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# Involvement of WSSV–shrimp homologs in WSSV infectivity in kuruma shrimp: *Marsupenaeus japonicus*

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ABSTRACT

White spot syndrome virus (WSSV) is pathogenic and specific to shrimp, and is capable of producing a persistent infection in the host. Moreover, shrimp are capable of persistently carrying a single or multiple viruses, allowing them to survive for long periods with latent infections. In order to identify genes that are specially involved in the intricate WSSV–shrimp association, we focused on homologs between the WSSV and shrimp genomes. We here investigated whether homologous *Wssv*ORFs (*Wssv*ORF285, *Wssv*ORF332) and their homologs in the kuruma shrimp genome (*Mj*ORF16, *Mj*ORF18) are important for WSSV infectivity by utilizing dsRNA-mediated RNA interference, and further proposed potential roles of homologous *Wssv*ORFs associated with the persistent viral infection stage. Homologous *Mj*ORFs were found to be highly up-regulated in several tested tissues upon WSSV infection. Injection of dsRNAs specific to homologous *Mj*ORFs, followed by WSSV challenge, led to reduced and delayed shrimp mortality when compared to that of shrimp without dsRNA injection. Silencing of homologous *Wssv*ORFs by specific dsRNAs sharply increased shrimp survival. *Wssv*ORF332 may function as a latency gene especially associated with the persistent WSSV infection stage while *Wssv*ORF285 may be classified into the same group as *Wssv*VP28 and may play a role in virus penetration during the infection. Our results suggest that WSSV–shrimp homologs are involved in WSSV infectivity and support the hypothesis that homologous *Wssv*ORFs are related to WSSV latency and pathogenesis.



## 1. Introduction

Infections by viruses, especially white spot syndrome virus (WSSV), have caused disastrous diseases in crustaceans such as shrimp, resulting in reduced shrimp production and causing serious economic losses worldwide. Although several methods have shown some efficacy against WSSV under experimental conditions, no therapeutic strategies are available to effectively control WSSV in the field. WSSV contains a large circular double-stranded DNA of about 300 kbp, encodes at least 181 open reading frames (ORFs) (Escobedo-Bonilla et al., 2008; Leu et al., 2009; van Hulten et al., 2001a; Yang et al., 2001) and is classified as the sole member of the genus *Whispovirus*, family *Nimaviridae* (Mayo, 2002a,b). This virus is unique, with an infection strategy that does not match infection models of any other known virus, and is highly pathogenic and virulent only on shrimp, although it has a remarkably broad host range covering almost all crustaceans (Escobedo-Bonilla et al., 2008; Leu et al., 2009; Luo et al., 2003; Sritunyalucksana et al., 2006). WSSV targets almost all organs of the host, including hemocytes, heart, stomach, gills, intestine, hepatopancreas, lymphoid organ and the nervous system. The primary sites of WSSV replication are stomach and gills, which can be severely damaged in both the early and late stages of WSSV infection (Escobedo-Bonilla et al., 2008). Because of the large size of the genome and the uniqueness of the proteins, WSSV has not yet been fully characterized. In addition, there is a discrepancy between responses of shrimp and other crustaceans to WSSV infection and the reason for the discrepancy is unknown.

Shrimp are capable of persistently carrying a single or multiple viral pathogens at low levels without signs of diseases, allowing them to survive for long periods with persistent/latent infections (Flegel, 2007, 2009). Flegel (2007) proposed that shrimp actively accommodate viral pathogens as persistent infections that act as a "specific memory" that functions to specifically reduce the severity of the disease. The specific anti-viral responses, as hypothesized by Flegel (2009), may be based on viral sequences inserted into the shrimp genome. Some of these sequences generate antisense, immunospecific RNAs (imRNAs) capable of stimulating host RNA interference (RNAi) that suppresses viral propagation. This leads to low-level, active infections where the host exhibits no clinical symptoms of disease.

As a result, the interactions between shrimp and virus are now attracting much attention. Several studies using different

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techniques have examined on host–WSSV interactions in shrimp to search for genes whose expressions are induced by viral infections, and genes whose expressions are associated with the ability of shrimp to survive from viral infections (He et al., 2005; Liu et al., 2006; Luo et al., 2003; Ma et al., 2008; Rojtinnakorn et al., 2002; Sritunyalucksana et al., 2006; Wang et al., 2006, 2007; Zhao et al., 2007). Several genes have been identified that are potentially involved in anti-WSSV responses or interacting with WSSV in shrimp (Liu et al., 2009).

One approach to identifying and understanding the functions of genes that are specially involved in the host–virus interactions is to search for homologs in the host genome. We have constructed a bacterial artificial chromosome (BAC) library that has at least a 3× coverage of the 2000 Mbp kuruma shrimp (*Marsupenaeus japonicus*) genome. Sequencing of one BAC clone (Mj024A04) revealed 27 ORFs. Seven of these ORFs appeared to be homologs of predicted proteins of WSSV (Koyama et al., 2010). Two of the homologs in kuruma shrimp (*Mj*ORF16 and *Mj*ORF18), both of unknown function, and their homologs in WSSV (*Wssv*ORF332 and *Wssv*ORF285, respectively) were selected to investigate potential roles of WSSV–shrimp homologs in WSSV infectivity in shrimp. To do this, we injected shrimp with double-stranded RNAs (dsRNAs) specific for tested ORFs and then challenged the shrimp with WSSV. This is the first study to demonstrate the involvement of both WSSV and shrimp homologs in WSSV infectivity. It provides a thorough understanding on both sides of the complex shrimp–WSSV interactions, and should lead to a better understanding of the establishment of viral latency in shrimp.

## 2. Materials and methods

### 2.1. Experimental shrimp

Kuruma shrimp (average weight of 10 g) used in this study were purchased from a commercial shrimp farm (Miyazaki, Japan) and reared in a re-circulating water tank system maintained at about 22–25 °C and 30 ppt salinity prior to the experiment. The WSSV-free status of randomly selected samples of the experimental shrimp was confirmed by both Shrimple WSSV kit (EnBioTec Laboratories Co. Ltd., Japan) and PCR assay using gene-specific primers corresponding to 3 WSSV ORFs (*Wssv*ORF285, *Wssv*ORF332, and *Wssv*VP28) (Table 1). Only apparently healthy and WSSV-negative shrimp were picked and used in all experimental set-ups.

### 2.2. Preparation of virus stock and virus inoculum

Whole WSSV-infected moribund shrimp were homogenized in PBS (Phosphate Buffered Saline) and the pooled solution was centrifuged at 3000 × *g* for 20 min at 4 °C. The supernatant was re-centrifuged at 6000 × *g* for 15 min at 4 °C and the final supernatant was filtered through a 0.45 μm filter membrane (Millipore Corp., USA). The virus filtrate was then aliquoted and stored at −80 °C for use as virus stock. The experimental viral inocula were prepared from the virus stock at dilutions of $10^4$ and $10^5$ because these conditions, based on an *in vivo* viral titration assay, were expected to give optimal responses for testing the efficiency of anti-viral activity of dsRNAs during an experimental period of about 10 days, and to differentiate between severe and persistent WSSV infections in shrimp.

### 2.3. Design of RNAi-probe and synthesis of dsRNA

RNAi-probes including a T7 RNA polymerase promoter were designed with E-RNAi (http://e-rnai.dkfz.de/), a web application for designing dsRNA constructs of approximately 500 bp (Arziman et al., 2005). Briefly, primers specific for each target gene were designed and the T7 promoter sequence was then incorporated to the 5′ end of gene-specific primers to generate dsRNAs by using T7 RiboMAX express RNAi system (Promega, USA). For shrimp RNAi-probes (*Mj*ORF16-dsRNA and *Mj*ORF18-dsRNA), gene-specific primers were designed according to the sequences of *Mj*ORF16 and *Mj*ORF18 obtained from the clone Mj024A04 (Koyama et al., 2010). For WSSV RNAi-probes (*Wssv*ORF285-dsRNA and *Wssv*ORF332-dsRNA), viral gene-specific primers were designed based on sequences of WSSV ORFs obtained from GenBank (GenBank accession nos. NP_477807 and NP_477854, respectively). RNAi-probes were searched for sequence homology to their homologs to ensure that a given dsRNA does not target its homolog. Likewise, sets of primers specific for *Wssv*VP28 and eGFP proteins with the T7 promoter sequence were also made to produce *Wssv*VP28-dsRNA that served as a positive anti-viral dsRNA control and eGFP-dsRNA to serve as an un-related dsRNA control. The oligonucleotide sequences used to generate *Wssv*VP28-dsRNA were obtained from Sarathi et al. (2008b) who attained 100% survival for WSSV-challenged shrimp injected with this dsRNA.

Double-stranded RNAs (dsRNAs) were generated *in vitro* using T7 RiboMAX express RNAi system (Promega, USA) following the manufacturer's instructions. Briefly, two separate PCR reactions with a single T7 promoter were set up for each dsRNA to generate two separate single promoter PCR templates for *in vitro* transcription. The resulting PCR products were purified and subsequently sequenced to confirm that they corresponded to the target genes. Purified PCR products were quantified and transcribed to yield single-stranded RNAs (ssRNAs). Equal amounts of ssRNAs were annealed to produce dsRNAs which were further purified and quantified for the *in vivo* RNAi experiments. The oligonucleotide sequences used for synthesis of dsRNAs are shown in Table 2.

