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Isolation of cDNA, genomic organization and expression of *small androgen receptor-interacting protein 1* (*PmSARIP1*) in the giant tiger shrimp *Penaeus monodon*

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ABSTRACT

The full-length cDNA and genomic organization of *small androgen receptor-interacting protein 1* (*SARIP1*; also called *RWD-containing protein 1*, *Rwdd1*) in the giant tiger shrimp (*Penaeus monodon*) was isolated and characterized. *PmSARIP1* was 1366 bp in length containing an open reading frame (ORF) of 738 bp corresponding to a polypeptide of 245 amino acids. Its genomic sequence contained 5 exons, 4 introns and untranslated regions (UTRs) spanning 5937 bp in length. Tissue distribution analysis indicated that *PmSARIP1* was specifically expressed in gonads (ovaries > testes) but not in other tissues of wild *P. monodon* adults. The expression level of *PmSARIP1* was not differentially expressed during ovarian maturation in intact wild adults ($P > 0.05$). Eyestalk ablation resulted in up-regulation of *PmSARIP1* throughout the ovarian maturation of wild adults where the peak level was observed at stage I (previtellogenic) ovaries ($P < 0.05$). *PmSARIP1* mRNA was clearly localized in ooplasm of previtellogenic oocytes. Serotonin injection (5-HT, 50 μg/g body weight; 18-month-old shrimp) promoted the expression level of ovarian *PmSARIP1* at 6–72 h post injection (hpi) with the peak level at 12 hpi ($P < 0.05$). Exogenous progesterone administration (0.1 μg/g BW; 14-month-old shrimp) did not significantly affect the expression level of ovarian *PmSARIP1*. In contrast, 17β-estradiol treatment (0.01 μg/g BW) resulted in an increase of *PmSARIP1* in 14-month-old shrimp at 7 days post injection (dpi). Results from this study suggested that *PmSARIP1* should play an important role during ovarian maturation of *P. monodon*.

1. Introduction

In Thailand, the giant tiger shrimp (*Penaeus monodon*) has been intensively cultured for about three decades. Therefore, it is one of the most economically important species locally (Limsuwan, 2004; Withyachumnarnkul et al., 1998). However, the culture production of *P. monodon* in Thailand has significantly decreased since the last several years due to problems from the disease outbreak and the shortage of high quality brooders (Flegel and Sritunyalucksana, 2011; Limsuwan, 2004).

Difficulties in reproductive maturation of captive *P. monodon* limit the potential of domestication and selective breeding programs in this species (Benzie, 1998; Rosenberry, 1997). Removing of an eyestalk (unilateral eyestalk ablation) is practically used to induce ovarian maturation of wild shrimp but the technique leads to an eventual loss in egg quality and death of the spawners (Benzie, 1998; Wongprasert et al., 2006). In addition, the technique does not provide an equal effect when applied to captive shrimp. Therefore, the control of reproductive maturation and spawning of captive *P. monodon* without the use of eyestalk ablation is a long-term goal for the industry (Klinbunga et al., 2009; Preechaphol et al., 2010; Quackenbush, 2001).

In penaeid shrimp, oocytes are arrested at the first meiotic prophase and reach metaphase I after ovulation (Yano, 1995, 1998). It remains unknown whether crustaceans possess a gonadotropin homologue that can trigger the meiotic resumption and final oocyte maturation as in most vertebrates (Miura et al., 2006; Thomas, 2008). Accordingly, understanding the molecular functions of reproduction-related genes that are differentially expressed during ovarian maturation will be

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useful to increase the culture efficiency of this economically important species.

Exogenous serotonin (5-HT) injection induced ovarian maturation in the crayfish, *Procambarus clarkii* (Sarojini et al., 1995) and *Litopenaeus vannamei* (Vaca and Alfaro, 2000) at rates lower than unilateral eyestalk ablation. Simultaneous injections of 5-HT (25 μg/g body weight) and the dopamine antagonist-spiperone (1.5 or 5 μg/g body weight) stimulated ovarian maturation and spawning in wild *Litopenaeus stylirostris* and pond-reared *L. vannamei* (Alfaro et al., 2004). Recently, the effects of exogenous 5-HT on reproductive performance in domesticated *P. monodon* were reported. 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 (Wongprasert et al., 2006).

Understanding the induction mechanisms of reproduction-related genes during ovarian and oocyte maturation will be useful to develop methodologies that to effectively induce ovarian maturation in *P. monodon*. Molecular effects on administration of 5-HT at 50 μg/g body weight in domesticated *P. monodon* adults were recently reported. It clearly promoted the expression of various reproduction-related genes in ovaries of domesticated *P. monodon* for example, *Ovary-Specific Transcript* (*PmOST1*) in cultured 5-month-old shrimp at 12–78 h post injection (hpi, Klinbunga et al., 2009), *adipose differentiation-related protein* (*PmADRP*) and *Broad-complex* (*PmBr-c*; Buaklin, 2010) in domesticated 14-month-old shrimp at 48 (Sittikankaew et al., 2010) and 12 hpi (Buaklin, 2010).

Steroid hormones are functionally involved in shrimp sexual differentiation and reproduction (Cahill, 2007; Miura et al., 2006). Conjugated and unconjugated dehydroepiandrosterone and estrone, conjugated pregnenolone and 17β-estradiol as well as unconjugated progesterone and estrone were detected in ovaries of wild *P. monodon* (Fairs et al., 1990). Quinitio et al. (1994) examined the levels of 17β-estradiol and progesterone in the hemolymph, ovaries and hepatopancreas of captive *P. monodon* females. Levels of 17β-estradiol in ovaries were significantly increased in shrimp possessing vitellogenic (yolky) and cortical rod (mature) ovaries. Interestingly, 17β-estradiol in hemolymph was only observed in mature shrimp while the peak level in hepatopancreas was also observed at this stage. The progesterone level in hemolymph and hepatopancreas was significantly increased in shrimp possessing vitellogenic and cortical rod ovaries while that in ovaries was significantly increased in the mature stage.

Small androgen receptor-interacting protein (SARIP) was first identified in rat. It contains the RWD domain which has been named after three major RWD-containing proteins; RING finger-containing proteins, WD-repeat-containing proteins, and yeast DEAD (DEXD)-like helicases (Doerks et al., 2002; Kang et al., 2008a,b). In human, the RWD domain was found in proteins whose deficiencies are implicated in severe human diseases. The deletion of this region leads to arthrogryposis-like symptoms, mental retardation, hypertonia, and several facial anomalies (Orti et al., 2000).

