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Molecular characterization of shrimp harbinger transposase derived 1 (HARBI1)-like and its role in white spot syndrome virus and *Vibrio alginolyticus* infection

Baozhen Sun, Xiyi Qian, Fei Zhu*

College of Animal Science and Technology, Zhejiang Agriculture and Forestry University, Hangzhou 311300, China

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ABSTRACT

The role of the nuclease, HARBI1-like protein (mjHARBI1-like) in the innate immunity of *Marsupenaeus japonicus* was explored in this study. The 1361 bp cDNA sequence of mjHARBI1-like was cloned from *M. japonicus* using RACE. RT-qPCR analysis results showed that the gills and hepatopancreas of *M. japonicus* were the main tissues where mjHARBI1-like is expressed. In addition, it was also found that white spot syndrome virus (WSSV) or *Vibrio alginolyticus* challenge could stimulate mjHARBI1-like expression. After mjHARBI1-likewas inhibited, expression of immune genes such as toll, p53, myosin, and proPO were significantly downregulated ($P < 0.01$). However, in shrimp hemocytes, hemocyanin and tumor necrosis factor-α (TNF-α) were up-regulated significantly ($P < 0.01$). This study demonstrated that mjHARBI1-like plays a key role in the progression of WSSV and *V. alginolyticus* infection. Specifically, the cumulative mortality of WSSV-infected and *V. alginolyticus*-infected shrimp was significantly advanced by double-strand RNA interference (dsRNAi) of mjHARBI1-like. Apoptosis studies indicated that mjHARBI1-dsRNA treatment caused a reduction in hemocyte apoptosis in bacterial and viral groups. In addition, phagocytosis experiments illustrated that mjHARBI1-dsRNA treatment led to a lower phagocytosis rate in hemocytes of *V. alginolyticus*-challenged shrimp. It was also found that knockdown of mjHARBI1-like inhibited shrimp phenoloxidase (PO) activity, superoxide dismutase (SOD) activity, and total hemocyte count (THC) after WSSV or *V. alginolyticus* infection. These data indicate a regulative role of mjHARBI1-likein the immunity of shrimp in response to pathogen infection. Resultantly, it was concluded that mjHARBI1-like might have a positive effect on the *anti*-WSSV immune response of shrimp by regulating apoptosis, THC, PO activity, and SOD activity. Additionally, mjHARBI1-like might promote *anti-V. alginolyticus* infection by participating in regulating phagocytosis, apoptosis, SOD activity, PO activity, and THC.

1. Introduction

Kuruma shrimp (*Marsupenaeus japonicus*) represent a major marine product which contributes significantly to the aquaculture economy worldwide. Invertebrate animals lack antibodies and, as such, lack an adaptive immune response [1]. The innate immune system in arthropods is composed of a large variety of specific and non-specific responses that are activated in response to the presence of foreign agents. Outbreaks of viral and bacterial diseases in shrimp are on the rise due to high cultivation densities, particularly white spot syndrome disease (WSSD) and vibriosis [2]. Marine immunologists typically accept that crustacean species are protected solely by relying on a robust innate immune system [3].

Harbinger transposon genes are distantly related to transposases characterized by a 3 bp target site duplication (TSD), flanked by a 14–25 bp (or even up to50 bp) terminal inverted repeat (TIRs). The gene also displays a DDD or DDE domain containing a transposase sequence coded by theIS5-like group of bacterial transposons, such as IS5, IS112 and ISL2 [4–6]. This is the first superfamily of eukaryotic DNA transposons where all autosomal transposons, even those that are hosted by species from different kingdoms, encode two proteins: a superfamily-specific transposase and a DNA-binding protein [7]. The proposed target-site specificity of the HARBI1 proteins is indirectly supported by markably strong 17 bp consensus sequence of target sites where the non-autonomous Harbinger 3N_DR transposons are inserted. HARBI1 mRNAs are expressed in a wide variety of adult and embryonic tissues. Such a diverse set of tissues expressing HARBI1 at different developmental stages, implies that this gene is of paramount biological importance [4]. The DDE superfamily contains three carboxylate residues that are believed to be responsible for coordinating metal ions

* Corresponding author.
E-mail address: zhufei@zju.edu.cn (F. Zhu).

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Table 1
Universal and specific primers used in this study.

Primer Name	Nucleotide sequence (5′ →3′)	
3′ race GSP	GACGAGAGACTGAGAGTGTTTACC	first primer for 3′RACE
3′ race NGSP	ATGAAGAAATGGCTTAGACTGGACA	second primer for 3′RACE
5′race sp1	GTGTTGCCAAATATCGCAGAGT	first primer for 5′RACE
5′race sp2	TATCGCAGAGTAAGGGTCAGGC	second primer for 5′RACE
5′race sp3	GATGAAGGAGGTGATCAAATTGC	third primer for 5′RACE
realtime-F	GGATGTTGGTGCTTGTGGTCG	primer for expression
realtime-R	GTCTGAGTTGGGGAGTGTTGAGG	primer for expression
dsRNA-F	CCCAAGCTTGAACGCCTGACCCTTACTCTGC	primer for knock down
dsRNA-R	CTAGCTCGACCACAAGCACCAGGATCCCG	primer for knock down
hemocyanin-RT-R	AACCCTGAACAAAGAGTTGCCTAT	for hemocyanin expression
hemocyanin-RT-R	AACGGACGGTAAGTTGATGATGT	for hemocyanin expression
IMD-RT-F[a]	ATTCATCCGTCTACCTCCCTACA	for IMD expression
IMD-RT-R	GAGCTGAGTCTGTCTTAATGTTATCC	for IMD expression
L-lectin-RT-F	ATGTTATGCCATCTGCCTCGTATTT	for L- lectin expression
L-lectin-RT-R	CTTTCGCTGCTGCTCTTTCTGTT	for L- lectin expression
MAPK-RT-F	CGCATCACTGTTGAGGAGG	for MAPK expression
MAPK-RT-R	GCAGGTCATCAAGTTCCATCT	for MAPK expression
NOS-RT-F	CCAGGATCTTCTTGTTGGTGTTG	for NOS expression
NOS-RT-R	CCCTCATCTGTAGCATAAAGTTCTC	for NOS expression
p53-RT-F	TTCCTGCCTGGCTGACTCTA	for p53 expression
p53-RT-R	CACCCAATCTTCCAACATCACAT	for p53 expression
proPO-RT-F	TTCTACCGCTGGCATAAGTTTGT	for proPO expression
proPO-RT-R	TATCTGCCTCGTCGTTCCTCAC	for proPO expression
STAT-RT-F	TGGCAGGATGGATAGAAGACAAG	for STAT expression
STAT-RT-R	TGAATAAGCTGGGATACGAGGGA	for STAT expression
TNF-RT-F	ACAGACGGTCCAGAGTCCCAAAG	for TNF expression
TNF-RT-R	GCGACGAAGTGAGCCACAGTAA	for TNF expression

[a] IMD, immune deficiency.

needed for catalysis. These genes play a key role in DNA cleavage and transfer in the process of transposases. In addition, they also mediate the fracture and integration of DNA [8]. HARBI1 proteins may act as DNA nucleases involved in crucial cellular functions, including possible DNA rearrangements of the genome. Therefore, HARBI1 functions are expected to be quite general and universally important.

However, we know less about the role of HARBI1 in the innate immunity operating in marine invertebrates. In our previous study (the study is unpublished), *M. japonicus* expression levels of the putative nuclease HARBI1-like superfamily-specific transposase (mjHARBI1-like) significantly increased in response to white spot syndrome virus (WSSV) and *Vibrio alginolyticus* infection. In the present study, the role of mjHARBI1-like was explored in shrimp innate immunity after WSSV or *V. alginolyticus* infection.

