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Characterization and expression analysis of *Cyclin-dependent kinase 7* gene and protein in ovaries of the giant tiger shrimp *Penaeus monodon*

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ABSTRACT

The characterization of genes differentially expressed in ovaries is necessary for understanding the mechanisms involving ovarian developmental processes of the giant tiger shrimp (*Penaeus monodon*). Here, the full-length cDNA of *P. monodon Cyclin-dependent kinase 7* (*PmCdk7*) was characterized. It was 1407 bp in length (ORF of 1062 bp corresponding to 353 amino acids). The expression of *PmCdk7* in the ovaries was greater than that in the testes ($P < 0.05$). *PmCdk7* was significantly up-regulated in stage IV (mature) ovaries in wild intact broodstock ($P < 0.05$). In wild eyestalk-ablated broodstock, its expression was earlier increased in stage III (late vitellogenic) ovaries ($P < 0.05$). The expression level of *PmCdk7* during ovarian development (stages I–IV ovaries) in eyestalk-ablated broodstock was greater than that of the same ovarian stages in intact broodstock ($P < 0.05$). 5-HT (50 μg/g body weight) injection promoted its expression level in ovaries of domesticated 18-month-old shrimp at 6–48 h post injection (hpi). The rPmCdk7 protein and its polyclonal antibody were successfully produced. The positive immunoreactive band (67 kDa) was not observed in premature ovaries of juveniles. Western blot analysis revealed differential expression of PmCdc7 in stages II–IV ovaries compared with stage I ovaries in intact broodstock but it was comparably expressed among different ovarian developmental stages in eyestalk-ablated broodstock. Immunofluorescence revealed the positive signals of PmCdk7 in the ooplasm of previtellogenic oocytes and its subsequent nucleo-cytoplasmic translocation during oocyte development. Results indicated that PmCdk7 seems to play functional roles in the development and maturation of oocytes/ovaries in *P. monodon*.

1. Introduction

Reduced reproductive performance of the giant tiger shrimp (*Penaeus monodon*) in captivity prevents the potential application of domestication and selective breeding program for genetic improvement of this economically important species (Preechaphol et al., 2007; Withyachumnarnkul et al., 1998). Farmers use unilateral eyestalk ablation to induce ovarian maturation of *P. monodon* but this technique leads to stress and the death of spawners (Benzie, 1998; Okumura and Sakiyama, 2004; Wongprasert et al., 2006). To avoid the use of eyestalk ablation, understanding molecular mechanisms controlling the development and maturation of ovaries/oocytes is essential for finding an alternative approach to trigger the reproductive maturation of *P. monodon* without the undesirable effects from eyestalk ablation (Hiransuchalert et al., 2013; Preechaphol et al., 2010; Quackenbush, 2001).

The meiotic maturation of animal oocytes is controlled by the maturation-promoting factor (MPF), a complex of Cdc2 and Cyclin B (Okano-Uchida et al., 1998). In most species, cytoplasmic MPF is maintained in the inactive form (called pre-MPF) by inhibitory phosphorylation of Cdc2 at Thr14 and Tyr15 by Myt1 kinase and at Thr161 by cyclin-activating kinase (CAK), a complex of Cyclin-dependent kinase 7 (Cdk7)/Cyclin H or Cdk7/Cyclin H/Mat 1 (Harper and Elledge, 1998; Patel and Simon, 2010; Tassan et al., 1995). Dephosphorylation of Thr14 and Tyr15 residues of Cdc2 by Cdc25 phosphatase leads to the resumption of meiotic maturation of oocytes (Clarke et al., 1992;

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Dunphy and Kumagai, 1991; Dunphy et al., 1988; Jessus et al., 1991; Mueller et al., 1995). Alternatively, a different mechanism of oocyte resumption has been reported in some amphibians and fishes where Cdc2 presents as a monomer with no phosphorylation due to the absence of Cyclin B in immature oocytes. Only Thr161 phosphorylation by CAK is required for MPF activation (Hirai et al., 1992; Honda et al., 1993; Kobayashi et al., 1991; Yamashita et al., 1995). In addition, CAK also acts as a transcriptional regulator in association with the transcription factor II H (TFIIH) (Nigg, 1996; Sclafani, 1996). In zebrafish, the function of CAK is especially important during the early development and *Cdk7* and *Cyclin H* mRNAs were shown to be maternally loaded (Liu et al., 2007).

Recently, *Penaeus monodon Cdc2* (*PmCdc2*) was characterized (Phinyo et al., 2013). Its ORF was 900 bp in length corresponding to a polypeptide of 299 amino acids with the conserved Thr14, Tyr15 and Thr161 residues. The expression level of *PmCdc2* transcript in wild intact broodstock was significantly increased in stages II (vitellogenic) and III (late vitellogenic) ovaries relative to stage I (previtellogenic) ovaries and peaked in stage IV (mature) ovaries ($P < 0.05$). Eyestalk ablation significantly promoted the expression level of *PmCdc2* during ovarian development of *P. monodon*. Using phospho-Cdc2 (Thr161) polyclonal antibody, the immunoreactive signal of 34 kDa (active PmCdc2) was observed in all ovarian stages but the most intense signal was found in stage IV ovaries (Phinyo et al., 2013). This indirectly suggested the important role of CAK on the activation of oocyte development in *P. monodon*.

To examine the molecular mechanisms of Cdk7 involvement in ovarian (and oocyte) development of *P. monodon*, the expression patterns of *P. monodon Cdk7* (*PmCdk7*) during ovarian development in wild intact and eyestalk-ablated shrimp were examined. In addition, the effects of serotonin (5-HT) on the expression of *PmCdk7* mRNA in domesticated shrimp were illustrated. The recombinant PmCdk7 protein and its polyclonal antibody were produced. Expression patterns of the PmCdk7 protein during ovarian development of *P. monodon* were examined. Localization of the PmCdk7 protein in different stages of oocytes was determined by immunofluorescence.

2. Materials and methods

2.1. Experimental animals

For examining the expression profiles of *PmCdk7* mRNA during ovarian development, female broodstock were wild-caught from the Andaman Sea and acclimated under the farm conditions for 2–3 days. The post-spawning group (stage V ovaries) was immediately collected after the shrimp were spawned. The ovaries were dissected out from each broodstock and weighed. For the eyestalk ablation group, wild broodstock were acclimated for 7 days prior to unilateral eyestalk ablation. The ovarian developmental stage of each shrimp was assessed by external visualization. Ovaries of eyestalk-ablated shrimp were collected at 2–7 days after ablation. The gonadosomatic index (GSI, ovarian weight/body weight $\times$ 100) of each shrimp was calculated. The ovarian developmental stages of wild shrimp were classified according to the GSI values: <1.5, 2–4, 4–6 and >6% for stages I (previtellogenic), II (vitellogenic), III (late vitellogenic) and IV (mature) ovaries, respectively. The ovarian developmental stages of wild shrimp were confirmed by conventional histology (Qiu et al., 2005). In addition, domesticated juveniles (4-month-old) were collected from the Broodstock Multiplication Center (BMC), Burapha University (Chanthaburi, Thailand) and included in the experiments.

