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Molecular Cloning and Expression Analysis of the *Mitogen-Activating Protein Kinase 1* (*MAPK1*) Gene and Protein During Ovarian Development of the Giant Tiger Shrimp *Penaeus monodon*

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SUMMARY

Isolation and characterization of genes and/or proteins differentially expressed in ovaries are necessary for understanding ovarian development in the giant tiger shrimp (*Penaeus monodon*). In this study, the full-length cDNA of *P. monodon mitogen-activating protein kinase 1* (*PmMAPK1*) was characterized. *PmMAPK1* was 1,398 bp in length containing an open reading frame of 1,098 bp that corresponded to a polypeptide of 365 amino acids. *PmMAPK1* was more abundantly expressed in ovaries than in testes of *P. monodon*. Quantitative real-time PCR revealed differential expression levels of *PmMAPK1* mRNA during ovarian development of intact broodstock, where it peaked in early cortical rod (stage III) ovaries ($P < 0.05$) and slightly decreased afterwards ($P > 0.05$). Likewise, the expression level of *PmMAPK1* in early cortical rod and mature (IV) ovaries was significantly greater than that in previtellogenic (I) and vitellogenic (II) ovaries of eyestalk-ablated broodstock ($P < 0.05$). The *PmMAPK1* transcript was localized in ooplasm of previtellogenic oocytes. In intact broodstock, the expression of the PmMAPK1 protein was clearly increased from previtellogenic ovaries in subsequent stages of ovarian development ($P < 0.05$). In contrast, the level of ovarian PmMAPK1 protein was comparable during oogenesis in eyestalk-ablated broodstock ($P > 0.05$). The PmMAPK1 protein was localized in ooplasm of previtellogenic and vitellogenic oocytes. It was also detected around the nuclear membrane of early cortical rod oocytes in both intact and eyestalk-ablated broodstock. Results indicated that *PmMAPK1* gene products seem to play functional roles in the development and maturation of oocytes/ovaries in *P. monodon*.

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Abbreviations: ERK, extracellular-regulated protein kinase; GVBD, germinal vesicle breakdown; MAPK, mitogen-activating protein kinase; MEK, MAP kinase–ERK kinase; MPF, maturation promoting factor; PKC, cAMP-dependent protein kinase C; S_TKc, serine/threonine protein kinase.

INTRODUCTION

Poor reproductive maturation of the economically important giant tiger shrimp (*Penaeus monodon*) in captivity limited its potential for domestication and selective breeding programs (Withyachumnarnkul et al., 1998; Preechaphol et al., 2007). Unilateral eyestalk ablation is used in practice to induce ovarian maturation and spawning of female penaeid shrimp as it reduces the secretion of gonad inhibiting hormone (GIH) from the sinus gland. The technique results in rapid vitellogenesis and nutrient accumulation in ovaries (Yano, 1984; Huberman, 2000; Okumura, 2004; Marsden et al., 2007), but the spawners become detrimentally exhausted with deterioration in egg quality and quantity leading to the death of spawners (Benzie, 1998). Therefore, predictable induction of maturation and spawning of captive penaeid shrimp without the use of eyestalk ablation is a long-term goal for a sustainable shrimp aquaculture (Quackenbush, 1992).

During oogenesis in eukaryotes, oocytes are naturally arrested at prophase I (Okano-Uchida et al., 1998). Oocyte maturation is resumed by a specific hormone, such as progesterone in lower vertebrates (Liang et al., 2007), that signals the oocyte to undergo germinal vesicle breakdown (GVBD), chromosome condensation, and reorganization of microtubules to form a bipolar spindle (Lazar et al., 2002). Generally, ovaries of penaeid shrimp are histologically categorized to five stages; previtellogenic (PV), vitellogenic (VG), early cortical rod (ECR), late cortical rod (LCR or mature), and post-spawning stages (Yano, 1988; Tan-Fermin and Pudadera, 1989; Tahara and Yano, 2004). Previtellogenic ovaries are predominantly composed of oogonia and primary oocytes in the chromatin nucleolus and/or perinucleolus stage. Vitellogenic ovaries are characterized by the presence of yolky oocytes. Early cortical rod ovaries are distinguished by the appearance of yolky oocytes with round, rod-like bodies at the peripheral cytoplasm. Mature ovaries are characterized by the occurrence of nuclear envelope breakdown (GVBD) of oocytes. The post-spawning (or spent) stage can be distinguished from undeveloped ovaries by the presence of few oocytes with yolky substance and/or cortical rods, thicker layers of follicle cells, and few darkly stained, irregularly shaped primary oocytes (Tan-Fermin and Pudadera, 1989). In kuruma prawn (*Marsupenaeus japonicus*), GVBD continued for several hours and completed after ovulation prior to spawning (Yano, 1988). The mature eggs, which are still in metaphase I, are fertilized by spermatozoa released from the spermatophore held in the female thelycum (Yano, 1995).

Induction of mitosis and meiosis in the eukaryotic cell cycle requires the coordinated activation of multiple M phase-inducing protein kinases and are also contributed by the dynamics of the mitogen-activating protein kinase (MAPK) pathway. Induction leads to the activation of maturation promoting factor (MPF), a cdc2-cyclin B complex (Matten et al., 1996; Gross et al., 2000; Islam et al., 2005) that initiates meiotic resumption of oocytes (Kishimoto, 2003; Liang et al., 2007), which is marked by rapid accumulation of vitellin, a yolk protein, and the presence of the cortical rods, spherical or rod-like bodies that appear in the peripheral cytoplasm of oocytes of penaeid shrimp.

The MAPK family is divided into three subfamilies; MAPK (also called extracellular signal-regulated kinases, ERK), c-Jun amino terminal kinases (JNK), and the p38-subfamily. The MAPK subfamily members contain a TEY activation domain; the JNK members possess a TPY activation domain; and the p38-isoforms have a TGY activation domain (Frödin and Gammeltoft, 1999; Johnson and Lapadat, 2002; Bogoyevitch and Court, 2004; Roux and Blenis, 2004; Johnson et al., 2005; Krens et al., 2006a,b).

In *Xenopus* oocytes, signal transmission involving multiple pathways related to MAPK phosphorylation occurs after hormonal stimulation, and affects the interaction between MAPK and MPF (Liang et al., 2007). Recently, the involvement of MAPK with meiotic cell-cycle progression of insects has been reported (Yamamoto et al., 2008). The ability of oocyte cytoplasm to phosphorylate MAP kinase is a prerequisite for the occurrence of GVBD (Sun et al., 1999; Lu et al., 2001). Mos kinase indirectly triggers MAPK activation through MAP kinase kinase phosphorylation (Kishimoto, 1999, 2003). *Xenopus* MAPK1 (42 kDa) was activated during the G2/M phase and, in turn, promotes the activity of Cdc25, a phosphatase functionally necessary for activation of MPF (Shibuya et al., 1992; Wang et al., 2007). The activation of MAPK cascade is initiated by extracellular stimuli, usually through G protein-coupled receptors (e.g., Ras) followed by the activation of several sets of cytoplasmic kinases resulting in proliferation, differentiation, development, or apoptosis of cells (Shaul and Seger, 2007).

Recently, *P. monodon cyclin B* was isolated and characterized. Expression levels of *PmCyB* in ovaries of broodstock were much greater than those of juveniles. During ovarian development, the level of *PmCyB* in mature ovaries (stage IV) was greater than that of previtellogenic (I) ovaries of *P. monodon* ($P < 0.05$) (Visudtiphole et al., 2009). The differential expression profiles of *PmCyB* indirectly suggested the possible roles of the MAPK cascade in the control of oocyte meiotic resumption in *P. monodon*.