2. Materials and methods

2.1. Shrimp challenge and tissue collection

Kuruma shrimp (*M. japonicus*; 15 g/shrimp) were purchased from the marine product market located in Hangzhou, China and cultured briefly in artificial seawater (23°) with air-pump before the works. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Zhejiang A & F University. WSSV (accession no. AF 332093.3) and *Vibrio alginolyticus* were cultured and used to challenge the shrimp following the previous report, respectively [9]. The1×10^4 copies/shrimp of WSSV, and 1×10^4 CFU/shrimp of *V. alginolyticus* dissolved in hyperhaline PBS (NaCl, 12.585 g/L; KCl, 0.315 g/L; KH_2PO_4, 0.27 g/L; Na_2HPO_4, 0.948 g/L; PH, 7.4) were utilized to inject shrimp, respectively. And the hyperhaline PBS injection was used as the control.

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

Total RNA for RACE was isolated immediately using the mirVana miRNA™ Isolation Kit (Ambion, USA), according to the manufacturer's protocol. The RACE experiment was performed based on the known middle fragment using 5′/3′ RACE Kit, 2nd Generation (Roche, Germany), according to the protocol of the manufacturer. The synthetic cDNAs were used for the 3′/5′ -RACE PCR and nest-PCR with 3′ gene-specific primer (3GSP, 3NGSP) or 5′ GSP (5SP1, 5SP2, 5SP3) designed on the core sequence. The following steps were performed as described previously [10]. All primers designed using Primer Premier 5.0 are shown in Table 1.

2.3. Nucleotide sequence and bioinformatics analyses

The assembled sequence was used to examine nucleotide sequence similarities basing on the online BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/). The protein prediction was performed using the open reading frame (ORF) Finder tool (https://www.ncbi.nlm.nih.gov/orffinder/). And the phylogenetic trees based on the amino acid sequences were performed by the neighbor-joining method using ClustalX 1.8 and MEGA7.0.

2.4. Tissue collection and quantitative real-time RT-PCR (qRT-PCR) analysis

The different tissue templates such as gills, hemolymph, intestines, heart, digestive gland, muscles, were collected from normal or challenged shrimp and immediately used for RNA extraction to prevent degradation of the total RNA. The gene expression was analyzed by Two-step real-time quantitative PCR using SYBR Green qRT-PCR assay (Promega, USA). Total RNA of different tissues of normal/infected shrimps was extracted by EASY spin tissue/cell RNA extraction kit (Aidlab, China), 200 ng total RNA was applied for cDNA synthesis by ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Japan). The qRT-PCR was carried out in a Bio-Rad Two Color Real-Time PCR Detection System (Bio-Rad, CA, USA). The amplification cycles of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal control. The $2^{-\Delta\Delta CT}$ method was used to analyze the qRT-PCR data, and the data were shown as means standard deviations (SD). The *t*-test was used to analyze the significant difference in the PCR data [11].

2.5. Prokaryotic expression, purification of mjHARBI1-dsRNA

The primers (shown in Table 1) with specific restriction sites (*Hin*d III and *Bam*H I) were designed from the cloned nucleotide sequence. PCR product digested with *Hin*d III/*Bam*H I was subcloned into LIMTUS 38i Vector (NEB, UK) digested with the same enzymes to gain constructed L38-HARBIplasmid. The L38-HARBI was verified by restriction enzyme digestion and DNA sequencing. Meanwhile, a LIMTUS L38i-EGFP (L38i-EGFP) plasmid was also constructed as the negative control. The recombinant plasmid L38-HARBI was transformed into HT115 (DE3) cells knocked out of RNase III. The following steps were performed as described previously [10].

2.6. Knock down of mjHARBI1-like by RNAi and challenge experiments

MjHARBI1-dsRNA (5 μg/shrimp) was purified and intramuscularly injected into shrimp, and knockdown effects were checked by qRT-PCR. Normal shrimps were randomly distributed into different groups, each group contained 20 individuals. And then, Shrimp were divided into six groups: intramuscular injection with 100 μL PBS alone; intramuscular injection with 100 μL mjHARBI1-dsRNA, followed by 100 μL PBS; intramuscular injection with 100 μL WSSV (10^5 copies/mL) challenge, followed by 100 μL; injection with mjHARBI1-dsRNA for 12 h, followed by 100 μL WSSV challenge; intramuscular injection with 100 μL *V. alginolyticus* (10^5 CFU/mL) challenge, followed by 100 μL; injection with mjHARBI1-dsRNA for 12 h, followed by 100 μL *V. alginolyticus* challenge. Shrimp mortality was monitored every 12 h after the last injection.

2.7. Detection of WSSV copies

Shrimp were injected with WSSV or mixture of WSSV and mjHARBI1-dsRNA. The whole-genome was extracted from shrimp hemocytes collected at 12, 24, and 48 h post injection using DNA extraction Kit (Tiangen, China), according to the manufacturer protocol. To figure the WSSV copies, shrimp gDNAs of each sample were detected by WSSV vp28 probe in Two Color Real-Time PCR Detection System (Bio-Rad, CA, USA). The primer sequences are shown in Table 1. Standard curve was made based on previous experiment [12].

2.8. Determination of immune parameters after RNAi

The immune parameters determined included total hemocyte count (THC), PO activity and SOD activity. THC was determined as previously described [13]. 500 μL hemolymph was collected and mixed with 500 μL 20 mM of EDTA solution to determine PO and SOD activities from individual. Based on the formation of dopachrome from the substrate L-3, 4-dihydroxyphenylalanine (L-DOPA), the PO activity was quantified from the hemolymph mixture as previously described [14]. The SOD activity was quantified from hemocytes isolated from 300 μL of the hemolymph mixture according to the improved method of Beauchamp & Fridovich [15]. Data were presented as a percentage of normal control.

2.9. Apoptosis analysis with annexin V by flow cytometry

Apoptosis assays of shrimp treated with/without mjHARBI1-dsRNA were conducted with BD PhrmingenTM FITC Annexin V Apoptosis Kit (Invitrogen, USA), following to the manufacturer's protocol. The hemolymph was drawn using 2 mL syringe with 20 mM of EDTA at a ratio of 1:1, and hemocytes were collect from the mixture which was centrifuged at 300 g at 4 °C for 5 min. Subsequently, acquired hemocytes were suspended with PBS, counted and adjusted with PBS to a cell density of 5×10^6 cells/mL. And then, the cells were stained and assessed by FACScan at wavelengths of 530 nm and 575 nm. The cell numbers on quadrant 2 and 4, with high annexin V staining, were considered as apoptotic. The data, presented as means ± standard deviation (SD), were derived from at least three independent experiments.

2.10. Phagocytosis detection by flow cytometry

Normal shrimp were separated into two groups, each group contained at least three shrimp. Injecting hyperhaline PBS as control, the other group was injected with mjHARBI1-dsRNA as knock down treatment. 24 h post injection, anti-coagulant soaked syringe was used to collect approximately 1.0 mL shrimp hemolymph of each group. Then, the mix was centrifugalized at 300 g for 5 min, 4 °C, in a horizontal centrifuge and abandoned supernatant to collect hemocytes. After re-suspending the hemocytes softly with precooled sterilized high saline PBS, the hemocytes were counted to adjust the cell of both treatment to the same density (a suitable density about $3–5 \times 10^6$ cells). Subsequently, hemocytes of normal group or knock down group were divided into three groups. Add 20 μL high saline PBS or 20 μL WSSV-FITC/*V. alginolyticus* -FITC into the normal or knock down hemocytes separately, gently mix up, then, incubate the hemocytes at 28 °C for 30 min for phagocytosis. After that, samples were centrifuged at 300 g for 5 min, 4 °C, in a horizontal centrifuge to move the residual FITC-pathogens, repeat twice. In the end, 1% paraformaldehyde in high saline PBS was used to immobilize hemocytes for flow cytometry detection [16].