To determine whether *PmCdk7* mRNA was differentially expressed between the gonads of male and female shrimp, the ovaries and testes of cultured juveniles and wild broodstock ($N = 5$ for each group) were collected, immediately placed in liquid N_2 and kept at −80 °C until needed.

For examining the effects of serotonin (5-HT) on the expression of *PmCdk7* mRNA, domesticated 18-month-old *P. monodon* broodstock were sampled and acclimated for 7 days at laboratory conditions (28–30 °C and 30 psu seawater under natural daylight) in 500-liter aquaria. Eight groups of female shrimp (average body weight = 106.80 ± 21.42 g) were injected intramuscularly into the first abdominal segment with 5-HT (50 μg/g body weight; the working solution = 25 μg/μL in 0.85% NaCl). The specimens were collected at 0, 1, 3, 6, 12, 24 and 48 h post injection (hpi). Shrimp injected with the 0.85% saline solution (at 0 hpi) were included as the vehicle control (VC).

For determining the expression profiles of PmCdk7 protein during ovarian development, wild intact adults having different stages of ovarian development were collected ($N = 3$ for each stage). Eyestalk-ablated broodstock were prepared as described previously ($N = 3$ for each stage).

2.2. Total RNA extraction and first strand cDNA synthesis

Total RNA was extracted from the ovaries of *P. monodon* using TRI Reagent following the protocol recommended by the manufacturer (Molecular Research Center). The concentration of extracted total RNA was spectrophotometrically measured (Sambrook and Russell, 2001). Ten micrograms of total RNA was 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 and subjected to a phenol–chloroform extraction. One and a half microgram of DNase I-treated total RNA was reverse-transcribed using an ImProm-II™ Reverse Transcription System (Promega).

2.3. Identification of the full-length cDNA of PmCdk7

The partial cDNA of *Cdk7* was originally identified from the ovarian cDNA library of *P. monodon* (clone no. OV-N-N01-0378-W; Preechaphol et al., 2007). The full-length cDNA of *PmCdk7* was identified by further sequencing of the original EST clone by a primer walking approach. The nucleotide sequence of an EST clone was searched against previously deposited sequences in GenBank using Blast*N* and Blast*X* (Altschul et al., 1990; available at http://ncbi.nlm.nih.gov). The p*I* value and molecular weight of the deduced PmCdk7 protein were examined using ProtParam (http://www.expasy.org/tools/protparam.html). The protein domain in the deduced PmCdk7 protein was predicted using SMART (http://smart.embl-heidelberg.de).

2.4. RT-PCR analysis

Expression of $PmCdk7_{123}$ in ovaries and testes of cultured juveniles and wild broodstock ($N = 5$ for each group) was analyzed by RT-PCR (Sittikankaew et al., 2010) with primers $PmCdk7_{123}$-F/R (5′-TCTTTCCT GCTGCCAGTGAT-3′ and 5′-GGACAGGCTTATTGCTGAAAT-3′). *EF-1*α_{500} (F: 5′-ATGGTTGTCAACTTTGCCCC-3′ and R: 5′-TTGACCTCCTTGATCACA CC-3′) amplified from the cDNA template of the same individuals was included as the positive control. The thermal profiles were 94 °C for 3 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 45 s and extension at 72 °C for 45 s. The final extension was carried out at 72 °C for 7 min. The amplicon was electrophoretically analyzed on a 2.0% agarose gel and visualized with a UV transilluminator after ethidium bromide (0.5 μg/mL) staining (Sambrook and Russell, 2001).

2.5. Quantitative real-time PCR

Standard curves representing 10^2–10^8 copies of recombinant plasmids of $PmCdk7_{123}$ and the internal control, *EF-1*α_{214} (F: 5′-TCCGTCTT

CCCCTTCAGGACGTC-3′ and R: 5′-CTTTACAGACACGTTCTTCACGTTG-3′), were constructed. Each concentration was carried out in triplicate. The expression of *PmCdk7*$_{123}$ and *EF-1*α_{214} in the ovaries of each shrimp was amplified in a 10 μL reaction volume containing 5 μL of 2× LightCycler 480 SYBR Green I Master (Roche), 100 ng of the first strand cDNA template and 0.2 μM of each primer. The thermal profile for quantitative real-time PCR was 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s. Real-time PCR of each specimen was carried out in duplicate.

2.6. In vitro expression of recombinant (r) PmCdk7 protein

The rPmCdk7 protein was expressed in the bacterial expression system. Plasmid DNA containing the complete ORF of *PmCdk7* was used as the template for amplification of the insert using primers rPmCdk7-*Nde*I-F (5′-CCG*CATATG*GAAGTAGAACAAGAGAAG-3′) and rPmCdk7-*Nco*I + HIS-R (5′-CGG***CCATGG***CTA**ATGATGATGATGATGATG**GAATTGAAGCTTCTTTGCTA-3′) containing a *Nde* I site (italicized) and a *Nco* I site (boldfaced and italicized) and six His encoded nucleotides (boldfaced), respectively. The amplification product was digested and electrophoretically analyzed. The gel-eluted product was ligated into *Nde* I/*Nco* I-digested pET29a vector and transformed into *E. coli* JM109 (Sambrook and Russell, 2001). The positive clones were sequenced to confirm the orientation of an insert and subsequently transformed into *E. coli* BL21-CodonPlus (DE3)-RIPL. A single colony of the recombinant clone was inoculated into 3 mL of LB medium supplemented with 50 μg/mL of kanamycin and 50 μg/mL of chloramphenicol at 37 °C. The overnight culture (50 μL) was transferred to 50 mL of LB medium containing kanamycin and chloramphenicol and further incubated to an OD_{600} of 0.4–0.6. One OD_{600} milliliter was time-interval taken at 0, 1, 2, 3, 6, 12 h and overnight after IPTG induction (1.0 mM final concentration). The culture was centrifuged at 12,000 *g* for 1 min, resuspended with 1× PBS and analyzed by 15% SDS-PAGE (Laemmli, 1970). In addition, 20 mL of the IPTG-induced culture was centrifuged, resuspended in lysis buffer (50 mM Tris-HCl; pH 7.4, 500 mM urea, 50 mM NaCl, 50 mM EDTA; pH 8.0 and 1 mg/mL lysozyme) and sonicated 2–3 times at 15% amplitude (pulse on 20 s and pulse off 20 s) for 5 min. The protein concentration of both soluble and insoluble fractions was measured (Bradford, 1976). Overexpression of the rPmCdk7 protein was analyzed by 12% SDS-PAGE. For Western blot analysis, the electrophoresed proteins were transferred to a PVDF membrane (Towbin et al., 1979) and analyzed against anti-6X His MAb as previously described (Sittikankaew et al., 2010).