To better understand molecular aspects of the MAPK pathway in reproductive maturation of *P. monodon*, the *mitogen-activating protein kinase 1* (*MAPK1*) cDNA sequence was characterized. The effect of eyestalk ablation on expression levels of ovarian *PmMAPK1* in *P. monodon* broodstock was examined. Localization of *PmMAPK1* mRNA and protein in different stages of oocytes was examined by *in situ* hybridization and immunohistochemistry, respectively. The expression profiles of PmMAPK1 in ovaries of *P. monodon* were examined by Western blot analysis.

RESULTS

Isolation of the Full-Length cDNA of *PmMAPK1*

The full-length transcript of *PmMAPK1* was isolated by rapid amplification of cDNA ends-polymerase chain

reaction (RACE-PCR). *PmMAPK1* was 1,398 bp in length with 5′UTRs of 18 bp and 3′UTRs of 254 bp (excluding the poly A tail; GenBank accession no. GU324353). The ORF of *PmMAPK1* was 1,098 bp, corresponding to a polypeptide of 365 amino acids (Fig. 1). The nucleotide sequence of the amplified fragment covering the ORF of *PmMAPK1* (1,139 bp) was identical to that of the full-length cDNA inferred from RACE-PCR. Two predicted polyadenylation sites were observed at 52 and 143 bp upstream from the poly A tail. The deduced protein with the closest match to this sequence was *MAPK1* of *M. japonicus* (*E*-value = 0.0). Two potential *N*-linked glycosylation sites (NXS/T; positions 43–45 and 156–158) were found in the deduced PmMAPK1 protein. A conserved TEY motif for mitogen-activated protein kinase kinase (MEK) phosphorylation (Ramos, 2008), and the predicted serine/threonine protein kinase (S_TKc) domain (*E*-value = 1.84e−95) were found at positions 183–185 and 23–311, respectively. Two predicted phosphorylation sites for cAMP-dependent protein kinase C (PKC, a consensus sequence of [K/R][K/R]X[S/T]) were

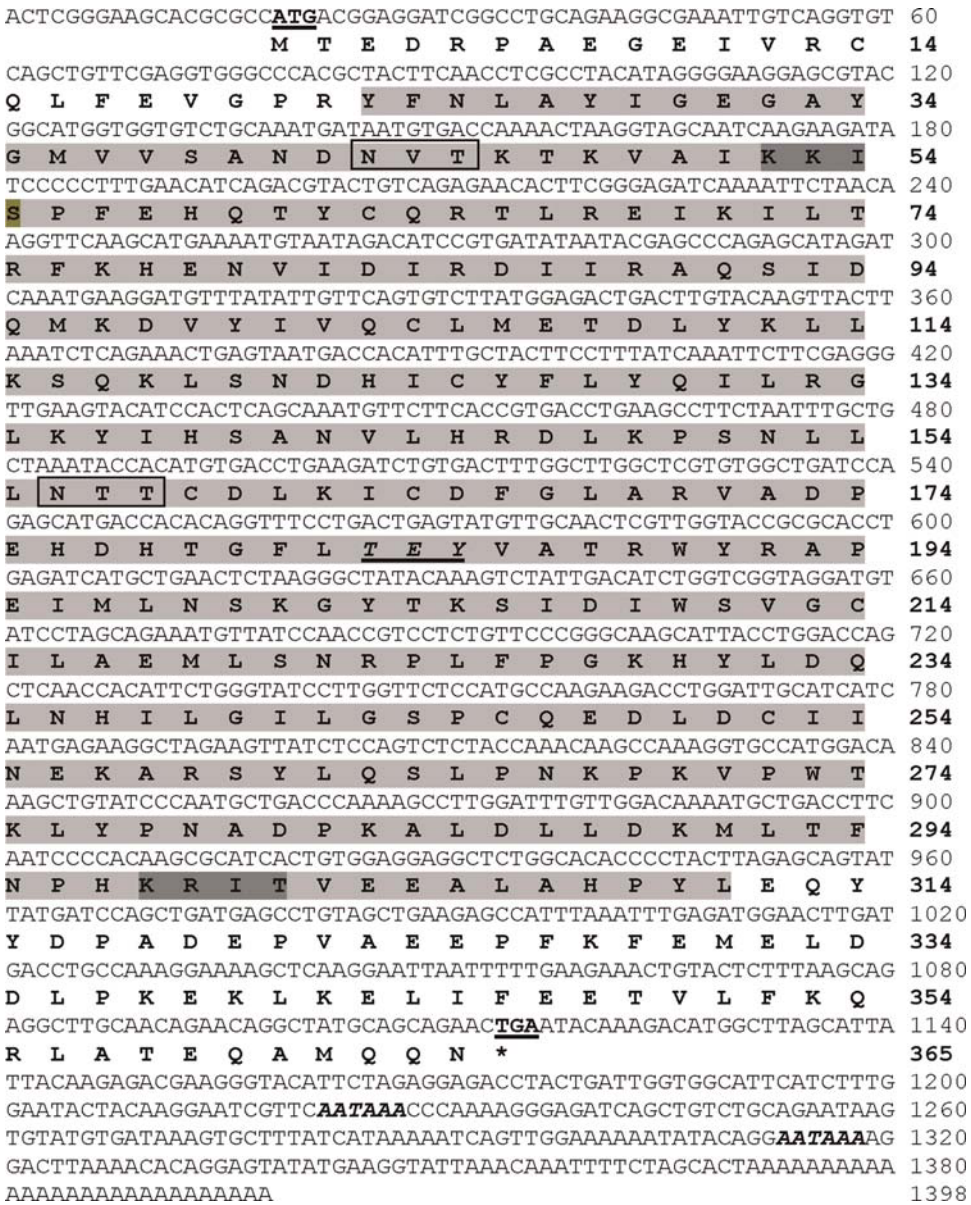

Figure 1. The nucleotide and deduced amino acid sequences of *P. monodon MAPK1*. Start (ATG) and stop (TGA, asterisk) codons are illustrated in boldface and underlined. The eukaryotic polyadenylation signals (AATAAA) are italicized. The S_TKc domain was gray-shaded (residues 23–311). The TEY domain typically found in the MAPK subfamily is italicized and underlined. The predicted cAMP-dependent protein kinase C phosphorylation sites ([K/R]/[K/R]-X-S/T) are highlighted. The putative *N*-glycosylation sites are boxed. [Color figure can be viewed in the online issue which is available at wileyonlinelibrary.com]

found at positions 52–55 and 298–301 of the deduced PmMAPK1 protein. The predicted molecular mass and p*I* value of PmMAPK1 were 42.36 kDa and 5.92, respectively.

Sequence Alignments and Phylogenetic Analysis

Multiple sequence alignments between *MAPK1*/*ERK2* and *ERK1b* revealed that the MAPK1 protein subfamily is highly conserved across taxa (Fig. 2). Phylogenetic analysis indicated a distinct difference between the vertebrate and invertebrate MAPK1 protein subfamily. *PmMAPK1* was more phylogenetically related to *M. japonicus* and *Scylla serrata MAPK1* than insect *MAPK1*/*ERK2*. Therefore, it should be recognized as a new member of the MAPK1 subfamily (Fig. 3).