Kang et al. (2008b) characterized a novel thymus aging related gene, *Rwdd1* in mouse using differential display reverse transcription-polymerase chain reaction (DDRT-PCR) and expressed sequenced tag (EST) segment ligation. Its open reading frame encoded a protein of 243 amino acid residues which contained an RWD domain at the N terminus and named *Rwdd1*. It has been suggested that androgen receptor (AR) is related to thymus involution in rats as testosterone (a ligand of AR) appears to inhibit thymus development (Kumar et al., 1995). The expression level of *Rwdd1* affected the transactivation activity of androgen receptor (AR) in thymic epithelial cells. Accordingly, it is considered as an AR co-regulator.

In the present study, *P. monodon SARIP1* (*PmSARIP1*) initially identified by EST analysis (Preechaphol et al., 2007) was further characterized. The full-length cDNA and genomic organization of this gene were isolated and reported for the first time in crustaceans. Localization of *PmSARIP1* transcript was examined by *in situ* hybridization. Expression patterns of *PmSARIP1* during ovarian maturation of intact and eyestalk-ablated adults of wild *P. monodon* and those of domesticated shrimp under serotonin (5-HT) and steroid hormone (progesterone and 17-β estradiol) induction were examined by quantitative real-time PCR.

2. Materials and methods

2.1. Experimental animals

Juvenile shrimp (4-month-old) were purchased from a commercial farm in eastern Thailand ($N = 5$). Female adults were live-caught from the Andaman Sea (west of peninsular Thailand) and acclimated under the farm conditions for 2–3 days ($N = 31$). The post-spawning group (stage V) was immediately collected after ovulation ($N = 5$). Ovaries were dissected out from juveniles and intact adults and weighed. For the eyestalk-ablated group, wild shrimp were acclimated for 7 days prior to unilateral eyestalk ablation. The reproductive maturation stages of ablated shrimp were visualized externally. Ovaries of eyestalk-ablated shrimp were collected at 2–7 days after the ablation to obtain stages I–IV ovaries ($N = 27$). The ovarian developmental stages of wild *P. monodon* were classified into four stages according to gonadosomatic indices (GSI, ovarian weight/body weight × 100): <1.5, 2–4, 4–6 and >6% for stages I (previtellogenic ovaries, $N = 10$ and 6 for intact and eyestalk-ablated adults, respectively), II (vitellogenic ovaries, $N = 5$ and 5), III (early cortical rod ovaries, $N = 7$ and 10) and IV (mature ovaries, $N = 9$ and 9) ovaries, respectively. The ovarian stage of each shrimp was further confirmed by conventional histology (Qiu et al., 2005; Yano, 1988). For tissue distribution analysis, various tissues of wild females and testes of wild males ($N = 4$ for each sex) were collected, immediately placed in liquid N_2 and kept at −70 °C until needed.

To determine effects of serotonin (5-HT) on *PmSARIP1* gene expression, domesticated shrimp (18-month-old, average body weight, BW = 74.18 ± 1.85 g) were collected and acclimated at the laboratory conditions (28–30 °C and 15 ppt seawater) in fish tanks (1000 l) for 7 days. Eight groups of female shrimp were injected intramuscularly into the first abdominal segment with 5-HT (50 μg/g body weight, $N = 4$ for each group). Specimens were collected at 0, 1, 3, 6, 12, 24, 48 and 72 h post injection (hpi). Ovaries of each shrimp were dissected out, immediately placed in liquid N_2 and kept at −80 °C until needed. Non-injected shrimp and those injected with the saline solution (0.85% at 0 hpi) were included as the negative (NC) and vehicle (VC) controls, respectively.

To determine effects of progesterone on *PmSARIP1* gene expression, four groups of acclimated female shrimp (14-month-old, average BW = 64.06 ± 3.20 g) were injected intramuscularly into the first abdominal segment with progesterone (0.1 μg/g body weight, $N = 4$ for each group). Specimens were collected at 12, 24, 48 and 72 hpi. Non-injected shrimp and those injected with absolute ethanol (at 0 and 12 hpi) were included as the negative (NC) and vehicle (VC) controls, respectively.

To determine effects of 17-β estradiol on *PmSARIP1* gene expression, three groups of acclimated shrimp (14-month-old, average BW = 48.99 ± 4.96 g) were injected intramuscularly with 17β-estradiol (0.01 μg/g of BW, $N = 4$ for each group) into the first abdominal segment of each shrimp. The injection was repeated with the original doses at 3 and 6 days post initial injection and specimens were collected at 7, 14 and 28 days post initial injection. Non-injected shrimp and those injected with 5% ethanol (at 7, 14 and 28 days after the initial injection; $N = 4$ for each group) were included as the negative (NC) and vehicle (VC) controls, respectively. In addition, unilaterally eyestalk-ablated shrimp were collected at the same time intervals. Ovaries of each shrimp were sampled and immediately placed in liquid N_2. The samples were stored at −80 °C until needed. Notably domesticated

shrimp can be used as brooders in the breeding programs since 14 months after cultivation. Accordingly, we used either 14- or 18-month-old domesticated shrimp in the hormone/neurotransmitter-injected experiments.

2.2. Genomic DNA extraction

Genomic DNA was extracted from a piece of pleopod of *P. monodon* adults using a phenol-chloroform-proteinase K method (Klinbunga et al., 2001). The concentration of extracted DNA was spectrophotometrically estimated. DNA was stored at 4 °C until used.

2.3. Characterization of cDNA and genomic DNA sequence of PmSARIP1

A homologue of *SARIP1* was originally identified from the ovarian cDNA library of *P. monodon* (Preechaphol et al., 2007). In the present study, the full-length cDNA of *PmSARIP1* was isolated by further sequencing of the original EST clone from the 3′ direction by a primer walking approach. Nucleotide identity was searched using Blast*N* and Blast*X* (Altschul et al., 1990; available at http://ncbi.nlm.nih.gov). The predicted protein domain was identified using SMART (http://smart.embl-heidelberg.de/). Molecular weight and p*I* of the deduced PmSARIP1 protein were examined using Protparam (http://www.expasy.org/tools/protparam.html).

Genomic organization of *PmSARIP1* was identified by overlapping PCR using primers spanning positions 12–111, 86–263, 239–574, 588–712 and 616–1319 of the full-length cDNA (Table 1). The amplification reaction was carried out in a 25 μl reaction volume composed of 25 ng genomic DNA, 1× BD Advantage® 2 PCR Buffer (40 mM Tricine-KOH; pH 8.7, 15 mM KOAc, 2.5 mM $Mg(OAc)_2$, 3.75 μg/ml BSA, 0.005% Tween 20, 0.005% Nonidet-P40), 200 μM each dNTP, 0.2 μM each of primers gPmSARIP-F1/R1, gPmSARIP-F2/R2, gPmSARIP-F3/R3, gPmSARIP-F4/R4 and gPmSARIP-F5/R5 (Table 1) and 1×BD Advantage® 2 Polymerase Mix (BD Biosciences). PCR was initially performed by predenaturation at 94 °C for 2 min followed by 35 cycles of a 94 °C denaturation for 30 s, a 60 °C annealing for 1 min and a 72 °C extension for 3 min. The final extension was carried out at 72 °C for 7 min. The amplicon was electrophoretically analyzed, eluted out from agarose gels, ligated with pGEM®-T easy (Promega) or pCR®-XL-TOPO (Invitrogen) vector and transformed into Premade Z-competent™ *Escherichia coli* DH5α cells (Zymo Research). The recombinant clone of each insert was sequenced for both directions.

