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Molecular cloning of Kuruma shrimp *Marsupenaeus japonicus* endonuclease-reverse transcriptase and its positive role in white spot syndrome virus and *Vibrio alginolyticus* infection

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ABSTRACT

This study investigated the function of endonuclease-reverse transcriptase (mjERT) in *Marsupenaeus japonicus*. The 1129 bp cDNA sequence of mjERT was cloned from *M. japonicus* using rapid amplification of cDNA ends (RACE) PCR, and RT-qPCR analysis indicated that mjERT was highly expressed in the gills and hepatopancreas of *M. japonicus*. We also found that white spot syndrome virus (WSSV) or *Vibrio alginolyticus* challenge could enhance the expression of mjERT. When mjERT was inhibited, immune genes such as toll, p53, hemocyanin and tumor necrosis factor-α (TNF-α) were significantly down-regulated ($P < .01$) in the hemocytes of shrimp, while myosin was significantly up-regulated ($P < .01$). We demonstrated that mjERT is very important for the progression of WSSV infection and that the cumulative mortality of WSSV-infected and *V. alginolyticus*-infected shrimps was significantly increased following mjERT RNA interfere (RNAi). Apoptosis data provided information to suggest that mjERT-dsRNA challenge caused less apoptosis in hemocytes in both the disease-free and viral group. We also revealed that mjERT-dsRNA treatment resulted in a lower phagocytosis rate in the hemocytes of *V. alginolyticus*-challenged shrimp. Finally, we found that the absence of mjERT had an significantly negative impact upon shrimp phenoloxidase (PO) activity, superoxide dismutase (SOD) activity and total hemocyte count (THC) following WSSV or *V. alginolyticus* infection, indicating a regulative role for mjERT in the innate immunity of shrimp in response to pathogenic infection. In summary, we concluded that mjERT might promote the anti-WSSV immune response of shrimp by regulating apoptosis, PO activity, THC and SOD activity, and also exert a positive role in the immune response against *V. alginolyticus* by regulating phagocytosis, SOD activity, PO activity and THC.

1. Introduction

In 1970, the identification of reverse transcriptase (RT) led to a revolution in biological research in that, for the first time, we could investigate and manipulate the passage of RNA to DNA [1]. The life cycle of retroviruses, which look and behave like other animal viruses, can be divided into several stages: virus attachment and entrance into cells; an eclipse or latent period and a period of virus formation and release. RT is a multifunctional enzyme with RNA-directed DNA polymerase, DNA-directed DNA polymerase and ribonuclease hybrid (RNase H) activities. As predicted from their life cycle, some retroviruses have managed to infected the germ line of some hosts to become permanent residents of the host genome and are thus passed on to offspring by Mendelian mechanisms [2].

Including approximately half of the human genome, a remarkable portion of other eukaryotic genomesis generated by RTs, which are present in two major types: telomerases, which synthesize the ends of linear eukaryotic chromosomes, and retrotransposon RTs, which deposit copies of themselves throughout the genetic landscape and thus generate pseudogenes with spectacularly diverse roles in gene regulation [3]. RT manifests as acombination of protease and integrase domains in both retroviruses and long-tandem repeat (LTR) retrotransposons, while RT is juxtaposed to endonuclease domains in target-primed (TP) retrotransposons and some group II introns.

Group II intron RNAs are unusual retro-elements in that they represent ribozymes that encode RT. After self-splicing, the intron can then reverse-splice into a DNA target and be reverse transcribed by TPRT, in a manner similar to TP-retrotransposition and telomere synthesis [3–5]. Endonuclease-reverse transcriptase, featuring an endonuclease domain and a Group II introndomain, has been predicted to exist in the genome of several invertebrates, such as *Bombyx mori*, *Lasius niger*, *Schistosoma mansoni*, and fungi, such as *Nosema ceranae*, *Pseudoloma neurophilia* [6–9].

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Table 1
Universal and specific primers used in this study.

Primer Name	Nucleotide sequence (5′ → 3′)	Purpose
3′ race GSP	CGGAGAAAATGGGATGAAGACA	first primer for 3′RACE
3′ race NGSP	ACAGAACCATTGCTCTAATATCG	second primer for 3′RACE
5′race SP1	CAATCAAAAGCCTTGCTGTAGTC	first primer for 5′RACE
5′race SP2	TGGCAAGCATTGGTGTTCCTGATA	second primer for 5′RACE
5′race SP3	TTTCTGCTTGAGGTAGAGACGCC	third primer for 5′RACE
realtime-F	CTCCTCTTCACATCATCCAACTACT	primer for expression
realtime-R	TACCTATTTCAAACCAGGGCGT	primer for expression
dsRNA-F	CCCAAGCTTACGGTCAGTTTACATACCAATTCCTA	primer for knock down
dsRNA-R	TTGAACAGAGTTGGCGAAAGGAGGATCCCG	primer for knock down
hemocyanin-F	AACCCTGAACAAAGAGTTGCCTAT	for hemocyanin expression
hemocyanin-R	AACGGACGGTAAGTTGATGATGT	for hemocyanin expression
IMD-F	ATTCATCCGTCTACCTCCCTACA	for IMD expression
IMD-R	GAGCTGAGTCTGTCTTAATGTTATCC	for IMD expression
L-lectin-F	ATGTTATGCCATCTGCCTCGTATTT	for L- lectinexpression
L-lectin-R	CTTTCGCTGCTGCTCTTTCTGTT	for L- lectinexpression
MAPK-F	CGCATCACTGTTGAGGAGG	for MAPK expression
MAPK-R	GCAGGTCATCAAGTTCCATCT	for MAPK expression
NOS-F	CCAGGATCTTCTTGTTGGTGTTG	for NOS expression
NOS-R	CCCTCATCTGTAGCATAAAGTTCTC	for NOS expression
p53-F	TTCCTGCCTGGCTGACTCTA	for p53 expression
p53-R	CACCCAATCTTCCAACATCACAT	for p53 expression
proPO-F	TTCTACCGCTGGCATAAGTTTGT	for proPOexpression
proPO-R	TATCTGCCTCGTCGTTCCTCAC	for proPOexpression
STAT-F	TGGCAGGATGGATAGAAGACAAG	for STAT expression
STAT-R	TGAATAAGCTGGGATACGAGGGA	for STAT expression
TNF-F	ACAGACGGTCCAGAGTCCCAAAG	for TNF expression
TNF-R	GCGACGAAGTGAGCCACAGTAA	for TNF expression

Kuruma shrimp (*Marsupenaeus japonicus*) is a major marine product and accounts for a large proportion of world aquaculture. However, outbreaks of viruses and bacterial diseases in shrimp populations are becoming more and more prevalentdue to high cultivation densities, particularly white spot syndrome disease (WSSD) and vibriosis [10]. At present, the general consensus of opinion considers that crustaceans lack an adaptive immune system and protect themselves by relying solely upon an innate immune system [11]. Exploring the innate immunity of shrimp is therefore a significant priority.

In our previous study, we showed that the endonuclease-reverse transcriptase (ERT) expression level in *M. japonicas* was significantly increased in white spot syndrome virus (WSSV) and *Vibrio alginolyticus* infection. In the present study, we investigated the role of mjERT in the shrimp innate immune system following infection by WSSV or *V. alginolyticus*.

2. Materials and methods

2.1. Shrimp challenge with WSSV and tissue collection

Kuruma shrimp (*M. japonicus*; 15g/shrimp) were got from the aquaculture market located in Hangzhou, Zhejiang Province, China, and then cultured briefly in artificial seawater (23°) with air-pump before the experiment. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Zhejiang A & F University. WSSV (accession no. AF 332093.3) was purified and used in challenge experiments as described previously [12]. *Vibrio alginolyticus* was cultured and used to challenge the shrimp following the previous report [13]. The shrimp were injected with 1×10^4 copies/shrimp of WSSV, and 1×10^4 CFU/shrimp of *V. alginolyticus*, respectively, and the hyperhaline PBS buffer injection was used as the control. The different tissue templates were collected from healthy or challenged shrimp. The samples were immediately used for RNA extraction, in order to prevent total RNA degradation.

2.2. Total RNA extraction and rapid amplification of cDNA ends (RACE)

The PureLinkTM RNA Mini Kit (Ambion, USA) was used to extract total RNAs collected from different tissues, according to the manufacturer's instructions. The following steps were performed as described previously [12]. The RACE technique was utilized to clone the full-length cDNA sequence of a gene, based on the known middle fragment using 5'/3′ RACE Kit, 2nd Generation (Roche, Germany), according to the protocol of the manufacturer. The synthesized cDNAs were kept at −20 °C, used for the 3′/5′ -RACE PCR with 3′ gene-specific primer (3GSP, 3NGSP) or 5′ GSP (5SP1, 5SP2, 5SP3), designed on the basis of middle sequence (the primer's sequences are shown in Table 1). The following steps were performed as described previously [12]. All primers used in this experiment were designed using Primer Premier 5.0.

