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A novel white spot syndrome virus-induced gene (MjVIG1) from *Marsupenaeus japonicus* hemocytes

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ABSTRACT

cDNA of a newly recognized white spot syndrome virus (WSSV)-induced gene (MjVIG1) was characterized from *Marsupenaeus japonicus* hemocytes; this gene encodes a protein that lack similarity to any known characterized protein. To identify this novel gene, we mainly conducted transcript level analysis, immunostaining and flow cytometry after WSSV infection. MjV1G1 transcript levels were also measured after Yellow head virus (YHV) and *Vibrio parahaemolyticus* infection tests. In non-infected and WSSV-infected shrimp, MjVIG1 was observed in granule-containing hemocytes. In addition, the MjVIG1 transcript level and ratio of MjVIG1-positive hemocytes both significantly increased, and number of MjVIG1-positive hemocytes slightly increased after WSSV infection. In contrast, MjVIG1 transcript level did not change after YHV and *V. parahaemolyticus* infection. These results indicated that MjVIG1 might be a WSSV-specific induced gene in *M. japonicus* hemocytes.

1. Introduction

Shrimp aquaculture production has rapidly expanded over the last few decades, and shrimp is considered the most economical commodity in fishery production [1]. However, invasive pathogens are a critical risk factor for the shrimp aquaculture industry. The vast majority of shrimp loss is caused by viral pathogens, including white spot syndrome virus (WSSV) and yellow head virus (YHV) [2]. WSSV is a rod-shaped, enveloped DNA virus that belongs to family *Nimaviridae*, which is highly lethal to penaeid shrimp [3,4]. YHV is a bacilliform, enveloped, positive-sense single-stranded RNA virus classified in the family *Roniviridae*, which is also reported to cause mass mortality in shrimp aquaculture [5,6].

Information regarding the viral defense system in penaeid shrimp is still limited. As invertebrates, crustaceans including penaeid shrimp solely depend on their innate immune systems for defense against pathogenic microorganisms [7,8]. Hemocytes are the major immune cells of shrimp and play an essential role in both cellular and humoral immune responses [9,10]. Three different types of hemocytes (granular, semigranular and hyaline cells) have been classified in shrimp hemolymph based on morphology [11–13]. Additionally, different types of hemocytes in crustaceans revealed specific but partially overlapping functions, such as phagocytosis in hyaline cells; encapsulation, phagocytosis, presence of ProPO system and cytotoxicity in semigranular cells (SGCs); and presence of ProPO system in granular cells [14–16].

In response to viral infections, the induction of host cell's gene expression is a primary step in the initiation of the host defense system. Viruses induced the up-regulation of many genes encoding for proteins that have antiviral activity [17]. Recently, several molecules have been reported to combat against WSSV involved in shrimp immunity, including antimicrobial peptides (AMPs), prophenoloxidase (ProPO) system and proteinase inhibitors [16,18,19].

In addition, to understand the interaction between host cell and virus, various viral responsive genes (VRP) have been identified in virus-infected shrimp and virus-resistant shrimp [20–23]. Particularly, in response to WSSV, an antiviral gene PmAV was identified from WSSV-resistant shrimp *Penaeus monodon* by mRNA differential display technique [24]. Another WSSV-strongly induced gene of *P. monodon* (PmERP15), was identified by microarray screening and its presence was found to be essential for the survival of WSSV-infected shrimp [25]. In *Marsupenaeus japonicus*, the VRP gene expression was up-regulated after knockdown with non-specific double strand RNA following WSSV infection, suggesting its essential role in viral-defense system [26]. Moreover, a previous study reported that MjVRP gene expression was significantly increased after WSSV infection, which concluded MjVRP + hemocytes have a supporting role in the pathogenesis of WSSV [27].

Apart from these characterized viruses responsive genes, there are

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still numerous virus-related genes with unknown function which are needed to be further investigated. A previous study separated hemocytes into two sub-populations by Percoll gradient centrifugation and transcriptome analysis using next-generation sequencing [28]. Some contigs were differentially expressed among hemocyte sub-populations; among them, contig c15785_g1 was strongly expressed in the upper layer [28]. In this study, we aimed to characterize and analyze the novel contig c15785_g1. This novel gene was found to be the first WSSV induced gene in *M. japonicus* hemocytes after performing different assays, and is hereafter referred to as MjVIG1.

2. Materials and methods

2.1. Experimental animals

Apparently healthy kuruma shrimp (*Marsupenaeus japonicus*) were purchased from a commercial shrimp farm in Okinawa, Japan. Approximately 15–30 g shrimp were kept in water-recirculated tanks that were maintained at 25 °C and 29–32 ppt, and fed daily with a commercial diet before the experiment.

2.2. cDNA cloning and sequence analysis of MjVIG1

Total RNA was extracted from different tissues of kuruma shrimp with RNAiso reagent (Takara Bio Inc. Japan) following the manufacturer's instructions. One microgram of the RNA was used to synthesize cDNA with a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Japan). Primer sets to clone the MjVIG1 gene were based on an Expressed sequence tag database previously obtained by our laboratory [28]. The full-length cDNA sequence was cloned by 5′-and 3′-rapid amplification of cDNA ends (RACE)-PCR using a SMARTer RACE cDNA Amplification kit following the manufacturer's protocol (Clontech, Japan), and analyzed with GENETYX version 11.0.4 (Software Development Co. Ltd. Japan). The signal peptide was predicted by SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/). The protein conserved domain was predicted by Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/) and NCBI-CDD (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi/). Transmembrane helix was predicted by TMHMM Server version 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). The UniProt Database (http://www.uniprot.org/blast/) was used to find homologs of the deduced amino acid sequences translated from the nucleotide sequences.

