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Molecular characterization of troponin T in *Scylla paramamosain* and its role in *Vibrio alginolyticus* and white spot syndrome virus (WSSV) infection

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ABSTRACT

This study investigated the function of Troponin T (TnT) in the mud crab, *Scylla paramamosain*. The 1952 bp cDNA sequence of TnT was cloned from *S. paramamosain* using rapid amplification of cDNA ends (RACE) PCR. The quantitative real-time PCR analysis showed that TnT was highly expressed in the muscle and heart of *S. paramamosain*. Challenging with white spot syndrome virus (WSSV) or *Vibrio alginolyticus* (VA), two common pathogens that infect mud crabs, enhanced the expression of TnT in *S. paramamosain*. Knockdown of TnT using TnT-dsRNA led to up-regulating the expression of immune-related genes, such as c-type-lectin, toll-like-receptor, crustin antimicrobial peptide and prophenoloxidase. The cumulative mortality of WSSV- and VA-infected crabs was significantly increased following TnT knockdown. After WSSV or VA infection, TnT knockdown caused a significant reduction in phenoloxidase (PO) activity, superoxide dismutase (SOD) activity and total hemocyte count (THC), indicating a regulatory role of TnT in the innate immune response of *S. paramamosain* to pathogens. Apoptosis of hemocytes was higher in crabs treated with TnT-dsRNA compared with control crabs treated with phosphate-buffered saline. Knockdown of TnT increased apoptosis of hemocytes following VA infection, but reduced hemocyte apoptosis following WSSV infection. In summary, TnT may enhance the immune response of *S. paramamosain* to WSSV infection by regulating apoptosis, THC, PO activity and SOD activity. And TnT may play a positive role in the immune response against VA infection by regulating apoptosis, THC, SOD activity and PO activity.

1. Introduction

Troponin is an important component of the thin filaments that allow striated muscles to contract in response to calcium [1,2]. The troponin complex is composed of troponin I (TnI), troponin C (TnC) and troponin T (TnT) [3]. TnI and TnT are myofibrillar proteins of skeletal muscle fibers in the ghost crab, *Ocypode quadrata* [4]. TnT is present in striated muscles of vertebrates and invertebrates and interacts with TnC and TnI of the troponin complex, as well as tropomyosin and actin, which are proteins of the myofibrillar thin filament [5]. The expression of TnT is regulated by different genes in human skeletal and myocardial muscle, so that troponin is different in myocardial and skeletal muscle [6]. In white prawn *Exopalaemon carinicauda,* the full length cDNA of TnT, EcTnC and EcTnI were found to be 1373 base pairs (bp), 692 bp, and 1475 bp, respectively [7]. Expression of all three genes was highest in abdominal muscle, with much lower levels of expression in the gill and hepatopancreas. In crayfish and lobster, TnT expression is associated with synaptic efficacy in slow fibers of the claw and leg opener muscles [8].

The immune system in vertebrates comprises an innate and an adaptive response, whereas all crustaceans, including the mud crab, *S. paramamosain*, rely solely on innate immunity to provide a highly efficient defense against pathogens [9,10]. Crustaceans have a non-specific immune response that is able to recognize and eliminate invading pathogens and also responds to osmotic stress [11]. Vibriosis, which is caused by marine bacteria, such as *Vibrio alginolyticus* (VA), is the most serious disease in mud crabs and has caused irreversible damage to the mud crab culture industry worldwide. Mud crabs can also be infected with WSSV by feeding on WSSV-infected crustaceans or by artificial infection. At present there are no efficient measures to control either of the two pathogens. In the present study, we investigated the role of TnT in the innate immune system of *S. paramamosain* response to WSSV or VA infection.

2. Methods and materials

2.1. Crabs and tissue preparation

Scylla paramamosain (150 g/crab) were got from the aquaculture

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market located in Hangzhou, Zhejiang Province, China and then cultured briefly in artificial seawater (23 ˚C) 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 according to the previous report [12]. *Vibrio alginolyticus* was cultured and used to challenge crabs following the previous report [12]. The crabs were injected with 1×10^6 WSSV/crab and *V. alginolyticus* at 1×10^5 CFU/crab, respectively. And the crabs were injected with hyperhaline PBS buffer were used as the control. The muscles, hepatopancreas, gills, heart, intestines and hemocytes were collected from health or challenged crabs. The samples were used immediately for RNA extraction, aiming to prevent total RNA degradation.

2.2. The rapid amplification of cDNA ends (RACE)

Total RNA was extracted from hemocytes of the *S. paramamosain* using PureLink™ RNA Mini Kit (Ambion, USA), following the protocol of the manufacturer. The concentration and quality of total RNA were determined by the Nanodrop Trace Spectrophotometer and 1% agarose gel electrophoresis detection, respectively. The RACE technique was utilized to clone the full length cDNA sequence of the gene, based on the known middle fragment using SMARTer® RACE 5′/3′Kit, following the protocol of the manufacturer. The synthesized cDNA were kept at −20 ˚C, used for the 3′/5′-RACE PCR with 3′ gene specific primer (3GSP1, 3NGSP1) or 5′ GSP (5GSP2, 5NGSP2), designed on the basis of middle sequence (the primers sequences are shown in Table 1). The PCR products were purified using MiniBEST DNA Fragment Purification Kit Ver.3.0 (Takara, Japan), following the manufacturer's instruction. Amplified cDNA fragments were transferred into the pMD19-T vector (Takara, Japan). Recombinant bacteria were identified by blue/white screen and confirmed by PCR. And then it was sent to sequencing company (Sangon, China). Nucleotide sequences of the cloned cDNA were sequenced by double pass. All primers used in this experiment were designed using Primer Premier 5.0 (Premier, Canada).

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 (Lynnon Biosoft, USA). The protein prediction was performed using the open reading frame (ORF) finder tool. Multiple sequence alignment was created by using the DNAMAN 5.0. And the phylogenetic trees based on the amino acid sequences were performed by the neighbor-joining method using Molecular Evolutionary Genetics Analysis, MEGA7.1.

2.4. The quantitative real-time PCR

The mRNA expression levels of Troponin T in various tissues were measured by qRT-PCR using a SYBR II® Premix Ex Taq (Tli Rnase Plus) (TaKaRa, Japan). Total RNA was isolated from various tissues of healthy adult crabs and hemocytes of crabs challenged by intramuscular injection of 0.2 mL of viral or bacterial suspension including WSSV (10^5 copies/mL) or *V. alginolyticus* (10^5 colony-forming units [CFU]/mL), respectively, for different times, using the EASY spin tissue/cell RNA extraction kit (Aidlab, China) according to the manufacturer's instructions. Experiments were performed in triplicate and at least three crabs were analyzed for each tissue type. The cDNA synthesis was carried out using 200 μg of total RNA with the ReverTra AceqPCR RT Master Mix with gDNA Remover (Code: FSQ-301; Toyobo, Japan). The synthetic cDNA was kept at −20 ˚C. The qRT-PCR was carried out in Bio-Rad Two Color Real-Time PCR Detection System and the data were calculated according to the $2^{-\Delta\Delta CT}$ comparative CT method, with GAPDH amplification as the internal control. The design and synthesis of the qRT-PCR primers were entrusted to Generay (Shanghai, China), based on the open reading frame (ORF). Table 1 lists the primers that were designed and synthesized by Generay.

2.5. Prokaryotic expression and purification of troponin T-dsRNA

The primers with specific restriction sites (*Hin*d III in the forward primer and *Sal*I in the reverse primer) were designed from the cloned nucleotide sequence. PCR product digested with Hind III/*Sal*I was subcloned into LIMTUS 38i Vector (NEB, MA, USA) digested with the same enzymes to gain plasmid L38-Troponin T. The constructed L38-Troponin T was verified by restriction enzyme digestion and DNA

Table 1
Universal and specific primers used in this study.

