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The crab Relish plays an important role in white spot syndrome virus and *Vibrio alginolyticus* infection



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ABSTRACT

Relish is a transcription factor and forms an important part of the immune deficiency signaling pathway. In the current study, a Relish homolog was cloned from the hemolymph of Scylla paramamosain using RT-PCR and RACE. The full length cDNA of Relish consists of 4263 base pairs (bp), including a 3552 bp open reading frame encoding a 1184 amino acid protein. The data showed that Relish was highly expressed in the gonad and digestive organs of S. paramamosain. Furthermore, the expression of Relish was up-regulated by infection with white spot syndrome virus (WSSV) or Vibrio alginolyticus. When Relish was knocked down, immune genes such as Janus Kinase, signal transducer and activator of transcription, crustin antimicrobial peptide, prophenoloxidase, C-type-lectin and myosin-II-essential-light-chain-like-protein were significantly down-regulated (P < 0.01), and Toll-like receptor was significantly up-regulated (P < 0.01) in hemocytes. The mortality of WSSV-infected or V. alginolyticus-infected crabs was enhanced following Relish knockdown. Thus, Relish is very important in the progression of WSSV and V. alginolyticus infection. It was found that Relish knockdown caused the highest level of apoptosis in the disease-free group, and higher levels of apoptosis in the WSSV group and V. alginolyticus group compared with that in the control group. Knockdown of Relish influenced the activity of phenoloxidase (PO) and superoxide dismutase (SOD), and total hemocyte count (THC) following WSSV or V. alginolyticus infection, indicating that Relish plays a regulatory role in the immune response to WSSV or V. alginolyticus infection in crabs. Thus, we conclude that Relish may anticipate host defense mechanisms against pathogen infection by affecting apoptosis, THC, PO activity and SOD activity.

1. Introduction

Innate immunity and acquired immunity are two components of the host immune system [1]. Due to the lack of complexity of the adaptive immune system, all crustaceans including *Scylla paramamosain* rely solely on innate immunity to maintain a highly efficient defense system against infections [2,3]. Immediate responses to pathogenic infection in animals involve innate immunity, which consists of humoral and cellular responses, such as the blood coagulation system [4], agglutination, nodule formation, phagocytosis, prophenoloxidase activating system [5] and various immune active proteins. Indeed, antimicrobial proteins are a group of immune active proteins in humoral response capable of inhibiting and eradicating pathogens [6,7].

The synthesis of antimicrobial proteins in innate immunity in response to infection is activated by two signaling pathways, the Toll and immune deficiency (IMD) pathways [8,9]. The κ B-like transcription factor (NF- κ B)/Rel transcription factors are central regulators of

mammalian immunity and are also implicated in the induction of cecropins and other antibacterial peptides in insects [10]. Three Rel/NFκB transcription factors, Relish, Dorsal [11] and Dif [12], are involved in the two aforementioned signal transduction pathways of innate immune reactions. Dorsal and Dif are activated in the successive signaling cascade of the Toll pathway for antifungal and antibacterial responses [13-15]. Relish is required for the IMD pathway to activate the gene expression of antimicrobial peptides (AMPs) [10]. Relish is a nuclear factor NF-KB whose N-terminal part enhances the synthesis of AMPs in Drosophila [16]. A Relish transcript is also detected in early embryos, suggesting that it acts in both immunity and embryogenesis. The presence of a compound Rel protein in Drosophila indicates that similar proteins were likely present in primordial immune systems and may serve unique signaling functions [17]. Relish is specifically required for induction of the humoral immune response, including both antibacterial and antifungal peptides [10].

As two of the most serious diseases in crabs, vibriosis and white spot

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syndrome virus (WSSV) infection have caused irreversible damage to the crab culture industry worldwide [18]. *Vibrio alginolyticus* is an important pathogen in oceans. Similar to other pathogens, many types of virulence factors that are based on the virulence gene mainly result in its pathogenicity [19]. The identification of effective treatments or preventive measures is essential for crustacean aquaculture.

In our previous study, we found that the expression level of Relish in *S. paramamosain* was up-regulated following infection with WSSV or *V. alginolyticus* [20]. In the present study, we aimed to investigate the role of Relish in the innate immune system of crabs.

2. Materials and methods

2.1. Crabs and tissue preparation

The normal adult *S. paramamosain* (approximately 100 g) were obtained from a aquatic product market of Hangzhou. 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, digestive organs, gonad and hemolymph were collected from healthy 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 [21]. *V. alginolyticus* was cultured and used to challenge the crabs according to the previous report [22].

2.2. Rapid amplification of cDNA ends (RACE)

Total RNA was extracted from hemocytes of *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 [23] 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 with 3′ gene-specific primer (3GSP1, 3NGSP1) or 5′ GSP (5GSP1, 5NGSP1), designed on the basis of middle known sequence (the primers sequences are shown in Table 1).

Table	1
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Primer sequences and purpose.

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 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 using clustalx and Molecular Evolutionary Genetics Analysis, MEGA 7.1, with neighbor-joining method.

2.4. The quantitative real-time PCR

Relative MCM7 mRNA expression levels 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 normal adult crabs and hemocytes of crabs challenged by intramuscular injection of 0.2 mL of viral or bacterial suspension including WSSV (10⁵ copies/mL) or V. alginolyticus (10⁵ colony-forming units [CFU]/mL) [24,25], 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 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 subpackaged and kept at -20 °C. 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 by Office Excel [24], with GAPDH amplification as the internal control. The design and synthesis of the RT-qPCR primers were

Name	Nucleotide Sequence (5' to 3')	purpose
3' race GSP1	GTTGGTGGGGGAAAGCAAGAAAG	first primer for 3' RACE
3' race NGSP1	TGAAAGCCAAGAAAGTGCTGGGGAG	second primer for 3' RACE
5' race GSP2	CCACACCTCTCTGTCATTCTCATCC	first primer for 5' RACE
5' race NGSP2	GTCATACTGGAAAGCCTGGAACCGC	third primer for 5' RACE
Relish-realtime-F	CAGGTACACCTTTGTGACCGT	for Relish expression
Relish-realtime-R	CCTTCTACTTAGGGCATTTCG	for Relish expression
GAPDH-realtime-F	ACCTCACCAACTCCAACAC	for GAPDH expression
GAPDH-realtime-R	CATTCACAGCCACAACCT	for GAPDH expression
Relish dsRNA F	AGCTTGCGTGACACAAGGGAGATAG	for Relish RNAi
Relish dsRNA R	TCGACTATCTCCCTTGTGTCACGCA	for Relish RNAi
JAK -F	ATTGCTGAGGGGATGGATT	for JAK expression
JAK -R	GCCCATCACATTCCCAAA	for JAK expression
STAT -F	GACTTCACTAACTTCAGCCTCG	for STAT expression
STAT -R	GAGCTGAGTCTGTCTTAATGTTATCC	for STAT expression
C-type-lectin-F	ACTGAGGGGAAAGTAGCC	for C-type-lectin expression
C-type-lectin-R	TGCCCGTGTTTATTCATC	for C-type-lectin expression
crustin-F	TCAGAGCACCCTGGTAAATGT	for crustin antimicrobial peptide expression
crustin-R	GGCAGAACTGCGAAAGAAAG	for crustin antimicrobial peptide expression
TLR-F	TGTTGCCAGAGCAGAAGGT	for toll-like receptor expression
TLR-R	TTCCGTGAATGAACGAAGG	for toll-like receptor expression
proPO-F	ATGAAAGAGGAGTGGAGATG	for prophenoloxidase expression
proPO-R	GTGATGGATGAGGAGGTG	for prophenoloxidase expression
myosin-F	GCCGAGATAAGTGTAGAGGAA	for myosin-II-essential-light-chain-like-protein expression
myosin-R	AGTGGGGTTCTGTCCAAG	for myosin-II-essential-light-chain-like-protein expression