2.11. Statistical analysis

Quantitative data were expressed as mean ± standard deviation (SD). The statistical differences were estimated by one-way analysis of variance (one-way ANOVA) followed by least-significant differences (LSD) and Duncan's multiple range test. All statistical analyses were carried out using SPSS Statistics version 19 and Graph Pad Prism 5.0. A probability level of 0.01 was used to indicate statistical significance ($P < 0.01$). The statistical significance of shrimp mortality was showed by A probability level of 0.05 ($P < 0.05$).

3. Results

3.1. Sequence, bioinformatics and phylogenetic analysis

MjHARBI1-likecDNA sequence, which had a full length sequence of 1361 bp (Fig. 1), had a 1263 bp open reading frame (ORF) encoding a 420 amino acid residue protein. MjHARBI1-like had a poly (A) tail in the C-terminus. In addition, mjHARBI1-like displayed an estimated molecular mass of 48.216 kDa and a theoretical *p*I of 9.67. The nucleotide and deduced amino acid sequences of the full-length cDNA were shown in Fig. 1.

We performed a comparison of the mjHARBI1-like DDE_Tnp_4domain amino acid sequences with the sequences of previously reported homologues of HARBI1. The analysis of mjHARBI1-like DDE_Tnp_4 domain revealed a 62.15% identity with HARBI1 of *Amphimedon queenslandica* (marine sponge, accession no. XP_011406655.1), a 49.10% identity with HARBI1 of *Lasius niger* (common black ant, accession no. KMQ89503) and a 48.48% identity with HARBI1 of *Xenopus tropicalis* (accession no. XP_017951152.1) (Fig. 2A). A condensed neighbor-joining phylogenetic tree based on the amino acid sequences was constructed by the neighbor-joining method using MEGA7.0 (Fig. 2B). The phylogenetic analysis

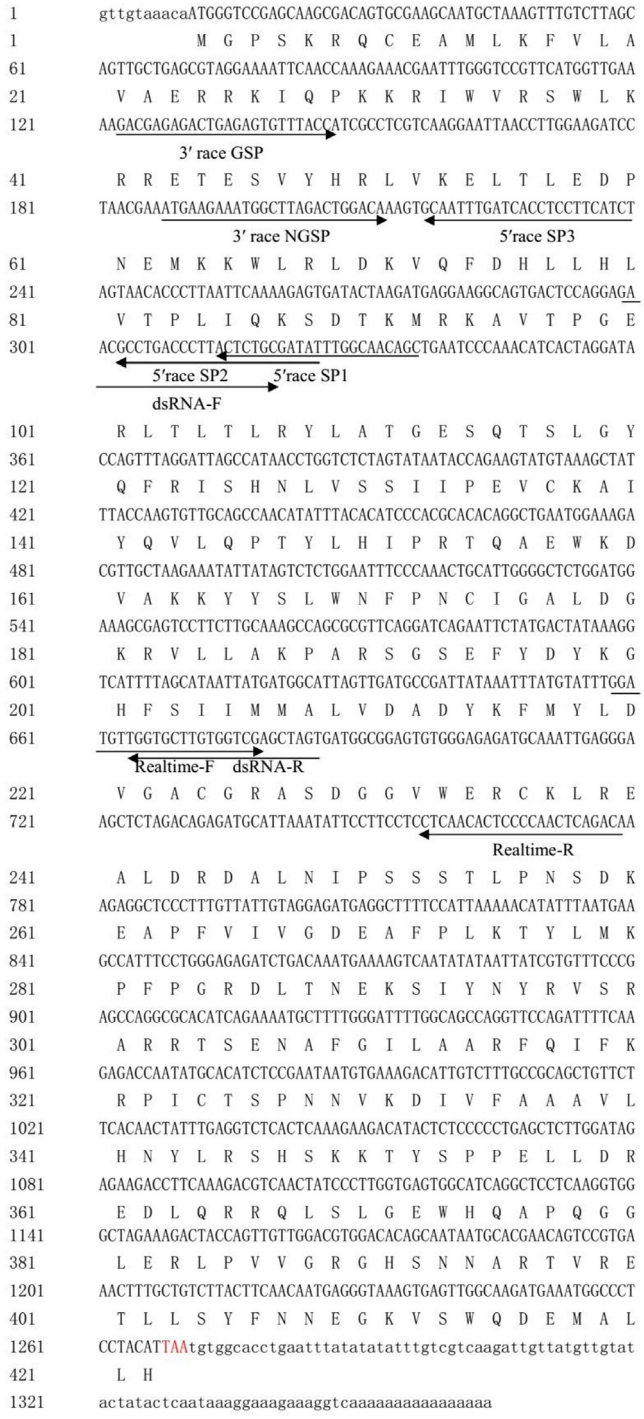

Fig. 1. Nucleotide and deduced amino acid sequence of mjHARBI1-like. The nucleotide sequence is displayed in the 5′–3′directions and numbered at the left. The deduced amino acid sequence is shown in a single capital letter amino acid code. 3′UTR and 5′UTR are shown with lowercase letters. Codons are numbered at the left with the methionine (ATG) initiation codon, and a red denotes the termination codon (TAA). RACE and real-time qPCR primers are marked with arrows.

showed that HARBI1 was relatively conserved among different species. In the amino acid sequence, conserved domains contained several highly conserved amino acid sites.

