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



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ABSTRACT

Troponin C (TnC) is one member of the EF-hand superfamily. In many species, this gene had been identified and related functions had been elucidated. The TnC gene was still blank in the *Scylla paramamosain*. We obtained the TnC gene for the first time in the *S. paramamosain*. And we systematically analyzed the possible role of this gene in the innate immunity of *S. paramamosain* while infected with white spot syndrome virus (WSSV) or *Vibrio alginolyticus*. The full-length 1427 bp sequence of TnC contains a 453 bp open reading frame (ORF) for encoding a 151 amino acid protein. Detection of tissue specificity of gene expression showed that the TnC was primarily expressed in muscle tissue. The expression of TnC was successfully inhibited by RNA interference technology, and several immune genes were affected. The activity of phenoloxidase and superoxide dismutase increased, and the total hemocytes counts increased after RNAi of TnC. It was found that after infection with *V. alginolyticus* and WSSV, the expression of TnC in hemocytes decreased. Infected with *V. alginolyticus* and WSSV, the cumulative mortality and apoptotic rate of hemocytes increased after silencing the TnC gene. Our results indicate that TnC takes participate in the innate immunity of *S. paramamosain* and may plays a different role in the antiviral and antibacterial immune response.

1. Introduction

The immune system covers innate and adaptive response. However, vertebrates are the only phylum that can mount an adaptive immune response [1]. As is known to all, *S. paramamosain* like other invertebrates rely solely on innate immunity to defend against foreign infection [2–4], which includes the surface barrier function, the filtration function, cellular immunity and humoral immunity. As two of the severest diseases in crabs, white spot syndrome virus (WSSV) and Vibriosis have brought a destructive loss to the crab culture industry worldwide. *S. paramamosain* is as highly susceptible to WSSV as marine shrimp and is a host or carrier of WSSV [5,6]. *Vibrio alginolyticus* is a sort of conditional pathogens in oceans [7]. The disease is prone to outbreak when the environment deteriorates or the immune function decreases [8]. In general, there is no effective method to control either of these two diseases till now.

Troponin C (TnC), known as having ability of binding Ca^{2+} , is one member of the EF-hand superfamily. Troponin C interacts with troponin I and troponin T, forming a troponin complex which takes part in regulating muscle contraction [9,10]. In invertebrate TnC amino acid

sequences, there are usually four potential EF-hand motif that can bind Ca^{2+} [11]. TnC plays an important and extensive role in regulating muscle contraction which is divided into two subtypes in vertebrate: fast skeleton muscular troponin C and cardiac muscular troponin. However, the subtypes of TnC in invertebrates and insects remain mysteries [11]. Recently, it was found that TnC has a close relationship with tumor [12–14], which suggests TnC may work in non-muscle tissues. The function and mechanism of TnC has been widely studied in humans, mice, pigs and other animals, but the possible function and role of TnC has not been explored in crustaceans.

In current project, the full length sequence of TnC gene was cloned from *S. paramamosain*. On this basis, TnC in crabs was specifically knocked down by RNAi technology. Infected with bacteria and virus, comparing with the wild type group, we studied a comprehensive evaluation of immune pathway gene expression, important immune parameters, mortality, hemocytes apoptosis and phagocytosis. We aimed to reveal the inseparable relationship between TnC gene and innate immunity of *S. paramamosain* upon viral and bacterial infection.

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2. Methods and materials

2.1. Crabs and tissue preparation

The healthy adult *S. paramamosain* (approximately 100 g) were obtained from a seafood market of Hangzhou. We ordered crabs from the same breeding area in the market to ensure the single population of experimental crabs. Short-term feeding observation and pathogen detection were carried out before the experiment. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Zhejiang A & F University (Hangzhou, China). 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. WSSV (AF 332093.3) was purified and used in challenge experiments, as described previously [15]. *V. alginolyticus* was cultured and used to challenge the crabs according to the previous report [16].

2.2. 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, 3NGSP1,3 GSP2,3NGSP2) or 5' GSP (5GSP, 5NGSP), designed on the basis of middle sequence (the primers sequences are shown in Table 1). The PCR products were purified using MiniBEST DNA

Table 1

Universal and specific p	primers used i	in this	study.
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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 screening and confirmed by PCR and 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.

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 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, MEGA 7.1.

2.4. The quantitative real-time PCR

Relative TnC mRNA expression levels in various adult tissues were measured by qRT-PCR using a SYBR II[®] Premix Ex Taq (TliRnase 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. cDNA synthesis

Name	Nucleotide Sequence (5'-3')	purpose
F1	ATCTTGAGGATGATGGGCGT	for the conservative fragment
R1	CATCATCKCCATGAACTCATC	for the conservative fragment
3' race GSP1	ACAAGGGAGGTGACGGCTACATC	first primer for 3'RACE
3' race NGSP1	CTACGACAAGGGAGGTGACGGCTAC	second primer for 3'RACE
3' race GSP2	GAGATGATGAGCGGTTGAAGGG	third primer for 3'RACE
3' race NGSP2	TCAGGGCAACCAGACTCAACATC	fourth primer for 3'RACE
5' race GSP	GATGTAGCCGTCACCTCCCTTGT	first primer for 5'RACE
5' race NGSP	CTCGTCAGTCTCCGCAATAACCT	second primer for 5'RACE
TnC dsRNA F	AAGCTTCGTGTGGTAAACTTAGCCTGC	for TnC RNAi
TnC dsRNA R	GAATTCCCCTCACAAGTTTTATTCGCC	for TnC RNAi
TnC-realtime-F	CGCTGCCAAGTTCCTGATT	for TnC mRNA expression
TnC-realtime-R	CGTCCTCGTCCACCTCTT	for TnC mRNA expression
GAPDH-realtime-F	ACCTCACCAACTCCAACAC	for GAPDH mRNA expression
GAPDH-realtime-R	CATTCACAGCCACAACCT	for GAPDH mRNA expression
C-type-lectin-F	ACTGAGGGGAAAGTAGCC	for C-type-lectin mRNA expression
C-type-lectin-R	TGCCCGTGTTTATTCATC	for C-type-lectin mRNA expression
crustin-F	TCAGAGCACCCTGGTAAATGT	for crustin antimicrobial
		peptide mRNA expression
crustin-R	GGCAGAACTGCGAAAGAAAG	for crustin antimicrobial
		peptide mRNA expression
JAK-F	ATTGCTGAGGGGATGGATT	for JAK mRNA expression
JAK-R	GCCCATCACATTCCCAAA	for JAK mRNA expression
proPO-F	ATGAAAGAGGAGTGGAGATG	for prophenoloxidase
		mRNA expression
proPO-R	GTGATGGATGAGGAGGTG	for prophenoloxidase
		mRNA expression
TLR-F	TGTTGCCAGAGCAGAAGGT	for toll-like receptor
		mRNA expression
TLR-R	TTCCGTGAATGAACGAAGG	for toll-like receptor
		mRNA expression
myosin-F	GCCGAGATAAGTGTAGAGGAA	for myosin-II-essential-light-chain-like-proteinmRNA expression
myosin-R	AGTGGGGTTCTGTCCAAG	for myosin-II-essential-light-chain-like-protein mRNA expression
STAT-F	GACTTCACTAACTTCAGCCTCG	for STAT mRNA expression
STAT-R	GAGCTGAGTCTGTCTTAATGTTATCC	for STAT mRNA expression

