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Molecular characterization of minichromosome maintenance protein (MCM7) in *Scylla paramamosain* and its role in white spot syndrome virus and *Vibrio alginolyticus* infection

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ABSTRACT

The minichromosome maintenance protein (MCM7) is a member of the MCM protein family which participates in the MCM complex by playing a role in the cell replication cycle and chromosome initiation in eukaryotes. The 2270 bp cDNA sequence of *MCM7*, including a 2127-bp open reading frame (ORF) encoding a 709-aa protein, was cloned from *Scylla paramamosain* using RT-PCR and RACE. Data showed that *MCM7* was highly expressed in the digestive organ and hepatopancreas of *S. paramamosain*. Furthermore, *MCM7* expression was down-regulated by infection with white spot syndrome virus (WSSV) or *Vibrio alginolyticus*. When *MCM7* was knocked down, immune genes such as Janus kinase (JAK) and crustin antimicrobial peptide (CAP) were down-regulated, and C-type-lectin (CTL) was up-regulated in hemocytes. The mortality of WSSV-infected or *V. alginolyticus*-infected crabs was enhanced following *MCM7* knockdown. It was demonstrated that *MCM7* is very important in the progression of WSSV and *V. alginolyticus* infection. We also investigated the effect of *MCM7* on apoptosis rate and phagocytic rate in *S. paramamosain*. *MCM7* knockdown caused higher levels of apoptosis in the hemocytes of the control, WSSV, and *V. alginolyticus* groups. *MCM7* knockdown influenced the activity of phenoloxidase (PO) and superoxide dismutase (SOD), and total hemocyte count (THC) after infection with WSSV or *V. alginolyticus*, which indicated that MCM7 plays a regulatory role in innate immunity of crabs. Thus, we conclude that MCM7 may participate in the anti-WSSV and *V. alginolyticus* immune response in crabs by regulating apoptosis and the activity of PO and SOD.

1. Introduction

The immune system is commonly divided into two major branches: innate and adaptive [1]. Because they lack the complexity of an adaptive immune system, all crustaceans including *Scylla paramamosain* rely solely on innate immunity to maintain a highly efficient defense system against infections [2,3]. As two of the most serious diseases in crabs, vibriosis and white spot syndrome virus (WSSV) have caused irreversible damage to the crab culture industry worldwide [4]. However, there are currently no efficient measures to control these diseases.

In all eukaryotes, the minichromosome maintenance protein has been first found in budding Yeast, *Saccharomyces cerevisiae* [5]. To initiating DNA replication, assembly of Cdc6, MCM protein complex, and Cdc45p is required to binding to the origin recognition complex (ORC) throughout the cell cycle [6–9]. MCM protein complex is consist of MCM2-7 [6,7,10], and three more members recently have been identified as MCM8 [11], MCM9 [12], and MCM10 [13]. MCM7 are key elements that function as part of a pre-replication complex to initiate DNA replication in eukaryotes [7,8,10]. MCM7 participates in the MCM complex by playing a critical role in the cell replication cycle and chromosome initiation in eukaryotes [14]. MCM7 is a marker for proliferation and is up-regulated in a variety of tumors including neuroblastoma, prostate, cervical, and hypopharyngeal carcinomas [15]. Notably, MCM7 is the only MCM family member whose promoter contains an E-box sequence, which is the binding site for members of the MYC transcription factor family [16]. MCM7 proteins were originally identified through a yeast genetic screen aimed to identify mutants defective in MCM [17]. Recent studies have reported a novel function of MCMs in cancer cells, where MCM7 was found to serve as an efficient prognostic marker and a potential therapeutic target in human cancer [17–20].

In our previous study, we found that MCM7 regulates phagocytosis in kuruma shrimp *Marsupenaeus japonicus* against WSSV [21]. In the current study, we aimed to explore the role of MCM7 in the innate

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immune system of crabs.

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. 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, sex glands and hemolymph 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 [22]. *V. alginolyticus* was cultured and used to challenge the crabs according to the previous report [23].

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) or 5′ GSP (5GSP2, 5NGSP2), designed on the basis of middle sequence (the primers sequences are shown in Table 1). The PCR products were purified using MiniBEST DNA Fragment Purification Kit Ver.3.0 (Takara, Japan), following the manufacturer's instruction. Amplified cDNA fragments were transferred into the pMD19-T vector (Takara, Japan). Recombinant bacteria were identified by blue/white 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 MCM7 mRNA expression levels in various adult tissues were measured by qRT-PCR using a SYBR II® Premix Ex Taq (Tli Rnase Plus) (TaKaRa, Japan). Total RNA was isolated from various tissues of healthy adult crabs and hemocytes of crabs challenged by intramuscular injection of 0.2 mL of viral or bacterial suspension including WSSV (10^5 copies/mL) or *V. alginolyticus* (10^5 colony-forming units [CFU]/mL), respectively, for different times, using the EASY spin tissue/cell RNA extraction kit (Aidlab, China) according to the manufacturer's instructions. Experiments were performed in triplicate and at least three crabs were analyzed for each tissue type. 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 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 $2^{-\Delta\Delta CT}$ comparative CT method by Office Excel, with GAPDH amplification as the internal control [24]. 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, purification of MCM7-dsRNA

The primers (shown in Table 1) with specific restriction sites (*Hin*d III in the forward primer and *Sal* I in the reverse primer) were designed from the cloned nucleotide sequence. PCR product digested with *Hin*d III/*Sal* I was subcloned into LIMTUS 38i Vector (NEB, MA, USA)

Table 1
Primer sequences and purpose.

Name	Nucleotide Sequence (5′ to 3′)	purpose
3′ race GSP1	TGGTGTAGGTCTCACTGCTGCTGTC	first primer for 3′ RACE
3′ race NGSP1	CTGGGATAATGACGAGCCTCAATGC	second primer for 3′ RACE
5′ race GSP2	GGGGCAATTGAAGTCGCTAGTT	first primer for 5′ RACE
5′ race NGSP2	TGCCTTGAAGCCTTGACGAACC	third primer for 5′ RACE
MCM7-realtime-F	TTTGACCTCCTTTGGCTTATTC	for MCM7 expression
MCM7-realtime-R	TTTGTTGTTGCGTGCTTCCTT	for MCM7 expression
GAPDH-realtime-F	ACCTCACCAACTCCAACAC	for GAPDH expression
GAPDH-realtime-R	CATTCACAGCCACAACCT	for GAPDH expression
MCM7 dsRNA F	*AGCTTGCCGCAAGCACTTCAAGTAG*	for MCM7 RNAi
MCM7 dsRNA R	*TCGACTACTTGAAGTGCTTGCGGCA*	for MCM7 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

Fig. 1. Nucleotide and deduced amino acid sequence of MCM7. 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 (GAA). RACE and real-time qPCR primers are marked with arrows.

digested with the same enzymes to gain plasmid L38-MCM7. The following process was prepared according to the previous reports [25].

