A farnesoic acid O-methyltransferase (FAMeT) from *Exopalaemon carinicauda* is responsive to *Vibrio anguillarum* and WSSV challenge

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Abstract Methyl farnesoate (MF), an analogue of the insect juvenile hormone III, is believed to play important roles in the regulation of the growth and reproductive development in crustaceans. Farnesoic acid O-methyltransferase (FAMeT) is the key enzyme in the juvenile hormone biosynthetic pathway, involved in the conversion of farnesoic acid (FA) to MF in the final step of MF synthesis. In this study, a FAMeT cDNA (named EcFAMeT) was cloned from the hemocytes of ridgetail white prawn *Exopalaemon carinicauda* by rapid amplification of cDNA ends (RACE) methods. The full-length cDNA of EcFAMeT was 1,620 bp, including contains a 5′-untranslated region (UTR) of 75 bp, 3′-UTR of 714 bp with a poly (A) tail, an open reading frame (ORF) of 831 bp, encoding a 276-amino-acid polypeptide with the predicted molecular weight of 31.57 kDa and estimated isoelectric point of 4.67. BLAST analysis revealed that amino acids of EcFAMeT shared high identity (75–90 %) with that of other crustaceans. Two conserved signatures domains of Methyltransf-FA superfamily were also identified in EcFAMeT. Real time quantitative RT-PCR analysis indicated that EcFAMeT could be detected in all the tested tissues and strongly expressed in hepatopancreas and ovary of *E. carinicauda*. After *Vibrio anguillarum* and WSSV challenge, EcFAMeT transcripts both in hemocytes and hepatopancreas increased significantly in the first 3 h, respectively. The results indicated that EcFAMeT might be associated with the immune defenses to *V. anguillarum* and WSSV in *E. carinicauda*.

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Introduction

The ridgetail white prawn *Exopalaemon carinicauda* is an economically important shrimp species naturally distributed in the coasts of the Yellow Sea and the Bohai Sea, China, which contributes to one third of the gross output of the polyculture ponds in eastern China (Xu et al. 2010). *E. carinicauda* have good reproductive performance, fast growth, and wide environmental adaptability, which make it a good experimental animal candidate for shrimp (Xu et al. 2010; Li et al. 2012; Wang et al. 2013). Due to its commercial value, "milky shrimp" disease caused by *Hematodinium* (Xu et al. 2010), immune gene discovery by expressed sequence tags (ESTs; Duan et al. 2013b) and identification of immune-related genes such as heat shock protein (HSP 90; Li et al. 2012), selenium dependent glutathione peroxidase (GPx; Duan et al. 2013a), and peritrophin (Wang et al. 2013) have been studied in *E. carinicauda*. However, with the development of intensive culture and the ecologic environmental deterioration, various diseases caused by bacteria and viruses have frequently occurred in cultured *E. carinicauda*, causing economic losses to commercial shrimp aquaculture (Xu et al. 2010). Shrimp lack an adaptive immune system and their defense mechanisms mainly rely on innate immune responses to protect them against invaders. Therefore, a better understanding of the innate immune abilities and immune defense mechanisms of shrimp will be beneficial to the development

of health management and disease control in shrimp aquaculture.

O-methyltransferase (OMT) is an enzyme that is ubiquitously present in diverse organisms and catalyses the methylation of both small and macromolecules for various functional and regulatory purposes (Li et al. 2006). OMT may play an important regulatory role in plant and animal growth, development, reproduction and defence, and is also implicated in human disease (Li et al. 2006; Chiron et al. 2000; Yao et al. 2003; Fan et al. 2005). In invertebrates, most studies of the kinds of OMT focus on farnesoic acid O-methyltransferase (FAMeT). FAMeT is the enzyme responsible for the conversion of farnesoic acid (FA) to methyl farnesoate (MF) in the final step of MF synthesis using the cofactor S-adenosyl-L-methionine (SAM; Burtenshaw et al. 2008; Silva Gunawardene et al. 2003; Hui et al. 2008). MF is a sesquiterpenoid, structurally similar to insect juvenile hormone III (JH III) and is an unepoxidized analogue of JH III, deemed the juvenile hormone of crustaceans (Silva Gunawardene et al. 2001, 2003; Hui et al. 2008; Holford et al. 2004). Previous studies have demonstrated that MF play a vital role in the regulation of growth and reproductive development, including morphogenesis, metamorphosis, reproduction, and molting, and the response to stress in crustaceans (Silva Gunawardene et al. 2001; Holford et al. 2004; Averbeck et al. 2000). The catalytic activity of FAMeT is thought to occur at a rate-limiting step in the JH biosynthetic pathway although its exact role is yet to be defined (Williamson et al. 2001).

The first study of FAMeT was in *Metapenaeus ensis* (Silva Gunawardene et al. 2001). To date, the FAMeT gene has also been isolated from some crustaceans (Silva Gunawardene et al. 2001, 2003; Hui et al. 2008; Holford et al. 2004; Yang et al. 2012; Kuballa et al. 2007; Ruddell et al. 2003). Research on FAMeT has mainly focused on the importance of this enzyme and its regulation of MF production (Hui et al. 2008). However, no studies on FAMeT in *E. carinicauda* and the gene in response to *Vibrio anguillarum* and white spot syndrome virus (WSSV) have been reported. *V. anguillarum* and WSSV caused the most serious disease leading to major losses in the shrimp aquaculture industry around the world (Toranzo et al. 2005; Lightner 2011; Spann and Lester 1997), but there are still no effective preventive and therapeutic measures against *V. anguillarum* and WSSV infection.

The present study isolated and characterized the full-length cDNA from hemocytes of *E. carinicauda*, compared its sequence with other known FAMeTs, investigated the expression pattern of FAMeT in various tissues of *E. carinicauda*, and evaluated this FAMeT expression in hemocytes and hepatopancreas of *E. carinicauda* after *V. anguillarum* and WSSV challenge. These results will be essential to better understand the physiological function of FAMeT in the shrimp immune response to bacterial and viral infection.

Materials and methods

Animal materials

Healthy adult *E. carinicauda*, averaging weight 1.19 ± 0.32 g, were collected from a commercial farm in Qingdao, China. They were cultured in filtered aerated seawater (salinity 20‰, pH 8.2) at 18 ± 0.5 °C for 7 days before processing. There were 30 prawns in each group. The prawns were fed daily with a ration of 10 % of body weight, and two-thirds of the water in each group was renewed once daily.

RNA extraction and cDNA synthesis

Hemocytes were collected with a syringe which contained an equal volume of anticoagulant solution (Söderhäll and Smith 1983), and centrifuged at $800 \times g$, 4 °C for 15 min. Total RNA was extracted from hemocytes using Trizol Reagent (Invitrogen, USA) following the manufacturer's protocol. The RNA samples were analyzed in 1.0 % agarose electrophoresis and quantitated at 260 nm, all OD_{260}/OD_{280} were between 1.8 and 2.0. The 3′ and 5′ ends RACE cDNA templates were synthesized using SMART^{TM} cDNA Kit (Clontech, USA) following the protocol of the manufacturer.