714	A	D	V	R	V	D	S	S	S	G	N	S	N	R	R	N	F	P	P	Q
2281	CGGC	CTG	AAG	GAC	AGT	CTC	AGC	AGC	GAC	TGI	'CCC	GAG	ATG	CAG	ATG	TGG	CCI	TCA	GGG	TG
734	R	P	Е	G	Q	S	Q	Q	R	L	S	R	D	A	D	V	A	F	R	V
2341	GCAG	TGA	GTG	GCTG	CCG	AGT	GTC	TCC	AAG	CAT	ACG	CAG	CGA	CTG	GGG	ACA	TAT	CGC	TGC	TC
754	A	V	S	А	A	Ε	С	L	Q	A	Y	A	A	Τ	G	D	I	S	L	L
2401	CTGG	CAA	CGC	ACC	GCI	ACC	TCC	TGO	CTG	TTC	AGA	ATA	ACC	AGG	GAG	ACA	CAC	GGC	CCT	CC
								-	_	_	-			-		-				

3' race NGSP1

774 LATHR YLL A VONNOGDTRP ŝ 2461 ACCAAGCCATCCCATAAGAACATGGAAGCCTTCAACAAAATTCTTAAAGCAAGTGAGAAA 794 TKPSHKNMEAFNKTLKASEK 2521 ATTAATCCAAGAGACCTCCTTAATGCCCAGAACTTTGCTCATGAAACGGCCCTCCACCAA 814 PRDLLNAQNFAHETALH N 0 2581 GCCATCCGTGGCAATGAGCTGACAATGGTACGTCGCTTGGTGGCCACACCAGGCTGCAAT AIRGNELTMVRRLVATPGCN 834 2641 GTGAGTTTAGTGGACAGCCAGGGCAACACCCCCATCCACAGTGCTGCTGGCCTGCAGGAC 854 V S L V D S Q G N T P I H S A A G L Q D 2701 874 PQCLDALLTQPINGARSALT 2761 CAGGCCATCAACATCTACAATTACCAAGGAGAGACACCTCTGCATGTGGCTGTGGTGTCT 894 O A I N I Y N Y O G E T P L H V A V V S 2821

Relish realtime-F

914	G	S	L	Ε	C	V	R	R	L	V	Ε	A	G	A	Q	V	Н	L	C	D
2881	CGT	AAG	AGA	GGA	GCA	AAC	CCT	CTCO	CACO	CTG	GCT	GCC	ATG	TTT	GGG	CGG	AGG	GAC	ATT	GCC
934	R	K	R	G	A	N	P	L	Н	L	A	A	М	F	G	R	R	D	I	A
2941	GCA'	TTC	CTC	ATT	GAT	CAT	ACG	AGTO	GTGA	ACT	GTA	GAG	GCA	GCC	ATG!	TTT	GAT	GGC.	AAC	ACA
954	A	F	L	I	D	H	Т	S	V	Т	V	E	A	A	М	F	D	G	N	Т
3001	GCC	CTC	CAT	CTG	GCA	GCC	CAA	AGC	AGAG	GAT	GCT	GAG	TTG	TGC	CGT	CTC	CTC	ATG	AGA	GCC
974	A	L	Н	L	Α	A	0	S	R	D	A	Е	L	C	R	L	L	М	R	A
3061	AAG	GCC	GAT	CCT	CAA	GTC	CGA	AATO	GCCC	CTA	AGT	AGA	AGG	AAA	AAG'	FCC'	FCA	GAA	TCA	GAA
							0	Rel	ish re	altin	ne-R									
994	K	А	D	Ρ	Q	V	R	N	A	L	5	R	R	K	K	S	s	Е	S	Е
3121	GAG	GAA	GAT	GAT	GAA	GTT(GAT	GAG	GAAG	GAG	GAG	GAG	GAT	TCT	GAA	GAG	GAA	CAT	CAA	GGA
1014	E	E	D	D	Е	V	D	Е	Е	E	Е	Е	D	5	Е	Е	E	H	Q	G
3181	TAC	ACC	CCA	TTG	GAC	TAT	GCT	GGA	GAT	GAT	GAT	GAG	ATC	CTG	GCA	ATC	CTG	CGT	GGA	GAG
1034	Y	Т	P	L	D	Y	A	G	D	D	D	E	I	L	A	I	L	F	G	E
3241	GAG	CCT	GTG	GAG	GTG	CAG	CTG	CAG	GGG.	AGG	TGT	TGG	TGG	GGG	AAA	GCA	AGA	AAG	AAG	CTC
1054	E	P	V	E	V	0	L	0	G	R	C	W	W	G	K	A	R	K	K	L
3301	CTC	AGC	ACT	CAG	CGC	TCG	ACT	CTG	GAA	TTG	ACA	TCT	CTG	TCA	CAG	ACA	TAC	AGA	GTT	CAG
1074	L	S	Т	0	R	S	T	L	E	L	Т	Ś	L	S	0	Т	Y	R	V	0
3361	AGC	TGG	GCC	TGT	GAT	GAA	AGC	CAA	GAA	AGT	GCT	GGG	GAG	CTG	AGT	GAG	CGT	ACT	CGA	GGA

1094 SWA C DESOESA G E L S E R T R G 3421 CACCTGGCAAGCCTCCTCAGCGGGGACACCTGGCAGCACCTTGCCCAGTTGCTTGACCTG 1114 H L A S L L S G D T W O H L A O L L D L 3481 GAGTTCCTGGTGCCATGCCTGGGCAAGGAAGCCACACCAGCCCACATGCTGCTTCAGCCG 1134 EFLVPCLGKEATPAHMLLQP 3541 GAGAACATGAAGGGTGTGTCCATGGAGAAGCTGCGGGAGTGCCTGGAGGTGCTGGGCTTG 1154 ENMKGVSMEKLRECLEVLGL AAGGACTGTGTCGCTGTGTGGACCAGGCATAG atgatactaagctgtccatgtgttggt3601 1174 K D C V A V L D Q A *

3661	ggtaaacaaaagttgtgaggctgcagcaaattctgggctgaggtgggcagcagtctggac
3721	aaatgatggatgatgtcatgaagggggttgtagatttggtggacaaatgatggatg
3781	catgaagggggttgtagatttggtggacaaatgatggatg
3841	gatttggtatatttgtattttgagattgtcaagcagcaaggagaggagaagcatgagtag
3901	ctcggagttgtgcaccattcccaaagtgcatcctgaaatggaaaatgctttcattatgtt
3961	catatttagaatgtggacgagtataataattacccagaatactttaattagcaggtaagt
4021	tataataataggaatgataggaaaattaaaaatatagatttttt
4081	gatattagttcaacaatatggtgtgaaggatgcagaggtcttgtattactatctttgtta
4141	cctgttgaatactttgatgcatgattaacaacactgactaagtcaaacagttggagagga
4201	tgagtagtgtcttgaaaactgtaactccacccccccccc
1261	tea



Fig. 1. Nucleotide and deduced amino acid sequences of Relish. The nucleotide sequence is displayed in the 5'-3' direction and numbered at the left. The deduced amino acid sequence is shown in a 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, and an asterisk denotes the termination codon (TAG). RACE and real-time qPCR primers are marked with arrows.

294

E.

