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Molecular characterization of diphthamide biosynthesis protein 7 in *Marsupenaeus japonicus* and its role in white spot syndrome virus infection

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ABSTRACT

Diphthamide biosynthesis protein 7 (Dph7) is a vital protein for diphthamide biosynthesis in archaea and eukaryotes. The 1143 bp cDNA sequence of Dph7 was cloned from the gills of *Marsupenaeus japonicus* using RT-PCR and RACE. Data showed that Dph7 was highly expressed in the gills and digestive gland of *M. japonicus*. Furthermore, the expression of dph7 was induced by infection with white spot syndrome virus (WSSV). When Dph7 was knocked down, immune genes such as toll, prophenoloxidase (proPO), p53, tumor necrosis factor-α (TNF-α) and signal transducer and activator of transcription (STAT) were significantly down-regulated ($P < 0.01$) in hemocytes. First, we demonstrated that Dph7 is very important in the progression of WSSV infection and that the time of death for WSSV-infected shrimp was significantly advanced following RNAi targeting of Dph7. We also investigated the effect of Dph7 on apoptosis rate in *M. japonicas* and found that Dph7-dsRNA treatment caused lower levels of apoptosis in hemocytes, both in the disease-free group and the WSSV group. Knock-down of Dph7 affected the activity of both phenoloxidase (PO) and superoxide dismutase (SOD), and total hemocyte count (THC) after infection with WSSV, indicating that Dph7 plays a regulatory role in the immunological reaction of shrimp in response to WSSV infection. Thus, we conclude that Dph7 may promote the anti-WSSV immune response of shrimp by regulating apoptosis, SOD and PO activity, and can influence the progression of WSSV infection.

1. Introduction

Kuruma shrimp (*Marsupenaeus japonicus*) is a major marine product in south-east Asia, and is associated with high economic benefit. However, the high cultivation densities of shrimp has led to outbreaks of viruses and bacterial diseases, particularly white spot syndrome disease (WSSD) and vibriosis [1]. Owing to the absence of the adapted immune system, invertebrates are generally thought to protect themselves by relying solely upon their innate immune system [2]. The properties of the innate immune system of shrimp to viruses and bacteria have therefore become a popular research field.

There are many genes playing a crucial role in host innate immune system. p53 can help to prevent cancer via a variety of mechanisms, including DNA damage recognition, DNA repair, and (in the event of failed DNA repair) by initiating programed cell death or apoptosis [3]. TNF-α can regulate many cellular processes, including immune function, inflammation, apoptosis, cell differentiation, proliferation, and the activation of various components of the immune system [4]. The modulation of STAT transcription in shrimp after WSSV infection suggested that JAK/STAT pathway might be crucial in shrimp responsive to virus infection [5]. Recent years, Tolls and Toll-like receptors (TLRs), as major PRRs, have been recognized and play an important role in microbes recognition during host defense [6]. The phenoloxidase (PO) is responsible for the activation of melanogenesis [7] is vital to anti-pathogen infection in invertebrates [8].

Protein synthesis elongation factor 2 (eEF-2), a GTPase, plays a role in the translocation of mRNA and tRNA on the ribosome during translation elongation in archaea and eukaryotes [9]. Diphthamide, the target of diphtheria toxin (DT) and *Pseudomonas* exotoxin A (ETA) [10], is a post-translational derivative of histidine that exists in eEF-2. The catalytic subunit of diphtheria toxin recognizes diphthamide and undergoes ADP-ribosylation to block protein synthesis, and thus causes cell death [10,11]. Recently, several reports have suggested that diphthamide plays a key role in maintaining translation fidelity in both yeast and mouse models by preventing frame shift [12,13]. In another paper, cells without diphthamide were presensitized to NF-κB and death-receptor pathways [14]. It was initially proposed that the diphthamide biosynthesis pathway involved four steps including Dph (Diphthamide biosynthesis protein) 1–7 [11,15–17]. Dph7, also referred to as WDR85, contains WD40 domains which are known to

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Table 1
Universal and specific primers used in this study.

Primer Name	Nucleotide sequence (5′ →3′)	Purpose
3′ race GSP	TGGACACCCCAGAAGAAGGAG	first primer for 3′RACE
3′ race NGSP	TGCCTGCTATTTTAGATATGAAGTG	second primer for 3′RACE
5′race SP1	ATGGCAAGCATTGGTGTTCCTGAT	first primer for 5′RACE
5′race SP2	CTCAAATTGGCAATGTGGTCTC	second primer for 5′RACE
5′race SP3	TGCGATATTAGAGCAATGGTTC	third primer for 5′RACE
Dph7 realtime-F	AAACGACTTGGGCGTCTCTACC	primer for expression
Dph7 realtime-R	GGCAAGCATTGGTGTTCCTGATA	primer for expression
Dph7 dsRNA-F	CCCAAGCTTCCTGAGAAAGCCTTGGACACCC	primer for RNAi
Dph7 dsRNA-R	CCAGTAGTCAAAGGCTGTTATCCAAGGGATCCCG	primer for RNAi
hemocyanin-F	AACCCTGAACAAAGAGTTGCCTAT	for hemocyanin expression
hemocyanin-R	AACGGACGGTAAGTTGATGATGT	for hemocyanin expression
IMD-F	ATTCATCCGTCTACCTCCCTACA	for IMD expression
IMD-R	GAGCTGAGTCTGTCTTAATGTTATCC	for IMD expression
L-lectin-F	ATGTTATGCCATCTGCCTCGTATTT	for L- lectinexpression
L-lectin-R	CTTTCGCTGCTGCTCTTTCTGTT	for L- lectinexpression
MAPK-F	CGCATCACTGTTGAGGAGG	for MAPK expression
MAPK-R	GCAGGTCATCAAGTTCCATCT	for MAPK expression
NOS-F	CCAGGATCTTCTTGTTGGTGTTG	for NOS expression
NOS-R	CCCTCATCTGTAGCATAAAGTTCTC	for NOS expression
p53-F	TTCCTGCCTGGCTGACTCTA	for p53 expression
p53-R	CACCCAATCTTCCAACATCACAT	for p53 expression
proPO-F	TTCTACCGCTGGCATAAGTTTGT	for proPOexpression
proPO-R	TATCTGCCTCGTCGTTCCTCAC	for proPOexpression
STAT-F	TGGCAGGATGGATAGAAGACAAG	for STAT expression
STAT-R	TGAATAAGCTGGGATACGAGGGA	for STAT expression
TNF-F	ACAGACGGTCCAGAGTCCCAAAG	for TNF expression
TNF-R	GCGACGAAGTGAGCCACAGTAA	for TNF expression