1	tggggagttgtgtgtcctgcccgcaagctcaacacatctcctcctcctcctcctcctcctcctcctc																	
61	tcgtc	atc	tct	ctca	actt	aaga	caaa	ATG	GAC	AAC	СТС	GAT.	AAG	GAC	CAG	ATC	GCA	GCCC
1								М	D	Ν	L	D	Κ	D	Q	I	А	A
121	TCAGG	AAG	GCC	TTCO	GAGT	CCTT	ГGAC	CATG	GAC	GGC	AAG	GGC	TAC	ATC.	ACCO	CCG	GAG/	ACCG
12	L R	Κ	А	F	Е	S F	D	М	D	G	Κ	G	Y	Ι	Т	Р	Е	Т
					F	1		_									-	
181	TGGGC	ACC	ATC	CTG	CGTA	TGAT	GGGC	GTC	AAG	ATC	ТСТ	GAG	AAG	AAC	CTG	CAG	GAG	GTTA
32	V G	Т	Ι	L	R	M M	G	V	Κ	Ι	S	Е	Κ	Ν	L	Q	Е	V
	5'	race	NG	SP										3'1	race	GS	P1	_
241	TTGCG	GAG.	ACT	GACO	GAGG	ACGG	ГТСС	CGGT	GAG	CTC	GAG	TTT	GAG	GAG	TTC	TGT	TCT	CTCG
52	IA	Е	Т	D	Е	D G	S	G	Е	L	Е	F	Е	Е	F	С	S	L
	ThC	C-rea	ltim	e-F	-													
301	CTGCC	'AAG'	TTC	CTG/	ATT G	AAGA	GGAC	GAG	GAG	GCC	CTG	AAA	GCT	GAG	CTG	CGT	GAG	GCTT
72	<mark>A A</mark>	K	F	L	Ι	ΕE	D	Е	Е	А	L	K	A	Е	L	R	Е	A
				+	5	' race	GSI	2										
		_		3	' race	e NGS	5P1		_									
361	TCCGT	'ATC'	TAC	GAC/	AGG	GAGG	ГGAC	CGC	TAC	ATC	ACC	ACC	GGC	ACC	CTG	AAG	GAA/	ATCT
92	FR	Ι	Y	D	K	G G	D	G	Y	Ι	Т	Т	G	Т	L	K	E	I
														Т	'nC-	- re:	altin	ne-R
421	TGAAG	GAA	CTG	GACA	AACA	GGCT	GACC	GAG	GCT	GAC	CTG	GAC	GGT	ATC.	ATC	GAA	GAG	GTGG
112	L K	Е	L	D	Ν	R L	Т	Е	A	D	L	D	G	Ι	Ι	Е	Е	V
									•			R1		3'	rac	e G	SP2	
481	ACGAG	GAC	GGC	TCCC	GGCA	CCCT	CGAT	TTT	GAC	GAG	TTC	R1 ATG	GAG	3' ATG	rac ATG	e GS	GGT	TGA <mark>a</mark>
481 132	ACGAG <mark>D E</mark>	GAC D	GGC G	TCCC S	GGCA G	CCCT T L	CGAT D	TTT F	GAC D	GAG E	TTC F	R1 ATG M	GAG. E	3' Atg. M	TAC ATG M	e GS AGC <mark>S</mark>	SP2 GGT G	TGA <mark>a</mark> *
481 132	ACGAG D E	GAC D	GGC G	TCCC S	GGCA G	CCCT T L	CGAT D	TTT F	GAC D	GAG E	TTC F	R1 ATG M	GAG. E	3' ATG M	ATG.	e GS AGC S	GGT GGT	TGAa *
481 132 541	ACGAG D E gggaa	GAC D	GGC G	TCC(S	GGCA G	CCCT TL caac	CGAT D agtt	TTT F act	GAC D cta	GAG E tct	TTC F tat	R1 ATG M tat	GAG E aat	3' ATG M	ATG M teta	e GS AGC S atc	GGT GGT	TGAa * tctt
481 132 541 601	ACGAG D E gggaa ctttc	GAC D	GGC G ccg ctto	TCCC S tgga ctta	GGCA G acgc agtt	CCCT TL caac	CGAT D agtt taga	TTT F act igac	GAC D cta taa	GAG E tct cat	TTC F tat tca	R1 ATG M tat	GAG E aat ctg	3' ATG M tat gta	ATG. M tcta atta	e GS AGC S atc att	GGT G gcat ttat	TGAa * tctt tcat
481 132 541 601 661	ACGAG D E gggaa ctttc ctttc	GAC D tat	GGC G ccg ctto	TCCO S tgga ctta ttt1	GGCA G acgc agtt tatt	CCCT TL caac tctt gata	CGAT D agtt taga tcat	TTT F act gac	GAC D cta taa ctc	GAG E tct cat tct	TTC F tat tca ctt	R1 ATG M tat: tct	GAG E aat ctg	3' ATG M tat gta tgt	ATG ATG M tct atta tct	atc att	GGT G gcat ttat	TGAa * tctt tcat cgtc
481 132 541 601 661 721 781	ACGAG D E gggaa ctttc ctttc tacta	GAC D tat tat	GGC G ccg cttc att	TCCC S tgga ctta ttt1	GGCA G acgc agtt tatt	CCCT TL caac: tctt gata attc	CGAT D agtt taga tcat tctg	TTT F act gac tct	GAC D cta taa ctc	GAG E tct cat tct ctc	TTC F tat tca ctt acc	R1 ATG M tat: tct tct	GAG E aat ctg ctt aaa	3' ATG M tat gta tgt act	M ATG M teta atta tet	atc att ttc	GGT GGT gcat ttat ccac gtct	TGAa * tctt tcat cgtc ttcc
481 132 541 601 661 721 781 841	ACGAG D E gggaa ctttc ctttc tacta aaatt	GAC D tat	GGC G ccg ctto att ctaa gcao	TCCC S tgga ctta ttt1 caag	GGCA G acgc agtt tatt tttt gcac	CCCT TL caac tctt gata attc attc	CGAT D agtt taga tcat tctg ctca	TTT F act gac tct gatg	GAC D cta taa ctc ccat cca	GAG E tct cat tct ctc cat	TTC F tat tca ctt acc cta	R1 ATG M tat: tct tct aca	GAG E aat ctg ctt aaa gtaa	3' ATG M tat gta tgt act aat	ATG ATG M tcta atta tct acto tga	atc atc att ttc tct	GGT G gcat ttat ccac gtct caca	TGAa * tctt tcat cgtc ttcc acca
