized rPmCOMT for 4 times

Dilution of serum	Rabbit anti-PmCOMT	
	Pre-immunized serum*	Immunized serum**
1:500	0.052	3.014
1:2000	0.020	2.220
1:8000	0.001	0.955
1:32000	-0.003	0.313

* Pre-immunized serum = serum from normal rabbit
** Immunized serum = serum from rabbit injected with the recombinant protein

Table 3.30 Titers of anti-PmFAMeT-1 after the rabbit was immunized rPmFAMeT-1 for 5 times

Dilution of serum	Rabbit anti PmFAMeT-1	
	Pre-immunized serum*	Immunized serum**
1:500	0.303	1.909
1:2000	0.153	1.021
1:8000	0.091	0.391
1:32000	0.075	0.166

* Pre-immunized serum=serum from normal rabbit
** Immunized serum=serum from rabbit injected with the recombinant protein

Table 3.31 Titers of anti-PmFAMeT-s after the rabbit was immunized rPmFAMeT-s for 3 times

Dilution of serum	Rabbit anti PmFAMeT-s	
	Pre-immunized serum*	Immunized serum**
1:500	0.115	2.392
1:2000	0.027	1.804
1:8000	0.007	0.841
1:32000	0.002	0.292

* Pre-immunized serum=serum from normal rabbit
** Immunized serum=serum from rabbit injected with the recombinant protein

3.8.7 Expression profiles of PmCOMT, PmFAMeT-l and PmFAMeT-s proteins during ovarian development of *P. monodon*

Non-purified anti-PmCOMT PAb generated the positive signals along with non-specific immunoreactive bands following western blot analysis (data not shown). Therefore, anti-PmCOMT and anti-PmFAMeT-l PAB were affinity-chromatographically purified.

Anti-PmCOMT PAb revealed a positive band of approximately 24 kDa suggesting no posttranslational modification (i.e. glycosylation) of this non-secretory ovarian protein. More intense signals of PmCOMT were observed in previtellogenic and vitellogenic ovaries than those in cortical rod and mature ovaries of intact *P. monodon* broodstock (Fig. 3.112).

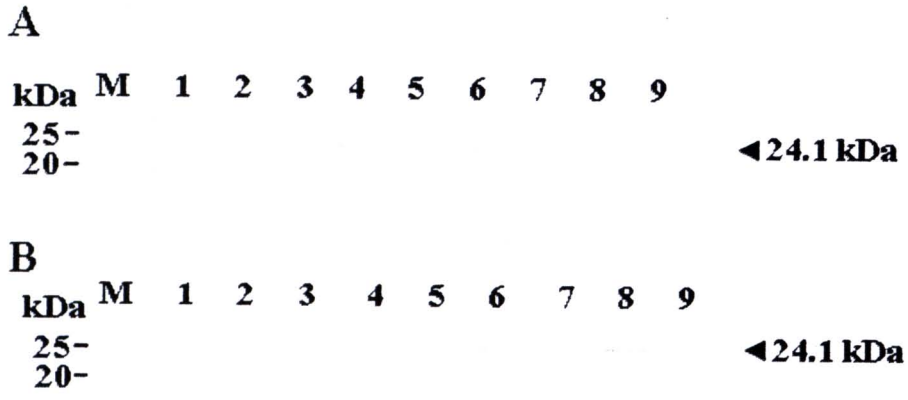


Figure 3.112 Western blotting analysis of anti-PmCOMT PcAb (dilution 1:300, expected MW of 24.1 kDa) using total proteins extracted from ovaries of intact (A) and eyestalk-ablated (B broodstock of wild *P. monodon.* Ovarian proteins (30 µg) were size-fractionated by 15% SDS-PAGE.

Lanes 1 = stage I ovaries (GSI =1.44%); lanes 2-3 = stage II ovaries (GSI = 2.95 and 2.15%, respectively); lanes 4-5, B = stage III ovaries (GSI = 4.62 and 5.37%); lane 6-7 = stage IV ovaries (GSI = 9.36 and 10.41%, respectively). Lanes M = protein standard.