2.7. Polyclonal antibody production and immunographic purification

The rPmCdk7 protein was purified using a His GraviTrap kit (GE Healthcare) under denaturing conditions (Sittikankaew et al., 2010). Each fraction of the washing and eluting fraction was analyzed by SDS-PAGE and Western blotting. The purified protein was stored at −20 °C. Anti-rPmCdk7 polyclonal antibody (PAb) was immunologically produced in a rabbit using the purified rPmCdk7 as an immunogen. The obtained anti-rPmCdk7 PAb was further purified using a HiTrap NHS-activated HP column following the protocol recommended by the manufacturer (GE Healthcare).

2.8. Specificity and sensitivity of purified anti-rPmCdk7 PAb

The sensitivity of detection for purified anti-rPmCdk7 PAb was examined against varying amounts (1.0, 0.8, 0.6, 0.4, 0.2, 0.1, 0.05, 0.03, 0.01 μg) of the rPmCdk7 protein. The electrophoretically separated protein was transferred onto a PVDF membrane (Hybond P, GE Healthcare) (Towbin et al., 1979) in 25 mM Tris, 192 mM glycine (pH 8.3) buffer containing 20% methanol at 100 V for 90 min. The membrane was treated with 5% BSA blocking solution (Sigma) overnight and incubated with purified anti-rPmCdk7 PAb (1:100 in the blocking solution) for 1 h at room temperature. The membrane was washed 3 times with 1× Tris Buffered Saline-Tween-20 (TBST; 50 mM Tris-HCl, 0.15 M NaCl, pH 7.4, 0.1% Tween-20) and incubated with goat anti-rabbit IgG (H + L) conjugated with alkaline phosphatase (Bio-Rad Laboratories) at 1:5000 for 1 h and washed 3 times with 1× TBST. Immunoreactive signals were visualized using NBT/BCIP (Roche) as the substrate.

Cross reactivity of purified anti-rPmCdk7 PAb was tested against 0.2 μg of other recombinant proteins of *P. monodon* previously produced in our laboratory including downstream of receptor kinase (rPmDRK), cAMP-dependent protein kinase, catalytic beta a-like (rPmPKACB), Cell division cycle 2 (rPmCdc2), Cyclin B (rPmCyclinB), Semaphorin (rPmSema) and Rpd3 histone deacetylase (rPmRpd3). Western blot analysis was carried out as previously described.

To illustrate the specificity of anti-rPmCdk7 PAb against the positive band of 67 kDa, an antigen-antibody competition experiment was carried out. Briefly, 0 (no competition), 1, 2.5, 5 and 10 μg of rPmCdk7 were separately added to 3.5 mL of purified anti-rPmCdk7 PAb (1:100). The reaction mixture was incubated at room temperature for 1 h. The resulting antibody was used for blotting against size-fractionated 2.5, 5, 10 and 20 μg total proteins extracted from the ovaries of a vitellogenic female (body weight = 255.16 g and GSI = 3.04%).

2.9. Western blot analysis of ovarian proteins

Approximately 0.5 g of frozen ovaries of *P. monodon* was ground to a fine powder in the presence of liquid N_2 and suspended in 1.2 mL of the TCA-acetone extraction buffer: 10% TCA in acetone containing 0.1% DTT and complete protease inhibitor cocktail (Roche), and left at −20 °C for 1 h. The mixture was centrifuged at 10,000 *g* for 30 min at 4 °C and the supernatant was discarded. The pellet was washed three times with acetone before being centrifuged at 10,000 *g* for 30 min at 4 °C. The resulting pellet was air-dried and dissolved in the lysis buffer (30 mM Tris–HCl, 2 M thiourea, 7 M urea, 4% CHAPS, w/v). The amount of extracted total proteins was measured by a dye binding assay (Bradford, 1976). Twenty micrograms of total ovarian proteins was heated at 100 °C for 5 min and immediately cooled on ice. Proteins were size-fractionated on a 12% SDS-PAGE (Laemmli, 1970). Electrophoretically

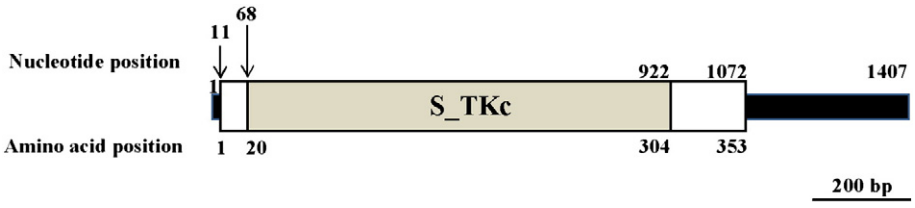


Fig. 1. A schematic diagram representing the full-length cDNA of *PmCdk7* (1407 bp containing an ORF of 1062 bp corresponding to a deduced polypeptide of 353 amino acids; accession no. KJ601727). The predicted S_TKc domain (positions 20–304) in the deduced PmCdk7 protein is indicated by label and shading.

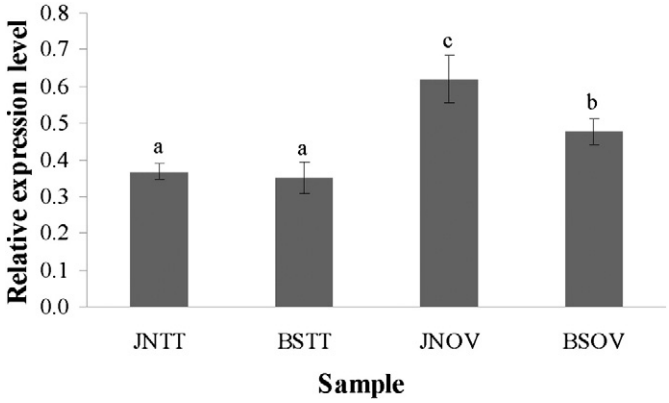


Fig. 2. Mean relative expression levels of *PmCdk7* in testes of cultured juveniles (JNTT) and wild broodstock (BSTT) and ovaries of cultured juveniles (JNOV) and wild broodstock (BSOV) of *P. monodon* analyzed by RT-PCR ($N = 5$ for each group). Each histogram corresponds to a particular ovarian stage. The same letters above the histograms reveal non-significant differences between groups of samples ($P > 0.05$).

separated proteins were transferred onto a PVDF membrane (Hybond P) (Towbin et al., 1979). The membrane was washed three times with 1× TBST (50 mM Tris–HCl, 0.15 M NaCl, pH 7.4, 0.1% Tween20) for 15 min, blocked with 20 mL of the blocking buffer (1.0 g of BSA in 20 mL of 1× TBST) and incubated overnight at room temperature with gentle shaking. The membrane was washed three times in 1× TBST and incubated with anti-rPmCdk7 PAb (1:100). The membrane was washed three times with 1× TBST and incubated with goat anti-rabbit IgG (H + L) conjugated with alkaline phosphatase (1:5000; Bio-Rad Laboratories) for 1 h and washed 3 times with 1× TBST. Immunoreactive signals were visualized using NBT/BCIP (Roche) as the substrate.