Cloning the full-length cDNA of EcFAMeT

An EST sequence was obtained from *E. carinicauda* hemocytes cDNA library of our laboratory (GenBank accession no. JK996877) and has been reported by Duan et al. (2013b). BLAST analysis showed that they had high identifies with FAMeTs of other shrimps. According to the EST sequence, a gene specific primer F1 was designed for 3′ RACE, and R1 was designed for 5′ RACE (Table 1).

Based on the EST sequence data of FAMeT, its 3′ and 5′ ends were obtained using SMART RACE cDNA Amplification Kit (Clontech, USA). For 3′ RACE, the PCR reaction was performed using the primer F1 and the anchor primer UPM (Table 1). The PCR reaction conditions were 5 cycles of 94 °C for 30 s, 72 °C for 3 min, 5 cycles of 94 °C for 30 s, 70 °C for 30 s, and 72 °C for 3 min, and 25 cycles of 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 3 min. For 5′ RACE, the PCR reaction was performed using the primer R1 and the anchor primer UPM (Table 1). The PCR reaction conditions were the same as those described above.

The PCR fragments were subjected to electrophoresis on 1.0 % agarose gel to determine length differences, and the target band was purified by PCR purification kit (Promega, USA). The purified products were cloned into PMD18-T vector, following the instructions provided by the manufacturer (TaKaRa, Japan). Recombinant bacteria were identified by blue/white screening and confirmed by PCR. Plasmids

Table 1 Primer sequences used in this study

Primer name	Sequence (5′-3′)
EcFAMeT	
F1 (forward)	TGAAATCAGAGTCGGGAA
R1 (reverse)	GTTTTCCCAACCACCAAT
F2 (forward)	CTCAGGTTCCAGGTCAAAGC
R2 (reverse)	TCCTCATCACAGCACAGAGC
UPM	CTAATACGACTCACTATAGGGCAA
	GCAGTGGTATCAACGCAGAGT
	CTAATACGACTCACTATAGGGC
18S rRNA	
18S-HF	TATACGCTAGTGGAGCTGGAA
18S-HR	GGGGAGGTAGTGACGAAAAAT

containing the insert were purified (Promega minipreps) and used as a template for DNA sequencing.

Sequence analysis

The nucleotide and deduced amino acid sequences of EcFAMeT cDNA were analyzed and compared using the BLAST search programs (http://www.blast.ncbi.nlm.nih.gov/Blast.cgi). The signal peptide was predicted by SignalP program (http://www.cbs.dtu.dk/services/SignalP/). The multiple sequence alignment of FAMeT amino acid sequences was performed using the programs of Vector NTI advance 10.3 (Invitrogen). A phylogenetic NJ tree of FAMeTs was constructed by the MEGA 4.0 software (Tamura et al. 2007).

Tissue expression of EcFAMeT

Hemocytes, gill, hepatopancreas, muscle, ovary, eyestalk, stomach, and intestine were dissected from unchallenged *E. carinicauda*. The mRNA expressions of EcFAMeT in different tissues were determined by quantitative real-time RT-PCR. Total RNA was extracted as described above. The RNA samples were analyzed in 1.0 % agarose electrophoresis and quantitated at 260 nm, all OD_{260}/OD_{280} were between 1.8 and 2.0. Total RNA (5 μg) was reverse transcribed using the PrimeScript™ Real time PCR Kit (TaKaRa, Japan) for real-time quantitative RT-PCR analysis. 18S rRNA gene of *E. carinicauda* (GenBank accession number: GQ369794) was used as an internal control.

Experimental design of *V. anguillarum* and WSSV challenge

The experiments were divided into the bacterial (*V. anguillarum*) challenged group, the virus (WSSV) challenged group and the control group. *V. anguillarum* strains was

obtained from Germplasm Resources and Genetic Breeding Laboratory, Yellow Sea Fisheries Research Institute (China), activating on marine agar 2611E. WSSV crude extract was obtained from 10 g WSSV-infected tissue from *Litopenaeus vannamei*, which was provided by the Mariculture Disease Control and Pathogenic Molecular Biology Laboratory, Yellow Sea Fisheries Research Institute, according to the methods of Xie et al. (2005). In the experiment, the challenged groups were injected individually with 20 μL live *V. anguillarum* suspended in sterile 0.9 % normal saline (2×10^8 CFU/mL) or 20 μL live WSSV crude extract, and the control group received individually an injection of 20 μL sterile 0.9 % saline solution. Then both the challenged and control prawns were returned to the PVC tanks of aerated seawater and fed at 25 °C as described above. The seawater waste was dealt with safely to make it harmless for the environment. Hemocytes and hepatopancreas of six prawns from each treatment (the challenged group and the control group) were randomly sampled at 0, 3, 6, 12, 24, 48, and 72 h post-injection respectively, then the samples were snap-frozen in liquid nitrogen. There were three replicates for each time point. Total RNA was extracted and the first strand cDNA was synthesized as described above.

Expression of EcFAMeT after *V. anguillarum* and WSSV challenge

Real time quantitative RT-PCR was performed on an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, USA) to investigate the expression of EcFAMeT. For the mRNA expression of EcFAMeT, the pair of specific primers F2 and R2 (Table 1) were used to amplify a PCR product of 593 bp. Two primers 18S-HF and 18S-HR (Table 1) were used to amplify an 18S rRNA gene of 147 bp as an internal control to verify the successful reverse transcription and to calibrate the cDNA template. The RT-PCR was carried out in a total volume of 20 μL, containing 10 μL SYBR® Premix Ex Taq™ II (2×) (TaKaRa), 2 μL of the 1:5 diluted cDNA, 0.8 μL each of F2 and R2 primer (or 18S-HF and 18S-HR to amplify the 18S rRNA), 0.4 μL ROX Reference Dye II (50×)*3, and 6 μL DEPC-treated water. The PCR program was 95 °C for 30 s, then 40 cycles of 95 °C for 5 s and 60 °C for 34 s, followed by 1 cycle of 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. DEPC-treated water for the replacement of template was used as negative control.

RT-PCR data from three replicate samples were analyzed with the ABI 7300 system SDS Software (Applied Biosystems, USA), for estimating transcript copy numbers for each sample. The comparative C_T method was to analyze the relative expression levels of EcFAMeT. The C_T for the target amplified products of EcFAMeT and internal control 18S rRNA was determined for each sample. The difference in

the C_T between the target and the internal control, called $\triangle C_T$, was calculated to normalize the differences in the amount of template and the efficiency of the RT-PCR. In the same challenge time, the $\triangle C_T$ of the control group was used as the calibrator, and the difference between the $\triangle C_T$ of the challenged group and the control group was called $\triangle\triangle C_T$. The expression level of EcFAMeT was calculated by the $2^{-\triangle\triangle CT}$ comparative C_T method (Livak and Schmittgen 2001). Statistical analysis was performed using SPSS software (Ver 11.0). Statistical significance was determined using one-way ANOVA (González-Rodríguez et al. 2012) and post hoc Duncan multiple range tests. Significance was set at $P < 0.05$.