Western blot analysis revealed the positive signals of ovarian PmFAMeT in juveniles and stages I and II but not in stages III and IV 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 only observed in stages I and II ovaries of broodstock.(Fig. 3.113).

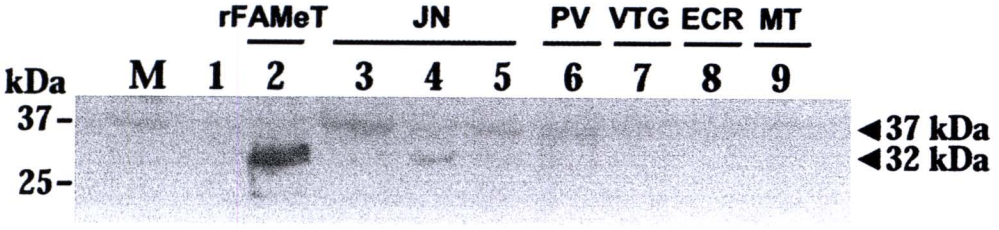


Figure 3.113 Western blot analysis of anti-PmFAMeT-1 PAb (1:300) against total proteins (25 µg) extracted form ovaries of different stages of *P. monodon*: JN = juveniles, PV = previtellogenic (stage I) ovaries, V = vitellogenic (II) ovaries, CR = cortical rod (III) ovaries, M = mature (IV) ovaries. Lane M: protein standard.

3.9 Localization of all proteins in ovaries of *P. monodon* broodstock

After affinity-chromatographic purification, anti-PmCOMT, anti-PmFAMeT-1 PAb and anti-PmFAMeT-s PAb were used to localize of the respective proteins in different stages of ovaries in both intact and eyestalk-ablated *P. monodon* broodstock.

The positive immunohistological signals of PmCOMT were detected in cytoplasm of previtellogenic and vitellogenic oocytes. Nevetheless, the positive signals were observed in cortical rods of stage III (early cortical rod) and IV (mature) oocytes in both intact (Fig. 3.114) and eyestalk-ablated broodstock(Fig. 3.115). However, clearer signals in the latter than the former were noticed. No immunoreactivity was found in ovaries

when incubated with the blocking solution (the negative control) and with the preimmnune serum (Figs. 3.114 and 3.115).

