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Two alpha-2 macroglobulin from *Portunus trituberculatus* involved in the prophenoloxidase system, phagocytosis and regulation of antimicrobial peptides

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ABSTRACT

Alpha-2 macroglobulin (A2M) is a ubiquitous protease inhibitor involved in the innate host defense system. Herein, two distinct A2M genes (designated as *PtA2M-1* and *PtA2M-2*, respectively) were isolated from the swimming crab *Portunus trituberculatus*. PtA2M-1 and PtA2M-2 encoded proteins with 1541 or 1516 amino acids, respectively, containing the typically functional domains of A2M. Unlike highly expressed in hemocytes of most arthropods, *PtA2M-1* and *PtA2M-2* were predominantly detected in gill, eyestalk and digestive tracks. During the embryonic stages, PtA2Ms were found to be expressed most highly in fertilized eggs, suggesting their maternal origin. After challenged with *Vibrio alginolyticus*, the transcripts of PtA2Ms showed similar time-dependent response expression pattern, while PtA2M-1 was more sensitive to *Micrococcus luteus* and *Pichia pastoris* infection than PtA2M-2. Knockdown of *PtA2M-1* or *PtA2M-2* could significantly enhance the expression of prophenoloxidase (proPO) associated genes (*PtproPO* and *PtPPAF*) and serine protease related genes (*PtcSP1-3* and *PtSPH*), however, *PtLSZ* and the phagocytosis-related genes (*PtMyosin* and *PtRab5*) were effectively inhibited. These results were further supported by the PO and lysozyme activities in hemolymph of the PtA2M-1- or PtA2M-2-silenced crabs. In addition, PtA2M-1 and PtA2M-2 could regulate the expression of antimicrobial peptide (AMP) genes (*PtALF1-3*, *PtCrustin1* and *PtCrustin3*) through the Toll and NF-κB pathways. Our findings together suggest that PtA2Ms might function in crab host defense via regulating the proPO system, phagocytosis and the expression of AMP genes.

1. Introduction

Alpha-2 macroglobulin (A2M), widely distributed from protostomes to deuterostomes, is a broad-spectrum protease inhibitor [1–3]. It performs the inhibition by physical trapping of target proteases within its protein fold [1,4]. When A2M is activated, the receptor binding domain of A2M is exposed and binds to low density lipoprotein receptor-related protein (LRP). The complex of A2M and LRP is ultimately internalized and cleared through the lysosome system [5].

A2M protein containing thioester bond belongs to a large evolutionarily conserved family, the thioester-containing protein (TEP) superfamily. This superfamily also comprises vertebrate complement components (C3, C4, and C5) and insect TEPs (iTEPs) [6,7]. In vertebrates, A2M is well studied and has been identified to regulate host cell apoptosis [8] and cancer cell metabolism [9], enhance prothrombin activation and thrombin potential [10], mediate T-cell proliferation [11], induce proliferation and activation macrophages [12], and so on.

Since originally identified from horseshoe crab *Limulus* sp. [13], mounting A2Ms have been successively isolated from insects [14,15], crustaceans [16–19], and molluscs [2,20]. Previous studies have shown that the peptidoglycan, lipopolysaccharide or pathogenic microbials could induce the mRNA expression of A2Ms [16,17,19,21]. Recently, LvA2M from *L. vannamai* has been proven to be necessary for activation of the prophenoloxidase (proPO) system [22]. For the pearl oyster, knockdown of *PfA2M* significantly reduces the phagocytosis of *Vibro*

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Table 1
Primer sequences used in this study.

Primers Name	Forward primer (5′–3′)	Reverse primer (5′–3′)
cDNA cloning		
PtA2M-1-1	TCGCCTCCCCTCTTTATGGA	CGTAGCTGGTGTAGTAGCCG
PtA2M-1-2	GCGTCTCACAAACCACCAAC	TCCGTGCCACTCTTGAAGTC
PtA2M-1-3	GGTGCCAGAAAACGGAACAC	CCACCTGTCATCTTCGCCTT
PtA2M-2-1	GCGTTGTGGAGGAGAGACAA	TAGTGACCGTAGGGATGCCA
PtA2M-2-2	CCCCTCACCATTCACCTCAC	GTCCTTGTGGCGATAGCGTA
PtA2M-2-3	CCGTCCATTATCCATCGCCA	TAACAAACTGGTCCGGGTCG
qRT-PCR		
PtA2M-1	TGGAGGACGTGAAACCCGGC	CGTCGTTTGGTGGCAGCGTG
PtA2M-2	GTGGTTGGCTACGGGACGGGT	ACGGCAATGTCATCACTGGGGAT
PtcSP1	ACTATGTCCAGCCAGCGTGT	GGAAGGACTCGCGCTCATAG
PtcSP2	TAAGGACATCGGACAGGAGACACT	TAAGGAAGTGAACGCTATCTCT
PtcSP3	AAGCCAGTCGAAATACAGGAG	CAGCATCTCCTTCCCAATTCC
PtSPH	CATCCTTGACCAGCCAGCA	CCCACCCAGACACAACACA
PtMyosin	CGTTGGCGAAGTAGGAGAGT	GAACAAGAGGCGTAATGAGGT
PtRab5	AACCCAGCATCCAGTCACCC	TACCCCTAAGCCCCTCAACC
PtLSZ	AGAAGTGGAAGGGAAAGGGG	GTCTGATGGGAACACGAGCG
PtproPO	CCTCTTCTTCACGACACTCAACTG	TCACGAGATAACACAAAACGCC
PtPPAF	GGACAGGACCAAGACCCAGT	GATTTGAGAAGGAACAAGCGTG
PtCrustin1	GGCAGTTGTGGCTACCATTGT	CGCTCGGTGTAAGGTGGATAG
PtCrustin3	AGTATCTCAGAATCGACCA	CCCTCTTAGTTTCTCTTGTT
PtALF1	ACGACGAGGAGGAGAAAGAGG	GGCACTGATGGTGGAAACTGA
PtALF2	GACGCTCTGAAGGACTTTATG	CGCCGAAACGCTTAGAAATAC
PtALF3	TAGTCGTGGTGAGAGGGCAA	CTTTGCTCTCTCATCAGGAC
PtgC1qR	ACGTGTCTTCAGGGGCGTGT	GCTCCCGGTCACCTCTCGTG
PtMBL	GGCACCGTCGGTCATCCAAC	GCTGGCTGCGACCAAACCTT
PtTEP	CTCTTCTCGCTGCTTTCCTTCATC	TTTTTGGGACTTTGCCACCACTT
PtTLR	CATTGAGGACAGCCACAGGAC	TGGTAGAGAGGTACAGCTTGAGTTC
PtMyD88	GGTCCTTGAAGCAACAGGTGGTAG	AGTGCTGGCTGACTAGGAGATGAC
PtPelle	ACTCTTGCCTTCCCTTGCTAAC	ACTGACCATGAATCATACCCCTG
PtRelish	CCAGAGTACGCAAGCCACATCAC	CCGCAGCACCACCTTGTTCAG
PtJNK	AGTGTGGCGGCTCAGCTGTT	CTCCACTCCGACTGCCTCGC
β-actin	TCACACACTGTCCCCATCTACG	ACCACGCTCGGTCAGGATTTTC
RACE		
PtA2M-1-3′RACE1	GATGGCTGCTTCACTGCTGTCG	
PtA2M-1-3′RACE2	TTACCAGGGTCCTCTCAATGTAGTCG	
PtA2M-1-5′RACE1	GGCGTGGTGACCCAAATCTCGG	
PtA2M-1-5′RACE2	CAGGGCAGCGAAAGCACGACAG	
PtA2M-2-3′RACE1	AAGCAGGTGGTTGGCTACGGGAC	
PtA2M-2-3′RACE2	TCGGGTTCTGATTTGGACGGGGT	
PtA2M-2-5′RACE1	GGAGTCAATGGGGAGGTCGGTG	
PtA2M-2-5′RACE2	GCACCGCCTTGAATCCTCCCAC	

alginolyticus by hemocytes in *vivo* [21]. Similar report is found in hard tick that provided the role of A2M in the phagocytosis of *Chryseobacterium indologenes* [15]. Moreover, the amphioxus A2M is found to be a maternal immune factor and protects developing embryos against pathogenic attack through maternal transfer of immunity [23]. These findings suggest that A2M is an important immune-relevant molecule in the host defense of invertebrates.