2.6. Knock down of MCM7 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. MCM7-dsRNAs (75 μg/crab) was immediately injected intramuscularly into the fourth pereopodcoxa of each crab [25], and MCM7 mRNA expression levels were detected by qRT-PCR following WSSV and *V. alginolyticus* challenges.

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 MCM7-dsRNA group received injections of MCM7-dsRNA alone, the WSSV group received injections of WSSV in PBS, and the MCM7-dsRNA + WSSV group received injections of MCM7-dsRNA and WSSV, the *V. alginolyticus* group received injections of *V. alginolyticus* in PBS, and the MCM7-dsRNA + *V. alginolyticus* group received injections of MCM7-dsRNA and *V. alginolyticus*. 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. Phagocytic rate counting by flow cytometry

Experimental and control groups were injected with dsRNA and PBS, respectively. In brief, 2.5 mL syringe was used to collect the hemolymph from the last walking legs of crabs which sterilized with 70% alcohol with half volume of pre-cooled anticoagulant solution (20 mM EDTA, PH 6.4), and the mixture was centrifuged at 2000 rpm at 4 °C for 10 min to collect hemolymph cells. The subsequent experiment was performed as described previously [26].

2.9. 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, counted and adjusted to a density of 3–5 × 10^6 cells/mL with PBS. The cells were stained using a BD Phrmingen™ FITC Annexin V Apoptosis Kit, and assessed by flow cytometry. The cell numbers on quadrant 4, with low PI and high annexin V staining, were considered as apoptotic. The data were presented as means ± standard deviation (SD) derived from at least three independent experiments.

2.10. 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 [27]. To determine PO and SOD activities, 500 μL of hemolymph was withdrawn into a 1 mL syringe containing 500 μL of 20 mM EDTA solution from each individual crab. PO activity was quantified in the hemolymph mixture based on the formation of dopa chrome from the substrate L-3, 4-dihydroxyphenylalanine (L-DOPA), as described previously [27]. SOD activity was quantified in hemocytes isolated from 300 μL of the hemolymph mixture, according to the improved method described by Beauchamp and Fridovich [28]. Data were presented as a percentage of the normal control.

2.11. Statistical analysis

Quantitative data were expressed as mean ± standard deviation (SD). Data from three independent experiments were analyzed by one-

