Genetics and Molecular Research

Molecular cloning and functional characterization of cyclin E and CDK2 from *Penaeus monodon*

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ABSTRACT. Reduced reproductive performance of the black tiger shrimp (*Penaeus monodon*) has caused economic losses and hampered the fishing industry. Detailed investigation of the molecular mechanism by which the cell cycle is regulated in this organism is needed to understand the development and maturation of ovaries and oocytes, with a view to improving reproductive capacity. Cell cycle progression is mainly determined by cyclin-dependent kinase (CDK) and cyclin complexes, the cyclin E/CDK2 complex playing a key role in G1/S transition. However, knowledge of the interplay between cyclin E and CDK2 in invertebrates remains limited. In this study, full-length *P. monodon cyclin E* (*Pmcyclin E*) and *CDK2* (*PmCDK2*) sequences were cloned. The open reading frame of *Pmcyclin E* was 1263 bp in

length and encoded a 47.9-kDa protein, while that of *PmCDK2* was 921 bp, encoding a protein of 34.9 kDa. Recombinant cyclin E and CDK2 proteins were expressed in *Escherichia coli* and purified by Ni-chelating affinity chromatography. In addition, a pull-down assay was performed to identify any interaction between Pmcyclin E and PmCDK2. This research provides a basis for the study of the functional mechanisms of the cyclin E/CDK2 complex in shrimp, further enriching our knowledge of invertebrate cell cycle regulation.

Key words: Cell cycle; Cyclin E/CDK2 complex; Prokaryotic expression; Pull-down; *Penaeus monodon*

INTRODUCTION

The cell cycle comprises the series of events occurring within a cell that result in cellular division (Liu et al., 2010). Cell cycle progression is strictly regulated by cyclin/cyclin-dependent kinase (CDK) complexes. Cyclins are expressed in a cyclical fashion during the cell cycle, while CDKs are expressed constitutively (Galderisi et al., 2003). Cyclins themselves do not exhibit enzymatic activity, but are able to bind CDKs, targeting them to specific subcellular locations. Thus, cyclins can regulate cell cycle progression by activating CDKs (Morgan, 1995). All cyclins are classified into one of two phylogenetically divergent subfamilies. The first contains cyclins A, B, D, E, F, and G, among others, and the second includes cyclins C and H (Lees and Harlow, 1993). As an important member of cyclins, cyclin E promotes G1/S phase transition (Dulić et al., 1992). CDK2 belongs to the CDK family of serine/threonine protein kinases, and is involved in the regulation of this cell cycle stage (Dulić et al., 1992). The activation of CDK2 through its interaction with cyclin E constitutes an indispensable event in the G1/S transition (Huang et al., 2012). The cyclin E/CDK2 complex promotes G1 progression by phosphorylating retinoblastoma protein (Rb; Hinds et al., 1992), causing the release of the transcription factor E2F from the Rb/E2F complex, which in turn promotes the expression of genes that drive the G1/S phase transition (Zhang et al., 1999). The cyclin/CDK inhibitors p27 and p21 can be phosphorylated by the cyclin E/CDK2 complex (Toyoshima and Hunter, 1994), as can Smad3, an important regulator of the TGF-β signaling pathway that hinders cell cycle progression (Zieba et al., 2012). Phosphorylation of Smad3 suppresses its transcriptional activity and eventually accelerates cell cycle progression (Zieba et al., 2012). Moreover, phosphorylation of CBP/p300 and E2F-5 by cyclin E/CDK2 can activate transcriptional events as the cell cycle progresses (Wang et al., 2013; Chen et al., 2014). Despite the important role played by cyclin E/CDK2 in the cell cycle, the functional characterization of this complex has predominately been focused on vertebrate species, particularly mammals (Sheaff et al., 1997; Kolupaeva and Basilico, 2012; Duong et al., 2013). In recent years, increasing attention has been paid to the activity of cyclin/CDK complexes in invertebrates, especially shellfish such as *Scylla paramamosain* (Zieba et al., 2012; Huang et al., 2013), the black tiger shrimp *Penaeus monodon* (Phinyo et al., 2014), and the thunberg *Crassostrea gigas* (Wang et al., 2011). However, compared to the progress made in studying the functional mechanism of the cyclin E/CDK2 complex in vertebrates, related research in invertebrates, particularly shrimp, is lacking.

P. monodon is one of the most commercially important aquatic animals in Asia, especially in southern China (Shi et al., 2016). However, reduced reproductive performance in aquacultures of black tiger shrimp has caused economic losses and hampered the fishing industry (Wen et al., 2015). In general, unilateral eyestalk ablation is used to induce *P. monodon* ovarian maturation, but this technique can lead to the death of parent shrimp and lower spawning quality (Benzie, 1998; Wongprasert et al., 2006; Phinyo et al., 2014). Therefore, the examination of alternative approaches is imperative. Detailed investigation of the molecular mechanism controlling the development and maturation of ovaries and oocytes is essential to develop such alternative methods, which could induce reproductive maturation in this organism without the detrimental effects of eyestalk ablation (Caillouet, 1972; Brady et al., 2012; Zhao et al., 2015).

Mitosis and meiotic maturation of animal oocytes is regulated by the cyclin/CDK complex (Lilly et al., 2000; Nguyen et al., 2002; Ortega et al., 2003; Phinyo et al., 2014). Therefore, functional analysis of this complex would improve our understanding of ovary development. In the present study, we successfully cloned both *P. monodon cyclin E* (*Pmcyclin E*) and *CDK2* (*PmCDK2*). Recombinant Pmcyclin E and PmCDK2 proteins were expressed in *Escherichia coli* and purified, and a pull-down assay was used to investigate their interaction.

MATERIAL AND METHODS

Experimental animals and sample collection

Healthy black tiger shrimp weighing an average of 100 ± 2 g were obtained from the testing ground of the South China Sea Fisheries Research Institute in Shenzhen, Guangdong, China. Before the experiments, shrimp were temporarily kept in tanks containing aerated 33‰ seawater for 3 days at 25° ± 1°C without feeding. Three randomly selected female shrimp were dissected, and ovary, heart, brain, lymph, hepatopancreas, and muscle tissues were collected. In addition, three shrimp were selected for each ovarian maturation stage, which were classified according to the morphological characteristics reported by Huang et al. (2005). The ovarian stages considered in this study were: ovogonium (I), chromatin nucleolus (II), perinucleolus (III), yolky (IV), and cortical rod (V). Each sample was tested in triplicate. All samples were snap frozen in liquid nitrogen and stored at -80°C before total RNA preparation.