3.2. Tissue expression difference and stress response

In order to understand the expression profile of the mjHARBI1-like

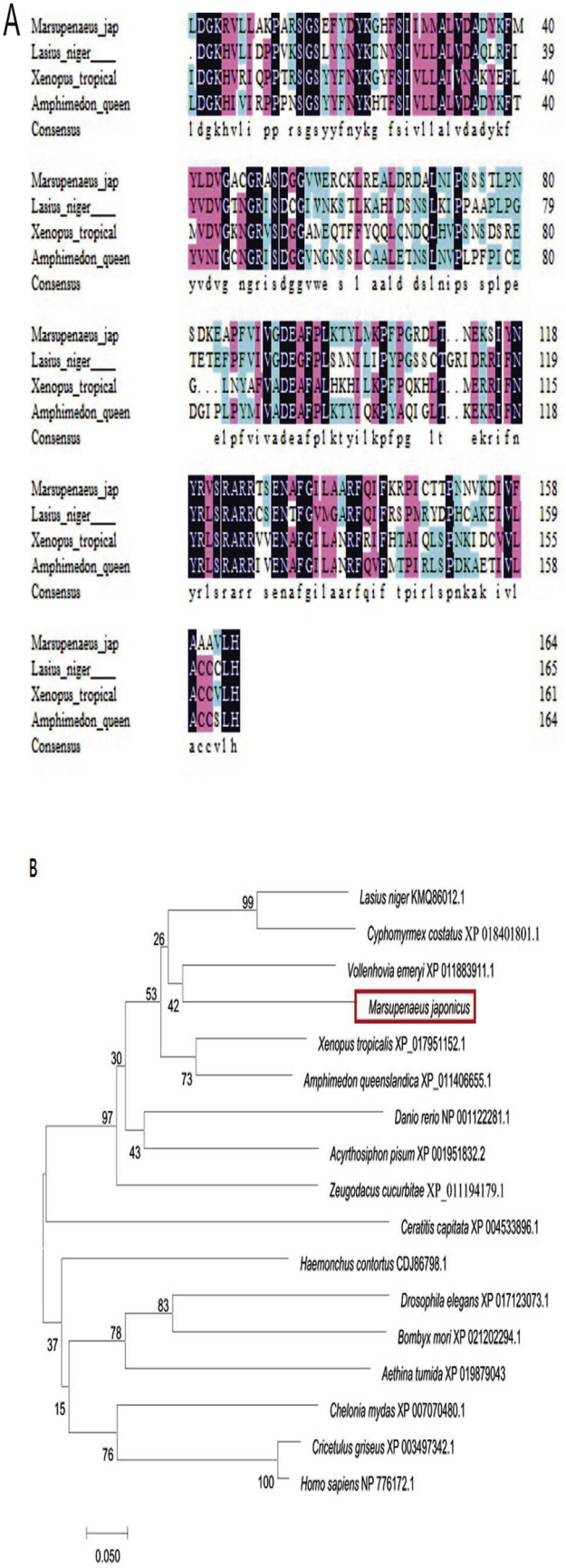

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Fig. 2. Sequence analysis. (A) Multiple alignments of DDE_Tnp_4 domain amino acid sequence of *M. japonicas* mjHARBI1-like with other HARBI1 sequences of Common animals: *Marsupenaeus japonicus* (in this study), *Amphimedon queenslandica* (XP_011406655.1), *Lasius niger* (KMQ89503), *Xenopus tropicalis* (XP_017951152.1). (B) The Neighbor-joining phylogenetic tree ofHARBI1 DDE_Tnp_4 domain from different organisms based on amino acid sequence comparisons. Species names and accession numbers of HARBI1 are listed on the right of the tree.

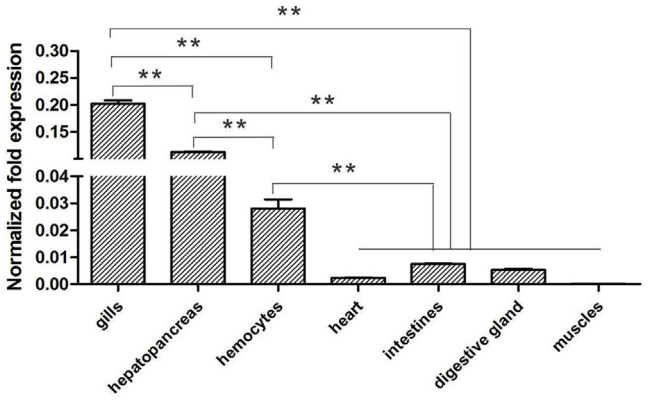

Fig. 3. Expression characterization of mjHARBI1-like, the various tissues were revealed by quantitative real-time PCR from normal shrimp. The amount of mjHARBI1-like mRNA was normalized to the GAPDH transcript level. Data are shown as mean ± SD (standard deviation) of three separate individuals in the tissues. Double asterisks indicate a significant difference ($P < 0.01$) between two samples.

gene in different tissues of *M. japonicas*, qRT-PCR was used (Fig. 3). Based on qRT-PCR analysis, there were significantly high expression levels in gills and hepatopancreas which were noticeably higher than that of other tissues. Among them, mjHARBI1-like showed the lowest expression level in muscle. According to statistical analysis, the expression of mjHARBI1-like in gills and hepatopancreas were significantly higher ($P < 0.01$) than that of other tissues.

After WSSV challenge, the relative mjHARBI1-like expression in the hemocytes was gradually up-regulated between 24 and 48 h post-infection (Fig. 4A), showing the highest level at 48 h post-infection ($P < 0.01$). A similar trend (Fig. 4B) was observed for mjHARBI1-like expression after *V. alginolyticus* challenge. The data indicated a significant up-regulation in shrimp hemocytes from 24 to 72 h after treatment ($P < 0.01$). These results indicated that mjHARBI1-like might play a significant role in combatting WSSV and *V. alginolyticus* infection in shrimp.

3.3. mjHARBI1-like RNAi by dsRNA and effect of mjHARBI1-like on expression of immune genes

The effect of mjHARBI1-dsRNA on the expression of mjHARBI1-like was detected using qRT-PCR. Results showed that the expression of mjHARBI1-like was significantly inhibited by mjHARBI1-dsRNA ($P < 0.01$) in shrimp hemocytes (Fig. 5A).

The expression of important immune genes was analyzed in the hemocytes of *M. japonicus* treated with mjHARBI1-dsRNAs. Of the twelve immune genes analyzed, p53, Toll, proPO, Rho, and myosin were significantly down-regulated ($P < 0.01$), while TNF-αand hemocyanin were significantly up-regulated ($P < 0.01$) at 24 h (Fig. 5B). These results suggested that the knockdown of mjHARBI1-like in shrimp might affect host PO activity, apoptosis, and phagocytosis.

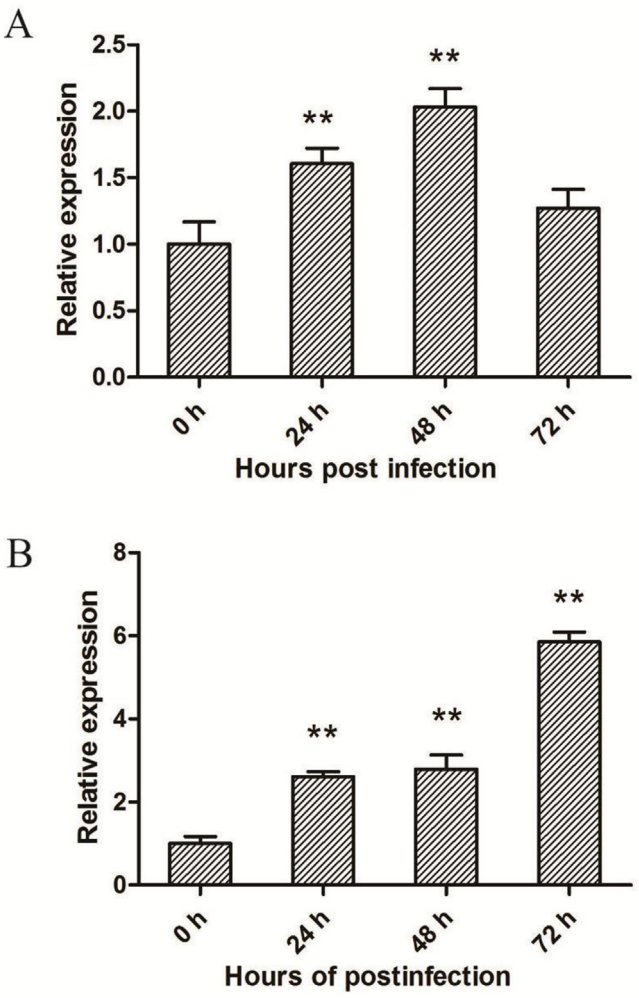

Fig. 4. (A) Real-time RT-PCR analysis of mjHARBI1-like expression in the hemocytes of *M. japonicus* challenged with WSSV. (B) Real-time RT-PCR analysis of mjHARBI1-like expression in the hemocytes of *M. japonicus* challenged with *V. alginolyticus*. The amount of mjHARBI1-like mRNA was normalized to the GAPDH transcript level. Data are shown as means ± SD (standard deviation) of three separate individuals in the tissues. Double asterisks indicate a significant difference ($P < 0.01$) between two samples.