mediate protein-protein interactions, and is a scaffold protein involved in the third step which converts methylated diphthine to diphthine so that Dph6 can create diphthamide [18]. However, there are few researches about effect of Dph7 on host immune system, especially in virus infection.

In our previous study, we found that the expression level of Dph7 in *M. japonicus* was up-regulated following infection with white spot syndrome virus (WSSV). In the present study, we investigated the role of Dph7 in the innate immune system of shrimp.

2. Materials and methods

2.1. Shrimp challenge with WSSV and tissue collection

Kuruma shrimp (*M. japonicus*; 15g) were purchased from a fishery market in Hangzhou, Zhejiang Province, China, and then cultured in air-pumped artificial seawater (23 °C) prior to the experiment. WSSV (GenBank accession no. AF332093.1) was purified and used in challenge experiments as described previously [19]. The shrimp were injected with 1×10^4 copies/shrimp of WSSV, and a hyperhaline phosphate-buffered saline (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) injection was used as the control [20]. Subsequently, shrimp tissues, including the heart, hepatopancreas, gills, stomachand intestines, were collected. Hemocytes were collected at 0, 12, 24 and 48 h post-injection. The hemolymph was extracted from the abdomen blood sinus of shrimp by using of a 2 mL syringe preloaded with 1 mL of anticoagulant (10 mM EDTA and 10 mM HEPES, dissolving with PBS, pH 7.45). The hemolymph was then centrifuged at 800 g for 10 min at 4 °C to collect the hemocytes. The hemocytes and other tissues were used for RNA extraction.

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

Total RNAs from different tissues (hemocytes, heart, hepatopancreas, gills, stomach and intestines) were extracted using a mirVanamiRNA™ Isolation Kit (Ambion, USA), following the manufacturer's instructions. The RACE technique was utilized to clone the full-length cDNA sequence of a gene, based on the known middle fragment using 5′/3′ RACE Kit, 2nd Generation (Roche, Germany), according to the protocol of the manufacturer. The synthesized cDNAs were kept at −20 °C, used for the 3′/5′ -RACE PCR with 3′ gene-specific primer (3GSP, 3NGSP) or 5′ GSP (5SP1, 5SP2, 5SP3), designed on the basis of middle sequence (the primer's sequences are shown in Table 1). The following steps were performed as described previously [19]. All primers used in this experiment were designed using Primer Premier 5.0.

2.3. Quantitative real-time RT-PCR (qRT-PCR) analyses

qRT-PCR was used to analyze the Dph7 mRNA expression levels at different time points after pathogen challenge. Total RNAs were extracted from different tissues using an Easy spin tissue/cell RNA extra kit (Aidlab, Shanghai China) according to the manufacturer's protocol. 200 μg total RNAs were used for cDNA synthesis by ReverTra Ace qPCR RT Master Mix with gDNA Remover Code: FSQ-301 (Toyobo, Japan). The cDNA was stored at −20 °C. A SYBR Green qRT-PCR assay (Promega, USA) was carried out in a Bio-Rad Two Color Real-Time PCR Detection System (Bio-Rad, CA, USA). The PCR profile was as follows: 95 °C for 30 s; 40 cycles of 95 °C for 5 s, 60 °C for 30 s; and then a melting period from 65 °C to 95 °C. The PCR data were analyzed using the $2^{-\Delta\Delta CT}$ method and are shown as means standard deviations (SD). The *t*-test was used to analyze the significance of differences in the PCR data [21].

2.4. Prokaryotic expression and purification of Dph7-dsRNA

The primers (shown in Table 1) with specific restriction sites (*Hin*d III in the forward primer and *Bam*H I in the reverse primer) 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 plasmid L38-Dph7. The constructed L38-Dph7 was verified by restriction enzyme digestion and

DNA sequencing. The recombinant plasmid L38-Dph7 was transformed into HT115 (DE3) cells with deficiency of RNase III. Single colonies of the above the engineering bacteria were separately inoculated to 5 mL of LB medium containing Amp (100 μg/mL), cultured at 37°Cwith shaking at 200 r/min for 12–16 h, and then inoculated to LB medium containing Amp by a proportion of 1%, cultured at 37 °C with shaking at 200 r/min for 2–3 h (OD600 ≈ 0.6), and added with IPTG (with a final concentration of 0.8 mmol/L) to induce the expression for 4 h.