2.3. Nucleotide sequence and bioinformatics analyses

The nucleotide sequence similarities were examined by BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/). The 5′ and 3′sequences from RACEs were assembled with the partial cDNA sequences corresponding to each fragmental sequence by DNAMAN 5.0. The protein prediction was performed using the open reading frame (ORF) Finder tool. Multiple sequence alignment was created using the ClustalX 1.8. And the phylogenetic trees based on the amino acid sequences were performed by the neighbor-joining method using Molecular Evolutionary Genetics Analysis, MEGA7.0.

2.4. Quantitative real-time RT-PCR (qRT-PCR) analysis

The mjERT mRNA expression levels at different time points after pathogens challenge were analyzed using qRT-PCR. Total RNAs were extracted from different tissues using an Easy spin tissue/cell RNA extra kit (Aidlab, Shanghai China) following the manufacturer's protocol. Subsequently, 200 μg total RNAs were used for cDNA synthesis by ReverTra Ace qPCR RT Master Mix with gDNA Remover Code: FSQ-301 (Toyobo, Japan). A SYBR Green qRT-PCR assay (Promega, USA) was

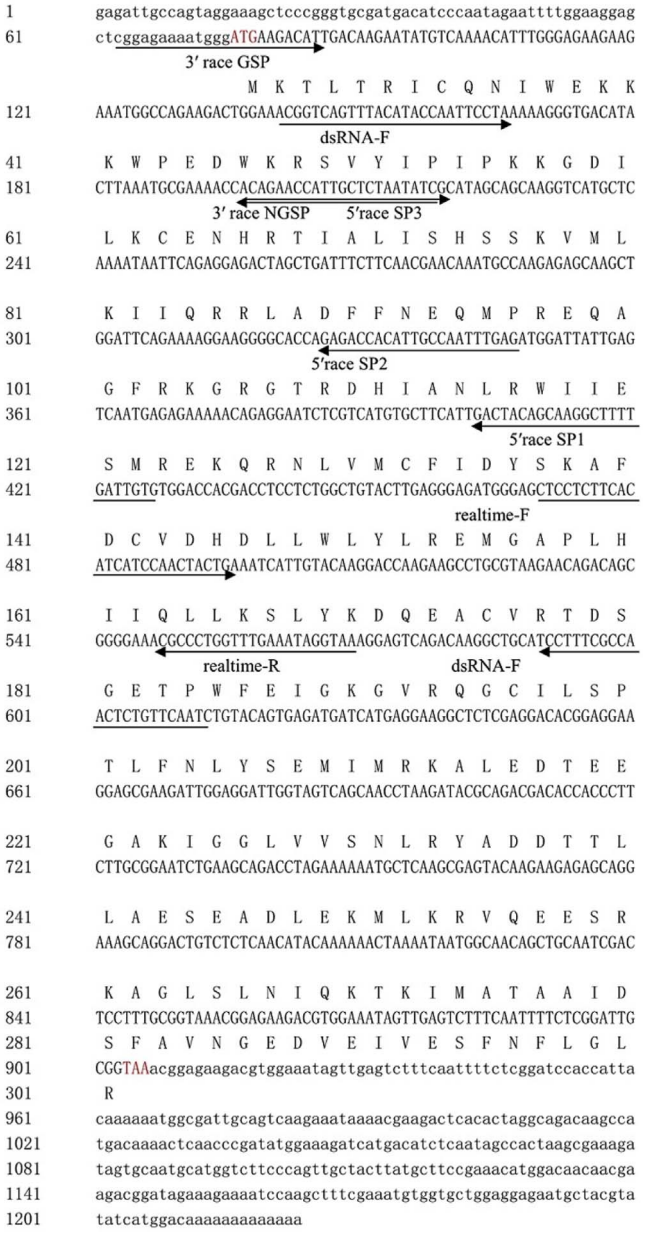

Fig. 1. Nucleotide and deduced amino acid sequences for mjERT. The nucleotide sequence is shown in the 5′–3′directions and numbered on the left. The deduced amino acid sequence is shown by a single capital letter amino acid code. The 3′UTR and 5′UTR are shown with lowercase letters. Codons are numbered on the left with the methionine (ATG) initiation codon, and the red annotation denotes the termination codon (TAA). RACE and real-time qPCR primers are marked with arrows.

carried out in a Bio-Rad Two Color Real-Time PCR Detection System (Bio-Rad, CA, USA). The $2^{-\Delta\Delta CT}$ method was used to analyze the qPCR data, and the data were shown as means standard deviations (SD). The *t*-test was used to analyze the significance of differences in the PCR data [14].

2.5. Prokaryotic expression, purification of mjERT-dsRNA

The primers (shown in Table 1) with specific restriction sites (*Hin*d III in the forward primer and *Bam*H I in the reverse primer) were designed from the cloned nucleotide sequence. PCR product digested with *Hin*d III/*Bam*H I was subcloned into LIMTUS 38i Vector(NEB, UK) digested with the same enzymes to gain plasmid L38-mjERT. The constructed L38-mjERT was verified by restriction enzyme digestion and DNA sequencing. The recombinant plasmid L38-mjERT was transformed into HT115 (DE3) cells knocked out of RNase III. The following steps were performed as described previously [12]. The EGFP-dsRNA was used as the control.

2.6. Knock down of mjERT by RNAi and challenge experiments

MjERT-dsRNA (30 μg/shrimp) was purified and immediately injected intramuscularly into shrimp, and mjERT mRNA expression levels were checked by qRT-PCR. And then, Shrimp were divided into eight groups: intramuscular injection with 100 μL PBS alone; intramuscular injection with 100 μL mjERT-dsRNA alone; intramuscular injection with 100 μL WSSV (10^5 copies/mL) challenge alone; injection with mjERT-dsRNA for 12 h, followed by 100 μL WSSV challenge; injection with EGFP-dsRNA for 12 h, followed by 100 μL WSSV challenge; intramuscular injection with 100 μL *V. alginolyticus* (10^5 CFU/mL) challenge alone; injection with EGFP-dsRNA for 12 h, followed by 100 μL *V. alginolyticus* challenge; injection with mjERT-dsRNA for 12 h, followed by 100 μL *V. alginolyticus* challenge. Shrimp mortality was monitored every 12 h after the last injection.

2.7. Detection of WSSV copies

Shrimp were injected with WSSV or mixture of WSSV and mjERT-dsRNA. The whole-genome was extracted from shrimp hemocytes collected at 12, 24 and 48 h post injection using DNA extraction Kit (Tiangen, China), according to the manufacturer protocol. To figure the WSSV copies, shrimp gDNAs of each sample were detected by WSSV vp28 probe in Two Color Real-Time PCR Detection System (Bio-Rad, CA, USA). The primer sequences are shown in Table 1. Standard curve was made based on previous experiment [15].

2.8. Determination of immune parameters after RNAi

The immune parameters determined included total hemocyte count (THC), PO activity and SOD activity. THC was determined as previously described [16]. 500 μL hemolymph was collected and mixed with 500 μL 20 mM of EDTA solution to determine PO and SOD activities from individual. Based on the formation of dopachrome from the substrate L-3, 4-dihydroxyphenylalanine (L-DOPA), the PO activity was quantified from the hemolymph mixture as previously described [17]. The SOD activity was quantified from hemocytes isolated from 300 μL of the hemolymph mixture according to the improved method of Beauchamp & Fridovich [18]. Data were presented as a percentage of normal control.

2.9. Apoptosis analysis with annexin V by flow cytometry

Apoptosis assays of shrimp with/without mjERT-dsRNA treatment were conducted with BD PhrmingenTM FITC Annexin V Apoptosis Kit (Invitrogen, USA), following to the manufacturer's protocol. The hemolymph was drawn using 2 mL syringe with 20 mM of EDTA at a ratio of 1:1, and hemocytes were collect from the mixture which was centrifuged at 300 g at 4 °C for 5 min. Subsequently, acquired hemocytes were suspended with PBS, counted and adjusted with PBS to a cell density of 5×10^6 cells/mL. And then, the cells were stained and assessed by FACScan at wavelengths of 530 nm and 575 nm. The cell numbers on quadrant 2 and 4, with high annexin V staining, were considered as apoptotic. The data, presented as means ± standard deviation (SD), were derived from at least three independent experiments.

