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# Molecular Cloning and Expression Analysis of *Ovary-Specific Transcript 1* (*Pm-OST1*) of the Giant Tiger Shrimp, *Penaeus monodon*

**Sirawut Klinbunga[1,2*], Kanchana Sittikankaew[1], Vasin Yuvanatemiya[3], Rachanimuk Preechaphol[3], Sirikan Prasertlux[1], Keisuke Yamano[4] and Piamsak Menasveta[2,5]**

[1]*Aquatic Molecular Genetics and Biotechnology Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), 113 Paholyothin Road, Klong 1, Klong Luang, Pathumthani 12120, Thailand*
[2]*Center of Excellence for Marine Biotechnology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand*
[3]*Faculty of Marine Technology, Burapha University, Chanthaburi 22170, Thailand*
[4]*National Research Institute of Aquaculture, Fisheries Research Agency, Mie 516-0193, Japan*
[5]*Department of Marine Science, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand*

**Isolation and characterization of genes specifically expressed in ovaries are necessary for understanding sex differentiation and ovarian development processes in the giant tiger shrimp, *Penaeus monodon*. In this study, a transcript that significantly matched the polehole precursor was further characterized by RACE-PCR. The sequence obtained was 5151 bp in length and contained a coding region of 5031 bp corresponding to 1677 amino acids. This transcript was only expressed in ovaries but not in testes of juveniles (*N*=10) and broodstock (*N*=22) of *P. monodon*. A tissue distribution analysis further confirmed ovary-specific expression of this transcript (called *P. monodon ovary-specific transcript 1*, *Pm-OST1*) in female broodstock. Expression levels of *Pm-OST 1* in ovaries of juvenile *P. monodon* upon 5-HT injection (33.9±6.40 g; 50 μg/g body weight) were significantly higher at 12–72 hours post injection (*P*<0.05). Quantitative real-time PCR indicated that *Pm-OST1* was comparably expressed throughout ovarian development in normal *P. monodon* broodstock (*P*>0.05). However, the expression level of *Pm-OST1* was significantly higher in stage-III ovaries in eyestalk-ablated broodstock (*P*<0.05). *Pm-OST1* was clearly localized in the ooplasm of previtellogenic and vitellogenic oocytes. Our results suggest that *Pm-OST1* plays a functionally important role in promoting the development of female germ cells and oocytes in *P. monodon*.**

**Key words:** RACE-PCR, real-time PCR, serotonin, *Penaeus monodon*, ovary-specific expression

## INTRODUCTION

Farming of *P. monodon* in Thailand relies almost entirely on wild-caught broodstock for a supply of juveniles because of poor reproductive maturation of cultured *P. monodon* females (Withyachumnarnkul et al., 1998; Preechaphol et al., 2007). As a result, breeding of pond-reared *P. monodon* is extremely difficult and rarely produce the quality of larvae required by the industry.

The high demand on wild female broodstock leads to overexploitation of natural populations of *P. monodon* in Thai waters (Klinbunga et al., 1999; Khamnamtong et al., 2005). The lack of high-quality wild and/or domesticated broodstock has caused a significant decrease in the farmed production of *P. monodon* in the last few years (Limsuwan, 2004). Reduced degrees of reproductive maturation in captive *P. monodon* females have limited the potential for genetic improvement, resulting in remarkably slow domestication and selective breeding programs for *P. monodon* in Thailand (Withyachumnarnkul et al., 1998; Clifford and Preston, 2006; Preechaphol et al., 2007).

The development of oocytes consists of a series of complex cellular events in which specific gene classes are expressed to ensure proper development and to store transcripts and proteins as maternal factors for early embryogenesis (Qiu and Yamano, 2005; Qiu et al., 2005). An understanding of sex determination and gonad development is necessary to manipulate the sex ratio and to control reproductive maturation of this economically important species (Benzie, 1998; Leelatanawit et al., 2009). Accordingly, the identification and characterization of the sex-specific expression of genes involved in ovary/oocyte development is

* Corresponding author. Phone: +66-2-6448150;
Fax : +66-2-6448190;
E-mail: sirawut@biotec.or.th
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an initial step toward understanding the molecular mechanism governing sex differentiation processes in *P. monodon* (Khamnamtong et al., 2006; Preechaphol et al., 2007).

Transcription in germ cells during oogenesis follows carefully regulated programs corresponding to a series of developmental events of oocytes (Grimes, 2004; Qiu and Yamano, 2005; Qui et al., 2005). Khamnamtong et al. (2006) identified sex-specific expression markers in ovaries and testes of *P. monodon* by RAP-PCR. Five (*FI-4*, *FI-44*, *FIII-4*, *FIII-39*, and *FIII-58*) unknown differential display-derived transcripts showed female-specific expression patterns in ovaries of 3-month-old juveniles and broodstock, implying that these unknown genes contribute to gonadal development and/or sex differentiation.