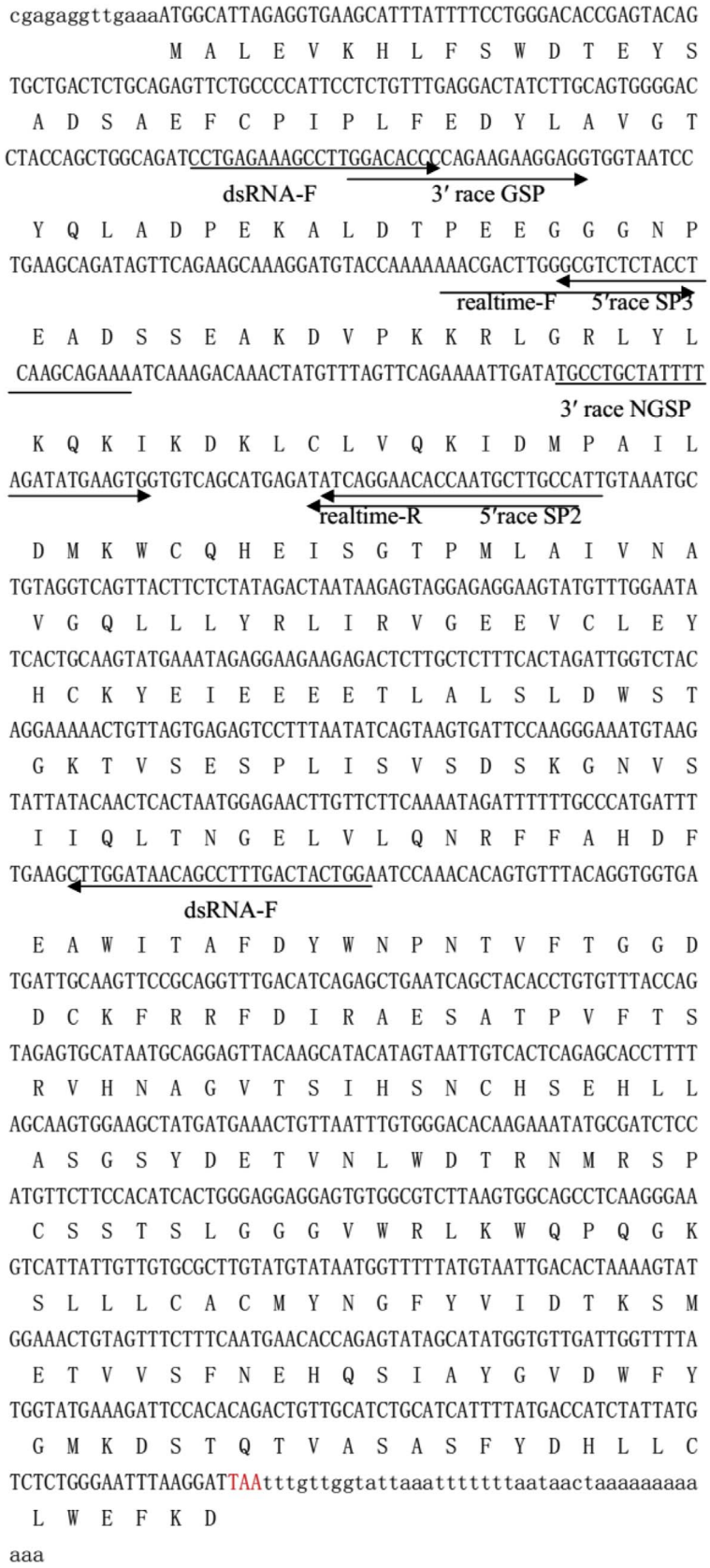

Fig. 1. Nucleotide and deduced amino acid sequence of Dph7. The nucleotide sequence is displayed in the 5′–3′direction and numbered on the left. The deduced amino acid sequence is shown in a single capital letter amino acid code. The 3′UTR and 5′UTR are shown with lowercase letters. Codons are numbered on 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

After purifying with mirVanamiRNA™ Isolation Kit (Ambion, USA), the dsRNAs were annealed and precipitated with 5 M sodium acetate and anhydrous alcohol.

2.5. Knock down of Dph7 by RNAi and challenge experiments

Dph7-dsRNA (30 μg/shrimp) was immediately injected intramuscularly into shrimp, and Dph7 mRNA expression levels were detected by qRT-PCR. Shrimp were divided into four groups: intramuscular injection with 100 μL PBS alone; intramuscular injection with 100 μL Dph7-dsRNA alone; intramuscular injection with WSSV (10^5 copies/mL) challenge, 30 μg/shrimp alone; injection with Dph7-dsRNA for 12 h, followed by 100 μL WSSV challenge. Shrimp mortality was monitored every 12 h after the last injection. Each group contained at least 25 shrimp to ensure adequate confidence levels, and treatments were replicated three times to avoid the influence of variations in weather, individual body condition, and injection-operation error.

2.6. Detection of WSSV copies

Shrimp were injected with WSSV or the mixture of WSSV and Dph7-dsRNA. Shrimp hemocytes were collected at different time post injection for whole-genome extraction using DNA extraction Kit (Tiangen, China), following 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 [22].

2.7. Determination of immune parameters after RNAi

The immune parameters determined included THC, PO activity and SOD activity. Total hemocyte count was determined as previously described [23]. To determine PO and SOD activities, 500 μL hemolymph was withdrawn into a 2 mL syringe containing 500 μL 20 mM of EDTA solution from individual shrimp. The PO activity was quantified from the hemolymph mixture based on the formation of dopachrome from the substrate L-3, 4-dihydroxyphenylalanine (L-DOPA) as previously described [24]. The SOD activity was quantified from hemocytes isolated from 300 μL of the hemolymph mixture according to the improved method for determining Beauchamp & Fridovich [25]. Data were presented as a percentage of normal control.