Primer Name	Nucleotide sequence (5′ → 3′)	Purpose
3′ race GSP1	CGGAGAAAATGGGATGAAGACA	first primer for 3′RACE
3′race GSP2	ACAGAACCATTGCTCTAATATCG	second primer for 3′RACE
5′race GSP2	CAATCAAAAGCCTTGCTGTAGTC	first primer for 5′RACE
5′race GSP1	TGGCAAGCATTGGTGTTCCTGATA	second primer for 5′RACE
5′race GSP2	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
prophenoloxidase –F	ATGAAAGAGGAGTGGAGATG	for prophenoloxidase expression
prophenoloxidase –R	GTGATGGATGAGGAGGTG	for prophenoloxidase expression
Myosin-F	TAGAGTGAGGGGTTGGTG	for Myosin expression
Myosin-R	GAGTGAAATGGAGAGATGGA	for Myosin expression
MELCLP-F	GCCGAGATAAGTGTAGAGGAA	for MELCLP expression
MELCLP-R	AGTGGGGTTCTGTCCAAG	for MELCLP expression
C-type-lectin-F	ACTGAGGGGAAAGTAGCC	For C-type-lectin expression
C-type-lectin-R	TGCCCGTGTTTATTCATC	For C-type-lectin expression
CAP-F	TCAGAGCACCCTGGTAAATGT	for CAP expression
CAP-R	GGCAGAACTGCGAAAGAAAG	for CAP expression
JAK-F	ATTGCTGAGGGGATGGATT	for JAK expression
JAK-R	GCCCATCACATTCCCAAA	for JAK expression
STAT-F	GACTTCACTAACTTCAGCCTCG	for STAT expression
STAT-R	GAGCTGAGTCTGTCTTAATGTTATCC	for STAT expression
Toll-like receptor –F	TGTTGCCAGAGCAGAAGGT	for Toll-like receptor expression
Toll-like receptor –R	TTCCGTGAATGAACGAAGG	for Toll-like receptor expression

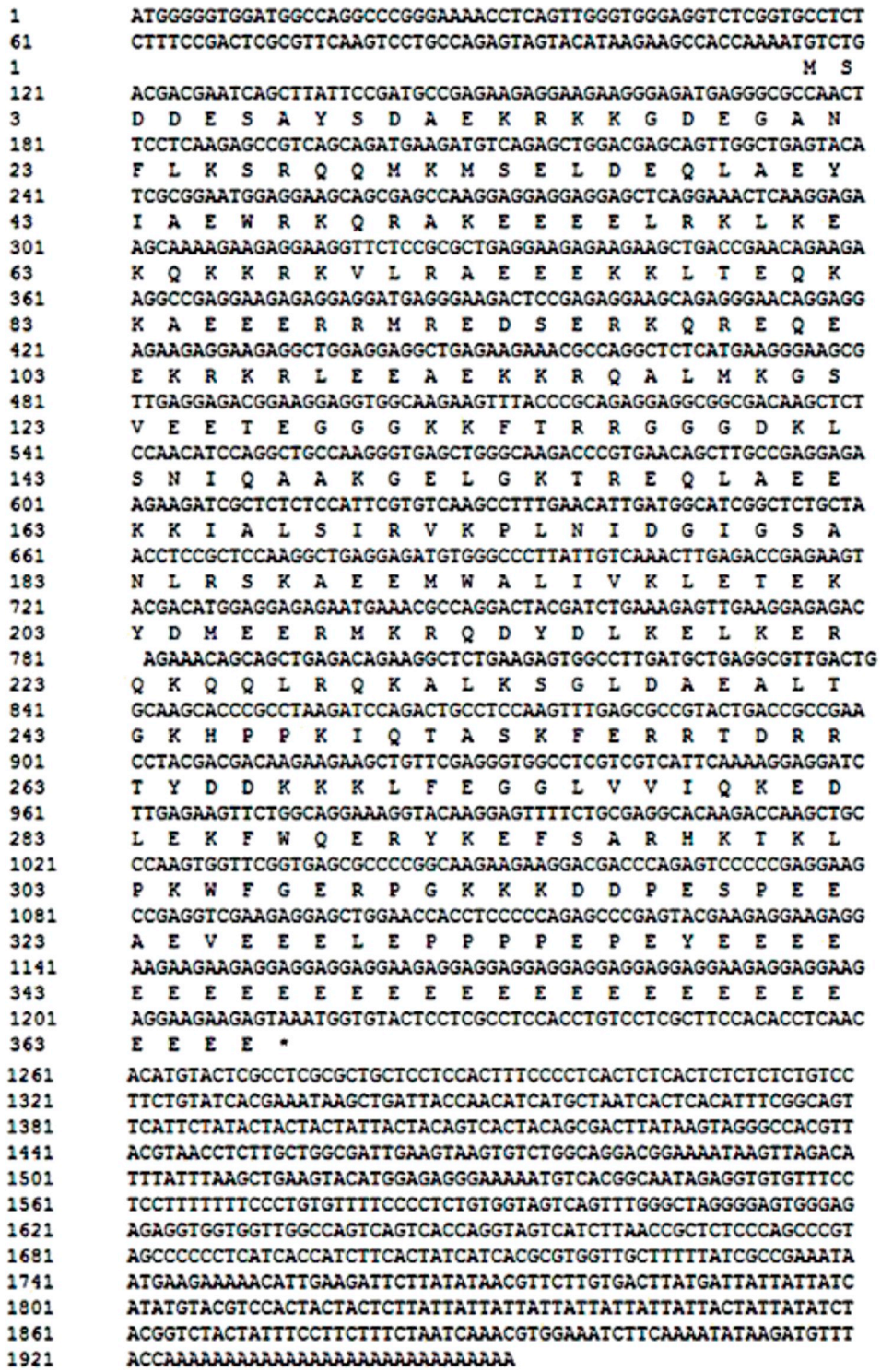

Fig. 1. Nucleotide and deduced amino acid sequences of *S. paramamosain* troponin T. The nucleotide sequence is displayed in the 5′–3′ direction. The deduced amino acid sequence is shown using the single letter amino acid code. The 3′-UTR and 5′-UTR regions are indicated by lower case letters. Codons are numbered from the initiating methionine (ATG) codon, with an asterisk denoting the termination codon (TAG).

sequencing. The recombinant plasmid L38-Troponin T was transformed into HT115 (DE3). Single colonies of the above engineering bacteria were separately inoculated to 5 mL of LB medium containing ampicillin (100 μg/mL), cultured at 37 ˚C with shaking at 200 rpm/min for 12–16 h, and then inoculated to LB medium containing Amp by a proportion of 1%, cultured at 37 ˚C with shaking at 200 rpm/min for 2–3 h (OD600 ≈ 0.6), and added with IPTG (isopropyl-β-*d*-thiogalactoside) (with a final concentration of 0.8 mM/L) to induce the expression for 4 h.

2.6. Knock down of troponin T by RNAi and challenge experiments

Troponin T-dsRNA (100 μg/crab) was purified according to the previous report [13] and immediately injected intramuscularly into crabs, and Troponin T mRNA expression levels were detected by qRT-PCR. And then, crabs were divided into eight groups: intramuscular