### 2.4. In vivo viral titration assay

This assay (data not shown) was done as a preliminary experiment to find out the appropriate WSSV dose for the viral challenge test. WSSV stock was diluted with PBS buffer in a series from $10^1$ to $10^9$ times to determine the dilution resulting in 90–100% mortality in shrimp. Healthy shrimp from the stocking tank were intramuscularly injected with several diluted viral inoculums (from $10^3$ to $10^9$ dilutions). Shrimp injected with PBS buffer served as the negative control. Ten shrimp were used and maintained at 25 °C and 30 ppt salinity in a separated tank for each dilution. The number of deaths was recorded daily and the cumulative percentage mortality was calculated. The presence of WSSV in dead shrimp was confirmed using a commercial kit (Shrimple WSSV Kit) and a PCR assay.

### 2.5. Assay for WSSV homologs in kuruma shrimp (MjORF16 and MjORF18)

#### 2.5.1. Expression of WSSV homologous MjORFs during WSSV infection in kuruma shrimp

To investigate whether homologous *Mj*ORFs interact with WSSV infection in shrimp, expression patterns of *Mj*ORFs were examined in various shrimp tissues upon time-course WSSV infection. Shrimp were infected with 50 μl of $10^6$ × diluted WSSV stock and maintained at 25 °C and 30 ppt salinity for time-course RNA sampling. Five live infected-shrimp were randomly collected at 0, 1, 3, and 5 days post-injection (d.p.i.). Several tissues (hemocytes, heart, lymphoid organ, intestine, stomach, hepatopancreas, nerve and gills) were dissected out from individual shrimp sam-

**Table 1**
Primers used for PCR and RT-PCR analysis.

| Primer name | Oligonucleotide sequence (5′–3′) | Sequence position | PCR (RT-PCR) product (bp) |
|---|---|---|---|
| *Wssv*ORF285-F | CTATTGTGCCGTTTCTGGGT | 220 | 477 |
| *Wssv*ORF285-R | CAGAAACCATGCGGAAGAAT | 696 | |
| *Wssv*ORF332-F | CCTGACCACATCAAGAGGGT | 1173 | 539 |
| *Wssv*ORF332-R | TCGTTGATGGGTGTTGAAGA | 1711 | |
| *Wssv*VP28-F | ATGGATCTTTCTTTCACTCTTTC | Full-length | 615 |
| *Wssv*VP28-R | TTACTCGGTCTCAGTGCCAG | | |
| *Wssv*ORF366-F | GAGACGTCGCTCATCAAAGATGGGGAAG | – | 210 |
| *Wssv*ORF366-R | GAAACCTGGACCATATTGAATACGGCCAG | | |
| *Mj*ORF16-F | CATTCGTGCCGGCTGAGA | 728 | 521 |
| *Mj*ORF16-R | CACGACTCGACTATCAGCGTA | 1248 | |
| *Mj*ORF18-F | GACAAAGTTGCCCCACAAGT | 1600 | 478 |
| *Mj*ORF18-R | CGAAATCCACTTAATGCCGT | 2077 | |
| *Mj*ORF11-F | CTTTTGGACACGGGAACACT | 2926 | 401 |
| *Mj*ORF11-R | TGTTTCTCGTGGCTGTGAAC | 3345 | |
| *Mj*ORF13-F | TGGATAGCCATAGATTCCGC | 2367 | 471 |
| *Mj*ORF13-R | CAAGAGTCCAGCATTGGGTT | 2837 | |
| *Mj*ORF14-F | CGAGTCTCTATCAGTGCCTCA | 3725 | 502 |
| *Mj*ORF14-R | TGTCTCCCAATGCACGTGA | 4227 | |
| *Mj*ORF15-F | GCGGACTCGACCAAAAATTA | 1006 | 418 |
| *Mj*ORF15-R | GTAGCCGATATTGTCCCCAA | 1423 | |
| *Mj*ORF17-F | TGCGTTTAATGCAGACCAAG | 1124 | 549 |
| *Mj*ORF17-R | TGGCTATTGGTAGCGGAATC | 1672 | |
| EF1α-F | ATGGTTGTCAACTTTGCCCC | – | 500 |
| EF1α-R | TTGACCTCCTTGATCACACC | | |
| DecaOIE-F | TGCCTTATCAGCTNTCGATTGTAG | – | 848 |
| DecaOIE-R | TTCAGNTTTGCAACCATACTTCCC | | |

ples. At each indicated time-course interval, each tissue sample was pooled from 5 selected-shrimp. Total RNA was isolated from pooled samples using RNAiso (Takara Bio., Japan), treated with RNAse-free DNase I (Promega, USA) and then used to extract mRNA using an Illustra QuickPrep micro-mRNA purification kit (GE healthcare, USA) according to the manufacturer's protocol. One hundred (100) ng of each mRNA sample was reverse transcribed to produce cDNA with M-MLV reverse transcriptase (Invitrogen, USA) following the manufacturer's recommendations. The expression patterns of *Mj*ORFs, upon time-course WSSV infection, were determined by RT-PCR using equal amounts of cDNAs as templates.

#### 2.5.2. Assay of sequence-specific gene silencing by homologous *MjORF-dsRNAs in kuruma shrimp in vivo*

Shrimp were injected with either 50 μl of PBS or each dsRNA (*Mj*ORF16-dsRNA and *Mj*ORF18-dsRNA and eGFP-dsRNA) dissolved in 50 μl of PBS (5 μg dsRNA per 1 g shrimp). The experiment was maintained at 25 °C and 30 ppt salinity. Two shrimp were randomly sampled from each experimental group at 0, 1, 3, 5 and

**Table 2**
Primers used for dsRNA production.

| Primer name | Oligonucleotide sequence (5′–3′) |
|---|---|
| *Wssv*ORF285-dsRNA | |
| T7*Wssv*ORF285-F | GGATCCTAATACGACTCACTATAGGGCTATTGTGCCGTTTCTGGGT |
| *Wssv*ORF285-R | CAGAAACCATGCGGAAGAAT |
| T7*Wssv*ORF285-R | GGATCCTAATACGACTCACTATAGGG CAGAAACCATGCGGAAGAAT |
| *Wssv*ORF285-F | CTATTGTGCCGTTTCTGGGT |
| *Wssv*ORF332-dsRNA | |
| T7*Wssv*ORF332-F | GGATCCTAATACGACTCACTATAGGGCCTGACCACATCAAGAGGGT |
| *Wssv*ORF332-R | TCGTTGATGGGTGTTGAAGA |
| T7*Wssv*ORF332-R | GGATCCTAATACGACTCACTATAGGGTCGTTGATGGGTGTTGAAGA |
| *Wssv*ORF332-F | CCTGACCACATCAAGAGGGT |
| *Mj*ORF16-dsRNA | |
| T7*Mj*ORF16-F | TAATACGACTCACTATAGGGAGACATTCGTGCCGGCTGAGA |
| *Mj*ORF16-R | CACGACTCGACTATCAGCGTA |
| T7*Mj*ORF16-R | TAATACGACTCACTATAGGGAGACACGACTCGACTATCAGCGTA |
| *Mj*ORF16-F | CATTCGTGCCGGCTGAGA |
| *Mj*ORF18-dsRNA | |
| T7*Mj*ORF18-F | TAATACGACTCACTATAGGGAGAGACAAAGTTGCCCCACAAGT |
| *Mj*ORF18-R | CGAAATCCACTTAATGCCGT |
| T7*Mj*ORF18-R | TAATACGACTCACTATAGGGAGACGAAATCCACTTAATGCCGT |
| *Mj*ORF18-F | GACAAAGTTGCCCCACAAGT |
| *Wssv*VP28-dsRNA | |
| T7*Wssv*VP28-F | TAATACGACTCACTATAGGGAGAATGGATCTTTCTTTCACTCTTTC |
| *Wssv*VP28-R | TTACTCGGTCTCAGTGCCAG |
| T7*Wssv*VP28-R | TAATACGACTCACTATAGGGAGATTACTCGGTCTCAGTGCCAG |
| *Wssv*VP28-F | ATGGATCTTTCTTTCACTCTTTC |
| eGFP-dsRNA | |
| T7eGFP-F | TAATACGACTCACTATAGGGAGAGTTCAGCGTGTCCGGCGAG |
| eGFP-R | GTTCTTCTGCTTGTCGGCC |
| T7eGFP-R | TAATACGACTCACTATAGGGAGAGTTCTTCTGCTTGTCGGCC |
| eGFP-F | GTTCAGCGTGTCCGGCGAG |

7 d.p.i. for RNA extraction. Total RNA was extracted from stomach of collected shrimp using RNAiso (Takara Bio., Japan), treated with RNase-free DNase I (Promega, USA), and then reverse transcribed to cDNA using M-MLV reverse transcriptase (Invitrogen, USA). The inhibitory effect and specificity of *Mj*ORF-dsRNAs were determined by RT-PCR analysis using equal amounts of cDNAs as templates.