RNA isolation and sequence cloning

Total RNA was isolated from the examined shrimp tissues (approximately 100 mg) using TRIzol reagent (Invitrogen, Shanghai, China) following the manufacturer protocol, and resuspended in diethylpyrocarbonate-treated water before being stored at -80°C. RNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), and RNA integrity was assessed by 1% agarose gel electrophoresis. Complementary DNA (cDNA) was synthesized from total RNA with a PrimeScript Reverse Transcriptase Kit (TaKaRa, Dalian, China) following the manufacturer protocol.

Partial *Pmcyclin E* and *PmCDK2* sequences were obtained from the *P. monodon* transcriptome established by our laboratory (Zhao C, Fu MJ, and Qiu LH, unpublished results). Full-length *Pmcyclin E* and *PmCDK2* cDNA sequences were determined using rapid amplification of cDNA ends (RACE). RACE-polymerase chain reaction (PCR) was

performed using a SMART RACE cDNA Amplification Kit following the manufacturer protocol (Clontech/TaKaRa, Dalian, China), with specific primers (Table 1).

Table 1. Polymerase chain reaction primers used in experiments.

Primer name	Sequence (5' to 3')	Experiment
3'-*PmcyclinE*-F1	ACGCCAACCTGACTACCT	3' RACE
3'-*PmcyclinE*-F2	CTACAGACTACATAGACCGCTAC	3' RACE
3'-*PmCDK2*-F1	ATGAAAGCCGTGGAGGATTACCGTTG	3' RACE
3'-*PmCDK2*-F2	GGAGTCTGGGTGCCATTTTTGCTGAG	3' RACE
PmcyclinE-F	ATGTCTCAGAATCAGAGAAGGCTGAAAAAT	Cloning *Pmcyclin E* ORF
PmcyclinE-R	CTATCCAGTATACTCTACATATCTAGTCTTCTC	Cloning *Pmcyclin E* ORF
PmCDK2-F	ATGTCGGTGCAGAATTACGAGA	Cloning *PmCDK2* ORF
PmCDK2-R	TCACCTAAGATTTGGTGGGACTAGCA	Cloning *PmCDK2* ORF
qRT*PmcyclinE*-F	AAAACCGATGAGAAGACT	qRT-PCR
qRT*PmcyclinE*-R	TCCTACAGAAGACACCCT	qRT-PCR
qRT*PmCDK2*-F	AACCTTCTTATAGATGCCAGA	qRT-PCR
qRT*PmCDK2*-R	CCAGACTCCACATATCAACT	qRT-PCR
qRT*PmEF*-F	AAGCCAGGTATGGTTGTCAACTTT	qRT-PCR
qRT*PmEF*-R	GCTTCGTGGTGCATCTCCACAGAC	qRT-PCR
PEcycEF	CGCGGATCCATGTCTCAGAATCAGAGAAGGCTGAAAAAT	Recombinant expression
PEcycER	CCGCTCGAGTCCAGTATACTCTACATATCTAGTCTTCTCATCGG	Recombinant expression
PECDK2F	CGCCATATGTCGGTGCAGAATTACGAGA	Recombinant expression
PECDK2R	CCCAAGCTTCCTAAGATTTGGTGGGACTAGCA	Recombinant expression

RACE = rapid amplification of complementary DNA ends, ORF = open reading frame, qRT-PCR = quantitative real-time polymerase chain reaction.

The open reading frames (ORFs) of *Pmcyclin E* and *PmCDK2* were cloned using specific primer pairs (*PmcyclinE*-F/*PmcyclinE*-R and *PmCDK2*-F/*PmCDK2*-R, respectively, Table 1) designed according to the respective cDNA sequences. PCRs were carried out on a Gradient Mastercycler system (Eppendorf, Hamburg, Germany). Each reaction (25.5 μL) consisted of 2.5 μL 10X reaction buffer with 15 mM $MgCl_2$, 2 μL 10 mM deoxynucleotide mix, 1.5 μL each primer (at 25 μM), 1 μL template cDNA, 16.5 μL Milli-Q water (Millipore, Billerica, MA, USA), and 0.5 μL 5 U/μL *Ex Taq* DNA Polymerase (TaKaRa). PCR cycling conditions were as follows: initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min, then a final extension at 72°C for 10 min. PCR products were analyzed by electrophoresis on a 1.2% agarose gel and subsequently purified using an agarose gel DNA purification kit (Sangon, Shanghai, China). The products were then ligated into the pMD18-T vector (TaKaRa) and sequenced (Invitrogen, Guangzhou, China).

Sequence analysis and multiple sequence alignment

Homology analysis of the *Pmcyclin E* and *PmCDK2* cDNA sequences was carried out using the National Center for Biotechnology Information Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi; Altschul et al., 1997). Amino acid sequences were deduced using BioEdit (https://www.bioedit.com/), and motifs were predicted with SMART (http://smart.embl-heidelberg.de/). Both molecular mass and theoretical isoelectric point were estimated using the Compute pI/Mw tool (http://web.expasy.org/compute_pi/), and subcellular localization predictions were made on the PSORT II server (http://psort.hgc.jp/form2.html). Multiple sequence alignments were created with the ClustalW software (http://www.clustal.org/; Thompson et al., 1997; Assavalapsakul and Panyim, 2012), and functional domains were predicted by InterPro (http://www.ebi.ac.uk/interpro/). The signal peptide was predicted with the Signal P 4.0 (http://www.cbs.dtu.dk/services/SignalP/).