Results

Analysis of the EcFAMeT sequence and the predicted protein

A full-length 1,620-bp cDNA of FAMeT (designated EcFAMeT) was obtained from the hemocytes of *E. carinicauda* by RACE method, and the nucleotide and deduced amino acid sequences were shown in Fig. 1. It contained an open reading frame (ORF) of 831 bp, 75 bp of 5′-untranslated region (UTR), 714 bp of 3′-UTR including a stop codon (TAA) polyadenylation signal (AATAAA) and a poly A tail. The EcFAMeT cDNA sequence has been submitted to the GenBank (GenBank accession number: KF534709).

The ORF of EcFAMeT encoded 276 amino acids without signal peptide analyzed by SignalP software, indicating that EcFAMeT did not belong to the family of secretory proteins. The calculated molecular mass was 31.57 kDa, and the estimated isoelectric point was 4.67. InterProScan analysis showed that the amino acid sequences [38]H-W[137] and [174]H-W[272] were the putative conserved domains of Methyltransf-FA superfamily.

Multiple sequences and phylogenetic analysis

Sequence analysis with the BLASTP program revealed that the deduced amino acid sequence of EcFAMeT exhibited a high identification with FAMeT of *Macrobrachium nipponense* (90 %), *Pandalopsis japonica* (83 %), *Marsupenaeus japonicus* (81 %), *Fenneropenaeus chinensis* (81 %), *Penaeus monodon* (81 %), *L. vannamei* (79 %), *M. ensis* (79 %), *Homarus americanus* (79 %), *Portunus trituberculatus* (78 %), *Cancer pagurus* (78 %), *Scylla paramamosain* (76 %), and *Eriocheir sinensis* (75 %) counterparts, respectively. Multiple sequence alignment of EcFAMeT with other animal FAMeTs revealed that they were highly conserved, especially in the regions of FAMeT family signatures. However, some shrimp FAMeTs consist of three or five additional amino acids in the amino acid sequences (Fig. 2).

Based on the amino acid sequences of FAMeTs, a Neighbor-Joining phylogenetic tree was constructed using MEGA 4.0 for phylogenetic analysis (Fig. 3). Shrimp and crab FAMeTs were separated and formed two distinct branches in the tree. In the branch of shrimp, all the shrimps FAMeTs were clustered together and formed three branches. All the FAMeTs of penaeid shrimp were clustered together and formed a subgroup, and FAMeTs of *H. americanus*, *Procambarus clarkia*, and *Cherax quadricarinatus* were classified into another subgroup. EcFAMeT of *E. carinicauda* showed closer evolutionary

Fig. 1 Nucleotide and deduced amino acid sequences of EcFAMeT cDNA of *E. carinicauda*. The *letters in the box* indicated the start codon (ATG), the polyadenylation signal sequence (AATAAA), and the stop codon (TAA). The two FAMeT superfamily signature motifs were underlined

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E. carinicauda FAMeT    MGDDNWPSHGTDENKEYRFRPIKGETLRFQVKAAHDAHIALTSADEETEPMLEVFIGGWEGAASAIRFNKGDDLVKVDTPDILSEEEYREFWIAF  95
M. nipponense FAMeT     MADNWPAYGTDENKEYRFRDIKGKTLRFQVKAAHDAHVALTSGAEETDPIVEVFIGGWEGAASAVRFKKADDLVKVDTPDILSEEEYREFWIAF  94
F. chinensis FAMeT      MGDSWASYGTDENKQYRFRDIKGKTLRFQVKAAHDAHIALTSGEEETDPMLEVFIGGWEGAASAIRFKKADDLVKVDTPDILSEEEYREFWIAF  94
L. vannamei FAMeT-S     MADNWPAYGTDENKQYRFRDIKGKTLRFEVKAAHDAHIALTSGEEETDPMLEVFIGGWEGAASAIRFKKADDLAKVDTPDILSEEEYREFWIAF  94
L. vannamei FAMeT-L     MADNWPAYGTDENKEYRFRDIKGKTLRFQVKAAHDAHIALTSGEEETDPMLEVFIGGWEGAASAIRFKKADDLAKVDTPDILSEEEYREFWIAF  94
P. monodon FAMeT-S                       RFRDIKGKTLRFQVKAAHDAHLALTSGEEETDPMLEVFIGGWEGAASAIRFKKADDLTKVDTPDILSEEEYREFWVAF  78
P. monodone FAMeT-L                      RFRDIKGKTLRFQVKAAHDAHLALTSGEEETDPMLEVFIGGWEGAASAIRFKKADDLTKVDTPDILSEEEYREFWVAF  78
F. merguiensis FAMeT-S                   RFRDIKGKTLRFQVKAAHDAHIALTSGEEETDPMLEVFIGGWEGAASAIRFKKADDLAKVDTPDILSEEEYREFWIAF  78
F. merguiensis FAMeT-L                   RFRDIKGKTLRFQVKAAHDAHIALTSGEEETDPMLEVFIGGWEGAASAIRFKKADDLAKVDTPDILSEEEYREFWIAF  78
S. paramamosain FAMeT-S MADEIPALGTDENKEYRFRQLHGKTLRFQVKAAHDCHVAFTTGAEETDPMVEVFIGGWEGAASAIRFKKADDLVKVDTPDIVTEAEYREFWIAV  94
S. paramamosain FAMeT-I MADEIPALGTDENKEYRFRQLHGKTLRFQVKAAHDCHVAFTTGAEETDPMVEVFIGGWEGAASAIRFKKADDLVKVDTPDIVTEAEYREFWIAV  94
S. paramamosain FAMeT-L MADEIPALGTDENKEYRFRQLHGKTLRFQVKAAHDCHVAFTTGAEETDPMVEVFIGGWEGAASAIRFKKADDLVKVDTPDIVTEAEYREFWIAV  94
S. serrata FAMeT-S                       RFRQLHGKTLRFQVKAAHDCHVAFTTGAEETDPMVEVFIGGWEGAASAIRFKKADDLVKVDTPDIVTEAEYREFWIAV  78
S. serrata FAMeT-I                       RFRQLHGKTLRFQVKAAHDCHVAFTTGAEETDPMVEVFIGGWEGAASAIRFKKADDLVKVDTPDIVTEAEYREFWIAV  78
S. serrata FAMeT-L                       RFRQLHGKTLRFQVKAAHDCHVAFTTGAEETDPMVEVFIGGWEGAASAIRFKKADDLVKVDTPDIVTEAEYREFWIAV  78
P. pelagicus FAMeT-S                     RFRQLHGKTLRFQVKTAHDCHVAFTSAAEETDPMVEVFIGAWEGAASAIRFKKADDLVKVDTPDIVTEAEYREFWIVV  78
P. pelagicus FAMeT-I                     RFRQLHGKTLRFQVKTAHDCHVAFTSAAEETDPMVEVFIGAWEGAASAIRFKKADDLVKVDTPDIVTEAEYREFWIAV  78
P. pelagicus FAMeT-L                     RFRQLHGKTLRFQVKTAHDCHVAFTSAAEETDPMVEVFIGAWEGAASAIRFKKADDLVKVDTPDIVTEAEYREFWIAV  78

