Targeting Essential Gene Utilizing RNA Interference to Protect the Ailing Shrimp/Prawn Industry Against WSSV

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Abstract

The white spot syndrome virus (WSSV) remains to be the most widespread and devastating infectious agent that has hit particularly the marine shrimp aquaculture industry worldwide. To date, there are no known effective strategies that can combat WSSV infection. This study aimed to elucidate host-pathogen interaction through the functional study of host - gene. Utilizing RNA Interference, the function of contig23 (c23) in the shrimp genome, identified to have high homology with WSSVORF-325, was determined. Three set-ups were prepared for treatment of c23-, GFP-dsRNA, and PBS using *Macrobrachium rosenbergii* freshwater prawns. Each treatment group was challenged with WSSV and survival rate was recorded. C23-, and GFP-dsRNA injected prawns showed a significant survival rate of 100%, in contrast to 20% of the PBS injected prawns at 10 days post-infection (dpi). Results showed that injection of c23- and GFP-dsRNA prior to challenge with WSSV, delayed and reduced mortality in contrast to PBS-treated prawns, which showed high mortality. Gene expression analysis showed silencing of both WSSV and c23 at day 3 post-WSSV challenge. This study proved that c23-dsRNA has a protective effect on WSSV-challenged prawns and highlights its involvement in the infectivity of WSSV in *M. rosenbergii*.

Keywords: WSSV, host-pathogen interaction, shrimp genome, gene expression analysis

Introduction

The inflicting diseases on today's aquaculture have wreaked havoc on the sustainable growth of the shrimp culture industry worldwide. In the Asia-Pacific countries, the industry reported annual losses of about 4 billion US dollars yearly. Shrimp is an indispensable source of revenue, as this commodity accounts for almost 20% of aquaculture commodities in trade worldwide. A large fraction of the damage to the industry is undoubtedly caused by viruses, which already accounted to have over 20 strains that of which affected both penaeid shrimp wild stocks and commercial production.

The white spot syndrome virus (WSSV) is one of the most potent and widespread pathogen (Flegel, 2006) affecting the shrimp culture industry. This virus can spread the disease rapidly in a span of 2-10 days post-infection and can bring 100% cumulative mortality (Flegel, 2006). The insufficient modes of sanitation and the growing number of shrimp cultures, coupled with uncontrolled trade movement

among countries, account for the optimum conditions for the virus to spread to almost all farming areas (de la Peña *et al.*, 2007). Although there are already some methods which displayed efficacy against the virus under experimental conditions, no effective treatments have been available to address WSSV problem in the field (Dang *et al.*, 2010).

Understanding the underlying molecular interaction between the host and pathogen is very critical in creating strategies to prevent diseases. A novel approach to understand host-pathogen interaction is the utilization of RNA interference (RNAi) technology. RNAi is a post-transcriptional gene silencing process in which double-stranded RNA (dsRNA) triggers the silencing of a cognate gene. Insurmountable evidences have pointed out the efficiency of RNAi in studying gene function and its implication in mounting antiviral responses in eukaryotes. In the shrimp system, a number of studies have demonstrated the effectiveness of RNAi in studying shrimp-pathogen interaction. Injection of WSSV gene-specific dsRNA efficiently suppressed viral replication in penaeid shrimp (Kim *et al.*, 2007; Robalino *et al.*, 2007) and suppression of yellow head virus (YHV) replication by cognate-dsRNA, significantly reduced mortality in the black tiger shrimp.

On the other hand, in the shrimp genome, several genes that can be potentially linked to anti-WSSV responses in shrimp had been identified (He *et al.*, 2005; Wang *et al.*, 2006; Zhao *et al.*, 2007). A bacterial artificial chromosome (BAC) library of the kuruma shrimp *Penaeus (Marsupenaeus japonicus)* genome revealed 27 open reading frames (ORFs) that are surprisingly homologous to the predicted proteins that code for WSSV. Two of the homologs found in kuruma shrimp (MjORF16 and MjORF18) and their WSSV homologs (WSSVORF-332 and WSSVORF-285, respectively) were utilized to demonstrate the involvement of both WSSV and shrimp homologs in the infectivity of WSSV in *Penaeus (Marsupenaeus) japonicus* (Dang *et al.*, 2010). One of the 27 ORFs identified in the genome of kuruma shrimp is contig 23 (c23), which has high homology to WSSVORF325 and codes for MjORF158 in the kuruma shrimp genome.

Here, we elucidated the function of contig 23 gene *in vivo* by utilizing RNA-interference technology.

Materials and Methods

Laboratory Set-up and Shrimp Acclimatization

Freshwater prawn *Macrobrachium rosenbergii* weighing 3-5 grams were purchased from Southeast Asian Fisheries Development Center (SEAFDEC) Binangonan, Rizal, Philippines. One hundred (100) juveniles were reared in filtered recirculating de-chlorinated tap water tanks maintained at 25-28°C and 0 ppt salinity. Feeding was *ad libitum* on a daily basis.