Quantitative real-time PCR (qRT-PCR) for gene expression analysis

qRT-PCR was used to detect the temporal expression profile of *Pmcyclin E* and *PmCDK2* in black tiger shrimp. cDNA sequences were synthesized using the PrimeScript Reverse Transcriptase Kit with gDNA Eraser (Perfect Real Time; TaKaRa), and used as templates in qRT-PCRs, which were performed using SYBR *Premix Ex Taq* II (TaKaRa) following the manufacturer protocol. The housekeeping gene *elongation factor-1 alpha* (*EF-1α*; GenBank accession No. DQ021452.1) was selected as an internal control. Details of the primers used in this experiment are given in Table 1 (qRT*PmcyclinE*-F/qRT*PmcyclinE*-R, qRT*PmCDK2*-F/qRT*PmCDK2*-R, and qRT*PmEF*-F/qRT*PmEF*-R). qRT-PCRs were performed in triplicate on a 384-well plate, each in a total volume of 10 μL, comprising 5 μL 2X SYBR *Premix Ex Taq*, 2 μL cDNA template, 0.8 μL 10 mM each forward and reverse primer, and 1.4 μL Milli-Q water. Reactions were performed under the following conditions: 94°C for 30 s, followed by 40 cycles of 94°C for 5 s, 60°C for 30 s, and 72°C for 30 s. Melt curve analysis was performed from 65° to 95°C at the end of each run to ensure that only single products were amplified. Relative expression levels of target genes were calculated via the $2^{-\Delta\Delta Ct}$ method, and normalized to *EF-1α* (Livak and Schmittgen, 2001; Assavalapsakul and Panyim, 2012).

Recombinant expression and purification of Pmcyclin E and PmCDK2

The prokaryotic expression primers PEcycEF/PEcycER and PECDK2F/PECDK2R (Table 1) were designed according to the ORFs of *Pmcyclin E* and *PmCDK2*, respectively. PEcycEF and PEcycER contain *Bam*HI and *Xho*I restriction sites, respectively, and PECDK2F and PECDK2R incorporate those for *Nde*I and *Hin*dIII, respectively (Table 1). After amplification, PCR products were ligated into the pMD18-T vector and verified by sequencing. The recombinant plasmid pMD18-T-*Pmcyclin E* was digested with *Bam*HI and *Xho*I (TaKaRa), and the *Pmcyclin E* fragment subsequently inserted into pGEX-4T-1 (GE Healthcare Life Sciences, Beijing, China), which had been treated with the same enzymes. Likewise, a restriction digest of pMD18-T-*PmCDK2* was carried out with *Nde*I and *Hin*dIII (TaKaRa), before placing the *PmCDK2* insert into pET21a+ (Invitrogen, Shanghai, China), which had been digested beforehand using the same enzymes. The recombinant plasmids pGEX-4T-1-*Pmcyclin E* and pET21a+-*PmCDK2* were transformed into *E. coli* BL21(DE3) cells, which were subsequently cultured at 37°C under constant shaking in lysogeny broth medium (10 mg/mL tryptone, 5 mg/mL yeast extract, and 10 mg/mL NaCl) containing 100 mg/mL ampicillin. When the optical density of the cultures at 600 nm reached 0.4-0.6, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration 1.0 mM to induce protein expression at 28°C overnight. Bacterial cells containing recombinant Pmcyclin E (rPmcyclin E) and PmCDK2 (rPmCDK2) were harvested by centrifugation at 16,000 *g* for 10 min at 4°C. The pellets were re-suspended in cold phosphate-buffered saline (PBS), and the resulting solutions subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 12% gel), followed by Coomassie Brilliant Blue R-250 staining. rPmcyclin E and rPmCDK2 proteins were transferred to nitrocellulose membranes, which were then blocked at 4°C for 12 h with 5% skim milk in PBS containing 0.3% Tween 20. rPmcyclin E and rPmCDK2 were detected by incubating membranes at 37°C for 1 h with horseradish peroxidase (HRP)-conjugated rabbit polyclonal antibodies (diluted 1:2000) against the glutathione *S*-transferase

(GST) tag (anti-GST; Huaan Biotechnology, Hangzhou, China) and the histidine (His) tag (anti-His; Huaan Biotechnology), respectively. The membranes were washed three times with PBS, and positive bands were visualized using HRP-diaminobenzidine substrate (Tiangen, Beijing, China). The rPmCDK2 protein was purified and refolded using a His-bind Purification Kit (Novagen, Shanghai, China) following the manufacturer protocol. Refolding of rPmCDK2 and rPmcyclin E were achieved by Ni-chelating affinity, as described by Suttnar et al. (1994). Before being stored at -80°C for use in subsequent experiments, the concentration of purified rPmCDK2 was ascertained using a bicinchoninic acid assay kit (Beyotime Biotechnology, Shanghai, China).

Analysis of the interaction between rPmcyclin E and rPmCDK2

A pull-down assay based on the MagneGST Pull-Down System (Promega, Beijing, China) was performed following the manufacturer protocol to determine whether rPmcyclin E interacts with rPmCDK2, as follows: 1) IPTG-induced *E. coli* BL21(DE3) cells transformed with pGEX-4T-1-*Pmcyclin E*, pGEX-4T-1, or pET21a+-*PmCDK2* were subjected to two freeze/thaw cycles, before bacterial pellets were lysed with MagneGST lysis reagent containing lyticase, DNase, and protease inhibitors for 40 min, and the resulting solution centrifuged at 14,000 *g* for 10 min; 2) supernatants from the *E. coli* BL21(DE3) cells expressing pGEX-4T-1-*Pmcyclin E* or pGEX-4T-1 were mixed with pre-equilibrated MagneGST beads at 4°C for 30 min, then washed with binding/wash buffer four times; 3) beads attached to GST-rPmcyclin E or GST were incubated with refolded rPmCDK2 at room temperature for 1.5 h; 4) after four washes with binding/wash buffer, the pulled-down proteins were separated by SDS-PAGE on a 12% gel and transferred to membranes, before being probed with anti-GST or anti-His antibodies.

