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Characterization and expression analysis of a cyclin B gene from black tiger shrimp (*Penaeus monodon*)

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ABSTRACT. The open reading frame of black tiger shrimp (*Penaeus monodon*) cyclin B (*Pmcyclin B*) was identified, based on cDNA sequence registered in GenBank (accession No. EF015590). The target sequence was 1206 bp, corresponding to 401 amino acids. Two conserved signature sequences of the cyclin B gene family were found in the *Pmcyclin B* deduced aa sequence. Temporal expression of *Pmcyclin B* in different tissues, including ovary, lymphoid organ, brain, blood, muscle, heart, gill, hepatopancreas, and intestine, were quantified by quantitative real time PCR. Messenger RNA expression levels of *Pmcyclin B* were greatest in the ovary, compared to other tissues ($P < 0.05$). Temporal expression of *Pmcyclin B* in the ovary at six different developmental stages was investigated by real-time PCR; no significant difference was observed ($P < 0.05$). Recombinant Pmcyclin B protein and its polyclonal antibody were

successfully produced. Western blot analysis revealed differential expression of Pmcyclin B in ovaries in developmental stages II to IV; a positive signal (45 kDa) was observed in all ovarian stages assessed, but was most intense at stage III. Pmcyclin B protein was assessed by immunohistochemistry and was localized to the cytoplasm of prophase oocytes at stage II and enriched in the nuclei of pro-metaphase oocytes at stages III and IV. Results from this study indicate that *Pmcyclin B* is constitutively expressed and plays an important role in ovarian maturation in *P. monodon*.

Key words: Cyclin B; Quantitative real time PCR expression; Western blot; Immunohistochemistry analysis; Black tiger shrimp; *Penaeus monodon*

INTRODUCTION

The cell cycle in eukaryotes is regulated by external signals and by internal regulators such as growth and mating factors, and fertilization (Westendorf et al., 1989). Cyclins, a group of cell cycle regulatory proteins, were identified in the early 1980s as proteins that degraded at the end of each M phase cycle in sea urchin embryos (Santala et al., 2015). Subsequently, cyclins have been described in diverse organisms, from yeasts to humans (Li et al., 2013). Cyclins are classified into groups based on their structure, functional period in the cell cycle, and regulated expression (Norbury et al., 1992). In animals, cyclins have been shown to contain a well-conserved amino acid (aa) sequence known as the cyclin box, and to have roles in cell cycle regulation (Pines and Hunter, 1989; Norbury et al., 1992).

The maturation promotion factor (MPF) is the master regulator of cell proliferation and can stimulate cell cycles of meiosis in eukaryotic cells (French et al., 2013). It is a heterodimer composed of cyclin B protein and cyclin-dependent kinase 1 (CDK1/CDC2); CDK1 is the catalytic, and cyclin B the regulative subunit (Clute and Pines, 1999). Catalytic activation of CDKs requires sufficient accumulation of cyclin protein at particular stages of the cell cycle (Pines and Hunter, 1990). Cyclin B is a protein kinase that is essential for G2/M phase transition and acts as an activator to CDK1 (Santala et al., 2015).

Oocyte maturation and early embryonic development require precise coordination between cell cycle progression and the developmental program. As the ubiquitous regulatory protein controlling eukaryotic cell division cycle, cyclin B plays a primary role in this process. Cyclin B accumulation and degradation are critical in driving the cell cycle, through activation and inactivation of CDK1 (Polański et al., 2012).

As a consequence of continual improvement in cloning techniques, cycline cDNAs have been cloned from various animals (Chapman and Wolgemuth, 1992; Qiu and Yamano, 2005; Polański et al., 2012; French et al., 2013; Fu et al., 2013). Two forms of cyclin B transcripts have been detected in the fruit fly (Dalby and Glover, 1992), mouse (Chapman and Wolgemuth, 1992), and nematode (Kreutzer et al., 1995). The primary structural difference among the forms of *cyclin B* lies in the 3'-untranslated region (UTR), which contains translation regulatory motifs - a cytoplasmic polyadenylation element (CPE) and translation control element (TCE) (Sheets et al., 1994; De and Richter, 1999). Although crustaceans are the third most species-diverse arthropod, including many commercially important species (for example: shrimp, prawns, lobster, crayfish, and crabs), the role of *cyclin B* in regulating ovarian maturation is still poorly understood in crustaceans, compared to other oviparous animals.