Preparation of Virus Stock Inocula and Median Lethal Dosage (LD50)

WSSV stock was isolated from WSSV-infected *Penaeus monodon* obtained from SEAFDEC, Iloilo, Philippines. WSSV infection was confirmed through Polymerase Chain Reaction (PCR) using WSSV-specific primers (Table 1). Viral isolation was done following the procedure

in the study of Rout *et al.,* (2007). One hundred (100) µL of the viral isolate was injected to 5 healthy *M. rosenbergii* juveniles, from which a new viral stock was isolated and used for the challenge test, following the same procedure mentioned above. The WSSV viral stock was then stored at -80°C until the commencement of the challenge test. The virus concentration used in the challenge test was determined based on the median lethal dose (LD_{50}).

dsRNA synthesis

For the production of double-stranded RNA (dsRNA), optimization of the conditions was based on the methods developed by Maningas (2008). dsRNAs were generated *in vitro* using the T7 RiboMAX Express Scale RNA Production System (Promega, USA) following the protocols provided by the manufacturer. Briefly, T7 promoter sequence was incorporated to gene specific primers for c23 (Table 1) to produce sense and antisense strands separately. 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 primers used to amplify the region are shown in Table 1. The resulting PCR products (with T7 promoter) were quantified through the use of Perkin Elmer Lambda 40 UV-VIS Spectrophotometer and normalized to attain similar concentration for the transcription experiment.

Transcription was performed by utilizing Promega Transcription T7 polymerase kit to yield single-stranded RNAs (ssRNAs). Two µL of PCR products were utilized for transcription and the reaction yielded 20 µL of ssRNA. Equal amounts of ssRNAs were mixed together to anneal the RNA strands and were incubated at 70°C for 10 minutes and allowed to cool at room temperature for about 20 minutes. After cooling to room temperature, the resulting dsRNAs were further purified following the protocol provided by T7 Ribomax Express Large Scale RNA Production System and were quantified again using the same spectrophotometer. The synthesized dsRNAs were quantified to make sure all samples were of equal concentration for the *in vivo* RNAi experiment (1 mg/mldsRNA).

Amplification, Sequencing and Analysis of c23

The target sequence, c23, was amplified through PCR and the primers used are in Table 1. The PCR mix contained the following: 1x buffer, 2 mMdNTPs, 0.6mM Primer (Forward and Reverse), 1 unit Taq, 5.7 ml ddH2O and 1 mg DNA template. The following thermocycler conditions were utilized: initial denaturation at 95°C for 5 min followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, 72 °C for 1 min and final extension at 72°C for 10 min. The PCR products were viewed in 1% agarose gel stained with ethidium bromide to check for the presence of amplified DNA.

The following reaction was prepared for capillary sequencing: 1 mLBigDye terminator reaction mix, 3.5 mL 5x BigDye sequencing buffer, 1 mL template DNA, 1 ml primer and 13.5 mL distilled water. The thermal profile used is as follows: one cycle hold at 95°C, 25 cycles 95 °C for 10 sec, 50 °C for 5 sec, 60 °C for 4 min. and final hold at 4 °C. The resulting product was purified then sequencing was carried out in Applied Biosystem 31301 Analyzer. The DNA sequence acquired was utilized for the Basic Local Alignment Search Tool (BLAST) search in order to establish its phylogenetic

Table 1. List of primer sequences for RT-PCR.

Primer name	Nucleotide Sequence
Contig-23	F 5' ACCGCTACTGACGACAACG3' R 5' CACTCGCTCCGTTAACAAGG3'
T7 Contig-23	F 5' TAATACGACTCACTATAGGACCGCTACTGACGACAACG3' R5'TAATACGACTCACTATAGGCACTCGCTCCGTTAACAAGG3'
GFP (Maningas, et al., 2008)	F 5'ATGGTGAGCAAGGGCGAGGA3' R 5'TTACTTGTACAGCTCGTCCA3'
T7GFP (Maningas, et al., 2008)	F5'TAATACGACTCACTATAGGATGGTGAGCAAGGGCGAGGA3' R 5'TAATACGACTCACTATAGGTTACTTGTACA GCTCGTCCA3'
WSSV (Flegel, 2006)	F 5'GTACGGCAATACTGGAGGAGGT3' R5'GGAGATGTGTAAGATGGACAAG3'
EF-1α (Maningas, 2006)	F 5'ATGGTTGTCAACTTTGCCCC3' R 5'TTGACCTCCTTGATCACACC3'
β-actin (Liu, et al. , 2006)	F 5'AACTCCCATGACATGGAGAACATC3' R 5'TCTTCTCACGGTTGGCCTTG3'

relationship with other genes available in GENBANK. The evolutionary history of the query sequence was inferred by using the Maximum Likelihood method based on the Tamura-Nei model in MEGA5.