#### 2.5.3. In vivo anti-viral RNAi assay of homologous MjORFs in kuruma shrimp

Protective efficiencies of tested *Mj*ORF-dsRNAs against WSSV infection were determined by intramuscular injection of dsRNAs in shrimp and challenge immediately with WSSV. Shrimp were injected with either PBS (negative and positive WSSV controls) or each dsRNA (5 μg dsRNA per 1 g shrimp) prior to the challenge with the virus inoculums of 100 μl of $10^4 \times$ diluted WSSV stock. Shrimp injected with eGFP-dsRNA and challenged with WSSV served as an un-related dsRNA control group. For each treatment, 10 shrimp were used and maintained at 25 °C and 30 ppt salinity in separated tanks. The number of dead shrimp was recorded daily up to 10 d.p.i. for the cumulative mortality assay. Cumulative mortality data were subjected to paired sample *t*-tests for statistical analysis. Differences were considered significant at $p < 0.05$.

### 2.6. Assay for WSSV genes homologous to hypothetical proteins of kuruma shrimp (WssvORF285 and WssvORF332)

#### 2.6.1. Assay of sequence-specific gene silencing by homologous WssvORF-dsRNAs in kuruma shrimp in vivo

Shrimp were injected with either 50 μl of PBS (negative and positive WSSV control groups) or each dsRNA (*Wssv*ORF285-dsRNA, *Wssv*ORF332-dsRNA, *Wssv*VP28-dsRNA and eGFP-dsRNA) dissolved in 50 μl of PBS (5 μg dsRNA per 1 g shrimp) prior to challenge with WSSV (100 μl of $10^5 \times$ dilution from the virus stock). The experiment was maintained at 25 °C and 30 ppt salinity. Two live shrimp were randomly sampled from each experimental group at 1, 3, 5 and 10 d.p.i. for RNA extraction. Total RNA was extracted from gills of collected shrimp to produce cDNA following the same protocol mentioned in Section 2.5.2. The inhibitory effect and specificity of *Wssv*ORF-dsRNAs were determined by RT-PCR analysis using equal amounts of cDNAs as templates.

#### 2.6.2. In vivo anti-viral RNAi assay of homologous WssvORFs in kuruma shrimp

Protective efficiencies of tested *Wssv*ORF-dsRNAs against WSSV infection were determined by intramuscular injection of dsRNAs in shrimp and challenge immediately with two different doses of WSSV. Shrimp were injected with either PBS (negative and positive WSSV control groups) or each dsRNA (5 μg dsRNA per 1 g shrimp) prior to the challenge with the virus inoculums of 100 μl of either $10^4$ or $10^5 \times$ diluted WSSV stock. Shrimp injected with *Wssv*VP28-dsRNA and eGFP-dsRNA and challenged with WSSV served as positive anti-viral dsRNA and unrelated dsRNA control groups, respectively. For each treatment, 10 shrimp were used and maintained at 25 °C and 30 ppt salinity in separated tanks. The number of dead shrimp was recorded daily up to 10 d.p.i. for the cumulative mortality assay. Cumulative mortality data were subjected to paired sample *t*-tests for statistical analysis. Differences were considered significant at $p < 0.05$.

#### 2.6.3. Assay for role of homologous WssvORFs associated with the persistent WSSV infection stage in kuruma shrimp

In order to investigate the potential role of homologous *Wssv*ORFs (*Wssv*ORF285 and *Wssv*ORF332) associated with persistent WSSV infection stage, expression of the two *Wssv*ORFs was detected by RT-PCR on samples of cDNA from 10-day WSSV-challenged shrimp (with and without dsRNA treatment) obtained from the assay of sequence-specific gene silencing by viral-dsRNAs (Section 2.6.1).

*Wssv*VP28 protein and *Wssv*ORF366 were determined parallel with the targeted genes in order to serve as genes for comparison. The *Wssv*VP28 is a major structural protein that plays an important role in infection process, especially in the attachment and penetration of WSSV into shrimp cells (Escobedo-Bonilla et al., 2008; Sarathi et al., 2008b; van Hulten et al., 2001b; Witteveldt et al., 2004; Yi et al., 2004; Zhang et al., 2002). The *Wssv*ORF366 is identified as a latency-associated gene of WSSV (Khadijah et al., 2003; Lu and Kwang, 2004).

### 2.7. PCR and time-course RT-PCR analysis

PCR was carried out to confirm the WSSV-free status of experimental shrimp (data not shown) and detect the presence of WSSV particles at the persistent infection stage in shrimp. Total DNA was extracted from gills of selected-shrimp by using a ZR viral DNA kit (Zymo Research Corp., USA) following the manufacturer's protocol and then used as templates for PCR analysis. Decapod-specific primers were employed as an internal control to confirm successful DNA extraction from the host shrimp samples and equal amounts of DNA used for PCR. Sense and anti-sense decapod-specific primers corresponding to 143F and 145R, respectively, were taken from OIE website (http://www.oie.int/eng/normes/fmanual/A_00048.htm, manual of diagnostic tests for Aquatic Animals, 2003). Time-course RT-PCR was applied to evaluate the interaction of homologous *Mj*ORFs with WSSV infections, to elucidate the sequence-specific gene silencing by both shrimp-dsRNAs and viral-dsRNAs, and to determine the expression of homologous *Wssv*ORFs for purpose of prediction of their role in the persistent WSSV infection stage in shrimp. Gene-specific primers used to produce RNAi-probes were also used here for PCR and RT-PCR. Likewise, sets of primers specific to the 5 remaining homologous *Mj*ORFs were also designed using the sequences obtained from the Mj024A04 BAC clone (Koyama et al., 2010), to investigate their expression during the WSSV infection. EF-1α primers corresponding to EF-1α protein were included to serve as an internal control to confirm successful cDNA synthesis from shrimp RNA samples and equal cDNA material used for RT-PCR. The EF-1α specific primers were taken from a previous publication (Maningas et al., 2008). The following thermocycler conditions were applied for all PCR and RT-PCR set-ups: an initial denaturation at 95 °C for 5 min, followed by 35 or 40 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min and a final extension at 72 °C for 5 min. Primers used for PCR, RT-PCR are shown in Table 1.

## 3. Results

### 3.1. Homology between shrimp ORFs and WSSV ORFs

From 27 shrimp ORFs (*Mj*ORFs) predicted from the Mj024A04 BAC clone, 7 *Mj*ORFs whose functions have not been characterized were found to be homologous to predicted proteins of WSSV (*Wssv*ORFs). Amino acid identities between the deduced amino acid sequences of *Mj*ORFs and *Wssv*ORFs were about 20–30% (data not shown). In the present study, two WSSV homologs in kuruma shrimp (*Mj*ORF16 and *Mj*ORF18) and their homologs in WSSV (*Wssv*ORF332 and *Wssv*ORF285, respectively) were of special interest for further study utilizing RNAi technology. The positions and directions of 2 homologous *Mj*ORFs on the Mj024A04 BAC clone,

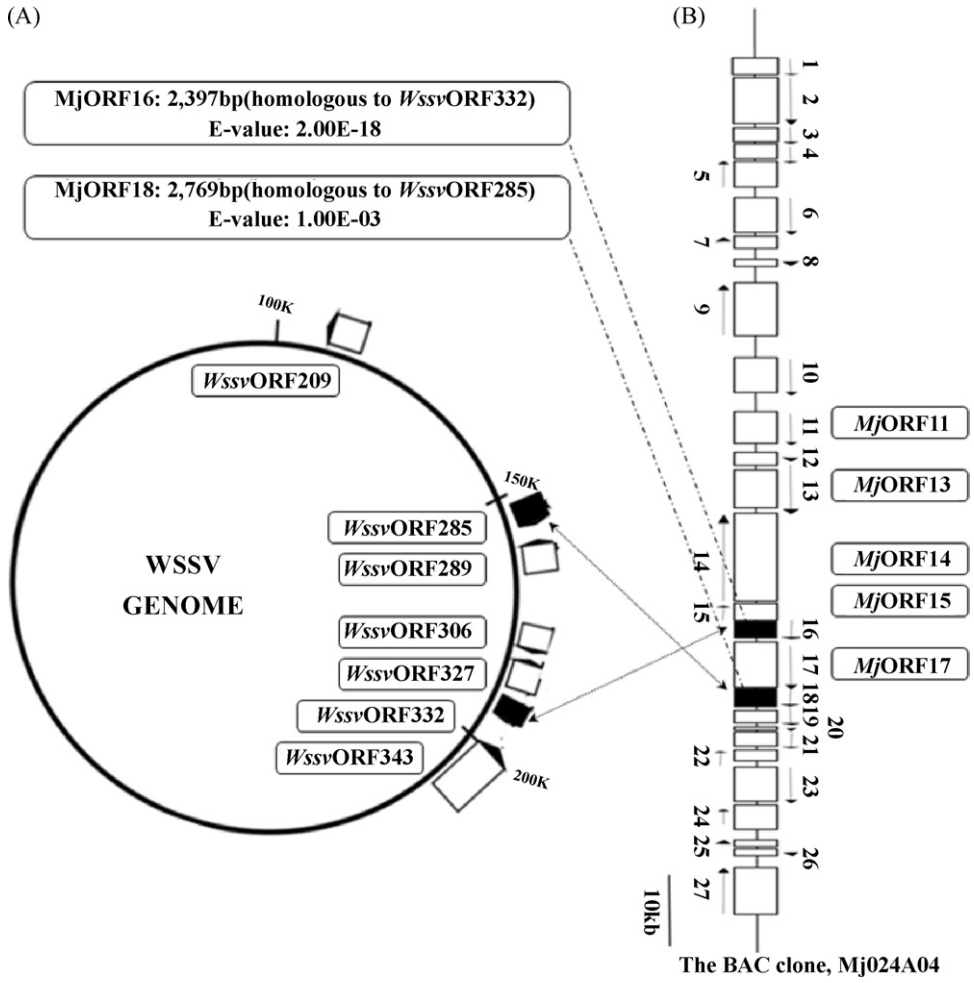


**Fig. 1.** Schematic diagram of *Mj*ORFs and *Wssv*ORFs. (A) Positions and directions of *Wssv*ORFs homologous to *Mj*ORFs in the WSSV genome (the genomic organization of WSSV was modified from Yang et al. (2001)). (B) Positions of *Mj*ORFs homologous to *Wssv*ORFs on the structure of Mj024A04 (the structure of Mj024A04 was modified from Koyama et al. (2010)). The targeted *Mj*ORFs and *Wssv*ORFs are shown in black.