Cathepsin C (dipeptidyl peptidase I) is a biologically active peptidase that plays a major role in intracellular protein degradation (Doughty and Gruenstein, 1987; Turk et al., 2001). In the kuruma prawn (*Marsupenaeus japonicus*), the *cathepsin C* transcript was more abundantly expressed in the early and middle cortical rod (CR) oocyte stages. In-situ hybridization revealed that the *cathepsin C* transcript was localized in both oocytes and follicle cells. This demonstrated multiple functions for a particular gene product during oogenesis (Qiu et al., 2005).

To assess the possible biological roles of *ovary-specific transcript 1*, which exhibits sequence similarity to the pole-hole precursor (*Pm-OST1*), in germ cell and ovary/oocyte development in *P. monodon*, the *Pm-OST1* cDNA sequence was further identified. The effects of serotonin (5-HT) administration and eyestalk ablation on expression levels of *Pm-OST1* in the ovaries of juveniles and broodstock, respectively, were examined. Localization of *Pm-OST1* in oocytes was examined by in-situ hybridization.

## MATERIALS AND METHODS

### Experimental animals and design

Female broodstock were caught from the Andaman Sea and acclimated under farm conditions for 2 or 3 days. The post-spawning group was immediately collected after the shrimp had ovulated ($N$=6). Ovaries were dissected out from cultured juveniles ($N$=5) and normal broodstock and weighed ($N$=34). For the eyestalk ablation group, shrimp were acclimated for 7 days prior to unilateral eyestalk ablation. Ovaries of eyestalk-ablated shrimp were collected 2–7 days after ablation ($N$=32). The gonadosomatic index (GSI, ovarian weight/body weight x 100) of each shrimp was calculated. Ovarian development was classified by conventional histology (Qiu et al., 2005) and divided into the previtellogenic (I, $N$=10 and 4 for normal and eyestalk-ablated broodstock, respectively), vitellogenic (II, $N$=7 and 7), early cortical rod (III, $N$=7 and 10), and mature (IV, $N$=10 and 11) stages.

In addition, juvenile (5-month-old) shrimp were purchased from a commercial farm and acclimatized to laboratory conditions (28–30°C and 30 ppt seawater) for 7 days in 150-L fish tanks. Four groups of female shrimp (average body weight 33.9±6.40 g) were injected intramuscularly into the first abdominal segment with 5-HT (50 μg/g body weight, $N$=5 for each group). Specimens were collected 12, 24, 48, and 72 hours post-injection (hpi). A normal saline injection (0.85% at 0 hpi) was included as a negative control. For a tissue-distribution analysis, various tissues from a broodstock female, and the testes from a male, were collected, immediately placed in liquid $N_2$, and kept at –70°C until needed.

### Total RNA and first strand cDNA synthesis

Total RNA was extracted from ovaries of *P. monodon* using TRI Reagent (Molecular Research Center). The concentration of extracted total RNA was measured spectrophotometrically. One microgram of DNase I-treated total RNA was reverse-transcribed using an Improm-II™ Reverse Transcription System (Promega).

### Rapid amplification of cDNA end-polymerase chain reaction (RACE-PCR) and sequence analysis

A gene-specific primer (OST1RACE-I: 5'-CGTTATTGCCACTTGTTCCCTC-3') was designed, and 5' RACE-PCR was carried out using a SMART RACE cDNA Amplification Kit following the protocol recommended by the manufacturer (BD Bioscience Clontech). The amplified fragment was electrophoretically analyzed, eluted from the gel, cloned into pGEM-T Easy vector and sequenced. Nucleotide sequences of EST and RACE fragments were assembled and searched with BlastX (Altschul et al., 1990; available at http://ncbi.nlm.nih.gov) for similar sequences previously deposited in GenBank. The protein domain and signal peptide in the deduced Pm-OST1 polypeptide were predicted by using SMART (http://smart.embl-heidelberg.de).

### 5-HT administration, semi-quantitative RT-PCR, and tissue distribution analysis

Expression levels of *Pm-OST1* in ovaries and testes of wild broodstock ($N$=11 for each sex) and cultured juveniles ($N$=5 for each sex) were analyzed by RT-PCR (forward, 5'-GCAATAACGGTGAACAAGGGA-3'; reverse, 5'-GCAACCACATTAGTAGCCATA-3'). *EF-1*$\alpha_{500}$ was included as a positive control (forward, 5'-ATGGTTGTCAACTTTGCCCC-3'; reverse, 5'-TTGACCTCCTTGATCACACC-3'). The thermal profiles were 94°C for 3 min followed by 25 cycles of 94°C for 30 s, 53°C for 45 s, and 72°C for 1 min, with a final extension at 72°C for 7 min. A tissue distribution analysis of *Pm-OST1* in various tissues of shrimp broodstock was performed for 30 cycles using the same conditions.