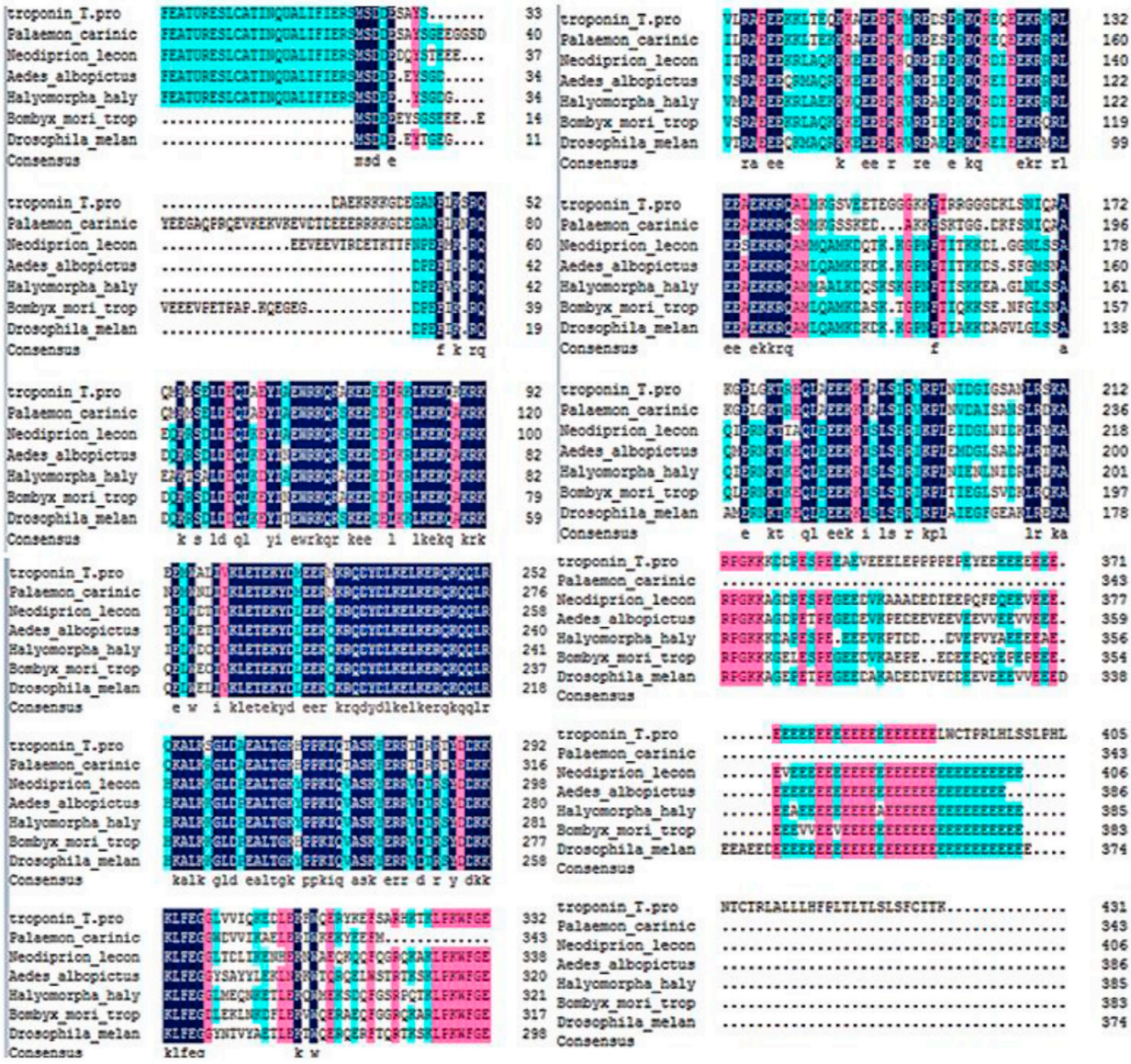

Fig. 2. Multiple alignment of the amino acid sequence of *S. paramamosain* Troponin T with *Palaemon carinicauda*, *Neodiprion lecontei*, *Halyomorpha halys*, *Bombyx mori*, *Aedes albopictus*, *Drosophila melanogaster*. The conserved cysteines are shaded and boxed.

injection with 100 μL of PBS alone; intramuscular injection with 100 μL of Troponin T-dsRNA alone; intramuscular injection with 100 μL of WSSV (10^5 copies/mL) challenge alone; injection with Troponin T-dsRNA for 12 h and followed by 100 μL WSSV challenge; intramuscular injection with 100 μL of *V. alginolyticus* (10^5 CFU/mL) challenge alone; injection with Troponin T-dsRNA for 12 h and followed by 100 μL of *V. alginolyticus* challenge. The mortality was monitored every 12 h after the last injection.

2.7. Quantitative analysis of WSSV

S. paramamosain were injected with WSSV or mixture of WSSV and Troponin T-dsRNA. The whole-genome was extracted from crab hemocytes collected at 12, 24 and 48 h post injection using DNA extraction Kit (Tiangen, China), according to the manufacturer protocol. To discover the WSSV copies, *S. paramamosain* 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 [14]. TaqMan real-time PCR was performed by using a Perfect Real Time premix (Takara, Japan) containing a high-performance Taq antibody, Takara Ex Taq HS, for hot start real-time PCR. Primers were designed using Primer 5.0 software and the TaqMan probe with the WSSV whole sequence. Primers WSSV-RT1 (5′-TTGGTTTCATGCCCGAGATT-3) and WSSV-RT2 (5′-CCTTGGTCAGCCCCTTGA-3′) produced a fragment of 154 bp after amplification. The TaqMan probe was synthesized and labeled with the fluorescent dyes 5-carboxyfluorescein (FAM) (5′-FAM-TGCTGCCGTCTCCAA-TAMRA-3′). PCR amplification was performed for 4 min at 50 °C, followed by 45 cycles of 45 s at 95 °C, 45 s at 52 °C and 45 s at 72 °C. Thermal cycling was performed on an icycle IQ5 real-time PCR detection system (Bio-RAD, CA, USA).

2.8. Determination of immune parameters after RNAi

The immune parameters determined included total hemocytes numbers (THC), PO and SOD activity. THC was determined as described previously [15]. To determine PO and SOD activities, 500 μL of hemolymph was withdrawn into a 1 mL syringe containing 500 μL of 20 mM EDTA solution from each individual crab. PO activity was quantified in the hemolymph mixture based on the formation of dopa

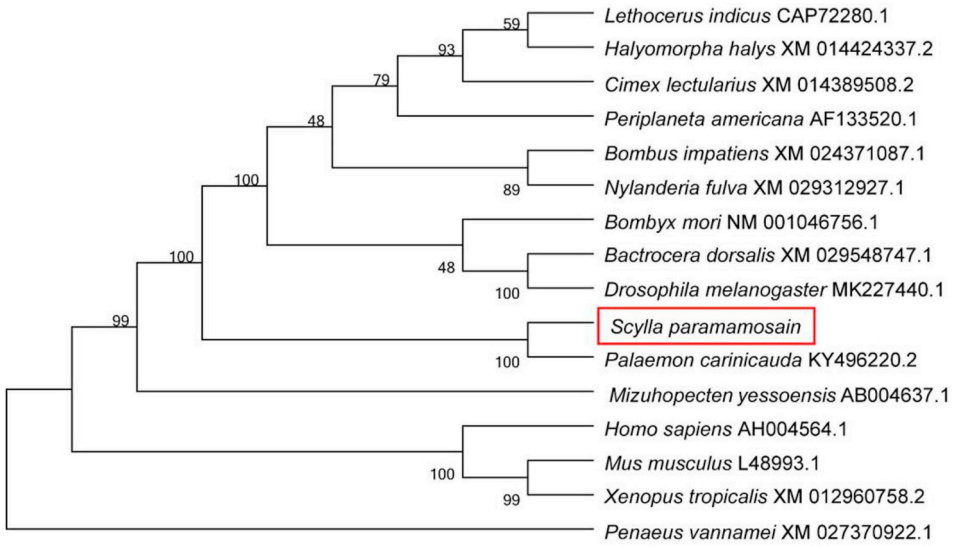

Fig. 3. The phylogenetic relationship of Troponin T from different organisms based on amino acid sequence comparisons.

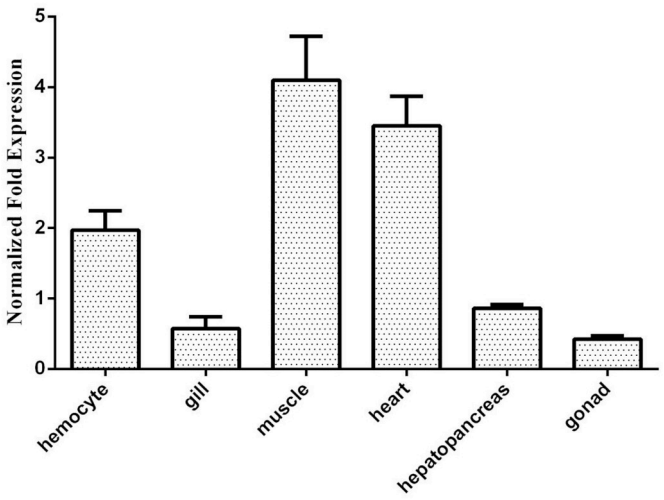

Fig. 4. Expression levels of Troponin T in various tissues of *S. paramamosain* were determined by quantitative real-time PCR. The amount of Troponin T mRNA was normalized to GAPDH transcript levels. Data are shown as the mean ± SD (standard deviation) from three individuals.

chrome from the substrate L-3, 4-dihydroxyphenylalanine (L-DOPA), as described previously [15]. SOD activity was quantified in hemocytes isolated from 300 μL of the hemolymph mixture, according to the improved method described by Beauchamp and Fridovich [16]. Data were presented as a percentage of the normal control.