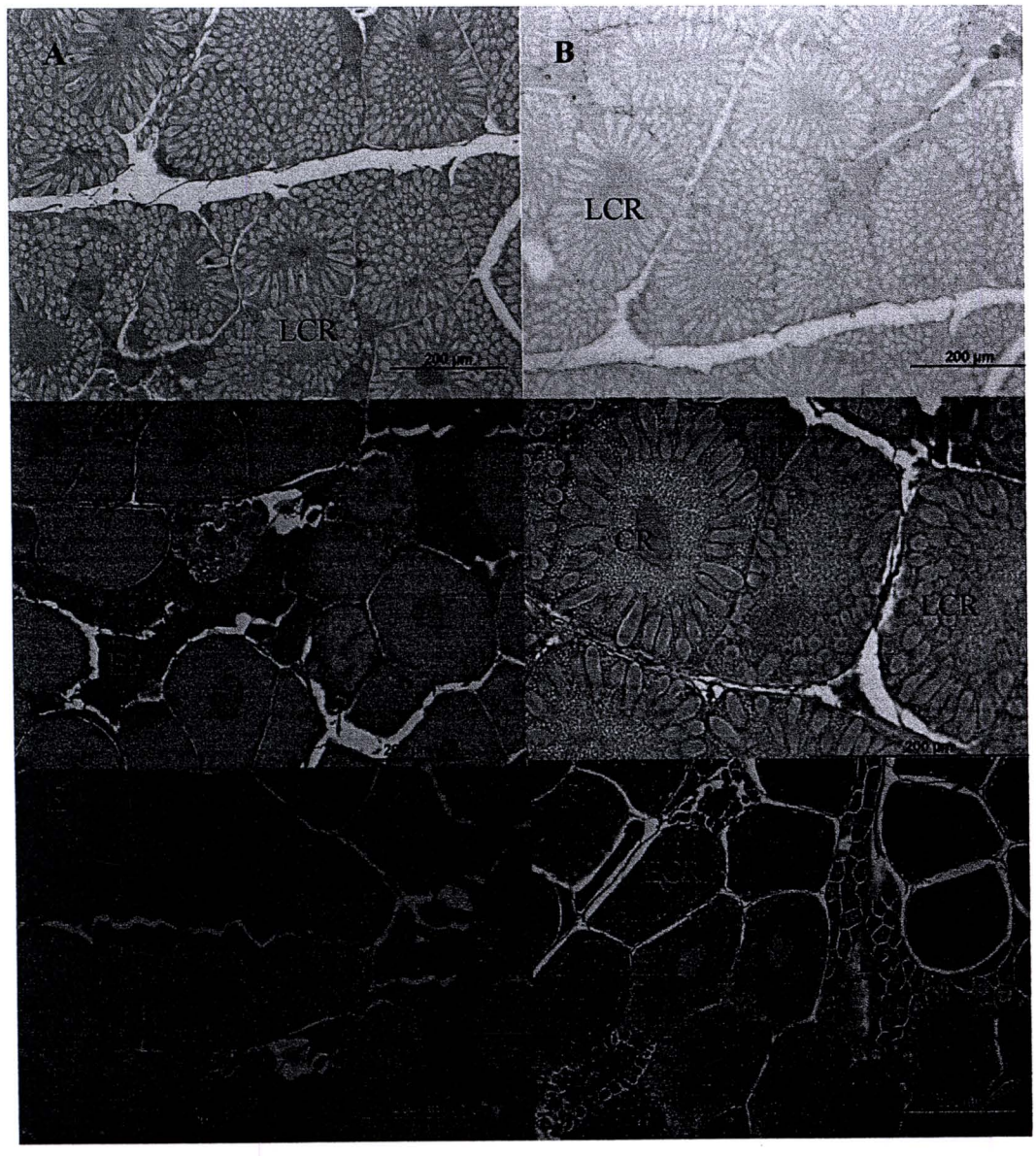


Figure 3.114 Immunohistochemical localization of the PmCOMT protein in ovaries of intact *P. monodon* broodstock (C and D). The blocking solution (A) and preimmune serum (B) were used as the negative control. The conventional HE staining (E) was carried out for identification of oocyte stages.

The anti-PmFAMeT-l PAb and anti-PmFAMeT-s PAb were used to localize the FAMeT protein in ovaries of *P. monodon* broodstock. The weak immunoreactivity was