and of 2 homologous *Wssv*ORFs on the WSSV genome are represented in Fig. 1.

### 3.2. Involvement of homologous MjORFs (MjORF16 and MjORF18) in WSSV infectivity in kuruma shrimp

#### 3.2.1. Expression of WSSV homologous MjORFs during WSSV infection in kuruma shrimp

The expression patterns of *Mj*ORF16 and *Mj*ORF18 in various tissues of kuruma shrimp following the time-course of WSSV infection were investigated by RT-PCR analysis using cDNAs derived from pooled mRNAs of 5 WSSV-injected shrimp at 4 time intervals (0, 1, 3 and 5 d.p.i.). In normal shrimp (0 d.p.i.), both *Mj*ORF16 and *Mj*ORF18 were abundantly expressed in the stomach, but were detected at low levels in all other tissues examined (hemocytes, heart, lymphoid organ, intestine, hepatopancreas, nerve and gills). However, WSSV infection induced the expression of the *Mj*ORFs. The expression levels were highly up-regulated in all tissues over the time-course of the virus infection (1, 3, and 5 d.p.i.) (Fig. 2A and B). Expressions of the 5 remaining homologous *Mj*ORFs also increased following viral infection (Fig. 2C). EF-1α was used as the internal reference control and was positively amplified, with similar levels, from all tissues of both normal and challenged shrimp (Fig. 2D), suggesting equal cDNA template levels and thus confirming the interaction of homologous *Mj*ORFs, particularly *Mj*ORF16 and *Mj*ORF18, with WSSV infection in shrimp.

#### 3.2.2. Sequence-specific gene silencing by homologous MjORF-dsRNAs

To determine the silencing efficiency of shrimp-specific dsRNAs (*Mj*ORF16-dsRNA and *Mj*ORF18-dsRNA) on the target *Mj*ORFs, expression levels of *Mj*ORFs were determined by RT-PCR in the stomach of shrimp at 1, 3, 5 and 7 days after injection with each dsRNA. The expression levels of *Mj*ORF16 and *Mj*ORF18 were significantly reduced when compared to those in normal shrimp (0 d.p.i.) and in shrimp injected with either PBS or other dsRNA at each indicated time point (Fig. 3A and B, respectively). The expression of EF-1α (Fig. 3C) was included as a reference. These results confirm that shrimp-dsRNAs specifically silenced the target genes.

#### 3.2.3. Effect of homologous MjORFs on WSSV infection in kuruma shrimp

To evaluate the protective effect of the tested *Mj*ORFs against WSSV infection, the mortality of shrimp treated with each *Mj*ORF-dsRNA was compared with that of control group for an interval of up to 10 days post-virus infection. The shrimp in positive WSSV control group died severely during 3–5 d.p.i. and all died at 7 d.p.i. However, mortalities were delayed and reduced in *Mj*ORF-dsRNA-injected shrimp upon WSSV challenge, although they were also delayed and reduced in shrimp injected with un-related eGFP-dsRNA. Shrimp mortality increased steadily from 3 to 10 d.p.i., ending about 80% for all shrimp groups injected with dsRNA and challenged with WSSV (Fig. 4). Shrimple–WSSV and PCR assays confirmed that shrimp

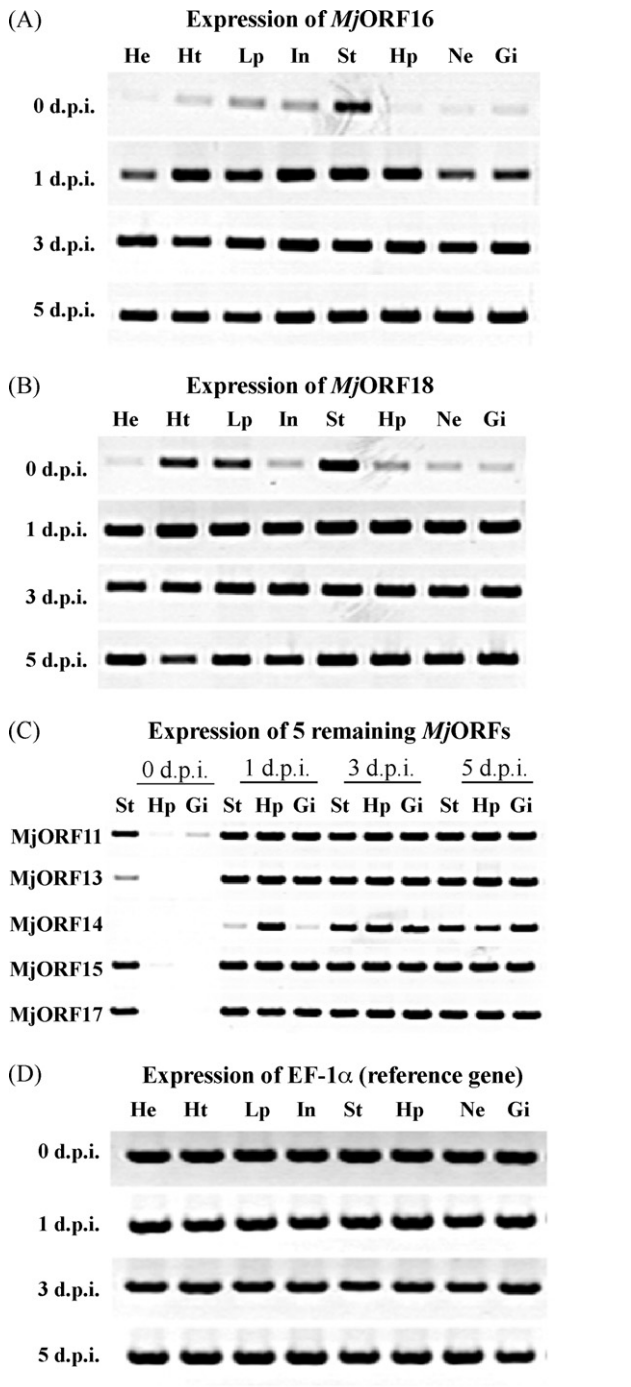


**Fig. 2.** RT-PCR analysis of interaction of homologous *Mj*ORFs with WSSV infection in shrimp. Shrimp were injected with WSSV and 5 live shrimp were randomly sampled at each indicated time points (0, 1, 3 and 5 d.p.i.). mRNAs were isolated from several tissues and then reverse transcribed to cDNAs for RT-PCR analysis. (A) Expression of homologous *Mj*ORF16; (B) expression of homologous *Mj*ORF18; (C) expression of 5 remaining homologous *Mj*ORFs and (D) expression of EF-1α as a reference gene. He: hemocytes; Ht: heart; Lp: lymphoid organ; In: intestine; St: stomach; Hp: hepatopancreas; Ne: nerve and Gi: gills. Each lane represents cDNA synthesized from pooled mRNAs of 5 shrimp.