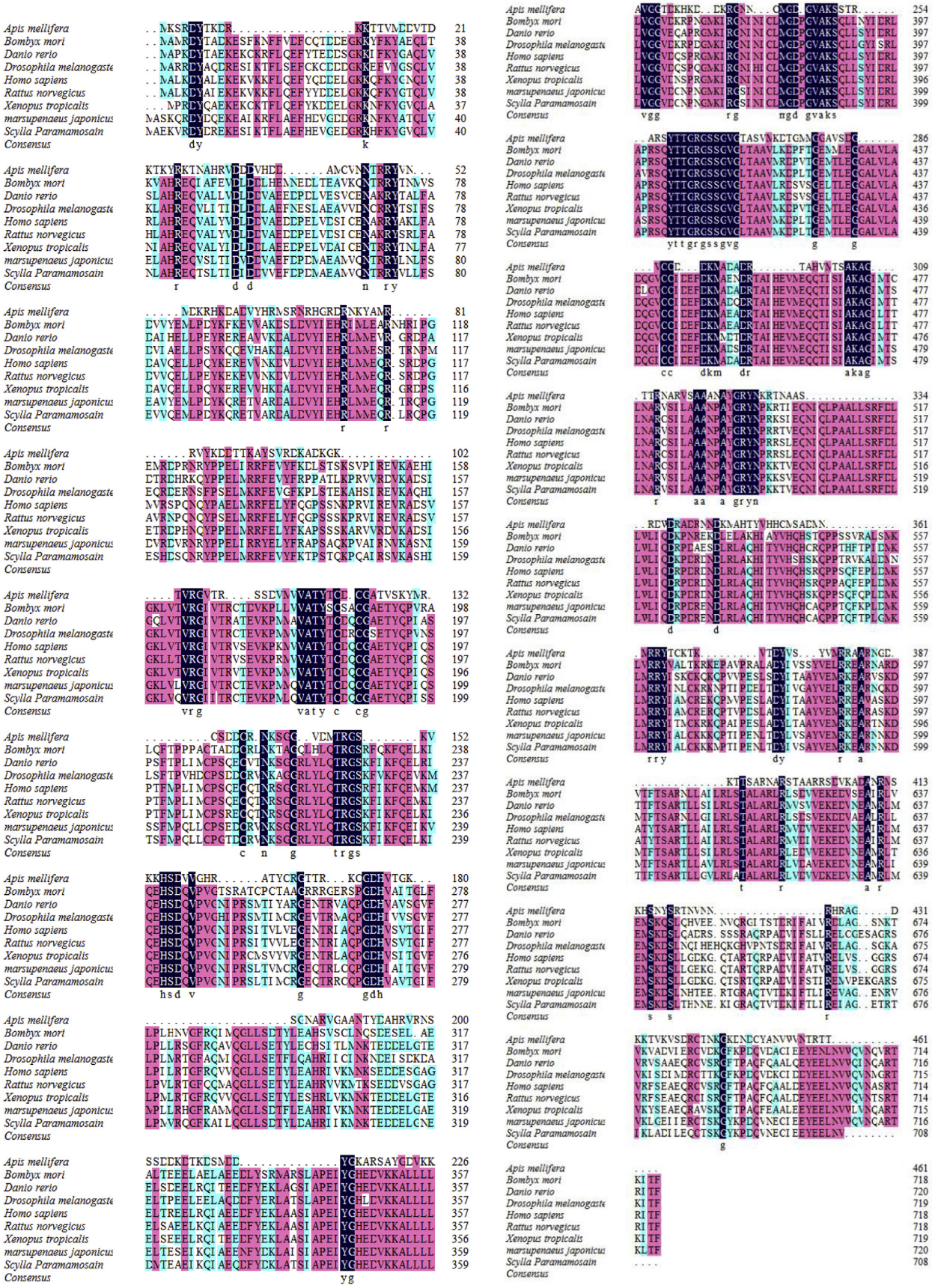

Fig. 2. Multiple alignments of the amino acid sequence of *Scylla paramamosain* MCM7 with other MCM7 sequences of common animals. *S. paramamosain* (in this study), *Apis mellifera* (XP_393469.2), *Bombyx mori* (XP_393469.2), *Danio rerio* (NP_997734.1), *Drosophila melanogaster* (NP_523984.1), *Homo sapiens* (AAH13375.1), *Rattus norvegicus* (NP_001004203.3), *Xenopus tropicalis* (NP_998877.1), and *Marsupenaeus japonicus*. Twelve conserved cysteines (C1 −C12) are shaded and boxed.

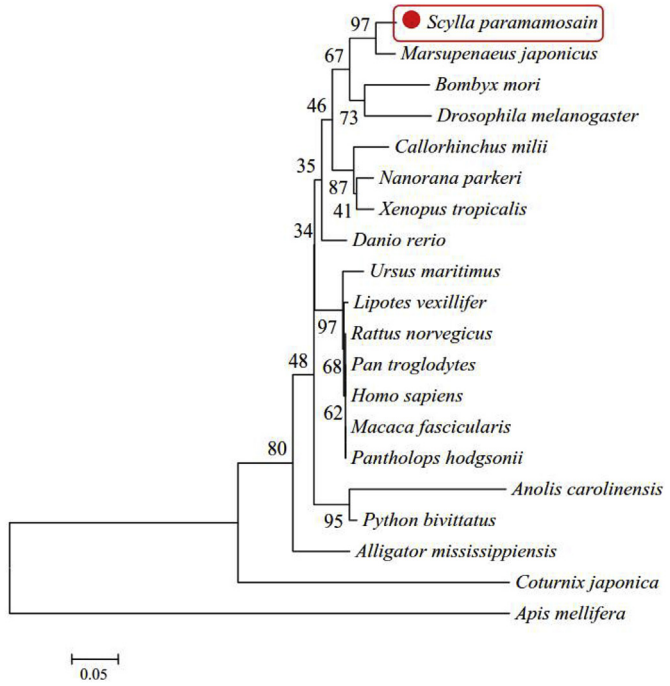

Fig. 3. The phylogenetic tree of MCM7 from different organisms based on amino acid sequence comparisons.

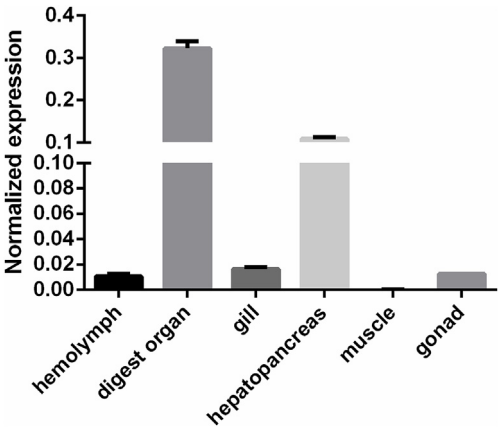

Fig. 4. Expression characterization of *MCM7* in various tissues from healthy *Scylla paramamosain* revealed by quantitative real-time PCR. The amount of *MCM7* mRNA was normalized to the *GAPDH* transcript level. Data are shown as means ± standard deviation of the tissues of three separate individuals. Capital letters indicate expression of *MCM7* in different adult tissues. Double asterisks indicate a significant difference ($P < 0.01$) between two samples.

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

3. Results

3.1. Characterization of MCM7 cDNA

The full-length *MCM7* cDNA sequence was 2270 base pairs (bp), including a 2127 bp open reading frame (ORF) encoding a 709 amino acid protein. The 5′ and 3′ untranslated regions (UTR) of *MCM7* were 124 bp and 19 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 amino acid sequences of MCM7 were then compared with sequences of MCM7 sequences of other species covering vertebrates and invertebrates using DNAMAN version 6.0. The results revealed a similarity of ~86% with *Marsupenaeus japonicus*, 72% with *Xenopus tropicalis*, 71% with *Danio rerio*, 70% with *Drosophila melanogaster*, 69% with *Homo sapiens*, 68% with *Rattus norvegicus* and 66% with *Bombyx mori* (Fig. 2).

A condensed phylogenetic tree based on the deduced amino acid sequences was constructed by the neighbor-joining method using MEGA7.1 (Fig. 3). Phylogenetic analysis showed that the *MCM7* sequence was conserved among different species. The amino acid sequence revealed that conserved domains contained several highly conserved amino acid sites. Among the known species, *S. paramamosain* MCM7 showed the closest relationship with that of *M. japonicus*.

3.3. Tissue distribution of MCM7 expression

Expression profiling of *MCM7* in different tissues of *S. paramamosain* was examined with quantitative real-time polymerase chain reaction (qRT-PCR) (Fig. 4). It was more highly expressed in the digestive organ compared with other tissues and the lowest expression was in muscle tissue. Expression levels of *MCM7* in digest organ were 32-, 20-, 1882.35-, 2.93- and 25-fold greater than that in the hemolymph, gills, muscle, hepatopancreas and gonad, respectively. *MCM7* expression in the digestive organ was thus significantly higher ($P < 0.01$) than in any other tissues.