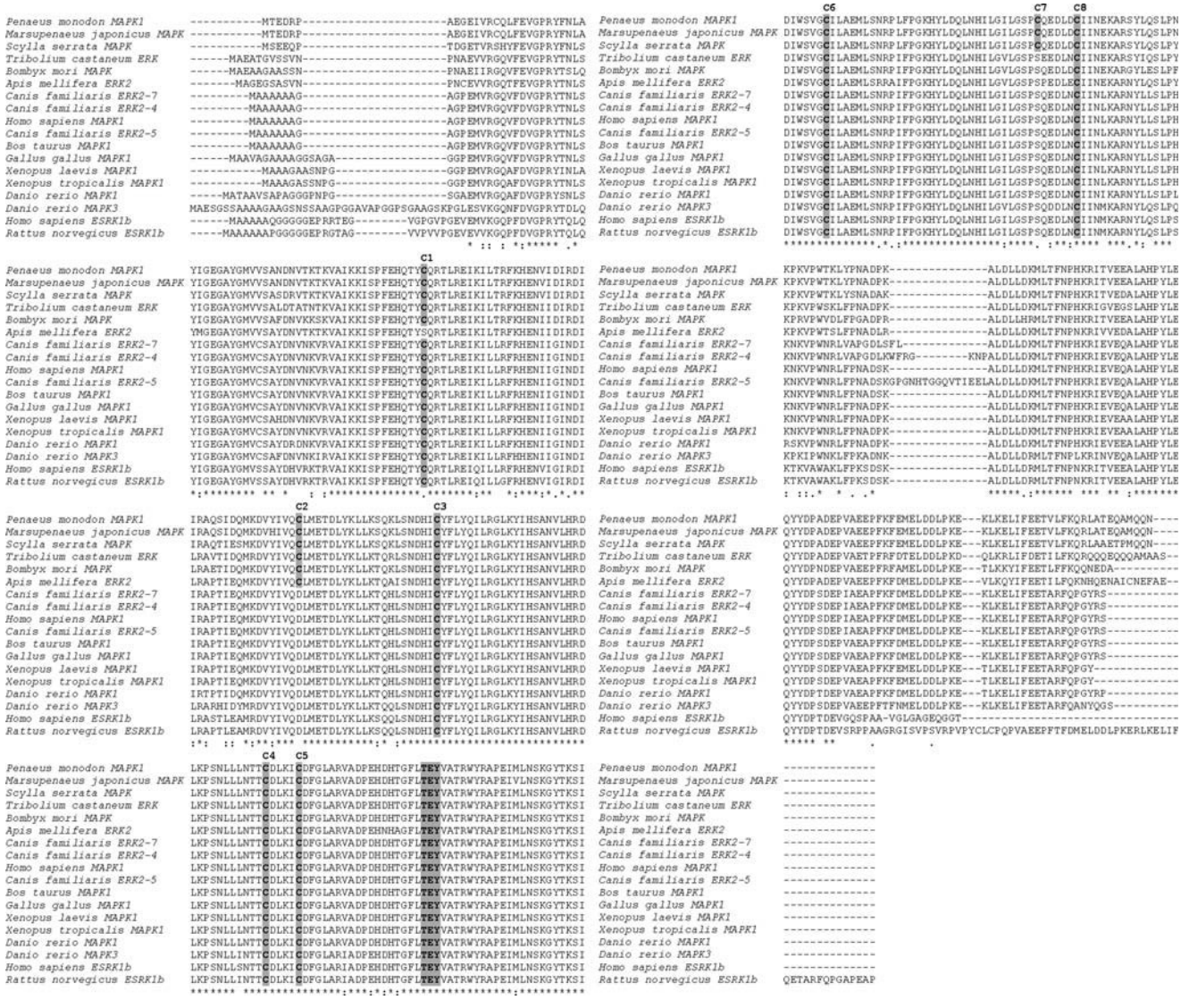

Figure 2. Multiple alignments of the deduced amino acid sequences of PmMAPK1, MAPK1/ERK2, MAPK3, and ERK1b proteins of various species. The analysis included PmMAPK1 (GenBank accession no. GU324353), MAPK1/ERK2 of *Marsupenaeus japonicus* (BAH86598), *Scylla serrata* (ACX32460), *Tribolium castaneum* (XP_966833), *Bombyx mori* (NP_001036921), *Apis mellifera* (XP_393029), *Xenopus laevis* (NP_001083548), *Xenopus (Silurana) tropicalis* (NP_001017127), *Gallus gallus* (NP_989481), *Bos taurus* (NP_786987), *Homo sapiens* (NP_620407), *Danio rerio* (AAH65868), *Canis familiaris* (XP_860651, XP_860682 and XP_860750); MAPK3 of *Danio rerio* (NP_958915) and ERK1b of *Homo sapiens* (AAK52329) and *Rattus norvegicus* (AAF71666). Asterisks, colons, and dots indicate residues identical in all sequences, conserved substitutions, and semiconserved substitutions, respectively. Dashes indicate gaps. Conserved cysteines and the TEY domain are highlighted.

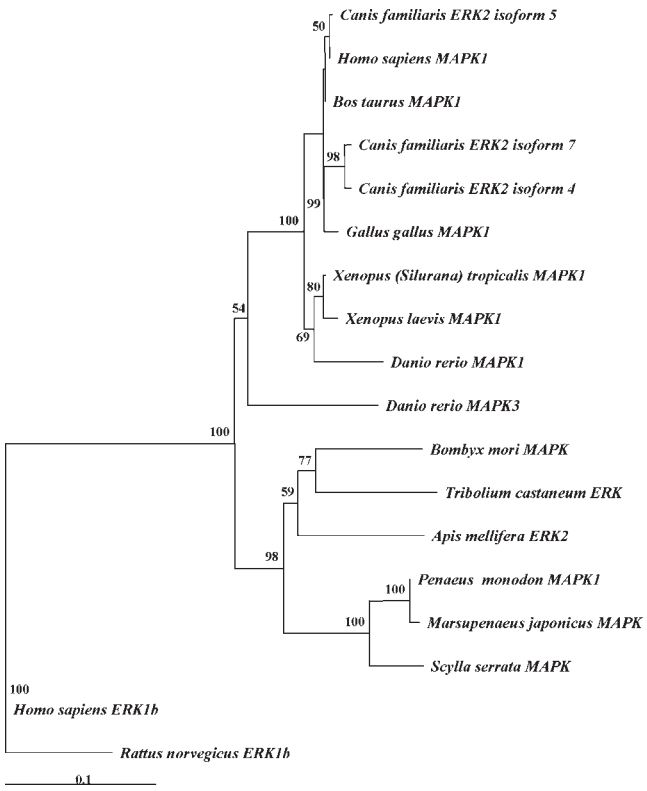

Figure 3. A bootstrapped neighbor-joining tree illustrating relationships between MAPKs/ERKs of various taxa. Values at the node represent the percentage of times that the particular node occurred in 1,000 trees generated by bootstrapping the original aligned sequences. The scale bar indicates 0.1 unit of the expected fraction of amino acid substitutions (1.0 unit = 100 PAMs).

Tissue Distribution Analysis and Expression of *PmMAPK1* During Ovarian Development of *P. monodon*

RT-PCR illustrated that the *PmMAPK1* transcript was constitutively expressed in various tissues of *P. monodon* broodstock. The expression of *PmMAPK1* in ovaries was greater than that in testes of both juveniles and broodstock of *P. monodon* (Fig. 4).

Quantitative real-time PCR revealed that the *PmMAPK1* mRNA in ovaries of juveniles was lower than that of broodstock ($P < 0.05$). In intact broodstock, *PmMAPK1* was up-regulated in early cortical rod (stage III) ovaries ($P < 0.05$) and slightly decreased in mature ovaries (stage IV) and after spawning (stage V) ($P > 0.05$). In contrast, *PmMAPK1* was up-regulated in both early cortical rod and mature ovaries in eyestalk-ablated broodstock ($P < 0.05$). Moreover, the expression level of *PmMAPK1* in mature ovaries of eyestalk-ablated broodstock was significantly greater than that in the same developmental stage of intact broodstock ($P < 0.05$, Fig. 5).