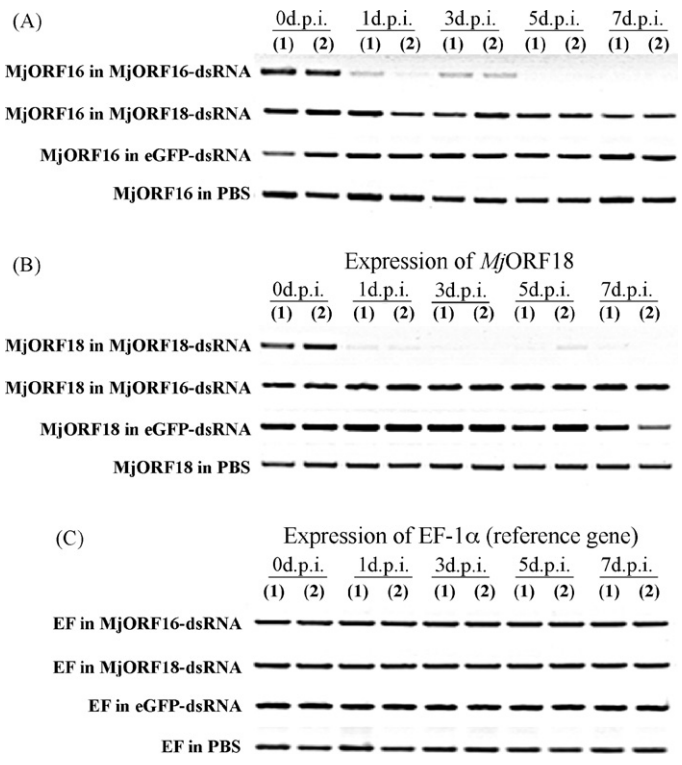


**Fig. 3.** RT-PCR analysis of sequence-specific gene silencing by shrimp-dsRNAs. Shrimp were injected with either PBS (negative control) or each dsRNA (*Mj*ORF16-dsRNA, *Mj*ORF18-dsRNA and eGFP-dsRNA). Total RNA was isolated from the stomachs of 2 live shrimp in each experimental group at indicated time points (0, 1, 3, 5 and 7 d.p.i.) for synthesis of cDNA for RT-PCR analysis. Each lane represents cDNA from a single shrimp. (A) Expression of *Mj*ORF16; (B) expression of *Mj*ORF18; (C) expression of EF-1α as a reference gene.

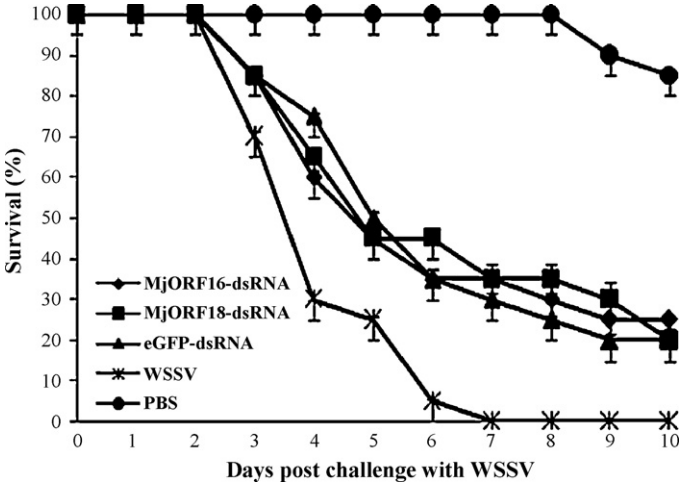


**Fig. 4.** Effects of homologous *Mj*ORF-dsRNAs on shrimp survival after WSSV infection. Shrimp were injected with either PBS (positive and negative WSSV controls) or each dsRNA (shrimp-RNAs and control-dsRNAs) and then immediately challenged with WSSV ($10^4$ dilution from stock). Data are representative of two independent experiments. Each point represents the mean ± s.d. (standard deviation).

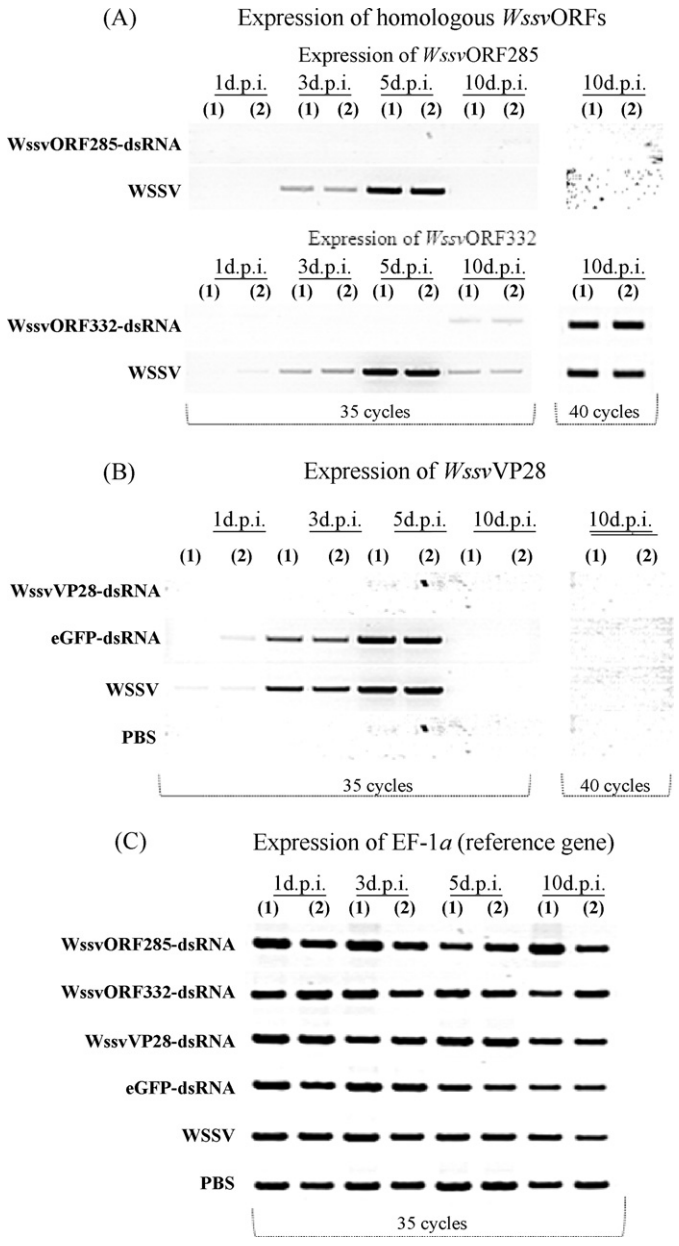


**Fig. 5.** RT-PCR analysis of sequence-specific gene silencing by viral-dsRNAs. Shrimp were injected with either PBS (positive and negative WSSV controls) or each dsRNA (viral-RNAs and control-dsRNAs) and then immediately challenged with WSSV. Total RNA was isolated from the gills of 2 live shrimp in each experimental group at indicated time points (1, 3, 5 and 10 d.p.i.) for synthesis of cDNA for RT-PCR analysis. Each lane represents cDNA from an individual shrimp. (A) Expression of homologous *Wssv*ORFs; (B) expression of *Wssv*VP28; and (C) expression of EF-1α as a reference gene.

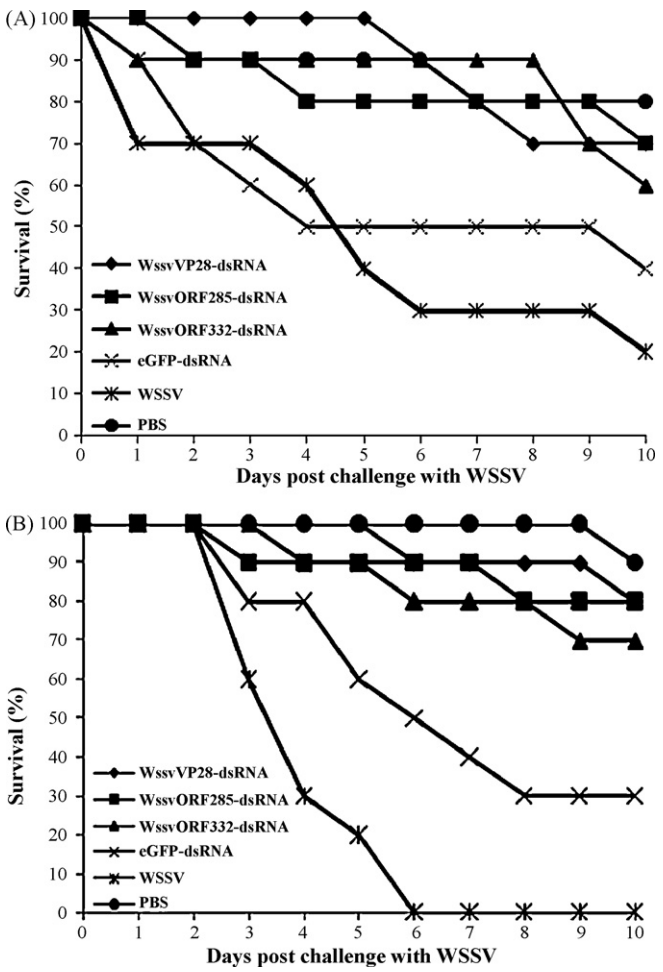


**Fig. 6.** Effects of homologous *Wssv*ORF-dsRNAs on shrimp survival after WSSV infection. Shrimp were injected with either PBS (positive and negative WSSV controls) or each dsRNA (viral-RNAs and control-dsRNAs) and then immediately challenged with WSSV. The data were from experiments of challenge with different WSSV doses. (A) Challenge with lower dose of WSSV ($10^5$ dilution from stock) and (B) challenge with higher dose of WSSV ($10^4$ dilution from stock).

mortality in all WSSV-challenged groups was due to WSSV infection (data not shown).