2.10. Immunofluorescence

Localization of the PmCdk7 protein was examined. Ovaries of intact and eyestalk-ablated *P. monodon* broodstock were fixed in 4% paraformaldehyde prepared in 0.1% phosphate-buffered saline (PBS, pH 7.4) 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 used. Conventional paraffin sections (5 μM) were prepared. The

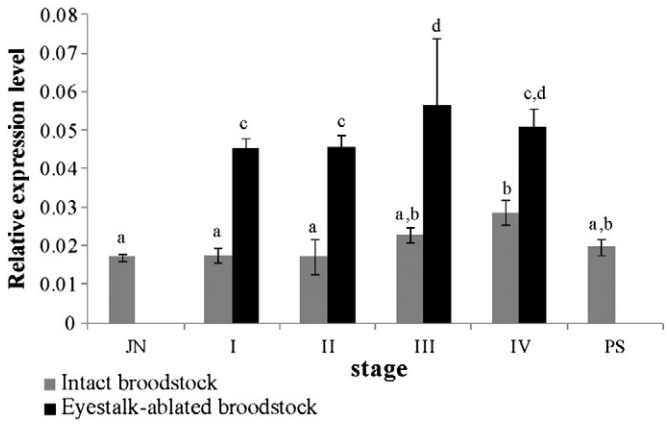


Fig. 3. Histograms showing mean relative expression levels of *PmCdk7* during ovarian development of intact and unilateral eyestalk-ablated broodstock of *P. monodon*. Bars labeled with the same letters above the histograms reveal non-significant differences between groups of samples ($P > 0.05$). JN = juvenile ovaries; I–IV = previtellogenic, vitellogenic, late vitellogenic, and mature ovaries, respectively; PS = ovaries of intact adults immediately collected after spawning (stage V).

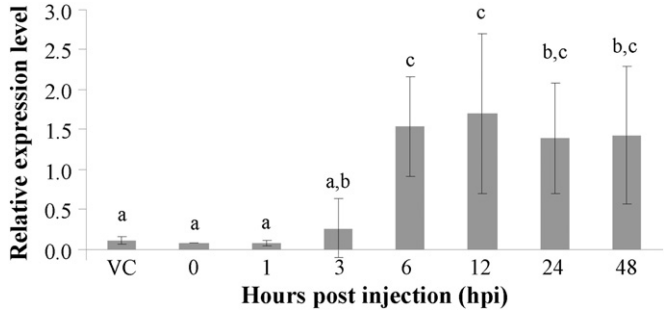


Fig. 4. Mean relative expression levels of *PmCdk7* in ovaries of domesticated 18-month-old shrimp injected with 5-HT and assessed at 0, 1, 3, 6, 12, 24, and 48 hpi ($N = 4$ for each group). Bars labeled with the same letters above the histograms reveal non-significant differences between groups of samples ($P > 0.05$). VC = shrimp injected with 0.85% NaCl at 0 hpi (vehicle control).

sections were deparaffinized and rehydrated with 95% and absolute ethanol for 5 min each and immersed in 1× PBST containing 1% glycine for 15 min. After treatment with normal goat serum (10% NGS in PBST) for 2 h, the sections were incubated with purified anti-rPmCdk7 PAb (1:10) for 2 h at room temperature, and rinsed with 1× PBST three times for 20 min each. Tissue sections were incubated with goat anti-rabbit IgG conjugated with Alexa 488 (1:200 in the blocking solution) for 2 h and rinsed with 1× PBST three times for 20 min each. The slides were viewed and image-captured by a confocal laser scanning microscope (Olympus Fluoview FV1000). Tissue sections were also incubated with preimmune rabbit serum as the negative control.

2.11. Statistical analysis

For expression analysis of *PmCdk7* in ovaries and testes of juveniles and broodstock ($N = 5$ for each group) using RT-PCR, the intensities of $PmCdk7_{123}$ and $EF\text{-}1\alpha_{500}$ of each specimen were quantified from gel photographs by using Quantity One software (Bio-Rad). The relative expression level of *PmCdk7* (intensity of the target/intensity of $EF\text{-}1\alpha_{500}$) in all experimental groups was calculated.

For quantitative real-time PCR analysis, the relative expression level of $PmCdk7_{123}$ and $EF\text{-}1\alpha_{214}$ mRNA of each specimen was evaluated from their standard curves. The relative expression levels (copy number of $PmCdk7_{123}$ and $EF\text{-}1\alpha_{214}$) between shrimp possessing different stages

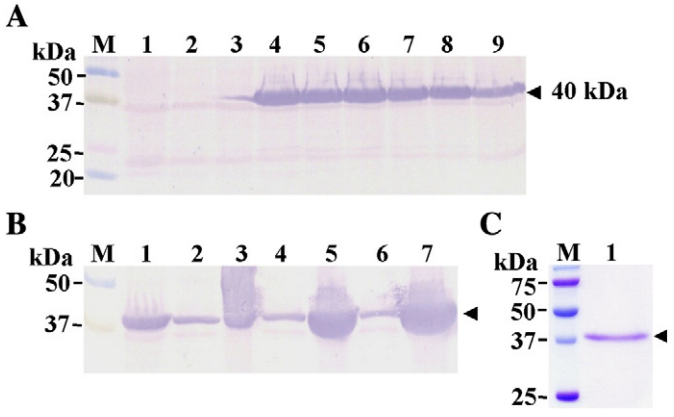


Fig. 5. A. Western blot analysis showing *in vitro* expression of the rPmCdk7 protein at 0, 1, 2, 3, 6, 12 and 24 h (lanes 3–9) after induction with 1 mM IPTG. *E. coli* BL21-CodonPlus (DE3)-RIPL and *E. coli* BL21-CodonPlus (DE3)-RIPL containing pET 29a vector (lanes 1–2) were included as the control. B. Western blot analysis using 20 μg each of proteins from whole cells of clone no. 1 (lane 1), soluble fractions (lanes 2, 4 and 6 for clones no. 1, 2 and 3, respectively) and insoluble fractions (lanes 3, 5 and 7 for clones no. 1, 2 and 3, respectively) against anti-6XHis MAb. C. SDS-PAGE of rPmCdk7 purified under the denaturing condition after culture at 37 °C and induced with 1 mM IPTG for 2 h.