Effects of 5-HT on expression of *Pm-OST1* were analyzed by semi-quantitative RT-PCR. Initially, non-quantitative RT-PCR was carried out using 100 ng of the first-strand cDNA as the template at various concentrations of primers (0.05, 0.075, 0.10, 0.15, 0.20, 0.25, 0.30, and 0.40 μM). The optimal concentration of $MgCl_2$ (among 1.0, 1.5, 2.0, 2.5, and 3.0 mM) was further tested using the optimized primer concentration. Finally, RT-PCR was performed for *Pm-OST1* for 18, 20, 22, 25, 28 30 and 35 cycles using the optimized primer and $MgCl_2$ concentrations. The number of cycles before the product reached an amplification plateau was chosen. Semi-quantitative RT-PCR was carried out under the following conditions: 94°C for 3 min; 20 cycles of 94°C for 30 s, 53°C for 45 s, and 72°C for 1 min; and 72°C for 7 min (with 0.15 μM and 1.5 mM primer and $MgCl_2$ concentrations, respectively). The amplicon was electrophoretically analyzed with 1.5% agarose gels and visualized with a UV transilluminator after ethidium bromide staining (Sambrook and Russell, 2001). The intensities of *Pm-OST1* and *EF-1*$\alpha$ were quantified from gel photographs by using Quantity One software (BioRad). The relative expression level of the transcript investigated (intensity of *Pm-OST1*/intensity of *EF-1*$\alpha$) in all experimental groups were statistically tested by using analysis of variance (ANOVA) followed by Duncan's new multiple-range test. Results were considered significant at $P$<0.05.

### Quantitative real-time PCR

*Pm-OST1* (primers $OST1_{238}$-F, 5'-AGACATAGAAGCAGAGACCACCTTT-3'; $OST1_{238}$-R, 5'-ATCCTCTGTAGCAGATTTCACATCC-3') and the internal control, *EF-1*$\alpha_{214}$ (forward, 5'-GTCTTCCCCTTCAGGACGTC-3'; reverse, 5'-CTTTACAGACACGTTCTTCACGTTG-3'), were amplified from ovaries of each shrimp in 10-μl reaction volumes containing 5 μl of 2x LightCycler 480 SYBR Green I Master (Roche), 50 ng of the first-strand cDNA as template, and 0.3 μM each of the gene-specific primers. The thermal profile for quantitative real-time PCR was 95°C for 5 min followed by 40 cycles of 95°C for 15 s, 58°C for 30 s, and 72°C for 30 s. Real-time PCR was carried

out in duplicate for each specimen. The relative expression levels (copy numbers of *Pm-OST1* and *EF-1α*) between shrimp at different stages of ovarian development were statistically tested ($P<0.05$).

**In-situ hybridization**

Ovaries from normal and eyestalk-ablated *P. monodon* broodstock were fixed overnight at 4°C in 4% paraformaldehyde prepared in 0.1% phosphate-buffered saline (PBS, pH 7.2). The fixed ovarian tissue was washed four times with PBS at room temperature and stored in 70% ethanol at –20°C until it was used. Conventional paraffin sections (5 μm) were made. Sense and anti-sense cRNA probes ($OST1_{1131}$-T7/F, 5'-TAATACGACTCACTATAGGGAATGACCTCTTCGTTAGTGGAACTG-3'; $OST1_{1131}$-R/SP6, 5'-ATTTAGGTGACACTATAGAAAGCATGTCTATTATGATCCGAGAGC-3') were