### 3.3. Involvement of homologous WssvORFs (WssvORF285 and WssvORF332) in WSSV infectivity in kuruma shrimp

#### 3.3.1. Sequence-specific gene silencing by homologous WssvORF-dsRNAs

To elucidate the sequence-specific gene silencing of homologous *Wssv*ORFs by viral-specific dsRNAs (*Wssv*ORF285-dsRNA and *Wssv*ORF332-dsRNA), the expressions of *Wssv*ORFs were determined in gills of shrimp injected with each dsRNA and challenged with WSSV at 1, 3, 5 and 10 d.p.i. by RT-PCR analysis. The expression levels of *Wssv*ORF285 and *Wssv*ORF332 in dsRNA-injected shrimp were completely silenced compared to those in shrimp challenged with WSSV alone at each indicated time point (Fig. 5A). Injection of shrimp with the positive anti-viral *Wssv*VP28-dsRNA also completely inhibited the expression of *Wssv*VP28 (Fig. 5B). The expression of EF-1α (Fig. 5C) was used as a reference. These results indicate that the viral-dsRNAs have specific and efficient inhibitory effects on expression of the target genes.

#### 3.3.2. Effect of homologous WssvORFs on WSSV infection in kuruma shrimp

Anti-WSSV activity of dsRNAs specific to *Wssv*ORF285 and *Wssv*ORF332 was detected in shrimp injected with dsRNA and challenged with 2 different WSSV dilutions ($10^4$ and $10^5$) for an interval of up to 10 d.p.i. In both cases, high survival rates were attained in shrimp injected with the tested viral-dsRNAs (Fig. 6A and B). For example, shrimp injected with *Wssv*ORF285-dsRNA and *Wssv*ORF332-dsRNA and challenged with $10^4$ × diluted WSSV displayed about 80% ($p < 0.001$) and 70% ($p < 0.001$) survivals, respectively, at 10 d.p.i., whereas the shrimp from the positive WSSV control group were all dead at 6 d.p.i. (Fig. 6B). Similarly, high survival rates were also significantly obtained ($p < 0.001$) in shrimp injected with the positive anti-viral *Wssv*VP28-dsRNA con-

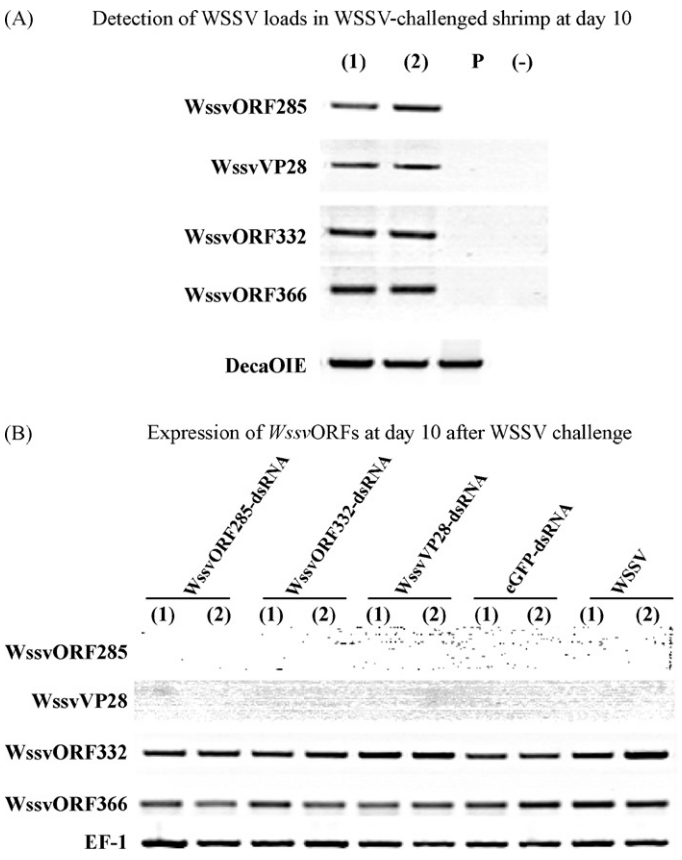


**Fig. 7.** Proposed role of homologous *Wssv*ORFs associated with the persistent WSSV infection stage in shrimp. (A) PCR analysis of WSSV loads in WSSV-challenged shrimp. Total DNA was extracted from the gills of 2 live shrimp taken from the positive WSSV control group at day 10 after WSSV challenge. PCR-amplified fragments of *Wssv*ORF285 (Line 1); full-length *Wssv*VP28 (Line 2); *Wssv*ORF332 (Line 3); *Wssv*ORF366 (Line 4); and DecaOIE as a reference gene (Line 5). (B) RT-PCR analysis of expression of *Wssv*ORFs in shrimp injected with viral-dsRNA and challenged with WSSV, and in shrimp challenged with WSSV alone at day 10 d.p.i. cDNA was synthesized from total RNAs obtained from the gills of 2 live shrimp in each experimental group. Expression of *Wssv*ORF285 (Line 1); Expression of *Wssv*VP28 (Line2); Expression of *Wssv*ORF332 (Line 3); Expression of *Wssv*ORF366 (Line 4); and expression of EF-1α as a reference gene (Line 5). Lanes (1) and (2) represent cDNA or DNA from a single shrimp; Lane (P) indicates PBS-injected sample and lane (−) indicates negative control sample (water template).

trol (Fig. 6), which is consistent with the results of a previous study (Sarathi et al., 2008b).

### 3.3.3. *Potential role of homologous WssvORFs associated with the persistent WSSV infection stage in kuruma shrimp*

As can be seen in Fig. 6, the positive WSSV control group exhibited high mortality during the period of 3–5 d.p.i. Subsequently, the mortality rate decreased. About 20% of the shrimp still survived at 10 d.p.i. and after that. These data suggest that the challenged shrimp were highly infected with WSSV during 3–5 d.p.i. and then became persistently infected with WSSV at 10 d.p.i. Shrimple WSSV kit and PCR analysis confirmed this finding.

The WSSV signals detected using Shrimple WSSV kit (data not shown) were strong at day 5 but undetectable at day 10 after the challenge. This could be due to the low copy numbers of WSSV particles in challenged shrimp at 10 d.p.i., a number that is beyond the detection limit of the kit. However, 4 *Wssv*ORFs (*Wssv*ORF285, 332, 366 and VP28) were amplified in the 10-day DNA samples taken from live shrimp of the positive WSSV control group using PCR assay (Fig. 7A), indicating the presence of WSSV in shrimp at day 10 after WSSV challenge. These results confirmed the finding in the cumulative mortality assay (Fig. 6) of a severe and persistent WSSV infection and provide further evidence that challenged shrimp are capable of carrying persistently WSSV at low-level, active infections at 10 d.p.i.

In terms of detection of *Wssv*ORF expression levels, by RT-PCR analysis, using 10-day cDNA samples taken from WSSV-challenged shrimp (with or without injection of any dsRNA) as templates, *Wssv*ORF332 and WssvORF366 were found to be highly expressed in all samples, while the expressions of *Wssv*ORF285 and *Wssv*VP28 were below the level detected (Fig. 7B). The results suggest that the homologous *Wssv*ORFs may be involved in WSSV latency and pathogenesis in shrimp although their functions appear to be different. Homologous *Wssv*ORF332 may function as a latency-associated gene while homologous *Wssv*ORF285 may function the same as *Wssv*VP28.

## 4. Discussion

To understand the pathogenesis of any disease, in particular viral diseases, knowledge of the interactions between virus and host is critical. Virus–host interactions may result in immune responses against the invader, and may also result in changes in the expression levels of host genes that favor virus replication (Wang et al., 2007). For the host, an effective defense strategy is to limit viral invasion by putting up innate and/or adaptive defenses. For the virus, it has evolved strategies to prevent infected cells from apoptosis and to evade defense responses of their hosts. Large DNA viruses, such as herpesviruses and iridoviruses, encode up to a few hundred ORFs that are essential for viral functions, and that are even involved in direct interaction with the host, affecting immune evasion and apoptosis control (Holzerlandt et al., 2002; Tidona and Darai, 2000).

In humans and in other animals, herpesviruses can produce a persistent carrier state in the host, rendering their control quite difficult. A number of herpesvirus/human homologs have been identified and most of them corresponded to proteins that were classified as being involved in host–virus interaction, primarily affecting immune and/or apoptosis controls (Holzerlandt et al., 2002). In marine invertebrates, WSSV, which is also a large DNA virus, is capable of producing a persistent infection in the host, as previously reported (Escobedo-Bonilla et al., 2008; Flegel, 2007, 2009; Luo et al., 2003) and revealed in this study. At least 78 species of decapod crustaceans have been reported as hosts or carriers of the WSSV either from culture facilities, the wild or experimentally infected animals (Escobedo-Bonilla et al., 2008). The persistence of WSSV has been reported in not only *M. japonicus* but also *P. monodon* (Withyachumnarnkul, 1999), *M. rosenbergii* (Sarathi et al., 2008a), and even specific-pathogen-free (SPF) shrimp (Khadijah et al., 2003) and crabs (Kanchanaphum et al., 1998). However, the molecular mechanisms underlying the WSSV life cycle and its modes of infectivity, especially WSSV latency, are still unclear.