Localization of *PmMAPK1* mRNA

The *PmMAPK1* transcript was found in the ooplasm of previtellogenic oocytes in both intact and eyestalk-ablated broodstock. Positive signals were not detected in the germinative zone, follicular cells, oogonia, and vitellogenic, early cortical rod, and mature oocytes in various stages of ovaries. No signal was found with the sense cRNA probe (Fig. 6).

Western Blot Analysis

Immunoblotting indicated that the MAPK1 protein in ovaries of juveniles and previtellogenic (I) ovaries was lower

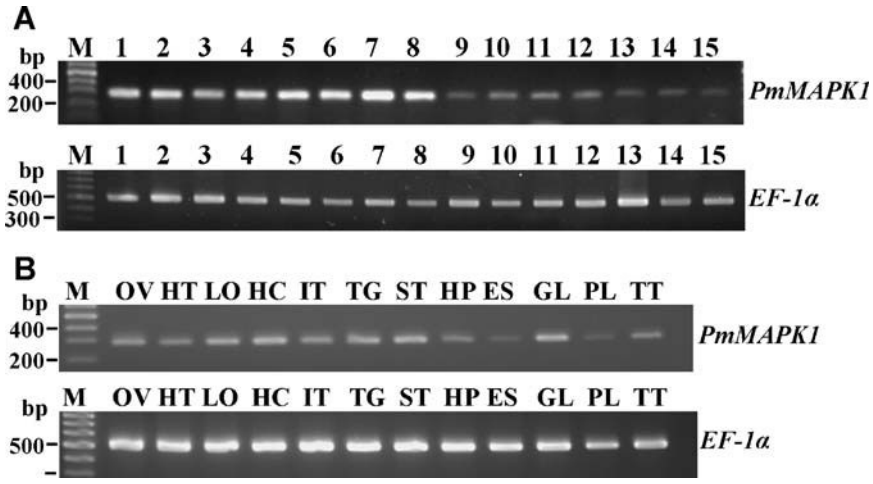

Figure 4. **A**: RT-PCR of *PmMAPK1* (top) and *EF-1*α (bottom) in ovaries (lanes 1–8) and testes (lanes 9–15) of *P. monodon* juveniles (lanes 1–4 and 9–12) and broodstock (lanes 5–8 and 13–15). **B**: Expression of *PmMAPK1* (top) in various tissues of a female (OV, ovaries; HT, heart; LO, lymphoid organs; HC, hemocytes; IT, intestine; TG, thoracic ganglion; ST, stomach; HP, hepatopancreas; ES, eyestalk; GL, gills; PL, pleopods) and in testes (TT) of a male *P. monodon* broodstock. *EF-1*α was successfully amplified from the same templates (A,B, bottom). Lanes M, 100 bp DNA marker.

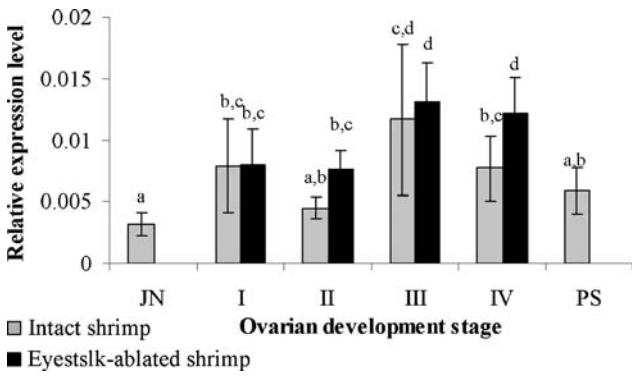

Figure 5. The relative expression profiles of *PmMAPK1* during ovarian development of intact and unilateral eyestalk-ablated *P. monodon* broodstock. The bars correspond to standard deviation (SD) of the means. The same letters above bars indicate that the expression levels were not significantly different ($P > 0.05$). JN = juvenile ovaries; I–IV = previtellogenic, vitellogenic, early cortical rod, and mature ovaries, respectively; PS = ovaries of intact broodstock immediately collected after spawning.

than that in vitellogenic (II), early cortical rod (III), and mature (IV) ovaries in intact broodstock ($P < 0.05$, Fig. 7A,B). In contrast, the expression levels of PmMAPK1 in different ovarian stages of eyestalk-ablated broodstock were not significantly different ($P > 0.05$, Fig. 7B,C).

Localization of the PmMAPK1 Protein

The immunoreactive signals of the PmMAPK1 protein were detected in all stages of ovarian development in intact broodstock. The signals were initially observed in ooplasm of previtellogenic oocytes, and more intense signals were found in vitellogenic and mature oocytes. The PmMAPK1 protein was also detected around the nuclear membrane of early cortical rod oocytes. Similar immunoreactive patterns, but more intense signals, of the PmMAPK1 protein were observed in eyestalk-ablated broodstock (Fig. 8). No positive signal was observed in oogonia and the follicular cells.

DISCUSSION

Isolation and Characterization of *PmMAPK1* cDNA

Molecular mechanisms underlying meiotic maturation of oocytes and ovarian development of penaeid shrimp are still unknown. In many animals, the meiotic cell cycle of arrested oocytes is resumed for acquisition of fertilization. MAPK and MPF, a complex of cyclin B and cdc2 (Cdk1) are activated and play essential roles during oocyte maturation (Yoshida et al., 2000; Kotani and Yamashita, 2002; Kishimoto, 2003).

In this study, the full-length cDNA of *MAPK1* was identified and reported for the first time in *P. monodon*. A TEY domain typically found in all characterized MAPK1/3 (ERK1/2) so far (Johnson and Lapadat, 2002; Krens et al., 2006a,b) was observed in the deduced PmMAPK1, indicating that it is a member of the MAPK1/3 subfamily. Ramos (2008) reported that phosphorylation of both threonine and tyrosine in the conserved TEY motif by MEK1/2 resulted in a conformation change in MAPK1 that allows the C-terminal portion to interact with the activation loop of MEK1/2. This dimerization would possibly promote a route for nuclear translocation. A serine/threonine kinase (S_TKc) catalytic domain was found in the deduced PmMAPK1 protein. Typically, the proteins containing the S_TKc domain functionally transfer the gamma phosphate from nucleotide triphosphates (often ATP) to one or more amino acid residues in the protein substrate side chain, affecting functions of the target proteins (Hanks et al., 1988). The S_TKc domain is typically found in the cell division cycle (Cdc) protein family. Previously, *P. monodon cell-dependent kinase 7* (*Cdk7*) and *check point kinase 1* (*Chk1*) were characterized, and their deduced amino acid sequences contained this phosphotransferase domain (Preechaphol et al., 2010a).

MAPK1 (also called ERK2) is evolutionarily conserved across various taxa. Six identical positions of cysteine (C1, C3, C4, C5, C6, and C8) residues were found in both vertebrate and invertebrate MAPK1 (except *Apis mellifera*, where C1 was replaced by S). An additional Cys (C2) was found in insects (*A. mellifera*, *Tribolium castaneum*, and *Bombyx mori*) and crustaceans (*P. monodon*, *M. japonicus*, and *S. serrata*). Interestingly, the C7 residue was only observed in crustaceans in this study. Two potential glycosylation sites were found in the deduced PmMAPK1 protein, one of which (NTT; positions 156–158) is conserved across the examined taxa.

PKC is reported to be involved in oocyte maturation following the resumption of meiosis, and regulates spindle organization in meiosis-I and -II until fertilization and embryogenesis (Kalive et al., 2010). The existence of a conserved phosphorylation site for PKC suggested that this enzyme may be involved in the cAMP-mediated MAPK activity regulation (Fan and Sun, 2004) by phosphorylation. Interestingly, the potential phosphorylation site is additionally found in crustaceans (*P. monodon*, *M. japonicus*, and *S. serrata*), *B. mori* and *A. mellifera*, implying that a single phosphorylation should be sufficient for the activation of *PmMAPK1*.