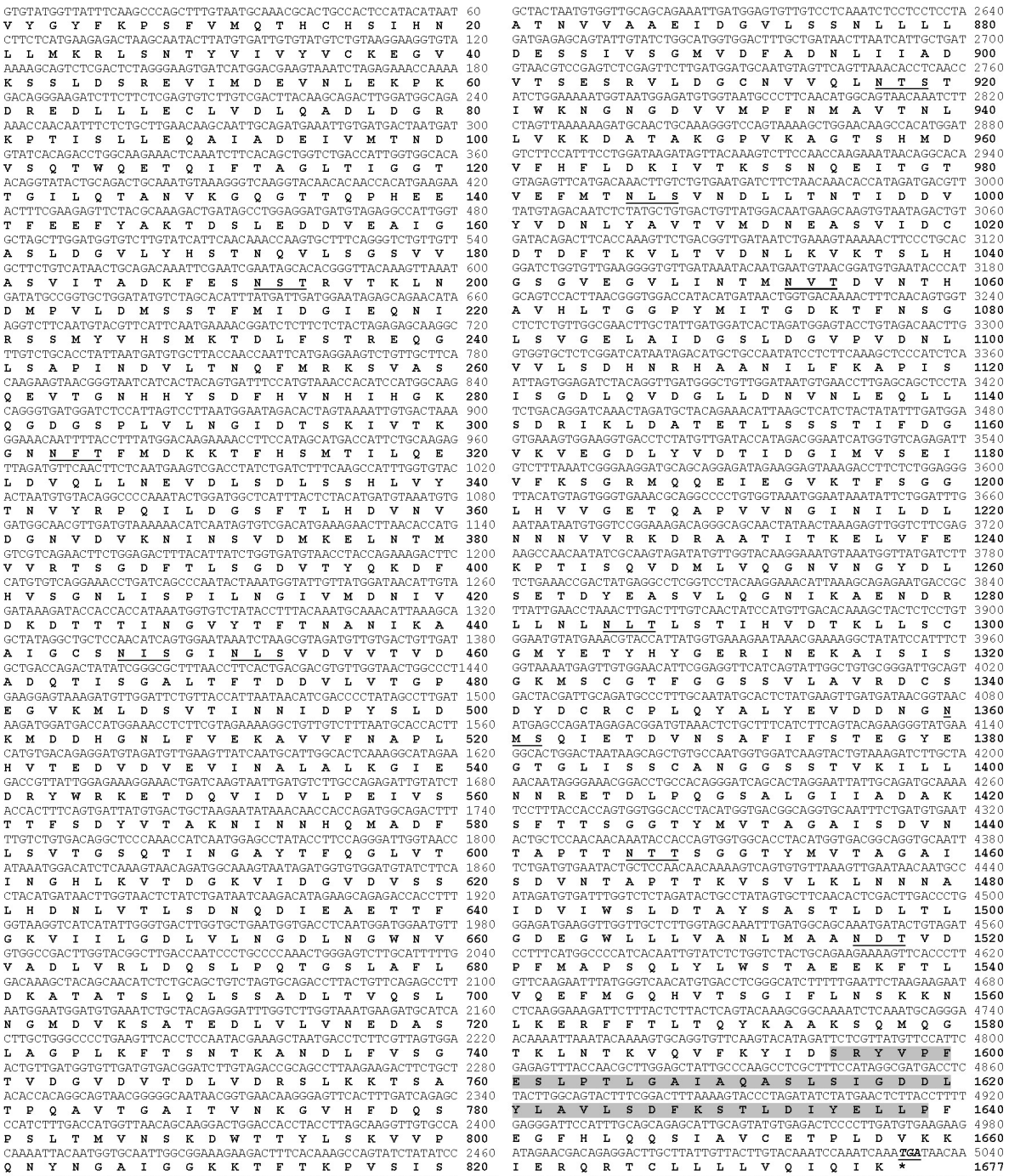

**Fig. 1.** Partial cDNA and deduced amino acid sequences of *Pm-OST1*. The putative stop codon (TGA) is in bold font, italicized, and underlined. A cytoplasmic polyadenylation element (CPE, T/ATTTTAT/A) is underlined and italicized. Predicted *N*-linked glycosylation sites (NXS/T) are underlined. The ESTP domain is highlighted.

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

## RESULTS

### Identification and characterization of *Pm-OST1*

The *Pm-OST1* transcript identified by RACE-PCR was 5151 bp in length, with a 3' UTR of 117 bp excluding the poly-A tail (GenBank accession no. FJ746694). No start codon was found in the longest open reading frame. The coding region of the *Pm-OST1* fragment covered nucleotides 1–5031 corresponding to 1677 amino acids (Fig. 1). A potential cytoplasmic polyadenylation element (CPE, T/ATTTTAT/A) was found at 22 nucleotides upstream from the poly-A tail. Twelve predicted *N*-linked glycosylation sites (NXS/T; positions 192–194, 303–305, 446–448, 451–453, 824–826, 917–919, 986–988, 1053–1055, 1285–1287, 1360–1362, 1446–1448, and 1516–1518) were found. A predicted epitempin (EPTP) domain was found at positions 1595–1639 (*E*-value=4.8e–02) of the deduced Pm-OST1 protein, which was most similar to the polehole precursor of *Drosophila melanogaster* (*E*-value=9e–28).