E. carinicauda FAMeT    DHGEIRVGKGGEWEPLMMCPIPEPFGITHFGYSTGWGAVGWWQFHNERKLHSDDSLTYQFEPAYGDTFRFSVACSNDAHLALTSGPEETTPMYEI  190
M. nipponense FAMeT     DHDEIRVGKGGEWEPLMMCPIPEPFGITHFGYSTGWGAVGWWQFHNERKFQSDDSLTYNFEPSYGDTFHFSVACSNDAHLALTSGPEETTPMYEI  189
F. chinensis FAMeT      DHDVVRVGKGGEWEPFMSATIPEPFDITHYGYSTGWGAVGWWQFHSEIQFQTEDCLTYNFIPVYGDTFSFSVACSNDAHLALTSGPEETSPMYEV  189
L. vannamei FAMeT-S     DHDVVRVGKGAEWEPFMSATIPEPFDITHYGYSTGWGATGWWQFHSEIHFQTEDCLTYNFIPVYGDTFSFSVACSNDAHLALTSGPEETSPMYEV  189
L. vannamei FAMeT-L     DHDVVRVGKGAEWEPFMSATIPEPFDITHYGYSTGWGATGWWQFHSEIHFQTEDCLTYNFIPVYGDTFSFSVACSNDAHLALTSGPEETSPMYEV  189
P. monodon FAMeT-S      DHDVIRVGKGGEWEPFMSATIPEPFDITHYGYSTGWGAVGWWQFHSEVHFQTEDCLTYNFIPVYGDTFTFSVACSNDAHLALTSGPEETTPMYEV  173
P. monodone FAMeT-L     DHDVIRVGKGGEWEPFMSATIPEPFDITHYGYSTGWGAVGWWQFHSEVHFQTEDCLTYNFIPVYGDTFTFSVACSNDAHLALTSGPEETTPMYEV  173
F. merguiensis FAMeT-S  DHDVVRVGKGGEWEPFMSATVPEPFDITHYGYSTGWGASGWWQFHSEMHFQTEDCLTYNFIPVYGDTFTFSVACSNDAHLALTSGPEETTPMYEV  173
F. merguiensis FAMeT-L  DHDVVRVGKGGEWEPFMSATIPEPFDITHYGYSTGWGASGWWQFHSEMHFQTEDCLTYNFIPVYGDTFTFSVACSNDAHLALTSGPEETTPMYEV  173
S. paramamosain FAMeT-S DHNEVRVGKGGEWEPLMQAPIPEPFEITHYGYSTGWGATGWWKFLNDRVLNTEDCLTYNFBPVYGDSISFSVACSNDAHLALTSGPEETTPMYEI  189
S. paramamosain FAMeT-I DHNEVRVGKGGEWEPLMQAPIPEPFEITHYGYSTGWGATGWWKFLNDRVLNTEDCLTYNFBPVYGDSISFSVACSNDAHLALTSGPEETTPMYEI  189
S. paramamosain FAMeT-L DHNEVRVGKGGEWEPLMQAPIPEPFEITHYGYSTGWGATGWWKFLNDRVLNTEDCLTYNFBPVYGDSISFSVACSNDAHLALTSGPEETTPMYEI  189
S. serrata FAMeT-S      DHNEVRVGKGGEWEPLMQAPIPEPFEITHYGYSTGWGATGWWKFLNDRVLNTEDCLTYNFBPVYGDSISFSVACSNDAHLALTSGPEETTPMYEI  173
S. serrata FAMeT-I      DHNEVRVGKGGEWEPLMQAPIPEPFEITHYGYSTGWGATGWWKFLNDRVLNTEDCLTYNFBPVYGDSISFSVACSNDAHLALTSGPEETTPMYEI  173
S. serrata FAMeT-L      DHNEVRVGKGGEWEPLMQAPIPEPFEITHYGYSTGWGATGWWKFLNDRVLNTEDCLTYNFBPVYGDSISFSVACSNDAHLALTSGPEETTPMYEI  173
P. pelagicus FAMeT-S    DHNEVRVGKAGEWEPLMQAPIPEPFEITHYGYSTGWGATGWWKFLNDRVLNTEDCLTYNFBPVYGDSIAFSVACSNDAHLALTSGAEETSPMYEI  173
P. pelagicus FAMeT-I    DHNEVRVGKAGEWEPLMQAPIPEPFEITHYGYSTGWGATGWWKFLNDRVLNTEDCLTYNFBPVYGDSIAFSVACSNDAHLALTSGAEETSPMYEI  173
P. pelagicus FAMeT-L    DHNEVRVGKAGEWEPLMQAPIPEPFEITHYGYSTGWGATGWWKFLNDRVLNTEDCLTYNFBPVYGDSIAFSVACSNDAHLALTSGAEETSPMYEI  173