Table 1
Lists of primers and primer sequences used for isolation of genomic DNA sequence and expression analysis of *PmSARIP1*.

Primer	Sequence (5′–3′)
Genomic organization (*primer walking*)	
gPmSARIP-F1	CAAGGAAGAACAGAATAACGAAATTG
gPmSARIP-R1	AGTAATTCTGAACTTGTGCCTTGGT
gPmSARIP-F2	AACCAAGGCACAAGTTCAGAATTAC
gPmSARIP-R2	TCATCCAGCTCTTCCTCTTCTATGT
gPmSARIP-F3	ACATAGAAGAGGAAGAGCTGGATGA
gPmSARIP-R3	TGAAGAGCTCTCTACCAGTTGGTTT
gPmSARIP-F4	TTTGAATGAGTCGGATCTTAGCTTC
gPmSARIP-R4	CTGGAACATAATCCTCGTCATCTTC
gPmSARIP-F5	GGTGAAGGAGAAGGTGAGGT
gPmSARIP-R5	TGCATCATACTGACTCCTCTA
RT-PCR and quantitative real-time PCR	
qPCR-SARIP-F	GAACGAAACTTAACGAGCAGTGTGA
qPCR-SARIP-R	ATCTCTTCCTGCTCTTTCTTCTTGC
EF-1α_{500}-F	ATGGTTGTCAACTTTGCCCC
EF-1α_{500}-R	TTGACCTCCTTGATCACACC
EF-1α_{214}-F	TCCGTCTTCCCCTTCAGGACGTC
EF-1α_{214}-R	CTTTACAGACACGTTCTTCACGTTG
In situ hybridization	
PmSARIP-T7-F[a]	**TAATACGACTCACTATAGGG**CTGGAGTCCATATATCCAGAGGAAT
PmSARIP-R-SP6[a]	**ATTTAGGTGACACTATAGAA**TCTTCATCCTCGAGGTCTAAGTCAT

[a] T7 and Sp6 promoter sequences are boldfaced and underlined.

2.4. In situ hybridization (ISH)

P. monodon ovaries were fixed in 4% paraformaldehyde prepared in 0.1% phosphate-buffered saline (PBS, pH 7.2) overnight at 4 °C. The fixed ovarian tissue was washed four times with PBS at room temperature and stored in 70% ethanol at −20 °C until further analyzed. The conventional paraffin sections (5 μm) were carried out. The *PmSARIP1* gene segment was amplified using gene-specific primers without the RNA polymerase recognition sequences (RPRS, Table 1). The amplified product of *PmSARIP* was diluted 100 fold and reamplified using the forward and reverse primers containing T7-RPRS (TAATACGACTCAC TATAGGG) and SP6-RPRS (ATTTAGGTGACACTATAGAA) (Table 1). The PCR product was purified using a MinElute PCR Purification Kit (Qiagen). The sense and antisense cRNA probes were synthesized using DIG RNA labeling mix (Roche). Tissue sections were dewaxed with xylene and dehydrated in absolute ethanol. The sections were prehybridized with 2× 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 hybridized with either the sense or antisense probe in the prehybridization solution overnight at 50 °C. After hybridization, the tissue sections were washed twice with 4× SSC for 5 min each and once with 2× 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 the RNase A buffer (37 °C, 10 min each) and 2× SSC (50 °C, 15 min each). High stringent washing was carried out twice in 0.2× SSC at 50 °C for 20 min each. The positive signals were detected using a DIG Wash and Block Buffer kit (Roche) (Preechaphol et al., 2010; Qiu and Yamano, 2005).

2.5. Total RNA extraction and first strand cDNA synthesis

Total RNA was extracted from ovaries of each shrimp using TRI Reagent (Invitrogen). Ten micrograms of total RNA were treated with RQ1 RNase-Free DNase (Promega; 0.5 U/μg total RNA at 37 °C for 30 min) to eliminate possible contamination of genomic DNA. The first strand cDNA was reverse transcribed from 1.5 μg DNase-treated total RNA using an Improm-II™ Reverse Transcription System (Promega).

2.6. RT-PCR and tissue distribution analysis

Expression of *PmSARIP1* (0.2 μM each of primers qPCR-SARIP-F/R, 157 bp; Table 1) in ovaries and other tissues of wild adults was analyzed by RT-PCR. The amplification was carried out in a 25 μl reaction volume containing 1× PCR buffer, 0.2 mM each dNTP, 0.2 μM each of primers qPCR-SARIP-F/R (Table 1), and 25 ng of the first strand cDNA template and 0.5 unit of EX *Taq*. *EF-1α* was included as a positive control. RT-PCR was initially performed by predenaturation at 94 °C for 5 min followed by 35 cycles of a 94 °C denaturation for 30 s, a 60 °C annealing for 30 s and a 72 °C extension for 1 min followed by a final extension of 72 °C for 7 min. The amplicon was electrophoretically analyzed on 1.5% agarose gels and visualized with a UV transilluminator after ethidium bromide staining (Sambrook and Russell, 2001).

2.7. Quantitative real-time PCR

The amplified *PmSARIP1* (primers qPCR-SARIP-F/R) and *EF-1α* (primers EF-1α_{214}-F/R) gene segments were cloned (Table 1). The standard curve of the target and reference sequences was constructed from 10^3 to 10^8 copies of each recombinant plasmid (1 kb = 6.6×10^5 Da; 1 μg of 1 kb cDNA contains 0.91×10^{12} molecules) in triplicate using

a LightCycler 480 SYBR Green I Master (Roche). The thermal profile for quantitative real-time PCR was 95 °C for 10 min followed by 40 cycles of 95 °C for 30 s, 58 °C for 1 min and at 72 °C for 1 min. Real-time PCR of each specimen was carried out in duplicate.

2.8. Statistical analysis

For quantitative real-time PCR analysis, the relative expression level of *PmSARIP1* and *EF-1α*$_{214}$ mRNA of each specimen was evaluated from their standard curves. The relative expression level (copy number of *PmSARIP1* and that of *EF-1α*$_{214}$) between shrimp possessing different ovarian stages of cultured juveniles ($N = 5$), wild ($N = 10$, 5, 7 and 9 and $N = 6$, 5, 10 and 9 for stages I–IV ovaries of intact and eyestalk-ablated adults and $N = 5$ for post-spawning intact adults) and domesticated shrimp in serotonin injection; (NC, VC and 0, 1, 3, 6, 12, 24, 48 and 72 hpi, $N = 4$ for each group), progesterone injection; (NC and VC at 0 and 12 hpi and 12, 24, 48 and 72 hpi $N = 4$ for each group) and 17β-estradiol injection; (NC and VC and 7, 14 and 28 dpi, $N = 4$ for each group and eyestalk-ablated shrimp (EA) at 7, 14 and 28 days, $N = 4$ for each group) were calculated.