To date, it is still very difficult to conduct *in vitro* functional studies of either shrimp genes or virus genes due to the lack of a shrimp cell culture system. In this context, an RNAi approach may be the best way to carry-out functional analysis of virus genes as well as endogenous genes of shrimp *in vivo*. RNAi can be activated by exogenous dsRNA for silencing the target gene in a sequence-dependent manner (Fire et al., 1998; Hannon, 2002), and has been widely applied in shrimp (Attasart et al., 2009; Kim et al., 2007; Ongvarrasopone et al., 2008; Robalino et al., 2004, 2009). In the present study, dsRNAs specific to our targets (*Mj*ORF16, *Mj*ORF18, *Wssv*ORF285 and *Wssv*ORF332) were designed to explore their involvement in WSSV infectivity. The specific and efficient inhibitory effects of dsRNAs were determined by time-course RT-PCR (Figs. 3 and 5).

Homologous *Mj*ORFs were highly up-regulated in all tested shrimp tissues during the WSSV challenge (Fig. 2) which suggest

that they are related to WSSV infection. The finding that treating shrimp with *Mj*ORF16-dsRNA and *Mj*ORF18-dsRNA followed by injection of WSSV delayed and reduced mortality (Fig. 4) suggest that suppression of host ORFs homologous to predicted WSSV proteins combat spread of the virus, and therefore homologous *Mj*ORFs may function in WSSV infectivity. However, similar results were observed in eGFP-dsRNA-injected shrimp (Figs. 4 and 6). Hence, we cannot rule out the possibility that the reduced mortality observed here is caused by other factors. One possibility is innate anti-viral immunity, which is known to be activated by injecting any dsRNA into shrimp (Robalino et al., 2005, 2009). In fact, increased survival was also observed in shrimp injected with un-related dsRNA (eGFP-dsRNA) when compared to the positive control group challenged with WSSV only, as found in our study as well as previously reported (Attasart et al., 2009; Kim et al., 2007; Shekhar and Lu, 2009). Another possibility is that other homologous *Mj*ORFs are induced by WSSV infection in shrimp and may function in anti-WSSV activity. Based on our data, there are at least 5 other *Mj*ORFs homologous to *Wssv*ORFs that were found in kuruma shrimp. These *Mj*ORFs were also strongly up-regulated (Fig. 2C). Thus, knock-down of only one *Mj*ORF may be less informative for functional analysis if the host has more than one form of each. In general, it could be concluded that our targeted *Mj*ORFs are involved in WSSV pathogenesis although further studies, such as challenge *Mj*ORF-dsRNA-injected shrimp with other WSSV conditions, are needed to determine whether their modulatory effect on viral infectivity is positive or negative.

Our results show that survival rates were significantly improved in shrimp injected with viral-specific dsRNAs corresponding to *Wssv*ORF285 and *Wssv*ORF332, followed by WSSV challenge (Fig. 6A and B). These results indicate that silencing of *Wssv*ORF285 and *Wssv*ORF332 by specific dsRNAs can protect shrimp from WSSV infection, thereby suggesting that the two homologous *Wssv*ORFs are involved in WSSV pathogenesis.

The finding that WSSV-challenged shrimp were highly susceptible to WSSV infection during the period of 3–5 d.p.i. and that the mortality rate subsequently decreased and some shrimp still survived over the period of the experiment indicate that infected-shrimp appear to become persistently infected with WSSV at 10 d.p.i., and after that. Our findings concur with the results of Sarathi et al. (2008a), which show that WSSV-injected prawns were highly susceptible to WSSV infection at 3 and 4 d.p.i. and that they survived without any mortality after this period. WSSV is also closely related to baculovirus in structure and morphology. *Mamestra brassicae* insects probably harbor the infectious baculovirus at very low levels, referred as a persistent infection (Hughes et al., 1993, 1997). Therefore, it is possible that WSSV also resides in hosts either in a quiescent state or by remaining as a persistent infection.

A comparison of *Wssv*VP28 protein and *Wssv*ORF366 showed that, in terms of detection of expression levels by RT-PCR (Fig. 7B), both *Wssv*ORF332 and *Wssv*ORF366 clearly exhibited high expression even though the expression of *Wssv*VP28 and *Wssv*ORF285 could not be detected. Concerning the functions of viral genes, it should be noted that latency genes are normally involved in the persistence of the virus and their function is to maintain a low number of viruses (Flint et al., 2000; Khadijah et al., 2003). Their expression can be detected even though the structural genes might not be active (Sanchez-Martinez et al., 2007). Because *Wssv*VP28 is involved in the attachment to, and penetration of, WSSV into host cells, its expression level may decrease during the persistent infection stage, which would reduce the spread of WSSV to un-infected cells as well as reduce the severity of the disease. In addition, it has been reported that the expression of *Wssv*ORF285 commences at a later stage of the virus infection (Wu et al., 2007), and that late-transcribed genes encode mainly structural proteins of viral particles (Chambers et al., 1999; Ebrahimi et al., 2003; Lua et al., 2005). On the other hand, *Wssv*ORF366 is identified as a latency-associated gene (Khadijah et al., 2003). Our results, taken together, suggest that homologous *Wssv*ORFs are involved in the persistent status of virus infection in shrimp but differ in function. *Wssv*ORF332 may function as a latency gene especially associated with the persistent WSSV infection stage while *Wssv*ORF285 could be classified into the same group as the *Wssv*VP28. This group of genes usually encodes the envelope and the nucleocapsid, and plays an important role in virus penetration during the infection.

In summary, our results suggest that both homologous *Wssv*ORFs and homologous *Mj*ORFs are involved in complex shrimp–WSSV interactions. We also propose that homologous *Wssv*ORFs are especially associated with the persistent infection stage in kuruma shrimp. Homologous *Wssv*ORF332 is proposed to function as a latency-associated gene while homologous *Wssv*ORF285 may function the same as *Wssv*VP28. Although more studies are needed to determine whether the effects of homologous *Mj*ORFs on WSSV infectivity are positive or negative, and more evidence is needed to ascertain the role of homologous *Wssv*ORFs, our results show the existence of intricate shrimp–WSSV interactions. These results may help to develop a more sensitive diagnostic method that uses *Wssv*ORF332 to detect the presence of viral particles in WSSV latency in shrimp and other crustaceans.

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## References

Arziman, Z., Horn, T., Boutros, M., 2005. E-RNAi: a web application to design optimized RNAi constructs. Nucleic Acids Res. 33, W582–W588.
Attasart, P., Kaewkhaw, R., Chimwai, C., Kongphom, U., Namramoon, O., Panyim, S., 2009. Inhibition of white spot syndrome virus replication in Penaeus monodon by combined silencing of viral rr2 and shrimp PmRab7. Virus Res. 145, 127–133.
Chambers, J., Angulo, A., Amaratunga, D., Guo, H., Jiang, Y., Wan, J.S., Bittner, A., Frueh, K., Jackson, M.R., Peterson, P.A., Erlander, M.G., Ghazal, P., 1999. DNA microarrays of the complex human cytomegalovirus genome: profiling kinetic class with drug sensitivity of viral gene expression. J. Virol. 73, 5757–5766.
Ebrahimi, B., Dutia, B.M., Roberts, K.L., Garcia-Ramirez, J.J., Dickinson, P., Stewart, J.P., Ghazal, P., Roy, D.J., Nash, A.A., 2003. Transcriptome profile of murine gammaherpesvirus-68 lytic infection. J. Gen. Virol. 84, 99–109.
Escobedo-Bonilla, C.M., Alday-Sanz, V., Wille, M., Sorgeloos, P., Pensaert, M.B., Nauwynck, H.J., 2008. A review on the morphology, molecular characterization, morphogenesis and pathogenesis of white spot syndrome virus. J. Fish Dis. 31, 1–18.
Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., Mello, C.C., 1998. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 391, 806–811.
Flegel, T.W., 2007. Update on viral accommodation, a model for host–viral interaction in shrimp and other arthropods. Dev. Comp. Immunol. 31, 217–231.
Flegel, T.W., 2009. Hypothesis for heritable, anti-viral immunity in crustaceans and insects. Biol. Direct. 4, 32.
Flint, S.J., Enquist, L.W., Krug, R.M., Racaniello, V.L., Skallka, A.M., 2000. Principles in Virology. ASM Press, Washington, DC, USA.
Hannon, G.J., 2002. RNA interference. Nature 418, 244–251.
He, N., Qin, Q., Xu, X., 2005. Differential profile of genes expressed in hemocytes of White Spot Syndrome Virus-resistant shrimp (Penaeus japonicus) by combining suppression subtractive hybridization and differential hybridization. Antiviral Res. 66, 39–45.
Holzerlandt, R., Orengo, C., Kellam, P., Alba, M.M., 2002. Identification of new herpesvirus gene homologs in the human genome. Genome Res. 12, 1739–1748.
Hughes, D.S., Possee, R.D., King, L.A., 1993. Activation and detection of a latent baculovirus resembling Mamestra brassicae nuclear polyhedrosis virus in *M. brassicae* insects. Virology 194, 608–615.
Hughes, D.S., Possee, R.D., King, L.A., 1997. Evidence for the presence of a low-level, persistent baculovirus infection of Mamestra brassicae insects. J. Gen. Virol. 78 (Pt 7), 1801–1805.