3.4. Kaplan-survival analyze and WSSV copies detection

To evaluate the effects of mjHARBI1-like on the mortality of shrimp challenged with virus or bacteria, mjHARBI1-dsRNA was injected into shrimp and then infected by WSSV and *V. alginolyticus*. The survival data of *V. alginolyticus*-infected shrimp were significantly reduced by the mjHARBI1-dsRNA from 24 to 108 h post challenge ($P < 0.05$) (Fig. 6C). The mjHARBI1-dsRNA and the negative control showed an indistinctively cumulative mortality (Fig. 6C), which indicated that mjHARBI1-dsRNA was not toxic to shrimp. A more remarkable trend (Fig. 6B) was observed for the relative mjHARBI1-like survival percentage after WSSV challenge such that cumulative mortality of WSSV-infected shrimp was significantly less than mjHARBI1-dsRNA + WSSV treated shrimp between 12 and 132 h post challenge. Comparing shrimp mortality between mjHARBI1-dsRNA + pathogen and pathogen only treatments, showed that mjHARBI1-like plays an essential role in WSSV and *V. alginolyticus* infection.

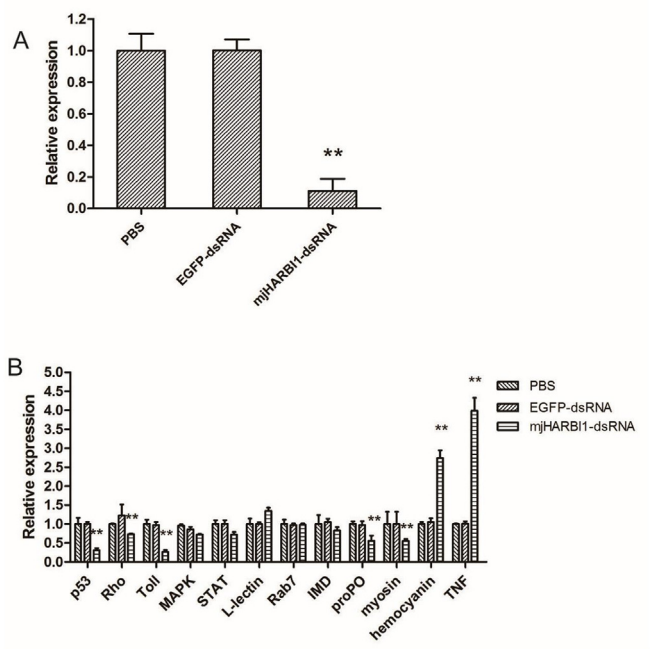

Fig. 5. (A) Real-time RT-PCR analysis of mjHARBI1-like expression in the hemocytes of *M. japonicus* treated with mjHARBI1-like dsRNA at 24 h post treatment. The amount of mjHARBI1-like mRNA was normalized to the GAPDH transcript level. (B) Real-time RT-PCR analysis of nine immune genes expression in the hemocytes of *M. japonicas* treated with mjHARBI1-dsRNA. The amount of mjHARBI1-like mRNA was normalized to the GAPDH transcript level. Data are shown as means ± SD (standard deviation) of three separate individuals in the tissues. Double asterisks indicate a significant difference ($P < 0.01$) between two samples.

With time, the number of WSSV copies rose after WSSV treatment. The number of WSSV copies was always significantly higher in the mjHARBI1-dsRNA-treated group than in the control (Fig. 6A). These results suggest that the presence ofmjHARBI1-like would significantly ($P < 0.01$) restrict the replication of WSSV.

3.5. Effect of mjHARBI1-like knockdown on shrimp immune parameters

The total hemocyte count (THC) in the WSSV-alone group was lower than that in the PBS group at 48 h. THC in the WSSV + mjHARBI1-dsRNA group significantly declined ($P < 0.01$) compared to the WSSV group at 24 and 48 h (Fig. 7A). However, THC in the *V. alginolyticus* group was higher than that in the PBS group and was significantly decreased ($P < 0.01$) in the mjHARBI1-dsRNA + *V. alginolyticus* group compared with shrimp injected with *V. alginolyticus* between 24 and 48 h (Fig. 7B). These results indicate that deficiency of mjHARBI1-likecaused a decrease in THC in both WSSV- or *V. alginolyticus* -challenged shrimp.

Relative PO activity in shrimp hemolymph was significantly lower ($P < 0.01$) in the WSSV + mjHARBI1-dsRNA group compared with the WSSV-alone at 24 h and 48 h post-injection (Fig. 7C). PO activity was reduced following *V. alginolyticus* infection compared with the PBS control group, while shrimp with *V. alginolyticus* + mjHARBI1-dsRNA showed a significant decrease ($P < 0.01$) in PO activity, compared with *V. alginolyticus*-treated shrimp (Fig. 7D). These data suggest that the absence of mjHARBI1-like significantly inhibited PO activity.

In this study, NBT photoreduction methods were used to detect the relative SOD activity in shrimp hemolymph. Compared to the PBS group, relative SOD activity following WSSV infection decreased significantly ($P < 0.01$). Conversely, shrimp with mjHARBI1-dsRNA + WSSV treatment at 24–48 h showed significantly ($P < 0.01$) lower levels of relative SOD activities than with WSSV alone (Fig. 7E). A similar trend was found in *V. alginolyticus*-infected shrimp when mjHARBI1-like was silenced (Fig. 7F). Inhibition of mjHARBI1-like expression lead to a significantly low SOD activity caused by WSSV or *V. alginolyticus* ($P < 0.01$). These results indicated that mjHARBI1-like had a positive effect on shrimp immune parameters.

3.6. Influence of mjHARBI1-like knockdown on hemocytes apoptosis

The apoptosis rate in the PBS group decreased from 41.19% to 25.3%after inhibition of mjHARBI1-like. Compared with the WSSV challenge alone group, the apoptosis result of the shrimp treated withmjHARBI1-dsRNA + WSSV was significantly decreased (33.8% *vs*. 20.62%) ($P < 0.01$) (Fig. 8). These results indicated that mjHARBI1-like was associated with virus-induced apoptosis. In addition, there was a similar trend in *V. alginolyticus* challenged groups. The down-regulation of apoptosis in shrimp hemocytes bymjHARBI1-like inhibition suggested that mjHARBI1-like could positively regulate WSSV-induced apoptosis.

3.7. Influence of mjHARBI1-like knockdown on hemocytes phagocytosis

The proportion of FITC-positive hemocytes in the WSSV groups was significantly up-regulated ($P < 0.01$) from 5.3% to 13.1% after mjHARBI1-like knockdown (Fig. 9) but significantly decreased ($P < 0.01$) from 76.3% to 58.4% after mjHARBI1-like knockdown in *V. alginolyticus* challenged groups (Fig. 9). The increase in FITC-positive cells represents a rise of hemocyte phagocytosis, as indicated by flow cytometry. This indicates that mjHARBI1-like was involved in the phagocytosis of shrimp hemocytes. The effect of the mjHARBI1-like on host phagocytosis was negative following WSSV infection but positive in *V. alginolyticus* challenged shrimp.

4. Discussion

To get the full length cDNA, rapid amplification of cDNA (RACE) was utilized. The cDNA of *M. japonicus* mjHARBI1-like with 1361 bp full length contained 420 amino acids, and was predominantly expressed in immune tissue, such as gills, hemocytes, and hepatopancreas, but it was most abundant in the gills. The report revealed that HARBI1 mRNAs are expressed in a wide variety of adult and embryonic tissues [4]. Such a diverse set of tissues expressing HARBI1 implies that this gene is of paramount biological importance. The mjHARBI1-like might have a similar biological role in shrimp. BLAST analysis of proteins and functional prediction showed that this amino acid sequence is relatively conserved. The neighbor-joining tree method revealed a close evolutionary relationship of this protein to other invertebrates. Pathogens (whether WSSV or *V. alginolyticus*) stimulation led to up-regulation of mjHARBI1-like, indicating its potential role in the innate immune system of shrimp. However, there are hardly any reports about the role of HARBI1 and its crucial DDE_Tnp_4 domain in viral or bacterial infection of arthropod, even other species.