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tcctgggtcttgctaagttgtgatctaagtaccgtgggtgttctgagtgcacaagat 61 cctgggacgtgattttgtgtcATGGTCAGAGGCGACAGCGGTGGGATGAATTCCCCCATAT MV RGDS G GMNS P Y AGTGGAGAGTCTGCCTCCCCTACTCCTCAGACCAGTCCATTGGGGTTCCTTCTCCTCCT 121 14 2 G ESAS p YS SDOSIG v p. 5 ACAGTGGTTGCTGCCTTCAACCGTCCATATGATGCCATCCACCATGATGGGCCATACATC 181 FNR PYDAIH 34 VAA H D G P AACATCCTGGAGCAACCACAGTCCAAGTTTCGATTCCGTTACAAAAGTGAGATGGTGGGG 241 EOP 54 SK F R Y K S E M L 0 R 301 ACACATGGACAGCTGAAGGCTGACCGAGCTGAGAAGAACAGAGCTGCCTTCCCTACAGTT 74 H G 0 L K A DRAEK NR A A F P T AAGCTTTCCAAATGGAACAGTGGTCCAGCCGTCATACGCCTCACTCTTTACACTGCTGAG 361 94 KI.SKWNSGPAVTRI.TI VTAP GACAATATTAACCAACGGAAGCGACATGTTCATGAACTGTCTGGGAAAAACTGCAACAAG 421 114 N 0 RKRH v Н E L S G K N C 481 GAGACAGGGATTTGTGAGGTGGTGGTGGATGAAAAAGTGACTATACAGCTGTGTTCCAG VDEKSDY 134 C E T E G A AATCTTGGAATCATCCACATTGCAAAGCGTGACACAAGGGAGATAATCAAACAACGAAAG 541

dsRNA-F

dsRNA-R

154 G T IHIAKRDTR E т KOR 601 CTTGAAGAGTTAATGACTCTTGCCAGAGTGCGTAAGCCACATCACAGCAATGAGGAGATC L E E L M T L A R V R K P H H S N E E I 174 CGTCGTAGCATCACCCCAACAGATCTTAAAAAGATTGATGAGGAAGCAGAAGAGGAAGCA 661 194 R R S I T P T D L K K I D E E A E E E A AAGAGTATGGACCTGAACAAGGTGGTGCTGCGGTTCCAGGCTTTCCAGTATGACAAGAAC 721

5'race NGSP2

214	K	S	М	D	L	N	Κ	V	V	L	R	F	Q	A	F	Q	Y	D	Κ	N
781	ATTO	AGA	GTT	ATA	GAC	CCA	TTA	CTC	TGC	CTG	TTG	ACT	CTG	ATG	TTG	TCT	TTA	ACC	TAA	AG
234	I	E	S	Y	R	Ρ	I	Т	L	Ρ	V	D	S	D	V	V	F	N	L	K
841	AATO	CC7	CTA	CTG	GCG	AGC	TAA	AGA	TAG	TGC	GGA	TGI	CTG	CAT	GCT	CTG	CCC	CCI	GCA	CT
254	N	A	Т	Т	G	E	L	K	I	v	R	М	S	A	C	S	A	P	C	Т
901	GGTO	GG7	CAG	AGA	TCT	GGC	TCC	TGG	TGG	AGA	AAG	TGC	GAA	GAA	ACA	ATG	TCC	ATA	TCA	AG
274	G	G	т	E	Ι	W	L	L	v	Е	K	v	R	R	N	N	V	H	I	K
961	TTCI	TTC	AGC	TGG	ATO	AGA	ATO	ACA	GAG	AGG	TGT	GGA	AAG	CTT	ATG	GAG	AGT	TTO	CTG	AT
				-	_	10.0		2.8-2.55	92			_								

5' race GSP2

FE DENDR EVWKAYG EFA D 1021 GCAGATGTTCACCACCAGTATGCTATTGTCTTCAGAACACCACGCTACAGGGTTACCAAT V Н Н F T P R 314 A D 0 A R 1081 CTGAGCACGTCCGTACGAGTGAAGGTACAACTTGAGCGCCCCACTGACAAAGACACAAGT 334 τ. S S VR v K VOLE RPT D D - गार 17 R. 1141 GAGGCACTGGATTTCACCTATCTCCCTGACAGTCTGAAGAGGCCCCGAATCAACCTTGAG 354 E Δ T. D F T Y L P D S L K R P R T N L F 1201 AACACCTTGGAAGAAGGGAAGCCGCACTTCAGTGACCTGCCTCCTGCCAAGAGATTTAAC 374 E Е G P H F S 1261 ATCAGCTCCTATACAAATGAGGCCATTGATCTCAGTAACAACACGAGGAATGTAGGCCAA 394 S S Y T N E A D L S N N T R N GAGGACTCGATGCCACCACCTGACATAATTGAGATGATCATTAGAAGTCCTGAGCTAACT 1321 414 SMP PPDTTEMT TRSP E. 17 D Τ. 1381 GACACCAGTGTGATGGGCCATTATGCACTGGAGGTGTCAAACCAGTCACCACAACATGTC S 434 v MG Н L E 37 S N Q 1441 GTCCCCTCACCAGCAATCCATGGGGACAGCAGCAATCAAGGCTTGTACTCCCCACTACAC 454 HG D S S N QG H A Т 1501 CCTGGGTCCACAGAAGTGACCTCCACCTACAGTAATGTGCTGCAGGTACCATCTCCAGTG G S T E V T S T Y S N V L O V P S P 474 P V CAGATGTCCCCCAGCTCCCCACAGTACAGCATGATGTCTCCTGGAGGGGAATCAGTTGGC 1561 494 M S P S S P Q Y S M M S P G G E S 0 C

1621 514 S Y PG SGGVVVTT T H TCACAGTCACCTCAGTATATTCAAGCACCCTCACCAGGGATGATGGTGCCTTCACCTGGC 1681 534 0 A G М 0 М TACCAGGATCCTTCCCCTCAGCTCATCACACAACAGCAGTTTTTACAGCAACAACAGCAG 1741 TO 554 0 D P S P OLI O O F L O 0 0 0 CAGCAGCAGCAACAACAACAACAGCAGCAGTTGAGACAAATACAGCTTCAGCAGCAACAA 1801 Q Q Q Q Q Q Q Q Q Q L R Q I Q L Q Q Q Q CAGCAACAGATGCTTAAAACTGAGCCTTTAGGATCACATGTGGTGCAGGGTGCTAGCAGT 574 1861 TEP HVV 594 0 0 M L K Τ. G S 0 GCTTGGGATGGCCTGCAGCAGGGAGGAGTCATGATGCAGCCCCTTACACAGCCACAGCCA 1921 V 614 D GG MMO P т A G L 0 0 L 0 0 GTCCTTCAGGTTCCAGAGTATGATTTGCCCAACTGCTTCAGTGTTGGTAACATGGAACAG 1981 V L Q V P E Y D L P N C F S V G N M E Q GAATCTTCACTGATGGATATCCTGGAAATTGCTGGTGAACAGCTTGGCTTTGAATCAGGA 634 2041 654 2 L M D т T. F AGEOLG c TTTACTAACCCAGACATCAAGACAGATTATGGTGGCAAGAAGAGTTCAGACTCTAAGAAG 2101 674 Т N P D I KTD YGGKKSSD K 2161 AAACTGAAGGAGCAACCCATTGGCTCAAATCCATCTGTGGATGCACTCAGCAAGATGATG K L K E Q P I G S N P S V D A L S K M M GCAGATGTCAGAGTGGACAGCTCATCAGGAACAGCAACAGGAGGAATTTCCCCCCCACAG 694 2221