481 132 541 601 661 721 781 841 901	ACGAG D E gggaa ctttc ctttc tacta aaatt ttatc	GAC D tat tat cat tct cta tac	GGC G ccg ctto att ctaa gcaa caaa	TCCO S tgga ctta ttt1 caag acto	GGCA G acgc agtt tatt tttt gcac	CCCT TL caac: tctt: gata attc atatc acgt	CGAT D agtt taga tcat tctg ctca ttat	TTTT F act gac tct gatg ictt	GAC D cta taa ctc cat cca cca	GAG E tct cat tct ctc cat cac	TTC F tat tca ctt acc cta cac	R1 ATG M tat: tct tct aca ttt	GAG E aat ctg ctt aaa gtaa cgt	3' ATG M tat gta tgt act act gcc	ATG ATG M tcta atta tct acto tga aaga	e GS AGC S atc att ttc cca tct aat	GGT G gcat ttat ccac gtct caca aatt	TGAa * tctt tcat cgtc ttcc acca tcgc
481 132 541 601 661 721 781 841 901	ACGAG D E gggaa ctttc ctttc tacta aaatt ttatc accca	GAC D tat tat cat tct cat tac gcg	GGC G cccg ctto ctat ctaa gcaa caaa caaa	TCCC S tgga ctta tttt tttt caag actc gatt	GGCA G acgc agtt tatt tatt gcac ccaa tcgg	CCCT TL caac: tctt gata attc attc acgt gccc	D agtt taga tcat tctg ctca ttat	TTT F act agac tct gatg actt cacc	GAC D cta taa ctc cat cca cca cca	GAG E tct cat tct cat cat cac gga	TTC F tat tca ctt acc cta cac tag	R1 ATG M tat. tct tct tct aca, ttt cca	GAG. E aaat ctg; ctt aaaa; gtaa cgt; tat	3' ATG. M tat gta tgt act. aat gcc: cta	M M ttta atta atta ttta tga aaga gta	e GS AGC S atc att ttc cca tct aat	GGT G gcat ttat ccac gtc tcac aatt taca	TGAa * tctt tcat cgtc ttcc acca tcgc aaaa
481 132 541 601 661 721 781 841 901	ACGAG D E gggaa ctttc ctttc tacta aaatt ttatc accca	GAC D tat tat cat tat cat tac cat gcg	GGC G cccg cttc att ctaa gcaa caaa caag	TCCC S ttgga ctta tttt attt caag actc gatt	GGCA G acgc agtt tatt tttt gcac ccaa tcgg	CCCT TL caac: tctt: gata attc acgt: gccc:	CGAT D agtt taga tcat tctg ctca ttat acaa	F F act gac tct gatg actt caac	GAC D cta taa ctc cat cca cca cca cca	GAG E tct cat ctc cat cac gga	TTC F tat tca ctt acc cta cac tag	R1 ATG M tat. tct tct tct tct tct tct tct tct	GAG. E aaat ctg; ctt aaaa; gtaa cgt; tat	3' ATG. M tat tgta tgta tgta act. act. act. cta	M M ttcta atta tct acto acto gta gta gta o	e GS AGC S atc att ttc cca tct aat cct C d	GGT G gcat ttat ccac aatt taca sRN	TGAa * tett teat egte ttec acca tege aaaa AF
481 132 541 661 661 721 781 841 901 961	ACGAG D E gggaa ctttc ctttc tacta aaatt ttatc accca tatac	GAC D ttat ttat ttat ctat ttat ctat ttac gcg	GGC G cccg ctto att ctaa gcaa ccaa ccaa ccaa	TCCC S ttgga ctta atti atti gati aggati	GGCA G acgc agtt tatt tttt gcac ccaa tcgg attc	CCCT TL caac: tctt gata attc acgt gccc; acat;	CGAT D agtt taga tcat tctg ctca ttat acaa atat	TTT F sact ugac utct gatg actt caac	GAC D cta taa ctc cca cca cca cca cca cca cca	GAG E tct cat cat cac gga cct	TTC F tat tca ctt cac cta cac tag gta	R1 ATG M tat. tct tct tct tct tct tct tct tct	GAG. E aat ctg ctt aaaa gtaa cgt tat	3' ATG. M tatt gtaa tgt act. aat gcc: ttt ttt	ATG ATG M teta atta teta acto gta gta gta gta	e GS AGC S atc att ttc cca ttct aat cct C d	GGT G gcal tttal ccac gtcl taca sRN gtac	TGAa * tett tett teet tee acca tege acca AF act
481 132 541 601 661 721 781 841 901 961 1021 1081	ACGAG D E gggaa ctttc ctttc tacta aaatt ttatc accca tatac tagcc	GAC D ttat ttat ccat: ttat cta; cta; gcg gcg gta	GGC G cccg cttt ctaa gcaa ccaa ccaa ccaa agt	TCCC S tgga ctta tttt att1 caag actc gat1 agga gtac	GGCA G acgc agtt tatt ttttt gcac ccaa tcgg attc ctat	CCCT TL caac: tctt gata attc attc acat: acat: acat: acat:	D agtt taga tcat tctg ctca ttat acaa atat	TTTT F actt ugacc uctt aaac uccc actt utaa	GAC D cta ctc cat cca cca cca caa cca caa caa	GAG E tct cat cat cac gga cct ggt	TTC F tat tca ctt cta cta cac tag gta ccg	R1 ATG M tat. tct tct tct tct tct tct tct tct tct t	GAG. E aat ctg; ctt aaaa cgt; tat gaa cgta ccta	3° ATG. M tat gta: tgt act: aat gcc: cta; ttt ttt	ATG ATG M teta atta tet acto tga acto gta cgt aata	e GS AGC S atc ttc cca tct cca tct cct d gtg act	GGT G gcat ttat ccac gtc tac aatt tac sRN gta	IGAa * tett tcat cgtc ttcc acca tcgc acaa AF aact taat