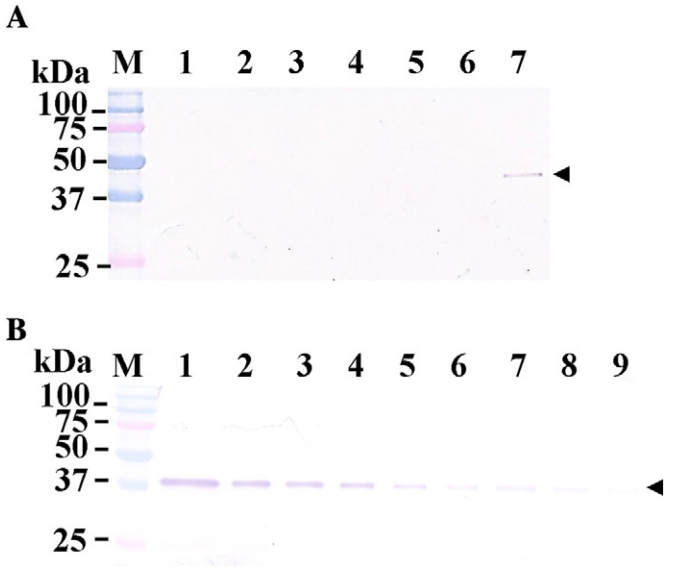


Fig. 6. Specificity of the anti-rPmCdk7 PAb was tested against various recombinant proteins (0.2 μg) of *P. monodon* including rPmDRK, rPmPKACB, rPmCdc2, rPmCyB, rPmSema, rPmRpd3 and rPmCdk7 (A). The sensitivity of anti-rPmCdk7 PAb was tested against varying amounts of rPmCdk7 protein (1, 0.8, 0.6, 0.4, 0.2, 0.1, 0.05, 0.03, 0.01 μg, lanes 1–9, respectively; B).

of ovarian development of cultured juveniles ($N = 5$), wild ($N = 10, 7, 7$ and 9 and $N = 4, 7, 9$ and 10 for stages I–IV ovaries of intact and eyestalk-ablated broodstock and $N = 6$ for post-spawning intact broodstock) and domesticated 18-month-old shrimp in serotonin injection (VC and 0, 1, 3, 6, 12, 24 and 48 hpi; $N = 4$ for each group), were calculated.

The relative expression levels of *PmCdk7* between shrimp possessing different stages of ovarian development and those in ovaries/testes of juveniles and broodstock were tested statistically using one way analysis of variance (ANOVA) and Duncan's new multiple range test ($P < 0.05$). To determine the effects of 5-HT injection on *PmCdk7* gene expression when the homogeneity test is invalid, a Kruskal–Wallis non-parametric analysis of variance test was applied (Kruskal and Wallis, 1952). Pairwise comparisons were performed by Dunn's multiple comparison test (Dunn, 1964). Results were considered significant when $P < 0.05$.

3. Results

3.1. Isolation and characterization of the full-length cDNA of PmCdk7

The full-length cDNA of *PmCdk7* was 1407 bp in length containing an ORF of 1062 bp corresponding to 353 amino acids with 5′ and 3′ UTRs of 10 and 335 bp, respectively (Fig. 1). Two poly A additional signals (AATA AA) was located between nucleotides 1150–1155 and 1372–1377 of the characterized transcript (GenBank accession no. KJ601727). Disregarding *Cdk7* previously identified from testes of *P. monodon*, the deduced amino acid sequence of *PmCdk7* showed the greatest similarity to *Cdk7* of *Bombus impatiens* (*E*-value = 2e-167). The deduced PmCdk7 protein contained a Ser/Thr protein kinase, catalytic domain located at positions 20–304. In addition, a cyclin binding site and phosphorylation within the T-loop (DFGLARFFGSPNRQYSHQVVTRWYRSPE, positions 164–191) were also found. The potential *N*-linked glycosylation sites were found at positions 64-66 (NRT) and 94-96 (NVS). The predicted molecular weight and p*I* of the deduced PmCdk7 protein was 40.12 kDa and 7.06, respectively. Three nucleotide variants were observed between *PmCdk7* from ovaries (this study) and testes (Leelatanawit et al., 2009) where A, G and C at positions 357, 573 and 996 of the former were synonymously substituted by G, A and T in the same positions of the latter.

3.2. Expression of PmCdk7 during ovarian development of P. monodon

PmCdk7 was more abundantly expressed in ovaries than that in testes in both cultured juveniles and wild broodstock of *P. monodon* ($N = 5$ for each group, $P < 0.05$) (Fig. 2).

Quantitative real-time PCR indicated that the expression level of *PmCdk7* in premature ovaries of juveniles was significantly lower than that in stage IV (mature) ovaries of non-ablated broodstock ($P < 0.05$). In intact broodstock, its expression level was significantly increased from stages I (previtellogenic) and II (vitellogenic) in stage IV (mature) ovaries ($P < 0.05$) and slightly reduced after spawning (stage V). The expression level of *PmCdk7* was earlier up-regulated in stage III ovaries compared to the previous stages of ovarian development in eyestalk-ablated broodstock ($P < 0.05$). Interestingly, the expression level of *PmCdk7* during ovarian development (stages I–IV) of eyestalk-ablated shrimp was greater than that of the same ovarian stages in intact broodstock ($P < 0.05$, Fig. 3).