The black tiger shrimp *Penaeus monodon* is widely distributed in the Indo-west Pacific Ocean (You et al., 2008) and, in South China, is an important aquacultural species. In 2012, production of

farmed *P. monodon* was 79, 244 metric tons in China (China fisheries statistics yearbook, 2013). Despite this, mechanisms of oocyte maturation in *P. monodon* are poorly understood.

The aims of the present study were to: characterize *Pmcyclin B* sequence in *P. monodon*; investigate temporal expression of *Pmcyclin B* in tissues, including the ovary at different stages of maturation; and explore the sub-cellular location of Pmcyclin B protein in the ovary. Results from the present study will improve our understanding of mechanisms of oocyte maturation in *P. monodon*.

MATERIAL AND METHODS

Experimental animals

Healthy *P. monodon* shrimp (wet weight 60-200 g) were purchased from Sanya, Hainan Province, P.R. China. Shrimp were acclimated at 25 ± 1°C for three days prior to the commencement of the experiment. For gene cloning and prokaryotic expression, RNA was isolated from the ovaries of three individual shrimp. For expression analysis, RNA was isolated from tissues, including ovary, lymphoid organ, brain, blood, muscle, heart, gill, hepatopancreas and intestine of three shrimp. Six shrimp according to six different development stages respectively, which were classified by anatomy and histology methods using a previously reported system (Huang et al., 2005), were used for total protein isolation from the ovary. The six developmental stages of ovary were: I, primordial germ cell; II, chromatin nucleolus; III, perinucleolus; IV, yolky; V, cortical rod; and VI, spent stages. Three shrimp were processed for paraffin section preparation and subsequent prokaryotic expression and immunohistochemistry (IHC) analysis.

Total RNA isolation and first strand cDNA synthesis

Total RNA was isolated from tissues (approximately 50 mg) using Trizol reagent following the manufacturer protocol (Life Technologies, 3175 Staley Road, Grand Island, NY 14072, USA). Isolated RNA samples were re-suspended in DEPC-treated water and stored at -80°C. Synthesis of cDNA was performed on 2 µg mRNA, using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase at 42°C for 50 min, with oligo-dT-adaptor primer (5'-GGCCACGCGTCGACTAGTACT17-3'), following the manufacturer protocol (Promega Corporation, 2800 Woods Hollow Road, Madison, WI 53711 USA). Resulting cDNA samples were used as templates for PCR.

Identification of full-length cDNA of *Pmcyclin B*

Expressed cDNA sequences of *Pmcyclin B* were cloned using specific primers designed against cDNA sequence registered in GenBank (accession No. EF015590). Primers forward (F, 5'-ATGTCTTTGAGAACCACCACGCATCT-3') and reverse (R, 5'-CAGAGAAGAGTGCGTCTTATG CATGA-3') were used to amplify a PCR product of 1206 bp. Cloned nucleotide sequence was searched against previously deposited sequences in GenBank, using BlastN and BlastX (Altschul et al., 1990; available at http://ncbi.nlm.nih.gov).