3'race GSP1

262

677 903

894 873 878

304 701

939 930 909

014

Apis mellifera Bombyx mori Litopenaeus vannamel Penaeus monodon Eriocheir sinensis Scylla Paramamosain Consensus	NSTTASECESESSESPCSYVSICSP. SCCVPCLTPELN MVRGESTGNNPYSGESASPYSEH. SLCVPSPTVVTA MVRGERGGSSPYSGESASPYSEH. SACVPSPTVVTA NSPYSGESASPYSEH. SACVPSPTVVTA NVRGESG6NSPYSGESASPYSSECSICVPSPTVVA	0 39 37 37 29 38	Apis mellifera Bombyx mori Litopenaeus vannamel Penaeus monodon Eriocheir sinensis Scylla Paramamosain	TDEBGESA. I TNALPPEERNDFTEVALSSVESFEEI DEEPNGWKFI RT ELPNCPN. GGI CGESSLEDLEI ASCGLEFGSGEGCNSD ELPSCPN VGGLEPERTLENEVADDCLAFESGLI CNSD ELPNCFSV. CNNECESSLEDLEI EI AGCLEFESGFT. NPD
Apis meilifera Bombyx mori Litopenaeus vannamei Penaeus monodon Eriocheir sinensis Scylla Paramamosain Consensus	LI YLENGSKRNRPFLRI I ECPCLYFRFRYASENACTHOCL FNRPYLAI HHDGYYI TI LECPCLYFRFRYASENACTHOCL YNRPYEAVHHDCAFI CI LECPCSKFRFRYSSIVCTHOCL YNRPYEAVHHDCAFI CI LECPCSKFRFRYSSIVCTHOCL FNRPYLAI HHDGYYI NI LECPCSKFRFRYSSIVCTHOCL	0 79 77 77 69 78	Consensus Apis meilifera Bombyx mori Litopenaeus vannamei Penaeus monodon Eriocheir sinensis Scylla Paramamosain	LÇMÇTDSA. RSRÇÇTPÇTSKPALKTVEPÇESSDRTELN IKEDYGÇKSSDSKKKÇRDÇPI GPSADVDELSKMSEVRV IKADYGÇKM. DSKKKÇKDÇAVSSÇÇDVDELTKMI SNVÇV IKADYGÇKM. DSKKKÇKDÇAVSSÇÇDVDELTKMI SNVÇV IKTDYÇÇKKSSDSKKKLKEÇPI GSNŞPU DALSKMADVRV
Apis meilifera Bombyx mori Litopenaeus vannamel Penaeus monodon Eriocheir stnensis Scylia Paramamosatn Consensus	LGKSSSTNKNKVHPTVELVNYTGAVI KCCI ACHKTPEE KARKARSNKTAPPTVKLSKVNNPAN RLTETTAEEN NO KARSENSANPPTVKLSKVNHOPAN RLITETAEEN NO KARSENSANPPTVKLSKVNGPAN RLITETAEEN NO KARRAENSRAPPTVKLSKVNSGPAN RLITETAEEN NO	0 118 117 117 109 118	Consensus Apis mellifera Bombyx mori Litopenaeus vannamei Penaeus monodon Eriocheir sinensis	VPSDAKDANEYSAYYNAQDGLEVKCLLRELTQIIRNKKVH EAPSCNNSRRSQPSQRLEGYNPQRVSRGADVAFRVAVSAA DDSRCNRPQGVDSNN, QHPTQSVDVAFRVATSAA EDSRCNRPQGVE, CYPTQSVDVAFRVATSAA
Bombys mori Litopenaeus vannamei Penaeus monodon Eriocheir sinensis Scylla Paramamosain Consensus	HPHKLFEEEQENEDREVSCI VPKQGI YKVGFGGNGI RKRNVHELSGKNCNKEFGI CEVV DEKSEFFAVFCNLGI RKRNVHELSGKQCCKEFGI CEVV DEKVETVAVFCNLGI RKRNVHELSGKQCCKEFGI CEVVUDEKVEYTAVFCNLGI RKRNVHELSGKNCNKEFGI CEVVUDEKSEYTAVFCNLGI	155 157 157 149 158	Scylia Paramamosain Consensus Apis meilifera Bombyx mori Litopenaeus vannamei Banonu mondon	KKCE KCKLKREFCI RLSNCCT YLHM LCS ECLQAYAATCDI SLLLATHRYLLAVCNCCT ALHTAVSN
Apis mellifera Bombyx mori Litopenaeus vannamei Penaeus monodon Eriocheir sinensis Scylla Paramamosain	HTAKNV. I AKRITREI I KÇRKLEELLTHARLKKPHISVEI RRSIT HI AKRITKLI I LÇRKKEELI THARLKKPHISVEI RRSIT HI AKRITKLI I LÇRKKEELI THARLKKPHISVEI RRSIT HI AKRITKEI I KÇRKLEELI TARVRKPHISVEI RRSIT	0 175 197 197 189 198	Eriocheir sinensis Scylla Paramamosain Consensus Apis mellifera Bowhy, mort	ECLQAVAATGEI SLLLATHRIVAAQANQGET ALHTAVSN ECLQAVAATGEI SLLLATHRIVLAVQNNQGET ALHTAVSN ECLQAVAATGEI SLLLATHRIVLAVQNNQGET RPSTKPSH
Consensus Apis mellifera Bombyx mori Litopenaeus vannamei Penaeus monodon Eriocheir sinensis Sculla Paramamosain	NI SNEELKI ÇCENNAKSI ELINI WEKEFSAHE. İ STDE PTENKKI DEEAEEASNELINAVVI BÇAÇHAHMEN ENYE ÇSINRAFEEAEEEASNELINAVVI BÇAÇHAKKI ESYE ÇSINRAFEEAEEEASNELINAVVI BÇAÇÇHAKI ESYE PTELIKKI DEFAEEASNELINAVVI BÇAÇÇHAKI ESYE	0 211 237 237 229 238	Litopenaeus vannamei Penaeus monodon Eriocheir sinensis Scylla Paramamosain Consensus	KNEAFNKI LKASEKI NPRDLI NACNFALE TALHCAVRCN KNI EAFNKI LKACEKI RPCDLI NACNFARE TALHCAVRCN KNI EAFNKI LKACEKI RPCDLI NACNFARE TALHCAVRCN KNEAFNKI LKASEKI NPRDLI NACNFAHE TALHCAI RCN
Consensus Apis meilifera Bombyx mori Litopenaeus vannamei Penaeus monodon Eriocheir sinensis Svila Paramamocain	EI CKPVF SEPI HSL SNASNULKI CRI SRE SGRPVGGEUV PI TLPVISUVFNI NATTGELKI VRSASA SAPCTGOTEI PVTLPVISUVVNI NATTGELKI VRSASA SAPCTGOTEI PVTLPVISUVVNI NATTGELKI VRSASA SAPCTGOTEI PVTLPVISUVNI NATTGELKI VRSASA SAPCTGOTEI PVTLPVISUVNI NATTGELKI VRSASA SAPCTGOTEI	0 251 277 277 269 278	Apis meitifera Bombyx mori Litopenaeus vannamei Penaeus monodon Ertocheir sinensis Scylla Paramamosain Consensus	LSHVVTLEVSK. GSSVSLCUVAGDTAFHYAAK. SHKO E ELTAVHRUATPGCNVSLUSSCCNPLHYAASLGEPOCD ETTAVRRUAPGCDVSIVTACCNPPHCAAEQSIQCE ETTAVRRUAPGCDVSIVTACCNPPHCAAEQSIQCE ETTAVRRUAPGCDVSIVTACCNPPHCAAQQSIQCE ELTAVRRUATPGCNVSLVSQCNTPIHSAAGLQDPQCD
Consensus Apis mellifera Bombys mori Litopenaeus vannamel Penaeus monodon Eriocheir sinensis Scylla Paramamosain	UT P VERVIKKNI CI REPELDENGRAVITAN VI P VERVIKKNI CI REPELDENGRAVITAN VI VERVRENNEN KEPELDENGRAVITAN VI VERVRENNEN CI REPELDENGRAVITAN VI VERVRENNCI REPELDENGRAVITAN VI VERVRENNCI REPELDENGRAVITAN VI VERVRENNCI REPELDENGRAVITAN ALA ALASSI	11 291 317 309 318	Apis mellifera Bombyx mori Litopenaeus vannamei Penaeus monodon Eriocheir sinensis Scylla Paramamosain Consensus	TAVKI PEAGACVDVLLFEKTDI EAYNDGWAPLHLÄAKAG PLLAAVKKINI PFELNTYNYEKCA. ALLTQPI KARSAVSQLAFAYYE. GETPLHVAVVSG ALLTRPVKUVSSAVTQAI NVYNYC. GETPLH AVI NG ALLTRPVKUVSSAVTQAI NVYNYC. GETPLHVAVVSG ALLTRPVKUVSSAVTQAI NI YNYC. GETPLHVAVVSG