The relative expression levels (copy number of *PmSARIP1* and that of *EF-1α*) between shrimp possessing different ovarian stages or between different groups of each hormone/neurotransmitter treatment were statistically analyzed using one way analysis of variance (ANOVA) and Duncan's new multiple range test. Results were considered significant when $P < 0.05$.

3. Results

3.1. Characterization of P. monodon small androgen receptor-interacting protein isoform 1 (PmSARIP1) cDNA and gene

The full-length cDNA of *PmSARIP1* was 1366 bp in length composing of an open reading frame (ORF) of 738 bp deducing to a polypeptide of 245 amino acids and the 5′ and 3′ UTRs of 71 and 557 bp, respectively (GenBank accession no. JX255376, Fig. 1). Three potential poly A additional signals located at 524, 278 and 13 bases upstream from the poly A tail were found. In addition, three cytoplasmic polyadenylation elements (CPEs; U/AUUUUAU/A) were also found at positions 943–949, 1211–1217 and 1244–1250 of *PmSARIP1*. The closest similarity of the deduced PmSARIP1 protein was RWD domain-containing protein 1 (small androgen receptor-interacting protein) of *Rattus norvegicus* (*E*-value = 2×10^{-40}). The predicted molecular mass and p*I* of the deduced PmSARIP1 protein was 28.54 kDa and 4.12, respectively.

Genomic organization of *PmSARIP1* was characterized by overlapping amplification of genomic DNA sequences using 5 primer pairs. The *PmSARIP1* gene was composed of 5 exons (73, 215, 144, 190 and 116 bp) and 4 introns covering 5937 bp in length (GenBank accession no. JX255377, Fig. 2). The length of each intron was 2646, 163, 453 and 1309 bp, respectively. The GC content of exons (41–49%) was much greater than that of introns (23–33%). All exon–intron boundaries of the *PmSARIP1* gene followed the GT/AG rule. Introns 1 and 4 of the *PmSARIP1* gene interrupt the ORFs between two codons (type 0 intron),

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GGCACGAGGGTCACGCTTTTGGGTTTCATGTGTTTCCTCCTAATTTCCTATTTTCTCTTC 60
AATTATTCGTCATGACAGACTACAAGGAAGAACAGAATAACGAAATTGAGGCCCTGGAGT 120
          M  T  D  Y  K  E  E  Q  N  N  E  I  E  A  L  E  S 17
CCATATATCCAGAGGAATTTGAGATAATTGATATAGAACCAAGGCACAAGTTCAGAATTA 180
  I  Y  P  E  E  F  E  I  I  D  I  E  P  R  H  K  F  R  I  T 37
CTGTCAAATCCGAAGGCTCTGATCCATATGATGAGATTCAGACGTTACCAGCAACTATTA 240
  V  K  S  E  G  S  D  P  Y  D  E  I  Q  T  L  P  A  T  I  I 57
TCCTCAACTTTGAATACACTCCAACGTATCCAGATGAACCCCCAGTCATGGAAGTCACAG 300
  L  N  F  E  Y  T  P  T  Y  P  D  E  P  P  V  M  E  V  T  A 77
CTGTTGAAAACATAGAAGAGGAAGAGCTGGATGATTTAAGAACGAAACTTAACGAGCAGT 360
  V  E  N  I  E  E  E  E  L  D  D  L  R  T  K  L  N  E  Q  C 97
GTGAGGAGAACCTGGGGATGGTCATGGTGTTCACGCTTGTCTCATACTCATTGGAGTGGC 420
  E  E  N  L  G  M  V  M  V  F  T  L  V  S  Y  S  L  E  W  L 117
TCACCACACACATGGAAGGTATTGCTCTCAGCACCAAAGAAGAATTGGATCGCAAGAAGA 480
  T  T  H  M  E  G  I  A  L  S  T  K  E  E  L  D  R  K  K  K 137
AAGAGCAGGAAGAGATAGATCGGAAAAAGTTTGAAGGTACCAGAGTGACTGTAGAAACGT 540
  E  Q  E  E  I  D  R  K  K  F  E  G  T  R  V  T  V  E  T  F 157
TTCTTGCTTGGAAAGCAAAGTTTGATACGGAGATGCAAGCACTCCGATCTGAGAAAGACA 600
  L  A  W  K  A  K  F  D  T  E  M  Q  A  L  R  S  E  K  D  R 177
GGGAGGATGAGAAGAACAAAAAACCAACTGGTAGAGAGCTCTTCATGAAGGACGTCACTT 660
  E  D  E  K  N  K  K  P  T  G  R  E  L  F  M  K  D  V  T  L 197
TGAATGAGTCGGATCTTAGCTTCCTTGGTGAAGGAGAAGGTGAGGTGACTGTTGATGAGA 720
  N  E  S  D  L  S  F  L  G  E  G  E  G  E  V  T  V  D  E  S 217
GTTTATTCCAAGACCTGGATGACTTAGACCTCGAGGATGAAGATGACGAGGATTATGTTC 780
  L  F  Q  D  L  D  D  L  D  L  E  D  E  D  D  E  D  Y  V  P 237
CAGGAGCGGATGATGACATATCTGATTAGAAACTTTAAATAAATATCATTTCATTATCAT 840
  G  A  D  D  D  I  S  D  *                                   245
ATCTTTTTTTTCTCTCTCACTCTCGTGGTTTCCCATCATGAATTTGTTTTAGTCATGGAT 900
ACTGGCTCATGAATTTTTTTTTTTTTCTTTCTTTCTTTCTTTTTTTTATACATCCGTTTT 960
TGCAGAAGATGTCTCTTCAAATGTAATGTTTGTTTGTGTTTCTTATTAGCTAAAATTAGC 1020
CCTCTTATTTATTTGGAAAAGTTGTTTTGATAATGCATTTGTAAATAAAACATTTAACAA 1080
AAATGGAGATTACTTCAGTTGTTTCAGAGGTTATTTAGTTGGGGAGTGTCACCGAAATTA 1140
TTATCTTTGTTTATTTGTTTTGCATACAGAATTAGATCATGCAAATAGACAGTTGCTTAT 1200
AAAGACCATTTTTTTATTTTTGTTTCATTCACCATGATTGTTATTTTTTATACAGTTTAAA 1260
CCATTTGTATCAAGTTGTTTTTACTTAGATTTCATCAATAGAGGAGTCAGTATGATGCAT 1320
TAGCCCTCAATAAAATGTTAAATCTGTAAAAAAAAAAAAAAAAAAAA              1366
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Fig. 1. The full-length cDNA and deduced protein sequences of *PmSARIP1* (1366 bp, ORF of 738 bp corresponding to a deduced polypeptide of 245 aa) further sequenced from the original EST by 3′ primer walking. The putative start (ATG) and stop (TAG) codons are illustrated in boldface and underlined. Three predicted CPEs are boldfaced. Three putative poly A additional signal sites are boldfaced, italicized and underlined. The predicted RWD (RING finger and WD repeat containing proteins) domain (positions 10–120) is highlighted.