Quantitative real-time PCR analysis

To investigate *Pmcyclin B* expression, real-time quantitative PCR was performed using SYBR Green 2X Supermix on an ABI 7300 real-time detection system (Life Technologies, 3175

Staley Road, Grand Island, NY 14072, USA). Primers F (5'-GTCACATCCTCCTCTATTCC-3') and R (5'-CACCAAAAGACAAACGGCAG-3') were used to amplify a PCR product of 110 bp. Primers F (5'-ATGGTTGTCAACTTTGCCCC-3') and R (5'-TTGACCTCCTTGATC ACACC-3') were used to amplify a 110 bp fragment of β-*actin* (GenBank accession No. EF087977), which was used as an internal control for qRT-PCR. The qRT-PCR program was 50°C for 2 min, 95°C for 10 min, and 40 cycles of 94°C for 30 s, 61°C for 30 s, and 72°C for 30 s. Analyses were based on cycle threshold (CT) values of PCR products. The relative abundance of cyclin B transcript was obtained after normalization to levels of β-*actin*. Statistical analyses were performed using SPSS19.0 software (IBM SPSS products, USA). Data were calculated as mean ± standard error of the mean (SEM). Data from qRT-PCR analysis were subjected to one-way analysis of variance (ANOVA) followed by an unpaired, two-tailed *t*-test. Differences were considered significant at $P < 0.05$.

In vitro expression of recombinant Pmcyclin B protein, and polyclonal antibody preparation

Expression vector PET-21a was a gift from Dr. Zhao Jianmin at the Yantai Institute of Coastal Zone Research, Chinese Academy of Science; *E. coli* strain BL21 (DE3) was stored by the authors' laboratory. *Pmcyclin B* was amplified by PCR using sense (CYCB-F, 5'-CATATGTCTTTGA GAACCACCAC-3') and antisense (CYCB-R, 5'-CTCGAGTCAGTGGTGGTGGTGGTGGTGTGCA TAAGACGCACTCTTCT-3') primers, which contained *NdeI* and *XhoI* restriction enzyme sites (underlined). Amplified product was purified and digested with *NdeI* and *XhoI*, then inserted into PET-21a, which was digested using the same enzymes. Following transformation and cloning into *E. coli* BL21 (DE3), fusion PET21a-cyclin B was expressed by induction with 0.6 mM isopropyl β-D-thiogalactoside (IPTG) at 37°C for 3, 4, 5, 6, 7, and 8 h, and overnight (24 h). Recombinant protein in the bacterial lysates was purified using His GRAVItrap kit (GE Healthcare Bio-Sciences, P.O. Box 643065 Pittsburgh, PA 15264-3065, USA) and analyzed on an SDS-PAGE (15%, w/v) gel.

Polyclonal antibody R-CYCB was raised in mice exposed to purified recombinant cyclin B, then purified using a HiTrap NHS-activated HP column, following the protocol recommended by the manufacturer (GE Healthcare).

Western blot analysis

Frozen ovary (approximately 0.5 g) was ground into fine powder in liquid N_2, suspended in 1.2 mL trichloroacetic acid (TCA)-acetone extraction buffer (10% TCA in acetone (w/v), containing 0.1% DTT and complete protease inhibitor cocktail (cOmplete Protease Inhibitor Cocktail Tablets, Roche, Basel, CH)), and held at -20ºC for 1 h. The mixture was centrifuged at 10,000 *g* and 4°C for 30 min. Supernatant was discarded, the pellet washed three times with acetone, and then the sample centrifuged at 10,000 *g* and 4°C for 30 min. The resulting pellet was air-dried and dissolved in lysis buffer (30 mM Tris-HCl; 2 M thiourea; 7 M urea; 4% 3-((3-Cholamidopropyl) dimethylammonium)-1-propanesulfonate (CHAPS), w/v). Extracted total protein was quantified by dye-binding assay (Bradford 1976). Total ovarian proteins (20 µg) were heated at 100°C for 5 min, then immediately cooled on ice. Proteins were size-fractionated on a 15% (w/v) SDS-PAGE. Electrophoretically-separated proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Hybond P, Amersham-GE). The membrane was washed three times for 15 min each in 1X Tris-Buffered Saline and Tween 20 (TBST) (25 mM Tris-HCl; 0.15 M NaCl, pH 7.4; 0.1% Tween20), and blocked with 20 mL blocking buffer (1.0 g bovine serum albumin (BSA) in 20 mL

1X TBST) at room temperature overnight with gentle shaking. The membrane was washed three times in 1X TBST and incubated for 1 h with the first antibody, R-CYCB, diluted 1:200 in blocking buffer. The membrane was washed three times with 1X TBST, and then incubated for 1 h with goat anti rabbit IgG (H + L, 1:5000) and the wash step repeated. Immuno-reactive signals were visualized using the substrate nitroblue tetrazolium/5-Bromo-4-Chloro-3-Indolyl Phosphate (NBT/BCIP) (Roche).