Consensus Apis meiliféra Bombyx mori Litopenaeus vannamei Penaeus monodon Eriocheir stnevnis Scylla Paramanosain Consensus	g f dvhh CVAI USETSPEALLNI TEPKENFI OLESSENSE CVAI USETA ANDERIKS NI NI ULEASSO CARSENESSE CVAI USETA ANDERIKS NI ULEASSO CARSENESSE CVAI USETANELINI. NIS VRIKV OLESSENDS CVAI USETANELINI. NIS VRIKV OLESSENDS CVAI USETANELINI. NIS VRIKV OLESSENDS CVAI USETANELINI. SI VRIKV OLESSENDS VII VII SI VII VII VII SI VRIKVOITENSENDS VII SI VII SI VRIKVOITENSENDS VII SI VRIKVOI	51 331 357 357 349 358	Apis melilifera Bombyx mori Litopenaeus vannamei Penaeus monodon Eriocheir sinensis Scylla Paramamosain Consensus	SYEAVCSLIHAGVENNTERFYGRTALHI RVECCHTN VE PLHURTGCNARH. SLECVRRIVEAGACVHICERKRGAPCHU AAFGRRTIAA LISVRAUVGAGACVHICERKRGAPCHU AVHGHREIAR NLESVRNIIDAGACVHICERKRGAPCHU AVHGHREIAR SLECVRRIVEAGACVHICERKRGAPCHU ANGRRTIAA Ih a
Apis mellifera Bombys: mori Litopenaeus vannamel Penaeus monodon Eriocheir sinensis Scylla Paramamosain Consensus	TYR PSL RII GRKRÇRI SHSGS BELSFILPI SNYSEN KYI AEPI YSN. TYL PSLKRSRI NLENTLEEGKRHF SLPPP AKRPAN GSYP TFN GTLKRRSNLI ENGEEGKRHF SLPP AKRPANF NYA FYL DTLKRRSNLI ENGEEGKRHF SLPP AKRPANF NYA TYL PUSLKRPRI NLENTLEEGKPHF SLLPPAKRPNI SSYT	87 341 397 397 389 398	Apis meilifera Bombyx mori Litopenaeus vannamei Penaeus monodon Eriocheir sinensis Scylla Paramamosain Consensus	YLLKKINI SVNRRISCNIAGE TE VVYTGVEAKELCALDL LLESGADHRVRCH GRUPT LAYDSRLAUVAAL VILDHGVVTEAANFOXIAT LA GYRDAE.CRLM VILDLISVTI EAGLFOXIAT LA GYRDSE.CRLM VILDHSVTI EAGLFOXIAT LA GYRDSE.CRLM FLICHTSVTVEAANFOXIAT LA GYRDSE.CRLM
Apis melilfera Bombyx mori Litopenzeus varnamei Penaeus monodon Eriocheir stuensis Scylla Paramamosain Consensus	ENI SUI LNSTE. NEAI ELSINNINVOQEESS CAPILI FENFI RIVPELALTINV SEAI ELSINNINVOQEESS PITULI ELLUVISIEPPINING SEAI ELSINNINVOQEESS PITULI ELLUVISIEPPINING NEAI ELSINTRIV. OQEESS PITULI ELLI RISPELITETSV	99 341 437 437 429 437	Apis melilfera Bombyx mori Litopenaeus vannamei Penaeus monodon Eriocheir sinensis Scylla Paramamosain	CHCG
Apis mellifera Bombyx mori Litopenaeus vannamei Penaeus monodon Eriocheir sinensis Scyila Paramamosain Consensus	SKKRRN NASCSTSPASSCS KSI SELPAL LCCYTLEVPNCSPCIAVPSPCSHCDS NCCI V91LPPCST LSPYTLENNCSPCIAVPSPCSHCDS STHE V91 IPCSV LSPYTLENCSPCHTVPSPCHPSDS STHE V91 IPCSV NCHYALEVSNCSPCHVPSPAI HCDSSNCCI V92LPPCST	99 370 477 477 469 477	Consensus Apis mellifera Bombyx mori Litopenaeus vannamei Penaeus monodon Eriocheir sinensis	d KVEVLAEDEKADËVI GÇSSFËLAKNEPCI GCLFNRÇYET KKADPNFHGACHESAVKNSAD KELRALLI GALAFEDGFE AEEEEEEESDE NÇQYTPLI YAQDDEEI DALI RGEAAA RKVEEEDDSEE PDCVTPLI VACNITE I SI LRGEAI QE RKVEEEDDSEE PDCVTPLI VACNITE I SI LRGEAI QE
Apis meliifera Bombyx mori Litopenaeus vannamei Penaeus monodon Eriocheir sinensis Scylla Paramamosain Consensus	IKI LSEG VILNNENDENNI EXPETELPAAA AAABELG CLABAL GTSAADATNI NNULQVSSPGCCSSSS PQYCI SSPSECS GTPNTEFTI NSSLLQVPSPGHPV. PNSPCTSVSSPGERS GTPNTEFTI NSSLLQVPSPGHPV. PNSPCTSVSSPGERS EV	107 409 517 516 508 511	Scylla Paramamosain Consensus Apis mellifera Bombyx mori Litopenaeus vannamei Penaeus monodon	EEEEEGAAGEEVVCTA.C. KAPLISALDSGI DI SVTDI (YSD
Apis mellifera Bombyx mori Litopenaeus vannamel Penaeus monodon Eriochetr sinensis Scyila Paramamosain Concemus	I TSPRCKEFLKNNEVENYLKFF. ENEENMLI TECHSTV MCE. IC PPYPCSGCVAVTTTHYTCP. SCSPCYI CAPSPCM WC PCSCYCCSCNFCCCMSCYCSCSCSPCYI CSPSPI M WC PCSCYCCSCNFCCCMSCYCSCSCSCPCI CSPSPVM WC PSYPCSGCVVTTTHYSCP. SCSPCYI CAPSPCM	144 413 554 556 548 548	Eriocheir stnensis Scylla Paramamosain Consensus Apis meilifera Bombyx mori Litonenceux yannamei	EEEECGAÇAÇEEVVGTAACPLHSALDSGI DI SVTDI (YSD VQLQGRCWVGKAR. KKLLSTQRSTLELTSLSQTYRVQS SEINV INNEH. KEKISIL DEGEETSG. STALIGAQQ'LGEVSSLLDRSGSVQEDAGR
Apis mellifera Bombyx mort Litopenaeus vannamet Penaeus monodon Eriochetr sinensis Scylla Paramamosain	CNCEEL NFAKNVETKVI CYNKNELKNVE. EY VPSPSYCEPAPCLI SCCYI PCCLCCHC CO VPSPSYCENATC ILNCYLCCPCCCCL CO VPSPSYCENATC ILNCYLCCPCCCCL CO VPSPSYCENATC ILNCYLCCPCCCCL CO	174 413 584 595 577 578	Penaeus monodon Eriocheir sinensis Scylla Paramamosain Consensus Apis mellifera	CPCEEDSV. CELSERVRERLASHLSCDT. VRHACL CPCEEDSV. CELSERVRERLASHLSCDT VRHACL VACDESCESAGELSERTRCHLASLLSCDT VCH LCC UKTCGVRKLAKHLNVEYLUKTFCBNST.
Consensus Apis mellifera Bombyx mori Litopenaeus vannamel Penaeus monodon Eriochetr stnensis Scylia Paramamosain	I CKLFKERSTYGESPLHAALRYGCREI VKYFLNLI SSNED SAVELHSVACNAM CCCCCCHVQELQI GLQCNCCPCQCNLRAEPLATCAVCET CCCCCCCCCFCCQCI GCFFKTQCLSCLCACHENET CCCCCCCCCFCCCCACCFFKTQCLSCVT GAVENULT CCCCCLRQT GCQCCCCLK	214 427 624 635 617 600	oomoyx mori Litopenaeus vannamei Penaeus monodon Eriocheir sinensis Scylla Paramamosain Consensus Apis meilifera	ALESELSE I GAUPS I INTELHELNY LAVIGAAL (QPEL DEDYNVPCI AREA PAGU HPENNIS VIVEK RECLET DEDYNVPCI ACEPS PAST UHPENNIS VISEK RECLTV DEDYNVPCI ACEPS PAST UHPENNIS VISEK RECLTV DEDEFLVPCI GREATPAHNEL (PENNIGVSNEK RECLEV
Consensus Apis meilifera Bombys mori Litopenaeus vannamei Penaeus monodon Eriocheir sthensis Scylla Paramamosain Consensus	CKAI VNCNSSGKTPLHYAI LCNCPEI TKALLNLGADPNR SPSAPVETN.SVECPPNLELNSTEFFK GSSVDELCCCVMHPCPRVCPCPCPCPCPCPPULCVPEF CPNN CLCC.CCACPCALCPCNNPEF CPNN CLCC.PCNVPEF EPLCSHVVCGASSAVDELCCGGVMCPLTCPEPVLCVPEY	254 453 664 661 643 640	Bombys: mori Litopenaeus vannamei Penaeus monodon Eriocheir sinensis Scylla Paramamosatn Consensus	LGLKECVAVLDR LGLKECVAVLDQ LGLKECVAVLDQ LGLKDCVAVLDQ