481 132 541 601 661 721 781 841 901 961 1021 1081 1141	ACGAG D E gggaa ctttc ctttc tacta aaatt ttatc accca tatac ccatc ccatc	GACC D ttat ttat cata ttac gcg gta ttac ttac	GGC G cccg ctto ctaa gcaa ccaa ccaa agta gtaa	TCCC S tgga ctta ttt1 caag actc gat1 agga gtac aggg	GGCA G acgc agtt tatt ttttt gcac ccaa attc ccaa attc gttg	CCCTT L caacc tcttt gata attc acatt gccc: acat: agac acat;	CGAT D agtt taga tcat tctg ctca ttat acaa atat tata gttt	TTTT F actt agac ttct actt actt accc acctt acctt acctt	GAC D cta taa ctc ccat ccat cca caa cgt agt tac	GAG E tct cat cat cat cat gga cct ggt aaa	TTC F tat tca ctt acc cta cac tag gta ccg tat	R1 ATG M tat: tct tct tct tct tct tct tct tct tct t	GAG. E aaat ctg; ctt aaaa; gtaa cgt; tat gaa ccgt; cta cag	3' MTG. M tat gta tgt act. aat gcc: cta ttt tat cat.	ATG ATG M teta atta teta aatta aag gta gta cgt aatta ttto	e G AGC S atc att ttc cca ttc cca tct cct gtg act ctt	GGTT G gcal ttat ccac aatt tac sRN gta tta caca gtc tta caca sRN	TGAa * tett teat egte ttec acca tege aaaa A F aact taat aata
481 132 541 601 661 721 781 841 901 961 1021 1081 1141 1201	ACGAG D E gggaa ctttc ctttc tacta aaatt ttatc accca tatac tatac ccate ccate	GACO D ttat ttat ctat ctat ttat ctat gcg gta ttac ttac	GGC G cccg ctto ctaa gcaa ccaa ccaa agti gtaa taa	TCCC S tgga ctta tttt attt caag gatt agga gtad aggg tcca	GGCA Gacgc agtt tatt ttttt ttttt gcac ccaa tcgg attc ctat gttg atct	CCCTT L caac: tett gata attc atat: acat: agac acat: agac acat; tagt:	CGAT D agtt taga tcat tctg ctca ttat acaa atat tata gttt agga	TTTT F actt agac actt actt accc accc accc accc	GAC D cta taa ctc cat cca cca caa cgt tac agt tac	GAG E tct cat cat cat gga cct gga cct gtt aaa act	TTC F tat tca ctt cta cca tag gta ccg tat tta	R1 ATG M tat. tct tct tct tct tct tct tct tct tct t	GAG. E aaat: ctt: aaaa: gtaa cgt; tato gaa cta cag acaa	3° M tat gta tgt act cta ttt cat ttt cat	M tcta atta atta acto gta gta cgt; aatta ttto taga	e G AGC S atc att ttc cca tct cct gtg act ctt aga	GGT G gcal ttai ccace aatt taca sRN gtaa tta cace gtgt	TGAa * tett teat cgtc ttec acca tegc acca tegc taata taat aata ttte
481 132 541 601 661 721 781 841 901 961 1021 1081 1141 1201	ACGAG D E gggaa ctttc ctttc tacta aaatt ttatc accca tatac ccatc ccatc ccatc	GACC D tati tati tati cata: tacc ggg gga tgc tac; acg	GGC G cccg ctto ctaa gcaa ccaa agta gtaa taa ccat	TCCC S tgga ctta ttt1 att1 acto gat1 agga gtac aggg tcca ttca tta1	GGCA G acgc agtt tatt tttt gcac ccaa tcgg attc gttg atct tgct	CCCTT L caac: tett gata attc acat: acat: acat: acat: tagt: ccat	CGAT D agtt taga tcat tctg ctca ttat tata atat tata gttt agga ctaa	TTTT F actt gatg uctt accc accc accc accc accc accc accc	GAC D cta taa ctc cca cca cca cca cca ca ca tac tac	GAG E tet cat cat cac gga cct ggt aaaa act aaa	TTC F tat tca ctt cac tag gta ccg tat tta ggc 3'	R1 ATG M tat. tct tct tct tct cca ttt tct aca ttt taa ttt taa ttt taa taa	GAG. E aat ctg ctt aaaa gaa cgt tat cag acaa cag acaa tat	3° M tat gta: tgt act. aat gcc: cta; ttt tat tat cat. tat	ATG ATG M tcta atta tct taata gtaa cgt aaaga gtaa cgt ttto taga aaac	e GS AGC S atc atc tct cca tct cct cct cct cct aat cct aat cct aat tct cct tct t	GGTT G gcat ttat ccac gtct tacac sRN gtat gtat gtat gtat	TGAa * tett teat tegte tege aaaa tege aaaa A F aact taat aata ttte teaagt
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Fig. 1. Nucleotide and deduced amino acid sequence of TnC. The nucleotide sequence is displayed in the 5'–3' directions and numbered at the left. The deduced amino acid sequence is shown with the single capital letter amino acid code. The 3'UTR and 5'UTR are shown with lowercase letters. Codons are numbered at the left with the methionine (ATG) initiation codon, an asterisk denotes the termination codon (TGA). Cloning the conservative fragment, RACE, real-time qPCR, and dsRNA primers are marked with arrows. Two predicted EF-hand domain amino acid sequences are highlighted in yellow. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