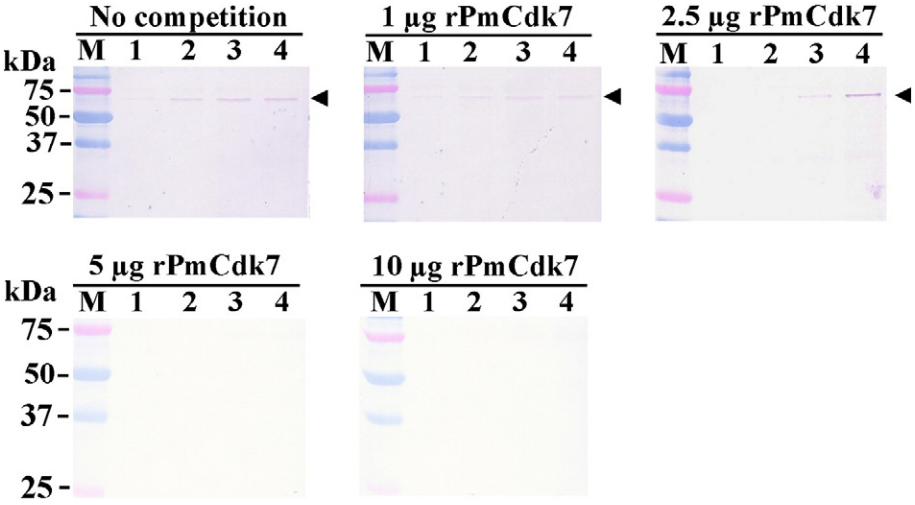


Fig. 7. Competitive binding assays with rPmCdk7 PAb were carried out using rPmCdk7 PAb. Western blot analysis of the purified anti-rPmCdk7 PAb (1:100) pre-mixed with 0, 1, 2.5, 5 or 10 μg of rPmCdk7 and used against 2.5, 5, 10 and 20 μg total ovarian proteins (lanes 1–4, respectively) of a female shrimp with vitellogenic ovaries (stage II) and showing complete competitive blocking at 5 and 10 μg rPmCdk7.

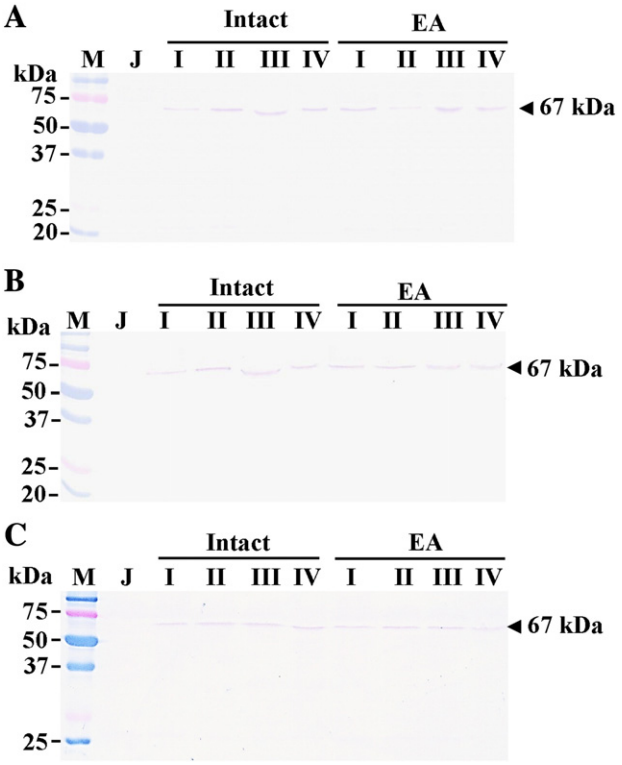


Fig. 8. Western blot analysis of anti-rPmCdk7 PAb (1:100) against 30 μg of total proteins extracted from different stages of ovaries of juvenile shrimp (J, $N = 3$), intact broodstock ($N = 3$ each of stages I–IV ovaries) and eyestalk-ablated broodstock (EA, $N = 3$ each of stages I–IV ovaries). Lane M = a protein standard.

3.3. Effects of 5-HT injection on PmCdk7 transcription in ovaries of domesticated P. monodon

The effects of 5-HT on expression of *PmCdk7* in ovaries of 18-month-old *P. monodon* were examined. The expression level of *PmCdk7* at 1–3 hpi was not different from that of the VC. Its expression level was significantly increased at 6–48 hpi ($P < 0.05$, Fig. 4).

3.4. In vitro expression of rPmCdk7 and polyclonal antibody production

Overexpression of rPmCdk7 was observed at 1 h after induction with 1 mM IPTG. (Fig. 5A). The rPmCdc7 protein was more abundantly expressed as an insoluble than soluble protein (Fig. 5B). A discrete band of 40 kDa was obtained after purification (Fig. 5C). Anti-rPmCdk7 PAb was successfully produced in rabbit with the relatively high titer (1:32000 with $OD_{450} = 0.161$ against 1 μg of purified rPmCdk7).

3.5. Specificity and sensitivity of anti-rPmCdk7 PAb

Anti-rPmCdk7 PAb gave the positive immunoreactive signal with the target (rPmCdk7) but did not cross-react with non-target proteins including rPmDRK (which contained the Src homologue domains, SH2 and SH3), PmPKACB (which contained a protein kinase domain), rPmCdc2 (which contained a S_TKc domain), rPmCyB (which contained a cyclin domain), rPmSema (which contained a semaphorin domain) and rPmRpd3 (which contained a Hist_deacetyl domain) (Fig. 6A). The detection sensitivity for the produced anti-rPmCdk7 PAb was tested and the positive immunoreactive band were observed with 0.03–1 μg of rPmCdk7 (Fig. 6B).

Western blot analysis using anti-rPmCdk7 PAb revealed a discrete band of 67 kDa in various stages of ovaries in both intact and eyestalk-ablated shrimp. Antigen-antibody competition experiment was carried out to determine the specificity of anti-rPmCdk7 PAb. The positive immmunoreactive band was observed from 2.5, 5, 10 and 20 μg total ovarian proteins whether or not the purified antibody was used in competition with 1 μg rPmCdk7. Increasing competition of rPmCdk7 to 2.5 μg resulted in the disappearance of the positive band in 2.5 and 5 μg total ovarian proteins. Disappearance of the positive signal was observed when the purified antibody was used in competition with either 5 or 10 μg rPmCdk7. This confirmed the specificity of anti-rPmCdk7 PAb against the 67 kDa band. (Fig. 7)

3.6. Expression of PmCdk7 protein during ovarian development of P. monodon

The positive immunoreactive signal of PmCdk7 protein (67 kDa) was not observed in juvenile ovaries. In intact broodstock, PmCdk7 seemed to be increased in stages II–IV compared with stage I ovaries. In contrast, this protein was not differentially expressed during ovarian development of eyestalk-ablated shrimp (Fig. 8).

3.7. Localization of PmCdk7 protein during ovarian development of P. monodon

Immunofluorescence signals were detected in follicular layers and ooplasm of previtellogenic oocytes in both intact and eyestalk-ablated shrimp (Fig. 9). In vitellogenic oocytes, PmCdk7 was localized in ooplasm and nucleus. Interestingly, PmCdk7 was found in nucleocytoplasmic compartments, the cytoskeletal architecture and at the cortical rods in early cortical rod and mature oocytes of both intact and eyestalk-ablated broodstock. No immunoreactivity was found in ovaries incubated with the preimmune serum (Fig. 9).