2.9. Kaplan–Meier survival analysis

For the pathogen challenge, healthy crabs were randomly distributed into six groups (n = 9 per group, three repeat). The control group received injections of PBS alone, the Troponin T-dsRNA group received injections of Troponin T-dsRNA alone, the WSSV group received injections of WSSV in PBS, and the Troponin T-dsRNA + WSSV group received injections of Troponin T-dsRNA and WSSV, the *V. alginolyticus* group received injections of *V. alginolyticus* in PBS, and the Troponin T-dsRNA + *V. alginolyticus* group received injections of Troponin T-dsRNA and *V. alginolyticus*. Each group of crabs was cultivated under the same condition. After every 12 h, the number of live and dead crab was counted. The survival data of crabs was arranged and analyzed in Microsoft GraphPad 5.0.

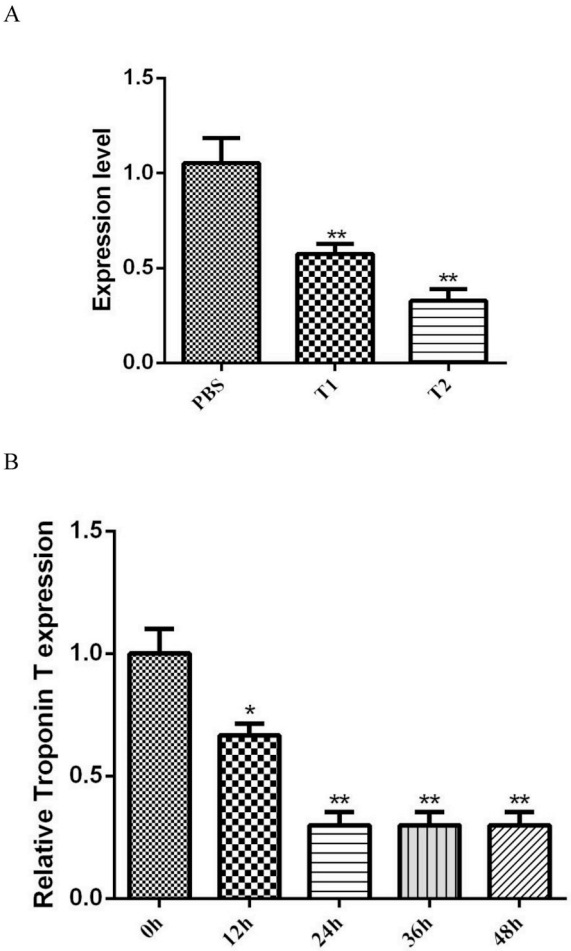

Fig. 5. (A) qRT-PCR analysis of troponin T (TnT) expression in hemocytes from *S. paramamosain* 24 h after treatment with TnT-dsRNA (T1, T2). The amount of TnT mRNA was normalized to the level of GAPDH mRNA. (B) qRT-PCR analysis of TnT expression in hemocytes from *S. paramamosain* at different times post treatment with TnT-dsRNA. Double asterisks indicate a highly significant difference ($P < 0.01$) from TnT levels prior to treatment with TnT-dsRNA. Single asterisks indicate a significant difference ($P < 0.05$) from TnT levels prior to treatment with TnT-dsRNA.

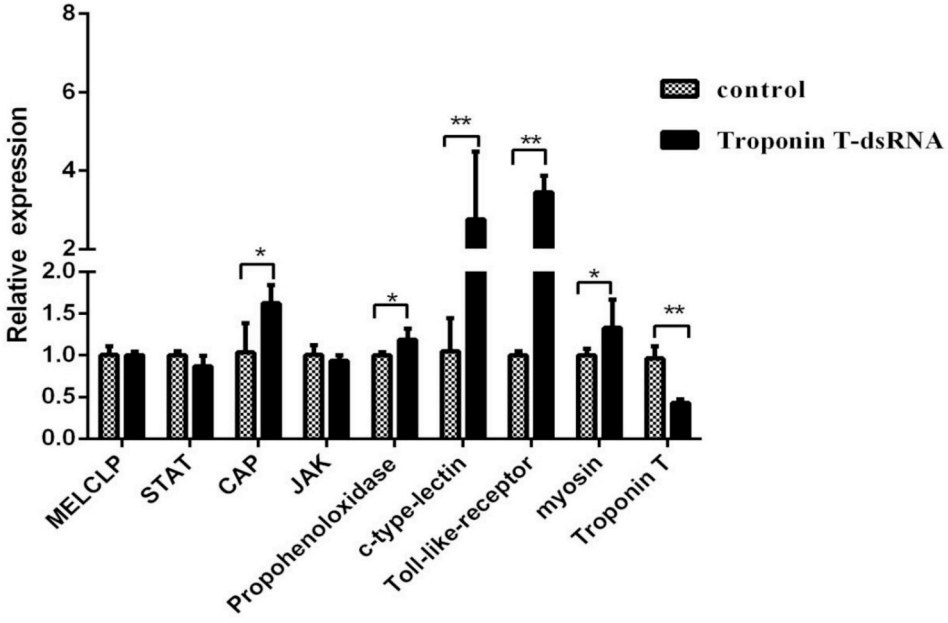

Fig. 6. The qRT-PCR analysis of expression of eight immune genes (JAK, STAT, crustin antimicrobial peptide (CAP), Toll-like receptor, prophenoloxidase, c-type-lectin, myosin–II–essential-light-chain-like-protein (MELCLP), and myosin) in hemocytes from *S. paramamosain* treated with TnT-dsRNA. The amount of TnT mRNA was normalized to the level of GAPDH transcripts. Data are presented as mean ± SD (standard deviation) of three animals. Double asterisks indicate a highly significant difference ($P < 0.01$) from levels prior to treatment with TnT-dsRNA. Single asterisks indicate a significant difference ($P < 0.05$) from levels prior to treatment with TnT-dsRNA.

2.10. Apoptosis assays by flow cytometry

The hemolymph was mixed with 20 mM EDTA at a ratio of 1:1, and the mixture was centrifuged at 2000 rpm at 4 °C for 10 min to collect hemolymph cells. The hemolymph cells were then suspended in highly alkaline PBS, counted and adjusted to a density of 3–5 × 10^6 cells/mL with PBS. The cells were stained using a BD Phrmingen™ FITC Annexin V Apoptosis Kit, and assessed by flow cytometry. The cells on quadrant 4, with low PI and high annexin V staining, were considered as apoptotic cells. The data was presented as means ± standard deviation (SD) derived from at least three independent experiments.

2.11. Statistical analysis

Quantitative data was expressed as mean ± standard deviation (SD). Data from three independent experiments was analyzed by one-way analysis of variance to calculate the means and standard deviations of the triplicate assays. Statistical differences were estimated using one-way ANOVA followed by least-significant differences (LSD) and Duncan's multiple range test. All statistics were measured using SPSS software version 19 (IBM, USA). A probability level of 0.01 was used to indicate statistical significance ($P < 0.01$).

3. Results

3.1. Characterization of the S. paramamosain troponin T cDNA

The full-length TnT cDNA sequence that we obtained was 1952 bp in length and contained an 1101 bp open reading frame, encoding a 367 amino acid protein. The 3′untranslated region (3′-UTR) was 738 bp in length and the 5′-untranslated region (5′-UTR) was 114 bp in length. The nucleotide and deduced amino acid sequence of the full-length cDNA are shown in Fig. 1.