2.10. Phagocytosis detection by flow cytometry

Health shrimp were separated into two groups, each group contained at least three shrimp. Inject high saline PBS as control, the other

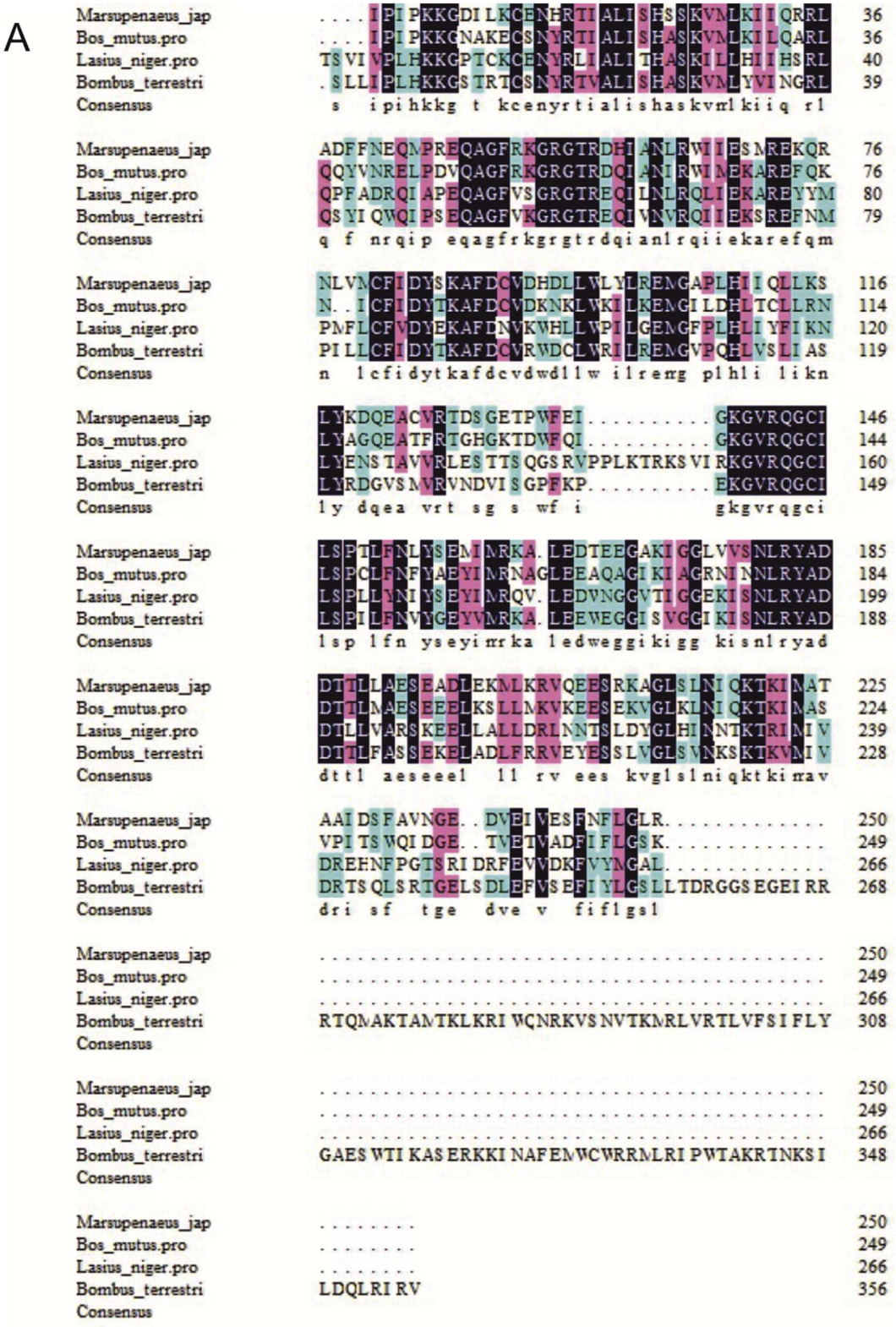

Fig. 2. Sequence analysis. (A) Multiple alignments ofthe RT_nLTR_like domain amino acid sequence of *M. japonicas* ERT with other ERT sequences from a range of other animals: *Marsupenaeusjaponicus* (in this study), *Bosmutus*(ELR61013), *Lasiusniger*(KMQ89503.1) and*Bombyxmori*(ADI61814.1). (B) Neighbor-joining phylogenetic tree of the ERT RT_nLTR_like domain from different organisms based upon amino acid sequence comparisons. Species names and accession numbers of ERT are listed on the right of the tree.

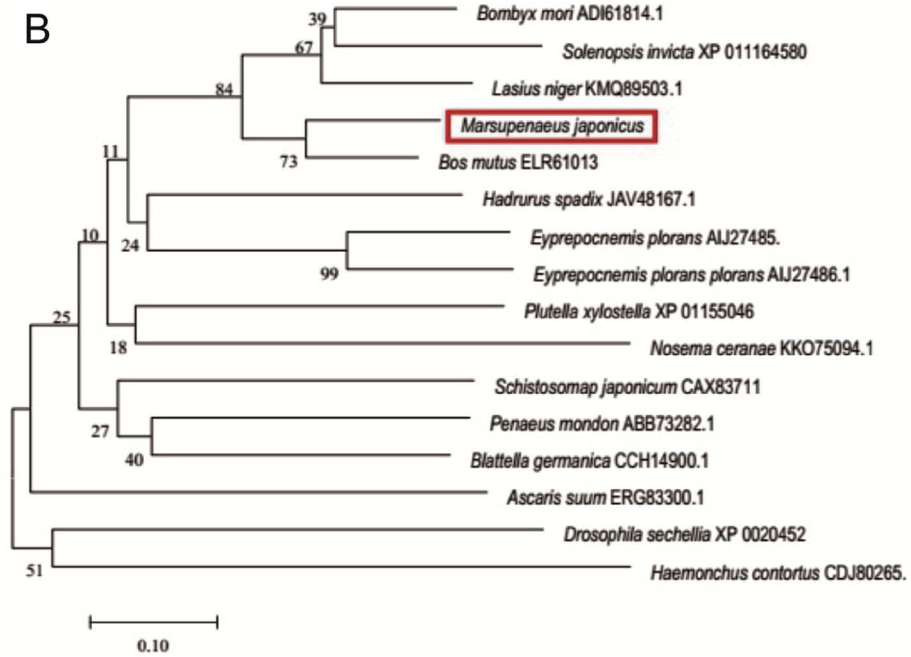

Fig. 2. (*continued*)

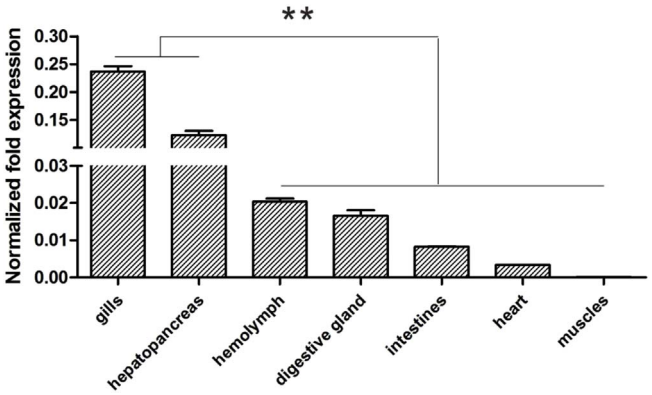

Fig. 3. Expression characterization of mjERT in various tissues as detected by quantitative real-time PCR from healthy shrimp. The amount of mjERT mRNA was normalized to the GAPDH transcript level. Data are shown as means ± standard deviation (SD) of three separate individuals for each tissue. Double asterisks indicate a significant difference ($P < .01$) between two samples.

group was injected with mjERT-dsRNA as knock down treatment. 24 h post injection, anti-coagulant soaked syringe was used to collect approximately 1.0 mL shrimp hemolymph of each group. Then, centrifugalize at 300 g for 5 min, 4 °C, in a horizontal centrifuge and abandon supernatant to collect hemocytes. Re-suspend the hemocytes softly with precooled sterilized high saline PBS, and the hemocytes were counted to adjust the cell of both treatment to the same density (a suitable density about 3–5 × 10^6 cells). Subsequently, hemocytes of normal group or knock down group were divided into three groups (200–300 μL per tube). Add 20 μL high saline PBS, 20 μL WSSV-FITC, or 20 μL *V. alginolyticus* -FITC into the normal or knock down hemocytes separately, gently mix up, then, incubate the hemocytes at 28 °C for 30 min for phagocytosis. After that, samples were centrifuged at 300 g for 5 min, 4 °C, in a horizontal centrifuge to move the residual FITC-pathogens, repeat twice. In the end, 1% paraformaldehyde in high saline PBS was used to immobilize hemocytes for flow cytometry detection.

2.11. Statistical analysis

Means and standard deviations of the results of triplicate assays were analyzed by one-way analysis of variance. Differences between treatments were analyzed using *t*-tests.

3. Results

3.1. Sequence, bioinformatics and phylogenetic analysis

The mjERT cDNA sequence, which is 1129 bp in length (Fig. 1) has a 1089 bp open reading frame (ORF) which encodes a 276 amino acid residue protein. mjERT, exhibiting a poly (A) tail at the C-terminal, includes a 131 bp 5′ untranslated region (UTR) and a 317 bp 3′UTR, respectively. MjERT has an estimated molecular mass of 32.926 kDa and a theoretical *p*I of 8.57. The nucleotide and deduced amino acid sequences of the full-length cDNA are shown in Fig. 1.