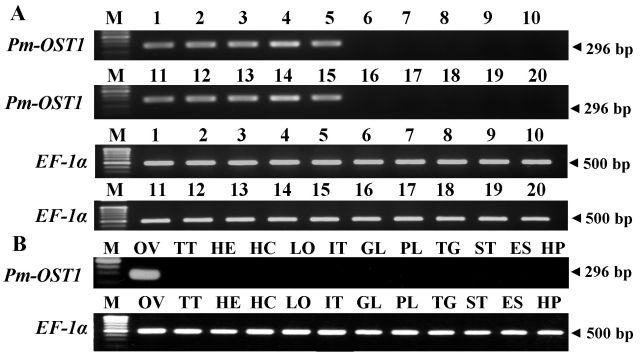


**Fig. 2.** **(A)** RT-PCR for *Pm-OST1* and *EF-1α* in ovaries (lanes 1–5 and 11–15, respectively) and testes (lanes 6–10 and 16–20, respectively) of *P. monodon*. The upper row for each gene represents juveniles, while the lower row represents broodstock. **(B)** Expression of the *Pm-OST1* transcript in various tissues of female (OV, ovaries; HE, heart; HC, hemocytes; LO, lymphoid organs; IT, intestine; GL, gills; PL, pleopods; TG, thoracic ganglion; ST, stomach; ES, eyestalk; HP, hepatopancreas) and in the testes of male *P. monodon* (TT, testes) broodstock. *EF-1α* was successfully amplified from the same templates. Lanes M, 100 bp DNA marker.

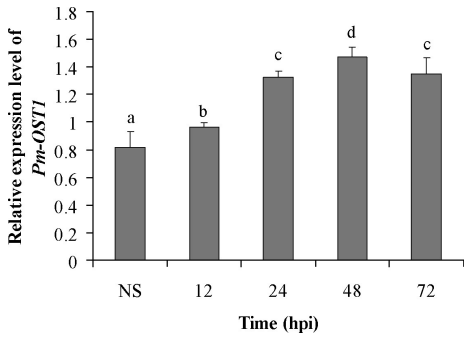


**Fig. 3.** Relative expression levels of *Pm-OST1* at 12, 24, 48, and 72 hours post-injection (hpi) with 5-HT. The negative control (NS; 0.85% NaCl at 0 hpi) is also shown. The same letters above bars indicate no significant difference between groups of samples ($P>0.05$).

### Ovary-specific expression of *Pm-OST1*

*Pm-OST1* was specifically expressed in the ovaries but not the testes of juvenile ($N=5$ for each sex) and broodstock ($N=11$ for each sex) *P. monodon* (Fig. 2A). A tissue distribution analysis further confirmed ovary-specific expression in female broodstock (Fig. 2B).

### Effects of 5-HT administration and eyestalk ablation on expression levels of *Pm-OST1*

The expression level of *Pm-OST1* in ovaries of juvenile *P. monodon* was significantly higher after injection with serotonin (5-HT) than after injection of the vehicle control ($P<0.05$). *Pm-OST1* was upregulated at 12–78 hour post injection (hpi, $P<0.05$), with the highest expression level observed at 48 hpi ($P<0.05$, Fig. 3).

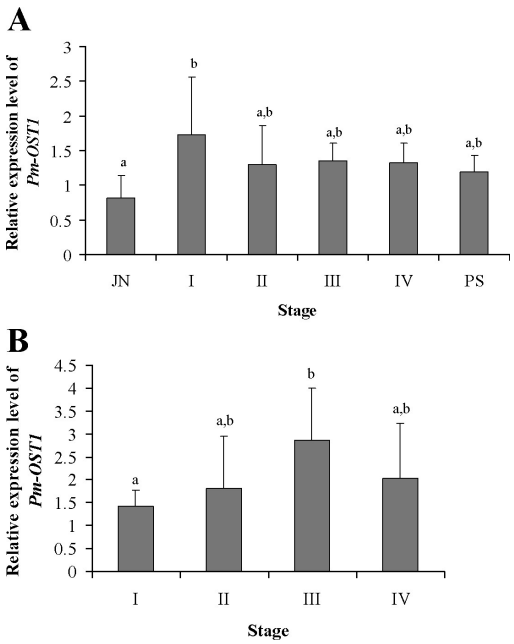


**Fig. 4.** Expression profiles of *Pm-OST1* in ovaries of *P. monodon*. **(A)** Normal (non-eyestalk-ablated). JN, cultured juveniles; I–IV, stages of ovarian development (I, previtellogenic; II, vitellogenic; III, early cortical rod; IV, mature ovaries); PS, post-spawning broodstock. **(B)** Unilateral eyestalk-ablated animals; ovarian stages as for (A). The same letters above bars indicate no significant difference between groups of samples ($P>0.05$).

Quantitative real-time PCR detected expression levels of *Pm-OST1* in the premature ovaries of juveniles that were significantly lower than in either normal or eyestalk-ablated broodstock ($P<0.05$). In normal adults, *Pm-OST1* was abundantly expressed at all stages of ovarian development. However, *Pm-OST1* was upregulated in stage-III ovaries in eyestalk-ablated shrimp ($P<0.05$). The expression level of *Pm-OST1* at this stage was also significantly greater than in various ovarian stages of normal broodstock ($P<0.05$). Therefore, unilateral eyestalk ablation affected expression levels of *Pm-OST1* in the ovaries of *P. monodon* broodstock (Fig. 4).