Keen research interest in the A2M molecules of *Portunus trituberculatus* is due to the high economic value and the continuing problem of infectious diseases in this crab. Understanding of the innate immunity may provide a basis for the development of new strategies for disease management in crab culture. Here, we reported the full-length cDNA sequences of two isoforms of A2M from the swimming crab *P. trituberculatus*. The expression profiles in embryonic stages and adult tissues, and temporal expression following pathogen challenge were investigated. The maternal mRNA of PtA2Ms was also detected in fertilized eggs and embryos. The involvement of PtA2Ms in the proPO activating system, phagocytosis and regulation of AMP genes was elucidated by the siRNA-mediated RNA interference.

2. Materials and methods

2.1. Animals, immune challenge and samples collection

Healthy crabs (100 ± 2 g) purchased from a commercial farm in Qingdao, China, were reared in filtered seawater (15 ± 2 °C) and fed with clams once daily at nightfall for one week before processing. Crabs were randomly separated into four groups and each crab of the challenge group was injected with 3×10^8 cells of live *Vibrio alginolyticus*, *Micrococcus luteus* and *Pichia pastoris* GS115 resuspended in 100 μl phosphate buffered saline (PBS). *V. alginolyticus*, *M. luteus* and *P. pastoris* were grown respectively in TSB medium at 28 °C, LB medium at 37 °C and YPD medium at 28 °C to logarithmic phase and diluted to 3×10^9 cells/ml with PBS. Crabs injected with 100 μl PBS were served as the control group. At each time point (0, 2, 4, 8, 12, 24, 48 and 72 h) post-injection, five crabs were randomly sampled to collect hemocytes. Briefly, hemolymph was harvested from the last walking leg with an equal volume of ice-cold anticoagulant buffer (27 mM sodium citrate, 336 mM NaCl, 115 mM glucose, 9 mM EDTA, pH 7.0). Then the hemolymph immediately centrifuged at 800 g, 4 °C for 5 min to isolate the hemocytes. For tissue-specific expression analysis, five untreated male crabs and five untreated female crabs were dissected to get hemocytes, gill, hepatopancreas, eyestalk, muscle, heart, intestine, stomach, ganglia thoracalia, ovary/testis and brain.

Meanwhile, ovigerous female crabs (*P. trituberculatus*) were collected during the breeding season and fertilized eggs (Fe) were collected immediately after discharge. The developmental stage of crab embryo was monitored under a dissecting microscope, and embryos from the cleavage stage (Cs), blastula stage (Bs), gastrula stage (Gs) and heart beating stage (Hs) were collected separately in 1.5 ml tubes from the same crab. The embryonic samples at each stage were collected from five berried crabs. Except hemocytes, tissues and embryos were immediately frozen in liquid nitrogen and stored at −80 °C for further RNA isolation.

2.2. RNA isolation, cDNA synthesis and cloning of PtA2Ms

The total (1 µg) RNA was extracted using TRIzol reagent according to the manufacture's protocol (Invitrogen). The RNA quantity and purity were tested using 1% agarose gel electrophoresis and Nanodrop 2000 (Thermo). The first-strand cDNA was synthesized for the quantitative real-time PCR (qRT-PCR) analysis using a PrimeScript™ first Strand cDNA Synthesis Kit (Takara, Dalian, China) with an oligo dT primer. To amplify the 3′- and 5′- ends of PtA2Ms cDNA sequence, the first strand cDNA was synthesized using the Clontech SMARTer™ RACE cDNA Amplification kit (Takara, Dalian, China) with 5′-CDS Primer A and SMARTer IIA oligo (5′-RACE-ready cDNA) and 3′-CDS Primer (3′-RACE-ready cDNA).

Two unigenes homologous to A2M of *Scylla paramamosain* (CCW43201.1) and *Pacifastacus leniusculus* (AEC50083.1) were found in our transcriptome dataset, designated as *PtA2M-1* and *PtA2M-2*, respectively. Gene-specific primers (Table 1) were designed to amplify partial cDNA sequences of *PtA2M-1* and *PtA2M-2*. The 3′- and 5′-ends were amplified using Universal Primer A mix combined with nested primers (Table 1). The PCR programs were performed as follows: 94 °C for 3 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at *T*a for 50 s, and elongation at 72 °C for 3 min, followed by a 10 min extension at 72 °C and cooling to 16 °C. The PCR products were gel-purified and cloned into pMD19-T simple vector (TaKaRa). After being transformed into the competent cells of *Escherichia coli* DH5a, positive recombinants were identified through anti-Amp selection prior to sequencing by a commercial company (Sangon, China). The complete cDNA sequences of PtA2Ms were obtained by overlapping the unigenes and the 5′ and 3′ fragments.

2.3. Sequence and phylogenetic analysis

Sequence alignment against the GeneBank protein database was performed using the BLAST at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST). Signal peptide was predicted using SignalP 4.0 program (http://www.cbs.dtu.dk/services/SignalP). The functional sites and domains in the deduced amino acid sequence were predicted with SMART program (http://smart.embl-heidelberg.de/). Multiple sequence alignment of PtA2Ms with other known A2M proteins was performed using the CLUSTALW program packaged in DNAMAN 8.0 software. The phylogenetic tree was constructed by the neighbor-joining (NJ) method using MEGA 7.0 software, applying the amino acid substitution type, poisson model and a bootstrapping procedure with 1000 times.

2.4. Quantification of gene expression by qRT-PCR

qRT-PCR was applied to analyze the expression of *PtA2M-1* and *PtA2M-2* in different tissues and embryos using two pairs of gene-specific primers (Table 1). To study their possible functions in *P. trituberculatus* innate immunity, the expression patterns of PtA2Ms in crab hemocytes after pathogen challenge were investigated using the same primers. Reactions were carried out on an ABI PRISM 7300 Sequence Detection System (Applied Biosystems, USA) using SYBR green II as fluorescent dye. The primers used for the qRT-PCR analysis were presented in Table 1, and β-actin was used as a reference for internal standardization.

For each PCR reaction, a total volume of 20 µl contained 10 µl of 2 SYBR Premix Ex Taq (TaKaRa), 0.4 µl 50 ROX Reference Dye, 4 µl of the diluted cDNA, 0.4 µl of each primer (10 µM), and 4.8 µl of sterile distilled H_2O. The PCR parameters were 95 °C for 30 s, 40 cycles of 95 °C for 5 s and 60 °C for 35 s. Each sample was examined in triplicate. Relative expression levels of PtA2Ms were quantified by $2^{-\Delta\Delta CT}$ methods [24].

2.5. Synthesis of siRNAs and RNAi assay

Small interfering RNA (siRNA) for RNA interference (RNAi) assays was synthesized using an in vitro transcription T7 kit (Takara, Dalian) according to the manufacturer's instructions. The sequence-specific PtA2M-1-siRNA (5′-GUGCACUAUGGAUUUGGCG-3′) and PtA2M-2-siRNA (5′-GUGCCUGACCAACUUGGUG-3′) were synthesized to silence the expression of the respective *PtA2M-1* or *PtA2M-2* gene, and the sequence of siRNA was scrambled to generate the PtA2M-1-random-siRNA sequence (5′-UGCUGUGGCUAUGGAUGCA-3′) and PtA2M-2-random-siRNA sequence (5′-UUACGACACUACGACCUGC-3′). The synthesized siRNAs were dissolved in siRNA buffer (50 mM Tris–HCl, 100 mM NaCl, pH 7.5), and examined by electrophoresis and spectrophotometry.