We compared themjERTRT_nLTR_like domain amino acid sequences with the sequences of previously reported mjERT isoforms. Our analysis of themjERTRT_nLTR_like domain revealed 62.15% identity with ERT of *Bos mutus* (accession number: ELR61013), 46.62% identity with ERT of *Lasius niger* (accession number: KMQ89503.1) and 37.54% identity with ERT of *Bombyx mori* (accession number: ADI61814.1)(Fig. 2A). A condensed neighbor-joining phylogenetic tree, based on the amino acid sequences, was constructed using the neighbor-joining method and MEGA6.0 software (Fig. 2B). This phylogenetic analysis showed that ERT was conservedacross several different species. Within the amino acid sequence, conserved domains contained several highly conserved amino acid sites.

3.2. Tissue expression differences and stress response

qRT-PCR was used to investigate expression of the mjERT gene in different *M. japonicus*tissues (Fig. 3). The qRT-PCR analysis showed that

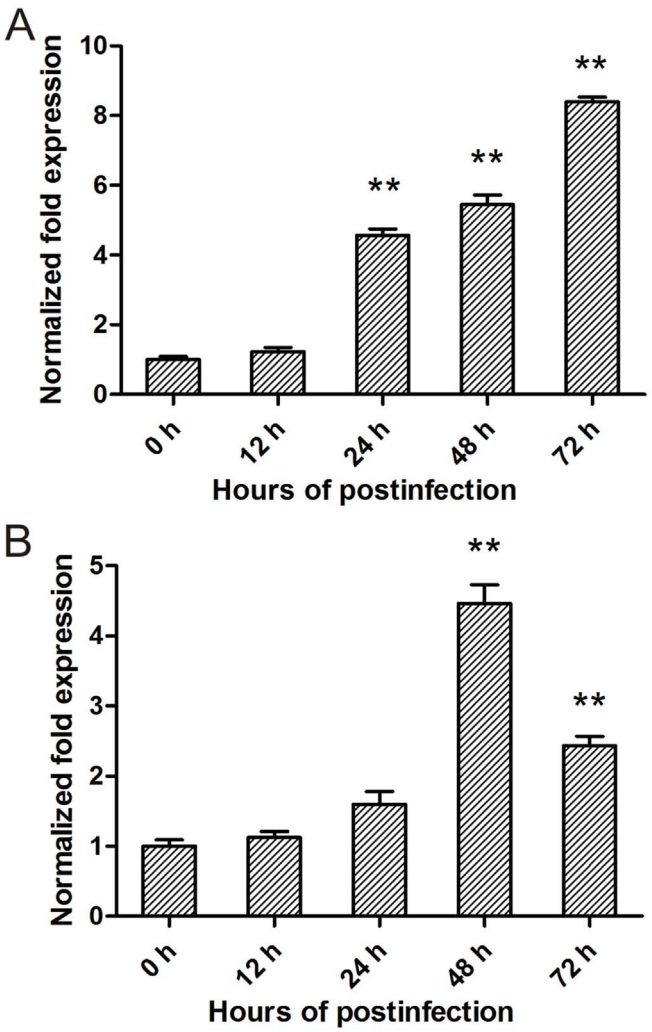

Fig. 4. (A) Real-time RT-PCR analysis of mjERT expression in the hemocytes of *M. japonicus*challenged with WSSV. (B) Real-time RT-PCR analysis of mjERT expression in the hemocytes of *M. japonicus*challenged with *V. alginolyticus*. The amount of mjERT mRNA was normalized to the GAPDH transcript level. Data are shown as means ± standard deviation (SD) of three separate individuals for each tissue. Double asterisks indicate a significant difference ($P < .01$) between two samples.

there were significantly higher expression levels in the gills and hepatopancreas than other tissues ($P < .01$). mjERTexpression was the lowest in muscle.

After WSSV challenge, the relative mjERT expression in the hemocytes was gradually up-regulated (Fig. 4A) and reached statistically significance, and the highest levels of expression, at 48 h post-infection ($P < .01$). A similar trend (Fig. 4B) was observed for the relative expression of mjERT expression after *V. alginolyticus* challenge. Following the challenge, expression levels were notably up-regulated in shrimp hemocytes from 24 to 72 h post-treatment ($P < .01$). These results implied that mjERT may have a potential role in the WSSV and *V. alginolyticus* infection of shrimp.

3.3. mjERTRNAi by dsRNA and the effects of mjERT upon the expression of immune genes

First, we evaluated the effect of mjERT-dsRNA on the expression of mjERT using qRT-PCR; data showed that the expression of mjERT was significantly inhibited by mjERT-dsRNA ($P < .01$) in shrimp hemocytes (Fig. 5A). Next, we analyzed the expression of a range of important immune genes in the hemocytes of *M. japonicus* treated with mjERT-dsRNA in order to explore the relationship between mjERT and these key immune genes. Of the twelve immune genes examined, p53, TNF, Toll and hemocyanin were significantly down-regulated ($P < .01$), while myosin was significantly up-regulated ($P < .01$) in the hemocytes of shrimp at 24 h post-injection (Fig. 5B). These results suggested that the knockdown of mjERT in shrimp might affect apoptosis and phagocytosis in the host.

3.4. Kaplan-survival analysis and WSSV copy detection

To evaluate the effects of mjERT on the mortality of shrimp challenged with either virus or bacteria, we injected mjERT-dsRNA into shrimp and then infected the shrimp with either WSSV or *V. alginolyticus*. The proportion (%) of surviving shrimp following *V. alginolyticus* infection was significantly reduced following mjERT-dsRNA from 24 to 108 h post-challenge ($P < .05$) (Fig. 6A). mjERT-dsRNA and the negative control showed no obvious effects upon cumulative mortality (Fig. 6A), which indicated that the mjERT-dsRNA did not exert toxicity on the shrimp. A similar trend (Fig. 6B) was observed for the relative proportion of surviving shrimp following WSSV challenge in that the cumulative mortality of WSSV-infected shrimp was significantly lower than shrimp treated with mjERT-dsRNA + WSSV between 12 and 120 h post challenge. By comparing shrimp mortalities between the mjERT-dsRNA + pathogen and pathogen only treatments, we revealed that mjERT plays an essential role in both WSSV and *V. alginolyticus* infection.

In all WSSV-challenged groups, the number of WSSV copies increased as time progressed, and the number of WSSV copies was always significantly higher in the mjERT-dsRNA-treated group than in the controls (Fig. 6C). Compared with the control group with WSSV challenge alone, the shrimp treated with mjERT-dsRNA + WSSV exhibited a higher copy number of WSSV, suggesting that the absence of mjERT significantly ($P < .01$) promoted the replication of WSSV.

3.5. Influence of mjERT knockdown on shrimp immune parameters

Total hemocyte count (THC) in the WSSV-alone group was lower than that in the PBS group at 24 and 48 h. Furthermore, THC in the mjERT-dsRNA + WSSV group fell significantly ($P < .01$) when compared to the WSSV group (Fig. 7A). In contrast, THC in the *V. alginolyticus* group was lower than that in the PBS group and there was a significant reduction ($P < .01$) of THC in the mjERT-dsRNA + *V. alginolyticus* group compared with shrimp injected with *V. alginolyticus* between 24 and 48 h (Fig. 7B). These results indicated that mjERT deficiency caused a reduction of THC in both the WSSV- and *V. alginolyticus*-challenged shrimp.

Relative PO activity in shrimp hemolymph was significantly lower ($P < .01$) in the WSSV + mjERT-dsRNA group compared with the WSSV-alone group at 24 h and 48 h post-injection (Fig. 7C). Similarly, PO activity was reduced following *V. alginolyticus* infection when compared with the PBS control group, and the shrimp treated with *V. alginolyticus* + mjERT-dsRNA exhibited a considerably lower ($P < .01$) PO activity compared with *V. alginolyticus*-treated shrimp (Fig. 7D). This suggested that the absence of mjERT significantly inhibited PO activity.

The NBT photoreduction method was used to evaluate the relative SOD activity of shrimp hemolymph in this study. Compared to the PBS group, relative SOD activity following WSSV infection increased significantly ($P < .01$). Shrimp receiving mjERT-dsRNA + WSSV treatment between 24 and 48 h showed significantly ($P < .01$) lower levels of SOD activities than the WSSV alone group (Fig. 7E). There was a similar trend in the *V. alginolyticus*-infected shrimp (Fig. 7F). Inhibition of mjERT expression thus caused a significant reduction in SOD activity caused by WSSV or *V. alginolyticus* ($P < .01$). These results indicated that mjERT had a positive effect upon shrimp immune parameters.

