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Identification and analysis of a *Marsupenaeus japonicus ferritin* that is regulated at the transcriptional level by WSSV infection

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ABSTRACT

Marsupenaeus japonicus is a shrimp species of great value in the Chinese aquaculture industry. Given the susceptibility to viral diseases, research efforts have focused on the molecular characteristics of the shrimp's immune mechanisms. Ferritin is well known for its iron storage function, but studies have also addressed its immune function in response to pathogens. In this study, an *M. japonicus ferritin* cDNA was identified by homology cloning and rapid amplification of cDNA ends-PCR. The full-length cDNA is 1244 bp long and contains an open reading frame (513 bp) that encodes a highly conserved protein of 170 amino acids. Quantitative real-time PCR detection of *ferritin* revealed high expression in eight tested tissues, with the highest levels in hemocytes—consistent with the iron storage capacity of ferritin. We infected *M. japonicus* with white spot syndrome virus and validated the model by viral copy analysis and histopathology, which demonstrated an increase in viral copies along with acute degeneration of tissues. Transcripts of *ferritin* increased by 3.1-fold, 2.1-fold, and 1.5-fold in the hepatopancreas, gill, and midgut at 24 h post-injection, suggesting that ferritin played an important role in the immune response of *M. japonicus*.

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1. Introduction

The kuruma shrimp *Marsupenaeus japonicus* is one of the principal *penaeid* shrimp cultures with great economic significance in China. The industry is threatened by infectious diseases, particularly those caused by viruses, which reduce shrimp production and cause severe economic losses (Escobedo-Bonilla et al., 2008). The white spot syndrome virus (WSSV) is one of the most virulent pathogens to affect the shrimp culture industry (Chiang and Lo, 1995). One approach to resolving this devastating pathogenic problem is to discover defense mechanisms against viral infection. Shrimp lack specific immunity and rely solely on innate, non-adaptive immunity. In recent years, shrimp studies have largely focused on immune-related genes and their functions (Li and Xiang, 2013; Zhao et al., 2007).

Ferritin, an iron storage protein, was first reported in *Philaenus spumarius*, in 1988 (Collin et al., 1988). Soon after, it was established that ferritin is ubiquitous in the animal kingdom. In higher vertebrates, ferritin is formed by a complex of 12 heavy and 12 light polypeptide chains encoded by separate genes (Orino et al., 1997). While in prokaryotes and plants it is composed of 24 equal subunits (Harrison and Arosio, 1996). Ferritin plays a key role in iron metabolism and binds iron in a soluble, bio-available, and non-toxic form (Andrews et al., 1992). Ferritin is a major component of the iron-withholding defense mechanism. It protects against iron toxicity (Torti and Torti, 2002; Weinberg and Miklossy, 2008), as excess Fe^{2+} causes oxidative stress by producing insoluble rust and soluble oxy-radicals (Theil, 2003). It also sequesters iron in the plasma, therefore reducing its availability to pathogens and prohibiting infection of host cells (Ong et al., 2006).

Ferritin mediates the inflammatory response to pathogens (e.g. bacteria, *Vibrio*, LPS) in various invertebrate and vertebrate species. Its expression is induced after pathogen challenge in arthropods (Kong et al., 2010; Ruan et al., 2010), annelids (Prochazkova et al., 2011), echinoderms (Beck et al., 2002; Ramírez-Gómez et al., 2008), mollusks (L. Zhang et al., 2013; Y. Zhang et al., 2013), cephalochordates (Li et al., 2008), and marine fish (Zhang et al., 2010; Zheng et al., 2010a, 2010b). Other investigations have demonstrated that *ferritin* expression increases under pH and heavy metal challenge in shrimp (Zhang et al., 2006; Zhou et al., 2008). These evidences suggested that ferritin may increase stress resistance (Y. Zhang et al., 2013). Genetic breeding programs have recently been initiated to improve productivity and stress resistance in *M. japonicus*. To improve our understanding of the function

Abbreviations: WSSV, white spot syndrome virus; bp, base pair(s); cDNA, DNA complementary to RNA; H&E, Mayer's hematoxylin and eosin; IRE, Iron Response Element; LPS, Lipopolysaccharides; ORF, open reading frame; qRT-PCR, quantitative real time PCR; RACE-PCR, rapid amplification of cDNA ends polymerase chain reaction; UTR, untranslated region; hpi, hours post-injection; SD, standard deviation; SE, standard error; pI, theoretical isoelectric point.

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of ferritin in this species, we cloned the full-length cDNA of *M. japonicus ferritin* and investigated its tissue expression patterns, particularly in response to WSSV challenge.

2. Materials and methods

2.1. Shrimp

M. japonicus were obtained in November 2011 from a commercial shrimp farm in Dongshan, Fujian, China. Intermolt shrimp, weighing 7.88 ± 2.0 g (n = 87, mean ± SD), were used for the study. Maintenance included feeding once per day with a formulated shrimp diet. About 30% water exchange was applied daily with isothermal seawater. Five shrimps were randomly chosen to verify the absence of WSSV by two-step PCR according to Wang et al. (1999).

2.2. Cloning and sequence analysis of ferritin cDNA

Total RNA extraction and first-strand cDNA synthesis of *M. japonicus* were performed as described (Feng and Zhang, 2012). cDNA fragments of *ferritin* were PCR-amplified with the primer pairs described in Table 1 (P1-F/P1-R and P2-F/P2-R). Primer design was based on the *ferritin* mRNA sequences of *Litopenaeus vannamei* (AY955373.1) and *Fenneropenaeus chinensis* (DQ205422.1). Cycling conditions were as follows: 95 °C for 5 min and 35 cycles of 95 °C for 30 s, 53 °C for 30 s, and 72 °C for 5 min, and a final extension at 72 °C for 10 min.