4. Discussion

4.1. Primary structure and expression of PmCdk7 during ovarian development of P. monodon

Molecular mechanisms on the activation of MPF is necessary for better understanding of the oocyte development of *P. monodon* to resolve the major constraint on reduced reproductive maturation of this economically important species in captivity (Klinbunga et al., 2009; Preechaphol et al., 2007). The full-length cDNA of ovarian *PmCdk7* was characterized in this study. Its deduced amino acid sequence was identical to that previously identified in the testes (Leelatanawit et al., 2009). Two potential polyadenylation signal sequences (AATAAA) were found in *PmCdk7* suggesting the possible selective polyadenylation usage. The T-loop (positions 164–191) which is a region of major conformational difference between active and inactive forms was found in the deduced PmCdc7 protein as in other Cdk proteins of *P. monodon*, for example, PmCdc2 (DFGLARAFGIPVRVYTHEVVTLWYRAPE located at positions 146–173, accession no. EU492538; Phinyo et al., 2013) and PmCdk2 (DFGLARAFCLPLRVYTHEVVTLWYRAPE located at positions 146–173, S. Klinbunga, unpublished data). T-loop phosphorylation favors a kinase conformation which allows the access of substrate to the active site (Morgan and De Bondt, 1994; Taylor et al., 1992). Cdk7 is unusual among Cdk's because dual phosphorylation in the T-loop (e.g. at S170 and T176 in *Xenopus* Cdk7 and at Ser164 and Thr170 in *Drosophila* Cdk7) is required for its activation (Larochelle et al., 2001; Martinez et al., 1997). Therefore, the actual positions for activating phosphorylation of PmCdk7 should be further determined.

The development of oocytes consists of a series of complex cellular events, in which different genes are expressed to ensure the proper development of oocytes and to store transcripts and proteins as maternal factors for early embryogenesis (Qiu et al., 2005). *PmCdk7* was more abundantly expressed in the ovaries than the testes in both juveniles and broodstock. Therefore, *PmCdk7* may play a more

important role in oogenesis (and ovarian development) than in spermatogenesis (and testicular development) of *P. monodon*. The expression profile of *PmCdk7* was similar to that of *PmCdc2* (Phinyo et al., 2013) where the peak level was found in mature ovaries. Like *PmCdc2*, eyestalk ablation significantly promotes expression of *PmCdk7* at all stages of ovarian development and also resulted in an earlier up-regulation of *PmCdk7* in stage III ovaries. This further indicated that *PmCdc7* should play an important role in reproductive development and eyestalk ablation affects *PmCdc7* transcription in ovaries of *P. monodon*.

4.2. Up-regulation of PmCdk7 in ovaries of domesticated P. monodon after 5-HT injection

Unilateral eyestalk ablation is used in practice to induce ovarian maturation in *P. monodon*. However, this technique affects egg quality and causes high mortality of spawners (Benzie, 1998; Okumura and Sakiyama, 2004). Therefore, the induction of reproductive maturation and spawning of captive *P. monodon* without the use of eyestalk ablation is a long-term goal for the industry (Quackenbush, 2001).

The effects of exogenous 5-HT injection on reproductive performance of the Pacific white shrimp, *Litopenaeus vannamei* (Vaca and Alfaro, 2000) and the giant freshwater prawn, *Macrobrachium rosenbergii* (Meeratana et al., 2006) have been reported. Additionally, domesticated 14-month-old *P. monodon* injected with 5-HT (50 μg/g body weight) exhibited ovarian maturation and spawning rates comparable to those in eyestalk-ablated shrimp but the hatching rate and the amount of nauplii produced per brooder were significantly higher in the 5-HT injected shrimp ($P < 0.05$; Wongprasert et al., 2006).

In this study, we injected 18-month-old shrimp with the same amount of 5-HT and found that 5-HT promoted the expression level of *PmCdk7* at 6–48 hpi ($P < 0.01$). Likewise, 5-HT injection (50 μg/g body weight) resulted in up-regulation of *GTP binding protein alpha subunit* G_o ($PmG_{\alpha o}$) which is functionally important in the meiotic signal transduction pathway of oocytes, at 6–12 hpi (Klinbunga, unpublished results) and *PmCdc2* which is functionally involved in meiotic maturation of oocytes, at 1 hpi (Phinyo et al., 2013). Results in the present study confirm the molecular effects of 5-HT on the transcription of genes functionally involved in the signal transduction and indicated that 5-HT may directly enhance meiotic maturation of oocytes in *P. monodon* by stimulation of the MPF (a complex of Cdc2 and Cyclin B) via the activity of CDK (PmCdk7 and its partner, Cyclin H and MAT1 which are still not identified and characterized in *P. monodon*).

4.3. Specificity and sensitivity of anti-rPmCdk7 PAb and expression of PmCdk7 protein during ovarian development of P. monodon

The rPmCdk7 protein (molecular weight approximately 40 kDa) and its polyclonal antibody were successfully produced. Anti-rPmCdk7 PAb gave the immunoreactive band against rPmCdk7 but not other recombinant proteins including PmCdc2 that also contains a S_TKc domain. The detection limit of anti-rPmCdk7 PAb was approximately 0.03 μg of rPmCdk7. The sensitivity of detection was slightly greater than that of anti-rPmCdc48-VCP PAb which was able to detect approximately 0.1 μg of rPmCdc48-VCP protein (Talakhun et al., 2014).

An immunoreactive band of 67 kDa was obtained when anti-rPmCdk7 PAb was tested against total ovarian proteins of *P. monodon* broodstock. Antigen-antibody competition experiments illustrated that the anti-rPmCdk7 PAb was specific to a 67 kDa protein. Western blot analysis showed that PmCdk7 was not expressed in premature ovaries of 4-month-old juveniles. In adults, the expression level of PmCdk7 seemed to be increased during the maturation stage of ovarian development in intact broodstock. The information agrees with the level of phosphorylated PmCdc2 (target protein) where the most intense signal of 34 kDa was also observed in mature (stage IV) ovaries (Phinyo et al., 2013).