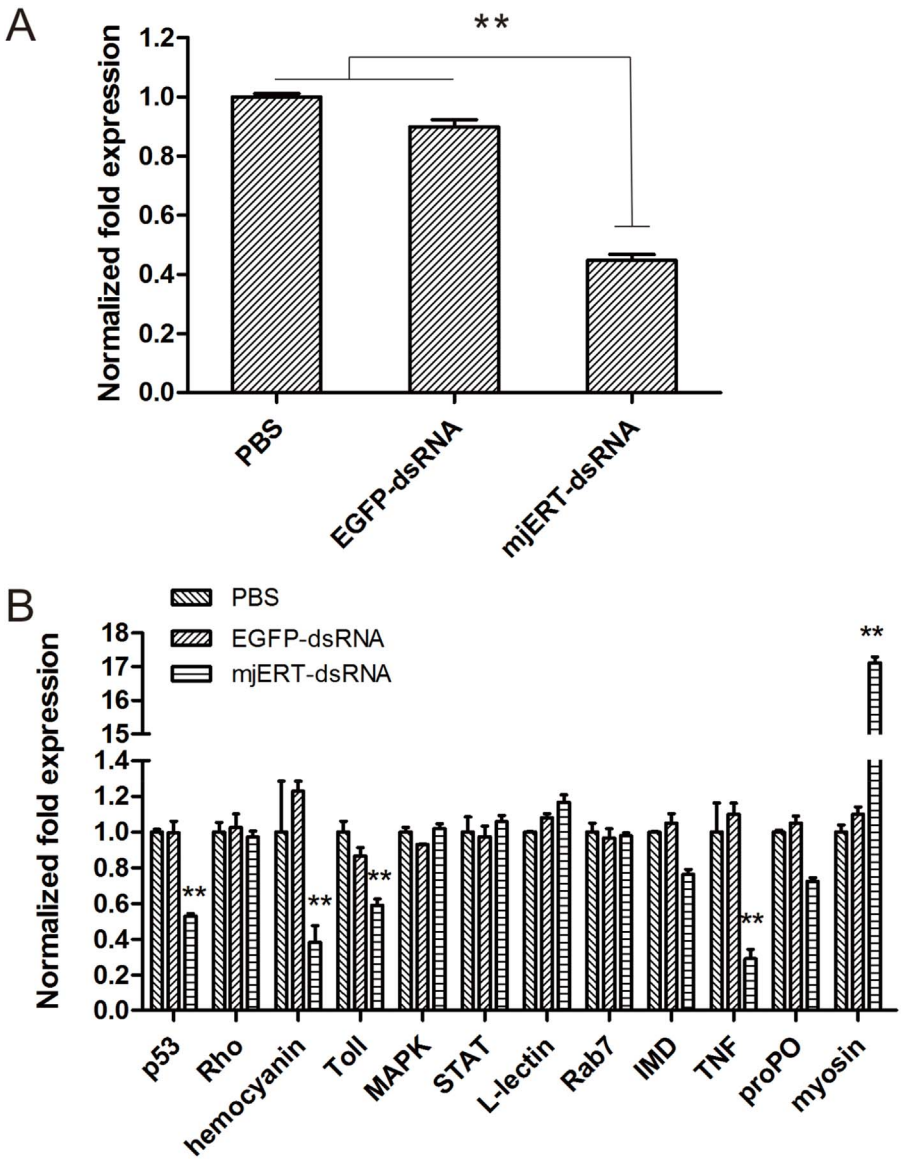

Fig. 5. (A) Real-time RT-PCR analysis of mjERT expression in the hemocytes of *M. japonicus*treated with mjERT-dsRNA at 24 h post-treatment. The amount of mjERT mRNA was normalized to the GAPDH transcript level. (B) Real-time RT-PCR analysis of the expression of nine immune genes in the hemocytes of *M. japonicas* treated with mjERT-dsRNA. The amount of mjERT mRNA was normalized to the GAPDH transcript level. Data are shown as means ± standard deviation (SD) of three separate individuals for each tissue. Double asterisks indicate a significant difference ($P < .01$) between two samples.

3.6. Influence of mjERT knockdown on apoptosis in hemocytes

The apoptosis rate in the PBS group was 41.19% and fell to 25.3% following the inhibition of mjERT. Compared with the control group with WSSV challenge alone, the apoptosis rate of the shrimp treated by mjERT-dsRNA + WSSV was remarkably lower (46.1% *vs*. 20.62%) ($P < .01$) (Fig. 8). These results indicated that mjERT was associated with viral-induced apoptosis. The down-regulation of apoptosis in shrimp hemocytes subjected to mjERT inhibition suggested that mjERT could positively regulate WSSV-induced apoptosis.

3.7. Influence of mjERT knockdown upon phagocytosis in hemocytes

The proportion (%) of fluorescein isothiocyanate (FITC)-positive hemocytes in the WSSV groups was significantly up-regulated ($P < .01$) from 5.3% to 12.6% following mjERT inhibition (Fig. 9) but was considerably lower (from 62% to 37.6%; $P < .01$) after mjERT knockdown in *V. alginolyticus* challenged groups (Fig. 9). The increase in the number of FITC-positive cells represented an increase in hemocyte phagocytosis, as indicated by flow cytometry, suggesting that mjERT deficiency affected the process of phagocytosis. These results suggest that mjERT was involved in the phagocytosis of shrimp hemocytes, and its effect upon phagocytosis was negative following WSSV infection but positive following *V. alginolyticus* infection.

4. Discussion

Surprisingly, in the 1970s, research identified that virions from retroviruses contained a form of reverse transcriptase that was able to transmit information from RNA to DNA [19]. This discovery broke the established Central Dogma, which stated that DNA to RNA was the only possible direction in terms of information flow, and therefore enriched our understanding of molecular biology [1]. Subsequently, reverse transcription has been identified in both retroviruses and para-retroviruses (hepatitis B-like viruses) [2]. The genomes of these organisms are integrated in the host genome and then permanently passed onto descendant organisms. The vertebrate genome contains DNA copies of retrovirus RNA, as well as retrotransposons and retrotranscripts. Found in eukaryotic genomes, endonuclease-reverse transcriptase is a form of Exonuclease-Endonuclease-Phosphatase (Exo_endo_phos_2) and RT_nLTR_like domain protein, which contains domains the following domains: Exonuclease-Endonuclease-Phosphatase (EEP), Exo_endo_phos_2, and RT_nLTR_like (long terminal repeat). However, few studies have investigated the function of this essential protein in