RNA interference (RNAi) using dsRNA to silence genes has been previously applied in studies of shrimp immunity [17–20]. In the present study, *in vitro* dsRNA was synthesized and used to silence the expression of shrimp mjHARBI1-like. The successful knockdown of mjHARBI1-like with unique mjHARBI1-dsRNA provided a practical way to demonstrate its potential function in the shrimp innate immune system.

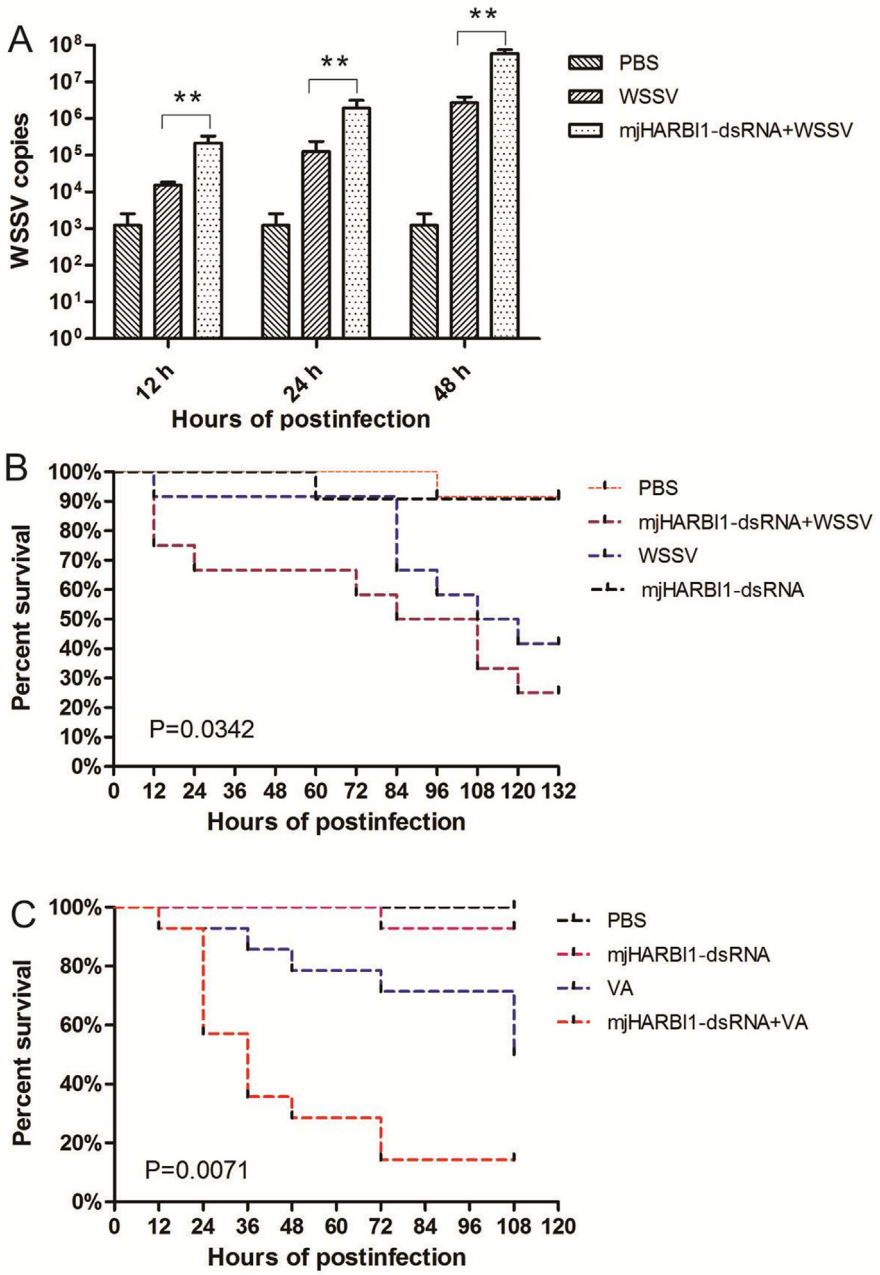

Fig. 6. Effects of mjHARBI1-like knockdown. (A) WSSV copies detected by TaqMan VP28 probe at different times post-infection. (B) Effects of WSSV challenge and mjHARBI1-like inhibition on shrimp mortality. Normal shrimp treated with high-saline PBS were used as control. Each group contained 20 shrimps to guarantee sufficient confidence levels, and treatments were repeated three times to avoid any influences of weather, individual body condition, and injection-operation error. (C) Effects of *V. alginolyticus* challenge and mjHARBI1-like inhibition on shrimp mortality. Primers used are listed in Table 1. (VA= *V. alginolyticus*).

The rapid and efficient innate system that shrimp rely on is sufficient to protect the host from intruding microorganisms [21]. Hemocytes play a key role in the shrimp cellular immune responses, such as phagocytosis, nodule formation, encapsulation and apoptosis. In addition, hemocytes are involved in humoral immune responses, such as the production of lectins, prophenoloxidase, and antimicrobial peptides (AMPs) [22,23]. As a result, analysis of shrimp hemocytes, in particular the production of several known crucial immune molecules, and signal transduction factors, was chosen examine the effect of mjHARBI1-like in shrimp.

Phenoloxidase (PO) is a member of the tyrosinase family of enzymes responsible for the activation of melanogenesis in invertebrates [24]. Furthermore, several studies have determined that PO is an important tool used against several pathogens [23]. The pathogen-recognition receptors (PRRs)recognize bacteria or virus patterns to initiate the proPO system in arthropods [25], and proPO is activated by prophenoloxidase-activating enzyme to convert to mature PO, one of the most important components of the immune defense system [26]. The invasion of WSSV usually cause the significantly change of PO activity in shrimp [10,27,28]. The result that the expression of proPO dropped