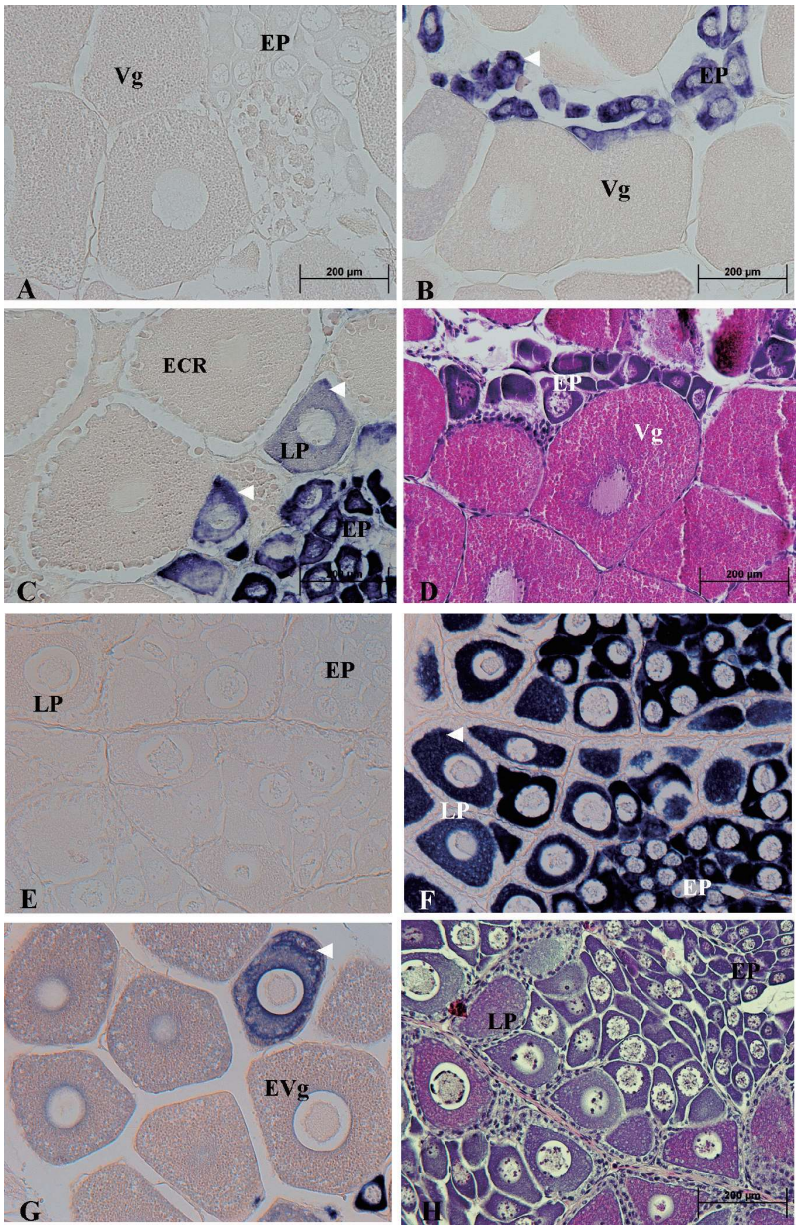


**Fig. 5.** Localization of *Pm-OST1* mRNA during ovarian development in normal **(A–D)** and eyestalk-ablated **(E–H)** *P. monodon* broodstock, visualized by in-situ hybridization using sense **(A, E)** and antisense **(B–C, F–G)** *Pm-OST1* probes. Oocyte stages were classified by a conventional hematoxylin/eosin staining **(D, H).** EP, early previtellogenic; LP, late previtellogenic; EVg, early vitellogenic; Vg, vitellogenic; ECR, early cortical rod oocytes. Arrowheads indicate positive hybridization signals (blue).

### Localization of *Pm-OST1* mRNA

From in-situ hybridization, the *Pm-OST1* transcript was found to be clearly localized in the ooplasm of previtellogenic and vitellogenic oocytes in both normal and eyestalk-ablated broodstock. No signal was found with the sense cRNA probe (Fig. 5).

## DISCUSSION

### *Pm-OST1* is specifically expressed in the ovaries in *P. monodon*

Basic information on sex differentiation, reproductive maturation, and oocyte development in *P. monodon* has long been of interest and can be directly applied to selective breeding programs for this economically important species (Benzie, 1998; Browdy, 1998; Leelatanawit et al., 2004; Preechaphol et al., 2007).

An EST significantly similar to the polehole precursor of *D. melanogaster* was previously identified from EST analysis of an ovarian cDNA library (Preechaphol et al., 2007). We further characterized this EST in the present study. The deduced amino acid sequence of *Pm-OST1* contains an EPTP domain that was originally found in proteins functionally associated with neurological disorders (Staub et al., 2002); however, no other conserved domains were found in *Pm-OST1* and its homologue (accession number CAD31790).

