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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