3.4. Time course of MCM7 expression after WSSV or V. alginolyticus challenge

We investigated the variation trend of *MCM7* expression in crabs

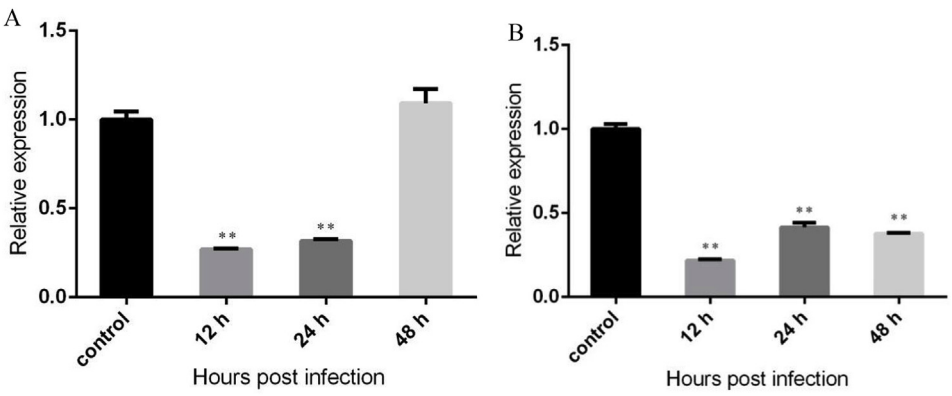

Fig. 5. Real-time RT-PCR analysis of *MCM7* expression challenged with WSSV or *Vibrio alginolyticus*. (A) Real-time RT-PCR analysis of *MCM7* expression in the hemocytes of *Scylla paramamosain* challenged with WSSV. (B) Real-time RT-PCR analysis of *MCM7* expression in the hemocytes of *S. paramamosain* challenged with *V. alginolyticus*. The amount of *MCM7* mRNA was normalized to the *GAPDH* transcript level. Data are shown as means ± standard deviation of three separate individuals. Double asterisks indicate a significant difference between two samples ($P < 0.01$).

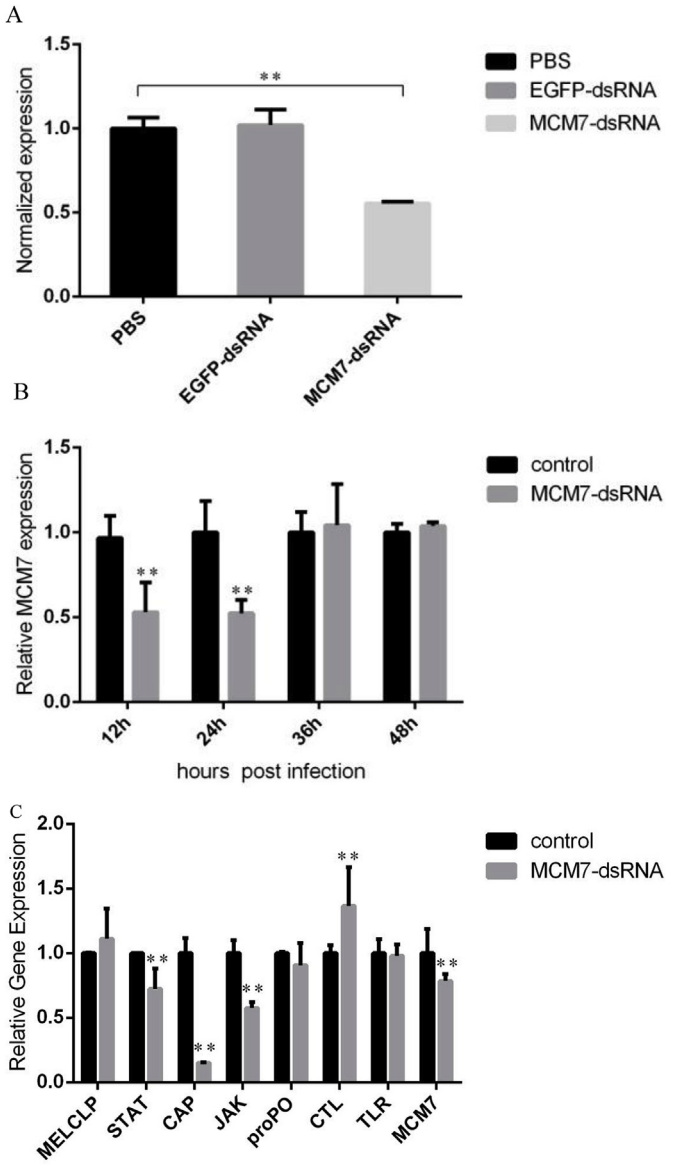

Fig. 6. Real-time RT-PCR analysis of *MCM7* and immune gene expression. (A) Real-time PCR analysis of MCM7 expression in the hemocytes of *Scylla paramamosain* treated with *MCM7* dsRNA (MCM7-dsRNA) at 24 h post-treatment. The amount of *MCM7* mRNA was normalized to the *GAPDH* transcript level. (B) Real-time RT-PCR analysis of *MCM7* expression of hemocytes of *S. paramamosain* treated with MCM7-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 the hemocytes of *S. paramamosain* treated with MCM7-dsRNA. The amount of *MCM7* mRNA was normalized to the *GAPDH* transcript level. Data are shown as means ± standard deviation of tissues in three separate individuals. Double asterisks indicate a significant difference between two samples ($P < 0.01$).

after the challenge with WSSV or *V. alginolyticus*. *MCM7* expression changed after the challenge in a time-dependent manner. *MCM7* expression was significantly down-regulated ($P < 0.01$) from 12 to 24 h post-infection in crabs with WSSV infection, with the lowest expression levels at 12 h, but gradually returned to control levels after 24 h post-challenge (Fig. 5A). *MCM7* expression was significantly up-regulated ($P < 0.01$) at 12 and 48 h post-challenge with *V. alginolyticus* (Fig. 5B). The results suggest that MCM7 may play an important role in the innate immunity of the crab in response to WSSV or *V. alginolyticus* infection.

3.5. Effects of MCM7 knockdown on expression of immune genes

We tested the effect of MCM7 double-stranded RNA (MCM7-dsRNA) on its mRNA expression using RT-PCR. *MCM7* mRNA expression in hemocytes was significantly knocked down by MCM7-dsRNA ($P < 0.01$) (Fig. 6A). We also detected the effect of MCM7-dsRNA on *MCM7* gene expression in hemocytes of *S. paramamosain* at different times post-treatment using RT-PCR. MCM7-dsRNA significantly inhibited the expression of *MCM7* mRNA in hemocytes from 12 to 48 h post-treatment ($P < 0.01$) (Fig. 6B). We also examined the relationship between *MCM7* expression and the expression levels of important immune genes in the hemocytes of crabs. Among seven immune genes, JAK and crustin antimicrobial peptide (CAP) were down-regulated significantly ($P < 0.01$), while C-type-lectin (CTL) were significantly up-regulated ($P < 0.01$) following MCM7-dsRNA treatment (Fig. 6C).