Fig. 2. Multiple alignments of the amino acid sequence of S. paramamosain Relish (in this study) with other Relish sequences of common animals including Apis mellifera (ACT66913.1), Bombyx mori (BAF74125.1), Litopenaeus vannamei (ABR14713.1), Penaeus monodon (AFH66691.1), and Eriocheir sinensis (ADM14334.1). Twelve conserved cysteines (C1 -C12) are shaded and boxed.



Fig. 3. The phylogenetic tree of Relish in different organisms based on amino acid sequence comparisons.

entrusted to Generay (Shanghai, China), based on the whole sequence. Table 1 lists all the primers that were designed and synthesized by Generay.

2.5. Prokaryotic expression, purification of Relish-dsRNA

The primers (shown in Table 1) with specific restriction sites (*Hind* 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-Relish. The constructed L38-Relish was verified by restriction enzyme digestion and DNA sequencing. The recombinant plasmid L38-Relish was transformed into HT115 (DE3) cells knocked out of RNase III. The following steps were performed as described previously [24].

2.6. Knock down of Relish by RNAi and challenge experiments

Total RNA was purified using an EASY spin tissue/cell RNA extraction kit (Aidlab, China), following the manufacturer's instructions. Relish-dsRNAs (75 μ g/crab) [25] was immediately injected intramuscularly into the fourth pereopodcoxa of each crab, and Relish mRNA expression levels were detected by qRT-PCR following WSSV and *V. alginolyticus* challenge.

2.7. Kaplan-Meier survival analysis

For the pathogen challenge, normal crab were randomly distributed into six groups (n = 9 per group, three repeats). The control group received injections of PBS alone, the Relish-dsRNA group received injections of Relish-dsRNA alone, the WSSV group received injections of WSSV in PBS, and the Relish-dsRNA + WSSV group received injections of Relish-dsRNA and WSSV, the *V. alginolyticus* group received injections of *V. alginolyticus* in PBS, and the Relish-dsRNA + *V. alginolyticus* group received injections of Relish-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



Fig. 4. Characterization of Relish expression in various tissues from normal *S. paramamosai*n determined with quantitative real-time PCR. The amount of Relish mRNA was normalized to the GAPDH transcript level. Data are shown as means \pm SD (standard deviation) of three separate individual tissues. Capital letters indicate expression of Relish in different adult tissues.

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 hemolymph cells. The hemolymph cells were then suspended in highly alkaline PBS (12.585 g/L NaCl, 0.315 g/L KCl, 0.27 g/L KH₂PO₄, 0.948 g/L Na₂HPO₄, add ddH₂O to 1 L), counted and adjusted to a density of $3-5 \times 10^6$ cells/mL with PBS. The cells were stained using a BD PhrmingenTM FITC Annexin V Apoptosis Kit, and assessed by flow cytometry. 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.

2.9. Determination of immune parameters after RNAi

The immune parameters determined included total hemocytes numbers (THC), PO and SOD activities. THC was determined as described previously [25]. To determine PO and SOD activities, $500 \,\mu$ L of hemolymph was withdrawn into a 1 mL syringe containing $500 \,\mu$ L EDTA (20 mM) 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 [26]. 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 [27].

2.10. 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 oneway 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). F. Zhu et al.



3. Results

3.1. Characterization of Relish cDNA

The full length Relish cDNA sequence was 4263 bp, including a 3552 bp open reading frame (ORF) which encoded a 1184 amino acid protein. The 5' and 3' untranslated regions (UTR) of Relish were 81 and 630 bp in length, respectively. The nucleotide and deduced amino acid sequences of the full-length cDNA are shown in Fig. 1.

3.2. Sequence homology and phylogenetic analysis

The putative intact amino acid sequence of Relish was then compared with the sequences of Relish in other species using DNAMAN version 6.0 (Lynnon Biosoft, USA). The results revealed a similarity of approximately 80% identity with that of *Eriocheir sinensis*, 67% with that of *Litopenaeus vannamei*, 66% with that of *Penaeus monodon*, and 22% with that of *Bombyx mori* (Fig. 2).

A condensed phylogenetic tree based on the deduced amino acid sequence was constructed by the neighbor-joining method using MEGA7.1 (Fig. 3). Phylogenetic analysis showed that *S. paramamosain Relish* showed the closest relationship to that of *E. sinensis*. The amino acid sequence showed that some highly conserved amino acid sites exist in it.