3.2. Sequence homology and phylogenetic analysis

The amino acid sequence of mud crab TnT was compared with that of other species using the NCBI website (Fig. 2). *S. paramamosain* TnT displayed 68.28% identity with that of *Palaemon carinicauda* (KY496220.2), 58.82% identity with that of *Nylanderia fulva* (XM029312927.1), 56.44% identity with that of *Bombyx mori* (NM001046756.1) and 54.7% identity that of *Drosophila melanogaster* (MK227440.1). A condensed phylogenetic tree, based on deduced amino acid sequences, was constructed by the neighbor-joining method using MEGA7.1 (Fig. 3). Phylogenetic analysis showed that the amino acid sequence of TnT was conserved among different species, with several highly conserved amino acid sites in the conserved domains. Among the species compared, TnT from *S. paramamosain* showed the closest relationship with that of *P. carinicauda*.

3.3. Tissue distribution of TnC expression

Quantitative real-time polymerase chain reaction (qRT-PCR) was used to measure the expression of TnT in different tissues from *S. paramamosain*. The expression level of TnT was significantly higher in muscle and heart than in other tissues ($P < 0\ 0.01$), and was low in the gonad (Fig. 4).

3.4. Effect of troponin T-dsRNA on troponin T expression

The effect of TnT double-stranded RNA (TnT-dsRNA) on the expression of TnT in hemocytes was measured using qRT-PCR. TnT mRNA expression in hemocytes was significantly reduced 24 h after treatment with TnT-dsRNA ($P < 0.01$) (Fig. 5A). Because TnT-dsRNA2 (T2) showed a better knockdown effect than TnT-dsRNA1 (T1), we chose T2 as the TnT-dsRNA for use in the following experiments. The qRT-PCR was also used to measure interference timelines and the effect of TnT-dsRNA was found to be significant at 24, 36 and 48 h after treatment ($P < 0.01$) (Fig. 5B).

3.5. Effects of troponin T knockdown on expression of immune genes

Having shown that expression of TnT was significantly knocked down by TnT-dsRNA ($P < 0.01$) in hemocytes, we next analyzed the expression of several important immune-related genes after treatment to explore the relationship between TnT and these immune genes. Of the eight immune genes examined, c-type-lectin and toll-like-receptor were very significantly up-regulated ($P < 0.01$), while crustin antimicrobial peptide (CAP), prophenoloxidase and myosin were significantly up-regulated ($P < 0.05$) at 24 h after treatment with TnT-dsRNA (Fig. 6). These results suggest that knockdown of TnT might affect the innate immunity in *S. paramamosain*.

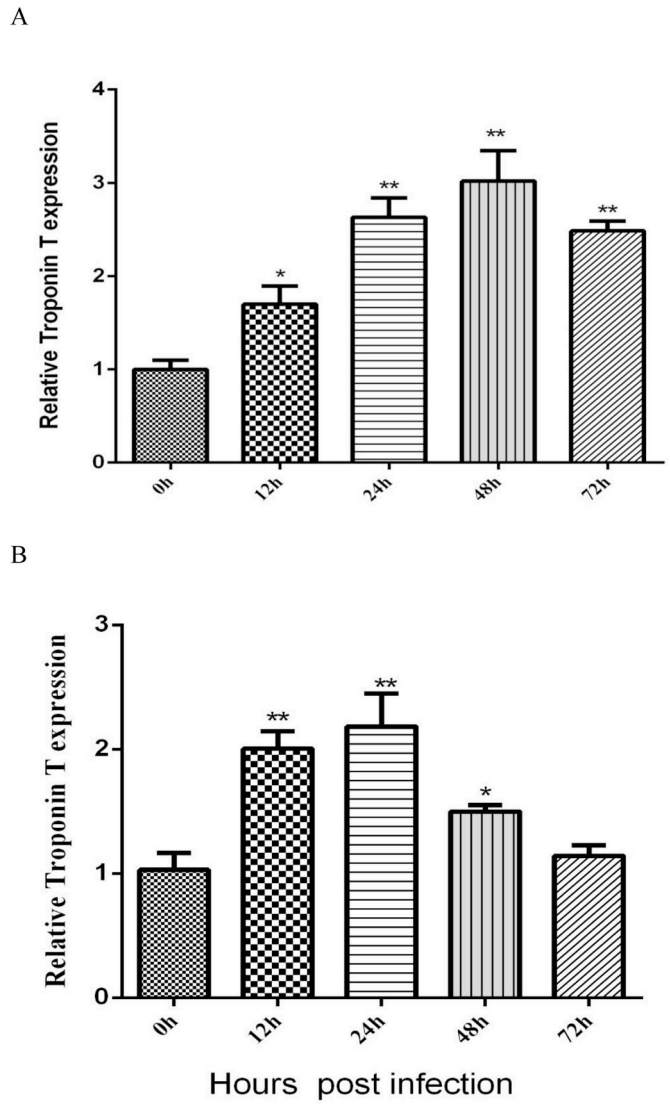

Fig. 7. The qRT-PCR analysis of Troponin T (TnT) expression in hemocytes from *S. paramamosain* infected with WSSV or VA. (A) qRT-PCR analysis of TnT expression in hemocytes from *S. paramamosain* infected with WSSV. (B) qRT-PCR analysis of TnT expression in hemocytes from *S. paramamosain* infected with VA. The level of TnT mRNA was normalized to the level of GAPDH transcripts. Data are shown as mean ± SD (standard deviation) of three animals. Double asterisks indicate a very significant difference ($P < 0.01$) from TnT levels prior to infection. Single asterisks indicate a significant difference ($P < 0.05$) from TnT levels prior to infection.

3.6. Time course of troponin T expression after WSSV or V. alginolyticus infection

TnT expression changed in a time-dependent manner after WSSV or VA infection in *S. paramamosain.* TnT expression was significantly up-regulated ($P < 0.01$) between 12 and 48 h post WSSV infection (Fig. 7A). The highest expression of TnT occurred at 48 h post infection, after which the expression of TnT gradually decreased. In contrast, the expression of TnT was significantly up-regulated ($P < 0.01$) at 12 and 48 h post VA infection (Fig. 7B), but gradually returned to the level of pre-infection at 72 h post infection. These results suggest that TnT may play an important role in the innate immune response to WSSV or VA infection in *S. paramamosain*.

3.7. Kaplan-survival analysis and WSSV copy detection

To evaluate the effects of TnT knockdown on the mortality of crabs challenged with WSSV or VA, crabs were injected with TnT-dsRNA before challenge. Following treatment with TnT-dsRNA, the percentage of crabs surviving VA infection was significantly reduced ($P < 0\ 0.01$) at 360 h post infection, compared with the control group that received PBS (Fig. 8A). This result indicates that TnT is very important for the crab's immune response to VA infection. A similar trend was observed in the result of WSSV challenge. The cumulative mortality of WSSV-infected crabs was significantly lower ($P < 0.01$) in the control group than in the group pretreated with TnT-dsRNA at 360 h post infection (Fig. 8B). Comparison of crab mortality between the groups pretreated with TnT-dsRNA and those treated with pathogen alone thus showed that TnT plays an essential role in the crabs' immune response to WSSV or VA infection.

In all WSSV-challenged groups, WSSV copy numbers in hemocytes increased with time and were always significantly higher in the TnT-dsRNA treated group than in the control group ($P < 0.01$) (Fig. 9), suggesting that the absence of TnT promotes replication of WSSV in the crabs.