3.6. Effects of MCM7 knockdown on immune parameters

The total hemocyte count (THC) in the WSSV group, the WSSV + MCM7-dsRNA group, *V. alginolyticus* group, and *V. alginolyticus* + MCM7-dsRNA group were significantly lower than that in the PBS group at 24 and 48 h ($P < 0.01$, Fig. 7 B, C). As in these challenge groups, THC was significantly reduced in the MCM7-dsRNA group compared with the PBS group ($P < 0.01$), and the THC of the MCM7-dsRNA group reached the lowest value at 48 h (Fig. 7A). The results indicated that the THC decreased in crabs after infection with WSSV or *V. alginolyticus* and that this was exacerbated by the inhibition of MCM7 expression.

Phenoloxidase (PO) activity in crab hemolymph was significantly reduced at 48 h after MCM7-dsRNA treatment compared with the PBS group ($P < 0.01$, Fig. 7A). PO activity was significantly reduced at 24 and 48 h post-WSSV infection compared with the PBS control group ($P < 0.01$), and was significantly higher in the WSSV + MCM7-dsRNA group compared with the WSSV group at 24 and 48 h (Fig. 7B). Data suggested that the absence of MCM7 significantly ($P < 0.01$) irritated PO activity after WSSV infection. However, PO activity was significantly ($P < 0.01$) increased at 24 h after *V. alginolyticus* infection compared with the PBS group and was significantly lower in the *V. alginolyticus* + MCM7-dsRNA group compared with the *V. alginolyticus* alone at 24 and 48 h post-infection (Fig. 7C). Data suggested that MCM7 knockdown significantly inhibited PO activity of hemolymph during *V. alginolyticus* infection ($P < 0.01$).

Crabs treated with MCM7-dsRNA showed a significantly ($P < 0.01$) lower SOD activity than the PBS group (Fig. 7). The WSSV group showed lower SOD activities than the controls at 24 and 48 h post-infection, but WSSV + MCM7-dsRNA group showed significantly ($P < 0.01$) higher SOD activities than the WSSV group at 48 h post-infection (Fig. 7B). Significantly lower levels of SOD activity were found in the *V. alginolyticus* and *V. alginolyticus* + MCM7-dsRNA groups after 24 and 48 h than in the PBS group ($P < 0.01$, Fig. 7C). These results indicate that MCM7 had exerted stimulatory properties on the immune parameters of the crab.

3.7. Effects of MCM7 knockdown on the survival of challenged crab

We evaluated the effects of MCM7 on the mortality of pathogen-challenged crabs by injecting them with MCM7-dsRNA and then challenging them with *V. alginolyticus* or WSSV. The results of the WSSV-infected mortality count agree with the WSSV copy assay. The mortality count of the WSSV + MCM7-dsRNA group was lower than the WSSV group from 24 to 96 h but showed a higher mortality count than the WSSV group from 96 to 216 h post-challenge (Fig. 8A). The negative control showed a similar mortality to the MCM7-dsRNA group, indicating that MCM7-dsRNA itself was non-toxic in crabs. However, MCM7-dsRNA had a different effect on the mortality of *V. alginolyticus*-infected crabs. The cumulative mortality following *V.*