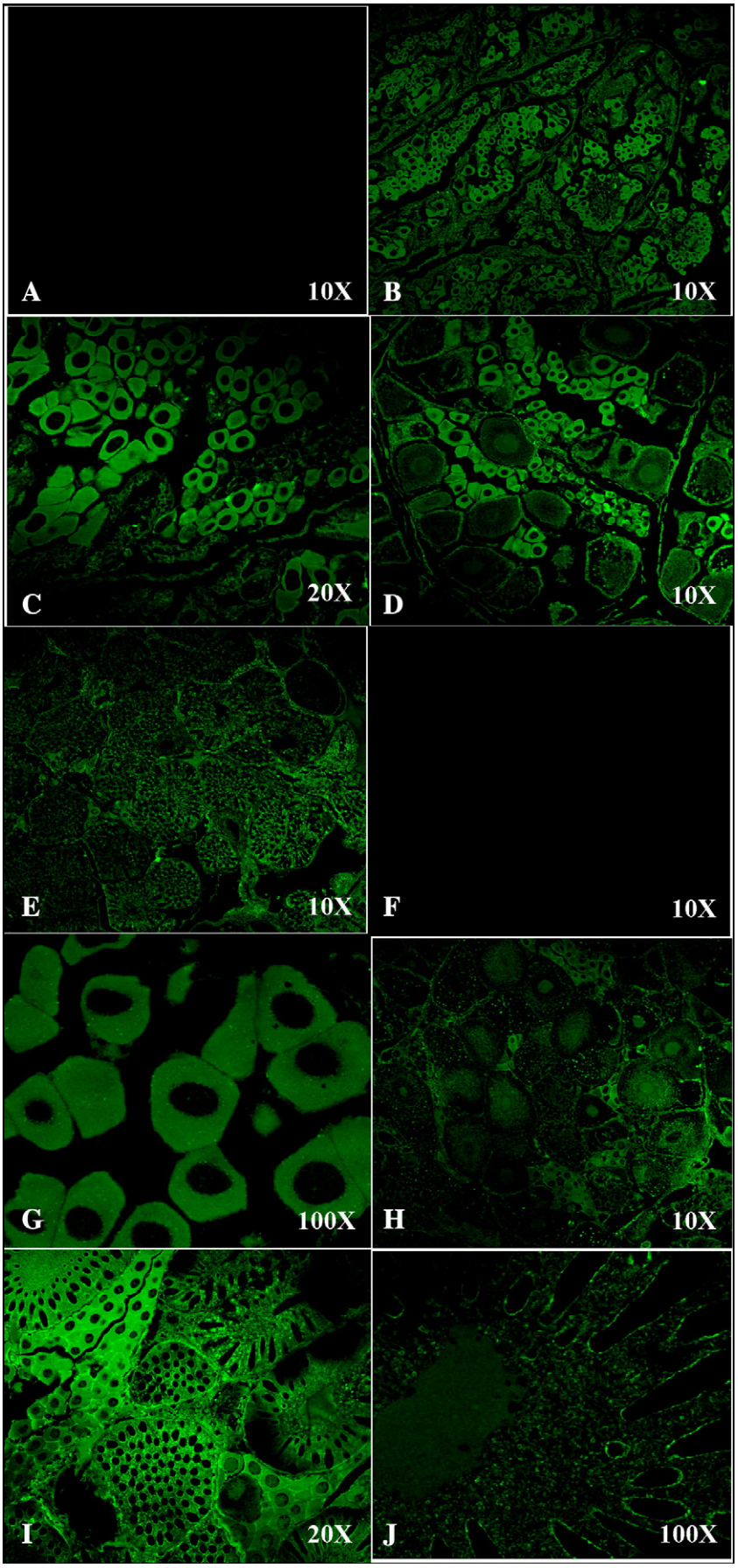
The expression profiles for *PmCdk7* mRNA and protein during ovarian development in non-ablated adults were similar. This suggested that phosphorylation of PmCdc2 may not be required during the premature ovarian stage in juvenile shrimp. Interestingly, *PmCdk7* mRNA and protein in intact shrimp were up-regulated in stages IV and II–IV ovaries. This suggested that more *PmCdk7* mRNA in oocytes is required for rapid translation of the PmCdk7 protein during vitellogenesis and the final maturation of ovarian development in non-ablated *P. monodon*. In eyestalk-ablated broodstock, greater levels of the *PmCdk7* transcript than those in intact broodstock were found but the PmCdk7 protein was comparable suggesting that the amount of *PmCdk7* mRNA in each ovarian stage was sufficient to maintain ovarian development in eyestalk-ablated broodstock. Therefore, *PmCdk7* gene and protein should play the functionally important role during development and maturation of oocytes/ovaries in female *P. monodon*. There is no difference between the expression level of the total (phosphorylated and non-phosphorylated) PmCdk7 protein in intact and eyestalk-ablated adults. Accordingly, the amount of active (phosphorylated) PmCdk7 should be further examined.

4.4. Localization of PmCdk7 during ovarian development of P. monodon

Immunofluorescence gave the interesting issue on the translocation of PmCdk7 protein during oogenesis in *P. monodon*. Immunoreactive signals for the PmCdk7 protein were observed in the ooplasm of previtellogenic oocytes. During vitellogenesis, PmCdk7 was found in the ooplasm and a translocation to the nucleus was also observed. Nuclear translocation of PmCdk7 is supported by its additional roles in transcription and DNA repair by association with the transcription factor TFIIH (Nigg, 1996; Svejstrup et al., 1996). After germinal vesicle breakdown (GVBD), the protein was observed at the nucleocytoplasmic compartment, the cytoskeletal architecture and cortical rods of oocytes. Cortical rods of penaeid shrimp are precursors of egg jelly investment composing of different proteins (e.g. thrombospondin and peritrophin) (Kruevaisayawan et al., 2007). Cortical rods are released during spawning when ovulated eggs contact with seawater and form a jelly investment around the eggs in penaeid species (Clark et al., 1990; Pongtippatee-Taweepreda et al., 2004; Yano, 1995). The functional roles of cortical rods in the induction of the sperm acrosome reaction in *P. monodon* were recently reported (Kruevaisayawan et al., 2007). The findings in this study further suggest that PmCdk7 protein may have been functionally involved in the fertilization and embryogenesis of *P. monodon*.

In this study, the full-length cDNA of *PmCdk7* was successfully identified. Expression profiles of *PmCdk7* mRNA and protein during ovarian development of *P. monodon* were examined. Localization of the PmCdk7 protein suggested a functionally important role in ovarian development, fertilization and embryo development in *P. monodon*. The further interesting issue is the identification of the PmCdk7 binding partners in the CDK complex.

Fig. 9. Localization of PmCdk7 protein revealed by immunofluorescence of anti-rPmCdk7 PAb against conventional ovarian tissue sections from wild intact (B–E) and eyestalk-ablated (G–J) *P. monodon* broodstock. Goat anti-rabbit IgG labeled with Alexa 488 was used as the second antibody. Ovarian tissue sections incubated with the preimmune serum were used as the negative control (A and F). B–C = stage I ovaries; D = stage II ovaries; E = stage IV ovaries of intact broodstock; G = stage I ovaries; H = stage II ovaries; I and J = stage IV ovaries of eyestalk-ablated broodstock.


A
10X
B
10X
C
20X
D
10X
E
10X
F
10X
G
100X
H
10X
I
20X
J
100X

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