was carried out using 200 µg of total RNA with the ReverTra Ace qPCR RT Master Mix with gDNA Remover (Code: FSQ-301; Toyobo, Japan). The synthetic cDNA was kept at -20 °C. RT-qPCR 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 [17] comparative CT method by Office Excel, with GAPDH amplification as the internal control. The design and synthesis of the RT-qPCR primers were entrusted to Generay (Shanghai, China), based on the open reading frame (ORF). Table 1 lists the primers that were designed.

2.5. Prokaryotic expression and purification of TnC-dsRNA

The primers (shown in Table 1) with specific restriction sites (Hind III in the forward primer and EcoR I in the reverse primer) were designed from the cloned nucleotide sequence. PCR product digested with Hind III/EcoR I was sub-cloned into LIMTUS 38i Vector (NEB, MA,

USA) digested with the same enzymes to gain plasmid L38-TnC. The following process was prepared according to the previous reports [18]. The constructed L38-TnC was verified by restriction enzyme digestion and DNA sequencing. The recombinant plasmid L38-TnC was transformed into HT115 (DE3) cells with deficiency of RNase III. Single colonies of the above the engineering bacteria were separately inoculated to 5 mL of LB medium containing Amp (100 µg/mL), cultured at 37 °C with shaking at 200 r/min for 12–16 h, and then inoculated to LB medium containing for 2-3 h (OD600 \approx 0.6), and added with IPTG (with a final concentration of 0.8 mmol/L) to induce the expression for 4 h. After purifying with mirVana miRNATM Isolation Kit (Ambion, USA), the dsRNAs were annealed and precipitated with 5 mol/L sodium acetate and anhydrous alcohol.

2.6. Knock down of TnC by RNAi and challenge experiments

Total RNA from crabs hemocytes was purified using an EASY spin tissue/cell RNA extraction kit (Aidlab, China), following the manufacturer's instructions. The total RNA solution was divided into 50 µL each tube and annealed by incubations at 95 °C for 2 min. During 45 min, the temperature slowly decreased from 95 °C to 25 °C and 25 °C for 10 min. The resulting TnC-dsRNA was ethanol-precipitated and resuspended in DEPC-treated H₂O. And TnC-dsRNA (100 µg/crab) was immediately injected intramuscularly into the fourth pereopodcoxa of each crab, and TnC mRNA expression levels were detected by RT-qPCR following WSSV and V. alginolyticus challenges. The crabs were divided into six groups: intramuscular injection with 100 µL PBS alone; intramuscular injection with 100 µL TnC-dsRNA alone; intramuscular injection with WSSV alone; injection with TnC-dsRNA for 12 h and followed by WSSV challenge; intramuscular injection with V. alginolyticus alone; injection with TnC-dsRNA for 12 h and followed by WSSV challenge. The mortality was monitored every 12 h after the last injection [19].

2.7. Kaplan-Meier survival analysis

For the pathogen challenge, healthy crab were randomly distributed into six groups (n = 9 per group, three repeat). The control group received injections of PBS alone, the TnC-dsRNA group received injections of TnC-dsRNA alone, the WSSV group received injections of WSSV in PBS, and the TnC-dsRNA + WSSV group received injections of TnCdsRNA and WSSV, the *V. alginolyticus* group received injections of *V. alginolyticus* in PBS, and the TnC-dsRNA + *V. alginolyticus* group received injections of TnC-dsRNA and *V. alginolyticus*. The copy number of WSSV injected to the crabs was 10⁴ each crab. And the cell density of injected *V. alginolyticus* was 10⁵ each crab. The above initial infection rates were consistent in this study. Each group of crabs was cultivated under the same condition. The number of live and dead crab was counted every 12 h. The survival data was arranged and analyzed in Microsoft GraphPad 5.0.

2.8. Apoptosis of crab hemocytes

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 hemocytes. The hemolymph cells were then suspended in highly alkaline PBS, counted and adjusted to a density of $3-5 \times 10^6$ cells/mL with PBS. The cells were stained using a BD PharmingenTM FITC Annexin V Apoptosis Kit, and assessed by flow cytometry. The cell numbers on quadrant 4, with low PI and high annexin V staining, were considered as apoptotic. The data were presented as means \pm standard deviation (SD) derived from at least three independent experiments.

2.9. Determination of immune parameters after RNAi

The immune parameters determined included total hemocytes

Scylla paramamosain	MDNLDKDQTAALRKAFESFDMDGKGYTTPET	31
Caenorhabditis elegans	MGDVVA <mark>DALEK.LSADQIEQFRKYE</mark> NMFDKE <mark>GKC</mark> YIRATQ	39
Drosophila melanogaster	MDNIDEDLTPEQIAVLQKAENSFDHQKTCSIFTEM	35
Penaeus monodon	MDSLDEEQUETLRKAENSEDTEGACSUNAET	31
Xenopus laevis	MPTDQQQDARSFLSEEMHAEFKAAFDMFDTDGGCDHSTKE	40
Consensus	i f fd g i	
Scylla paramamosain	VGTILEMMCVKISEKNIQEVIADTIEDCSCELEFEEFCSL	71
Caenorhabditis elegans	VGQILRTMCQAFEERDLKQLIKEFDADGSGEIEFEEFAAM	79
Drosophila melanogaster	VADILR <mark>LMCQPFDRQILDELID^DVD<mark>E</mark>DKSGRL<mark>E</mark>FEEEVQL</mark>	75
Penaeus monodon	VGVILR <mark>MMG</mark> VKISEKNLQEVIAFTDEDGSGMLEFEEFAEL	71
Xenopus laevis	LGTVMRMLCQTPTKEELDAIIEPVDEDGSGTIDFEEFLVM	80
Consensus	rg lieddsg feef	
Scylla paramamosain	AAKELIE.EDEEALKAELRDAFRIYDKGGDGYITTGTLKE	110
Caenorhabditis elegans	VANFVVNNENDEGLEEELREAFRLYDKEGNGYINVSC <mark>L</mark> RD	119
Drosophila melanogaster	AAKFIVE.EDDEAMQK <mark>ELRDAFR</mark> LYDKQGNGYIPTSCLKE	114
Penaeus monodon	AAKFLIE.EDEEALKADIRDAFRIYDKDCQGYITTDILKE	110
Xenopus laevis	MVRQMKE.DAQGKSEE <mark>ELAE</mark> CFRIFDKNADGYIDGEELAE	119
Consensus	el e fr dk gyi l	
Scylla paramamosain	IIKELDNRLTEADIDGIIEEVDEDGS <mark>C</mark> ILDFDEEMBMMSG	150
Caenorhabditis elegans	ILRALDDNVSEEELDEMIAEIDADGSGTVDFDEEMEMMSG	159
Drosophila melanogaster	IL <mark>KELDDQLTEQELDIMIEEID</mark> SDGS <mark>GTV</mark> DFDEEMEMMTG	154
Penaeus monodon	ILVELDPKLTPTDLEGIIEEVDEDGSGTLDFDEFMEMMSG	150
Xenopus laevis	ILRSSGESITDEEIEELMKDGDKNNDGKIDFDEFLKMMEG	159
Consensus	il d g dfdef mm g	
Scylla paramamosain		150
Caenorhabditis elegans	E	160
Drosophila melanogaster	E	155
Penaeus monodon		150
Xenopus laevis	V	160
Consensus		