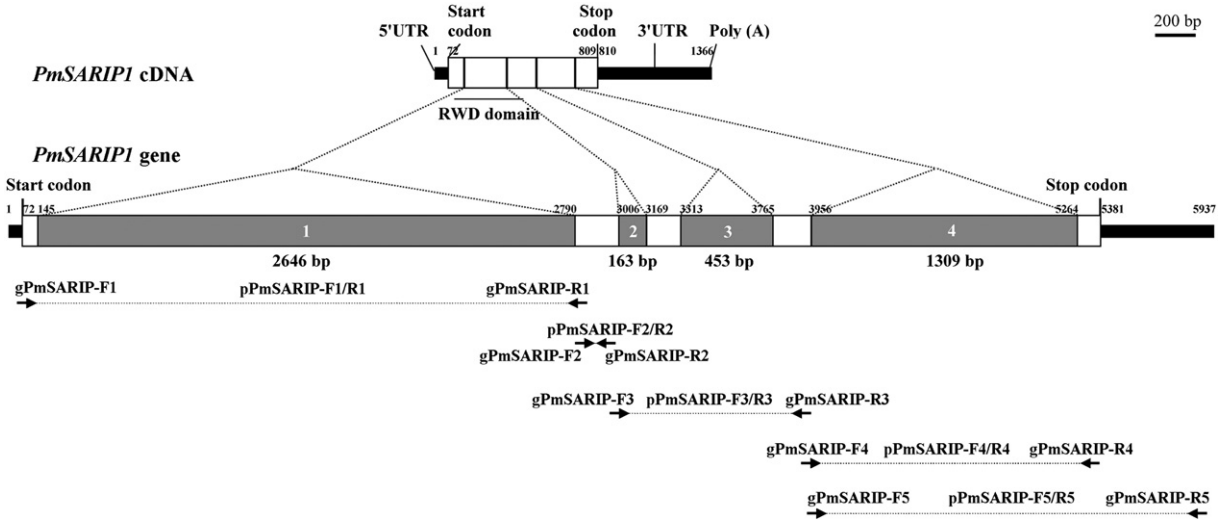

Fig. 2. Schematic diagram of the *PmSARIP1* gene. Complete cDNA was obtained by sequencing of the 3′ terminus of an EST clone whereas genomic DNA fragments were obtained from amplification of overlapping PCR fragments. Introns (with position numbers) are illustrated by gray-boxes. Names of primers and recombinant clones are shown.

whereas introns 2 and 3 interrupt the ORFs within the same codons (type 1 intron) (Fig. 2 and Table 2).

3.2. Tissue distribution analysis and expression levels of PmSARIP1 during ovarian maturation of P. monodon

PmSARIP1 was specifically expressed in gonads but not in other tissues where a greater expression level was observed in ovaries than testes of wild *P. monodon* adults (Fig. 3).

Quantitative real-time PCR revealed that the expression level of *PmSARIP1* in ovaries of intact captive juveniles (4-month-old shrimp) and intact wild adults was not significantly different at any of the analyzed stages ($P > 0.05$, Fig. 4). In intact wild adults, the expression levels of *PmSARIP1* were comparable during ovarian maturation and after spawning ($P > 0.05$). In eyestalk-ablated adults, *PmSARIP1* mRNA peaked in stage I (previtellogenic) ovaries and the expression was reduced in stages II (vitellogenic), III (early cortical rod) and IV (mature) ovaries ($P < 0.05$). Interestingly, the expression level of *PmSARIP1* in each ovarian stage of unilateral eyestalk-ablated adults was greater than that of the same ovarian stages in intact adults ($P < 0.05$, Fig. 4).

3.3. Localization of PmSARIP1 mRNA in ovaries of P. monodon

PmSARIP1 transcripts were localized in ooplasm of previtellogenic oocytes in both intact (Fig. 5B–C) and eyestalk-ablated (Fig. 5E–F) adults of wild *P. monodon* by *in situ* hybridization. Generally, oocytes at the late previtellogenic stage showed a weaker signal than did early previtellogenic oocytes. *PmSARIP1* was not detected in the germ line/germ cells, oogonia and more mature (vitellogenic, early cortical rod and mature) oocytes. No signal was observed from the sense probe (Fig. 5A and E).

3.4. Effects of 5-HT, progesterone and 17β-estradiol on transcription of PmSARIP1 in ovaries of domesticated P. monodon

Effects of 5-HT on expression of ovarian *PmSARIP1* in 18-month-old shrimp was examined. 5-HT administration resulted in a significant increase of the *PmSARIP1* expression at 6–72 hpi where the peak expression level was observed at 12 hpi ($P < 0.05$, Fig. 6).

In contrast, progesterone administration did not affect the expression of *PmSARIP1* in ovaries of 14-month-old domesticated shrimp during the treatment period (0–72 hpi, $P > 0.05$; Fig. 7).

The expression level of *PmSARIP1* in 14-month-old shrimp treated with 17β-estradiol for 7 days was greater than that of untreated shrimp and the vehicle control ($P < 0.05$) but was not significantly different from that in eyestalk-ablated shrimp ($P > 0.05$). At 14 days, the expression level of *PmSARIP1* in eyestalk-ablated shrimp was only significantly greater than the controls ($P < 0.05$). No difference among treatment was observed at 28 days of the experiments (Fig. 8).

4. Discussion

The major obstacle in the development of shrimp maturation technology is the limited knowledge of the molecular events of ovarian maturation (Benzie, 1998). The presence of vertebrate-type steroids has been documented in almost all invertebrate groups including crustaceans (Fingerman et al., 1993; Lafont, 1991; Lehoux and Sandor, 1970). Vertebrate type steroids (*e.g.* pregnenolone, testosterone, progesterone and 17α-hydroxyprogesterone) were identified in gonads

Table 2
GC content and length of exons and introns in the *PmSARIP1* gene.