RESULTS

Cloning and characterization of the *Pmcyclin E* and *PmCDK2* ORFs

The full-length *Pmcyclin E* and *PmCDK2* sequences were 1706 and 1679 bp, respectively. The *Pmcyclin E* ORF, consisting of 1263 bp encoding 420 amino acids, and that of *PmCDK2*, comprising 921 bp corresponding to 306 amino acids, were cloned from *P. monodon* based on the cDNA sequences submitted to GenBank under accession Nos. KF218604 and KT727915, respectively (Figure 1). The molecular weights of Pmcyclin E and PmCDK2 were 47.9 and 34.91 kDa, respectively. Bioinformatic analysis showed that the deduced polypeptide sequences possess several significant domains or motifs. That of Pmcyclin E contains a conserved CYCLIN domain (amino acids 143-228) and a Cyclin_C domain (amino acids 237-371), whereas PmCDK2 includes a conserved serine/threonine protein kinase catalytic domain (S_TKc; amino acids 6-290; Figure 2). SignalP 4.0 analysis revealed that neither Pmcyclin E nor PmCDK2 contain typical signal peptide sequences. Multiple sequence alignment showed that both Pmcyclin E (Figure 3) and PmCDK2 (Figure 4) are highly homologous to comparable sequences from other eukaryote species. Pmcyclin E shares 55, 46, 45, and 45% sequence identity with cyclin E proteins from *Daphnia pulex*, *Anolis carolinensis*, *Xenopus laevis*, and *Homo sapiens*, respectively; for PmCDK2, sequence identity values of 67, 67, 66, and 66% were recorded in relation to CDK2 proteins from *Alligator mississippiensis*, *A. carolinensis*, *X. laevis*, and *H. sapiens*, respectively.

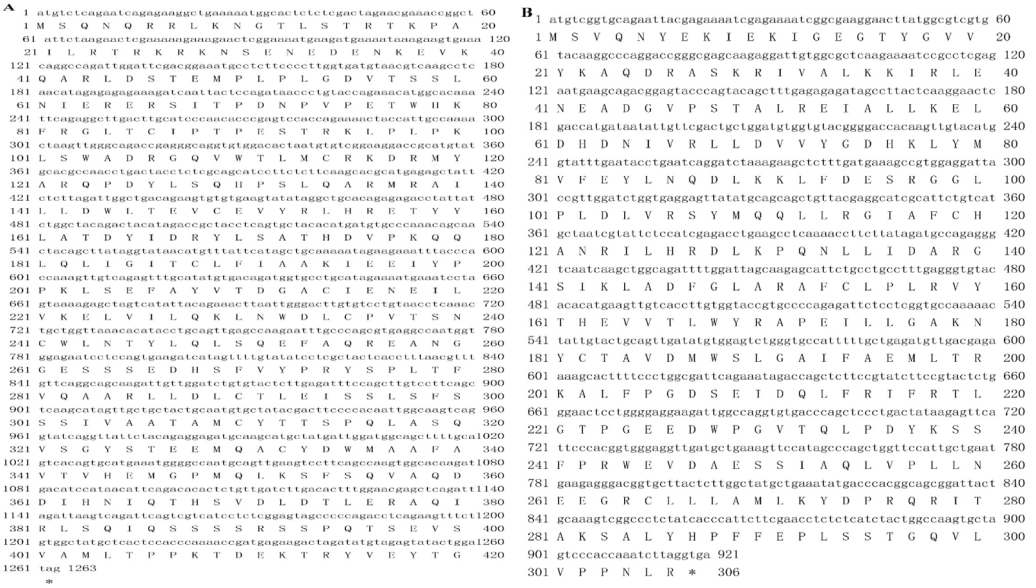

Figure 1. Nucleotide and deduced amino acid sequences of the *Pmcyclin E* (**A**) and *PmCDK2* (**B**) open reading frames. Numbers to the left and right of each row signify nucleotide or amino acid positions.

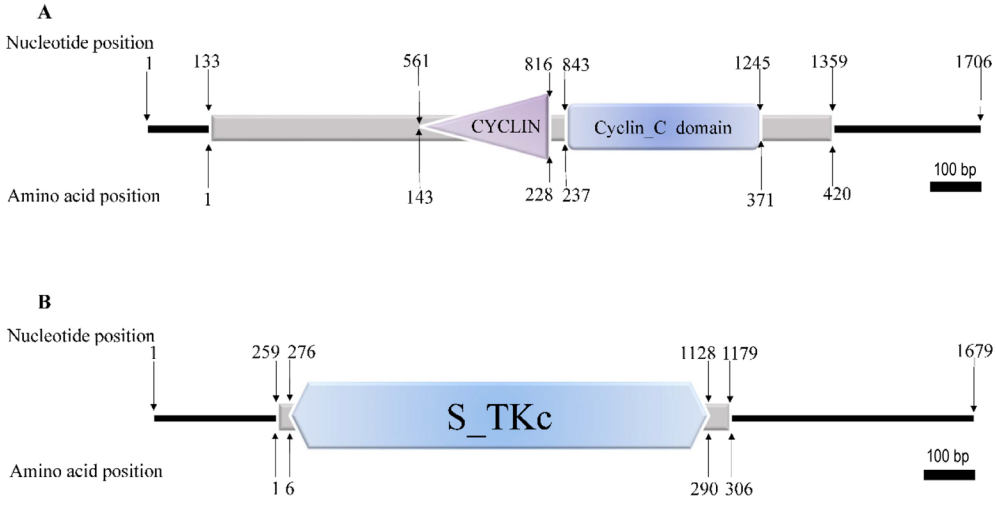

Figure 2. Schematic diagram representing the full-length complementary DNA sequences of *Pmcyclin E* and *PmCDK2*. **A.** *Pmcyclin E*, showing the predicted CYCLIN box (amino acids 143-228) and Cyclin_C domain (amino acids 237-371) in the deduced Pmcyclin E protein sequence. **B.** *PmCDK2*, containing a predicted serine/threonine protein kinase catalytic domain (S_TKc; amino acids 6-290) in the deduced PmCDK2 protein sequence.