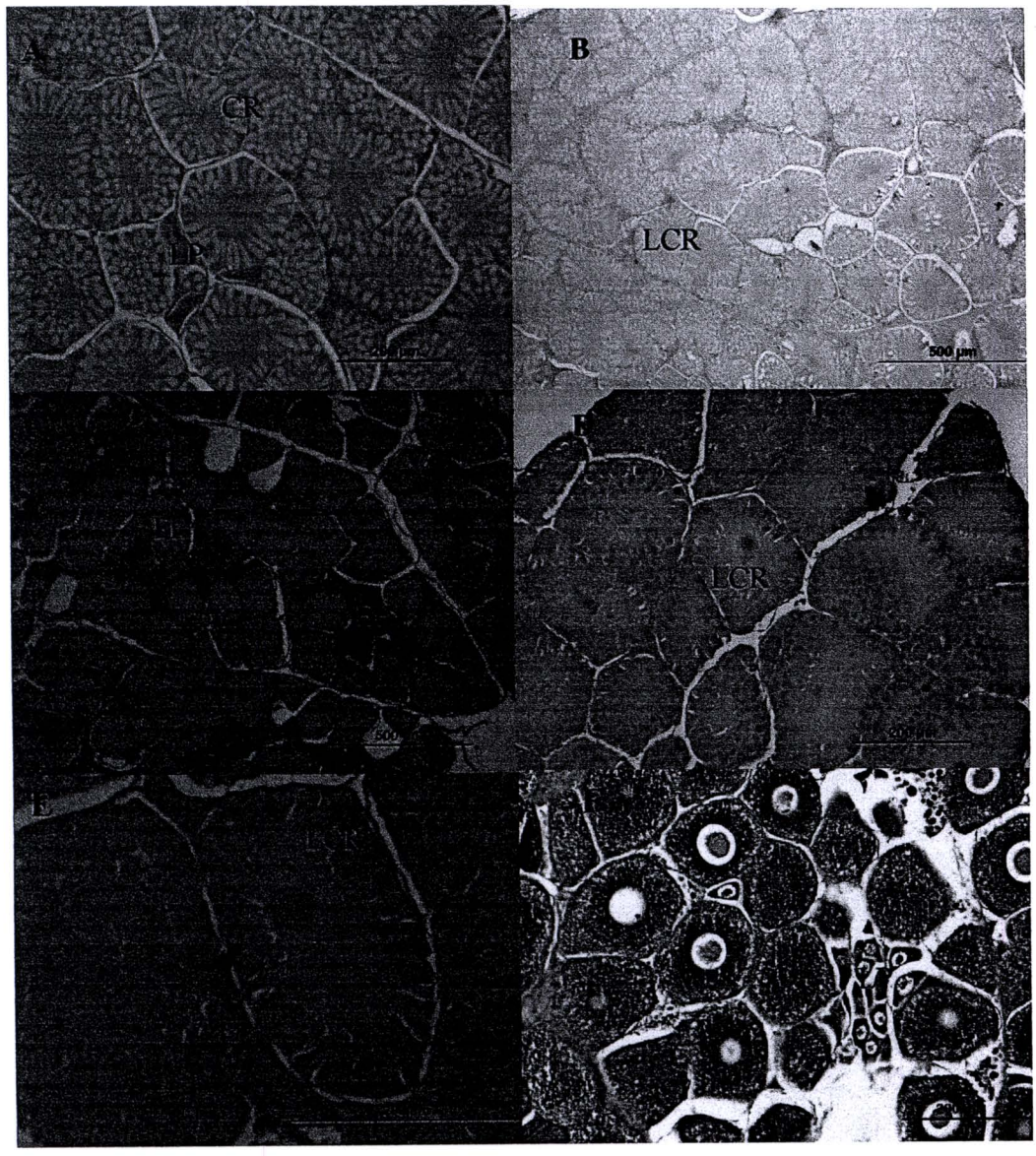


Figure 3.115 Immunohistochemical localization of PmCOMT protein in ovaries of eyestalk-ablated broodstock of *P. monodon* (C-D). The blocking solution (A) and preimmune serum (B) were used as the negative control. The conventional HE staining (E) was carried out for identification of oocyte stages.

observed when anti-PmFAMeT-s PAb was used (Figs 3.116 and 3.117). However, stronger signals were found when anti-PmFAMeT-1 PAb was used (Figs 3.118-3.119).

The positive immunohistological signals of PmFAMeT were detected in cortical rods of stages III and IV oocytes in both intact and eyestalk-ablated broodstock of *P. monodon* (Figs 3.118 and 3.119). No immunoreactivity was found in stages I and II oocytes and in ovaries incubated with the blocking solution and the preimmune serum (Figs 3.118 and 3.119).