Analysis of Pmcyclin B by immunohistochemistry

For IHC analysis, ovarian tissue samples from shrimp at developmental stages II to IV were processed according to conventional techniques for paraffin embedding and sectioning (Sloviter, 1982). Tissue sections (4 µm thick) were deparaffinized, rehydrated, and blocked for 30 min at room temperature in phosphate buffered saline (PBS) with 13% BSA (v/v). Sections were incubated in 20 µL R-CYCB (diluted 1:1000) for 1 h at room temperature; at this step, control tissues were incubated in an equivalent volume PBS. Sections were washes three times in PBS, incubated with biotinylated rabbit anti-mouse IgG, the wash step repeated, then incubated in streptavidin-alkaline phosphatase for 50 min at 37°C. Reactions were developed using an alkaline phosphatase substrate AP-Red kit (Santa Cruz Biotechnology, USA). Sections were counterstained with hematoxylin and examined using an Olympus microscope (Olympus Co., Ltd., Shanghai, China).

RESULTS

Isolation and characterization of the *Pmcyclin B* open reading frame

The *Pmcyclin B* open reading frame (ORF) consisted of 1206 bp, corresponding to 401 aa, and sequence differed from the reference sequence in GenBank (GenBank accession No. EF015590) at only 3 bases. The deduced Pmcyclin B protein contained a cyclin family signature motif located at aa position 170-201; the consensus sequence RXALGXIXN, known as the cyclin destruction box, at aa position 34-42; aa residues of pkA site RRXSK at aa position 276-280, which is characteristic for B-type cyclins (Minshull and Hunt, 1989); and the cyclin box motif at aa position 169-318 (Figure 1).

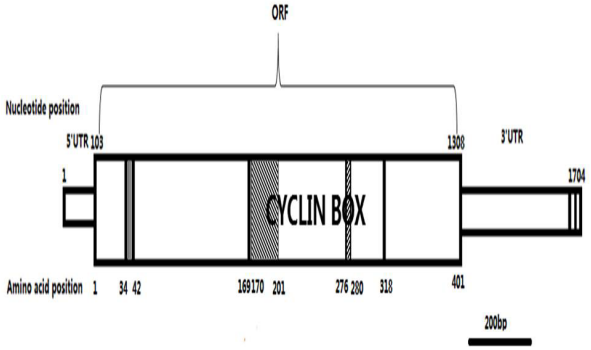

Figure 1. A schematic diagram representing the full-length cDNA of Pmcyclin B 1704 bp containing an ORF of 1206 bp corresponding to a deduced polypeptide of 401amino acids; accession no. EF015590. The predicted domain in the deduced Pmcyclin B protein is indicated by label and shading.

Expression studies in various tissues and at different ovarian maturation stages

Pmcyclin B transcript was constitutively expressed in a wide range of tissues, in which expression levels differed. Expression was predominantly detectable in ovarian tissues, and to a lesser degree, in lymphoid tissues and heart. Levels were very low in the hepatopancreas (Figure 2).