Phylogenetic analysis clearly assigned ERK1b and MAPK1/ERK2 to different groups, and allocated MAPK1/ERK2 of different taxa according to classical relationships; this distribution was observed with significant boostrapping values (>50%) for all branches. PmMAPK1 is recognized as a new member of invertebrate MAPK1, and similar to other invertebrate MAPK1s, shares an ancestral ortholog with vertebrate MAPK1. In addition, the bootstrapped neighbor-joining tree allocated *Danio rerio* MAPK3 (also called ERK1) in the vertebrate MAPK1/ERK2 group. Accordingly, the zebrafish MAPK3 (Krens et al., 2006a) should be recognized as a variant of MAPK1 rather than that of MAPK3.

Multiple sequence alignments and phylogenetic analysis strongly indicated that the protein sequences of *MAPK1*/

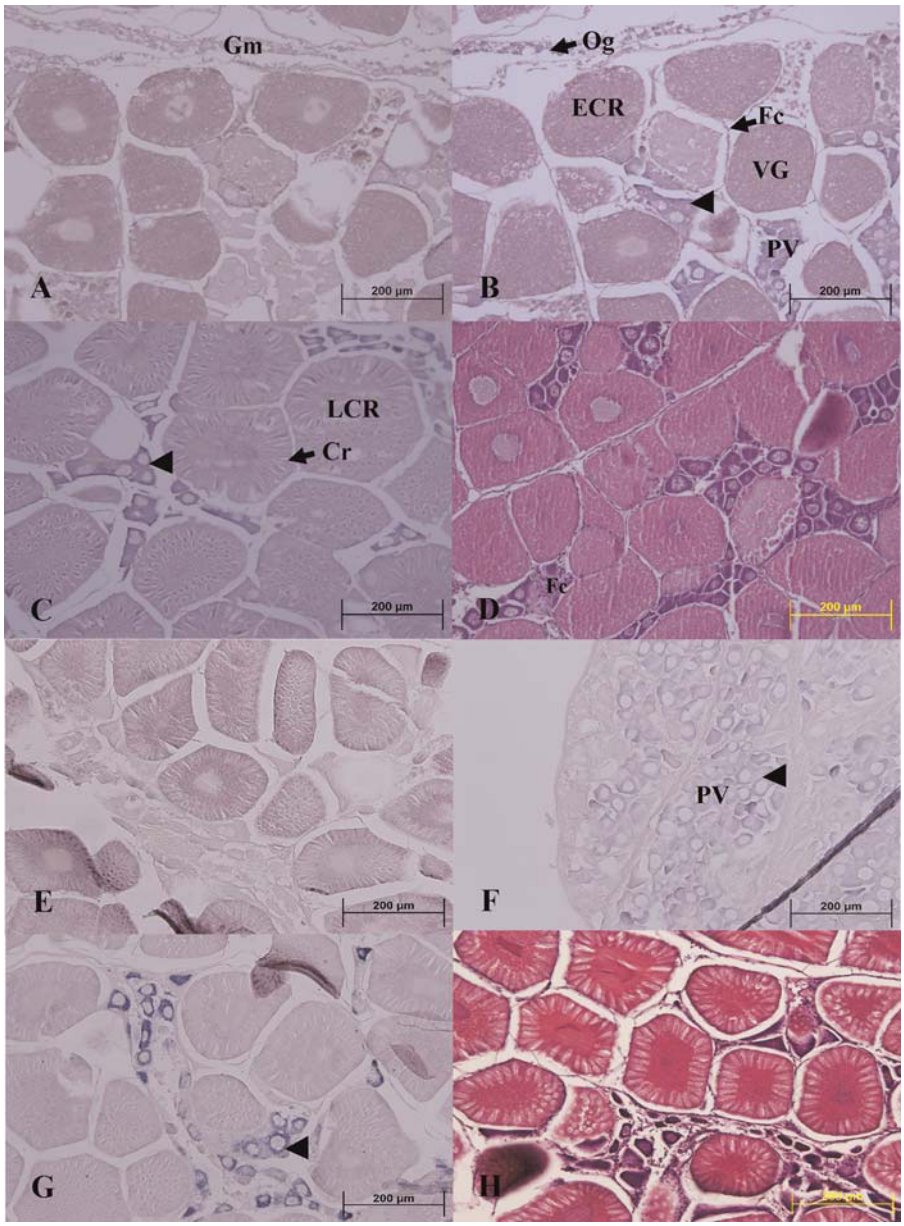

Figure 6. Localization of *PmMAPK1* mRNA during ovarian development in intact (**A–D**) and eyestalk-ablated (**E–H**) *P. monodon* broodstock, visualized by *in situ* hybridization using sense (A,E) and antisense (B,C, and F,G) *PmMAPK1* probes. Oocyte stages were classified by a conventional hematoxylin/eosin staining (D,H). Gm, germinative zone, PV, previtellogenic oocytes; VG, vitellogenic oocytes; ECR, early cortical rod oocytes; Fc, follicular cells; Og, oogonia; Cr, cortical rod; LCR, late cortical rod oocytes. Arrowheads indicate positive hybridization signals (blue). [Color figure can be viewed in the online issue which is available at wileyonlinelibrary.com]

ERK2 are conserved across distantly related taxa. Accordingly, *MAPK1* and *ERK2* are orthologous, and should be recognized under a single name, for example, as MAPK1 in this study. In zebrafish, *MAPK* genes contain 6–14 exons whereas *MAPK1* possesses 8 exons (Krens et al., 2006a). Currently, there has been no information on genomic organization of MAPK1 in invertebrates. It is interesting to determine whether or not the number of exons of invertebrate MAPK1 is conserved, particularly in crustaceans.

Expression Profiles and Localization of *PmMAPK1* Transcripts During Ovarian Development of *P. monodon*

Tissue distribution analysis revealed only a single isoform of *PmMAPK1* constitutively expressed in all examined

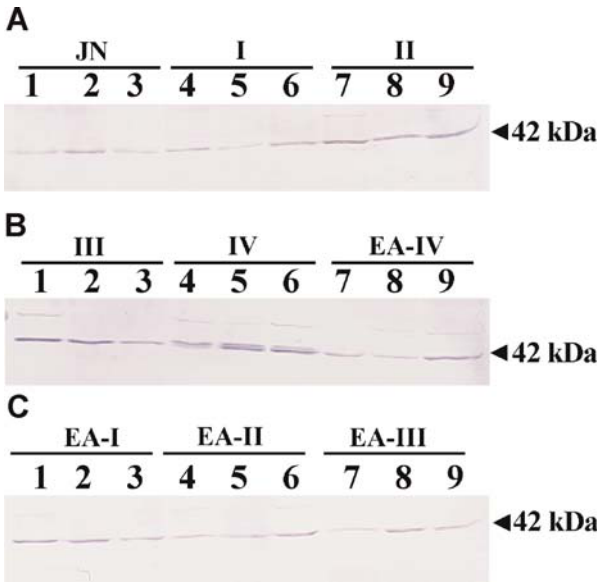

Figure 7. Western blotting analysis of PmMAPK1 using total ovarian proteins (20 μg) of juveniles and of intact and eyestalk-ablated (EA) *P. monodon* broodstock (panels **A**–**C**), as detected by mouse anti-rat MAPK1/3 MAb (1:1,000). Panel A: lanes 1–3, 4–6, and 7–9 = ovaries of juveniles and previtellogenic and vitellogenic ovaries of intact broodstock. Panel B: lanes 1–3 and 4–6 = early cortical rod and mature ovaries of intact broodstock, lanes 7–9 = mature ovaries of eyestalk-ablated broodstock. Panel C: lanes 1–3, 4–6, and 7–9 = previtellogenic, vitellogenic, and early cortical rod ovaries of eyestalk-ablated broodstock.

tissues of *P. monodon*. This suggested that a particular gene product may have multiple functions in diverse physiological processes. The *MAPK1* null mice died in utero because of a defect in trophoblasts, suggesting that MAPK1 was important for embryo development (Nishimoto and Nishida, 2006). MAPK1/3 modulates most cell activities, including cell-cycle progression, proliferation, cytokinesis, transcription, differentiation, cell death, migration, actin and microtubule networks, neural induction, and cell adhesion in mammalian cells (Ramos, 2008). These roles agree with the presence of *PmMAPK1* in all tissues examined.