3.3. Tissue distribution of Relish mRNA

Expression profiling of the Relish gene in different tissues of *S. paramamosain* was examined with quantitative real-time polymerase chain reaction (qRT-PCR) (Fig. 4). Relish was significantly highly expressed in gonad compared with other tissues, and showed lowest expression in muscle tissue. Relish expression levels in gonad were 1.85-, 6.50-, 2600-, 5.90-, 65- and 1.18-fold greater than that in hemolymph, gills, muscle, hepatopancreas, heart and digestive organs, respectively. Thus, Relish expression in gonad was significantly higher (P < 0.01) than in any other tissues examined.

3.4. Time course of Relish expression after WSSV or V. alginolyticus challenge

We investigated the variation in Relish expression in crabs after immune challenge with WSSV or *V. alginolyticus*. Relish expression changed after immune challenge in a time-dependent manner. Relish expression was significantly up-regulated (P < 0.01) from 12 to 48 h post-challenge in crabs with WSSV infection, with the highest expression levels at 24 h (Fig. 5A). Relish expression was also significantly upregulated (P < 0.01) at 12 and 24 h post-challenge with *V. alginolyticus*, but gradually returned to the control level at 48 h post-challenge (Fig. 5B). These results suggested that Relish may play an important role in crab innate immunity following infection with WSSV or *V*. **Fig. 5.** Real-time RT-PCR analysis of Relish expression challenged with WSSV or *V. alginolyticus*. (A) Real-time RT-PCR analysis of Relish expression in the hemocytes of *S. paramamosain* challenged with WSSV. (B) Real-time RT-PCR analysis of Relish expression in the hemocytes of *S. paramamosain* challenged with *V. alginolyticus*. The amount of Relish mRNA was normalized to the GAPDH transcript level. Data are shown as means \pm SD (standard deviation) of three separate individual tissues. Double asterisks indicate a significant difference (P < 0.01) between two samples.

alginolyticus.

3.5. Effects of Relish double-stranded RNA on the expression of immune genes

The effect of Relish-dsRNA on its mRNA expression was determined using RT-PCR. Relish expression in crab hemocytes was significantly knocked down by Relish-dsRNA (P < 0.01) (Fig. 6A). We also examined the effect of Relish-dsRNA on Relish gene expression in hemocytes of S. paramamosain at different times post-treatment. RelishdsRNA inhibited the expression of Relish mRNA in hemocytes from 24 to 48 h post-treatment (Fig. 6B). We also examined the relationship between Relish and other immune-related genes by analyzing the expression of a variety of immune genes in hemocytes under RelishdsRNA treatment. Among these genes, JAK, STAT, crustin antimicrobial peptide (CAP), prophenoloxidase (proPO), C-type-lectin (CTL) and myosin-II-essential-light-chain-like-protein (MELCLP) were significantly down-regulated (P < 0.01), while Toll-like receptor (TLR) was significantly up-regulated (P < 0.01) following Relish-dsRNA treatment (Fig. 6C). The expression levels of these genes in EGFPdsRNA-treated crabs showed no obvious differences compared with those in the control.

3.6. Determination of immune parameters

The THC in the Relish-dsRNA group was significantly (P < 0.01) decreased at 48 h post-treatment compared with the PBS group (Fig. 7A). THC was increased in the WSSV group compared with the PBS group, but was significantly (P < 0.01) decreased in the WSSV + Relish-dsRNA group compared with the WSSV group (Fig. 7A). THC was increased in the PBS group at 24 h post-challenge. However, THC was increased in the *V. alginolyticus* + Relish-dsRNA group compared with the V. alginolyticus (Fig. 7A). These results indicated that THC increased in crab after WSSV infection.

Phenoloxidase (PO) activity in the Relish-dsRNA group was significantly (P < 0.01) decreased at 24 h and 48 h post-treatment compared with the PBS group (Fig. 7B). PO activity was significantly (P < 0.01) higher in the WSSV + Relish-dsRNA group compared with the WSSV group at 24 h and 48 h post-challenge (Fig. 7B). These data suggested that Relish knockdown enhanced PO activity after WSSV challenge. However, PO activity was reduced at the 24 h and 48 h after *V. alginolyticus* challenge compared with that in the PBS group, but was significantly (P < 0.01) enhanced in the *V. alginolyticus* + Relish-dsRNA group compared with the *V. alginolyticus* group (Fig. 7B). These data suggested that Relish knockdown may increase PO activity in crabs infected with *V. alginolyticus*.

SOD activity in the Relish-dsRNA group was significantly (P < 0.01) decreased at 24 h and 48 h post-treatment compared with the PBS group (Fig. 7C). SOD activity was decreased in the WSSV group

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Fig. 6. Real-time RT-PCR analysis of the expression of Relish and immune genes. (A) Real-time PCR analysis of Relish expression in the hemocytes of *S. paramamosain* treated with Relish dsRNA (Relish-dsRNA) at 24 h post-treatment. The amount of Relish mRNA was normalized to the GAPDH transcript level. (B) Real-time RT-PCR analysis of Relish expression in hemocytes from *S. paramamosain* treated with Relish-dsRNA at different times post-treatment. (C) Real-time RT-PCR analysis of seven immune genes (JAK, STAT, crustin antimicrobial peptide [CAP], Toll-like receptor [TLR], prophenoloxidase [proPO], C-type-lectin [CTL] and myosin-II-essential-light-chain-like-protein [MELCLP]) expression in hemocytes from *S. paramamosain* treated with Relish-dsRNA. The amount of Relish mRNA was normalized to the GAPDH transcript level. Data are shown as means \pm SD (standard deviation) of three separate individual tissues. Double asterisks indicate a significant difference (P < 0.01) between two samples.

compared with the PBS group, and was significantly (P < 0.01) decreased in the WSSV + Relish-dsRNA group compared with the WSSV group (Fig. 7C). SOD activity was reduced at 24 h and induced at 48 h after *V. alginolyticus* challenge compared with that in the PBS group. However, SOD activity was significantly (P < 0.01) decreased in the *V. alginolyticus* + Relish-dsRNA group compared with the *V. alginolyticus* group (Fig. 7C). These results indicated that Relish knockdown may



A 100%

90%

Fig. 7. Effects of Relish knockdown on crab immune parameters, including THC, PO activity, and SOD activity, as determined in normal, WSSV-treated or VA (*V. alginolyticus*)-treated crab. (A) THC after Relish-dsRNA, WSSV, WSSV + Relish-dsRNA, VA, VA + Relish-dsRNA treatment. (B) Hemocyte PO activity after Relish-dsRNA, WSSV, WSSV + Relish-dsRNA, VA, VA + Relish-dsRNA treatment. (C) Relative SOD activity after Relish-dsRNA, WSSV, WSSV + Relish-dsRNA, VA, VA + Relish-dsRNA treatment. All treatments, at each time point, included at least three crabs, and all experiments were repeated three times. Each column represents the mean value of triplicate assays.

block SOD activity in crab.

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

The WSSV + Relish-dsRNA group showed a significantly higher mortality rate (P < 0.01) than the WSSV group at 216 h post-challenge (Fig. 8A). The negative control showed a similar mortality rate to the Relish-dsRNA group post-treatment, indicating that Relish-dsRNA itself was non-toxic in crabs. However, Relish-dsRNA showed a similar effect on the cumulative mortality of *V. alginolyticus*-infected crabs. The cumulative mortality in the Relish-dsRNA + *V. alginolyticus* group was significantly higher (P < 0.01) than that in the *V. alginolyticus* group at 216 h post-challenge (Fig. 8B). Overall, these results indicated that Relish knockdown enhanced the cumulative mortality of crabs infected with WSSV or *V. alginolyticus*.