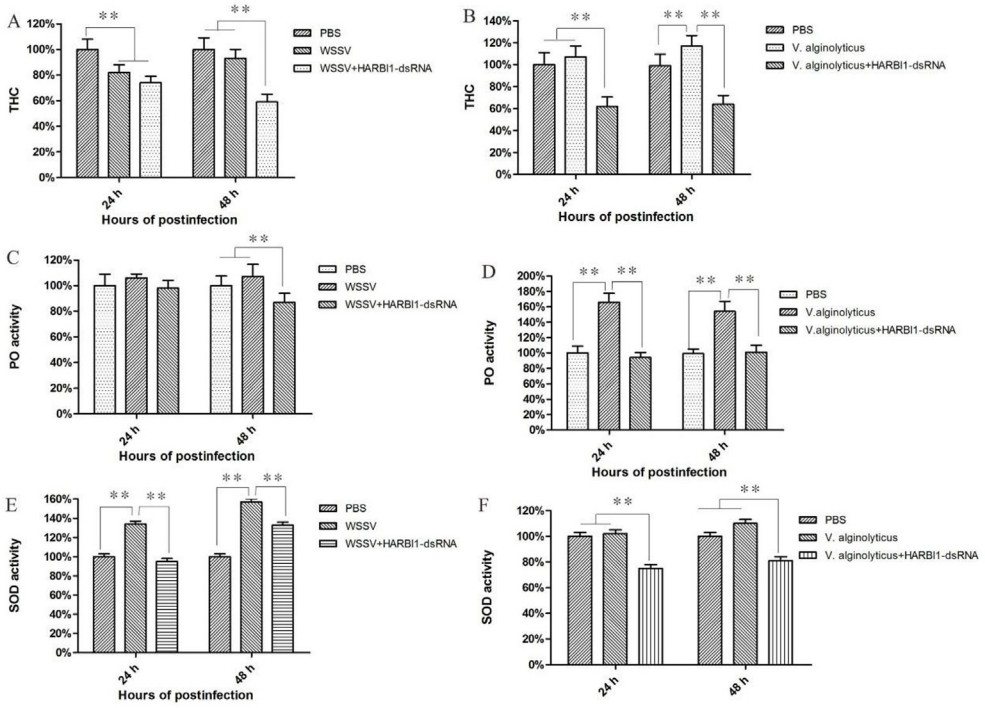

Fig. 7. Effects of mjHARBI1-like knockdown on shrimp immune parameters. Immune parameters including THC, PO activity, and SOD activity were determined in normal or WSSV-treated shrimp. (A) Relative THC after WSSV or WSSV + mjHARBI1-dsRNA treatment; (B) Relative THC after *V. alginolyticus* or *V. alginolyticus* + mjHARBI1-dsRNA treatment; (C) Relative PO activity after WSSV or WSSV + mjHARBI1-dsRNA treatment; (D) Relative PO activity after *V. alginolyticus* or *V. alginolyticus* + mjHARBI1-dsRNA treatment. (E) Relative SOD activity after WSSV or WSSV + mjHARBI1-dsRNA treatment; (F) Relative SOD activity after *V. alginolyticus* or *V. alginolyticus* + mjHARBI1-dsRNA treatment. Data were presented as a percentage of normal control. All treatments at each time point included at least three individuals, and all experiments were repeated three times.

significantly after mjHARBI1-like was silenced suggests mjHARBI1-like might have a positive effect on the regulation of the PO activating system. Furthermore, additional supporting evidence was derived from the detection of PO activity, which significantly decreased in WSSV- or *V. alginolyticus*-challenged shrimp after mjHARBI1-dsRNA treatment.

Toll-like receptors (TLRs) are key pattern recognition receptors of the innate immune system which can protect the host against pathogens in both arthropods and mammals [29]. The recognition of microbial PAMPs by PRRs leads to activation of specific signal pathways and a variety of cell dependent responses, including pro-inflammatory cytokine release, phagocytosis and antigen presentation. However, unlike that observed in vertebrates, an additional extracellular intermediary, called Spatzle, is essential for recognition of bacteria or virus patterns in the Toll pathway. The Toll-like receptor can also induce AMPs and mediate the NK-κB pathway [29]. Reports also show that expression analysis of FcToll showed that its transcriptional level in shrimp was modulated after *Vibrio* or WSSV stimulation [30]. The significant decrease of Toll expression in mjHARBI1-like-knockdown shrimp suggested the mjHARBI1-like might positively regulate expression of Toll to carry out a role in host immunity.

Hemocyanin is the carrier of oxygen and provides energy throughout the shrimp body. In arthropods, hemocyanin can be activated by clotting enzymes that cooperate with AMPs to participate in the innate immune response to pathogen infection and condition-caused stress [31,32]. Hemocyanin is sensitive to stimulations from external conditions as well as to internal stimulation from pathogen invaders [33].

Myosin is a key factor in the signal transduction motility pathway, participating in cell cytoskeleton construction and motility processes [34]. In Kuruma shrimp, myosin and myosin light chains have been reported as a WSSV interacting proteins involved in the antiviral defense mechanism in shrimp immune cells [35]. A role for myosin and myosin light chain in the hemocyte antiviral defense mechanism has been demonstrated by involvement in regulation of phagocytosis in the Kuruma shrimp, *Marsupenaeus japonicas* [36]. The small guanosine triphosphatase (GTPase) Rho, which includes regulation of the formation of stress fibers and focal adhesions, cell motility, cell aggregation, and cytokinesis, is implicated in the enhancement of Ca^{2+} sensitivity of smooth muscle contraction by GTP [37,38]. In the mjHARBI1-like knockdown experiments, the expression levels of the Rho declined significantly while myosin had a contrary result. The phagocytosis rate was shown to decrease in mjHARBI1-like-deficient cells in the *V. alginolyticus*-treated group suggesting that mjHARBI1-like could positively regulate hemocyte phagocytosis in the presence of bacteria. However, there was a distinct trend in WSSV-infected shrimp inhibiting hemocyte phagocytosis. This will be explored further in future studies.

As one kind of pre-inflammation factor, tumor necrosis factor-α (TNF-α) kinetin, is produced by macrophages [39]. TNF- α is one of the pro-inflammatory cytokines that can promote inflammation and the apoptotic response; in mammals, the up-regulation of TNF-α can lead to several neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease [40]. Likewise, the protein p53 can help to prevent cancer via initiating programed cell death, i.e. apoptosis [41]. Meanwhile, p53 connects, interacts, and cooperates with other proteins to regulate the process of programmed cell death, including phagocytosis-induced apoptosis [42]. In the present study, the expression of p53 was up-regulated by mjHARBI1-like while TNF-α had an opposite trend, indicating a positive role for mjHARBI1-like in apoptosis. Next, apoptosis of hemocytes was analyzed using the Annexin V by flow cytometry. The results showed that hemocyte apoptosis was inhibited when the expression of mjHARBI1-like was silenced in WSSV- or *V. alginolyticus*-challenged shrimp.

Kaplan-survival analysis showed that *V. alginolyticus*-challenged shrimp had a considerably lower survival rate after mjHARBI1-like-knockdown. There was a similar trend in WSSV-infected shrimp where the lack of mjHARBI1-like significantly increased the number of WSSV in shrimp at 12, 24and 48 h post challenge compared with the control. In WSSV-infected shrimp, as the mjHARBI1-like expression was ablated,