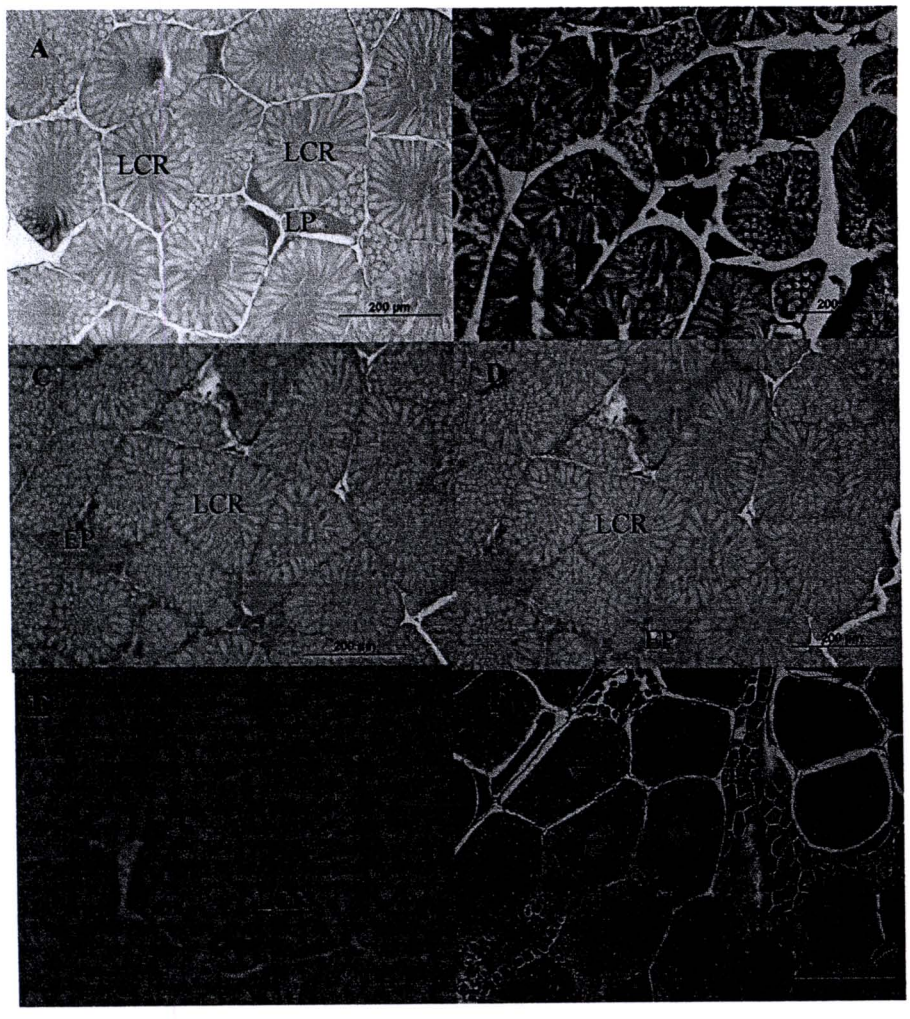


Figure 3.116 Immunohistochemical localization of the PmFAMeT-s protein in ovaries of eyestalk-ablated broodstock of *P. monodon* (C-E). The blocking solution (A) and preimmune serum (B) were used as the negative control. The conventional HE staining (F) was carried out for identification of oocyte stages.