Surprisingly, the polehole genes (not the polehole precursor) of *D. melanogaster* (accession numbers Nm_080308, Nm_001042793, and Nm_001103397), *D. simulans* (AY135135), and *D. mauritiana* (AY135030) contain three conserved domains: the Ras-binding domain (RBD) commonly found in proteins related to small GTPase (Ras), the PE-binding domain (phorbol ester binding domain, also called the protein kinase C conserved region 1 [C1] domain), and the protein kinase STYkc domain (Ono et al., 1989). The full-length cDNAs of three isoforms of the *D. melanogaster polehole* are 3090, 3420, and 3549 bp in length and contain ORFs of 2223, 2223, and 2352 bp that encode polypeptides of 740, 740, and 783 amino acid residues, respectively. Sequence similarity was low between *D. melanogaster* polehole and the deduced Pm-OST1 protein (data not shown). The lack of any conserved domain and large differences in sequence length and similarity between *Pm-OST1* and *Drosophila polehole* suggest that the former is not a member of the polehole protein family but should be regarded as a novel gene newly characterized in *P. monodon*. The significant similarity in amino acid sequence between the Pm-OST1 fragment and the polehole precursor of *D. melanogaster* must have occurred randomly.

In oocytes, part of the mRNA transcribed from several genes contains a conserved, U-

rich sequence called CPE in the 3' UTR (Nishimura et al., 2009). A predicted CPE was found in the 3' UTR of *Pm-OST1*. Cytoplasmic polyadenylation is one of the translational regulation mechanisms for maternal mRNAs during oocyte maturation (Katsu et al.,1999; Tremblay et al., 2005; Nishimura et al., 2009). This suggests the possible involvement of *Pm-OST1* in ovarian development in *P. monodon*.

Sex determination and differentiation mechanisms are problematic in studies of many species. The diploid chromosome number in *P. monodon* is 2*N*=88 (Benzie, 1998). Recent advances in genetic mapping (Li et al., 2003; Maneeruttanarungroj et al., 2006) and a sex-specific AFLP-derived marker (Staelens et al., 2008) suggested female heterogamy (ZW) in *P. monodon*. Nevertheless, because the sex chromosomes in this species are not obviously heteromorphic, little is known about the sex chromosomes or their segregation pattern (Khamnamtong et al., 2006). Although the genes *sex lethal protein* (*Sxl*) and *transformer 2* (*Tra-2*), which function in the *doublesex* (*dsx*) pathway, were recently identified in *P. monodon*, sex differentiation in this species is not understood at present (Leelattanawit et al., 2009).

The gene *vasa*, which encodes an ATP-dependent RNA helicase belonging to the DEAD-box protein family, was recently identified in the Pacific white shrimp, *Litopenaeus vannamei* (Alfalo et al., 2007), and the giant freshwater prawn, *Macrobrachium rosenbergii* (Nakkrasae and Damrongphol, 2007). *Vasa* was restrictively expressed in the gonads of adults of both species and was proposed to be a potential biomarker for germ cell development.

Gender-specific gene expression detected by RNA arbritary primed PCR (RAP-PCR) has been reported in the nodule worm *Oesophagostomum dentatum*. A total of 31 bands differentially expressed between the sexes were cloned and sequenced. A Northern blot analysis indicated that ESTs significantly matching *vitellogenin-5* and *endonuclease III* of *C. elegans* were expressed solely in *O. dentatum* females (Boag et al., 2000).

Tissue-specific transcription is important during the development and during maturation of specific cell types from stem cells in adults (Grimes, 2004). *Pm-OST1* was expressed only in the ovaries but not in the testes of *P. monodon* juveniles and broodstock. Ovary-specific transcription of *Pm-OST1* suggests an essential role in ovarian but not in testicular development in *P. monodon*. More importantly, our tissue expression analysis further indicated that this transcript contributes mainly to female germ cell development and can potentially be used as a biomarker for the elucidation of developmental and reproductive processes in the female germ line of *P. monodon*.

### Upregulated expression of *Pm-OST1* in the ovaries of juvenile *P. monodon* after 5-HT injection

Unilateral eyestalk ablation is used in practice to induce ovarian maturation in penaeid shrimp, but this technique leads to the eventual loss of egg quality and the death of spawners (Benzie, 1998; Okumura, 2004). Therefore, predictable maturation and spawning of captive penaeid shrimp without the use of eyestalk ablation is a long-term goal for the industry (Quackenbush, 2001).

Biogenic amines (e.g., serotonin or 5-HT, epinephrine, and dopamine) and peptide neuroregulators are known to modulate the release of neuropeptide hormones from the sinus gland (Fingerman, 1997; Sarojini et al., 1995; Okumura, 2004). Simultaneous injections of 5-HT (25 μg/g body weight) and the dopamine antagonist spiperone (1.5 or 5 μg/g body weight) induced ovarian maturation and spawning in wild *L. stylirostris* and pond-reared *L. vannamei* (Alfaro et al., 2004).