The RNAi assay was conducted in crab by the injection of siRNA into the arthrodial membrane of the last walking leg at 100 µg/crab using a syringe. In details, 50 µg of specific siRNA (or random-siRNA) was injected at a volume of 50 µl per crab. At 24 h after the first injection, the siRNA (50 µg) suspended in 50 µl PBS was injected into the same crab. At the same time, the crabs only injected with PBS was as a positive control. The amount of injected siRNA was chosen according to our pre-experiment and the RNAi assay in *Eriocheir sinensis* [25]. For each treatment, five crabs were used. At 24 h and 48 h after the last injection, the intestine and hemocytes from all crabs in the experimental groups and control groups were separately isolated and frozen in liquid nitrogen for RNA extraction and cDNA synthesis as described earlier. Then the expression of PtA2Ms was further determined by qRT-PCR to detect the efficiency of the RNAi. The assays described above were biologically repeated four times.

2.6. Expression patterns of immune-related genes in the PtA2Ms-silenced crabs

In order to explore the involvement of PtA2Ms in the serine protease cascades, phagocytosis, proPO activating system and complement-like pathways. The expression of twelve immune-related genes, including *PtcSP1-3* (clip domain serine protease, JF412648, JF412649 and JF412650), *PtSPH* (serine protease homologue, JF412651), *PtproPO* (FJ215871.1), *PtPPAF* (proPO-activating factor, GQ914996.1), *PtMyosin* (POR|c99862_g4), *PtRab5* (small GTP-binding protein, POR|c94716_g1), *PtLSZ* (lysozyme, FJ589729.2), *PtgC1qR* (complement C1q receptor, MK076886), *PtMBL* (mannose-binding lectin, POR|c92478_g1) and *PtTEP* (thioester-containing protein, MK076885), in response to PtA2M genes silencing was detected in the intestine using qRT-PCR. Meanwhile, the expression of AMP genes including *PtALF1-3* (anti-lipopolysaccharide factor, HM627757, HM627758 and GQ165621), *PtCrustin1* (FJ612106), *PtCrustin3* (JQ728425), and the key genes involved in the Toll, NF-κB and JNK pathways, including *PtTLR* (Toll-like receptor, KR108027.1), *PtMyD88* (myeloid differentiation factor 88, KM521426.2), *PtPelle* (POR|c93425_g1), PtRelish (MF624027.1) and *PtJNK* (c-Jun N-terminal kinase, POR|c99916_g3), was also investigated in the PtA2Ms-silenced crabs. The gene-specific primers used for qRT-PCR analysis were listed in Table 1 β-actin was used as the control.

2.7. Phenoloxidase (PO) activity in the PtA2Ms-silenced crabs

Total PO activity in hemolymph was detected after setting up the RNAi assay (Supplementary Fig. 3). Hemolymph was withdrawn from four PtA2M-1- or PtA2M-2-silenced crabs without the use of anticoagulant. Protein concentration was measured using a Bradford assay kit (Bio-Rad). L-3, 4-dihydroxyphenylalanine (L-dopa) was used to detect PO activity according to previous literature with slight modification [26]. Briefly, 2 mg of total hemolymph proteins in 435 μl of Tris–HCl (10 mM, pH 8.0) were mixed with 65 μl of freshly prepared chromogenic substrate L-3, 4-dihydroxyphenylalanine (3 mg/ml, Sigma). After incubation at room temperature for 30 min, 500 μl of 10% (v/v) acetic acid was added to each mixture. The reactions were measured the absorbance at 490 nm in precision microplate reader (Emax). The PBS and random-siRNA treated crabs was used as the black and negative control, respectively.

2.8. Lysozyme activity in the PtA2Ms-silenced crabs

After setting up the RNAi assay (Supplementary Fig. 3), lysozyme activity in hemolymph was measured based on the turbidimetric method [27] with slight modifications. Briefly, a solution of *M. luteus* (Nanjing Jiancheng, China) was prepared by dissolving 5 mg of the lyophilized cells in 20 ml sodium phosphate buffer (0.1 M, pH 6.2). 100 μl of hemolymph was withdrawn without the use of anticoagulant from four PtA2M-1- or PtA2M-2-knockdown crabs using a syringe and quickly added 1 ml of prepared *M. luteus* solution (MLS), and bathed at 37 °C for 15 min then received ice-bath for 3 min. The mixture of hemolymph and MLS was added to a 96-well plate (220 μl/well) in triplicate. 20 μl of double-distilled water and standard solution mixed with 200 μl MLS were added to 96-well plates as the black and standard, respectively. Plates were assessed immediately at the absorbance of 530 nm after the ice-bath. The PBS and random-siRNA treated crabs was used as the black and negative control, respectively.

2.9. Statistical analysis

Statistical analyses were processed using SPSS 16.0 software. The data were expressed as mean ± standard deviation (S.D.) and subjected to a one-way analysis of variance (ANOVA) followed by Duncan multiple comparison tests. Differences were considered statistical significance at $P < 0.05$ (*) and $P < 0.01$ (**).

3. Results

3.1. Structural features and phylogenetic analysis of PtA2Ms

The schematic structures and cloning strategies of PtA2M-1 and PtA2M-2 were shown in Supplementary Fig. 1. The full-length cDNA sequences of PtA2M-1 and PtA2M-2 were 5046 and 5079 bp, and deposited in GenBank under accession numbers MK076885 and MK076887, respectively. PtA2M-1 cDNA contained a 5′ untranslated region (UTR) of 15 bp, a 3′ UTR of 405 bp and an open reading frame (ORF) of 4626 bp encoding 1541 amino acid residues without signal peptide, while PtA2M-2 included a 136-bp 5′ UTR, a 392-bp 3′ UTR and a 4551-bp ORF encoding 1516 amino acid with the first 26 residues as the putative signal peptide (Supplementary Fig. 2). The theoretical mass of PtA2M-1 mature protein was calculated to be 170.96 kDa with an isoelectric point (pI) 5.62. The mature protein of PtA2M-2 has 1490 amino acid residues with a putative molecular mass of 165.04 kDa and a predicted pI of 5.15.

The conserved A2M-N-terminal domain (A2M-N), A2M_2 domain and receptor binding domain (RBD) were identified for PtA2M-1 and PtA2M-2 (Fig. 1). The bait sequence of PtA2M-1 and PtA2M-2 exhibited little similarity to that of other crustacean A2Ms, while the thioester motif (GCGEQNM) was highly conserved. Moreover, a histidine (His) residue (position at amino acid 1181 for PtA2M-1 and 1126 for PtA2M-2) responsible for binding specificity of thioester protein was found at the catalytic site about 110 amino acids downstream of the thioester domain. Lys residues used for receptor binding were also detected in the RBD (Fig. 1).

The complete amino acid sequences of A2Ms from different phyla of animals were chosen to construct a neighbor-joining (NJ) phylogenetic tree. Human complement components (C3, C4 and C5) and insect TEPs were employed as the outgroups. The tree topology can be separated into four distinct branches clades, including invertebrate A2Ms, vertebrate A2Ms, insect TEPs and human complement components, respectively (Fig. 2). Crustacean A2Ms were grouped together in the clade of invertebrate A2Ms. PtA2M-1 were clustered with SpA2M from *S. paramamosain*, while PtA2M-2 was closely related to FcA2M-2 from *Fenneropenaeus chinensis*.

3.2. Expression profiles of PtA2Ms in adult tissues and embryos

The basal expression of *PtA2M-1* and *PtA2M-2* was detected in all tested tissues and embryos stages (Fig. 3). The mRNA levels of *PtA2M-1* in gill, intestine and eyestalk of male crabs were significantly higher than those in females, but the transcripts exhibited a reverse trend in gonad (Fig. 3A). *PtA2M-2* was highly expressed in gill, eyestalk and digestive tract, and its expression in gill, stomach, eyestalk and ganglia thoracalia of male crabs were significantly higher than those in females (Fig. 3B). It is worth noting that the lowest expression of *PtA2M-1* and *PtA2M-2* was both detected in hemocytes of male and female crabs. During the embryonic stages, the highest mRNA levels of *PtA2M-1* and *PtA2M-2* were both detected in Fe, then decreased remarkably to the lowest level at the blastula stage, and increased slightly at the heart beating stage (Fig. 3C and D).