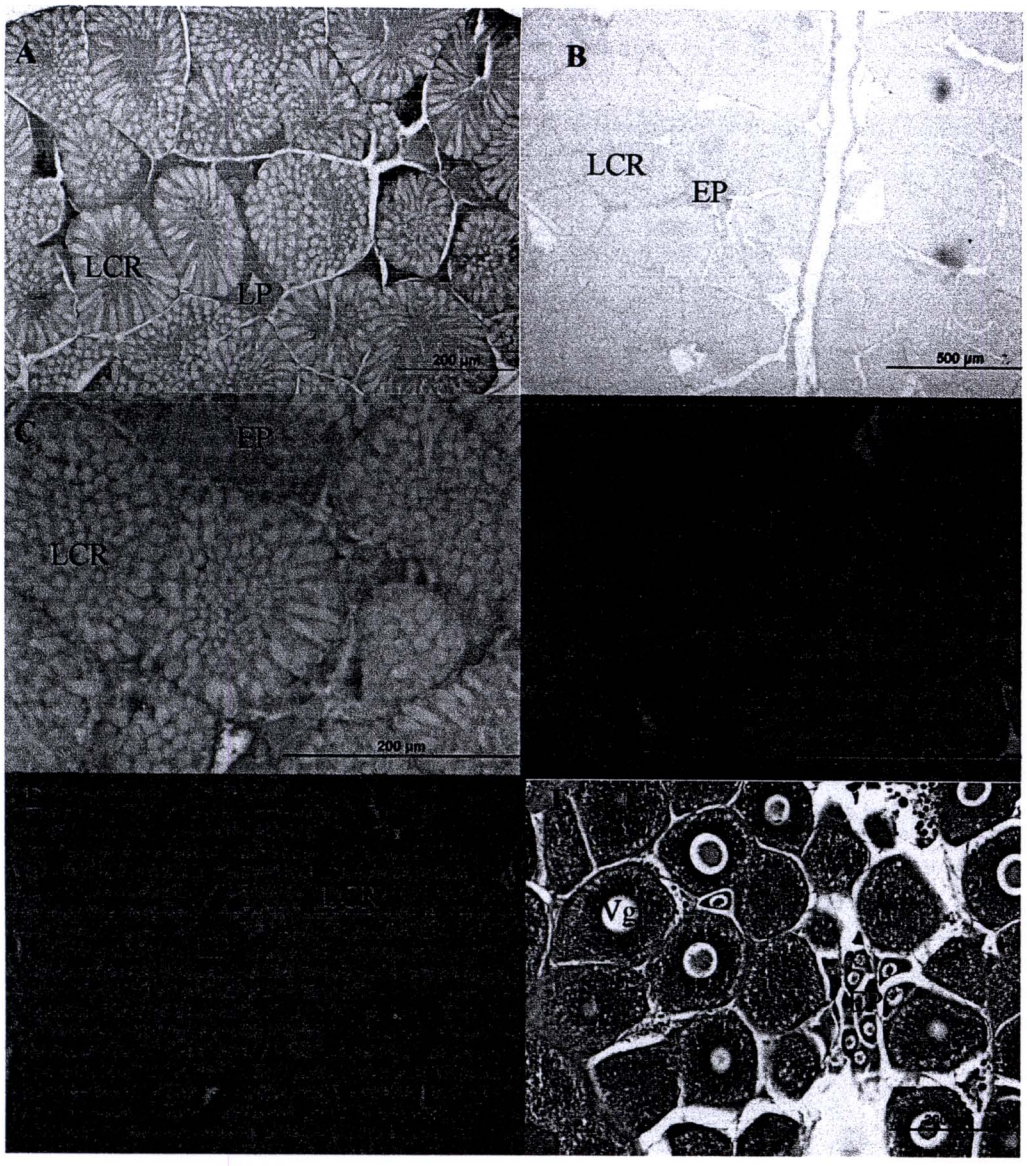


Figure 3.117 Immunohistochemical localization of the PmFAMeT-s protein in ovaries of eyestalk-ablated broodstock of *P. monodon* (C-E). The blocking solution (A) and preimmune serum (B) were used as the negative control. The conventional HE staining (F) was carried out for identification of oocyte stages.