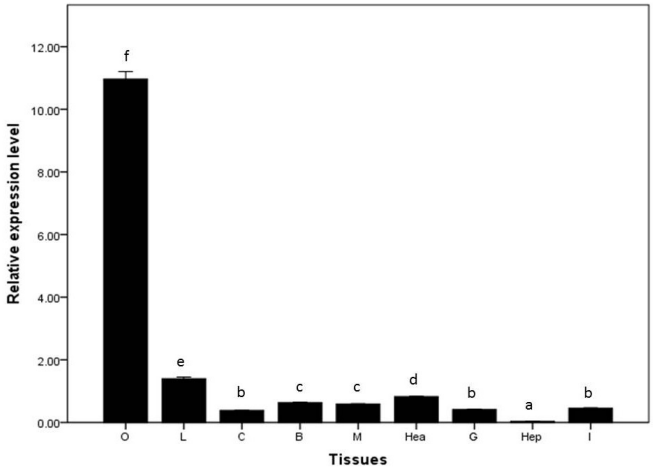

Figure 2. QRT-PCR analysis of Pmcyclin B expression in various tissues of *Penaeus monodon*. Vertical bars represented the meanclin B expression in various tissues of P indicated with different letters above vertical bars (P < 0.05). O: ovary; L: lymphoid organ; C: brain; B: blood; M: muscle; Hea: heart; G: gill; Hep: hepatopancreas; I: intestine;

Pmcyclin B mRNA expression levels differed throughout ovarian maturation, with abundance increasing among stage II and V, and reaching peak levels at stage III (P < 0.05; Figure 3).

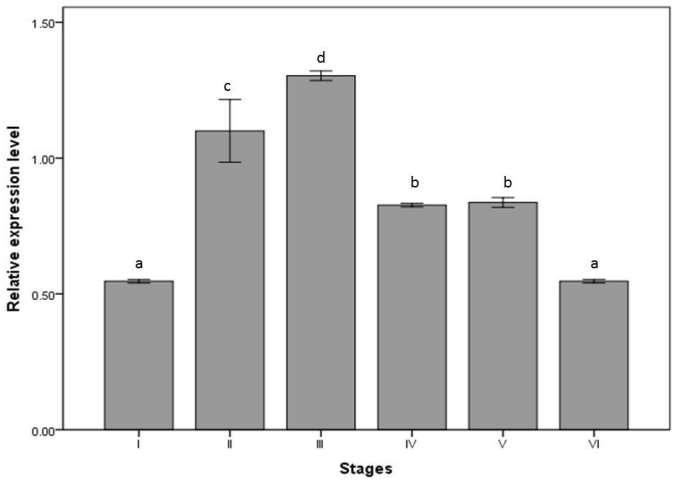

Figure 3. QRT-PCR analysis of Pmcyclin B expression in different ovarian development stages. Vertical bars represented the meanevelopmen3). Significant differences were indicated with different letters above vertical bars (P < 0.05). I: primordial germ cell stage; α: chromatin nucleolus stage; β: perinucleolus stage; χ: yolky stage; V: cortical rod stage; ε: spent stage.

Expression profiles of Pmcyclin B during ovarian development of *P. monodon*

Analysis by SDS-PAGE showed that Pmcyclin B was not detected in bacteria without induction (Figure 4, band 1), and the fusion protein was abundantly expressed as an insoluble protein (Figure 4, bands 2 to 8). A target band of 45 kDa was obtained following purification (Figure 4, band 9). The antibody R-CYCB was successfully prepared in mouse to a relatively high titer (1:10,000; Optical Density $(OD)_{450}$ = 0.602 against 1 μg purified recombinant Pmcyclin B protein).

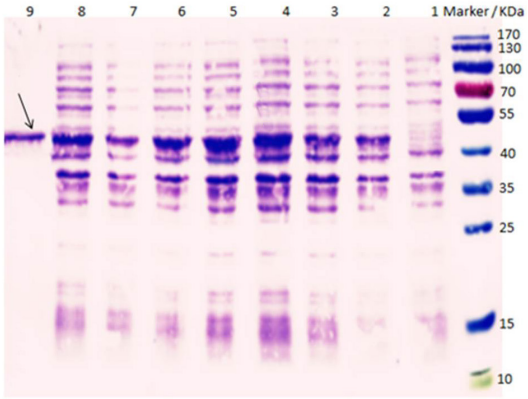

Figure 4. The expression and purification of Pmcyclin B recombinant protein From right to left: Maker/170 kDa; 1, uninduced; 2-8, showing *in vitro* expression of the recombinant Pmcyclin B protein at 3, 4, 5, 6, 7, 8 and 24 h after induction with 1 mM IPTG.; 9, the protein after purification; arrow indicates target protein.