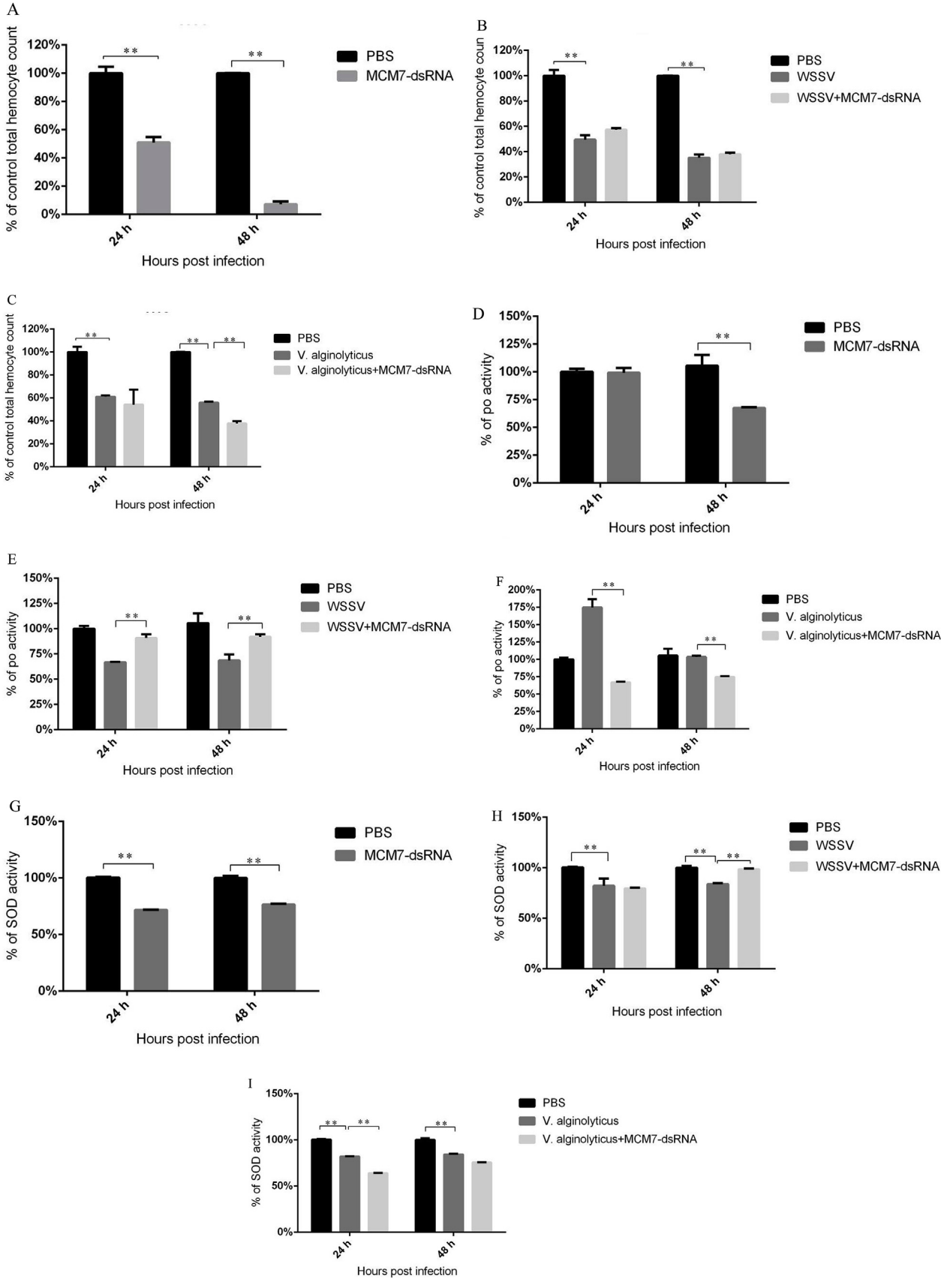

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Fig. 7. Effects of *MCM7* knockdown on crab immune parameters, including THC and the activity of PO and SOD, as determined in healthy, WSSV-treated, or *Vibrio alginolyticus*-treated crabs. (A) THC after PBS or MCM7-dsRNA treatment; (B) THC after WSSV or WSSV + MCM7-dsRNA treatment; (C) THC after *V. alginolyticus* or *V. alginolyticus* + MCM7-dsRNA treatment; (D) hemocyte PO activity after PBS or MCM7-dsRNA treatment; (E) hemocyte PO activity after WSSV or WSSV + MCM7-dsRNA treatment; (F) hemocyte PO activity after *V. alginolyticus* or *V. alginolyticus* + MCM7-dsRNA treatment. (G) Relative SOD activity after PBS or MCM7-dsRNA treatment; (H) Relative SOD activity after WSSV or WSSV + MCM7-dsRNA treatment; (I) Relative SOD activity after *V. alginolyticus* or *V. alginolyticus* + MCM7-dsRNA treatment. Data are presented as a proportion (%) of the normal control. 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.

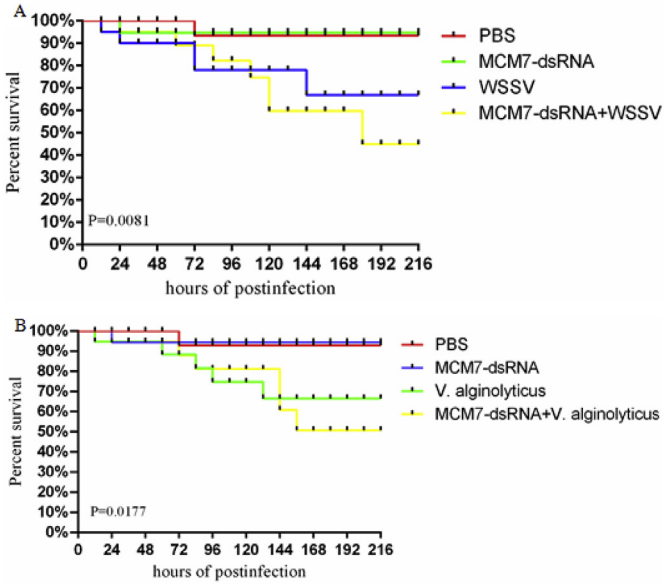

Fig. 8. The survival analysis of challenged crabs treated with MCM7-dsRNA. (A) The survival analysis from Kaplan–Meier of WSSV challenged crabs treated with MCM7-dsRNA. (B) The survival analysis from Kaplan–Meier of *V. alginolyticus* challenged crabs treated with MCM7-dsRNA. The solutions used for injection are shown on the left. There were nine individuals in each group.

alginolyticus + MCM7-dsRNA treatment was significantly higher than that of *V. alginolyticus* alone ($P < 0.01$), starting from 144 h post-challenge, while the MCM7-dsRNA group showed lower mortality count compared with the *V. alginolyticus* group from 96 to 144 h post-challenge (Fig. 8B). Overall, the results indicated that crab MCM7 was vital for the immune resistance to WSSV or *V. alginolyticus* infection.

3.8. Effects of MCM7 knockdown on hemocyte apoptosis

The apoptosis rate of MCM7-dsRNA group was significantly higher ($P < 0.01$) than that of the WSSV and PBS group (Fig. 9). However, the WSSV + MCM7-dsRNA group showed a higher apoptosis rate than the MCM7-dsRNA group (Fig. 9G). Similarly, the apoptosis rate was significantly ($P < 0.01$) increased in the *V. alginolyticus* + MCM7-dsRNA group compared with the *V. alginolyticus* group (Fig. 9H). These results suggested that MCM7 had an inhibitory effect on hemocyte apoptosis in crabs infected with *V. alginolyticus* or WSSV.

3.9. Effect of MCM7 knockdown on phagocytosis

The phagocytic rate was significantly increased in the WSSV + MCM7-dsRNA group compared with the WSSV group (37.2% vs. 31.4%, $P < 0.01$), according to flow cytometry (Fig. 10). The phagocytic rate was also significantly increased in the *V. alginolyticus* + MCM7-dsRNA group compared with the *V. alginolyticus* group (15.2% vs. 5.8%, $P < 0.01$, Fig. 10H). The phagocytic rate was similar in EGFP-dsRNA-treated groups and challenged control groups. The results suggest that knockdown of MCM7 may participate in the phagocytosis process and influence the phagocytosis of hemocytes.

4. Discussion

MCM7 belongs to the origin recognition complex with other MCM proteins and participates in cooperation with cell division cycle proteins to block cells in the S phase and to activate the cell cycle checkpoint [29]. In doing so, MCM7 regulates cell division from the S phase to the end of DNA replication, ensuring that only one single replication occurs during one cell cycle, thereby maintaining the preciseness of genetic material and the normal functioning of the cell [30]. Since the discovery of the MCM protein family, the extended function of this highly conserved family had been thoroughly studied to obtain insights into cell replication and regulation. In addition, considerable attention has been drawn to MCM7 over-expression in tumor cells and in virus-infected cells. MCM7 has been viewed as a novel and efficient biomarker for prognosis and medicine targeting [17–20,31]. However, few studies have investigated the function of this essential protein in invertebrates.