Exon	Nucleotides position	GC content (%)	Intron	Nucleotide position	GC content (%)	Intron type	GT/AG rule
1	72–144 (73 bp)	41	1	145–2790 (2646 bp)	33	1	Yes
2	2791–3005 (215 bp)	40	2	3006–3168 (163 bp)	30	0	Yes
3	3169–3312 (144 bp)	49	3	3313–3765 (453 bp)	29	0	Yes
4	3766–3955 (190 bp)	43	4	3956–5264 (1309 bp)	32	1	Yes
5	5265–5380 (116 bp)	45					

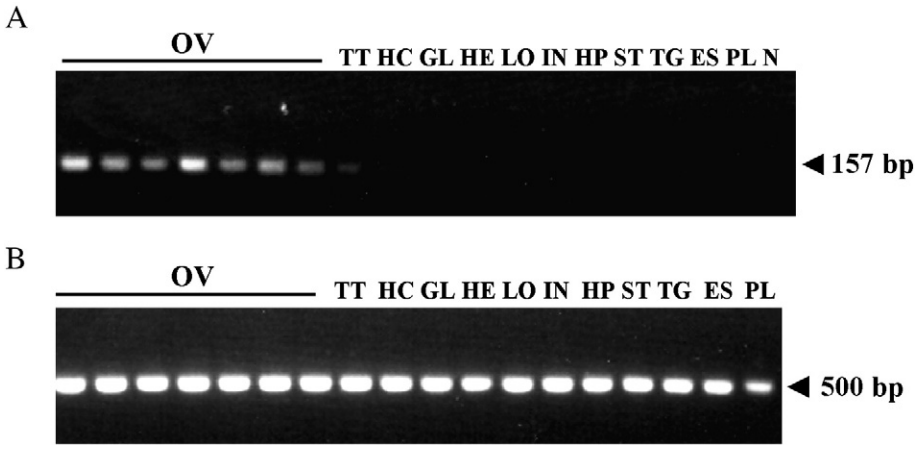

Fig. 3. (A) 1.5% ethidium bromide-stained agarose gel showing results from tissue distribution analysis of *PmSARIP1* using the first strand cDNA of ovaries of different individuals and various tissues of wild *P. monodon* adults. TT = testes, HC = hemocytes, GL = gills, HE = heart, LO = lymphoid organs, IN = intestine, HP = hepatopancreas, ST = stomach, TG = thoracic ganglion, ES = eyestalks and PL = pleopods. *EF-1α* was successfully amplified from the same template (B). Lane N is the negative control (without cDNA template).

of penaeid shrimp (Cardoso et al., 1997; Fairs et al., 1990). In addition, progesterone and 17β-estradiol was found in hemolymph, hepatopancreas and ovaries of captive *P. monodon* (Quinitio et al., 1994). Moreover, testosterone, pregnenolone, 17α-hydroxyprogesterone and progesterone were also found in gonads of *Marsupenaeus japonicus* (Cardoso et al., 1997).

Summavielle et al. (2003) showed that several key enzymes such as 17β-hydroxysteroid dehydrogenase (17β-HSD), C_{17}–C_{20} hydroxylase, 17α-hydroxylase and aromatase are present in *M. japonicus* ovaries and suggested that 17β-estradiol could be vitellogenin stimulating ovarian hormone (VSOH). This primarily suggested that sex steroids should mediate oocyte maturation in penaeid shrimp (Yano, 1985, 1987; Yano and Hoshino, 2006). In *Cancer pagurus*, 17β-HSD was detected in vitellogenic ovaries suggesting the ability to convert androstenedione to testosterone (Blanchet et al., 1972). Likewise, *17β-HSD* mRNA is found to be differentially expressed during ovarian maturation of wild *P. monodon* (S. Klinbunga, unpublished data).

Progesterone is a sex steroid hormone that plays the important roles in oocyte maturation (Miura et al., 2006; Rodríguez et al., 2002).

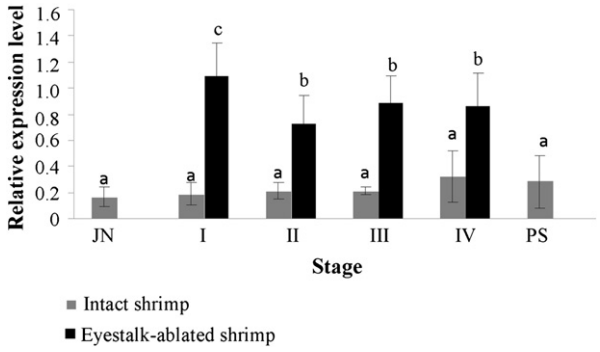

Fig. 4. Relative expression levels of *PmSARIP1* mRNA during ovarian maturation of wild intact and unilateral eyestalk-ablated *P. monodon* adults. The levels were measured as an absolute copy number of *PmSARIP1* and normalized by that of *EF-1α*. Each histogram corresponds to different stages of ovaries. The bars correspond to the standard deviation (SD) of the means. The same letters above bars indicate that the expression levels were not significantly different ($P > 0.05$). JN = juvenile ovaries; I–IV = previtellogenic, vitellogenic, early cortical rod, and mature ovaries, respectively; PS = ovaries of intact adults immediately collected after spawning (stage V). The number of individuals for each sample group is 10, 5, 7 and 9 individuals and 6, 5, 10 and 9 individuals for stages I–IV ovaries of intact and eyestalk-ablated adults and 5 individuals for 4-month-old juveniles and post-spawning intact adults, respectively.

Nevertheless, several steroids other than progesterone can also act as potent promoters of oocyte maturation *in vitro* (Smith and Ecker, 1971). Lutz et al. (2001) revealed that progesterone was nearly undetectable in the serum and ovaries of hCG-injected *Xenopus laevis* adults regardless the dose and time after injection but androstenedione and testosterone were significantly more abundant. In addition, progesterone was rapidly converted to androstenedione in isolated oocytes suggesting that androgens were also the dominant mediators of *Xenopus* oocyte maturation *in vivo*.

In this study, a homologue of *P. monodon SARIP1* cDNA and gene were successfully identified. The deduced PmSARIP1 protein contained an RWD domain which is functionally proposed as a protein–protein interaction domain (Doerks et al., 2002). A highly conserved YPXXXP motif of the RWD domain (Nameki et al., 2004) is also found in the deduced PmSARIP1 protein. Three potential polyadenylation signal sequences (AATAAA) were found in *PmSARIP1* suggesting the possible selective polyadenylation usage. In addition, the CPE motifs were observed in *PmSARIP1* as previously reported in *cyclin A* and *cyclin B* of *P. monodon* (Visudtiphole et al., 2009). In oocytes, mRNA of several genes contains a conserved U-rich sequence, called CPE, in their 3′ UTR (Nishimura et al., 2009). Cytoplasmic polyadenylation is one of the translational regulation mechanisms for maternal mRNAs during oogenesis (Katsu et al., 1999; Nishimura et al., 2009; Tremblay et al., 2005). Genomic organization of the *PmSARIP1* gene was also successfully characterized in this study allowing further examination whether exon/intron alternative splicing is existent in this gene.