A positive immunoreactive signal of Pmcyclin B protein (45 kDa) was observed by western blot. In test shrimp, Pmcyclin B was detected in ovarian stages II to IV, and reached peak levels at stage III (Figure 5).

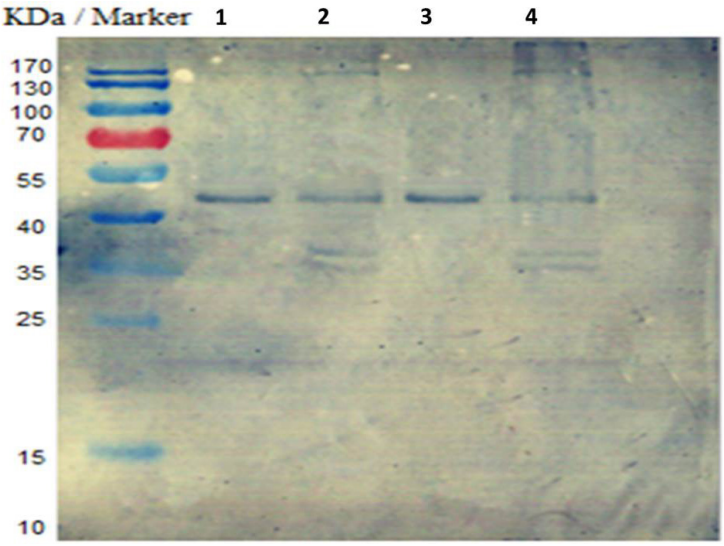

Figure 5. Western blot analysis of R-CYCB (1:200) against total proteins extracted from ovaries of *P. monodon*.

Analysis by IHC demonstrated that Pmcyclin B was localized in the cytoplasm of prophase oocytes at ovarian stage II (Figure 6 B and C), and was enriched in the nuclei of pro-metaphase oocytes at stages III (Figure 6 E and F) and IV (Figure 6 H and I). Positive signal was not detected in control tissues (Figure 6 A, D, and G).

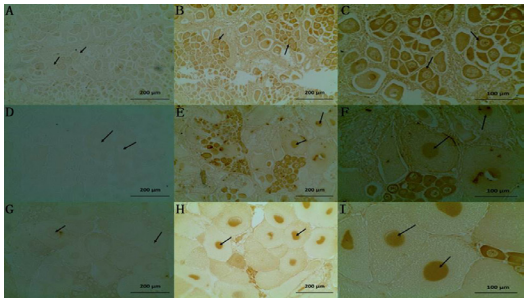

Figure 6. Representative immunohistochemcal staining image of cyclin B protein in ovary of the black tiger shrimp. B, C the second stage, arrow indicates cyclin B protein immunoreactivity in the cytoplasm of prophase oocytes; E, F the third stage, arrow indicates cyclin B protein immunoreactivity enriched in the nuclei of pro-metaphase oocytes; H, I the fourth stage, arrow indicates cyclin B protein immunoreactivity enriched in the nuclei of metaphase oocytes; A the result of the control at the second stage, arrow indicates prophase oocytes; D the result of the control at the third stage, arrow indicates the nuclei of pro-metaphase oocytes; G the result of the control at the fourth stage, arrow indicates the nuclei of metaphase oocytes.

DISCUSSION

An expressed cDNA sequence of a novel gene cyclin B was cloned and characterized in black tiger shrimp using specific primers designed against sequence previously registered in GenBank by our group, which was 1206 bp and encoded a polypeptide of 401 aa. The deduced Pmcyclin B protein contained a cyclin family signature motif, which is a signal for the degradation of the B-type cyclin at the transition from metaphase to anaphase known as the cyclin destruction box (Glotzer et al., 1991). Three bases differed from the reference sequence for Pmcyclin B by sequence alignment, but these changes were not located in the functional domain; this may have been due to sequencing error or to mutations in the gene itself. The functional domain of deduced protein was consistent among the species, suggesting that the primary structures of B-type cyclin proteins are highly conserved throughout evolution (Li et al., 2013).