3.3. Challenge-induced expression by microorganisms

Hemocytes are considered the main line of defense in invertebrates and rapidly respond to pathogenic invasion. Therefore, hemocytes were chosen as the tissue to evaluate the expression patterns of PtA2Ms in response to bacterial (*V. alginolyticus* and *M. luteus*) or fungal (*P. pastoris*) infection (Fig. 4). The expression of *PtA2M-1* increased significantly and reached to the highest level at 2 h post *V. alginolyticus* and *P. pastoris* infection (Fig. 4A). Subsequently, the mRNA level of *PtA2M-1* decreased and gradually recovered to the initial level at 8 h post-injection. After challenge with *M. luteus*, the mRNAs of *PtA2M-1* rose to the highest level at 4 h post injection, then decreased significantly and recovered at 24 h post-injection (Fig. 4A).

Comparatively, *PtA2M-2* was less sensitive to *M. luteus* and *P. pastoris* challenges. Its transcripts rose to a peak at 2 h after *V. alginolyticus* challenge, and recovered to the control level at 8 h post injection (Fig. 4B). When challenged with *P. pastoris*, the transcripts of *PtA2M-2* increased remarkably at 4 h post injection, and quickly recovered to the initial level at 8 h post injection, however, its expression level was significantly up-regulated again at 48 h and 72 h post-injection. After *M. luteus* challenge, the expression of *PtA2M-2* cannot be affected within 24 h post injection, and then up-regulated significantly at 48 h and 72 h post injection.

3.4. Expression analysis of immune-related genes after PtA2M genes silenced by RNAi

The silencing efficiency of synthesized PtA2M siRNA was shown in Fig. 5. The mRNA levels of *PtA2M-1* in the intestine of the RNA interference group was significantly suppressed at 24 h compared with PBS group and random-siRNA group (Fig. 5A), the silencing efficiency of PtA2M-1-siRNA was 66.5%, indicating that *PtA2M-1* could be specifically and efficiently silenced by the synthesized siRNAs. The expression of *PtA2M-2* in the intestine could be significantly knocked down by PtA2M-2-siRNA at 48 h post-injection, and the silencing efficiency reached up to 94.0% (Fig. 5B). Similarly, the expression of *PtA2M-1* and *PtA2M-2* in the hemocytes could also be manifestly inhibited at 48 h post the specific-siRNA injection (Supplementary Fig. 3). According to the results of specific-tissue expression, intestine was selected as the optimal tissue of PtA2Ms silencing for further experiments.

Under the conditions where the *PtA2M-1* gene was knocked down,

Fig. 1. Amino acid sequence alignment of A2Ms of crustacean. Arrows with short vertical bar indicated the start and end positions of A2M_N domain, A2M_2 domain and receptor binding domain. Sequences in black and red box were bait region and thioester motif, respectively. The black triangle indicated the amino acids at the corresponding catalytic His position. Lys residues were marked with black diamond. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

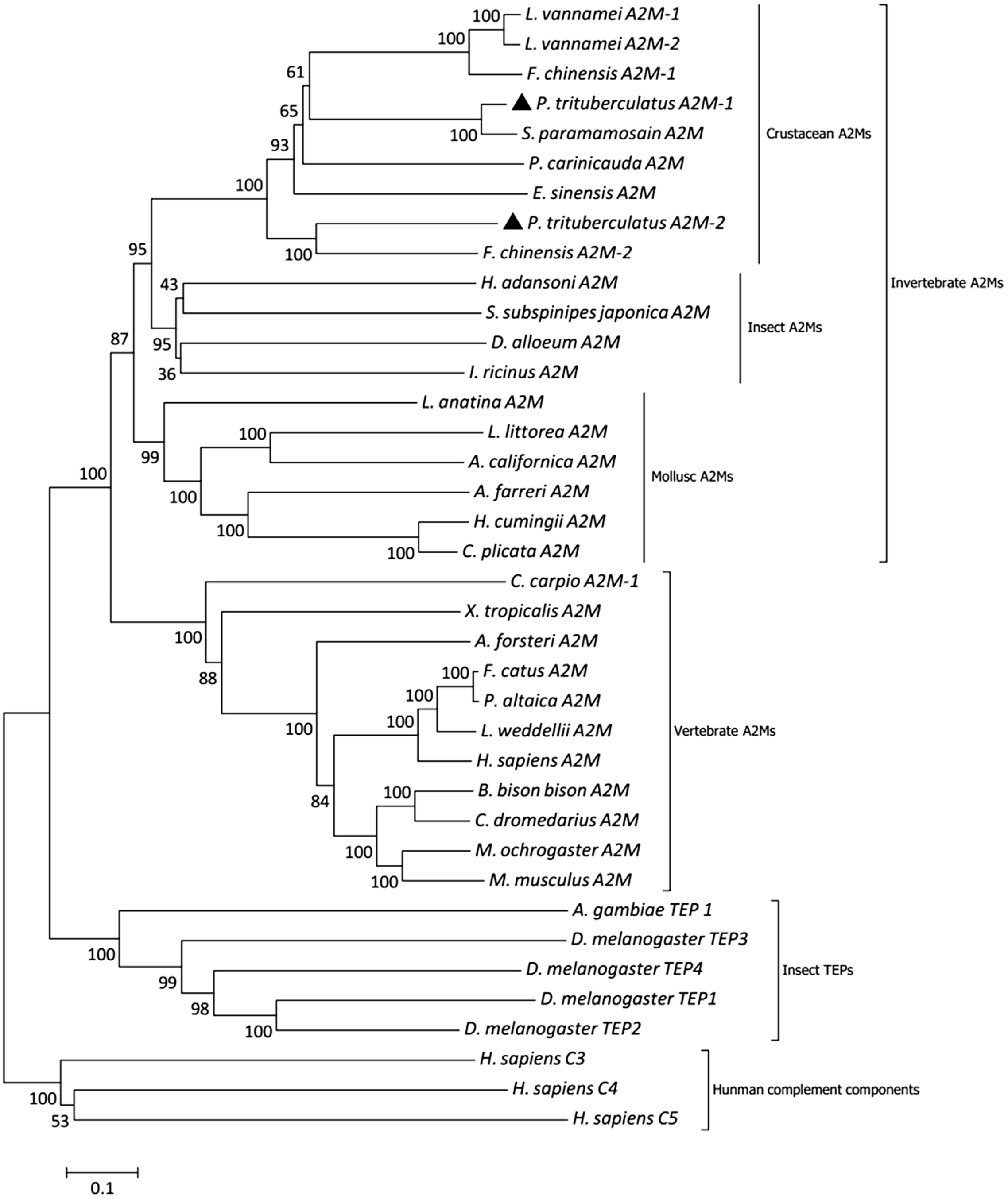

Fig. 2. Phylogenetic tree produced using the Neighbor-joining method. Human complement components (C3, C4 and C5) and insect TEPs were employed as the outgroup. PtA2M-1 and PtA2M-2 were marked by black triangle. A bootstrap analysis of 1000 replications was carried out on the tree and the values are shown at each branch of the tree. GenBank accession numbers are given in Supplementary Table 1.

the expression of serine protease related genes (*PtcSP1-3* and *PtSPH*) and proPO-associated genes (*PtproPO* and *PtPPAF*) were significantly upregulated compared with the control group in the intestine (Fig. 6A), but *PtLSZ*, phagocytosis related genes (*PtMyosin* and *PtRab5*) and complement-like gene (*PtTEP*) were remarkably suppressed. In the *PtA2M-2*-silenced crabs, the expression patterns of serine protease related genes (except *PtcSP3*), proPO-associated genes, phagocytosis related genes, *PtLSZ* and *PtTEP* genes exhibited similar trend compared with those in *PtA2M-1*-silenced crabs (Fig. 6B). Furthermore, the expression of *PtA2M-1* or *PtA2M-2* could be changed when *PtA2M-2* or *PtA2M-1* gene was silenced, while the expression of *PtgC1qR* and *PtMBL* could not be affected in the PtA2Ms-silenced crabs (Fig. 6).