E. carinicauda FAMeT    FIGGWENQKSAIRLSK.....GDDMIKVDAPDALCCDEERKFYVTFRNGHIRVGYCQDSDPFMEWTDPEPWKVTHVGFCTGWGAQGKWQFEF    276
M. nipponense FAMeT     FIGGWENQISAIRLSK.....GEDMIKVDTPDALCCDEERKFYVTFRNGHIRVGYCQDSDPFMEWTDPEPWKVTHVGFCTGWGANGKWQFEI    275
F. chinensis FAMeT      FIGGWENQHSAIRLSKDGRESGEDMIKVDTPDAVCCEEDRKFYVTFKDGHIRVGYCQDSDPFMEWTDPEPWKITHIGYCTGWGATGKWKFEF    280
L. vannamei FAMeT-S     FIGGWENQHSAIRLSK.....GEDMIKVDTPDVVCCEEERKFYISFKDGHIRVGYCQDSDPFMEWTDPEPWKITHIGYCTGWGASGKWKFEF    275
L. vannamei FAMeT-L     FIGGWENQHSAIRLSKEGRGSGEDMIKVDTPDVVCCEEERKFYISFKDGHIRVGYCQDSDPFMEWTDPEPWKITHIGYCTGWGASGKWKFEF    280
P. monodon FAMeT-S      FIGGWENQHSAIRLSK.....GEDMIKVDTPDVVCCEEERKFYVSFKDGHIRVGYCQDSDPF                                 229
P. monodone FAMeT-L     FIGGWENQHSAIRLSKEGRGSGEDMIKVDTPDVVCCEEERKFYVSFKDGHIRVGYCQDSDPF                                 234
F. merguiensis FAMeT-S  FIGGWENQHSAIRLSK.....GEDMIKVDTPDVVCCEEDRKFYVTFKDGHIRVGYCQDSDPF                                 229
F. merguiensis FAMeT-L  FIGGWENQHSAIRLSKEGRGSGEDMIKVDTPDVVCCEEDRKFYVTFKDGHIRVGYCQDSDPF                                 234
S. paramamosain FAMeT-S FIGGWENQHSAIRLNK.....GDDMAKVETPDVVCTEEERKFLVSFRNGQIKVGYKDTDPFLEWTDPEPWKITHVGYCTGWGATGKWRLDI    275
S. paramamosain FAMeT-I FIGGWENQHSAIRLNKDGK..GDDMAKVETPDVVCTEEERKFLVSFRNGQIKVGYKDTDPFLEWTDPEPWKITHVGYCTGWGATGKWRLDI    278
S. paramamosain FAMeT-L FIGGWENQHSAIRLNKDGKGTGDDMAKVETPDVVCTEEERKFLVSFRNGQIKVGYKDTDPFLEWTDPEPWKITHVGYCTGWGATGKWRLDI    280
S. serrata FAMeT-S      FIGGWENQHSAIRLNK.....GDDMAKVETPDVVCTEEERKFLVSFRNGQIKVGYKDTDPF                                 229
S. serrata FAMeT-I      FIGGWENQHSAIRLNKDGK..GDDMAKVETPDVVCTEEERKFLVSFRNGQIKVGYKDTDPF                                 232
S. serrata FAMeT-L      FIGGWENQHSAIRLNKDGKGTGDDMAKVETPDVVCTEEERKFLVSFRNGQIKVGYKDTDPF                                 234
P. pelagicus FAMeT-S    FIGGWENQHSAIRLNK.....GDDMAKVETPDVLCCEEERKFYVSFRNGQIKVGYKDTDPF                                 229
P. pelagicus FAMeT-I    FIGGWENQHSAIRLNKEGK..GDDMAKVETPDVLCCEEERKFYVSFRNGQIKVGYKDTDPF                                 232
P. pelagicus FAMeT-L    FIGGWENQHSAIRLNKEGKGTGDDMAKVETPDVLCCEEERKFYVSFRNGQIKVGYKDTDPF                                 234
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Fig. 2 Multiple alignment of EcFAMeT with other known FAMeTs: *M. nipponense* FAMeT (AFA26604); *F. chinensis* FAMeT (AFA28125); *L. vannamei*: FAMeT-S (AAZ22180), FAMeT-L (AAZ22181); *P. monodon* FAMeT-S (ABA86955), FAMeT-L (ABA86956); *F. merguiensis* FAMeT-S (ABA86957), FAMeT-L (ABA86958); *C. quadricarinatus* FAMeT-S (ABA86959), FAMeT-L (ABA86960); *S. paramamosain* FAMeT-S (AEE26197), FAMeT-I (ADK32330), FAMeT-L (AEE26196); *Scylla serrata*: FAMeT-S (ABA86954), FAMeT-I (ABA86953), FAMeT-L (ABA86952); *P. pelagicus* FAMeT-S (AAZ40196), FAMeT-I (AAZ40197), FAMeT-L (ABB22042). The inserted amino acids in various FAMeT informs were marked by *frame*

relationships with FAMeT of *M. nipponense*. The relationships displayed in the phylogenetic tree were in good agreement with the concept of traditional taxonomy.

Tissue expression of EcFAMeT gene

To investigate the expression profiles of EcFAMeT, quantitative real-time RT-PCR was employed to analyze their relative expression levels in different tissues, including hemocytes, gill, hepatopancreas, muscle, ovary, intestine, stomach, and eyestalk.

The mRNA transcripts of EcFAMeT could be detected in all the tested tissues, but showed different expression levels. EcFAMeT was highly expressed in hepatopancreas, followed by ovary, and the lowest expression was in intestine (Fig. 4).

Expression profiles of EcFAMeT in hemocytes and hepatopancreas after *V. anguillarum* challenge

Expression profiles of EcFAMeT mRNA in hemocytes and hepatopancreas of *E. carinicauda* after *V. anguillarum*

Fig. 3 Phylogenetic tree of different species FAMeTs on the basis of the amino acid sequence using neighbor-joining distance analysis. The protein sequences used for phylogenetic analysis were as follows: FAMeT: *M. nipponense* (AFA26604), *F. chinensis* (AFA28125), *P. japonica* (ADR64207), *M. japonicas* (BAE78496), *H. americanus* (AAA67080), *M. ensis* (AAK28535), *C. pagurus* (AAR00732), *P. trituberculatus* (AGH25488), *E. sinensis* (ACX30003), *P. clarki* (AEB54633); *L. vannamei* FAMeT-S (AAZ22180), FAMeT-L (AAZ22181); *P. monodon* FAMeT-S (ABA86955), FAMeT-L (ABA86956); *F. merguiensis*: FAMeT-S (ABA86957), FAMeT-L (ABA86958); *C. quadricarinatus* FAMeT-S (ABA86959), FAMeT-L (ABA86960); *S. paramamosain* FAMeT-S (AEE26197), FAMeT-I (ADK32330), FAMeT-L (AEE26196); *S. serrata* FAMeT-S (ABA86954), FAMeT-I (ABA86953), FAMeT-L (ABA86952); *P. pelagicus* FAMeT-S (AAZ40196), FAMeT-I (AAZ40197), FAMeT-L (ABB22042). The *numbers at the forks* indicated the bootstrap
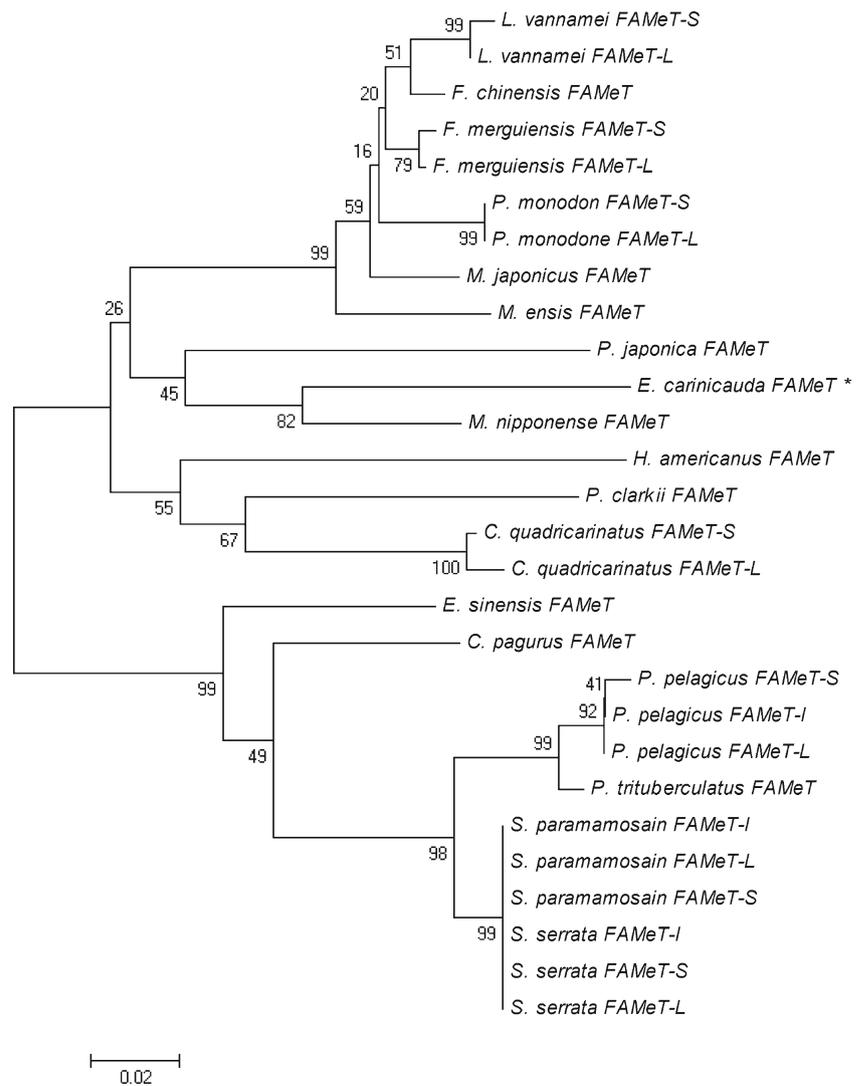
injection were shown in Fig. 5. Compared to the control, the expression of EcFAMeT in hemocytes increased significantly and reached a peak value at 6 h (3.87-fold of the control group, $P < 0.05$), then it decreased gradually and dropped to the lowest level at 48 h (0.13-fold of the control group, $P < 0.05$), then it up-regulated to the control group level at 72 h (Fig. 5a). EcFAMeT transcripts in hepatopancreas obviously increased to the first peak level at 3 h (2.94-fold of the control group, $P < 0.05$), after decreasing significantly from 3 h to 6 h (0.32-fold of the control group, $P < 0.05$), it up-regulated again to the second peak level at 12 h (4.64-fold of the control group, $P < 0.05$), then it decreased gradually and recovered to the control level at 72 h (Fig. 5b).