3.8. Effects of troponin T knockdown on immune parameters

Firstly, we investigated the effect of TnT knockdown in uninfected crabs. Here, knockdown of TnT produced no significance difference in total hemocyte count (THC) or phenoloxidase (PO) activity compared with the control (Fig. 10A and B). However, superoxide dismutase (SOD) activity was significantly higher at 24 h after TnT knockdown compared with that of control (Fig. 10C), indicating that knockdown of TnT has some affect on the innate immunity. We next investigated the effect of TnT knockdown on the immune response to infection. In VA-infected crabs, THC levels were significantly higher ($P < 0.05$) than in uninfected crabs at 12 and 24 h post infection. THC levels in VA-infected crabs with knockdown of TnT were very significantly higher ($P < 0.01$) at 36 h post infection than in control crabs (Fig. 10D). PO activity was not significantly different between VA-infected and uninfected crabs, but in infected crabs with TnT knockdown, PO activity was significantly higher ($P < 0.01$) at 24 h and 36 h post infection (Fig. 10E). Compared with the uninfected group, SOD activity was very significantly increased ($P < 0.01$) at 24 and 36 h post infection in VA-infected crabs and TnT knockdown significantly reduced ($P < 0.05$) SOD activity in VA-infected crabs (Fig. 10F). Finally, we investigated the effect of TnT knockdown on the immune response to WSSV infection in *S. paramamosain*. THC levels in WSSV-infected crabs were very significantly higher ($P < 0.01$) than in uninfected crabs at 24 and 36 h post treatment (Fig. 10G) and this response was diminished by TnT knockdown. PO activity was significantly increased ($P < 0.01$) in WSSV-infected crabs at 24 and 36 h post infection compared with that of control and this response was diminished by TnT knockdown (Fig. 10H). SOD activity in WSSV-infected crabs was significantly reduced ($P < 0.01$) at 24 and 36 h post infection compared with that of control and this increase was attenuated by TnT knockdown (Fig. 10I). The data showed that TnT knockdown would affect the innate immune response of *S. paramamosain.* Following VA infection, knockdown of TnT further increased levels of THC and PO activity but attenuated the increase in SOD activity. However, after WSSV infection, TnT knockdown augmented the increase in PO activity but reduced the increase in THC and SOD activity.

3.9. Effects of troponin T knockdown on apoptosis of hemocytes

Apoptosis of hemocyte was significantly increased ($P < 0.05$) in TnT knockdown crabs compared with control crabs (Fig. 11E). The increased apoptosis in TnT knockdown crabs suggests that TnT can inhibit the apoptosis of hemocyte. Apoptosis was significantly increased

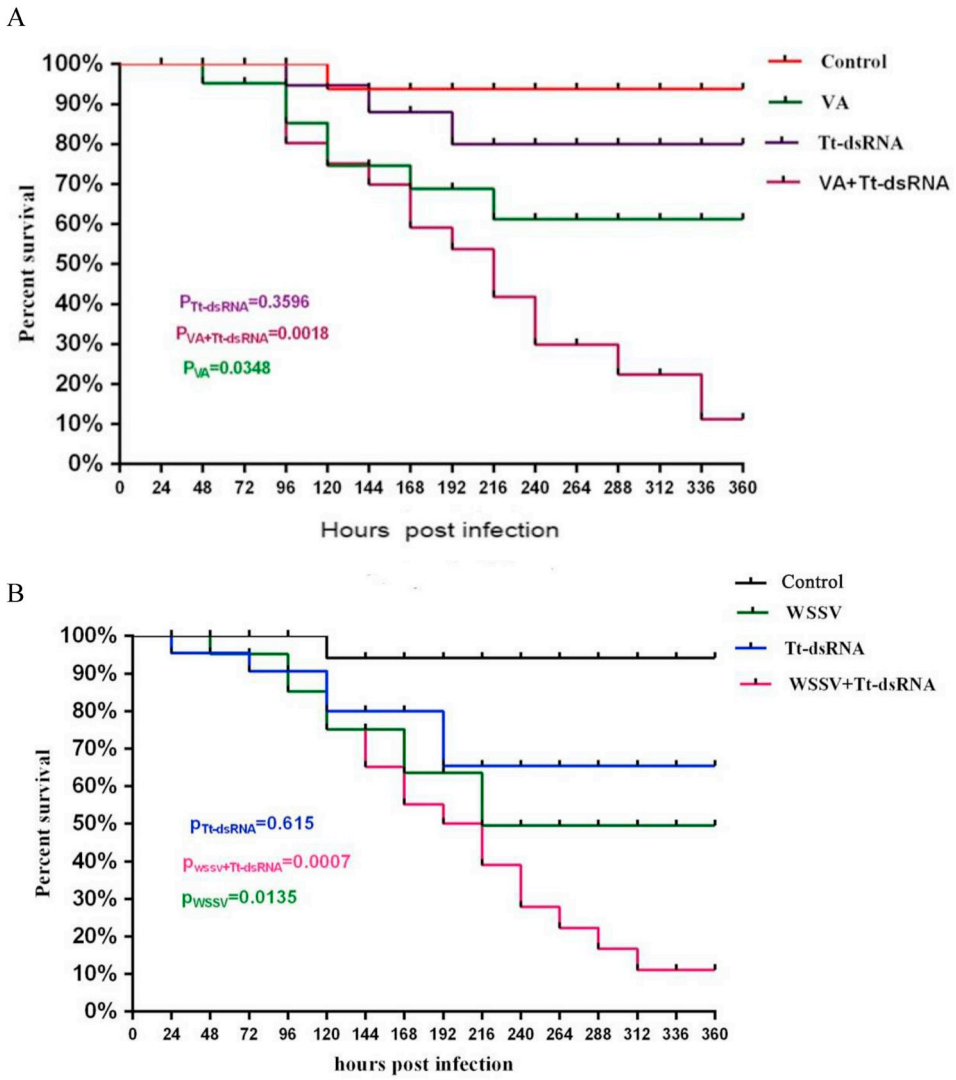

Fig. 8. Kaplan-Meier survival analysis of *V. alginolyticus*-infected crabs pretreated with Troponin T-dsRNA (A). Kaplan-Meier survival analysis of WSSV-infected crabs pretreated with Troponin T-dsRNA (B). The treatment regime applied to each group is indicated.

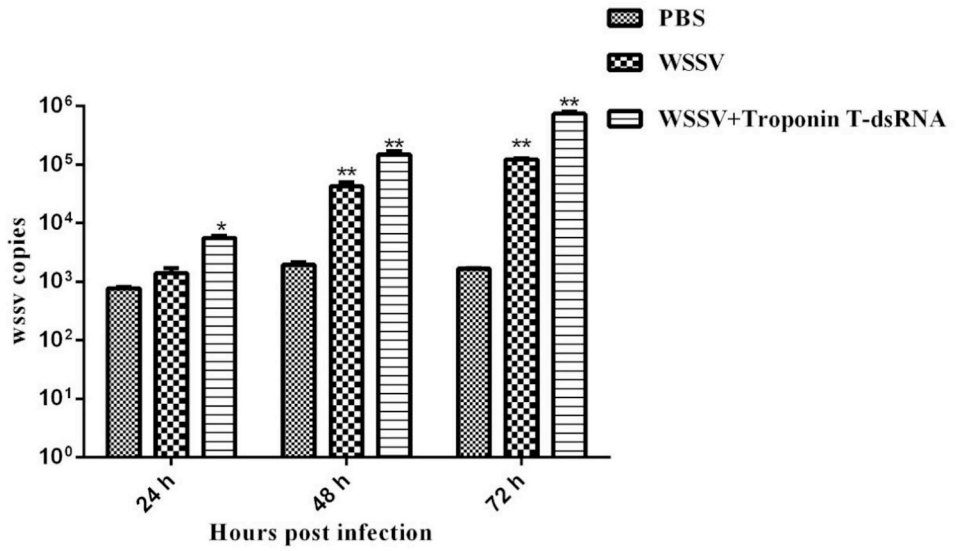

Fig. 9. Detection of WSSV copy numbers in hemocytes at different times post infection. Double asterisks indicate a very significant difference ($P < 0.01$) from troponin T (TnT) levels prior to infection. Single asterisks indicate a significant difference ($P < 0.05$) from TnT levels prior to infection.