Tissue-specific transcription is important during the development and maturation of specific cell types (Grimes, 2004). *Vasa* which encodes an ATP-dependent RNA helicase belonging to the DEAD-box protein family, was isolated in the Pacific white shrimp, *L. vannamei* (Alfalo et al., 2007), and the giant freshwater prawn, *Macrobrachium rosenbergii* (Nakkrasae and Damrongphol, 2007). *Vasa* was only expressed in gonads of adults of both species. Likewise, gonad-specific expression of *PmSARIP1* suggested its essential role in oogenesis and spermatogenesis of *P. monodon*.

Recently, the full-length cDNAs of *progestin membrane receptor component 1* (*PmPgmrc1*) were isolated from both testes (Leelatanawit et al., 2008) and ovaries (Preechaphol et al., 2010) of *P. monodon*. Unilateral eyestalk ablation resulted in a greater expression of *PmPgmrc1* in vitellogenic, early cortical rod and mature ovaries compared to that in the same ovarian stages of intact adults. Immunohistochemistry revealed the positive signals of the PmPgmrc1 protein in the follicular layers and cell membrane of follicular cells and various stages of oocytes (Preechaphol et al., 2010). However, the presence of nuclear androgen receptor(s) and associated proteins have not been reported in penaeid

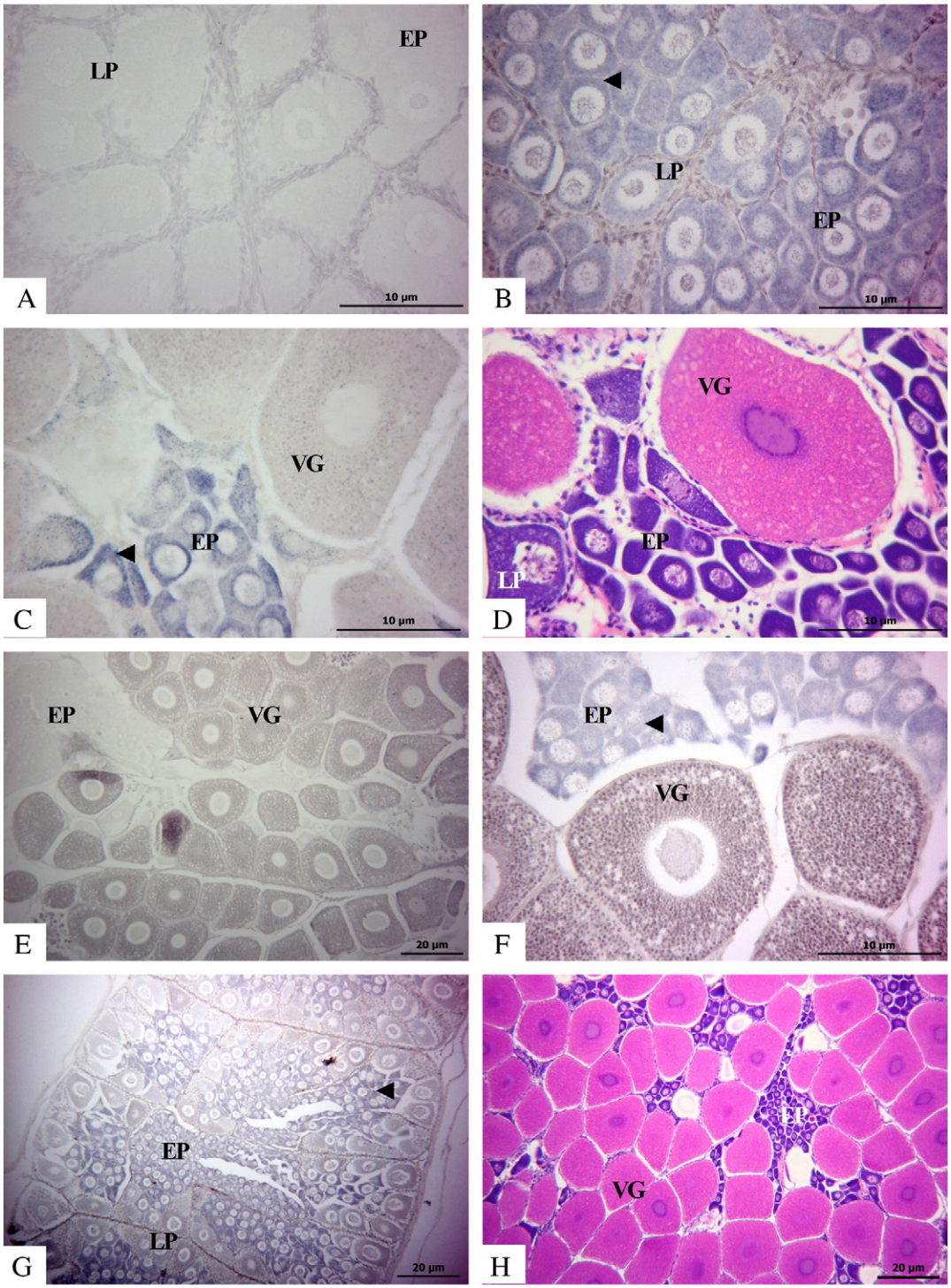

Fig. 5. Localization of the *PmSARIP1* transcript during ovarian maturation of intact (A–C) and eyestalk-ablated (E–G) adults of wild *P. monodon* visualized by *in situ* hybridization using the sense (A and E) and antisense cRNA probes (B–C and F–G). Conventional hematoxylin/eosin (HE) staining was carried out for classification of oocyte stages (D and H). EP = early previtellogenic oocytes; LP = late previtellogenic oocytes; VG = vitellogenic oocytes. Arrowheads indicate the positive hybridization signals.

shrimp. In the present study, we showed that the expression level of ovarian *PmSARIP1* in each stage of intact adults was significantly lower than that of the same stages in eyestalk-ablated adults ($P < 0.05$). The results suggest that the *PmSARIP1* gene/protein is involved in maturation of oocyte and ovaries of *P. monodon*.

Eyestalk ablation significantly promotes expression of *PmSARIP1* during vitellogenesis and final ovarian maturation of *P. monodon*. Apparently, the expression profiles of *PmSARIP1* and *PmPgmrc1* (Preechaphol et al., 2010) in eyestalk-ablated adults were similar suggesting that these transcripts positively responded to eyestalk-ablation and gonad inhibiting hormone (GIH) affects transcription of both *PmPgmrc1* and *PmSARIP1*. Accordingly, the expression profiles of *PmSARIP1* may be used as molecular indicators for investigation of the progression in reproductive maturation of female *P. monodon* adults as a consequence of maturation-inducing feed and/or exogenous hormone/neurotransmitter administration.