Expression profiles of EcFAMeT in hemocytes and hepatopancreas after WSSV challenge

The temporal expression of EcFAMeT mRNA in hemocytes and hepatopancreas of *E. carinicauda* after WSSV challenge was shown in Fig. 6. Compared with the control group, EcFAMeT

transcripts in hemocytes were significantly induced by WSSV challenge, and reached to the peak level at 6 h (3.32-fold of the control group, $P < 0.05$), then dropped to the lowest level at 12 h (0.27-fold of the control group, $P < 0.05$). Afterwards, the transcript level of EcFAMeT increased gradually and reached the control level at 72 h (Fig. 6a). In hepatopancreas, the EcFAMeT mRNA expression level increased significantly and reached to the peak level at 3 h (2.89-fold of the control group, $P < 0.05$), after a significant decrease at 6 h (0.14-fold of the control group, $P < 0.05$), it increased again to the second peak level at 12 h post-challenge (1.80-fold of the control group, $P < 0.05$). Afterwards, the EcFAMeT mRNA expression level decreased significantly at 24 h, and increased gradually to the control level at 72 h (Fig. 6b).

Discussion

Invertebrates lack an adaptive immune system and their defense mechanisms mainly rely on innate immune responses

Fig. 4 Tissue specific expression of EcFAMeT mRNA related to hepatopancreas expression by the real-time PCR. The reference gene is 18S rRNA. *Vertical bars* represent the mean±SD ($N=3$). Significant differences ($P<0.05$) in EcFAMeT expression between different tissues were indicated with *different letters*
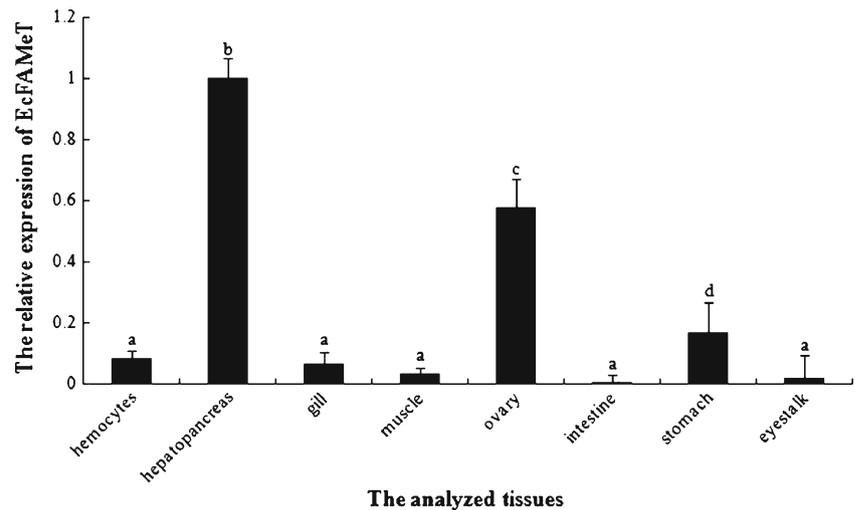
for protecting them against invaders (Zhang et al. 2008; Iwanaga and Lee 2005). The identification and characterization of genes involved in immune responses are essential for the elucidation of immune defense mechanisms and for disease control (Zhao et al. 2007). In the present study, a new full-length 1,620-bp of FAMeT cDNA (EcFAMeT) was isolated and sequenced from *E. carinicauda*. The EcFAMeT sequence showed a high identification (75–90 %) with FAMeTs of other crustaceans. Multiple sequence alignment analysis revealed that the FAMeT sequences were highly conserved. Phylogenetic analysis revealed that EcFAMeT of *E. carinicauda* was clustered with FAMeT of other crustaceans. The sequence alignment and phylogenetic analysis further suggested that EcFAMeT was a member of the FAMeT family and should have the same function as FAMeT from other species due to the conserved sequences and motifs.

In penaeid shrimp *L. vannamei*, *P. monodon*, and *Fenneropenaeus merguiensis*, two different isoforms of FAMeT (long and short isoform) were found which were named FAMeT-L and FAMeT-S respectively, and they were almost identical except for the additional five amino acids in the FAMeT-L isoform (Fig. 2). In the crabs *S. serrata*, *S. paramamosain*, and *Portunus pelagicus*, three different isoforms (long, intermediate and short isoform) were found in the amino acid sequence of FAMeT, and there were three or five different amino acids between the various types of isoform. EcFAMeT lacked the five inserted amino acids, indicating that EcFAMeT was more closely related to the FAMeT short isoform (FAMeT-S) of other crustaceans (Fig. 2). But in our study, as there were no other isoforms of FAMeT found in *E. carinicauda*, it might be related to the total RNA extracted from different tissues in the process of gene cloning. We will extract total RNA from different tissues in the future, to prove whether there are other isoforms of FAMeT in *E. carinicauda*.