In the present study, *MCM7* was characterized from *S. paramamosain* and it was highly expressed in the digestive gland and hepatopancreas of crabs. In fact, for a long time, the digestive system was considered to be a complete and individual system, separated from the immune system, as well as the blood circulation and respiratory systems. Moreover, research has demonstrated that there are remarkable similarities and shared functions in both nutrient acquisition and host defense [32]. BLAST analysis of proteins showed that this amino acid sequence is highly conserved. The neighbor-joining tree method revealed a close evolutionary relationship of this protein to *M. japonicus*. Pathogen (WSSV or *V. alginolyticus*) stimulation led to under-expression of *MCM7* in the hemolymph of crabs.

RNA interference has been previously applied in studies of immunity in many invertebrate models to investigate the function of certain target proteins [33]. In the present study, we successfully inhibited *MCM7* expression with specific dsRNA, thus providing a practical way to reveal the role of MCM7 in the innate immune system, and subsequently, healthy and MCM7-inhibited crabs were then subjected to further experiments.

As the absence of a whole genome sequence for crabs, the genes crucial to the immune system are being slowly identified from crabs. The current study identified several genes crucial to the immune system of crabs, and the expression levels of these genes could reflect changes in different immune processes. Knockdown of *MCM7* mRNA led to significant down-regulation of the innate immune factors JAK and CAP, while CTL was significantly up-regulated. Our study provides more evidence and novel understanding of the possible functions of the MCM7 protein. Changes in the expression levels of the aforementioned genes indicate that MCM7 is likely to be associated with the innate immunity, and may be involved in antiviral processes.

Next, we found that crabs injected with MCM7-dsRNA and then challenged with WSSV showed a lower mortality count than the WSSV group between 24 and 96 h. To explore whether MCM7 exerted any other effects upon the innate immunity of crabs, we investigated a variety of functional parameters to evaluate immune potential. In *V. alginolyticus*-infected crabs, the THC and the activity of PO and SOD were reduced as the expression of *MCM7* was inhibited, whereas in the WSSV-infected crabs the THC and activity of PO and SOD were not greatly increased compared with the WSSV only group. In this study, apoptosis was increased in MCM7-dsRNA-treated crabs after WSSV or

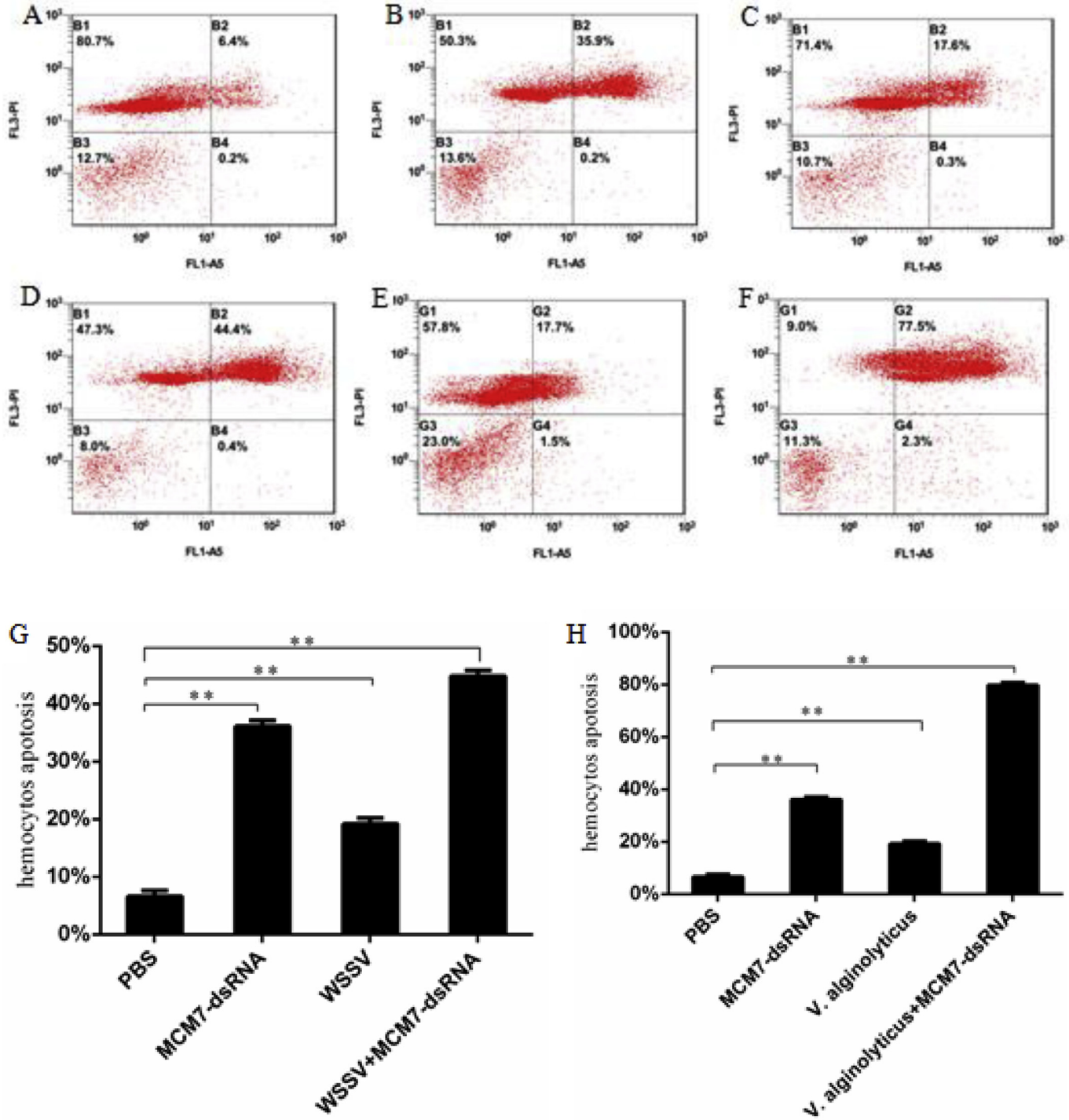

Fig. 9. Flow cytometry assay of apoptosis. (A) PBS; (B) MCM7-dsRNA; (C) WSSV; (D) WSSV + MCM7-dsRNA; (E) *V. alginolyticus*; (F) *V. alginolyticus* + MCM7-dsRNA; (G) Bar graph of phagocytosis of WSSV; (H) Bar graph of phagocytosis of *V. alginolyticus*. Double asterisks indicate a significant difference ($P < 0.01$) between the sample and the challenge only.