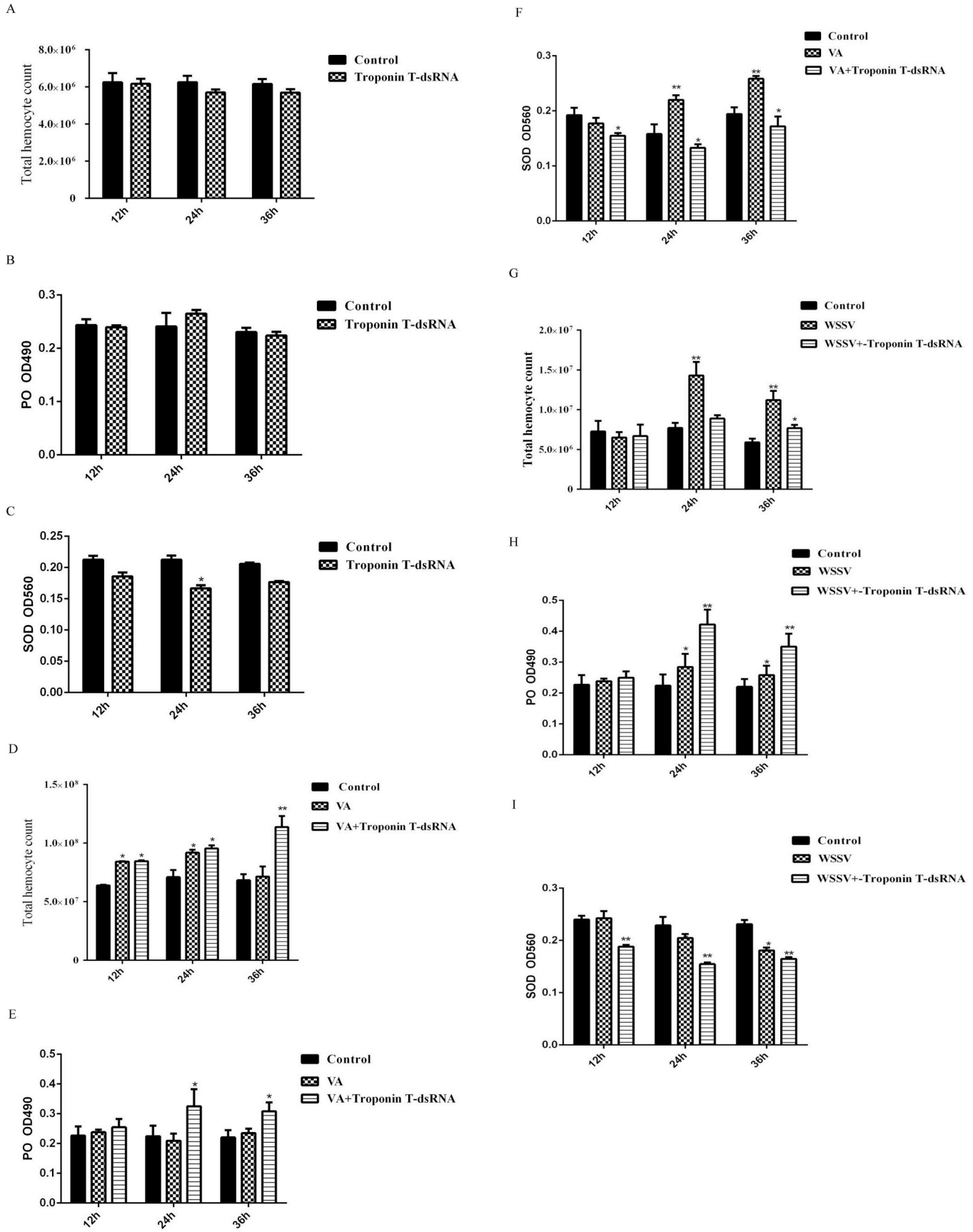

Fig. 10. Effects of troponin T (TnT) knockdown on components of the immune response. Activity of (A) THC, (B) PO and(C) SOD (C) in crabs treated with TnT-dsRNA; Activity of (D) THC, (E) PO and (F) SOD in VA-infected crabs pretreated with TnT-dsRNA; Activity of (G) THC, (H) PO and (I) SOD in WSSV-infected crabs pretreated with TnT-dsRNA. Double asterisks indicate a very significant difference ($P < 0.01$) from TnT levels prior to infection. Single asterisks indicate a significant difference ($P < 0.05$) from TnT levels prior to infection.

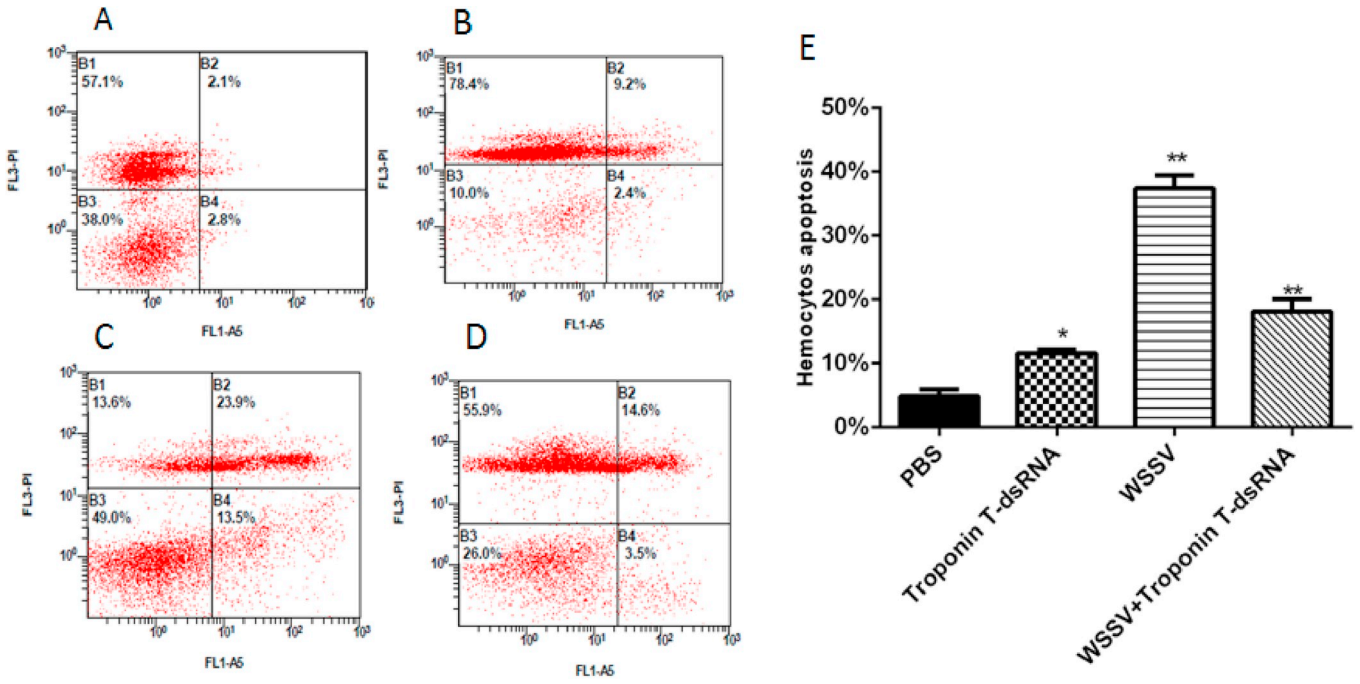

Fig. 11. Detection of hemocyte apoptosis in WSSV-infected *S. paramamosain* by flow cytometry. (A) PBS control; (B) Treatment with troponin T (TnT)-dsRNA; (C) Infection with WSSV; (D) Infection with WSSV after treatment with TnT-dsRNA; (E) Bar graph showing hemocyte apoptosis. Data are shown as mean ± SD. Columns sharing superscript letters are not significantly different, as determined by Tukey's test. Double asterisks indicate a highly significant difference ($P < 0.01$) compared with the PBS control for a given time period. Single asterisks indicate a significant difference ($P < 0.05$) compared with the PBS control.