FAMeT is one of the most abundant cellular proteins and multiple tissues of crustaceans contain RNA transcripts (Ruddell et al. 2003; Silva Gunawardene et al. 2001, 2002). In our study, tissue-specific differences in levels of constitutive FAMeT were observed. The EcFAMeT mRNA level was high in hepatopancreas and ovary as compared with other six tested tissues (hemocytes, gill, muscle, stomach, intestine, and eyestalk), which implied that EcFAMeT might have a certain relationship with metabolism, genital organ development, and maturation. Previous studies have demonstrated that FAMeT might be involved in many important biological functions in crustaceans, including metamorphosis, reproduction, and molting (Holford et al. 2004; Silva Gunawardene et al. 2001; Averbeck et al. 2000). In the insects *Manduca sexta*, *Helicoverpa zea*, and *Vanessa cardui*, only the reproductive tract of males contained the FAMeT, but the female had none, indicating that FAMeT was a sex-specific expression gene (Bhaskaran et al. 1988). In the eyestalk of *M. ensis*, FAMeT was expressed in the adult shrimp during all stages of reproductive development; whereas in the juveniles, it was only expressed in male shrimp and not in the females, which showed that FAMeT might be important for the regulation of eyestalk neuropeptides and involved in the regulation of the reproduction and molting processes in crustaceans through the synthesis of MF (Silva Gunawardene et al. 2001). After double stranded RNA (dsRNA) injection, the expression of FAMeT in the eyestalk of *L. vannamei* was knocked down and the shrimp did not advance to the final stage of the molt cycle. Moreover, the molt-related genes (cathepsin-L and hemocyanin) were disturbed and a 100 % mortality of the shrimp was observed, all of which revealed that FAMeT might be involved in the control of molting in shrimp (Hui et al. 2008). A similar conclusion was also reached in *S. paramamosain* (Yang et al. 2012). Many studies focus principally on the function of FAMeT in the reproduction and

Fig. 5 The mRNA expression levels of EcFAMeT in hemocytes (**a**) and hepatopancreas (**b**) of *E. carinicauda* at different time intervals after *V. anguillarum* challenge treatment. The reference gene is 18S rRNA. *Vertical bars* represented the mean±SD (*N*=3). Significant differences (*P*< 0.05) in EcFAMeT expression between the challenged and the control group were indicated with *asterisks*
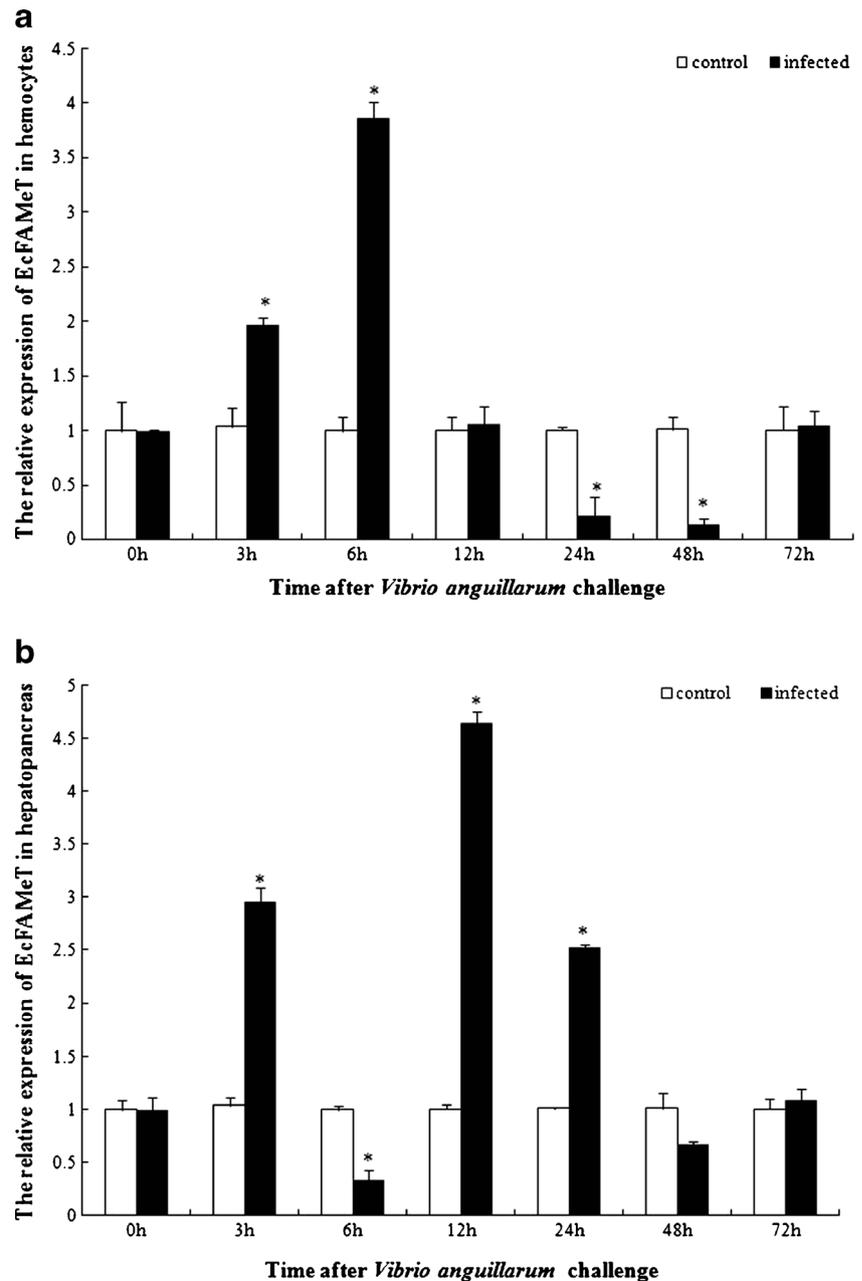
molting processes, but whether FAMeT is related to the immune response of shrimp has not yet been studied.

Virulent pathogens have a major impact on aquaculture. *V. anguillarum* and WSSV are both extremely virulent pathogens that seriously affect shrimp aquaculture (Toranzo et al. 2005; Lightner 2011; Spann and Lester 1997). When pathogens enter the body of the shrimp, they will encounter the innate immune system (Soonthornchai et al. 2010). Previous studies have demonstrated that hemocytes and hepatopancreas are the main cells and tissue involved in the immune response, and the major site for the synthesis of immune defense

molecules involved in eliminating pathogens or other particulate matter in crustaceans. Information on the expression profile of the EcFAMeT gene in these tissues after *V. anguillarum* and WSSV challenge would be helpful to better understand its immunological function.