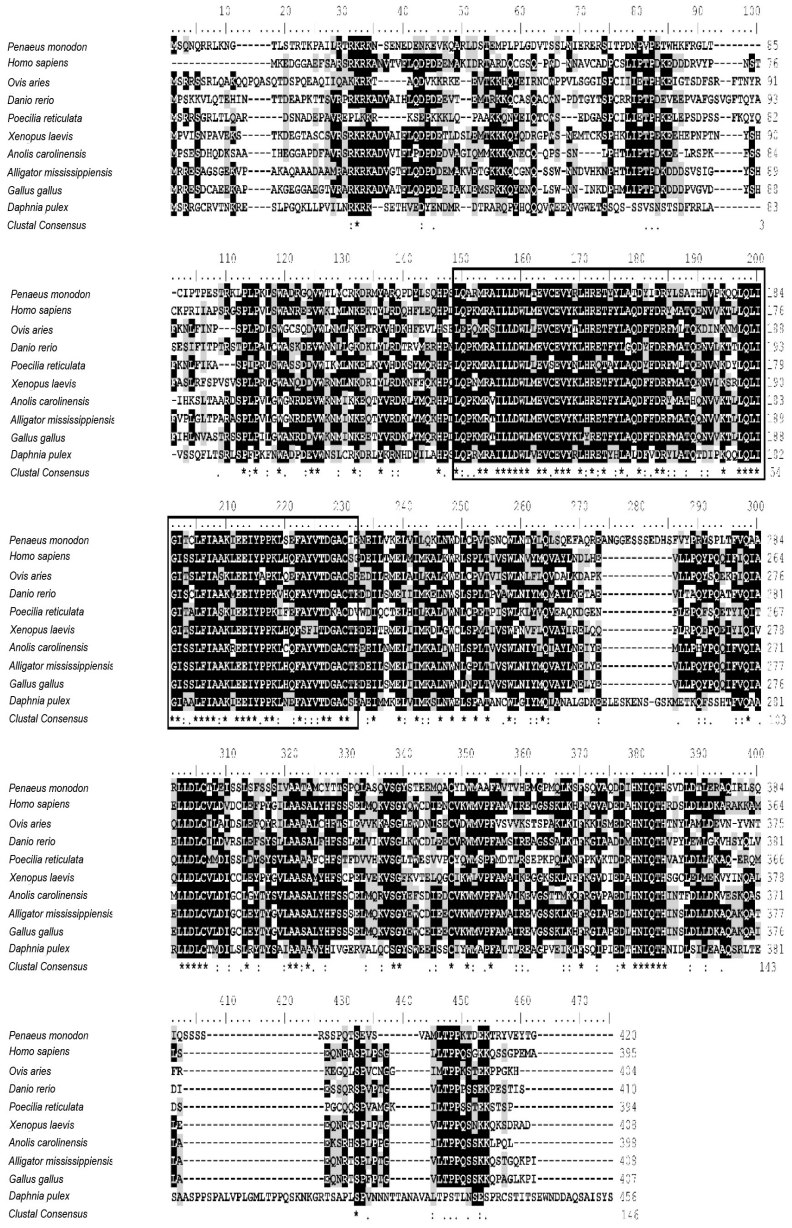

Figure 3. Multiple alignment of Pmcyclin E with other known cyclin E amino acid sequences performed using ClustalW. Identical and similar sites are shown with asterisks and dots or colons, respectively. The boxed-off area represents the conserved CYCLIN domain in different species. Species names and GenBank accession Nos. are as follows: *Penaeus monodon* (AGW23550.1), *Homo sapiens* (AAM54043.1), *Ovis aries* (XP_012039495.1), *Danio rerio* (CAA58574.1), *Poecilia reticulata* (XP_008429012.1), *Xenopus laevis* (CAA78370.1), *Anolis carolinensis* (XP_008115996.1), *Alligator mississippiensis* (KQL94667.1), *Gallus gallus* (AAA74981.1), and *Daphnia pulex* (EFX67141.1).

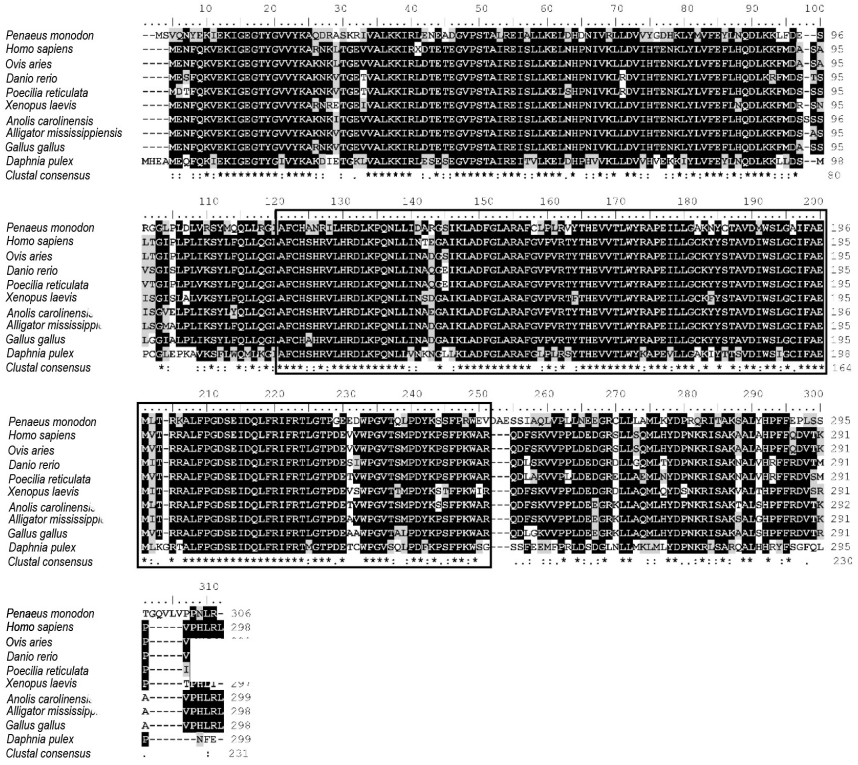

Figure 4. Multiple alignment of the deduced amino acid sequence of PmCDK2 with other known CDK2 proteins performed using ClustalW. Identical and similar sites are indicated with asterisks and dots or colons, respectively. The boxed-off area represents the phylogenetically conserved serine/threonine protein kinase catalytic domain in different species. Species names and GenBank accession Nos. are as follows: *Penaeus monodon* (KT727915), *Homo sapiens* (CAA43985.1), *Ovis aries* (ACJ53943.1), *Danio rerio* (NP_998571.1), *Poecilia reticulata* (XP_008408225.1), *Xenopus laevis* (NP_001084120.1), *Anolis carolinensis* (XP_008112629.1), *Alligator mississippiensis* (XP_006267726.1), *Gallus gallus* (ABM55710.1), and *Daphnia pulex* (EFX72524.1).