Fig. 2. Multiple alignments of the amino acid sequence of *S. paramamosain* TnC with other TnC sequences of common animals. *S. paramamosain* (in this study), *Caenorhabditis elegans* (NP_496251.1), *Drosophila Melanogaster* (AHN56108.1), *Penaeus Monodon* (ADV17344.1) and *Xenopus Laevis*(XP_018092657.1). Forty-two conserved amino acids are shaded and boxed.

numbers (THC), PO and SOD activities. THC was determined as described previously [19]. To determine PO and SOD activities, 500 μ L of hemolymph was withdrawn into a 1 mL syringe containing 500 μ L of 20 mM EDTA solution from each individual crab. PO activity was quantified in the hemolymph mixture based on the formation of dopa chrome from the substrate L-3, 4-dihydroxyphenylalanine (L-DOPA), as described previously [19]. SOD activity was quantified in hemocytes isolated from 300 μ L of the hemolymph mixture, according to the improved method described by Beauchamp and Fridovich [20]. Data were presented as a percentage of the normal control.

2.10. Detection of WSSV copies

The crab was injected with WSSV or mixture of WSSV and TnCdsRNA. The whole-genome was extracted from crab hemocytes collected at 12, 24 and 48 h post injection using DNA extraction Kit (Generay, China), according to the manufacturer protocol. To figure the WSSV copies, crab 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.

2.11. Statistical analysis

Quantitative data were expressed as mean \pm standard deviation (SD). Data from three independent experiments were analyzed by oneway analysis of variance to calculate the means and standard

deviations of the triplicate assays. Statistical differences were estimated using paired *t*-test 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 TnCcDNA

The full-length TnC cDNA sequence was 1427 base pairs (bp), containing a 453 bp open reading frame (ORF) encoding a 151 amino acids protein. The Pfam website predicted it had two EF-hand function domains. In addition to the ORF, 86 bp and 888 bp, respectively, are untranslated regions (UTR) of the TnC 5' and 3' ends. The full length cDNA sequence and the amino acid sequence deduced from cDNA are shown in Fig. 1.

3.2. Sequence homology and phylogenetic analysis

To analysis the homology of the *S. paramamosain* TnC gene, the putataive amino acid sequence. The amino acid sequence of the *S. paramamosain* TnC was compared with multiple model organisms by using the DNAMAN version 6.0, including *Caenorhabditis elegans* (NP_496251.1), *Drosophila melanogaster* (AHN56108.1), *Penaeus monodon* (ADV17344.1) and *Xenopus laevis* (XP_018092657.1). The



Fig. 3. The phylogenetic tree of TnC from different organisms based on amino acid sequence comparisons.

results showed that the homology with *C. elegans* was 51.25%, 62.58% with *D. melanogaster* and 36.02% with *X. laevis.* It has the highest homology with *P. monodon*, which is 82% (Fig. 2).

Based on the deduced amino acid sequence, a bootstrapped neighjoining tree was constructed by the neighbor-joining method using MEGA7.1 (Fig. 3). Through the phylogenetic tree we can see that the evolutionary of TnC was conserved among different species. In the arthropods, the evolution of TnC in *S. paramamosain* was backward. The amino acid sequence homology analysis revealed that the conserved domain contains multiple conserved sites. The *S. paramamosain* TnC had the highest affinity with *Homarus americanus*.

3.3. Tissue distribution of TnC expression

Expression profiling of TnC in different tissues of *S. paramamosain* was examined by reverse transcription-quantitative PCR (RT-qPCR) (Fig. 4-A). It was more highly expressed in the muscle tissue compared with other tissues and the lowest expression was in heart. Expression levels of TnC in muscle tissue were greater than that in the intestines, hemocytes, hepatopancreas, gills and heart, respectively. The expression level of TnC in muscle is 45 times that of intestine, 285 times that of hemocyte, and 9933 times that of hepatopancreas. This difference is actually 23,500 times compared to myocardial tissue. The expression of TnC in the muscle tissue was thus significant higher (P < 0.01) than in any other tissues.

3.4. Time course of TnC expression after WSSV or V. alginolyticus infection

We investigated the variation trend of TnC expression in crabs after infection with WSSV or *V. alginolyticus*. TnC expression changed after the infection in a time-dependent manner. TnC expression was significant down-regulated (P < 0.01) from 12 to 72 h post-infection in crabs with WSSV infection. The lowest expression levels occurred at 12 h, after which TnC expression gradually recovered but still not to the pre-infection levels after 72 h post-challenge (Fig. 4-B). Similarly, TnC expression was significant down-regulated (P < 0.01) from 12 to 48 h following infection with *V. alginolyticus* (Fig. 4-C). The following results showed that the presence of TnC was not conducive to anti-infection. The decrease of TnC expression after pathogen infection was beneficial to enhance the anti-infection ability of crabs. These results suggested that TnC may be used by pathogens after infection.

3.5. Effects of TnC knockdown on expression of immune genes

We tested the effect of TnC double-stranded RNA (TnC-dsRNA) on its mRNA expression using RT-qPCR. TnC mRNA expression in hemocytes was significantly knocked down by TnC-dsRNA (P < 0.01) (Fig. 5-A). We detected the effect of TnC-dsRNA on TnC gene expression in hemocytes of *S. paramamosain* at different times post-treatment using RT-qPCR as well. TnC-dsRNA significantly inhibited the expression of TnC mRNA in hemocytes from 24 to 72 h post-treatment (P < 0.01). Among them, the 24-h knockdown effect was the most significant, and the subsequent 36 h and 48 h gradually increased (Fig. 5-B). We also examined the relationship between the expression of TnC and the other Y. Zhao, et al.