3.5. Expression analysis of AMP genes and the key genes involved in immune pathways after PtA2M genes silenced by RNAi

To figure out whether PtA2Ms can regulate AMP through specific immune pathway, the expression of AMP genes and the effector genes involved in the Toll, NF-κB and JNK pathways were detected in the PtA2Ms-silenced crabs. Results showed that knockdown of *PtA2M-1* could significantly enhance the expression of AMP genes (*PtALF1-3*, *PtCrustin1* and *PtCrustin3*) and the genes (*PtTLR*, *PtMyD88*, *PtPelle* and *PtRelish*) involved in the Toll and NF-κB pathways (Fig. 7A). However, the expression of the above-mentioned genes was significantly suppressed in the PtA2M-2-silenced crabs (Fig. 7B). There was no significant effect on the *PtJNK* gene in the crabs with the *PtA2M-1* or

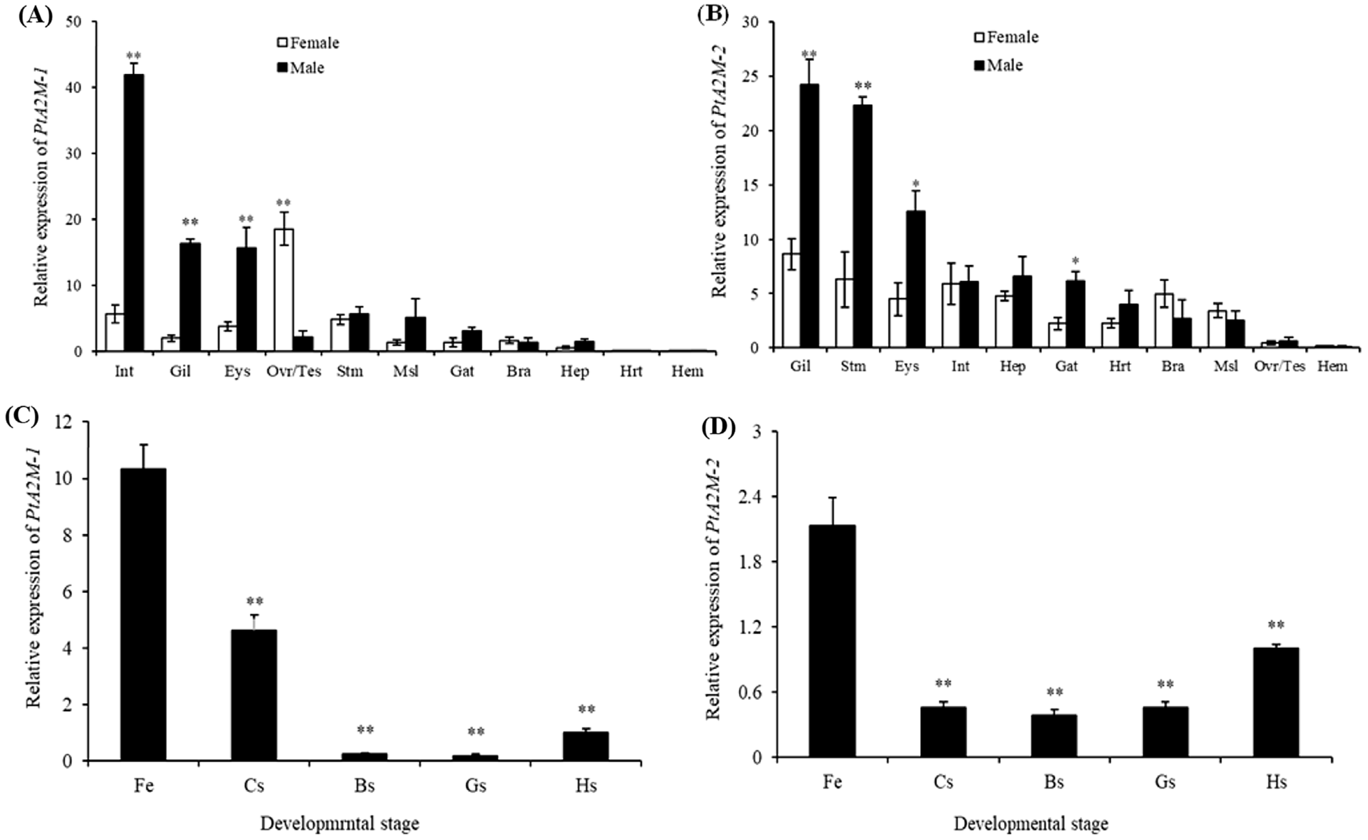

Fig. 3. The expression profiles of PtA2Ms in different tissues and embryos determined by qRT-PCR. A: tissue distribution of *PtA2M-1*; B: tissue distribution of *PtA2M-2*; C: the expression of *PtA2M-1* in embryos; D: the expression of *PtA2M-2* in embryos. Tissue names were shortened as: msl for muscle, gil for gill, hep for hepatopancreas, hrt for heart, int for intestine, stm for stomach, gat for Ganglia thoracalia, ovr for ovary, tes for testis, eys for eyestalk, bra for brain and hem for hemocyte. Fe: fertilized eggs, Cs: cleavage stage, Bs: blastula stage, Gs: gastrula stage, Hs: heart beating stage. Each column represented the mean of triplicate assays within ± S.D. Statistically differences were represented with asterisk ($*P < 0.05$, $**P < 0.01$).

PtA2M-2 gene knockdown (Fig. 7).

3.6. Effect of PtA2M genes silencing on PO and lysozyme activities

To further investigate the possible roles of PtA2Ms in the proPO system and basal immunity, hemolymph was collected from the *PtA2M-1*- or *PtA2M-2*-silenced crabs and subjected to PO and lysozyme enzymic activity assay. The results showed that silencing of *PtA2M-1* gene (Fig. 8A) could significantly increase PO activity by 42% when compared with that of the random-siRNA injection group, and a significant increase (31%) of total PO activity in the *PtA2M-2*-silenced crabs (Fig. 8B). However, lysozyme activities exhibited an opposite trend, decreased significantly in respective *PtA2M-1*- or *PtA2M-2*-silenced crabs compared with that of PBS group and random-siRNA injection group (Fig. 9).

4. Discussion

In the present study, two A2M genes, designated as *PtA2M-1* and *PtA2M-2*, were isolated for the first time in *P. trituberculatus*. Phylogenetic analysis showed that PtA2M-1 had higher similarity with SpA2M compared to PtA2M-2. Such phenomenon has also been found in FcA2Ms of *F. chinesis* [18], suggesting that PtA2Ms diverged before the differentiation of those crab species. The bait region of PtA2Ms showed great diversity in both sequence and length when compared with other crustacean A2Ms, which is consistent with the results identified from vertebrates and invertebrates [19,21,28]. This diversity makes A2Ms able to react with a wide spectrum of proteinases [18]. The thioester domains were completely conserved across species, which suggests the conserved function of A2Ms for protease entrapment [21]. Besides, five conserved Lys residues found in the receptor-binding domain of PtA2Ms may be responsible for binding with a cell surface receptor to activate receptor-mediated endocytosis [29]. In vertebrates, a highly conserved asparagine (Asn) residue was observed in A2Ms corresponding to the catalytic His in complement C3 [30]. However, most invertebrate A2Ms identified so far have a serine (Ser) residue at the catalytic position instead [17,18,21]. Herein, both PtA2M-1 and PtA2M-2 had a His residue at the comparable position suggesting their possible C3-like opsonic activity.