The expression levels of *PmMAPK1* mRNA in ovaries were significantly higher in broodstock than in juveniles. In addition, it was up-regulated in early cortical rod (stage III) ovaries in intact broodstock, suggesting that *PmMAPK1* should be functionally involved in ovarian development of *P. monodon*. Eyestalk ablation resulted in high expression levels of *PmMAPK1* in both cortical rod (III) and mature (IV) ovaries of *P. monodon* broodstock. Accordingly, abundant expression of the PmMAPK1 gene products may be required for the final maturation of shrimp ovaries (see below).

In situ hybridization was used to localize the *PmMAPK1* transcript in ovaries of *P. monodon*. The *PmMAPK1* transcript was found in ooplasm, and the hybridization signal was more intense in the early previtellogenic than late previtellogenic oocytes in all ovarian stages of both intact and eyestalk-ablated female broodstock. The finding further suggested that *PmMAPK1* plays a role in oogenesis and ovarian development of *P. monodon*. Typically, stages of oocytes within a single ovarian lobe are not synchronous (Medina et al., 1996). Accordingly, the ovarian developmental stage was determined based on the predominant oocyte type in a particular specimen. Contradictory results from quantitative real-time PCR and *in situ* hybridization on the disappearance of *PmMAPK1* hybridization signals in ooplasm of more mature stages of oocytes may have been due to the significant increase in oocyte size as oogenesis proceeds. Moreover, quantification of *PmMAPK1* mRNA profiles was examined based on cDNA template from the ovarian tissue, whereas *in situ* hybridization revealed subcellular localization of the *PmMAPK1* transcript. Technically, *in situ* hybridization detects gene expression with much lower sensitivity than real-time quantitative PCR (Klinbunga et al., 2009).

PmMAPK1 Protein Profiles During Ovarian Development of *P. monodon*

The expression profiles of *PmMAPK1* mRNA and protein were different in both intact and eyestalk-ablated shrimp. In contrast to results at the transcriptional level, the PmMAPK1 protein level in vitellogenic, early cortical rod, and mature (stages II–IV) ovaries was significantly greater than that in previtellogenic (stage I) ovaries in intact broodstock ($P < 0.05$). The level of PmMAPK1 was comparable during ovarian development of eyestalk-ablated broodstock. In contrast, the *PmMAPK1* mRNA was up-regulated in stage III, and stages III and IV in respective groups of broodstock. This suggested that the accumulated *PmMAPK1* mRNA in oocytes is probably required for its rapid translation during the late stages (III and IV) of ovarian development of *P. monodon*.

Eyestalk ablation affects protein and lipid metabolism in shrimp ovaries (Marsden et al., 2007). Different mRNA and protein expression profiles of *adipose differentiation-related protein*, a gene functionally involving lipid accumulation, during ovarian development of *P. monodon* were recently reported (Sittikankaew et al., 2010). Apparently, the expression profiles of the *PmADRP* and *PmMAPK1* transcripts in both ablated and non-ablated female broodstock of *P. monodon* showed a similar pattern. However, the ovarian PmADRP protein was observed in vitellogenic, early cortical rod, and mature ovaries, but not in previtellogenic ovaries of intact broodstock and juvenile ovaries. Eyestalk ablation clearly promoted earlier expression of PmADRP in the previtellogenic ovaries of eyestalk-ablated *P. monodon* broodstock (Sittikankaew et al., 2010). The high levels of the ovarian PmMAPK1 protein, present from early developmental stages onward in ablated broodstock, suggest that eyestalk ablation results in earlier and greater expression of the PmMAPK1 protein during oogenesis of *P. monodon*.

In *Xenopus*, MAPK activates a protein kinase, p90rsk by phosphorylation and, in turn, p90rsk down-regulates Myt1

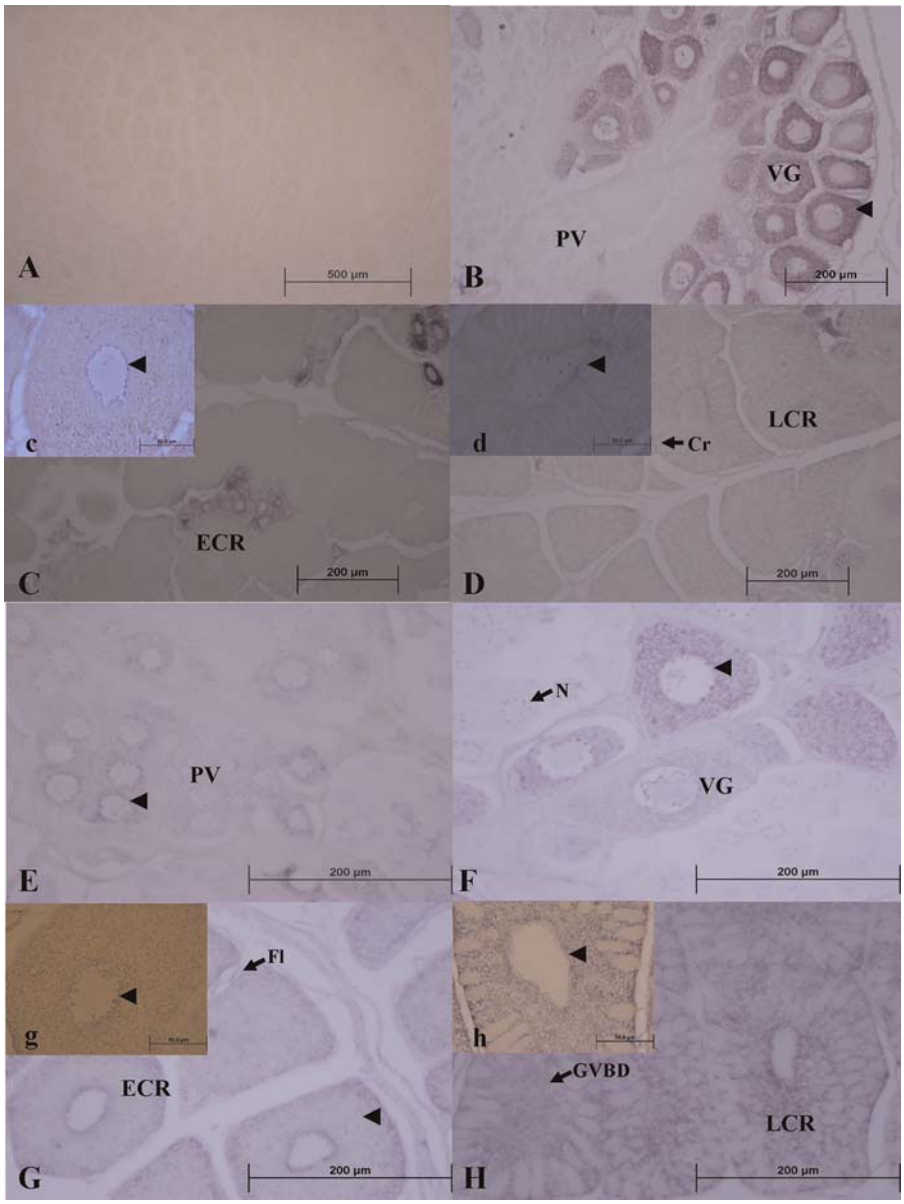

Figure 8. Localization of the PmMAPK1 protein in ovaries of intact (**B**–**D**) and eyestalk-ablated (**E**–**H**) *P. monodon* broodstock determined by immunohistochemistry. The blocking solution (**A**) was used as the negative control. Arrowheads indicate the positive immunoreactive signals. The insert panels with a higher magnification (100×) showed localization of PmMAPK1 around the nucleus of early cortical rod oocytes (c, d, g, and h). PV, previtellogenic oocytes; VG, vitellogenic oocytes; ECR, early cortical rod oocytes; LCR, late cortical rod oocytes; Cr, cortical rod; N, nucleolus; Fl, follicular layer; GVBD, germinal vesicle breakdown.