The effects of exogenous 5-HT on reproductive performance in *P. monodon* were reported. Domesticated shrimp injected with 5-HT (50 μg/g body weight) exhibited ovarian maturation and spawning rates comparable to those in eyestalk-ablated shrimp. Interestingly, the hatching rate and the amount of nauplii produced per brooder were significantly higher in the 5-HT-injected shrimp ($P<0.05$) (Wongpraset et al., 2006). 5-HT also induced ovarian maturation and spawning in *L. vannamei* (Vaca and Alfaro, 2000). Nevertheless, the effects of 5-HT on the expression levels of genes and/or proteins in the ovaries of penaeid shrimp have not been reported.

Due to the high cost (~$200 USD/brooder) of a gravid female of *P. monodon* (Preechaphol et al., 2007), 5-month-old shrimp, regarded as early subadults, were used to examine the effects of 5-HT on *Pm-OST1* expression. The injection of 5-HT clearly promoted expression of *Pm-OST1* in the ovaries of *P. monodon* subadults at 12–72 hpt. The molecular effects of 5-HT on this expression should be further examined in both wild and domesticated broodstock to evaluate the use of 5-HT in place of eyestalk ablation for enhancing ovarian/oocyte development in *P. monodon*.

### Expression patterns and localization of *Pm-OST1* during ovarian development in *P. monodon*

The expression levels of *Pm-OST1* in ovaries were significantly higher in broodstock than in juveniles ($P<0.05$), suggesting that the *Pm-OST1* gene product is involved in oogenesis. The steady-state amounts of *Pm-OST1* mRNA may be sufficient to maintain the translational product throughout oogenesis in non-ablated shrimp.

Meiotic oocyte maturation is regulated in all animals by maturation promoting factor (MPF), a complex of cdc2 (Cdk1), cyclin B, and other Cdk/cyclin complexes (Kishimoto, 1999, 2003). Characterization of the full length cDNA and genomic organization of *P. monodon cyclin B* (PMCyB) showed it to have an identical ORF of 1206 bp with 8 exons and 7 introns, and length polymorphism in the UTR spanning 4181, 4307, and 4940 bp for short, medium, and long isoforms, respectively). During ovarian development and following spawning in normal shrimp broodstock, the level of *cyclin B* was greater in stage-IV than in stage-I ovaries ($P<0.05$). Unilateral eyestalk ablation had no effect on the transcription of *PMCyB* ($P>0.05$) (Visuthiphole et al., 2009).

In contrast, eyestalk ablation caused an increase in the mRNA levels of *vitellogenin* and *cortical rod protein* in ovaries of *M. japonicus* (Tsutsui et al., 2005; Okumura et al., 2006). Likewise, the increase in *Pm-OST1* mRNA during ovarian development in eyestalk-ablated female broodstock suggests that gonad inhibiting hormone (GIH; Meusy and Payen, 1988) affects *Pm-OST1* transcription. Yamano et al. (2004) found that in most cases, ovaries of *M. japonicus*

start to develop in the reproductive season but fail to reach the full growth requisite for the formation of cortical rods (CRs). This is also a major constraint in *P. monodon*. Upregulation of *Pm-OST1* in stage-III ovaries as a result of unilateral eyestalk ablation suggests that the expression level of this gene may be used to reflect the effects of neurotransmitters (e.g., 5-HT and dopamine; Alfaro et al., 2004; Wongpresert et al., 2006), steroid hormones (e.g., progestins and estradiols; Yano and Hoshina, 2006) and/or maturation feed (e.g., that supplemented with EPA and DHA; Yano, 1995) on ovarian development in *P. monodon*.

In-situ hybridization signals from the *Pm-OST1* transcript in immature (early>late previtellogenic) oocytes were more intense than in vitellogenic (primary>secondary) oocytes. No localization of *Pm-OST1* was observed in the more mature (early and late cortical rod) stages of oocytes and follicular cells. This further indicates cell-type specific expression of *Pm-OST1* in the ovaries of *P. monodon* broodstock. Contradictory results from quantitative real-time PCR and in-situ hybridization on the disappearance of *Pm-OST1* hybridization signals from the ooplasm in oocytes at later stages may have been due to a significant increase in oocyte size as oogenesis proceeded. In addition, real-time PCR detects gene expression with much greater sensitivity than in-situ hybridization.

Considering all information, ovary-specific *Pm-OST1* likely plays a main role in the development of female germ cells and oocytes in *P. monodon*. The expression levels and localization of the Pm-OST1 protein during ovarian development and/or oogenesis should be further examined for an unambiguous conclusion on the functions of this gene product. The basic knowledge obtained in this study will allow characterization of the function of *Pm-OST1* in sex differentiation in this economically important species.

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