Hemocytes are considered as the main site for the synthesis of A2M in crustaceans [16,17,19], and the presence of A2M transcripts in different tissues may originate from infiltrated hemocytes [16,21]. However, the mRNAs of *PtA2M-1* and *PtA2M-2* were detected in all tested tissues with the low expression in hemocytes, which is consistent with that observed in *FcA2M-2* of *F. chinesis* [18]. PtA2Ms were most highly expressed in the major immune-related tissues, including gill, intestine and stomach, suggesting their potential defensive role against pathogens. Similar to that reported in amphioxus A2M [23], the high expression of PtA2Ms was observed in crab fertilized eggs. Together with the mRNA level in ovary, our results suggest PtA2Ms might be maternally-transferred immune factors deposited in the eggs or embryos of *P. trituberculatus*. As embryonic development proceeds, the elimination of maternal gene products and zygotic genome activation (ZGA) occur during the maternal-to-zygotic transition [31,32]. In this study, the transcripts of *PtA2M-1* and *PtA2M-2* exhibited a significant decrease at the cleavage stage followed by a manifest increase at the heart beating stage, which also favors the view that PtA2Ms may be maternally derived immune factors.

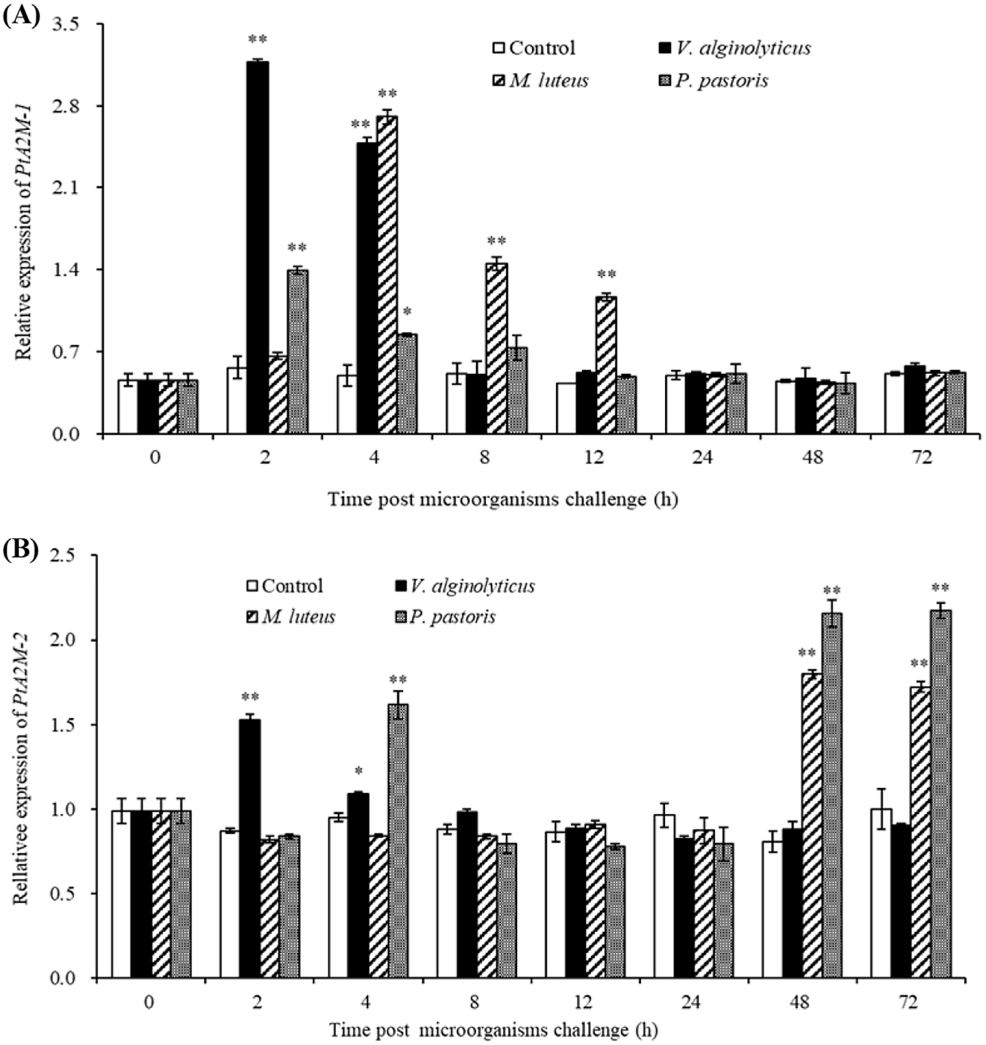

Fig. 4. The temporal expression profiles of *PtA2M-1* (A) and *PtA2M-2* (B) in crab hemocytes challenged with *V. alginolyticus*, *M. luteus*, *P. pastoris* and PBS (control group) were determined by qRT-PCR. Each column represented the mean of triplicate assays within ± S.D. Expression values were normalized to those of β-actin using the $2^{-\Delta\Delta CT}$ method. Statistically differences were represented with asterisk (*$P < 0.05$, **$P < 0.01$).

The transcripts of PtA2Ms can be induced by the Gram-negative bacterium, the Gram-positive bacterium and fungi, suggesting the wide-ranging defensive roles of PtA2Ms in crab immune response. The mRNA abundance of PtA2Ms in hemocytes increased significantly at 2 h post *V. alginolyticus* injection, indicating PtA2Ms are inducible acute-phase molecules. It is in good agreement with the significant upregulation of PfA2M in *Pinctada fucata* at early time point after *V. alginolyticus* infection [21]. Moreover, compared with PtA2M-1, PtA2M-2 was more sensitive to Gram-negative bacterium compared to Gram-positive bacterium and fungi. The varying expression patterns of *PtA2M-1* and *PtA2M-2* in crab hemocytes after being challenged with the three microorganisms indicates that they each perform different roles. This

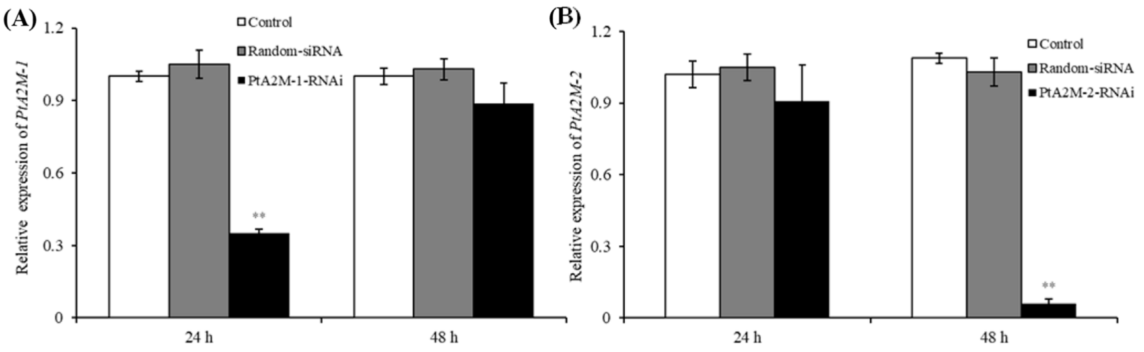

Fig. 5. The silencing efficiency of siRNA on transcription level of *PtA2M-1* (A) and *PtA2M-2* (B) in intestine of *P. trituberculatus* were determined by qRT-PCR. Control: positive control group after PBS only injection; Random-siRNA: negative control group after random-siRNA injection; RNAi: experimental group after sequence-specific siRNA injection. Each column represented the mean of triplicate assays within ± S.D. Expression values were normalized to those of β-actin using the $2^{-\Delta\Delta CT}$ method. The statistically differences between experimental group and control group were represented with asterisk (*$P < 0.05$, **$P < 0.01$).