(membrane-associated tyrosine- and threonine-specific cdc2-inhibitory kinase) by binding to its C-terminal regulatory domain. This results in down-regulation of Myt1, leading to the activation of MPF (Palmer et al., 1998).

Progesterone (P4) and 17α-hydroxyprogesterone (17α-OHP4) stimulated ovarian maturation and yolk protein synthesis of penaeid shrimp in vivo (Kulkarni et al., 1979; Yano, 1985, 1987; Quackenbush, 1992). Recently, the activity of progesterone and 17α-hydroxyprogesterone extracted from the polychaetes in comparison with the synthetic hormones (0.4, 0.7, and 1.0 ng/ml for P4 and 1.0, 2.0, and 3.0 ng/ml for 17α-OHP4) were tested in vitro against previtellogenic ovaries of *P. monodon* for 24 hr. Synthetic steroid hormones gave similar results to steroid hormone extracted from polychaetes. P4 was more effective in enhancing the final maturation of oocytes, while 17α-OHP4

had more effects on vitellogenic oocytes (Meunpol et al., 2007).

More recently, *progestin membrane receptor component 1* (*PmPGMRC1*) in ovaries of *P. monodon* was identified and characterized. The expression level of *PmPGMRC1* mRNA was up-regulated in mature (stage IV) ovaries in intact broodstock ($P < 0.05$). Unilateral eyestalk ablation resulted in an earlier up-regulation of *PmPGMRC1* since the vitellogenic (II) ovarian stage (Preechaphol et al., 2010b). Progesterone administration promotes the expression level of *PmPGMRC1* in ovaries of 14-month-old shrimp at 72 hr post-treatment (S. Klinbunga, unpublished work). Accordingly, further studies to determine if the expression of *PmMAPK1* mRNA and/or protein are affected by P4 and if the PmMAPK1 protein plays a similar role in meiotic maturation of *P. monodon* oocytes, as previously reported in many species, should be carried out.

The positive immunoreactive signals for PmMAPK1 protein were observed around the oocyte nucleus, suggested a temporal nucleo-cytoplasmic transport of PmMAPK1 during oogenesis in *P. monodon*. Typically, MAPK is activated in the cytoplasm and subsequently translocated into the nucleus where it induces gene expression and promotes cell-cycle entry (Yu et al., 2007).

In the present study, the full-length cDNA sequence of *P. monodon MAPK1* was characterized. The *PmMAPK1* gene products were differentially expressed during oogenesis of *P. monodon*. Taken together, *PmMAPK1* gene products should be functionally involved in ovarian development and maturation of *P. monodon* oocytes. The basic knowledge from this study allows for further analysis of the activation/suppression of PmMAPK1 by different signal transduction cascades, and examination of its functional roles on reproductive maturation of female *P. monodon*.

MATERIALS AND METHODS

Experimental Animals

Female broodstock were live-caught from the Andaman Sea, and acclimated under farm conditions for 2–3 days. The post-spawning group (stage V) was immediately collected after the shrimp had ovulated ($n = 6$). Ovaries from cultured juveniles ($n = 5$) and intact broodstock ($n = 31$) were dissected and weighed. For the eyestalk ablation group, shrimp were acclimated for 7 days prior to unilateral eyestalk ablation. Ovaries of eyestalk-ablated shrimp were collected 2–7 days after ablation ($n = 31$). The ovarian developmental stages of *P. monodon* were classified into four stages according to gonadosomatic indices (GSI, ovarian weight/body weight $\times$ 100): <1.5, 2–4, >4–6, and >6% for previtellogenic (stage I, $n = 8$ and 6 for intact and eyestalk-ablated broodstock, respectively), vitellogenic (II, $n = 7$ and 4), early cortical rod (III, $n = 7$ and 10), and mature (IV, $n = 9$ and 11) stages, respectively. The ovarian developmental stage of each shrimp was further confirmed by conventional histology (Qiu et al., 2005). Various tissues of a female and testes of a male broodstock were collected, immediately placed in liquid N_2, and kept at -70°C for tissue distribution analysis of *PmMAPK1*.

Total RNA and First Strand cDNA Synthesis

Total RNA was extracted from ovaries of *P. monodon* using TRI Reagent (Molecular Research Center). The concentration of the extracted RNA was spectrophotometrically measured (Sambrook and Russell, 2001). One and a half micrograms of DNase I-treated total RNA was reverse-transcribed using an Improm-IITM Reverse Transcription System (Promega, Madison, WI).

Rapid Amplification of cDNA End-Polymerase Chain Reaction (RACE-PCR) and Sequence Analysis

Gene-specific primers (5′-PmMAPK1 and 3′-PmMAPK1; Table 1) were designed from an ovarian expressed sequence tag (EST) of *P. monodon* that significantly matched MAPK1 (extracellular-regulated MAP kinase) of *B. mori* (*E*-values = 1e−85, Preechaphol et al., 2007), and 5′ and 3′ rapid amplification of cDNA end (RACE)-PCRs were carried out using a SMART RACE cDNA Amplification Kit following the protocol recommended by the manufacturer (BD Bioscience Clontech, Mountain View, CA). The primary amplification product was diluted 50-fold, and a secondary PCR was carried out using a nested universal primer (nUPM) and the original gene-specific primer. The resulting product was visualized by gel electrophoresis, and purified from the agarose gel, cloned into pGEM-T Easy vector (Promega). Plasmid DNA was extracted from the positive clone and sequenced from both directions. Nucleotide sequences of EST and RACE-PCR fragments were assembled. Subsequently, primers PmMAPK1-ORF-F/R were designed to amplify the complete ORF of *PmMAPK1*. The amplified *PmMAPK1* ORF was cloned and sequenced. The nucleotide sequence was searched with BlastX (Altschul et al., 1990; available at http://ncbi.nlm.nih.gov) for similar sequences previously deposited in GenBank. The protein domain and signal peptide in the deduced PmMAPK1 polypeptide were predicted using SMART (http://smart.embl-heidelberg.de). Molecular weight and p*I* of deduced PmMAPK1 protein were examined using Protparam (http://www.expasy.org/tools/protparam.html).