2.8. Apoptosis analysis with annexin V by flow cytometry

Apoptosis assay of shrimp with/without at 4 °C knockdown was conducted with Annexin V (Invitrogen, USA) according to the manufacturer's protocol. The hemolymph was mixed with 20 mM of EDTA at a ratio of 1:1, and the mixture was centrifuged at 300 g at 4 °C for 5 min to collect hemocytes. The hemocytes were suspended in highly alkaline PBS, counted and adjusted with PBS to a cell density of 3-5 × 10^6 cells/mL. Subsequently, the cells were stained using BD Phrmingen TM FITC

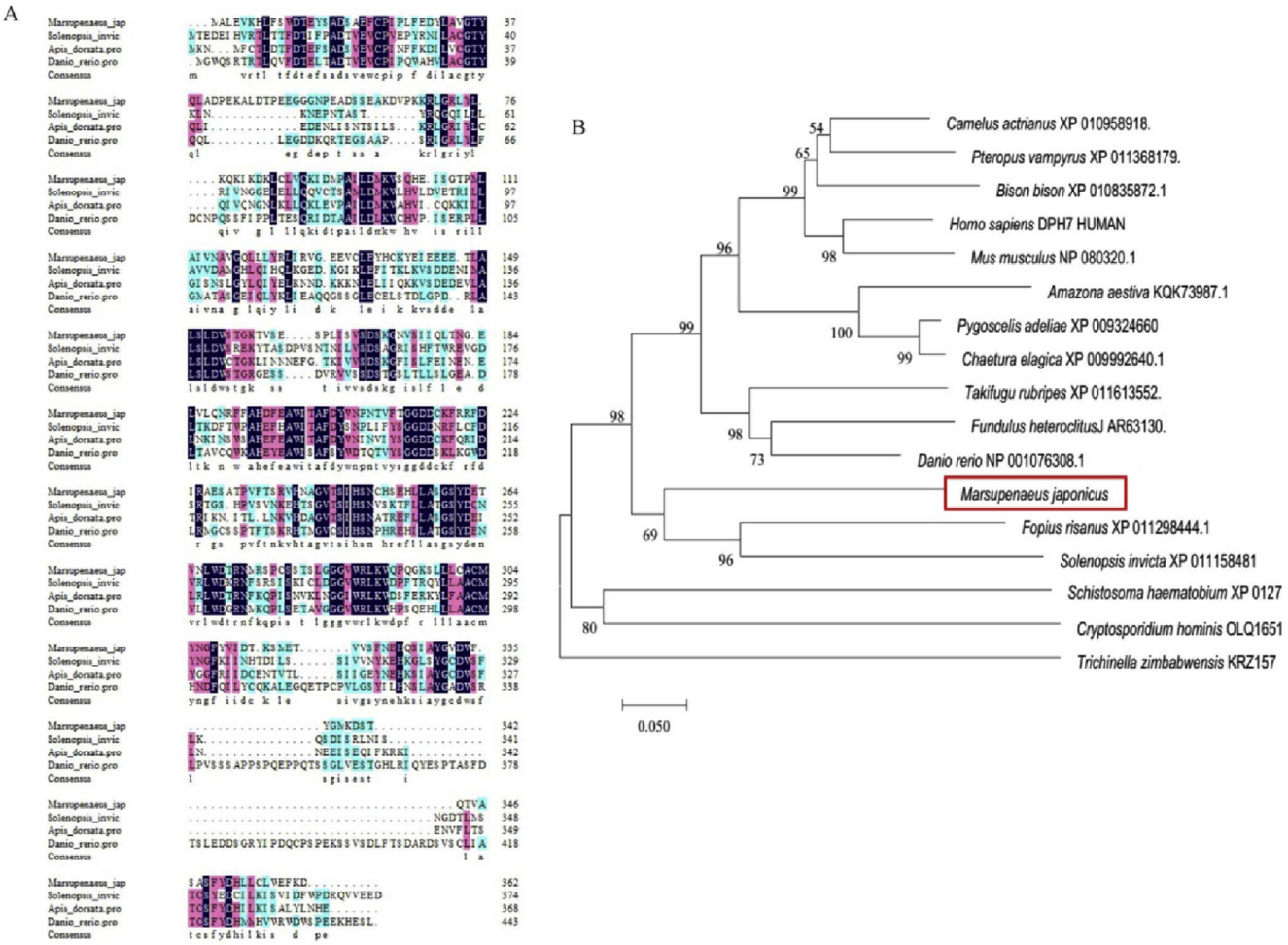

Fig. 2. Sequence analysis. (A) Multiple alignments of the amino acid sequence of *M. japonicas* Dph7 with Dph7 sequences from *Solenopsis invicta* (XP_011158481.1), *Danio rerio* (NP_001076308.1) and *Apis dorsata* (XP_006616681.1). (B) The neighbor-joining phylogenetic tree of Dph7 from different organisms based upon amino acid sequence comparisons. Species names and accession numbers for Dph7 are listed on the right of the tree.

Annexin V Apoptosis Kit, 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 were presented as means ± standard deviation (SD) derived from at least three independent experiments.

2.9. Statistical analysis

Quantitative data were expressed as mean ± standard deviation (SD). The statistical differences were estimated by one-way analysis of variance (ANOVA) followed by least-significant differences (LSD) and Duncan's multiple range test. All statistical analyses were carried out using SPSS Statistics version 19.

3. Results

3.1. Sequence analysis, bioinformatic analysis and phylogenetic analysis

The full length of the Dph7 cDNA sequence was 1143 bp (Fig. 1), and included a 1089 bp open reading frame (ORF) encoding a protein composed of 362 amino acids. The 5′ and 3′ untranslated regions (UTR) of Dph7 were 13 bp and 41 bp in length, respectively. The cloned sequence also included a poly (A) tail. The estimated molecular mass of Dph7 was 40.857 kDa, with a theoretical *p*I of 4.68. The nucleotide sequence, and the deduced amino acid sequence, for the full-length cDNA are shown in Fig. 1.

We compared the Dph7 amino acid sequence with the published sequences of different types of Dph7. Our analysis of the Dph7 ORF revealed 42.34% identity with *Daniorerio* Dph7 (accession no. NP_001076308.1), 34.43% identity with *Solenopsis invicta* Dph7 (accession no. XP_011158481.1) and 33.92% identity with *Apis dorsata* Dph7 (accession no. XP_006616681.1) (Fig. 2A). A condensed neighbor-joining phylogenetic tree, based on the amino acid sequences, was constructed by the neighbor-joining method using MEGA6.0 (Fig. 2B). Phylogenetic analysis showed that Dph7 was conserved across different species. In the amino acid sequence, the conserved domains contained several highly conserved amino acid sites. *M. japonicus* Dph7 showed the closest relationship with *Danio rerio* Dph7.