Pmcyclin E and *PmCDK2* expression profiles in *P. monodon*

The expression of *Pmcyclin E* and *PmCDK2* mRNA in various tissues is shown in Figure 5A. qRT-PCR revealed that both genes were widely expressed, their transcription being detected in all examined tissues. The highest *Pmcyclin E* expression was observed in the ovaries, and the lowest in the hepatopancreas, whereas *PmCDK2* was most highly expressed in the brain, and its lowest levels were detected in the heart. However, both genes demonstrated elevated expression in the ovaries.

The relative levels of *Pmcyclin E* and *PmCDK2* mRNA at different ovarian developmental stages in *P. monodon* were detected by qRT-PCR (Figure 5B). Maximum expression of both transcripts was seen in stage III ovaries. Although levels gradually declined from their peaks at stage III, expression of both genes was still higher at stages IV and V than during stage II.

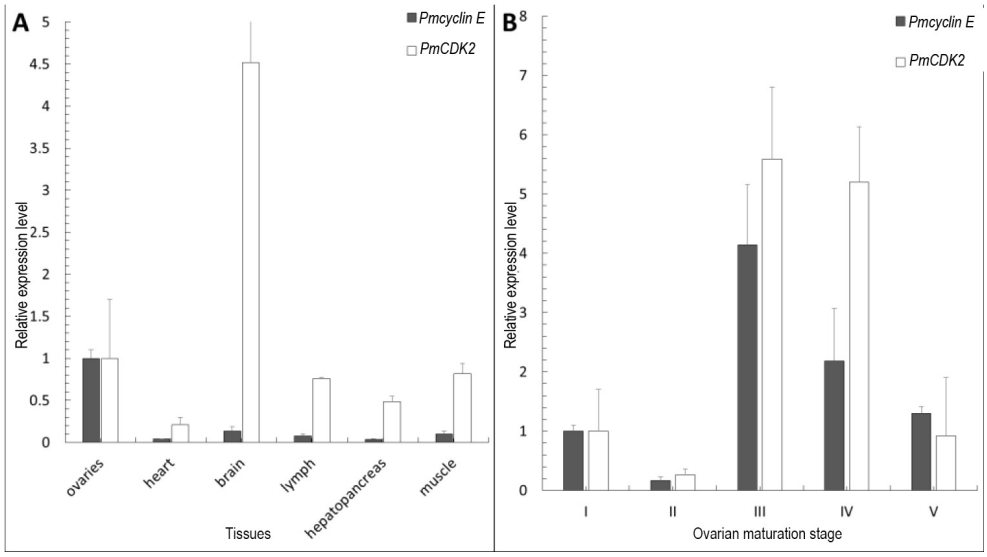

Figure 5. Relative expression of *Pmcyclin E* and *PmCDK2* transcripts in different tissues (**A**) and at different ovarian maturation (**B**) stages. Relative expression levels were calculated with the $2^{-\Delta\Delta Ct}$ method. Bars represent means ± standard deviations (N = 3). Different letters above bars indicate significant differences ($P < 0.05$).

Recombinant expression and western blot analysis of Pmcyclin E and PmCDK2

In order to express Pmcyclin E and PmCDK2 *in vitro*, the recombinant plasmids pMD18-T-*Pmcyclin E* and pMD18-T-*PmCDK2* were successfully constructed and double digested with *Bam*HI/*Xho*I and *Nde*I/*Hin*dIII, respectively (Figure 6). The *Pmcyclin E* and *PmCDK2* fragments resulting from the restriction digest were then inserted into pGEX-4T-1 and pET21a+ vectors, respectively, digested using the same restriction enzymes. This yielded the two recombinant plasmids pGEX-4T-1-*Pmcyclin E* and pET21a+-*PmCDK2*. These plasmids were transformed into *E. coli* BL21(DE3) cells, then rPmcyclin E tagged with GST (GST-Pmcyclin E; Figure 7A) and rPmCDK2 carrying a His tag (His-PmCDK2; Figure 8A) were detected by SDS-PAGE. To verify the identity of the fusion proteins based on their GST or His tag, western blot analysis was performed, which showed specific protein bands of approximately 47.9 (Figure 7B) and 34.9 kDa (Figure 8B), respectively. Moreover, His-PmCDK2 was successfully purified and refolded using affinity chromatography, and a single target band was detected by SDS-PAGE (Figure 9).

In vitro interaction between Pmcyclin E and PmCDK2 proteins

A GST pull-down experiment was carried out to assess *in vitro* interactions between Pmcyclin E and PmCDK2. GST and GST-Pmcyclin E were immobilized on MagneGST beads, then incubated with His-PmCDK2. Anti-His and -GST antibodies were used to visualize the target proteins in a western blot analysis. As shown in Figure 10B, His-PmCDK2 was expressed and was present in the reaction mixture (input). In addition, both GST-Pmcyclin E

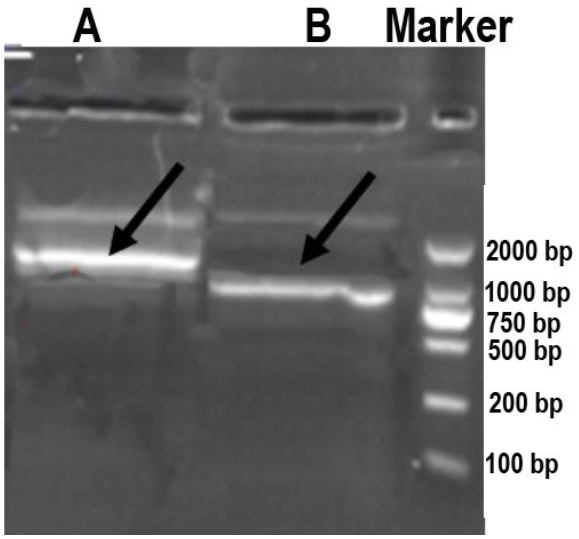

Figure 6. Double restriction enzyme digest results. **A.** *Bam*HI and *Xho*I digestion of pMD18-T-*Pmcyclin E*; **B.** *Nde*I and *Hin*dIII digestion of pMD18-T-*PmCDK2*. Arrows indicate the gene inserts.