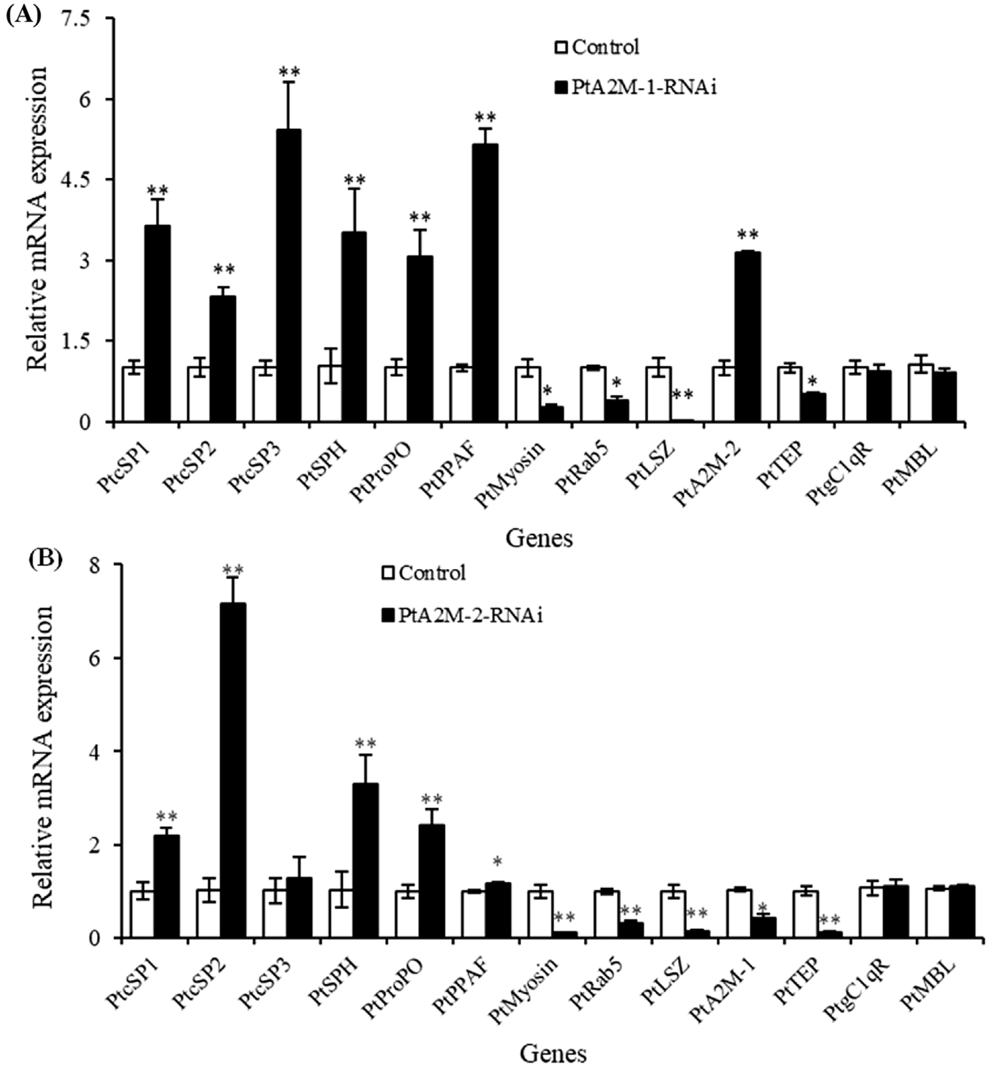

Fig. 6. Expression analysis of immune related genes in the *PtA2M-1* (A) or *PtA2M-2* (B) silenced crabs was determined by qRT-PCR. Control: negative control group after random-siRNA injection, RNAi: experimental group after sequence-specific siRNA injection. cSP: clip domain serine protease, SPH: serine protease homologue, proPO: prophenoloxidase, PPAF: proPO-activating factor, Rab5: small GTP-binding protein, LSZ: lysozyme, TEP: thioester-containing protein, gC1qR: globular receptor for complement C1q, MBL: mannose-binding lectin. Each column represented the mean of triplicate assays within ± S.D. Expression values were normalized to those of β-actin using the $2^{-\Delta\Delta CT}$ method. The statistically differences between experimental group and control group were represented with asterisk (*$P < 0.05$, **$P < 0.01$).

might because they possess different structural characteristics (such as signal peptide and bait region) or participate in their specific immune pathways. Similar results have been identified in FcA2M-1 and FcA2M-2 of *F. chinesis* [18], indicating that different types of A2M in *P. trituberculatus* might provide multiple immune functions.

In invertebrates, serine proteinase cascades are involved in humoral immune responses against pathogens mainly through activation of the proPO-activating system. However, the active proteases are hazardous to the tissues and needed to be controlled by serine protease inhibitors [22,33]. Herein, we discovered that the expression of serine protease-related genes increased significantly in the crabs after knockdown of PtA2Ms gene, which validates that PtA2Ms act as a kind of serine protease inhibitor. In addition, with the suppression of PtA2M gene, the PO activity was significantly higher than the control treatments, which is consistent with the results reported in LvA2M from *L. vannamai* [22]. Meanwhile, silencing of PtA2Ms led to a significant upregulation of proPO-associated genes. These data together indicate that PtA2Ms may act as modulators of the proPO activating system.

In this study, knockdown of *PtA2M* gene could result in significantly lower expression of phagocytosis-related genes, suggesting that PtA2Ms might have an opsonic function in regulating the phagocytosis. As reported in *P. fucata*, silencing of *PfA2M* or chemical inactivation by methylamine can significantly reduce the phagocytosis of hemocytes as well [21]. However, whether A2Ms from invertebrates regulate the phagocytosis like mammal complement C3 molecule remains still unclear. In the meantime, we also observed that C3-like genes (*PtTEP*, *PtA2M-1* or *PtA2M-2*) were changed remarkably in the *PtA2M-1*- or *PtA2M-2*-silenced crabs, which is similar to the relationship of C3-like molecules found in vertebrate complement pathways [34]. Besides, knockdown of *PtA2M* gene could not affect the expression of *PtgC1qR* and *PtMBL*, while the expression of *PtTEP*, *PtA2M-1* and *PtA2M-2* genes was changed significantly by suppression of *PtgC1qR* or *PtMBL* (in press). These results together suggest that PtA2Ms may function as the downstream genes of PtgC1qR and PtMBL in crab with similarities to the classical and lectin pathways in vertebrates.

It is well established that antimicrobial peptides display important roles in the crab innate immune in response to bacterial attack [35,36]. Recent report shows that a mutant fly line lacking the four immune-inducible TEPs (TEP1-4) can reduce Toll pathway activation upon microbial infection, resulting in lower expression of AMP genes [37]. In this study, we also observed that PtA2M-1 and PtA2M-2 can regulate the expression of AMP genes in an opposite manner. Furthermore, with the suppression of *PtA2M* expression, the changing trend of AMP genes was consistent with the key genes of the Toll and NF-κB pathways in crabs, which indicates PtA2Ms may regulate the expression of AMP genes through the Toll and NF-κB pathways. As known to us, lysozyme is a broad-spectrum defensive molecule involved in the lysis of the bacterial cell wall peptidoglycans. The basal expression of lysozyme gene was suppressed significantly in the PtA2Ms-silenced crabs. This result is also supported by the significant reduction of lysozyme activities in hemolymph of the crabs with silencing of *PtA2M* gene. Therefore, it is tempting to speculate that PtA2Ms may play an essential role