3.2. Tissue expression difference and stress response

The expression profiling of the Dph7 gene in different *M. japonicus* tissues was investigated using qRT-PCR (Fig. 3). The expression level of

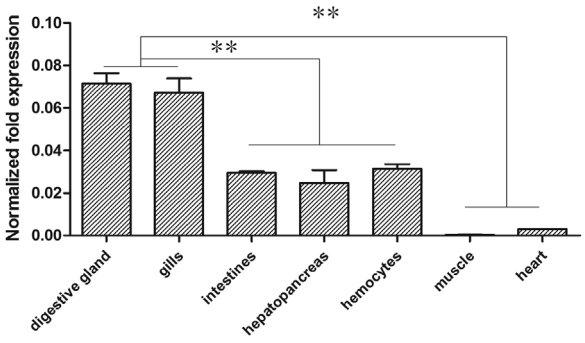

Fig. 3. Expression characterization of Dph in various tissues as revealed by quantitative real-time PCR from healthy shrimp. The amount of Dph7 mRNA was normalized to GAPDH transcript levels. For each tissue, data are shown as means ± SD (standard deviation) of three separate individuals. Double asterisks indicate a significant difference ($P < 0.01$) between two samples.

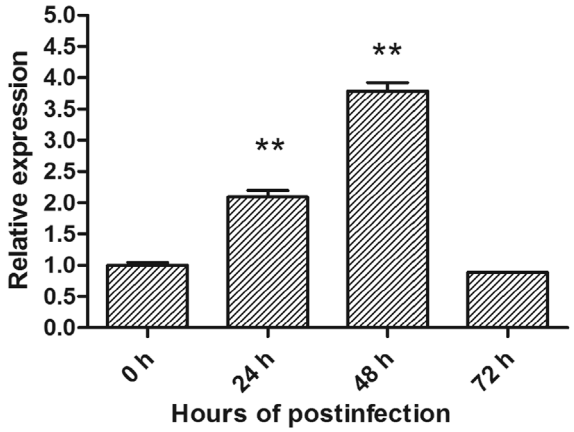

Fig. 4. (A) Real-time RT-PCR analysis of Dph7 expression in the hemocytes of *M. japonicas* challenged with WSSV. The amount of Dph7 mRNA was normalized to GAPDH transcript levels. For each tissue, data are shown as means ± SD (standard deviation) of three separate individuals. Double asterisks indicate a significant difference ($P < 0.01$) between two samples.

Dph7 was highly expressed by the gills and the digestive gland; expression levels in these tissues were significantly higher than in any of the other tissues tested, based on qRT-PCR analysis. The lowest expression level of Dph7 was in the muscle and heart. The expression level of Dph7 in the gills was 2.28-fold greater than the expression of Dph7 in hemocytes. The expression level of Dph7 in the gills was 2.89-, 172.90-, and 23.72-fold higher than that in the hepatopancreas, intestine, muscle and heart, respectively. According to our statistical analysis, the expression of Dph7 in the gills and digestive gland were significantly higher ($P < 0.01$) than that of the other tissues.

After WSSV challenge, the relative Dph7 expression in the hemocytes was gradually up-regulated (Fig. 4), and had a significant highest level at 48 h post infection ($P < 0.01$). Apparently, there were remarkably up-regulated expressions in shrimp hemocytes from 24 to 48 h treatment ($P < 0.01$). Above result claimed that Dph7 might have a potential role in WSSV infection of shrimp.

3.3. Dph7 RNAi by dsRNA and the effect of Dph7 on the expression of immune genes

First, we tested the effect of Dph7-dsRNA on the expression of Dph7 using qRT-PCR. Data showed that the expression of Dph7 was significantly knocked down by Dph7-dsRNA ($P < 0.01$) in shrimp hemocytes (Fig. 5A).

Next, we studied the relationship between Dph7 and important immune genes by analyzing the expression of important immune genes in the hemocytes of *M. japonicus* treated with Dph7-dsRNA. Of the eight immune genes tested, p53, TNF, Toll, proPO and STAT were significantly down-regulated ($P < 0.01$) in shrimp hemocytes (Fig. 5B).

3.4. Mortality count and the detection of WSSV copies

To evaluate the effects of Dph7 on the mortality of virus-challenged shrimp, Dph7-dsRNA was injected into shrimp, followed by WSSV challenge. The proportion of WSSV-infected shrimp that survived was significantly reduced by treatment with Dph7-dsRNA from 24 to 120 h post-challenge ($P < 0.05$) (Fig. 6A). The groups of Dph7-dsRNA alone and the negative control showed indistinctive cumulative mortality (Fig. 6A), which indicated that the Dph7-dsRNA had no toxic effects