Fig. 4. (A) Expression characterization of TnC in various tissues from healthy S. paramamosain revealed by quantitative real-time PCR. And realtime RT-PCR analysis of TnC expression challenged with WSSV or V. alginolyticus. (B) Real-time RT-PCR analysis of TnC expression in the hemocytes of S. paramamosain challenged with WSSV. (C) Realtime RT-PCR analysis of TnC expression in the hemocytes of S. paramamosain challenged with V. alginolyticus. The amount of TnC mRNA was normalized to the GAPDH transcript level. Data are shown as means \pm standard deviation of the tissues of three separate individuals. Capital letters indicate expression of TnC in different adult tissues. Double asterisks indicate a significant difference (P < 0.01) between two samples.

important immune genes in the hemocytes of crabs. Among the seven immune genes, crustin antimicrobial peptide (CAP) and prophenoloxidase (proPO) were down-regulated (P < 0.01), while Toll-like receptor (TLRs) was significantly up-regulated (P < 0.01) following TnC-dsRNA treatment (Fig. 5-C).

3.6. Effects of TnC knockdown on immune parameters

The total hemocyte count (THC) was significantly increased in the TnC-dsRNA group compared with PBS group (P < 0.01), and the THC of the TnC-dsRNA group reached the highest value at 24 h (Fig. 6-A). The THC in *V. alginolyticus* group, *V. alginolyticus* + TnC-dsRNA group and WSSV group were significantly lower than that in the PBS group at 24 h and 48 h (P < 0.01), but WSSV + TnC-dsRNA group was on the opposite (Fig. 6-B,C). The results indicated that the THC decreased in crabs after infection with WSSV or *V. alginolyticus* and hemocyte proliferated after the inhibition of TnC expression.

Phenoloxidase (PO) activity in crab hemolymph was significantly enhanced at 24 h and 48 h, after TnC-dsRNA treatment compared with the PBS group (P < 0.01), and PO activity of the TnC-dsRNA group reached the highest value at 24 h (Fig. 6-D).

Crabs treated with TnC-dsRNA showed a significantly higher SOD activity than the PBS group at 24 h and 48 h (P < 0.01). The SOD activity of the TnC-dsRNA group reached the highest value at 24 h

(Fig. 6-E).

3.7. Effects of TnC knockdown on the survival of challenged crab

We evaluated the effects of TnC on the mortality of pathogenchallenged crabs by first injecting them with TnC-dsRNA and then challenging them with V. alginolyticus or WSSV. The negative control group had a cumulative mortality of zero which showed a similar mortality to the TnC-dsRNA group, indicating that TnC-dsRNA itself was non-toxic in crabs (Fig. 7). There was no significant difference in the cumulative mortality between V. alginolyticus-treated group and V. alginolyticus + TnC-dsRNA group within 180 h after injection. However, the cumulative mortality in crabs first treated with TnC-dsRNA then infected with V. alginolyticus was significantly higher than that infected with V. alginolyticus (P < 0.01) starting from 180 h post infection (Fig. 7-B). The WSSV + TnC-dsRNA group show higher mortality than the WSSV-treated group between 12 and 96 h, but thereafter, from 96 to 156 h post challenge, it had a lower mortality (Fig. 7-A). We measured the replication of WSSV in crab in three time periods (24 h, 48 h, and 72 h). The results showed that after knocking down TnC, the WSSV copy number of WSSV + TnC-dsRNA group was significantly lower at 48 and 72 h post challenge than WSSV alone group (Fig. 7-C). Overall, these results demonstrated that TnC knockdown decreased the copy number of WSSV and the mortality of crabs following WSSV

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Fig. 5. Real-time RT-PCR analysis of TnC and immune gene expression. (A) Real-time PCR analysis of TnC expression in the hemocytes of S. paramamosain treated with EPGF dsRNA (EGFPdsRNA) and TnC dsRNA (TnC-dsRNA) at 24 h post-treatment. The amount of TnC mRNA was normalized to the GAPDH transcript level. (B) Real-time RT-PCR analysis of TnC expression of hemocytes of S. paramamosain treated with TnCdsRNA at different times post-treatment. (C) Realtime RT-PCR analysis of seven immune genes [crustin antimicrobial peptide (CAP), C-typelectin (CTL), JAK, prophenoloxidase (proPO), tolllike receptor (TLR), myosin-II-essential-lightchain-like-protein (MELCLP) and STAT] expression in the hemocytes of S. paramamosain treated with TnC-dsRNA. The amount of TnC mRNA was normalized to the GAPDH transcript level. Data are shown as means \pm standard deviation of tissues in three separate individuals. Double asterisks indicate a significant difference between two samples (P < 0.01).

infection. In contrast, TnC knockdown increased the mortality of crabs following *V. alginolyticus* infection.

3.8. Effects of TnC knockdown on hemocyte apoptosis

We investigated the role of TnC in apoptosis of crab hemocytes using flow cytometry. The apoptosis rate of TnC-dsRNA group was significantly higher than that of the PBS group (Fig. 8-G). Similarly, the *V. alginolyticus* + TnC-dsRNA group showed a higher apoptosis rate than the *V. alginolyticus* group and TnC-dsRNA group (Fig. 8-G). The rate of apoptosis was higher in the WSSV + TnC-dsRNA group compared with the WSSV group (Fig. 8-H). And the difference in the WSSV group with the WSSV + TnC-dsRNA group is slightly smaller than in the *V. alginolyticus* group with the *V. alginolyticus* = TnC-dsRNA (Fig. 8). These results indicated that TnC had an inhibitory effect on hemocyte apoptosis in crabs treated with WSSV.

3.9. Effects of TnC knockdown on phagocytosis

The effect of TnC knockdown on phagocytosis in WSSV-treated group was measured, the phagocytosis rate was small increased (Fig. 9-F). In the phagocytic assay of *V. alginolyticus*, knockdown of TnC was slightly increased compared to the PBS group, though the differences

were not considerable significant (Fig. 9-E). This indicates that the presence of TnC has an inhibitory effect on phagocytosis of WSSV and *V. alginolyticus* during infection.

4. Discussion

Troponin is an important regulatory protein that is primarily present in muscle tissue cells. In vertebrates, it is known that troponin contains two subtypes, namely fast response type, slow response type or cardiac troponin. A troponin complex consists of three subunits, TnI, TnT and TnC, respectively. As a molecular switch, TnC is a sensor of Ca^{2+} , which can detect the rapid changes of Ca^{2+} in cells, and has completed intracellular signal transduction and corresponding cellular responses [21]. As a member of the EF hand protein domain family, TnC typically has four Ca^{2+} binding sites. A small number of subtypes have less than four Ca^{2+} binding sites [11]. In past studies, research on TnC has focused on vertebrates. In recent years, the acquisition of invertebrate TnC gene clones such as sandworm, mussel, and barnacle provides the basis of gene sequences for the study of TnC in invertebrates [22].