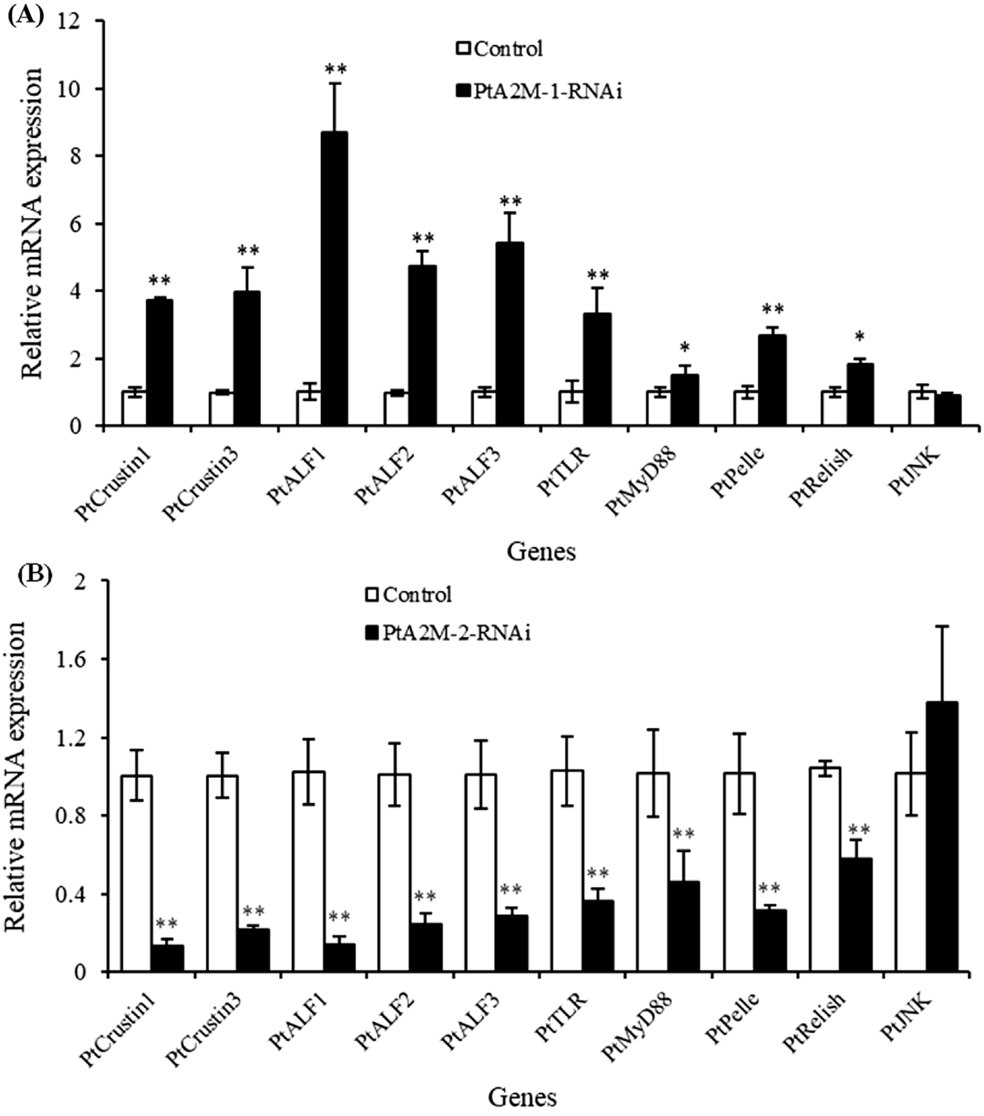

Fig. 7. Regulation expression of AMP genes mediated by the Toll and NF-κB pathways in the *PtA2M-1* (A) or *PtA2M-2* (B) silenced crabs. Control: negative control group after random-siRNA injection, RNAi: experimental group after sequence-specific siRNA injection. ALF: anti-lipopolysaccharide factor, TLR: Toll-like receptor, MyD88: myeloid differentiation factor 88, PtJNK: c-Jun N-terminal kinase. Each column represented the mean of triplicate assays within ± S.D. Expression values were normalized to those of β-actin using the $2^{-\Delta\Delta CT}$ method. The statistically differences between experimental group and control group were represented with asterisk ($*P < 0.05$, $**P < 0.01$).

in the basal immunity of crab.

In summary, the characterization, distribution and functional studies of two distinct alpha-2 macroglobulin were carried out in this study. PtA2Ms may be maternally-transferred molecules and can be induced by bacteria and fungi. PtA2Ms functioned in regulating the proPO activating system, in which limit the extent of PO activity probably by preventing relevant serine proteases from activating the protease cascades. Silencing of *PtA2M-1* or *PtA2M-2* gene leads to a reduction in lysozyme activity and a lower expression of phagocytosis-associated genes. Moreover, PtA2Ms might be involved in the transcriptional regulation of AMP genes mediated by the Toll and NF-κB pathways. Accordingly, it is suggested that PtA2Ms are immune-

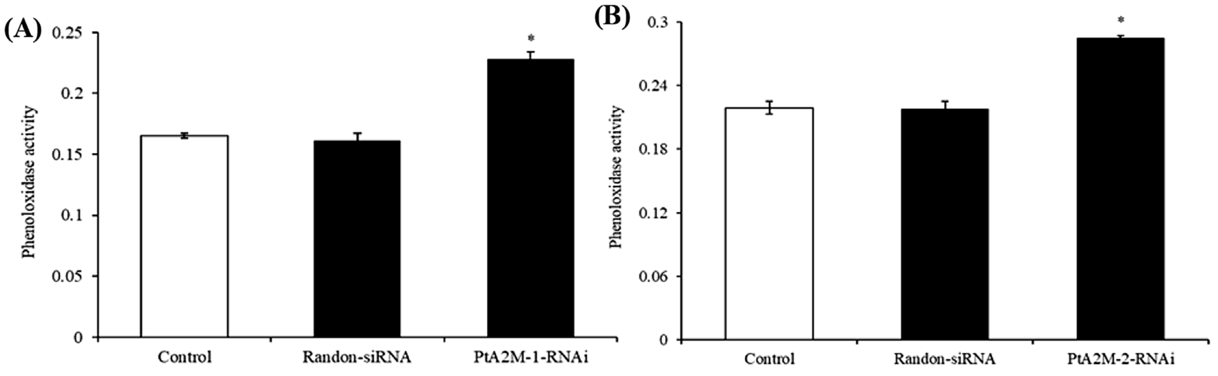

Fig. 8. Phenoloxidase (PO) activity analysis in the *PtA2M-1* (A) or *PtA2M-2* (B) silenced crabs. Control: positive control group after PBS only injection; Random-siRNA: negative control group after random-siRNA injection; RNAi: experimental group after sequence-specific siRNA injection. Each column represented the mean of triplicate assays within ± S.D. The asterisk indicated significant difference ($P < 0.05$) compared with the control groups.

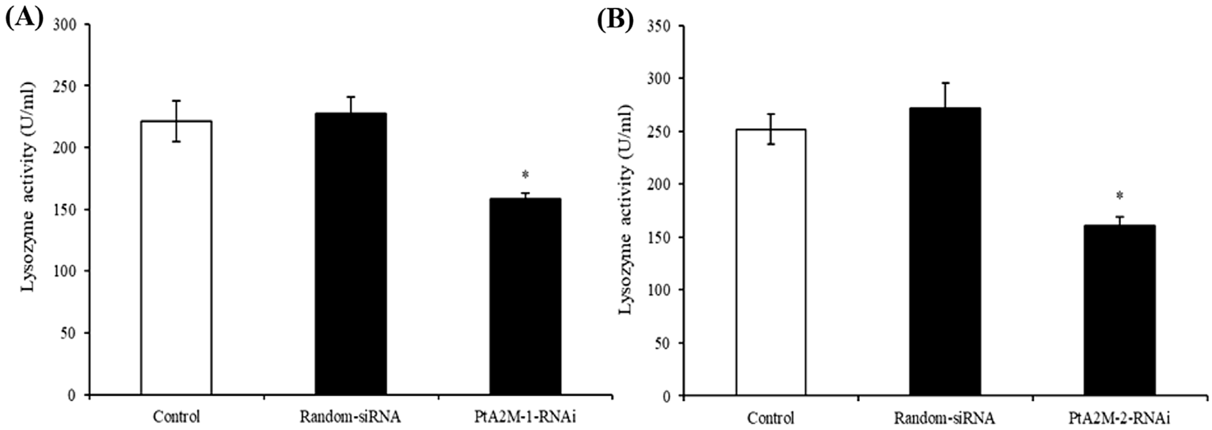

Fig. 9. Lysozyme activity analysis in *PtA2M-1* (A) and *PtA2M-2* (B) silenced crabs. Crabs injected with random-siRNA and PBS were used as negative control group and black control group. Each column represented the mean of triplicate assays within ± S.D. The asterisk indicated significant difference ($P < 0.05$) compared with the control groups.

relevant molecules and may perform an important role in the basal immunity of crab.

Disclosure statement

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.04.033.

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