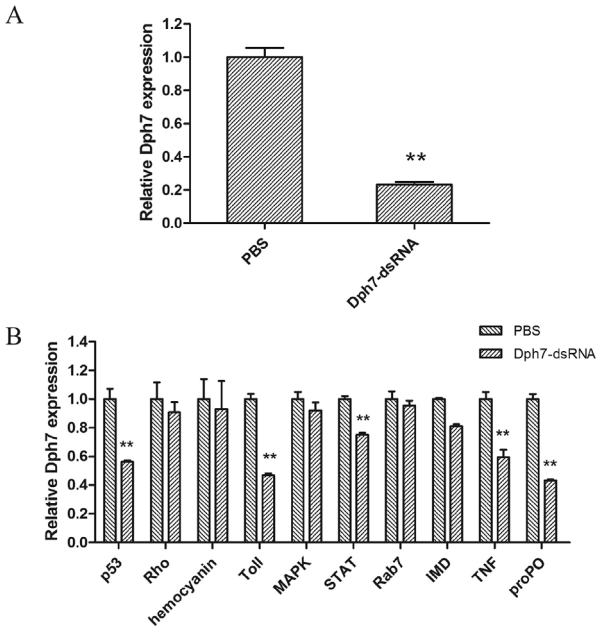

Fig. 5. (A) Real-time RT-PCR analysis of Dph7 expression in the hemocytes of *M. japonicas* treated with Dph7 dsRNA at 24 h post-treatment. The amount of Dph7 mRNA was normalized to GAPDH transcript levels. (B) Real-time RT-PCR analysis of the expression of nine immune genes in the muscle of *M. japonicas* treated with Dph7-dsRNA. The amount of Dph7 mRNA was normalized to GAPDH transcript levels. For each tissue, data are shown as means ± SD (standard deviation) of three separate individuals. Double asterisks indicate a significant difference ($P < 0.01$) between two samples.

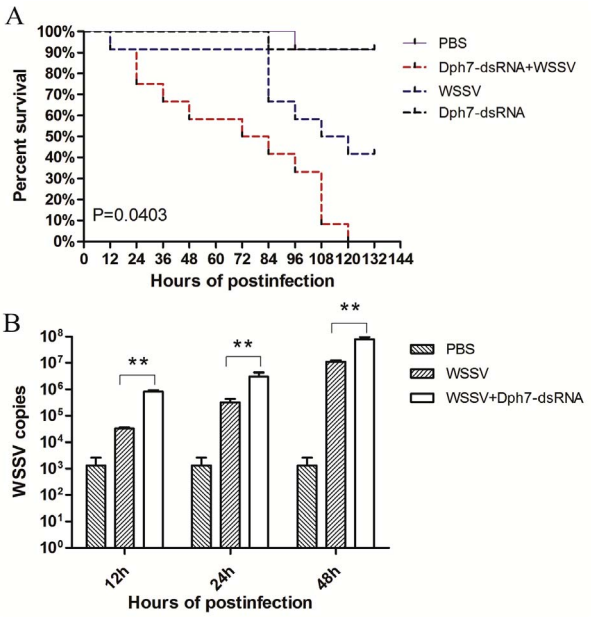

Fig. 6. Effects of Dph7 knockdown. (A) Effects of WSSV challenge and Dph7 inhibition on shrimp mortality. Healthy shrimp treated with high-saline PBS were used as controls. Each group contained at least 25 shrimp to ensure adequate confidence levels, and treatments were replicated three times to avoid the influence of variations in weather, individual body condition, and injection-operation error. (B) WSSV copies detected by TaqMan VP28 probe at different times post-infection. A list of the primers used is given in Table 1.

upon the shrimp. The result of shrimp mortality revealed that Dph7 is very important for WSSV infection.

In all groups, the number of WSSV copies increased as time progressed (Fig. 6B). Compared with the control group with WSSV challenge alone, the shrimp that were injected with Dph7-dsRNA + WSSV had a higher copy number of WSSV, suggesting that the absence of Dph7 significantly ($P < 0.01$) promoted the replication of WSSV.

3.5. Influence of Dph7 knockdown on shrimp immune parameters

The total hemocyte count (THC) in the WSSV-alone group was lower than that in the PBS group at 24 and 48 h, while the THC in the WSSV + Dph7-dsRNA group was significantly lower than in the WSSV group ($P < 0.01$, Fig. 7). These results indicated that the THC decreased in shrimp after WSSV infection, and that the inhibition of Dph7 expression exacerbated the reduction in THC.

PO activity in shrimp hemolymph was reduced at the 48 h following WSSV infection compared with the PBS control group, and was significantly lower in the WSSV + Dph7-dsRNA group compared with the WSSV-alone and PBS groups at 24 h and 48 h (Fig. 7). Data suggested that the absence of Dph7 significantly ($P < 0.01$) inhibited PO activity.

In present study, we used the NBT photoreduction method to detect the relative SOD activity of shrimp hemolymph, as described in a previous study. Shrimp treated with Dph7-dsRNA for 24 and 48 h showed significantly ($P < 0.01$) lower levels of superoxide dismutase (SOD) activities than the controls (Fig. 7). Compared to the PBS group, relative SOD activity following WSSV infection increased significantly to 141% ($P < 0.01$) at 24 h, then increased to 166% at 48 h. Inhibition of relative SOD activities in the WSSV + Dph7-dsRNA group were 121% at 24 h and 116% at 48 h. Dph7 expression thus significantly reduced the SOD activity caused by WSSV at 24 and 48 h ($P < 0.01$). These results indicated that Dph7 had exerted stimulatory properties on the immune parameters of shrimp.

3.6. Influence of Dph7 knockdown on hemocytes apoptosis

The apoptosis rate in the PBS group was 63.4%, which fell to 51.3% following the inhibition of Dph7. Compared with the control group with WSSV challenge alone, the shrimp injected with Dph7-dsRNA + WSSV exhibited a higher rate of apoptosis (Fig. 8). These results indicated that Dph7 was involved in virus-induced apoptosis. The down-regulation of virus-induced apoptosis suggested that Dph7 could positively regulate virus-induced apoptosis.

4. Discussion

Further studies demonstrated that the expression level of Dph7 mRNA was significantly up-regulated in WSSV-challenged shrimp. Hence, our study contributes to elucidating the effect of Dph7 in shrimp innate immune response to WSSV infections.

In the present study, Dph7, possessing a 1089 bp ORF, was identified in the kuruma shrimp (*M. japonicus*). Meanwhile, Dph7 was highly expressed in digestive gland and gills of shrimp. Actually, in a long time, the digestive system is considered to be a complete and individual system, separated from immune system, as well as the blood circulation system, and respiratory system. Besides that, the researcher demonstrated that there are remarkable similarities and shared function in both nutrient acquisition and host defense [26]. In order to explore the expression of Dph7 after viral challenge, shrimp were infected with WSSV and qRT-PCR confirmed that Dph7 was remarkably up-regulated at 24 h and 48 h post-WSSV infection. The high expressions in the two tissues and the up-regulated expression after virus infection suggested Dph7 might have a potential effect in innate immune system of shrimp.