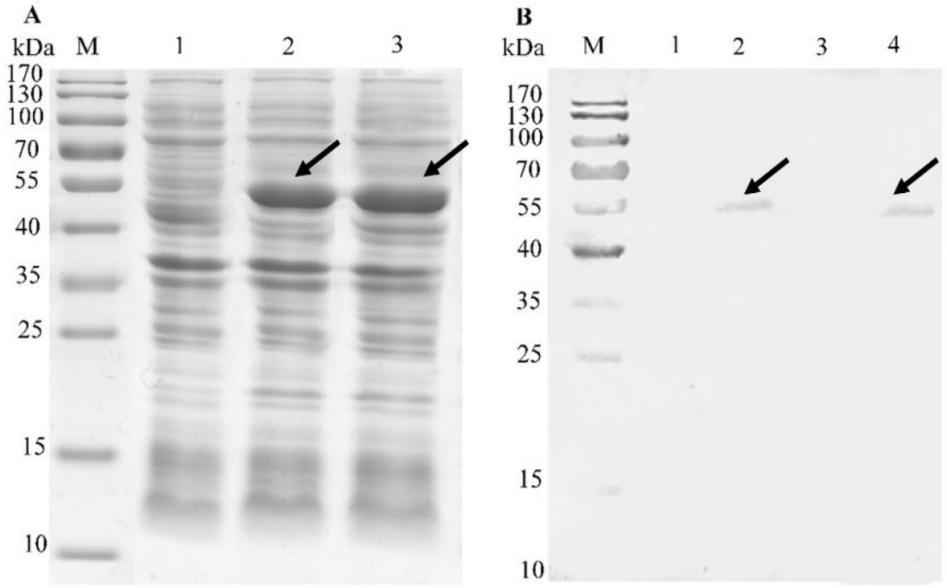

Figure 7. Expression of recombinant Pmcyclin E (arrows) in *Escherichia coli*, and western blot analysis. **A.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of expressed protein. *Lane M*: Pre-stained molecular weight marker; *lane 1*: non-induced transformed cells; *lane 2*: transformed cells induced for 8 h; *lane 3*: transformed cells induced for 12 h. **B.** Western blotting of glutathione *S*-transferase-tagged Pmcyclin E from: *lane 1*: non-induced cells; *lane 2*: cells induced for 8 h; *lane 3*: non-induced cells; *lane 4*: cells induced for 12 h.

and GST proteins were successfully pulled down using MagneGST beads (Figure 10C). A band corresponding to His-PmCDK2 was observed in the presence of GST-Pmcyclin E, whereas no such band was detected in the GST group (Figure 10A). In conclusion, His-PmCDK2 can be pulled down by GST-Pmcyclin E, but cannot be precipitated with GST alone.

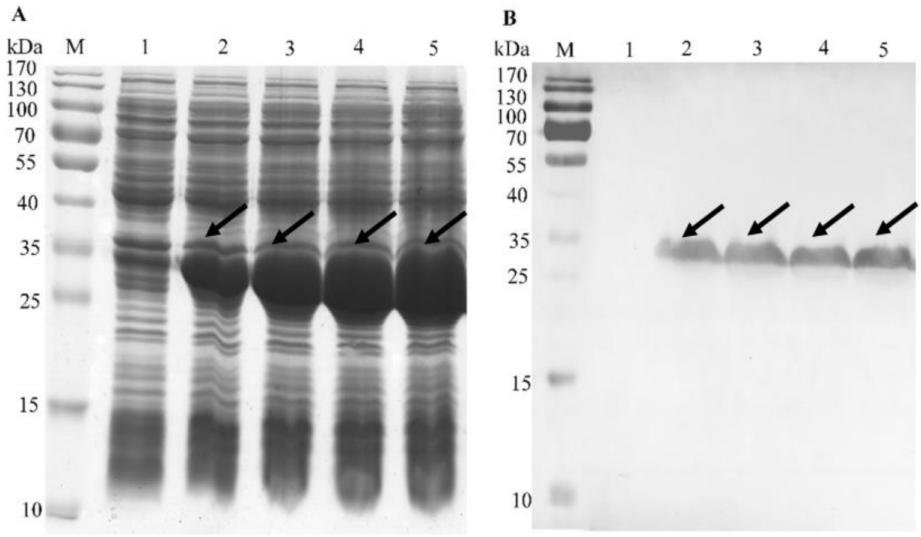

Figure 8. Expression of recombinant PmCDK2 (arrows) in *Escherichia coli*, and western blot analysis. **A.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of expressed protein. *Lane M*: pre-stained molecular weight marker; *lane 1*: non-induced transformed cells; *lane 2*: transformed cells induced for 6 h; *lane 3*: transformed cells induced for 8 h; *lane 4*: transformed cells induced for 10 h; *lane 5*: transformed cells induced for 12 h. **B.** Western blotting of histidine-tagged PmCDK2 from: *lane 1*: non-induced cells; *lane 2*: cells induced for 6 h; *lane 3*: cells induced for 8 h; *lane 4*: cells induced for 10 h; *lane 5*: cells induced for 12 h.

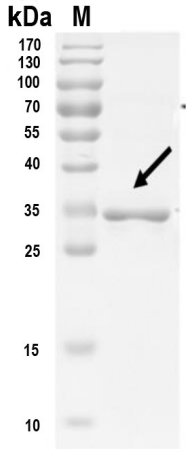

Figure 9. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of histidine-tagged PmCDK2 protein purified by Ni-affinity chromatography from bacterial lysate. *Lane M*: pre-stained molecular weight marker. The arrow indicates the target protein.