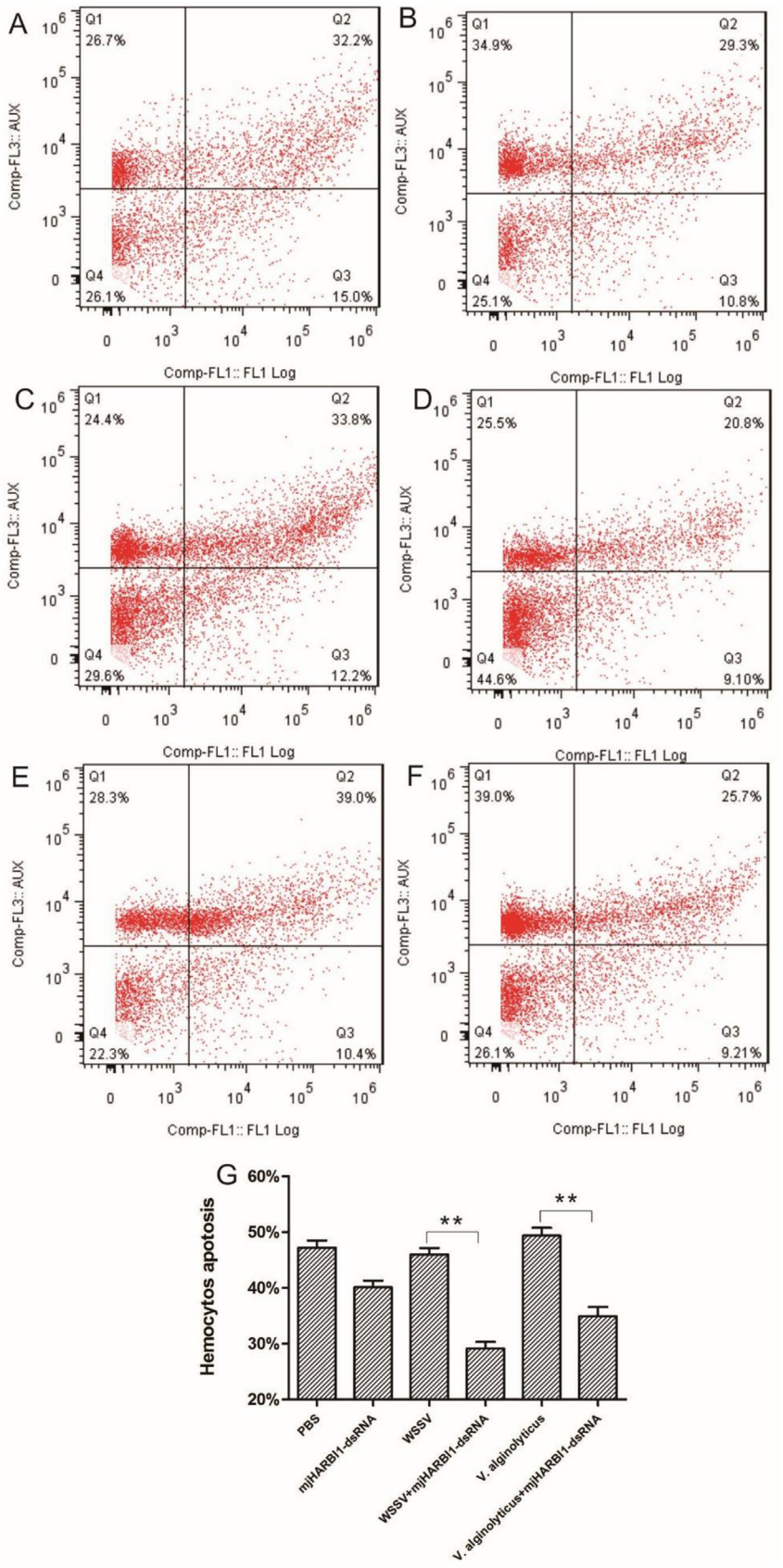

Fig. 8. Effects of mjHARBI1-dsRNA interference on hemocyte apoptosis. Samples were taken at 24 h post-injection. (A) PBS treatment; (B) mjHARBI1-dsRNA treatment; (C) WSSV treatment; (D) WSSV + mjHARBI1-dsRNA treatment; (E) *V. alginolyticus* treatment; (F) *V. alginolyticus* + mjHARBI1-dsRNA treatment; (G) column chart of apoptosis. Q1 area represents false positives caused by cell damage, Q2 represents late-stage apoptotic hemocytes, B4 represents negative (normal) hemocytes, and B3 represents early-stage apoptosis. The annexin V peak represents the sum of the Q2 and Q4 areas (total value of annexin V-positive hemocytes), and is shown in the column chart. The percentage of annexin V-positive hemocytes represents the apoptosis rate in each group. All treatments included at least three shrimp individuals, and all experiments were repeated three times. Each column represents the mean value of triplicate assays.

THC and SOD activities were also significantly inhibited. Similar results were found in *V. alginolyticus*-challenged shrimp as well.

These findings indicate that shrimp mjHARBI1-like plays an active and essential role in the innate immune system of shrimp. This protein could regulate the host's defense against viral infection through regulating apoptosis, PO activity, and SOD activity, while also having a

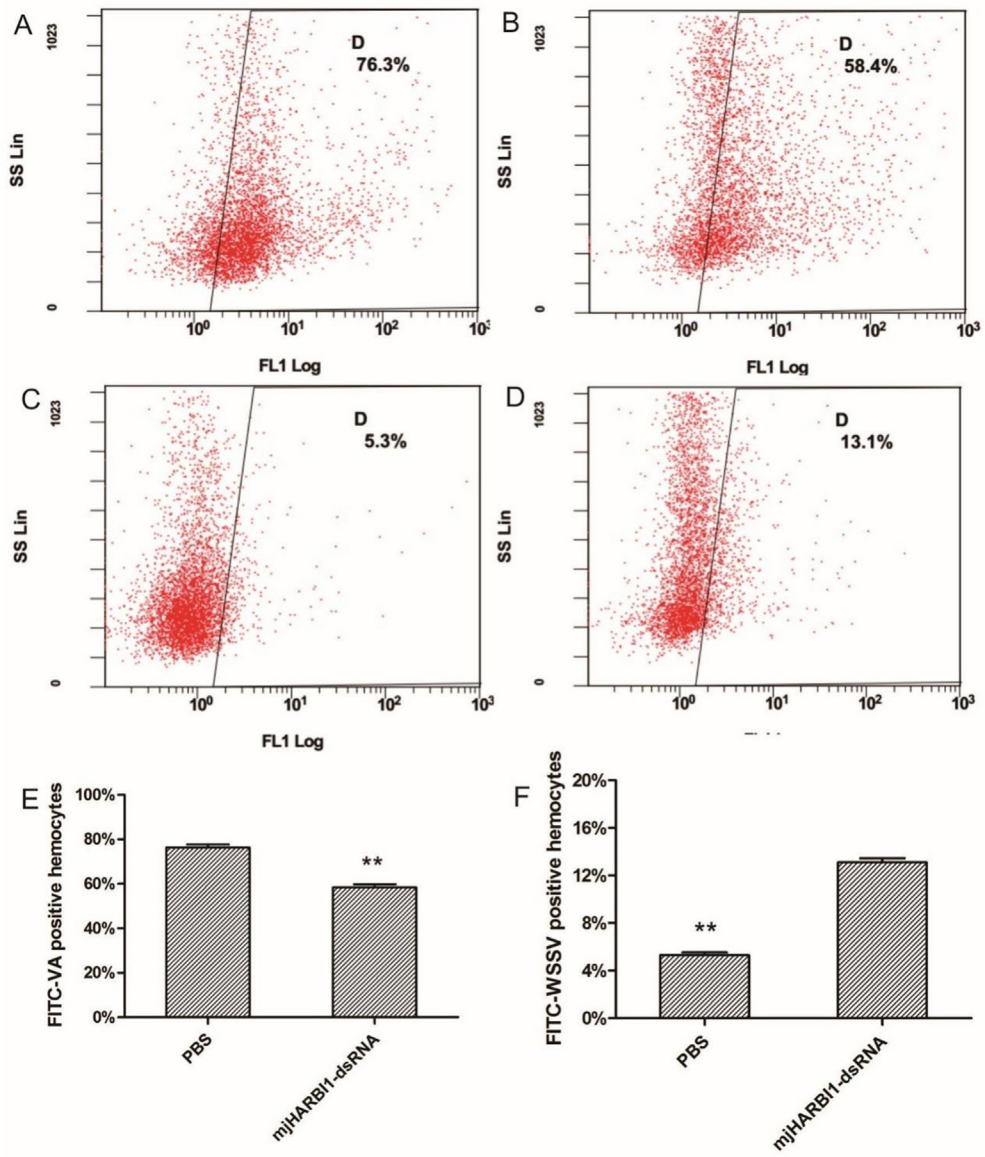

Fig. 9. Influence of mjHARBI1-like knockdown on hemocyte phagocytosis detected by flow cytometry. Hemocyte phagocytosis was detected in hemolymph from normal shrimp treated with high-saline PBS or mjHARBI1-dsRNA. FITC-labeled WSSV virions or *V. alginolyticus* particles were added to normal or mjHARBI1-inhibited hemocytes. (A) WSSV treatment; (B) WSSV + mjHARBI1-dsRNA treatment; (C) *V. alginolyticus* treatment; (D) *V. alginolyticus* + mjHARBI1-dsRNA treatment; (E) Column chart of phagocytosis after WSSV or WSSV + mjHARBI1-dsRNA; (F) Column chart of phagocytosis after *V. alginolyticus* or *V. alginolyticus* + mjHARBI1-dsRNA. All treatments included at least three shrimp individuals, and all experiments were repeated three times. Each column represents the mean value of triplicate assays.

positive *anti-V. alginolyticus* effect by regulating phagocytosis, apoptosis, PO activity, and SOD activity.

Conflicts of interest

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

Acknowledgments

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