In this study, we obtained the full-length TnC cDNA sequence of the *S. paramamosain*. Through amino acid alignment analysis and phylogenetic tree construction, the TnC gene of *S. paramamosain* is highly conserved, and has the height of the homology with other invertebrates,

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Fig. 6. Effects of TnC knockdown on *S. paramamosain* immune parameters, including THC and the activity of PO and SOD, as determined in healthy, WSSV-treated, or *V. alginolyticus*-treated crabs. (A) THC after PBS or TnC-dsRNA treatment; (B) THC after WSSV or WSSV + TnC-dsRNA treatment; (C) THC after *V. alginolyticus* or *V. alginolyticus* + TnC-dsRNA treatment; (D) the PO activity after PBS or TnC-dsRNA treatment; (E) Relative SOD activity after PBS or TnC-dsRNA treatment. All treatments, at each time point, included at least three individual crabs, and all experiments were repeated three times. Each column represents the mean value of triplicate assays.

especially P. monodon. TnC expression levels of S. paramamosain vary in different tissues, with extremely high levels in the muscle tissue, second highest in the intestine and with relatively low levels in the hemocytes, hepatopancreas, gills and heart. There is no doubt that TnC is highly expressed in muscle tissue because of its function to muscles contract. However, there is such a large difference in the expression between muscle tissue and myocardium. Based on this, we suspect that in S. paramamosain, there may be two or three types of TnC in muscle tissue and myocardium. In known vertebrates, it has been demonstrated that two common subtypes sTnC and cTnC are located in muscle tissue and myocardium, respectively [23,24]. Since there are no reports about other TnC subtypes present in the S. paramamosain, this inference remains to be confirmed. Relatively high expression of TnC in the intestine, a known immune organ in crustaceans, indicates that the various expressions of TnC in different tissues may reflect various roles in the crab. It was reported that Troponin I is present in human cartilage and inhibits angiogenesis [25]. It also provides some reference ideas for our research on immune-related research in the S. paramamosain.

RNA interference (RNAi) is a phenomenon of post transcriptional gene silencing specifically mediated by double-strand RNA (dsRNA) sequences in vivo [26]. RNAi has been widely applied in studies of an immune response in many invertebrate models to investigate the antibacterial and antiviral mechanisms of certain target proteins [27,28]. In the present study, we successfully inhibited TnC expression by injecting specific dsRNA into *S. paramamosain*, thus providing a practical method to characterize the function of TnC in the innate immune system.

We then identified the effects of TnC-dsRNA on the expression of several genes crucial to the innate immune system of crabs. Among the seven immune genes, knockdown of TnC mRNA resulted in significant down-regulation of CAP and proPO, while TLRs was significantly upregulated. The proPO system is recognized as an important invertebrate defense cascade against parasites, bacteria and fungi [29–31]. TLRs are type I transmembrane proteins involved in innate immunity by recognizing microbial conserved structures [32]. Changes in the expression levels of these genes reflect changes in the immune process. Our study suggests that TnC may be involved in innate immunity and have obvious antibacterial and antiviral activities.

We also found that the crabs treated with TnC-dsRNA then challenged with *V. alginolyticus* had a higher mortality count than the crabs only injected with *V. alginolyticus*, while the reverse was true in the WSSV-treated group and the WSSV + TnC-dsRNA group. To explore whether TnC has other effects on innate immunity in crabs, we investigated multiple functional parameters to assess immune potential.



Fig. 7. The survival analysis of challenged crabs treated with TnC-dsRNA. (A) The survival analysis from Kaplan–Meier of WSSV challenged crabs treated with TnC-dsRNA. (B) The survival analysis from Kaplan–Meier of *V. alginolyticus* challenged crabs treated with TnC-dsRNA. The numbers in brackets indicate the significance of difference from PBS group. (C) WSSV copies detected by a TaqMan VP28 probe at different times post-infection. The solutions used for injection are shown on the right. There were eight individuals in each group.

The THC and the activity of PO and SOD were significant increased by knock-down TnC, which indicated that TnC can regulate hemocyte proliferation and the activity of PO and SOD. In our study, apoptosis was increased in WSSV-treated crabs which pretreated with TnC-dsRNA compared with the WSSV group. At the same time, the crab infected with *V. alginolyticus* and pretreated with TnC-dsRNA also increased

apoptosis compared with the *V. alginolyticus* group. Apoptosis is highly regulated, programmed cell death process which plays a critical role in limiting virus infection [33]. The troponin C had been found to be involved in apoptosis of mammals [34]. The apoptosis data counts the number of caspase 3/7 activated cells, and many hemocytes with complete shape are also included in apoptosis. But the total hemocyte



Fig. 8. Flow cytometry assay of apoptosis. (A) PBS; (B) TnC-dsRNA; (C) *V. alginolyticus*; (D) *V. alginolyticus* + TnC-dsRNA; (E) WSSV; (F) WSSV + TnC-dsRNA; (G) Bar graph of phagocytosis of *V. alginolyticus*; (H) Bar graph of phagocytosis of WSSV. Double asterisks indicate a significant difference (P < 0.01) between the sample and the challenge only.



Fig. 9. Flow cytometry assay of phagocytosis. Inactivated WSSV and *V. alginolyticus* virions were labeled with FITC. (A) PBS (*V. alginolyticus*); (B) TnC-dsRNA (*V. alginolyticus*), (E) Bar graph of phagocytosis of *V. alginolyticus*. (C) PBS (WSSV); (D) TnC-dsRNA (WSSV), (F) Bar graph of phagocytosis of WSSV. Double asterisks indicate a significant difference (P < 0.01) between the sample and the challenge only.

count (THC) only counts the number of hemocytes with complete shape. So the data from hemocyte apoptosis and hemocyte count were both increased following the WSSV + TnC-dsRNA treatment.

These results indicate that TnC not only regulates THC and the activity of PO and SOD in crabs but also influences the apoptosis in hemocytes. Virus invasion and bacterial infection are two different modes and therefore can trigger different immune mechanisms. The mortality rate showed different changes after WSSV or *V. alginolyticus* challenged. However, in this study, in the hemocyte apoptosis, the infection against viruses and bacteria showed consistency.

In conclusion, our present study suggests that TnC plays a key role on the innate immune system of crabs against bacterial and viral infection. The finding reveals a more potential function of TnC and provides an initial basis for further research into the role of TnC in the innate immunity of invertebrates.

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