V. alginolyticus challenges. The findings of this study suggest that MCM7 not only regulates THC and the activity of PO and SOD in crabs but also influences the apoptosis and phagocytosis process in hemocytes. Virus invasion and bacterial infection are two very different processes, so they will change different immune parameters. Under this influence, the activity of PO showed different change after WSSV or *V. alginolyticus* challenged. Crab MCM7 plays a completely opposite role in PO activity in viral infection and bacterial infection. However, pathogenic virus and bacteria can cause harm to the host because they are all pathogenic microorganisms. Under this influence, THC, SOD and apoptosis were decreased after the two pathogens challenge. Crab MCM7 plays a positive role in inhibiting the apoptosis of hemocytes after the two pathogens challenge. Infected hosts promote apoptosis so as to enhance host resistance to viral infection [34]. Viruses can induce apoptosis by triggering cellular sensors in the host that initiate cell death; these play an important protective role in eliminating virus-infected cells [35]. The results of the infection experiment revealed that MCM7 may play a positive role in the anti-WSSV and *V. alginolyticus* immune response of

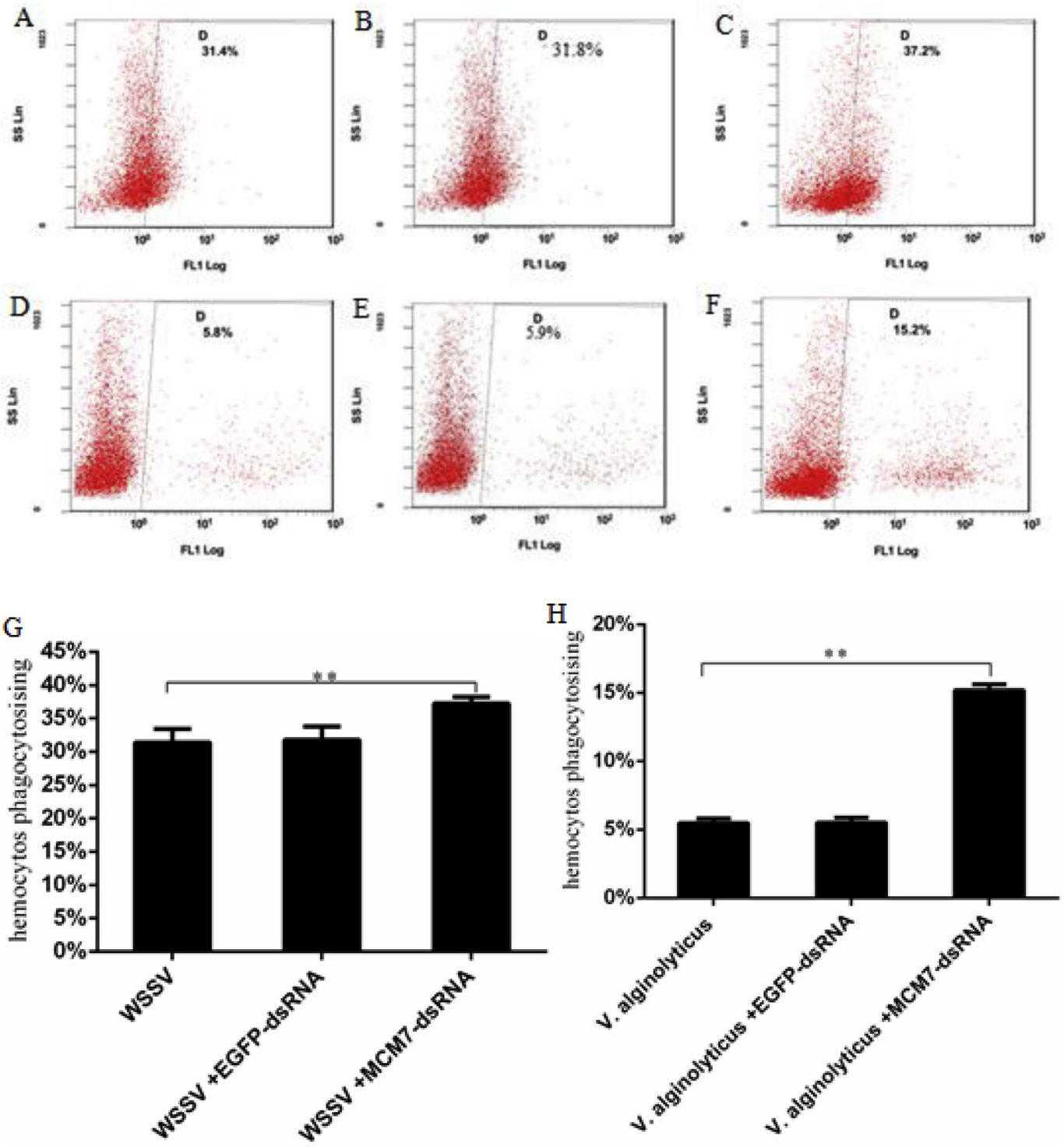

Fig. 10. Flow cytometry assay of phagocytosis. Inactivated WSSV and *V. alginolyticus* virions were labeled with FITC. (A) WSSV; (B) WSSV + EGFP-dsRNA; (C) WSSV + MCM7-dsRNA, (D) Bar graph of phagocytosis of WSSV; (E) *V. alginolyticus*; (F) *V. alginolyticus* + EGFP-dsRNA; (G) *V. alginolyticus* + MCM7-dsRNA; (H) bar graph of phagocytosis of *V. alginolyticus*. Double asterisks indicate a significant difference ($P < 0.01$) between the sample and the challenge only.

crabs and reduce mortality.

In conclusion, our current findings indicate that crab MCM7 plays an important role on the innate immune system of crabs. Crab MCM7 could regulate host defense mechanisms against bacterial and viral infection by regulating apoptosis, phagocytosis, THC, and the activity of PO and SOD.

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