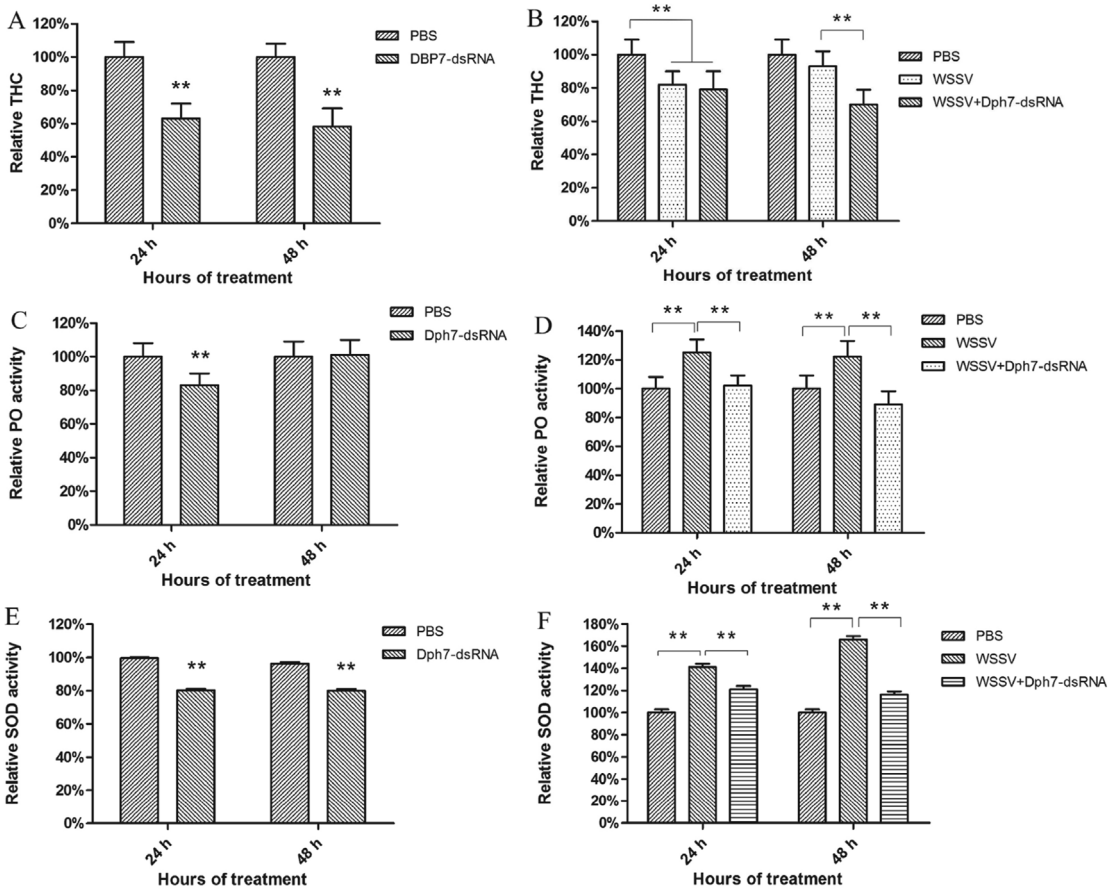

Fig. 7. Effects of Dph7 knockdown on shrimp immune parameters, including THC, PO activity, and SOD activity, as determined in healthy or WSSV-treated shrimp. (A) THC after PBS or Dph7-dsRNA treatment; (B) THC after WSSV or WSSV + Dph7-dsRNA treatment; (C) hemocyte PO activity after PBS or Dph7-dsRNA treatment; (D) hemocyte PO activity after WSSV or WSSV + Dph7-dsRNA treatment. (E) Relative SOD activity after PBS or Dph7-dsRNA treatment; (F) relative WSSV or WSSV + Dph7-dsRNA. Data are presented as a proportion (%) of the normal control. All treatments, at each time point, included at least three shrimp individuals, and all experiments were repeated three times. Each column represents the mean value of triplicate assays.

RNA interference (RNAi) has been previously applied in studies of shrimp immunity in order to investigate the function of certain target proteins [27]. In the present study, we successfully inhibited the expression of Dph7 with specific dsRNA, thus providing a practical way to reveal the role of Dph7 in the innate immune system, and subsequently, healthy and Dph7-inhibited shrimp were then subjected to further experiments.

Up to now, owing to the absence of shrimp whole genome, the genes crucial to the immune system are being progressively identified in shrimp. The current study identified several genes crucial to the immune system of shrimp, which expression levels could reflect changes in different immune processes. p53 is a known tumor suppressor that plays crucial roles in multicellular organisms. TNF also plays a key role in the innate immune defense system of kuruma shrimp [28]. Previous papers reported that the loss of diphthamide rendered cells hypersensitive to TNF-mediated apoptosis [14]. Our study provides more evidence and novel understanding to the possible functions of the Dph7 protein. STAT has been shown to be an important part of *Drosophila* and shrimp's defense responses to viral infection [5,29,30]. Our data showed that inhibition of Dph7 expression resulted in strong down-regulation of toll, proPO, p53, TNF-α and STAT. Changes in the expression levels of these genes listed above indicated that Dph7 is likely to be associated with apoptosis in shrimp innate immunity, and may be involved in anti-viral processes.