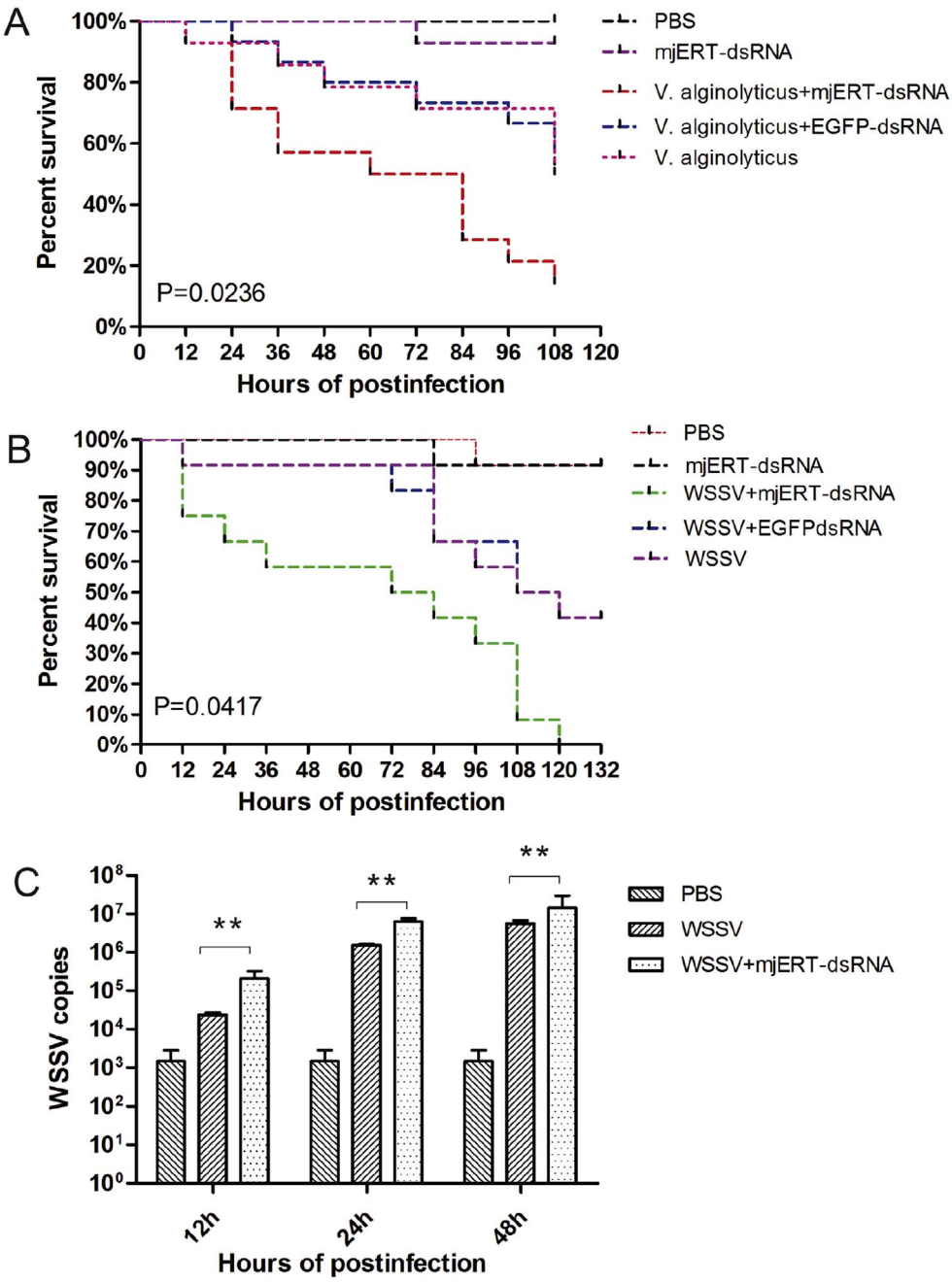

Fig. 6. Effects of mjERT knockdown. (A) Effects of *V. alginolyticus* challenge and mjERT inhibition on shrimp mortality. Healthy shrimp treated with high-saline PBS were used as a control. Each group contained at least 25 shrimp to ensure adequate confidence levels, and treatments were repeated three times to avoid any influence of weather, individual body condition, and injection-operation error. (B) Effects of WSSV challenge and mjERT inhibition on shrimp mortality. (C) WSSV copies detected by a TaqMan VP28 probe at different times post-infection. The primers used for this part of the study are listed in Table 1.

either vertebrates or invertebrates.

In the present manuscript, the full-length cDNA, which included the open reading frame (ORF) encoding for a modular protein consisting of an RT_nLTR_like domain, was cloned using rapid amplification of cDNA ends (RACE). Analysis showed that the cloned cDNA was 1129 bp in length, with a putative ORF encoding a 276 amino acid residue alkalescent protein. Protein sequence similarities were analyzed by BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/) and along with structural and functional prediction, showed that this amino acid sequence is relatively well conserved. The neighbor-joining tree method revealed a close evolutionary relationship of this protein among different species. mjERT was expressed in a range of tissues, including the gills, hepatopancreas, hemocytes, intestines, heart, and digestive gland, but was expressed at the highest levels in the gills. The high levels of mjERT expression in the gills, hemocytes and hepatopancreas indicated a role for mjERT in the immune defense system. Stimulation with pathogens (either WSSV or *V. alginolyticus*) led to the over-expression of mjERT, indicating its potential role in the innate immune system of shrimp.

To illustrate the function of this protein in shrimp, we successfully inhibited mjERT using dsRNA interference methodology. RNA interference (RNAi) using dsRNA to silence genes has been previously applied in studies of shrimp immunity [20–23]. Using the principle that viruses take advantage of host peptides and energy to spread their own genetic materials into the host cell to interfere with host DNA, previous researchers discovered an efficient way to manipulate this system and developed a tool to manually block or inhibit the expression of a specific target gene. In the present research, dsRNA was synthesized *in vitro* and used to silence the expression of shrimp mjERT.

The successful knockdown of mjERT with a specially-designed mjERT-dsRNA provided a very practical and efficient means of demonstrating its potential function in the shrimp innate immune system.

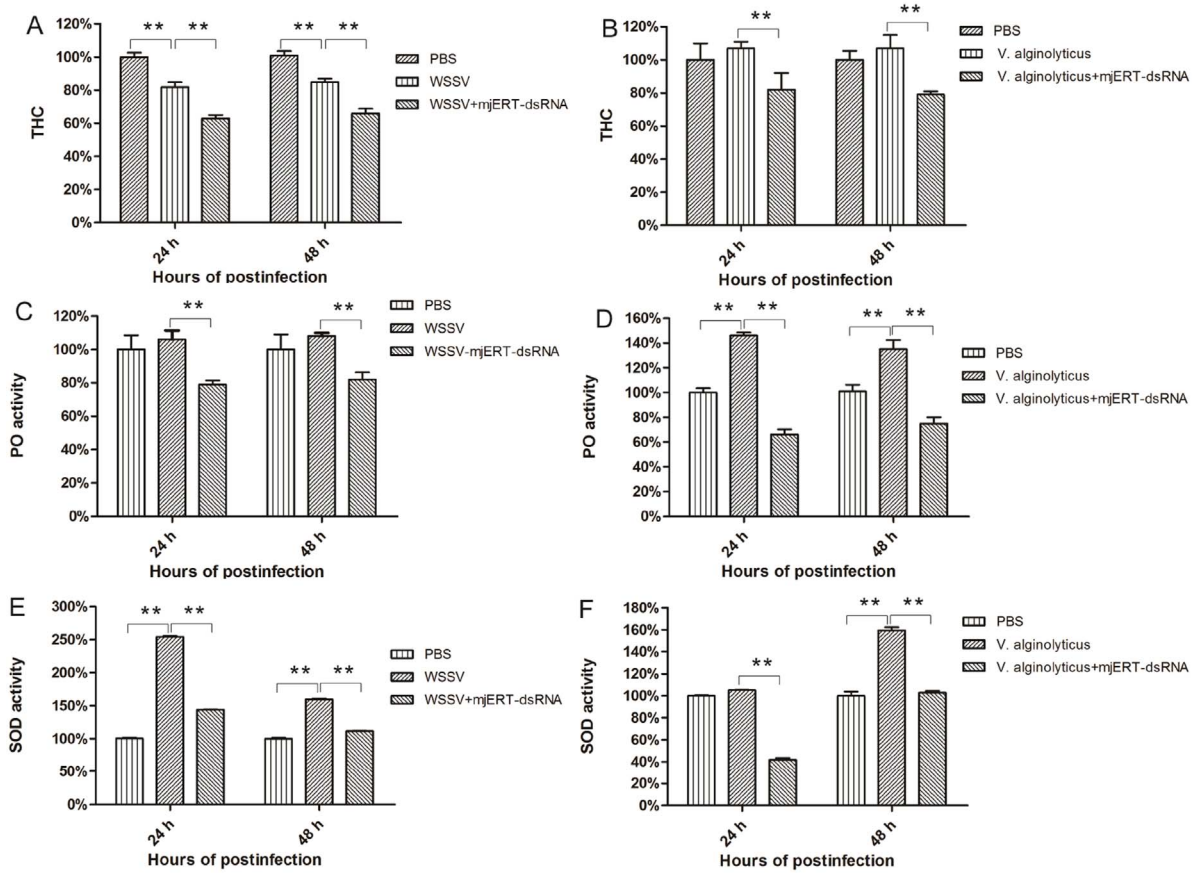

Fig. 7. Effects of mjERT knockdown on shrimp immune parameters. Immune parameters including THC activity, PO activity, and SOD activity were determined in healthy or WSSV-treated shrimp. (A) Relative THC after WSSV or WSSV + mjERT-dsRNA treatment; (B) Relative THC after *V. alginolyticus* or *V. alginolyticus* + mjERT-dsRNA treatment; (C) Relative PO activity after WSSV or WSSV + mjERT-dsRNA treatment; (D) Relative PO activity after *V. alginolyticus* or *V. alginolyticus* + mjERT-dsRNA treatment. (E) Relative SOD activity after WSSV or WSSV + mjERT-dsRNA treatment; (F) Relative SOD activity after *V. alginolyticus* or *V. alginolyticus* + mjERT-dsRNA treatment. Data were presented as a proportion (%) of the normal control. All treatments at each time point included at least three shrimp individuals, and all experiments were repeated three times.

The rapid and efficient innate system that shrimp rely upon is sufficient to protect hosts from intruding microorganisms [24]. Cellular immune responses including phagocytosis, nodule formation, encapsulation and apoptosis in invertebrates are carried out by hemocytes which are found in hemolymph, the open circulatory system of the shrimp. Hemocytes play a positive rolein humoral immune responses because they produce a range of immune factors such as lectins, prophenoloxidase; furthermore, antimicrobial peptides (AMPs) cannot function without hemocytes [25,26]. As a result, shrimp hemocytes represent a key focus for future research. In the present study, we selected a range of crucial immune molecules and signal transduction factors and investigated the variation in expression level of these factors in order to predict the potential effect of ERT in shrimp after silencing. Data showed that the knockdown of mjERT led to an up-regulation of myosin, a comprehensive and essential innate immune factor. Myosin is a key factor in the energy signal transduction pathway, participating in cell skeleton construction and motility processes [27]. However, hemocyanin, toll, p53 and tumor necrosis factor-α (TNF-α) all showed reduced expression after mjERT was inhibited in shrimp hemocytes. Hemocyanin is sensitive to stimulation from external conditions, as well as to internal stimulation from invading microorganisms [28]. As one aspect of pre-inflammation, tumor necrosis factor-α (TNF-α) kinetin is produced by macrophages [29]. p53 can also help to prevent cancer via initiating programed cell death, i.e. apoptosis [30]. The significant changes observed in the expression of these essential immune related factors after mjERT silencing suggested that mjERT might be associated with shrimp apoptosis, phagocytosis, and the immune pathway.