Multiple Sequence Alignments and Phylogenetic Analysis

Phylogenetic analysis of the deduced amino acid sequence of *P. monodon MAPK1* and *MAPK1*/*ERK2*, *MAPK3* and *ERK1b* from other species previously deposited in GenBank was performed. Multiple alignments were carried out using ClustalW (Thompson et al., 1994). A bootstrapped neighbor-joining tree (Saitou and Nei, 1987) was constructed with the Seqboot, Prodist, Neighbor, and Consense routines in Phylip (Felsenstein, 1993), and illustrated with Treeview (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

TABLE 1. Nucleotide Sequences of Primers Used for Isolation, Characterization, and Expression Analysis of *PmMAPK1*

Primer	Sequence
5′RACE-PCR	
5′-PmMAPK1	5′-TCTTCTTGATTGCTACCTTAGTTTTGG-3′
3′RACE-PCR	
3′-PmMAPK1	5′-CTTTGGCTTGGCTCGTGTGGCTG-3′
PmMAPK1 ORF	
PmMAPK1-ORF-F	5′-ATGACGGAGGATCGGCCT-3′
PmMAPK1-ORF-R	5′-CCCTTCGTCTCTTGTAATAATGCTA-3′
RT-PCR	
PmMAPK1-F	5′-ATTCTAACAAGGTTCAAGCAT-3′
PmMAPK1-R	5′-GAGCCAAGCCAAAGTCACAG-3′
EF-1α-F	5′-ATGGTTGTCAACTTTGCCCC-3′
EF-1α-R	5′-TTGACCTCCTTGATCACACC-3′
Quantitative real-time PCR	
qPmMAPK1-F	5′-CTCTACCAAACAAGCCAAAGGTG-3′
qPmMAPK1-R	5′-GGCTCTTCAGCTACAGGCTCATC-3′
qEF-1α-F	5′-TCCGTCTTCCCCTTCAGGACGTC-3′
qEF-1α-R	5′-CTTTACAGACACGTTCTTCACGTTG-3′
In situ hybridization	
$PmMAPK_{957}$-T7-F[a]	5′-**TAATACGACTCACTATAGGG**GTCTGCAAATGATAATGTGACCAAA-3′
$PmMAPK_{957}$-SP6-R[a]	5′-**ATTTAGGTGACACTATAGAA**GCAAGCCTCTGCTTAAAGAGTACAG-3′

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

RT-PCR and Tissue Distribution Analysis

Expression of *PmMAPK1* (primers PmMAPK1-F/R, 247 bp) in ovaries and testes of wild broodstock (n = 4 and 3, respectively) and those of cultured juveniles (n = 4 for each sex) were analyzed by RT-PCR. *EF-1*α_{500} was included as a positive control (Table 1). The thermal profiles were 94°C for 3 min, followed by 25 cycles of 94°C for 30 sec, 53°C for 45 sec, and 72°C for 1 min, with a final extension at 72°C for 7 min. Expression of *PmMAPK1* in various tissues of a female and testes of a male broodstock was performed for 30 cycles using the same PCR conditions.

Quantitative Real-Time PCR

Standard curves representing 10^3–10^8 copies of *PmMAPK1* in triplicate (primers qPmMAPK1-F/R; 186 bp) and the internal control, *EF-1*α_{214} (primers qEF-1α-F and qEF-1α-R, 214 bp; Table 1), were constructed. *PmMAPK1* and *EF-1*α_{214} were amplified from ovaries of each shrimp in a 10-μl reaction volume containing 5 μl of 2× LightCycler 480 SYBR Green I Master (Roche, Indianapolis, IN), 50 ng of the first-strand cDNA template, and 0.2 μM each of gene-specific primers. The thermal profile for quantitative real-time PCR was 95°C for 10 min followed by 40 cycles of 95°C for 15 sec, 58°C for 30 sec, and 72°C for 30 sec. Real-time PCR was carried out in duplicate for each specimen. The relative expression levels (copy numbers of *PmMAPK1* and *EF-1*α_{214}) between shrimp possessing different stages of ovarian development were compared using one-way analysis of variance (ANOVA) and Duncan's new multiple range test ($\alpha = 0.05$).

In Situ Hybridization

Pieces of ovaries from 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 three times each with PBS (136.89 mM NaCl, 0.9 mM Na_2HPO_4, 1.76 mM KH_2PO_4, 2.68 mM KCl, pH 7.3) and 50% ethanol at room temperature, and stored in 70% ethanol at −20°C until used. Conventional paraffin sections (5 μm) were carried out. The sense and antisense cRNA probes ($PmMAPK_{957}$-T7-F and $PmMAPK_{957}$-SP6-R) were synthesized using DIG RNA labeling mix (Roche). Tissue sections were dewaxed with xylene and dehydrated in absolute ethanol. The sections were prehybridized with 2× SSC containing 50% deionized formamide, 1 μg/μl yeast tRNA, 1 μg/μl salmon sperm DNA, 1 μg/μl BSA, and 10% (w/v) dextran sulfate at 50°C for 30 min, and hybridized with either the antisense or sense probes in the prehybridization solution overnight at 50°C. After hybridization, the tissue sections were washed twice with 4× SSC for 5 min each, and once with 2× SSC containing 50% formamide for 20 min at 50°C. The sections were immersed in prewarmed RNase A buffer (0.5 M NaCl, 10 mM Tris–HCl, pH 8.0, 1 mM EDTA) at 37°C for 5 min, and treated with RNase A (20 μg/ml) at 37°C for 30 min. Tissue sections were washed four times with RNase A buffer (37°C, 10 min each), and then with 2× SSC (50°C, 15 min each). High stringency washing was carried out twice in 0.2× SSC at 50°C for 20 min each. The bound probes were detected with a DIG Wash and Block Buffer Kit (Roche) (Qiu and Yamano, 2005).

Western Blotting

Ovaries of juveniles and intact and eyestalk-ablated broodstock of *P. monodon* (N = 3 for each stage) were homogenized in the ice-cold PBS buffer. The protein concentration was measured by a dye binding assay (Bradford, 1976). Ovarian proteins (20 μg) were resolved in a 12% polyacrylamide gel under reducing conditions (Laemmli,

1970), and electroblotted onto a PVDF membrane (Towbin et al., 1979) in a 25 mM Tris, 192 mM glycine (pH 8.3) buffer containing 20% methanol at 100 V for 90 min. The membrane was treated with the blocking solution (5% BSA in Tris buffered saline (TBS) containing 0.1% Tween-20 (TBST); 20 mM Tris–HCl, 0.9% NaCl, pH 7.4, 0.1% Tween -20), and incubated with mouse anti-rat MAPK1/MAPK3 monoclonal antibody (1:1,000, Abcam, Cambridge, MA) for 1 hr at room temperature; sequence similarity between the immunogen (the synthetic peptide of positions 324–345 of rat MAPK3, EALAHPYLEQYYDPTDEPVAEE; accession no. P21708) and a homologous region in PmMAPK1 was 95.45%. The membrane was washed three times with 1× TBST, and incubated with goat anti-rabbit IgG conjugated with alkaline phosphatase (1:10,000, Bio-Rad, Hercules, CA) for 1 hr. The hybridized signal was detected using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (NBT/BCIP) (Roche) as a substrate. The intensity of PmMAPK1 bands was quantified from the scanned photographs using the Quantity One software (Bio-Rad). The expression level of PmMAPK1 in each ovarian stage was statistically analyzed.

Immunohistochemistry

Ovaries were dissected out from *P. monodon* broodstock and fixed in Davidson's fixative overnight at 4°C. Standard paraffin sections (6 μm) were carried out. IHC was carried out essentially described by Qiu and Yamano (2005). Briefly, deparaffinized sections were immerged in 0.01 M citric acid buffer (pH 6.0) containing 0.1% Tween-20 and autoclaved for 5 min. After treatment in a blocking solution (Roche) for 2 hr, the sections were incubated with mouse anti-rat MAPK1/3 MAb (1:30, Abcam) overnight, and rinsed with TBST three times for 5 min each. The tissue sections were incubated with goat anti-rabbit IgG conjugated with phosphatase (1:1,000) for 30 min and rinsed with TBST. Immunoreactive signals were visualized using NBT/BCIP (Roche) as the substrate. Tissue sections were also incubated with the blocking solution as the negative control.

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