Kanchanaphum, P., Wongteerasupaya, C., Sitidilokratana, N., Boonsaeng, V., Panyim, S., Tassanakajon, A., Withyachumnarnkul, B., Flegel, T.W., 1998. Experimental transmission of white spot syndrome virus (WSSV) from crabs to shrimp Penaeus monodon. Dis. Aquat. Organ. 34, 1–7.

Khadijah, S., Neo, S.Y., Hossain, M.S., Miller, L.D., Mathavan, S., Kwang, J., 2003. Identification of white spot syndrome virus latency-related genes in specific-pathogen-free shrimps by use of a microarray. J. Virol. 77, 10162–10167.

Kim, C.S., Kosuke, Z., Nam, Y.K., Kim, S.K., Kim, K.H., 2007. Protection of shrimp (Penaeus chinensis) against white spot syndrome virus (WSSV) challenge by double-stranded RNA. Fish Shellfish Immunol. 23, 242–246.

Koyama, T., Asakawa, S., Katagiri, T., Shimizu, A., Fagutao, F.F., Mavichak, R., Santos, M.D., Fuji, K., Sakamoto, T., Kitakado, T., Kondo, H., Shimizu, N., Aoki, T., Hirono, I., 2010. Hyper-expansion of large DNA segments in the genome of kuruma shrimp, Marsupenaeus japonicus. BMC Genomics 11, 141.

Leu, J.H., Yang, F., Zhang, X., Xu, X., Kou, G.H., Lo, C.F., 2009. Whispovirus. Curr. Top. Microbiol. Immunol. 328, 197–227.

Liu, H., Jiravanichpaisal, P., Söderhäll, I., Cerenius, L., Söderhäll, K., 2006. Antilipopolysaccharide factor interferes with white spot syndrome virus replication in vitro and in vivo in the crayfish Pacifastacus leniusculus. J. Virol. 80, 10365–10371.

Liu, H., Söderhäll, K., Jiravanichpaisal, P., 2009. Antiviral immunity in crustaceans. Fish Shellfish Immunol. 27, 79–88.

Lu, L., Kwang, J., 2004. Identification of a novel shrimp protein phosphatase and its association with latency-related ORF427 of white spot syndrome virus. FEBS Lett. 577, 141–146.

Lua, D.T., Yasuike, M., Hirono, I., Aoki, T., 2005. Transcription program of red sea bream iridovirus as revealed by DNA microarrays. J. Virol. 79, 15151–15164.

Luo, T., Zhang, X., Shao, Z., Xu, X., 2003. PmAV, a novel gene involved in virus resistance of shrimp Penaeus monodon. FEBS Lett. 551, 53–57.

Ma, T.H., Benzie, J.A., He, J.G., Chan, S.M., 2008. PmLT, a C-type lectin specific to hepatopancreas is involved in the innate defense of the shrimp Penaeus monodon. J. Invertebr. Pathol. 99, 332–341.

Maningas, M.B., Kondo, H., Hirono, I., Saito-Taki, T., Aoki, T., 2008. Essential function of transglutaminase and clotting protein in shrimp immunity. Mol. Immunol. 45, 1269–1275.

Mayo, M.A., 2002a. A summary of taxonomic changes recently approved by ICTV. Arch. Virol. 147, 1655–1663.

Mayo, M.A., 2002b. Virus taxonomy—Houston 2002. Arch. Virol. 147, 1071–1076.

Ongvarrasopone, C., Chanasakulniyom, M., Sritunyalucksana, K., Panyim, S., 2008. Suppression of PmRab7 by dsRNA inhibits WSSV or YHV infection in shrimp. Mar. Biotechnol. (NY) 10, 374–381.

Robalino, J., Browdy, C.L., Prior, S., Metz, A., Parnell, P., Gross, P., Warr, G., 2004. Induction of antiviral immunity by double-stranded RNA in a marine invertebrate. J. Virol. 78, 10442–10448.

Robalino, J., Bartlett, T., Shepard, E., Prior, S., Jaramillo, G., Scura, E., Chapman, R.W., Gross, P.S., Browdy, C.L., Warr, G.W., 2005. Double-stranded RNA induces sequence-specific antiviral silencing in addition to nonspecific immunity in a marine shrimp: convergence of RNA interference and innate immunity in the invertebrate antiviral response? J. Virol. 79, 13561–13571.

Robalino, J., Carnegie, R.B., O'Leary, N., Ouvry-Patat, S.A., de la Vega, E., Prior, S., Gross, P.S., Browdy, C.L., Chapman, R.W., Schey, K.L., Warr, G., 2009. Contributions of functional genomics and proteomics to the study of immune responses in the Pacific white leg shrimp Litopenaeus vannamei. Vet. Immunol. Immunopathol. 128, 110–118.

Rojtinnakorn, J., Hirono, I., Itami, T., Takahashi, Y., Aoki, T., 2002. Gene expression in haemocytes of kuruma prawn, Penaeus japonicus, in response to infection with WSSV by EST approach. Fish Shellfish Immunol. 13, 69–83.

Sanchez-Martinez, J.G., Aguirre-Guzman, G., Mejia-Ruiz, H., 2007. White spot syndrome virus in cultured shrimp: a review. Aquac. Res. 38, 1339–1354.

Sarathi, M., Nazeer Basha, A., Ravi, M., Venkatesan, C., Senthil Kumar, B., Sahul Hameed, A.S., 2008a. Clearance of white spot syndrome virus (WSSV) and immunological changes in experimentally WSSV-injected Macrobrachium rosenbergii. Fish Shellfish Immunol. 25, 222–230.

Sarathi, M., Simon, M.C., Ahmed, V.P., Kumar, S.R., Hameed, A.S., 2008b. Silencing VP28 gene of white spot syndrome virus of shrimp by bacterially expressed dsRNA. Mar. Biotechnol. (NY) 10, 198–206.

Shekhar, M.S., Lu, Y., 2009. Application of nucleic-acid-based Therapeutics for Viral Infections in Shrimp Aquaculture. Mar. Biotechnol. (NY) 11, 1–9.

Sritunyalucksana, K., Wannapapho, W., Lo, C.F., Flegel, T.W., 2006. PmRab7 is a VP28-binding protein involved in white spot syndrome virus infection in shrimp. J. Virol. 80, 10734–10742.

Tidona, C.A., Darai, G., 2000. Iridovirus homologues of cellular genes—implications for the molecular evolution of large DNA viruses. Virus Genes 21, 77–81.

van Hulten, M.C., Witteveldt, J., Peters, S., Kloosterboer, N., Tarchini, R., Fiers, M., Sandbrink, H., Lankhorst, R.K., Vlak, J.M., 2001a. The white spot syndrome virus DNA genome sequence. Virology 286, 7–22.

van Hulten, M.C., Witteveldt, J., Snippe, M., Vlak, J.M., 2001b. White spot syndrome virus envelope protein VP28 is involved in the systemic infection of shrimp. Virology 285, 228–233.

Wang, B., Li, F., Dong, B., Zhang, X., Zhang, C., Xiang, J., 2006. Discovery of the genes in response to white spot syndrome virus (WSSV) infection in Fenneropenaeus chinensis through cDNA microarray. Mar. Biotechnol. (NY) 8, 491–500.

Wang, H.C., Wang, H.C., Leu, J.H., Kou, G.H., Wang, A.H., Lo, C.F., 2007. Protein expression profiling of the shrimp cellular response to white spot syndrome virus infection. Dev. Comp. Immunol. 31, 672–686.

Withyachumnarnkul, B., 1999. Results from black tiger shrimp penaeus monodon culture ponds stocked with postlarvae PCR-positive or -negative for white-spot syndrome virus (WSSV). Dis. Aquat. Organ. 39, 21–27.

Witteveldt, J., Cifuentes, C.C., Vlak, J.M., van Hulten, M.C., 2004. Protection of Penaeus monodon against white spot syndrome virus by oral vaccination. J. Virol. 78, 2057–2061.

Wu, J., Lin, Q., Lim, T.K., Liu, T., Hew, C.L., 2007. White spot syndrome virus proteins and differentially expressed host proteins identified in shrimp epithelium by shotgun proteomics and cleavable isotope-coded affinity tag. J. Virol. 81, 11681–11689.

Yang, F., He, J., Lin, X., Li, Q., Pan, D., Zhang, X., Xu, X., 2001. Complete genome sequence of the shrimp white spot bacilliform virus. J. Virol. 75, 11811–11820.

Yi, G., Wang, Z., Qi, Y., Yao, L., Qian, J., Hu, L., 2004. Vp28 of shrimp white spot syndrome virus is involved in the attachment and penetration into shrimp cells. J. Biochem. Mol. Biol. 37, 726–734.

Zhang, X., Huang, C., Xu, X., Hew, C.L., 2002. Identification and localization of a prawn white spot syndrome virus gene that encodes an envelope protein. J. Gen. Virol. 83, 1069–1074.

Zhao, Z.Y., Yin, Z.X., Weng, S.P., Guan, H.J., Li, S.D., Xing, K., Chan, S.M., He, J.G., 2007. Profiling of differentially expressed genes in hepatopancreas of white spot syndrome virus-resistant shrimp (Litopenaeus vannamei) by suppression subtractive hybridisation. Fish Shellfish Immunol. 22, 520–534.