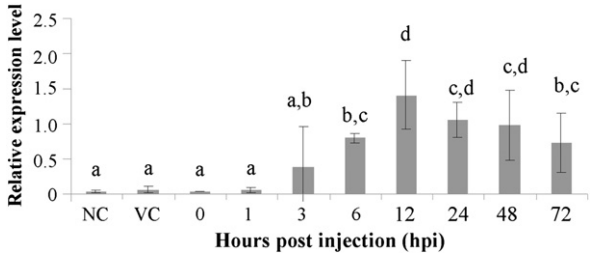

Fig. 6. Time-course relative expression levels of ovarian *PmSARIP1* in 18-month-old domesticated shrimp at 0, 1, 3, 6, 12, 24, 48 and 72 h after 5-HT injection (50 μg/g body weight, $N = 4$ for each stage). Acclimated shrimp without any treatment and those injected with the saline solution (0.85% NaCl) at 0 hpi were included as the negative (NC) and vehicle (VC) controls, respectively. The bars correspond to the standard deviation (SD) of the means. The same letters above bars indicate that the expression levels were not significantly different ($P > 0.05$).

In situ hybridization signals from the *PmSARIP1* transcript were clearly observed in previtellogenic oocytes suggesting that it may have a function in meiosis. The signal of *PmSARIP1* was not observed in follicular cells, oogonia and more mature (vitellogenic, early and late cortical rod) oocytes. It should be noted that oocyte stages within a single ovarian lobe are not synchronous (Medina et al., 1996). The ovarian maturation stages were determined based on the predominant oocyte type found in ovaries. Generally, previtellogenic, vitellogenic, early cortical rod and mature oocytes are approximately 25–50, 100, 200 and 250–300 μm in diameter. Our study showed contradictory results from quantitative real-time PCR and *in situ* hybridization on the disappearance of *PmSARIP1* hybridization signals from the ooplasm in oocytes at later stages that may be due to a significant increase in oocyte sizes as oogenesis proceeded. In addition, real-time PCR detects gene expression with much greater sensitivity than *in situ* hybridization.

Results in the present study further confirmed positive molecular effects of 5-HT on transcription of *PmSARIP1* in domesticated shrimp at 6–72 hpi with more prominent stimulating effects (4–15 fold) than that previously examined in *PmOST1*, *PmBr-c* and *PmADRP* (1.5–2.0 fold, Buaklin, 2010; Klinbunga et al., 2009; Sittikankaew et al., 2010). Accordingly, 5-HT seems to affect the transcription of several pathways and could be used for stimulation of ovarian maturation of *P. monodon*.

Molecular effects of steroid hormonal induction on oocyte maturation in shrimp is not well understood at present (Yano and Hoshino, 2006). Although progesterone is recognized as maturation inducing hormone (MIH) that promotes meiotic resumption of oocytes in *Xenopus* and other lower vertebrates (Hammes, 2004; Miura et al., 2006), progesterone injection (0.1 μg/g body weight; 14-month-old shrimp) did not result in a significant increase on the transcription of *PmSARIP1* mRNA in the present study.

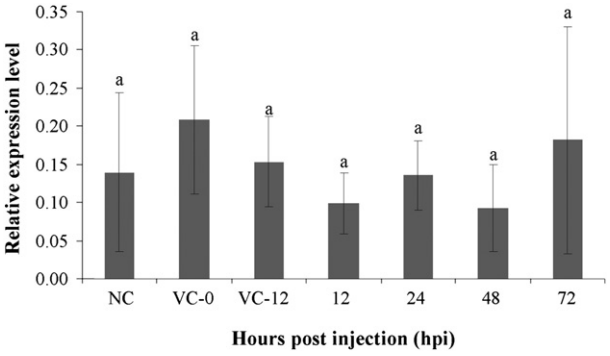

Fig. 7. Time-course relative expression levels of *PmSARIP1* in ovaries of 14-month-old domesticated shrimp at 12, 24, 48 and 72 hpi after progesterone injection (0.1 μg/g body weight, $N = 4$ for each stage). Acclimated shrimp without any treatment and those injected with absolute ethanol at 0 and 12 hpi were included as the negative (NC) and vehicle (VC-0 and VC-12) controls, respectively. The bars correspond to the standard deviation (SD) of the means. The same letters above bars indicate that the expression levels were not significantly different ($P > 0.05$).

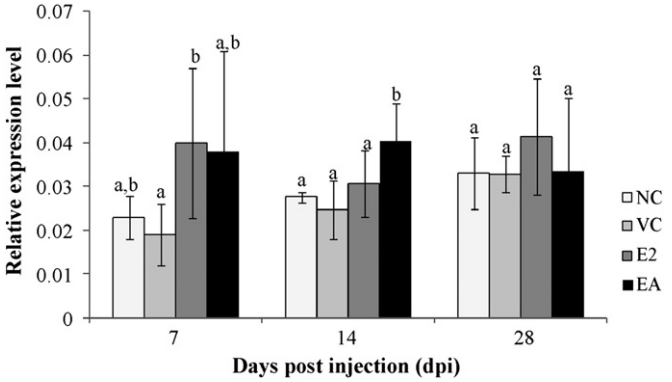

Fig. 8. Relative expression levels of *PmSARIP1* in ovaries of 14-month-old domesticated shrimp at 7, 14 and 28 days after injection with 17-β estradiol (0.01 μg/g body weight, $N = 4$ for each group). Non-injected shrimp, those injected with 5% ethanol and eyestalk-ablated shrimp were included as the negative, vehicle and positive controls, respectively. The bars correspond to the standard deviation (SD) of the means. The same letters above bars indicate that the expression levels were not significantly different ($P > 0.05$).

The effects of 17β-estradiol on induction of vitellogenin synthesis and oocyte maturation of *M. japonicus* were investigated *in vitro*. Vitellogenin (Vg) concentrations in primary vitellogenic oocytes in immature ovaries cultured in M199 containing 17β-estradiol (3.6, 36.7, 367 and 3671 nM) for 3 days were significantly greater than those of the controls (Yano and Hoshino, 2006). In the present study, exogenous 17 β-estradiol administration (at 0.1 μg/g body weight) seemed to promote the expression of *PmSARIP1* at day 7 after treatment compared to the non-injected and the vehicle controls ($P < 0.05$). The inducing effect of 17β-estradiol treatment on the expression of *PmSARIP1* in domesticated *P. monodon* was not as rapid as the serotonin treatment but seemed to be similar as that from eyestalk ablation ($P > 0.05$). The findings in this study open the possible use of serotonin for initial induction followed by repeated injection of 17β-estradiol for more effective promotion of ovarian maturation of *P. monodon* in captivity.

Taking all the information together, *PmSARIP1* seems to play a role on oocyte maturation in *P. monodon*. Functional studies should be carried out to determine whether the PmSARIP1 protein enhances any transactivation activity of a nuclear receptor pathway in *P. monodon*.

Acknowledgments

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