Subsequently, Kaplan-survival analysis showed that *V. alginolyticus*-challenged shrimp had a considerably lower survival rate after mjERT knockdown. There was a similar trend for WSSV-infected shrimp, and the lack of mjERT significantly increased the number of WSSV copies in shrimp at 12, 24, and 48 h post infection compared with controls. Then, in order to explore other potential roles of mjERT on shrimp innate immunity, we evaluated a range of other functional parameters as indicators of immune potential. Following mjERT silencing in WSSV-infected shrimps, there was a significant inhibition of THC, PO and SOD activity. Similar results were obtained for *V. alginolyticus*-challenged shrimp. Meanwhile, the knockdown of mjERT led to a remarkable reduction in healthy or WSSV-treated shrimp. Because of the mjERT deficiency, the lower phagocytosis rates in *V. alginolyticus*-infected shrimp suggested that mjERT could positively regulate phagocytosis in hemocytes. Interestingly, there was an opposite phagocytosis rate in mjERT-dsRNA treated group in WSSV-challenged shrimp. The results suggested WSSV might utilize mjERT to inhibit phagocytosis of hemocytes, and more evidences would be indispensable to account for phenomenon in the following work.

Collectively, these findings indicate that shrimp mjERT plays an active essential role in the innate immune system of shrimp. This host protein could regulate the host's defense system against viral infection by regulating apoptosis, THC, PO and SOD activity, while exerting a positive effect upon anti-*V. alginolyticus* via phagocytosis, THC, PO activity and SOD activity.

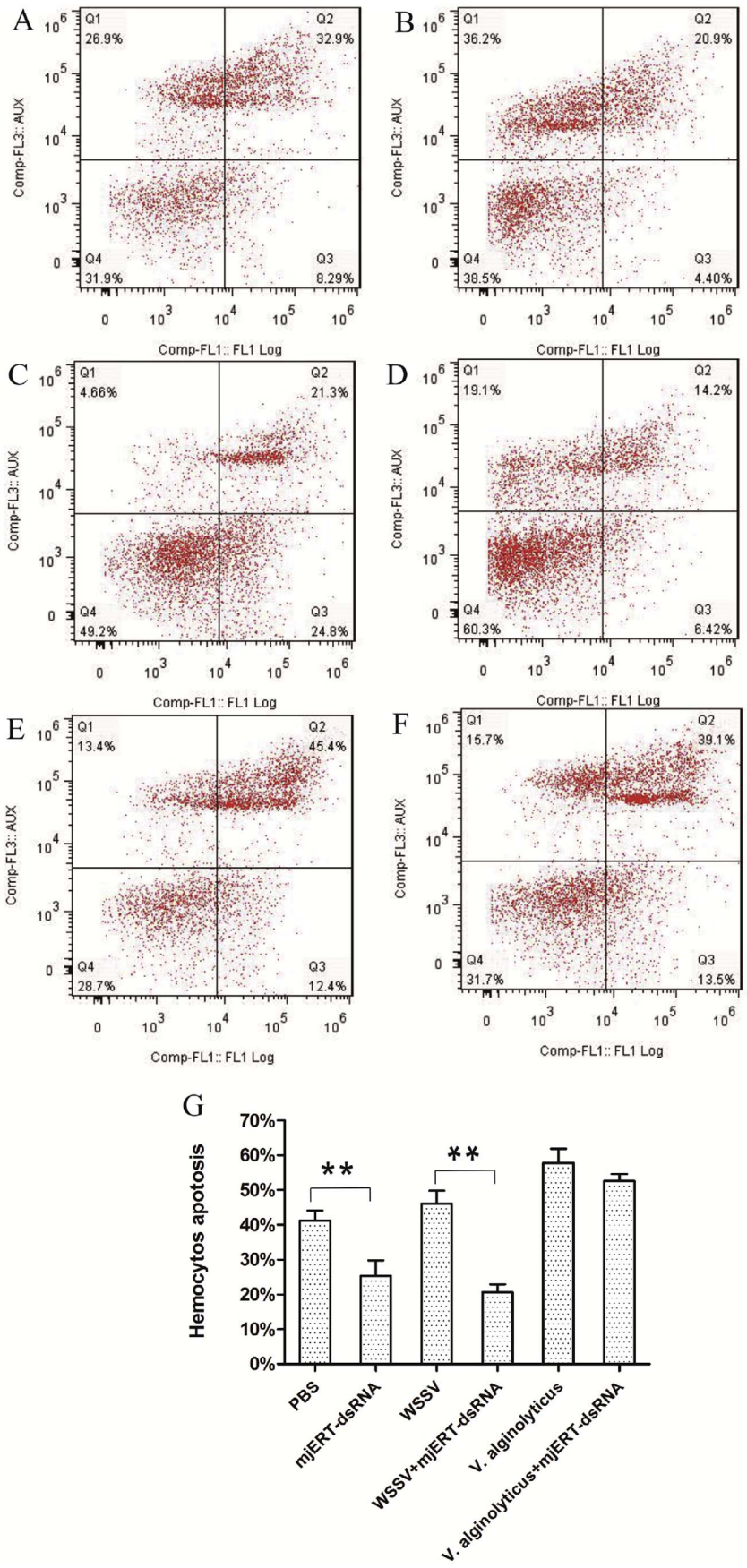

Fig. 8. Effects of mjERT-dsRNA interference on apoptosis in hemocytes. Samples were taken at 24h post-injection. (A) PBS treatment; (B) mjERT-dsRNA treatment; (C) WSSV treatment; (D) WSSV + mjERT-dsRNA treatment; (E) *V. alginolyticus* treatment; (F) *V. alginolyticus* + mjERT-dsRNA treatment; (G) Column chart showing apoptosis. The area marked B1 represents false positives caused by cell damage, B2 represents late-stage apoptotic hemocytes, B3 represents negative (normal) hemocytes, and B4 represents late-stage apoptosis. The annexin V peak represents the sum of the B2 and B4 areas (total value of annexin V-positive hemocytes), and is shown in the chart. The proportion (%) of annexin V-positive hemocytes represents the apoptosis rate in each group. All treatments included at least three shrimp individuals, and all experiments were repeated three times. Each column represents the mean value of triplicate assays.