2.3. MjVIG1 gene expression

MjVIG1 transcript level was determined by Real-Time PCR (qRT-PCR). Sequence specific primers (MjVIG1_qPCR_F and MjVIG1_qPCR_R) were set shown in Table 1. Elongation factor-1 alpha (EF-1a) was used as an internal control gene. qRT-PCR was conducted using THUNDERBIRD SYBR qRT-PCR Mix (TOYOBO, Japan) and conditions were as follows: 95 °C for 1 min and 40cycles of 95 °C for 15 s and 60 °C for 1 min, followed by dissociation analysis. The Ct values (relative measure) of target genes were analyzed with one-way ANOVA with Tukey multiple comparisons tests on the GraphPad Prism v 6.0 (GraphPad Software, Inc. USA), where $p^{<} 0.05$ was considered as statistically significant.

2.4. Production of recombinant protein MjVIG1 and rabbit polyclonal antiserum

The sequence that corresponded to the MjVIG1 mature protein was amplified with the specific primers MjVIG1_rF and MjVIG1_rR (Table 1) from hemocyte cDNA of kuruma shrimp. The PCR fragment was cloned into the pET32a vector by adding a His-tag. Recombinant proteins (rMjVIG1) were produced in the *Escherichia coli* BL21 (DE3) strain.

Table 1
List of primers and their sequences used in experiments.

Primer name	Primer sequence (5′-3′)	Usage
3′RACE_ngsp1	GGGCAACCAACTCCAGGCAGAAGAAGGG	RACE PCR
3′RACE_gsp1	GTGATGCTACTGGCGGCGATCGCTGC	RACE PCR
5′RACE_ngsp2	GCGTTACTTAGTTGACCGACCCTCTGGG	RACE PCR
5′RACE_gsp2	CTGTTCGACCTCTTGCTTGCTGGGTCCG	RACE PCR
MjVIG1_rF (NdeI[a])	CATATGGCTTCGCTGGGCAACCAACTCC	Recombinant protein
MjVIG1_rR (NotI[a])	GCGCCGCTCTTCCAGTCACAATCTGCCTAG	Recombinant protein
MjVIG1_qPCR_F	GGTCGAACAGATTGCTAATGGAA	qRT-PCR
MjVIG1_qPCR_R	GGCTGTTATGGCCGAAGATG	qRT-PCR
EF1α_qPCR_F	ATTGCCACACCGCTCACA	qRT-PCR
EF1α_qPCR_R	TCGATCTTGGTCAGCAGTTCA	qRT-PCR
VP28_qPCR_F	GATCCGCAATGGAAAGTCTG	qRT-PCR
VP28_qPCR_R	AGATTCTGCCCCACAGTCAC	qRT-PCR
YHV_qPCR_141F	CGTCCCGGCAATTGTGAT	qRT-PCR
YHV_qPCR_206R	CCAGTGACGTTCGATGCAATA	qRT-PCR

[a] Restriction enzyme site is underlined at the start of the sequence.

Then recombinant protein was purified by a Ni-NTA agarose column (QIAGEN, Germany), and protein concentration was measured with a Qubit 2.0 fluorometer (Invitrogen by Life Technologies, USA). Rabbit polyclonal antiserum (anti-rMjVIG1) against the purified rMjVIG1 was prepared by Eurofins Genomics (Japan).

2.5. Western blotting

Anti-rMjVIG1 specificity determination by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting followed the protocol described in a previous report [29]. However, the primary antibody was changed to anti-rMjVIG1 serum (1:5000 diluted in 3% skim milk).

2.6. Immunostaining

Kuruma shrimp hemolymph was collected from a control (0 h), and 24, 48 and 72 h after WSSV infection (three per group) using an anticoagulant composed of 10 mM EDTA (pH 7) diluted in kuruma shrimp PBS (KPBS) (480 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4{\cdot}12H_2O$, 1.47 mM KH_2PO_4, pH 7.4), which contained 2% (w/v) paraformaldehyde. Collected hemolymph was fixed at room temperature for 15min, while the total hemocyte counts (THCs) were counted by a C-chip hemocytometer (NanoEntek, Germany). Then, hemocytes were collected by centrifugation and washing twice with 0.5% bovine serum albumin in 1 × KPBS in 0.05% tween-20 (BSA-KPBST). Then, hemocytes were mixed with rabbit polyclonal anti-rMjVIG1 serum and anti-VP28 antibody which is specific to the VP28 capsid protein of WSSV (1:100 diluted in 5% BSA-KPBST) for 1 h at room temperature and washed three times. Then, hemocytes were incubated with Alexa fluor 488-conjugated goat anti-rabbit IgG (1:100 diluted in 5% BSA-KPBST, Biomedical Technologies Inc. USA) at room temperature for 30 min in the dark. Following another round of centrifugation and being washed twice, the pellet was suspended in 500 μl 1 × KPBS. Hoechst staining solution (Invitrogen, USA) was diluted 1:1000 in distilled water and it was used to stain hemocyte nuclei. These hemocytes were spread onto a glass slide (Matsunami Glass Ind. Ltd, Japan) and subjected to cytospin centrifugation for 2 min at 200 × *g*. Then, the slides were analyzed under afluorescence microscope (Nikon Eclipse Ci, DS-Ril camera, Japan).

2.7. Flow cytometry analysis

The hemocytes described in section 2.6 were analyzed with a BD FACSCalibur flow cytometer (Becton-Dickinson, USA) with Cell Quest