Next, we found that Dph7-dsRNA treatment significantly increased the number of WSSV copies in shrimp, and the increase of WSSV-induced shrimp mortality corroborated this positive effect of Dph7. Based on these results, we draw a conclusion that Dph7-dsRNA treatment could enhance WSSV replication, and thus contribute the cumulative mortality of WSSV infected individuals. To explore whether Dph7 exerted any other effects upon shrimp innate immunity, we investigated a variety of functional parameters to evaluate immune potential. In WSSV-infected shrimp, as Dph7 expression was inhibited, the THC, PO activity and SOD activity were significantly reduced. The same results were obtained in the apoptosis analysis. Infected hosts promote apoptosis so as to enhance host resistance to the viral infection [31]. Viruses can induce apoptosis by triggering cellular sensors in the host that initiate cell death; these play an importantly protective role in eliminating virus-infected cells [32]. Obviously, our study provided new evidence that Dph7 might be able to take advantage of diphthamide biosynthesis pathway to regulate shrimp hemocytes apoptosis. WSSV is capable of inducing hemocytes apoptosis of shrimp; our study revealed that Dph7 positively regulates the process of hemocytes apoptosis in shrimp.

In conclusion, our current findings indicate that shrimp Dph7 plays an essential effect on the innate immune system of shrimp. This host protein could regulate host defense mechanisms against viral infection by regulating apoptosis, THC, PO activity and SOD activity.

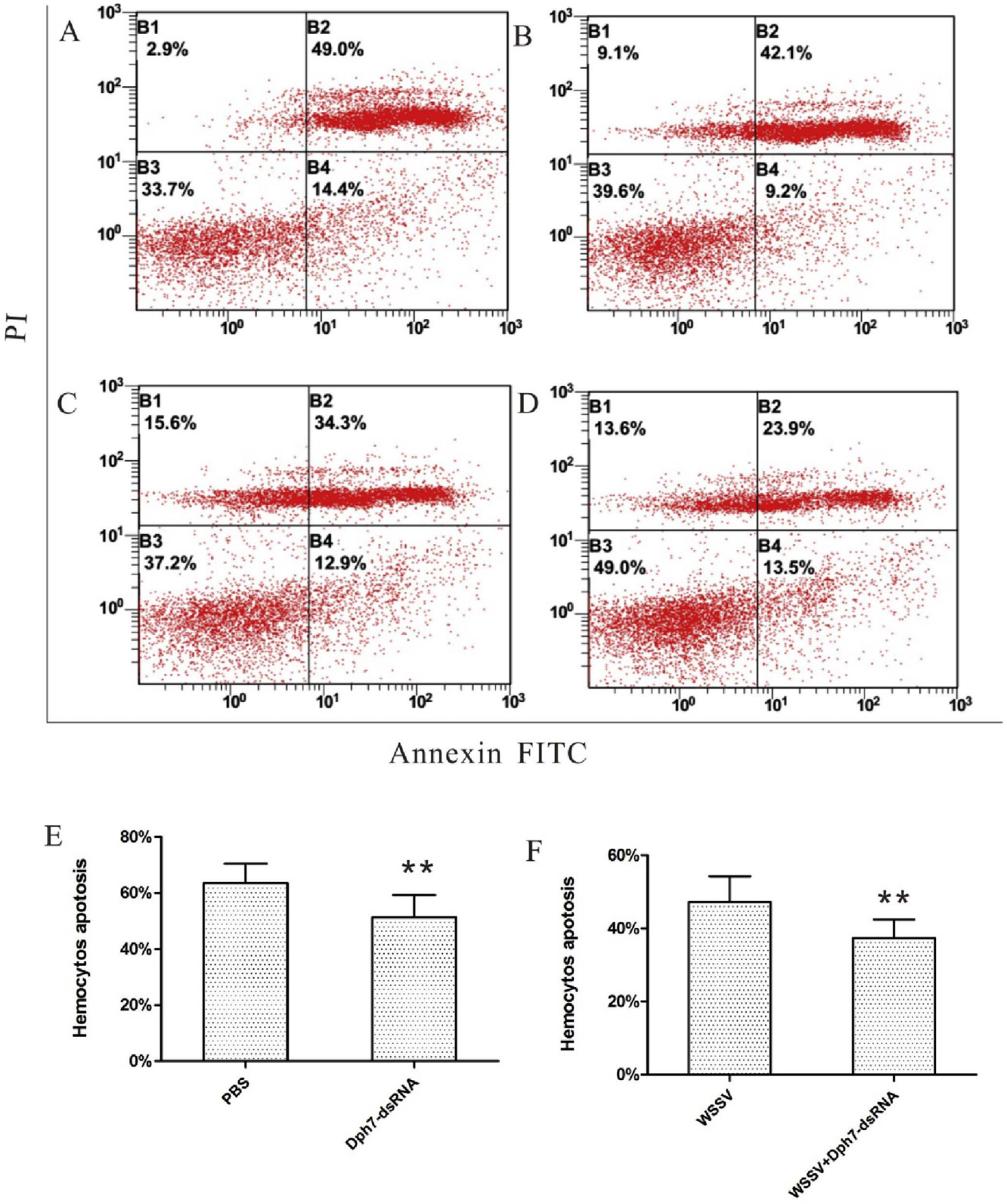

Fig. 8. Effect of Dph7-dsRNA interference on apoptosis of hemocytes. Samples were taken at 24 h post-injection. (A) PBS treatment; (B) Dph7-dsRNA treatment; (C) WSSV treatment; (D) WSSV + Dph7-dsRNA treatment; (E) column chart of apoptosis after PBS or Dph7-dsRNA treatment; (F) column chart of apoptosis after WSSV or WSSV + Dph7-dsRNA. The B1 area represents false positives caused by cell damage, B2 represents late-stage apoptotic hemocytes, B3 represents negative (normal) hemocytes, and B4 represents late-stage apoptosis. The annexin V peak represents the sum of the B2 and B4 areas (total value of annexin V-positive hemocytes), and is shown in the column chart. The proportion (%) of annexin V-positive hemocytes represents the apoptosis rate for 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.

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