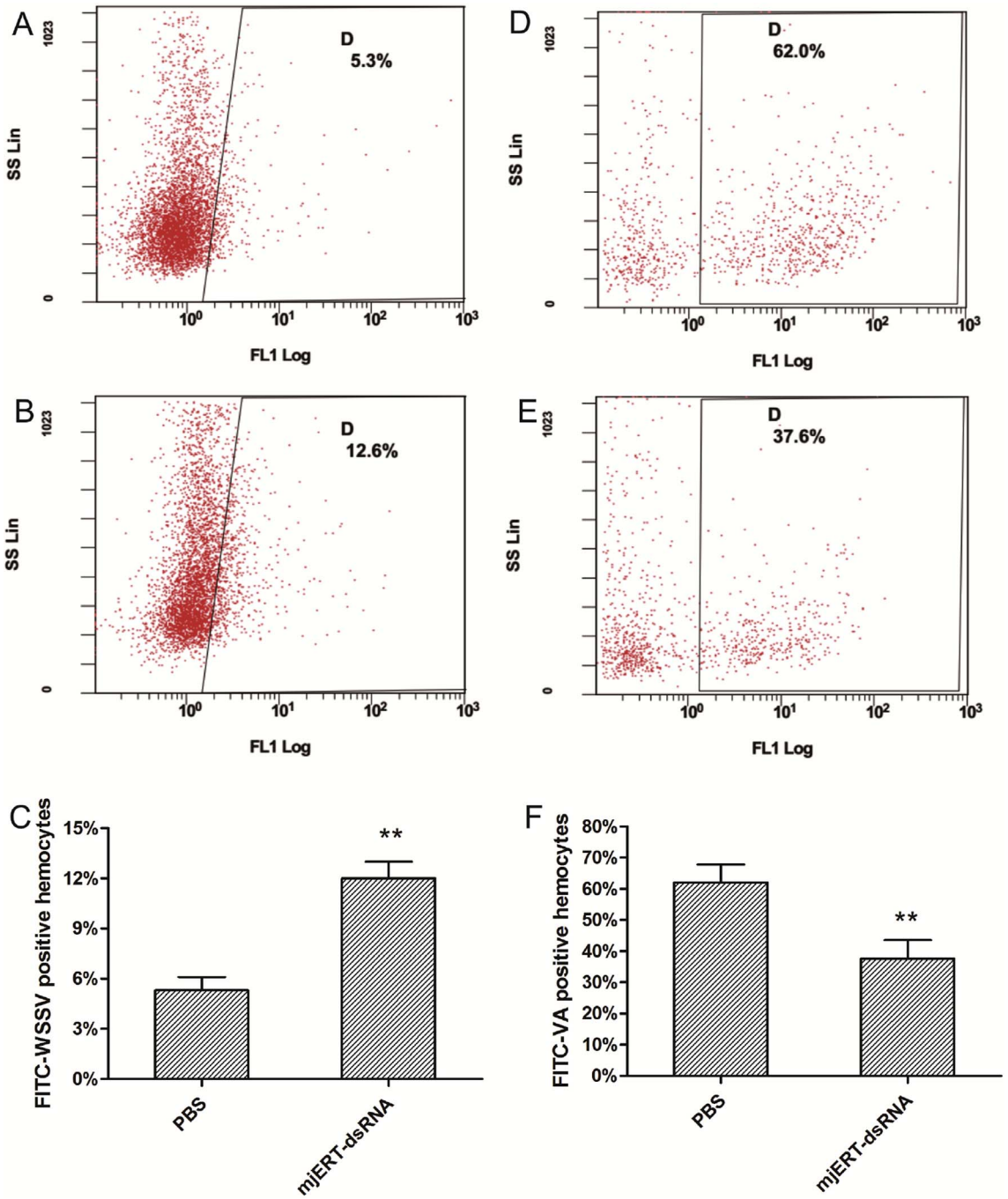

Fig. 9. Influence of mjERT knockdown on hemocyte phagocytosis as detected by flow cytometry. Hemocyte phagocytosis was detected in hemolymph from healthy shrimp treated with high-saline PBS or mjERT-dsRNA. FITC-labeled WSSV virions or *V. alginolyticus* particles were added to healthy or mjERT-inhibited hemocytes. (A) WSSV treatment; (B) WSSV + mjERT-dsRNA treatment; (C) Column chart of phagocytosis after WSSV or WSSV + mjERT-dsRNA; (D) *V. alginolyticus* treatment; (E) *V. alginolyticus* + mjERT-dsRNA treatment; (F) Column chart of phagocytosis after *V. alginolyticus* or *V. alginolyticus* + mjERT-dsRNA. All treatments included at least three shrimp individuals, and all experiments were repeated three times. Each column represents the mean value of triplicate assays.

Conflict of interest

There are no patents, products in development or marketed products to declare. This does not alter our adherence to all the gene policies on sharing data and materials.

Acknowledgments

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References

[1] S.M.D.B.H. Temin, A DNA-depenent DNA polymerase and a DNA endonuclease in virions of Rous sarcoma virus, Nature 228 (1970) 424–427.
[2] H.M. Temin, Reverse transcription in the eukaryotic genome: retroviruses, para-retroviruses, retrotransposons, and retrotranscripts, Mol. Biol. Evol. 2 (1985) 455–468.
[3] M. Belfort, M.J. Curcio, N.F. Lue, Telomerase and retrotransposons: reverse transcriptases that shaped genomes, Proc Natl Acad Sci U S A 108 (2011) 20304–20310.
[4] A. Beauregard, M.J. Curcio, M. Belfort, The take and give between retrotransposable elements and their hosts, Annu. Rev. Genet. 42 (2008) 587–617.
[5] A.M. Lambowitz, S. Zimmerly, Group II introns: mobile ribozymes that invade DNA, Cold Spring Harb Perspect Biol. 3 (2011) 003616.

[6] W.T. Tay, G.T. Behere, P. Batterham, D.G. Heckel, Generation of microsatellite repeat families by RTE retrotransposons in lepidopteran genomes, BMC Evol. Biol. 10 (2010) 144.
[7] R. DeMarco, A.A. Machado, A.W. Bisson-Filho, S. Verjovski-Almeida, Identification of 18 new transcribed retrotransposons in *Schistosoma mansoni*, Biochem. Biophys. Res. Commun. 333 (2005) 230–240.
[8] A. Pelin, M. Selman, S. Aris-Brosou, L. Farinelli, N. Corradi, Genome analyses suggest the presence of polyploidy and recent human-driven expansions in eight global populations of the honeybee pathogen *Nosema ceranae*, Environ. Microbiol. 17 (2015) 4443–4458.
[9] S. Ndikumana, A. Pelin, A. Williot, J.L. Sanders, M. Kent, N. Corradi, Genome analysis of pseudoloma neurophilia: a microsporidian parasite of zebrafish (*Danio rerio*), J. Eukaryot. Microbiol. 64 (2017) 18–30.
[10] T.W. Flegel, Historic emergence, impact and current status of shrimp pathogens in Asia, J. Invertebr. Pathol. 110 (2012) 166–173.
[11] P.H. Wang, T. Huang, X. Zhang, J.G. He, Antiviral defense in shrimp: from innate immunity to viral infection, Antivir. Res. 108 (2014) 129–141.
[12] Z. Wang, F. Zhu, MicroRNA-100 is involved in shrimp immune response to white spot syndrome virus (WSSV) and *Vibrio alginolyticus* infection, Sci. Rep. 7 (2017) 42334.
[13] M. Huang, Y. Liu, C. Xie, W.N. Wang, LvDJ-1 plays an important role in resistance against *Vibrio alginolyticus* in *Litopenaeus vannamei*, Fish Shellfish Immunol. 44 (2015) 180–186.
[14] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, Methods 25 (2001) 402–408.
[15] Y. Gong, C. Ju, X. Zhang, The miR-1000-p53 pathway regulates apoptosis and virus infection in shrimp, Fish Shellfish Immunol. 46 (2015) 516–522.
[16] K. Wongprasert, T. Rudtanatip, J. Praiboon, Immunostimulatory activity of sulfated galactans isolated from the red seaweed *Gracilaria fisheri* and development of resistance against white spot syndrome virus (WSSV) in shrimp, Fish Shellfish Immunol. 36 (2014) 52–60.
[17] Z. Zhao, C. Jiang, X. Zhang, Effects of immunostimulants targeting Ran GTPase on phagocytosis against virus infection in shrimp, Fish Shellfish Immunol. 31 (2011) 1013–1018.
[18] C.O. Beauchamp, I. Fridovich, Isozymes of superoxide dismutase from wheat germ, Biochim. Biophys. Acta 317 (1973) 15.
[19] D. Baltimore, Viral RNA-dependent DNA polymerase: RNA-dependent DNA polymerase in virions of RNA tumour viruses, Nature 226 (1970) 1211–1213.
[20] M.C. Saleh, R.P.V. Rij, A. Hekele, A. Gillis, E. Foley, P.H. O'Farrell, R. Andino, The endocytic pathway mediates cell entry of dsRNA to induce RNAi silencing, Nat. Cell Biol. 8 (2006) 793–802.
[21] D.H. Kim, M.A. Behlke, S.D. Rose, M.S. Chang, S. Choi, J.J. Rossi, Synthetic dsRNA Dicer substrates enhance RNAi potency and efficacy, Nat. Biotechnol. 23 (2005) 222–226.
[22] W. Tirasophon, Y. Roshorm, S. Panyim, Silencing of yellow head virus replication in penaeid shrimp cells by dsRNA, Biochem. Biophys. Res. Commun. 334 (2005) 102–107.
[23] P. Posiri, C. Ongvarrasopone, S. Panyim, A simple one-step method for producing dsRNA from E. coli to inhibit shrimp virus replication, J. Virol Meth. 188 (2013) 64–69.
[24] S.Y. Lee, K. Söderhäll, Early events in crustacean innate immunity, Fish Shellfish Immunol. 12 (2002) 421–437.
[25] S.K. Syed Musthaq, J. Kwang, Reprint of "evolution of specific immunity in shrimp - a vaccination perspective against white spot syndrome virus, Dev. Comp. Immunol. 48 (2015) 342–353.
[26] L. Cerenius, B.L. Lee, K. Soderhall, The proPO-system: pros and cons for its role in invertebrate immunity, Trends Immunol. 29 (2008) 263–271.
[27] T.W. Flegel, K. Sritunyalucksana, Shrimp molecular responses to viral pathogens, Mar. Biotechnol. 13 (2011) 587–607.
[28] K.S. Kallaya Sritunyalucksana, The proPO and clotting system in crustaceans, Aquaculture 191 (2000) 53–69.
[29] J. Crespo, A. Cayon, P. Fernandez-Gil, M. Hernandez-Guerra, M. Mayorga, A. Dominguez-Diez, et al., Gene expression of tumor necrosis factor alpha and TNF-receptors, p55 and p75, in nonalcoholic steatohepatitis patients, Hepatology 34 (2001) 1158–1163.
[30] W.P. Roos, B. Kaina, DNA damage-induced cell death: from specific DNA lesions to the DNA damage response and apoptosis, Canc. Lett. 332 (2013) 237–248.