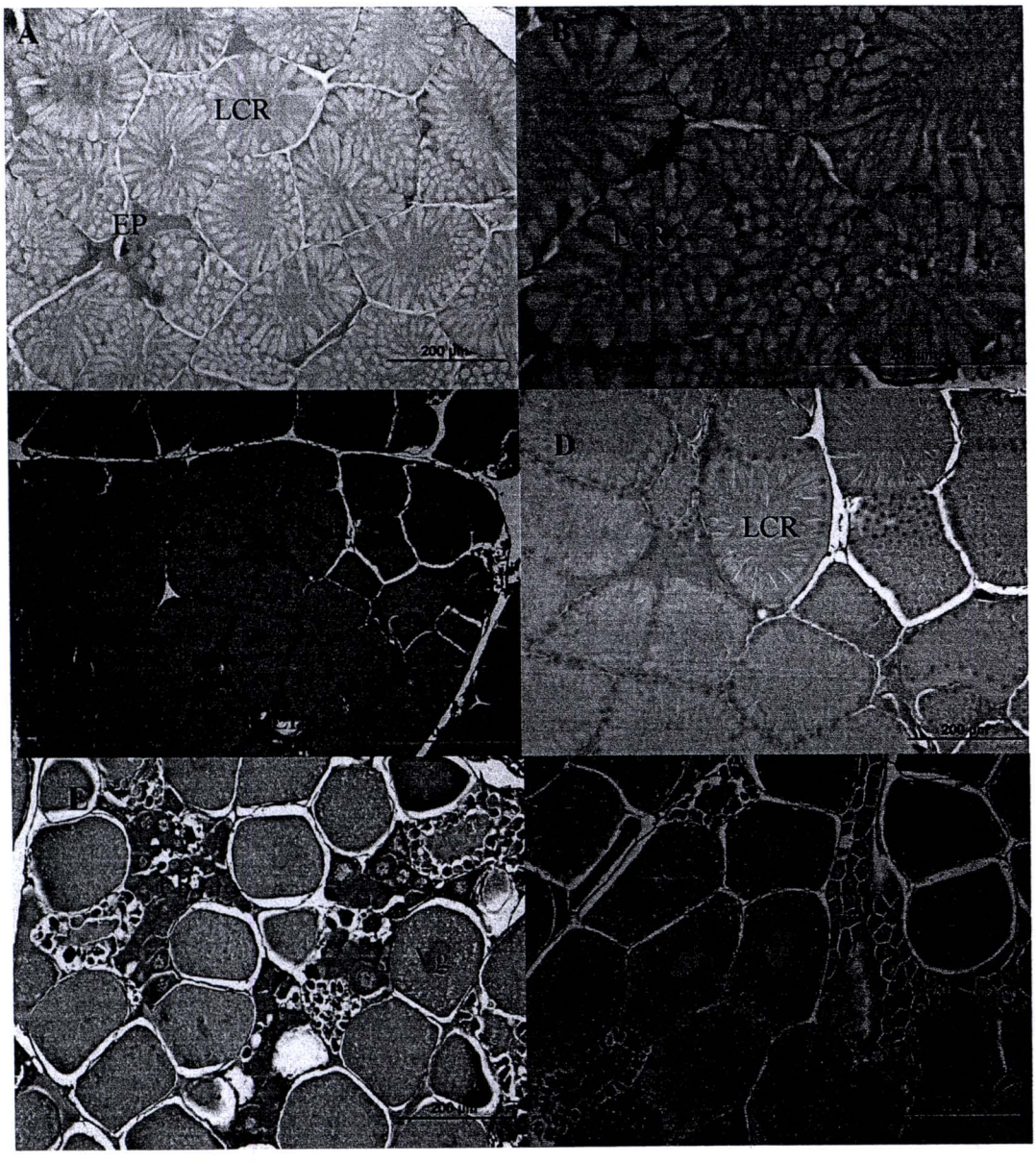


Figure 3.118 Immunohistochemical localization of the PmFAMeT-1 protein in ovaries of intact *P. monodon* broodstock (C-E). The blocking solution (A) and preimmune serum (B) were used as the negative control. The conventional HE staining (F) was carried out for identification of oocyte stages.