After determination of the partial cDNA sequences, rapid amplification of cDNA ends polymerase chain reaction (RACE-PCR) was performed to obtain the full-length cDNA. All primers are described in Table 1. The gene-specific primers RACE-5′ and RACE-5′-nest were used to clone the 5′-end cDNA. RACE-3′ and RACE-3′-nest were used to clone the 3′-end. The 5′ and 3′-RACE-Ready cDNA was synthesized from total RNA with the Clontech BD SMART™ RACE cDNA Amplification Kit according to manufacturer's protocols.

PCR products of predicted sizes were sequenced and analyzed as described (Ma et al., 2012). Sequencing primers P3-F and P3-R (Table 1) were designed according to the composed sequence to amplify the full length of *M. japonicus ferritin*.

2.3. WSSV infection and sampling

The WSSV stock solution (10^8 virions/μl) was provided by Key Laboratory of Marine Biogenetic Resources, Third Institute of Oceanography, China. WSSV particle purification procedures were described by Xie et al. (2005). After 10,000-fold dilution in 0.9% saline (NaCl, 9 g/l), WSSV solution (10^5 virions per 1.0 g shrimp) was injected intramuscularly in the fourth abdominal segment of each shrimp. Control shrimp received 100 μl 0.9% saline solution.

Different tissues of 4 to 6 randomly chosen shrimp from each group (treatment and control) were collected at 0, 3, 6, 12, 24, 48, and 72 h post-infection (hpi) and stored in RNAstore solution (TIANGEN, China). At the same time, tissues were collected from another two shrimp, fixed in Davidson's Alcohol Formalin Acetic acid Fixative for 24 h, and transferred to 70% ethyl alcohol for preservation (Lightner, 1996). Muscle tissues were collected from the first abdominal segment of each shrimp and stored in 95% ethanol.

2.4. Histopathology

Standard histological procedures were performed according to Lightner (1996). Briefly, the tissues were dehydrated in a series of graded ethanol (70% to 100%), then transferred through xylene into liquid paraffin wax (melting point 53–54 °C) and embedded in paraffin wax. Blocks were sectioned at 5-μm thickness and stained with Mayer's hematoxylin and eosin (H&E). The sections were screened with an Olympus CX31 microscope (Olympus, Japan).

2.5. Quantitative real-time PCR

2.5.1. Tissue distribution of ferritin mRNA in normal M. japonicus

Relative quantitative real-time PCR (qRT-PCR) was performed to measure *ferritin* expression in the hepatopancreas, hemocytes, eyestalk, gill, midgut, muscle, heart, and stomach of WSSV-free *M. japonicus*. Three samples for each tissue were collected and cDNAs were prepared. The specific primers Fer-F and Fer-R (Table 1) were designed based on the cDNA sequence of *ferritin*. A pair of 18S rRNA (GenBank accession: AF463512.1) primers, 18S-F and 18S-R (Table 1), served as the reference for internal standardization. Amplification was performed in a 20-μl reaction with 0.4 μl primers (10 mM) and 2 μl cDNA, with SYBR® Premix Ex Taq™ (TaKaRa, Japan), and an ABI 7500 real-time PCR system (Applied Biosystems, USA) provided by the Open and Sharing Platform of Equipment and Technology (OSPET, College of Ocean and Earth Sciences, Xiamen University). PCR assays were performed in triplicate for each cDNA sample. qRT-PCR cycling conditions were as follows: 95 °C for 10 s, followed by 40 cycles of 95 °C for 5 s, and 60 °C for 34 s. Melting curve analysis was performed at the end of the reaction to confirm that only one PCR product was amplified and detected.

2.5.2. Ferritin mRNA levels in different tissues after WSSV challenge

To examine the change of *ferritin* mRNA expression after WSSV challenge, tissue samples from the hepatopancreas, gill, and midgut were collected at several stages of infection and used to prepare cDNA templates. These tissues are important in immune response and are sensitive to WSSV infection. qRT-PCR was performed as described in Section 2.5.1.

Table 1
PCR primers for cloning and qRT-PCR.

Primers	Sequence (5′–3′)	Product length	Sequence information
P1-F	TCACCAGTGTGTGGACGAG	452	Gene fragment
P1-R	AAGAGAGACTGATTGACCTGC		Gene fragment
P2-F	CAAGAGTGGGGTACTGGTCT	396	Gene fragment
P2-R	GCCTTTGGGATGTTTTCAG		Gene fragment
P3-F	TCACCAGTGTGTGGACGAG	1152	Gene fragment
P3-R	TTCCAGAAGCCAACACCA		Gene fragment
RACE-5′	GCCACCACGCTTGTTCTGGTACTTCA	354	5′-RACE PCR
RACE-5′-nest	ATCGTCTTCTGGATTCGTGGGAGGT	125	5′-RACE PCR
RACE-3′	ACTCCATAGCACTGCAAGTGGCAACA	777	3′-RACE PCR
RACE-3′-nest	AAGCTGAAGCGTGCTGGCCCAA	665	3′-RACE PCR
Fer-F	CAAGTTCTTCAAGGAGTCCAGTG	191	qRT-PCR
Fer-R	CCAAGAGAGACTGATTGACCTGC		qRT-PCR
18S-F	GTTCGTTCGTTCGTTCGTTC	154	qRT-PCR control
18S-R	GTACGTCCACCGCTGCATTA		qRT-PCR control

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