Cyclin B is induced during the S phase of the cell cycle, and expressed at maximum levels in phases G2 to M, by combination with catalysis subunit Cdc2 kinase to form the complex cyclin B-Cdc2 kinase, also termed maturation promotion factor (MPF) (Gautier et al., 1988; Masui, 1992; Dunphy et al., 1998; Yamashita, 1998; Pines, 1999; Ito, 2000). In most eukaryotic cells including higher plant orders, B-type cyclin genes are expressed specifically during the G2/M phase of the cell cycle (Ito, 2000). The expression of *cyclin B* is highly related to cell proliferation status (Masui and Markert, 1971; Re et al., 1995). In the present study, the highest level of *Pmcyclin B* mRNA expression was detected in ovary, consistent with massive cell proliferation during ovarian development and maturation (Li et al., 2013). We also observed that the abundance of *Pmcyclin B* mRNA increased among ovarian maturation stages II (the chromatin nucleolus stage) and IV (the yolky stage), and reached peak levels at stage III. This expression pattern is consistent with that previously described for cyclin B protein in mud crabs (Li et al. 2013). Up-regulation of *Pmcyclin B* at stage II is consistent with the proposed role of *cyclin B* in mitosis proliferation of oogonia (Li et

al. 2013). Oocytes undergo active yolk accumulation at ovarian maturation stages III and IV (the developing and almost-ripe stages) and mature oocytes resume meiosis and, finally, arrest at the metaphase of meiosis I at stages V and VI (the ripe and spent stages) (Shangguan and Hunt, 1992; Huang et al., 2005; Li et al., 2013).

It is known that cyclin B-CDK1 complexes exist in an inactive state in the cytoplasm of early prophase cells (Pines and Hunter, 1990; Ookata et al., 1993). Most of these complexes are translocated rapidly from the cytoplasm to the nucleus in late prophase (Clute and Pines, 1999; Hagting et al., 1999). Similar to the sea urchin and mud crab, cyclin B protein in black tiger shrimp was found to be localized in the cytoplasm of prophase oocytes during stage II, was partially expressed in the cytoplasm and nuclei at stage III, and was enriched in the nuclei of pro-metaphase oocytes at stage IV. Results from our study suggest that cyclin B protein may have different roles during different developmental stages in the ovary.

Mechanisms of cell cycle-regulated transcription of B-type cyclin genes seem to be evolutionarily divergent, whereas the timing of expression during the cell cycle is well conserved among eukaryotes (Li et al., 2013). Cell cycle-dependent expression of *cyclin B* is critical for the proper timing of the initiation of mitosis. In mammals, *cyclin B* shows periodic expression during the cell cycle (Irniger et al., 1995). In the present study, *Pmcyclin B* displayed various expression levels throughout ovarian maturation, and the pattern of *Pmcyclin B* expression reflected phase-specific regulatory function of respective gene products. Generally, expression patterns of *cyclin B* transcripts during oogenesis vary in animals. For instance, Kuruma prawn *cyclin B* transcripts are expressed continuously throughout oogenesis, from an oogonium to a mature oocyte (Qiu et al., 2005). In *Drosophila*, gene expression is only detectable in the presumptive oocytes until stages VII to VIII of oogenesis (Dalby and Glover, 1992). In mice, *cyclin B* transcripts were predominant in the ovary but undetectable in resting oocytes that had not entered the growth phase (Chapman and Wolgemuth, 1992). Results from the present study indicate that *Pmcyclin B* may be related to cellular proliferation in the ovary, and play an important role in oocyte maturation in the black tiger shrimp.

Conflicts of interest

The authors declare no conflicts of interest.

ACKNOWLEDGMENTS

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