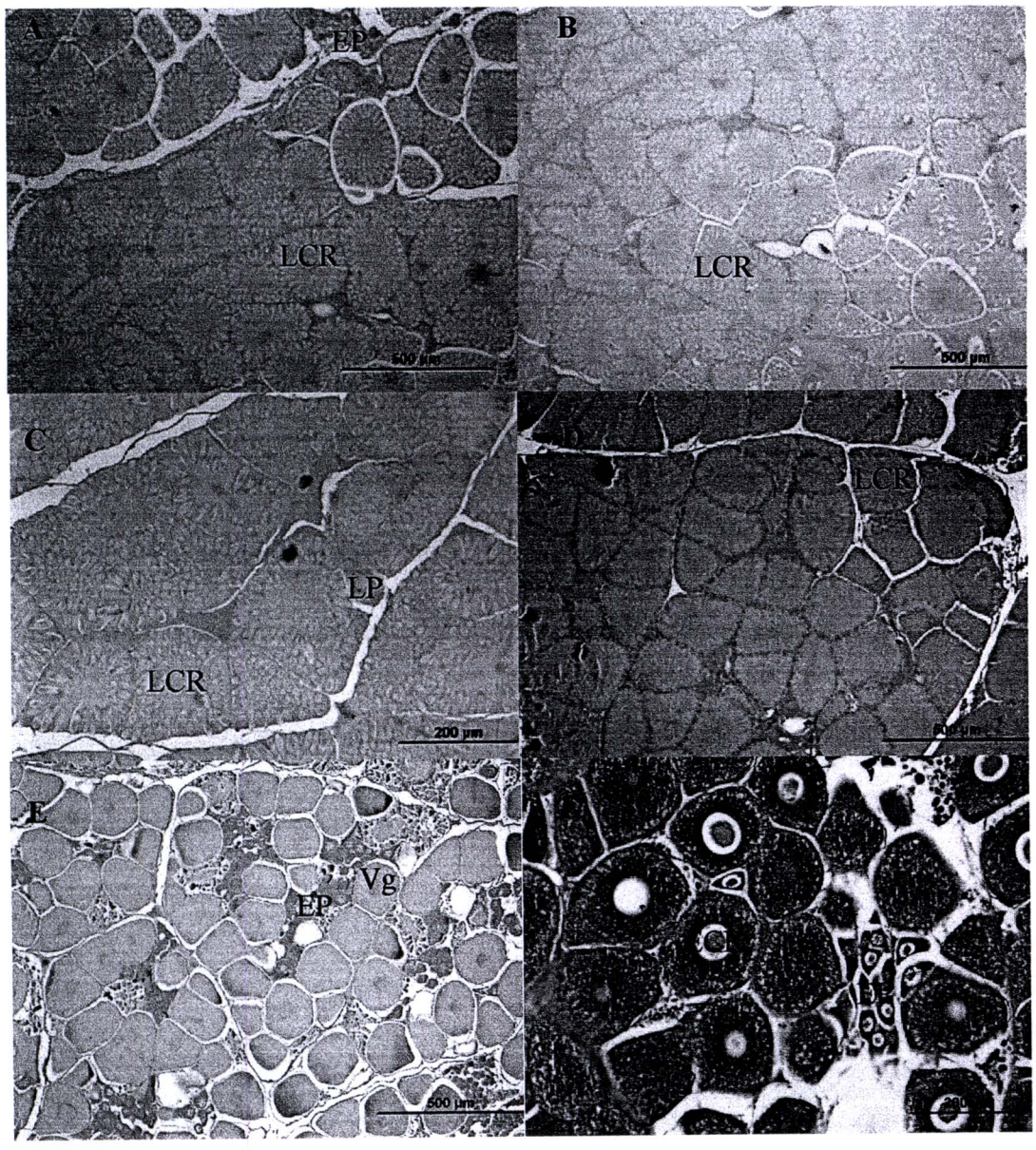


Figure 3.119 Immunohistochemical localization of the PmFAMeT-1 protein in ovaries of eyestalk-ablated broodstock of *P. monodon* (C-E). The blocking solution (A) and preimmune (B) were used as the negative control. The conventional HE staining (F) was carried out for identification of oocyte stages.