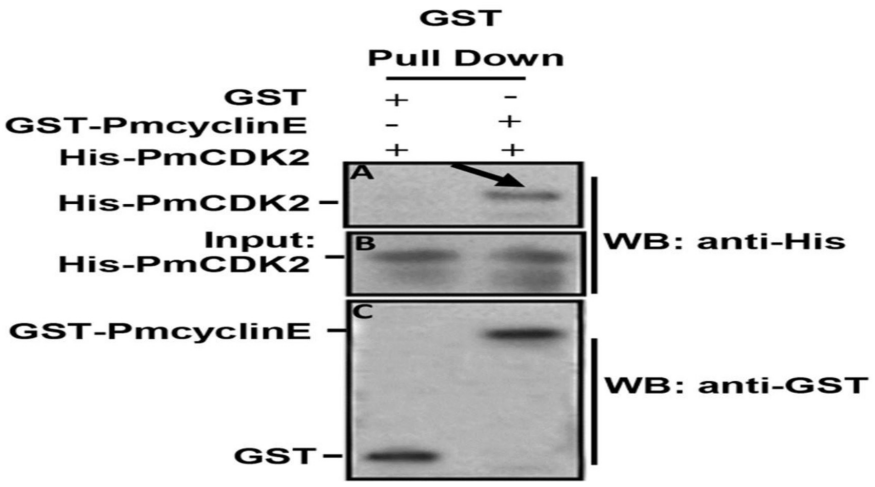

Figure 10. *In vitro* binding of Pmcyclin E with PmCDK2. **A.** Pull-down assay. Histidine-tagged (His) PmCDK2 was incubated with glutathione *S*-transferase (GST) and GST-Pmcyclin E proteins immobilized on magnetic beads. After washing, protein complexes were dissociated from the beads and subjected to western blotting (WB) with an anti-His antibody; **B.** His-PmCDK2 added to the reaction mixtures was detected by WB analysis using an anti-His antibody; **C.** WB with an anti-GST antibody of GST and GST-Pmcyclin E proteins expressed by *Escherichia coli* BL21 (DE3) cells and immobilized on MagneGST beads.

DISCUSSION

Cell division is a fundamental process of all unicellular and multicellular organisms (Korsisaari, 2002), and cell cycle progression is tightly regulated by cyclin/CDK complexes (Kaffman et al., 1994). In view of the important role that such complexes play in ovarian development, and the reduced reproductive performance observed in aquacultures of black tiger shrimp, we intended to study the interaction between cyclin E and CDK2 in this organism. Research into cell cycle regulation may improve our knowledge of the molecular mechanisms controlling development and maturation of ovaries and oocytes in *P. monodon* (Phinyo et al., 2014).

In the present study, we cloned and characterized the cDNA sequences of *Pmcyclin E* and *PmCDK2*. Bioinformatic analysis indicated that sequences homologous to a CYCLIN box and an S_TKc domain are present in the Pmcyclin E and PmCDK2 proteins, respectively, in accordance with previous studies (Lees and Harlow, 1993). Multiple sequence alignment further suggested that Pmcyclin E and PmCDK2 belong to the cyclin and CDK families, respectively. qRT-PCR experiments were carried out to examine the expression pattern of *Pmcyclin E* and *PmCDK2* in different tissues and at various periods during ovarian development. Both genes were widely expressed, with mRNA being detected in all examined tissues, and were transcribed at relatively high levels in the ovaries. This result indirectly demonstrates that the Pmcyclin E/PmCDK2 complex may play important roles in *P. monodon*, which differ depending on tissue type. The development of oocytes consists of a series of complex cellular events, in which temporal and spatial differences in gene expression ensure their proper maturation (Qiu et al., 2008). Huang et al. (2005) divided the process of ovarian maturation into six morphological stages. During stage III (perinucleolus), a large number of cells begin

to proliferate, and vitellogenin starts to accumulate in the yolk cell cytoplasm (Huang et al., 2005). The expression of *Pmcyclin E* and *PmCDK2* peaked at this stage, suggesting that the Pmcyclin E/PmCDK2 complex may perform a key function in oogenesis and ovarian development. This is consistent with previous reports, in which the highest expression of *cyclin A* and *cyclin B* in *P. monodon* was recorded at stage III (Visudtiphole et al., 2009).

In order to further elucidate the potential function of the Pmcyclin E/PmCDK2 complex in this animal, recombinant forms of these proteins were expressed in *E. coli* BL21 (DE3) cells. GST-tagged Pmcyclin E and His-tagged PmCDK2 were successfully generated *in vitro* and detected by SDS-PAGE and western blot. In mammalian cells, it has been shown that G1/S progression is regulated by the activity of cyclin E, which associates with CDK2 subunits, whereas subsequent cell cycle transitions require cyclin A/CDK2, cyclin A/cdc2, and cyclin B/cdc2 (Koff et al., 1992; Sheaff et al., 1997). However, literature concerning the relationship between cyclin E and CDK2 in invertebrates is currently relatively scarce. In this study, a pull-down experiment was carried out to assess the interplay between Pmcyclin E and PmCDK2 *in vitro*. From this, a direct interaction between these two *P. monodon* proteins was observed. This finding implies a mechanism involving the binding and activation of PmCDK2 by Pmcyclin E to promote G1/S transition, as demonstrated in other species (Koff et al., 1992). The CDK subunit is inactive as a protein kinase in the absence of the cyclin subunit (Morgan, 1995). The binding of the latter confers basal kinase activity to the complex (Koff et al., 1992), and phosphorylation of a threonine residue in the former by CDK-activating kinase results in full enzymatic functionality (Kaldis et al., 1996).

In conclusion, Pmcyclin E and PmCDK2 may play functional roles in *P. monodon* ovarian development. The basic findings obtained in this study lay the foundation for clarifying the interplay between Pmcyclin E and PmCDK2 in *P. monodon*. However, the detailed mechanism by which these proteins might regulate ovarian development should be further explored in future study.

Conflicts of interest

The authors declare no conflict of interest.

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