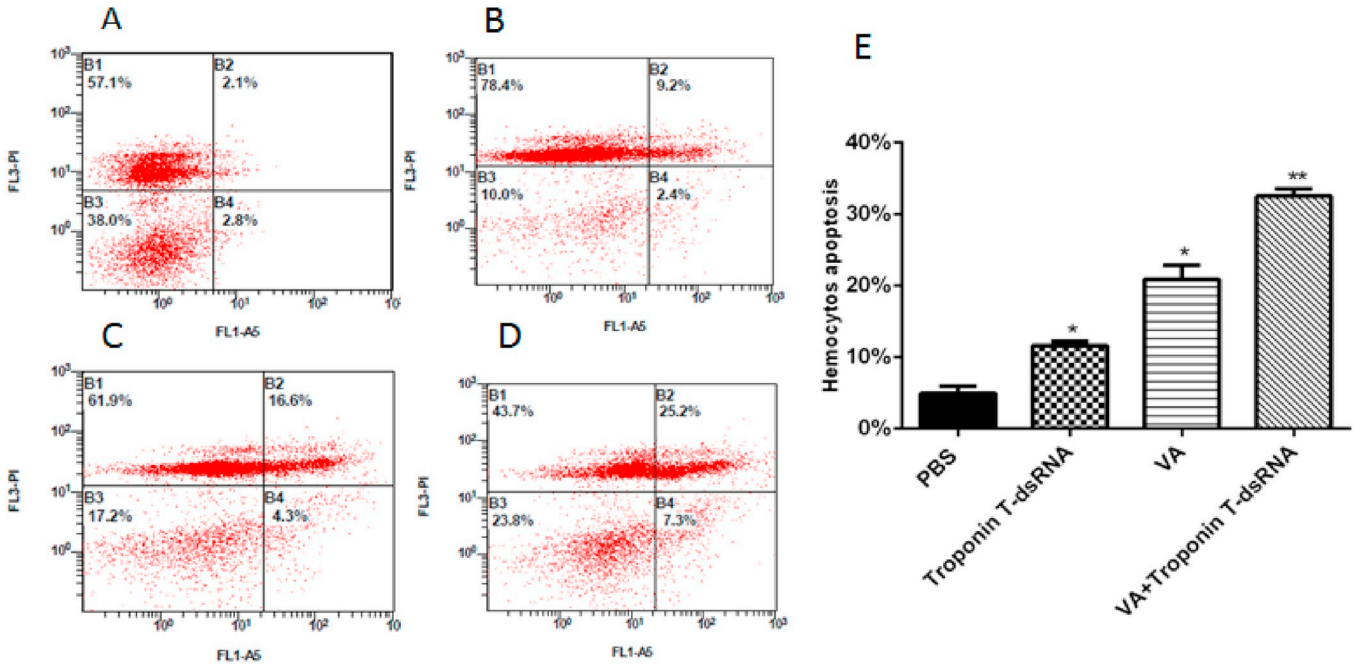

Fig. 12. Detection of hemocyte apoptosis in *V. alginolyticus* (VA)-infected *S. paramamosain* by flow cytometry. (A) PBS control; (B) Treatment with troponin T (TnT)-dsRNA; (C) Infection with VA; (D) Infection with VA after treatment with TnT-dsRNA; (E) Bar graph showing hemocyte apoptosis. Data are shown as mean ± SD. Columns sharing superscript letters are not significantly different, as determined by Tukey's test. Double asterisks indicate a very significant difference ($P < 0.01$) compared with the PBS control for a given time period. Single asterisks indicate a significant difference ($P < 0.05$) compared with the PBS control.

($P < 0.01$) in both normal and TnT knockdown crabs following WSSV infection, but the increase was significantly attenuated ($P < 0.01$) by TnT knockdown (Fig. 11E). These results indicate that TnT can regulate WSSV-induced apoptosis in *S. paramamosain*. Apoptosis of hemocyte was also significantly increased ($P < 0.01$) in crabs infected with VA than in control crabs, but the increased apoptosis was augmented by TnT knockdown (Fig. 12E). These results indicate that TnT plays an important role in pathogen-induced apoptosis of hemocytes and the role is different in response to WSSV or VA infection.

4. Discussion

Human troponin T was first discovered by the German physician Hugo A. Katus and shown to be a useful diagnostic marker for acute

myocardial infarction [17]. Troponin T is a part of the troponin complex expressed in skeletal and cardiac myocytes. Cardiac TnT is a preferred biomarker for the diagnosis of acute myocardial infarction [18]. The cardiac subtype of TnT is particularly useful for the laboratory diagnosis of heart attack since it is released into the blood-stream when damage to heart muscle occurs [19]. Concentrations of high-sensitivity cardiac TnT are a useful biomarker of cardiac and pulmonary dysfunctionevenin patients with stable chronic obstructive pulmonary disease [20]. In contrast to the wealth of information on the role of TnT in striated muscle, few studies were performed to investigate the role of TnT in the host immune response to viral infection. Mean TnT levels were found to be increased in the patients infected by parvovirus B19 [21] and muscle TnT level was up-regulated in the orange-spotted grouper, *Epinephelus coioides,* infected with nervous necrosis virus [22]. The role of TnT in the innate immune response of crustacean has not been reported till now.

TnT from *S. paramamosain* consists of 1952 nucleotides, which contain an open reading frame coding for 367 amino acid residues. Phylogenetic analysis by construction of a neighbor-joining tree revealed a close evolutionary relationship between TnT of *S. paramamosain* and that of *P. carinicauda*. *S. paramamosain* TnT is expressed at high levels in muscle and heart, and at relatively low levels in gills, hemolymph, gonad and hepatopancreas. The high expression of TnT in the muscle is consistent with the previous reports [4,5]. TnT is a myofibrillar protein of skeletal muscle fiber in the ghost crab *O. quadrata* [4], and is present in striated muscle of vertebrates and invertebrates [5]. We infected *S. paramamosain* with WSSV or with VA and then examined the expression levels of TnT by qRT-PCR. TnT expression was up-regulated between 12 and 72 h post WSSV infection, with the highest expression level at 48 h post infection. Expression of TnT was up-regulated at 12 and 48 h post VA infection, with the highest expression level at 24 h post infection. These results suggest that TnT is involved in the crab immune response to both WSSV and VA.

Then we examined the role of TnT in the innate immunity of *S. paramamosain* by knocking down TnT expression using TnT-dsRNA. We determined the effects of TnT-dsRNA on the expression of several genes known to be involved in the innate immuny. Knockdown of TnT expression resulted in the up-regulation of immune-related genes, including c-type-lectin, toll-like-receptor, myosin, prophenoloxidase and CAP. These results suggest that TnT maybe affect apoptosis, phagocytosis and PO activity in crab hemocytes. In order to explore the roles of TnT in the innate immunity of *S. paramamosain*, we evaluated the effect of knockdown on components of the immune response. TnT knockdown had no significant effect on levels of THC or PO activity, but SOD activity was significantly higher at 24 h after knockdown. However, the levels of THC, PO and SOD activity were significantly changed by TnT knockdown after WSSV or VA infection. Apoptosis is a highly regulated, programmed cell death process which plays a critical role in limiting viral infection [23–25]. In this study, TnT knockdown was shown to increase apoptosis of hemocyte in normal crabs but showed very different effects on apoptosis of hemocyte in WSSV-infected and VA-infected *S. paramamosain*.

Kaplan-Meier survival analysis showed that VA-infected *S. paramamosain* had a significantly lower survival rate after TnT knockdown and a similar trend was shown in WSSV-infected crabs. The increased mortality of TnT knockdown crabs following infection with pathogens indicates that TnT plays an essential role in the crabs' resistance to pathogens. Knockdown of TnT also significantly increased WSSV copy numbers in WSSV-infected crabs, indicating that TnT plays an important role in inhibiting WSSV replication. In conclusion, TnT plays an essential role in the innate immune response of *S. paramamosain* by regulating the host immune system against pathogens through modulating apoptosis and regulating the levels of THC, PO activity and SOD activity.

CRediT authorship contribution statement

Fei Zhu: Conceptualization, Methodology, Investigation, Writing - review & editing. **Xiongchao Ma:** Investigation, Data curation, Writing - original draft, Validation, Software.

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