V. anguillarum is a major virulent pathogen for cultured shrimp worldwide (Toranzo et al. 2005). As far as we know, the effect of *V. anguillarum* on the expression of FAMeT has not been evaluated in crustaceans. In the present study, live *V. anguillarum* were chosen to challenge the shrimp, so that the shrimps' health would be affected severely by the production

Fig. 6 The mRNA expression levels of EcFAMeT in hemocytes (**a**) and hepatopancreas (**b**) of *E. carinicauda* at different time intervals after WSSV challenge treatment. The reference gene is 18S rRNA. *Vertical bars* represented the mean±SD ($N=3$). Significant differences ($P< 0.05$) in EcFAMeT expression between the challenged and the control group were indicated with *asterisks*
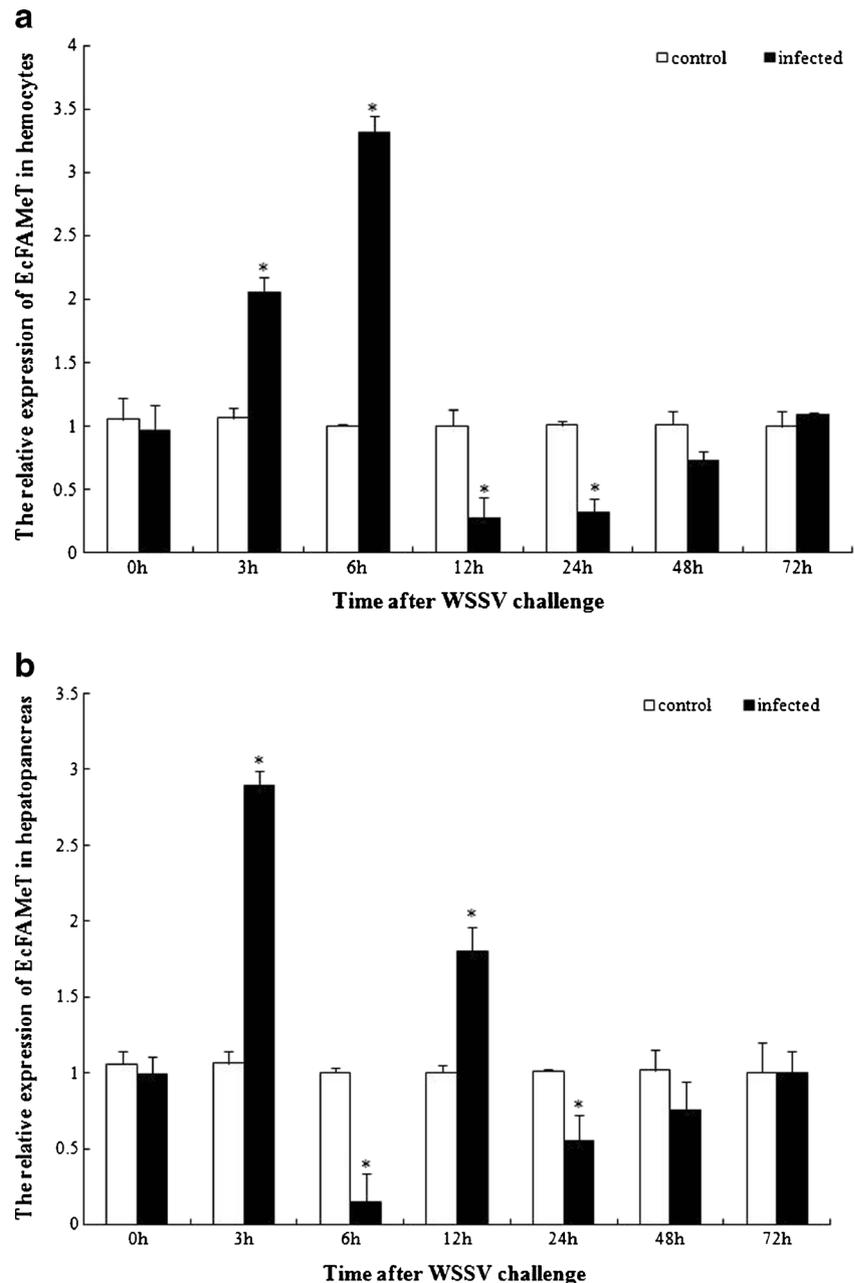
of *V. anguillarum*. When injected with mixtures of *V. anguillarum* and *Staphylococcus aureus*, the expression level of OMT was up-regulated in the hepatopancreas and hemocytes of *F. chinensis* (Li et al. 2006). In our study, the expression of EcFAMeT increased at 3 h significantly in hemocytes and hepatopancreas respectively, which indicated that EcFAMeT could be induced by *V. anguillarum*. As time progressed, the expression of EcFAMeT dropped to a low level at 48 h in both hemocytes and hepatopancreas, perhaps because the infection progress brought more bacteria, and destroyed the normal function of the shrimps' cells and finally caused the expression of EcFAMeT in the challenged group to

decrease gradually (Chen et al. 2011). The results showed that EcFAMeT might be involved in an immune response to *V. anguillarum* stimulation. The expression profiles of the EcFAMeT gene with *V. anguillarum* challenge indicated that it was inducible and might be involved in the immune response against bacterial infection in *E. carinicauda*.

White spot syndrome (WSS) is a lethal disease that affects cultured shrimp species (Lightner 2011). WSSV is a large DNA virus with a broad host range among shrimps and the causative agent of White Spot disease of shrimp, causing mass mortalities and economic losses in shrimp aquaculture (Lightner 2011; Escobedo-Bonilla et al. 2008; Leu et al. 2009).

However, there are still no effective preventive and therapeutic measures against WSSV infection. In our study, the transcriptional profile of EcFAMeT was analyzed in hemocytes and hepatopancreas of *E. carinicauda* when shrimps were injected with WSSV. The transcript levels of EcFAMeT following the WSSV injection ascended at 6 h in hemocytes and at 3 h in hepatopancreas, respectively, demonstrating that it might play important roles in the immune response against WSSV infection in shrimp. The data indicated that the expression profiles of EcFAMeT were very similar when shrimp were challenged by *V. anguillarum* and WSSV. But whether in the *V. anguillarum* challenged group or in the WSSV challenged group, the expression profiles of EcFAMeT in hemocytes were different with hepatopancreas, which might be caused by the different function of hemocytes and hepatopancreas in the immune defense system. It has been proved that hemocytes are key cells for invertebrates' innate defense reactions (Vazquez et al. 2009; Iwanaga and Lee 2005) and play an important role in the host immune functions when the organism is attacked by bacterias or viruses (Lee and Söderhäll 2002; Lavine and Strand 2002). In the presence of stressors such as elevated temperature, anoxia and diluted seawater, the hemolymph titer of MF in the crab increased (Lovett et al. 2001; Borst et al. 2001).

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