3.8. Effect of Relish knockdown on apoptosis

We investigated the role of Relish on apoptosis of crab hemocytes at 24 h post-treatment using the Annexin V-FITC Apoptosis Detection Kit I. The apoptosis rate was significantly enhanced (P < 0.01) in the RelishdsRNA group compared with the PBS group as shown by flow cytometry (Fig. 9). In addition, the WSSV + Relish-dsRNA group showed a significantly higher apoptosis rate (P < 0.01) than the PBS group and WSSV group. The apoptosis rate was significantly decreased (P < 0.01) in the *V. alginolyticus* group compared with the PBS group, and the *V.*

alginolyticus + Relish-dsRNA group showed a higher apoptosis rate than the *V. alginolyticus* group. These results suggested that Relish had an inhibitory effect on hemocyte apoptosis in crabs irrespective of whether they were infected with pathogens.

4. Discussion

Relish has the capacity to activate cecropin gene transcription, as does Dorsal and Dif, and the Relish gene is very strongly induced after infection, much more than either Dorsal or Dif [11,12,15]. Relish is a transcription factor and forms an important part of the IMD signaling pathway [13]. We examined the relationship between Relish and other immune-related genes by analyzing the effects of Relish-dsRNA on the expression levels of immune genes in crab hemocytes. The results strongly suggest that Relish is involved in affection of the immune response in S. paramamosain. To date, due to the absence of crab whole genome, the genes crucial for the immune system are being progressively characterized in crabs. In this experiment, Relish, possessing a 3552 bp ORF, was characterized from S. paramamosain. Relish was found to be highly expressed in the gonad, digestive gland and hepatopancreas of S. paramamosain. The digestive system is considered to be a complete and individual system, separate from the immune system, as well as the blood circulation system, and respiratory system. The mud crab intestine contains diverse microorganisms, and these microorganisms produce and secrete a variety of enzymes to maintain microbial metabolism and aid the conversion and absorption of food and medicine. In addition, it has been demonstrated that there are remarkable similarities and shared function in both nutrient acquisition and host defense. BLAST analysis of proteins showed that the amino acid sequence is highly conserved between crustaceans. The neighborjoining tree method revealed a close evolutionary relationship between S. paramamosain Relish and E. sinensis Relish.

In crustaceans, it has been shown that Relish is involved in immune responses against bacteria, fungi and WSSV [23,27,28]. Previous research reported that the expression of Relish gene was positively stimulated by *Vibrio harveyi*, as well as WSSV or yellow head virus [29].

Fig. 8. The survival analysis of challenged crabs treated with Relish-dsRNA. (A) The Kaplan–Meier survival analysis of WSSV-challenged crabs treated with Relish dsRNA (Relish-dsRNA). (B) The Kaplan–Meier survival analysis of *V. alginolyticus*-challenged crabs treated with Relish-dsRNA. The solutions used for injection are shown on the left. Each group consisted of 9 individuals, respectively.



PBS

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Fig. 9. Flow cytometry assay of apoptosis. (A) PBS; (B) Relish-dsRNA; (C) WSSV; (D) WSSV + RelishdsRNA; (E) *V. alginolyticus*; (F) *V. alginolyticus* + Relish-dsRNA; (G) bar graph of apoptosis due to WSSV; (H) bar graph of apoptosis due to *V. alginolyticus*. Double asterisks indicate a significant difference (P < 0.01) between the sample and the challenge only.

The expression profile of the Dorsal homolog (FcDorsal), a type of Rel/ NF- κ B transcription factor similar to Relish, in *Fenneropenaeus chinensis* hemocytes was apparently modulated when shrimp were stimulated by bacteria or WSSV [30]. In the present study, similar results were observed and pathogen (WSSV or *V. alginolyticus*) stimulation led to upregulation of Relish mRNA expression in the hemolymph of *S. paramamosain*. RNA interference has previously been used in the study of immunity in many invertebrate models in order to investigate the function of certain target proteins [23,31]. In the present study, we successfully inhibited the expression of Relish with specific doublestrand RNA (Relish-dsRNA), which provided a practical way of examining the role of Relish in the innate immune system, and then normal and Relish-inhibited crabs were subsequently subjected to further experiments.

The Janus family tyrosine kinase and signal transducer and activator of transcription (JAK/STAT) signaling pathway has been proved to have a very important role in the antiviral process of vertebrates [32,33] and invertebrates [34-37]. In insects, such as mosquitoes and Drosophila, the JAK/STAT pathway also showed antiviral activity and in the Pacific white shrimp [38,39]. Toll-like receptors (TLRs) are key pattern recognition receptors (PRRs) of the innate immune system and can protect the host against pathogens in both arthropods and mammals [40-42]. The recognition of microbial pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) leads to activation of specific signaling pathways and a variety of cell-dependent responses, including pro-inflammatory cytokine release, phagocytosis and antigen presentation. Reports also show that the expression analysis of FcToll at the transcriptional level in shrimp was modulated after Vibrio or WSSV stimulation [43]. RNAi knock-down of shrimp Litopenaeus vannamei Toll gene would reduce the expression of Relish [44]. In this study, he significant change in Toll expression in Relish-knockdown crab suggested that Relish may affect the expression of Toll. Knockdown of Relish gene led to significant down-regulation of the innate immune factors JAK, STAT, CAP, proPO, CTL and MELCLP (P < 0.01), while TLRs were significantly up-regulated (P < 0.01) in the hemocytes of S. paramamosain. PO is a member of the tyrosinase group, and this enzyme is responsible for the activation of melanogenesis in invertebrates [45]. It has also been documented that PO is an important tool used against several pathogens [46]. Myosin is a key factor in the energy signal transduction pathway, participating in cell skeleton construction and motility processes [47]. In Kuruma shrimp (Penaeus (Marsupenaeus) japonicus), myosin and its light chain has been reported as a WSSV interacting protein involved in the antiviral defense mechanism of shrimp immune cells [48]. The role of myosin light chain and its partner (myosin) in the hemocyte defense mechanism has been demonstrated by its involvement in the regulation of phagocytosis in Kuruma shrimp [49].

Our study provides more evidence and a novel understanding of the function of Relish. Changes in the expression levels of the immune genes listed above indicated that Relish is likely to be associated with the innate immunity of crab. To determine whether Relish exerted any other effects on crab innate immunity, we investigated a variety of functional parameters to evaluate immune potential. We found that WSSV or V. alginolyticus-challenged crabs pretreated with Relish-dsRNA showed a higher mortality rate than that of the WSSV or V. alginolyticus group at 216 h post-challenge. In WSSV or V. alginolyticus-infected crabs, as Relish expression was inhibited, PO activity was enhanced, while SOD activity was reduced compared with the WSSV or V. alginolyticus group, THC activity was reduced compared with the WSSValone group in WSSV-infected crabs, and increased in V. alginolyticusinfected crabs. In this study, apoptosis was increased in Relish-dsRNAtreated crabs after WSSV or V. alginolyticus challenge. Apoptosis of crab hemocyte increases a lot with Relish-dsRNA treatment but not in conjunction with WSSV or V. alginolyticus infection. We suspect that WSSV or V. alginolyticus may affect the process of apoptosis to a great extent in crabs. This is an interesting phenomenon that we found, and it will be the next direction in our study. The results of the V. alginolyticus infection experiment revealed that Relish may play a positive role in the antibacterial process in crab. The findings of the present study suggest that Relish not only plays a positive role in bacterial infection, but also influences the immune response to WSSV in S. paramamosain.

In conclusion, our findings indicate that *S. paramamosain* Relish plays an important role in the innate immunity of crabs. This host protein could affect host defense mechanisms against pathogenic infections by regulating apoptosis, THC, PO activity and SOD activity.

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