In vivo gene silencing of c23-dsRNA

Three set-ups were prepared in the Wet Laboratory and each contained 12 *M. rosenbergii* as test animals. The set-ups corresponded to experimental (c23-dsRNA treated), treated control (GFP-dsRNA) and untreated control (PBS). One group was injected with 5mg of c23-dsRNA (suspended in 100 uL PBS) and another with 5 mg of GFP-dsRNA (suspended in 100 uL PBS). The negative control group was injected with 100µL of PBS. After dsRNA/PBS injection, the animals were left untouched for 24 hours to recuperate, then injection with WSSV followed. Two hours after WSSV infection corresponded to Day 0 post infection (p.i.). All the animals were injected intramuscularly at the 3rd abdominal segment. The tanks were maintained at 22-25°C and 0 ppt salinity. There were four sampling days for the gene expression analysis: day 0, 1, 3, and 7. Tissues (hemocytes and gills) were collected from three randomly sampled shrimps for RNA extraction. Total RNAs were reverse transcribed to cDNA using M-MLV reverse transcriptase following the protocol provided by the manufacturer (Invitrogen, USA). The inhibitory effect and specificity of the c23-dsRNA were determined by RT-PCR using equal amounts of cDNA as templates.

PCR and RT-PCR analysis

PCR was carried out to confirm the WSSV-free status of experimental animals and detect the presence of WSSV particles at the persistent infection stage.

Time-course RT-PCR was utilized to evaluate the interaction of homologous shrimp, c23, with WSSV infections and to elucidate the sequence-specific gene silencing by c23-dsRNA. Gills and hemocytes were dissected out from three individual samples for each set-up and sampling day (Day 0, 1, 3, 7 p.i). Total RNAs were isolated using Trizol (Invitrogen, USA)

and quantified by UV Spectrophotometer to ensure that all samples were of equal concentration when used for RT-PCR analysis. One microgram of each sample was reverse transcribed to produce single-strand cDNA with the use of SuperScript™ First-Strand Synthesis System following the protocols recommended by the manufacturer. Transcripts were visualized in 1.5% agarose gel stained with ethidium bromide.

The following thermocycler conditions were followed for all PCR and RT-PCR set-ups: initial denaturation, 95°C for 5 minutes, followed by 30 cycles of (1) denaturation at 95°C for 30 seconds, (2) annealing at 55°C for 30 seconds and (3) extension 72°C for 1 minute then final DNA extension at 72°C for 5 minutes. The transcripts were visualized and analyzed in a gel electrophoresis using 1.5% agarose. The expression of EF served as the reference gene.

Challenge Test

Three set-ups, each with ten (10) *M. rosenbergii*, were prepared for the challenge test and were housed in the Wet Laboratory of TARC. A total of six plastic aquaria (72.3 cm x 52 cm x 44 cm) equipped with re-circulating water tank system were utilized for the set-up. The aquaria were maintained at about 22–25°C and 0 ppt salinity prior to the experiment. Protective efficiencies of c23-dsRNAs against WSSV infection were determined by intramuscular injection at the 3rd abdominal segment. The juveniles were injected with either 100 mL of PBS or 5µg of c23-dsRNA or GFP-dsRNA suspended in 100 mL PBS prior to challenge with the viral inoculum (100µL of 10^{-2} diluted WSSV stock). The injection of GFP-dsRNA served as the unrelated dsRNA control group. Cumulative mortality rate was recorded daily up to 10 days after infection.

Statistical Analysis

The survival data were analyzed through the use of Kaplan-Meier survival with a chi-square test using GraphPad Prism Software and differences were considered significant at $p<0.05$.

Results

Phylogenetic analysis

The query sequence (c23) consisted of 597 base pairs and was compared with other sequences from NCBI (Figure 1). In addition to the other homologous sequences from the database, c23 sequence obtained from *Penaeus (Marsupenaeus) japonicus* was also included in the analysis. Sequencing results showed that c23 is part of the *Macrobrachium rosenbergii* genomic DNA and the sequence has 27% identity (having an E value of $1e^{-5}$) with WSSVORF-325. The sequence was aligned with other sequences obtained from BLAST and a Phylogenetic tree was constructed. Figure 1 shows related sequences with c23, and WSSVORF-325 was one of those which branched out from the c23 sequences obtained from *M. japonicus* and *M. rosenbergii*. WSSVORF-325, homologous to vp25, is an envelope protein responsible for the systemic infection of its host by the virus. In constructing the phylogenetic tree, the Maximum Likelihood (ML) method was utilized for the analysis of the sequences. In addition to this, the test of phylogeny made use of the Bootstrap method using 1000 Bootstrap replicates and gaps were not included in the analysis.

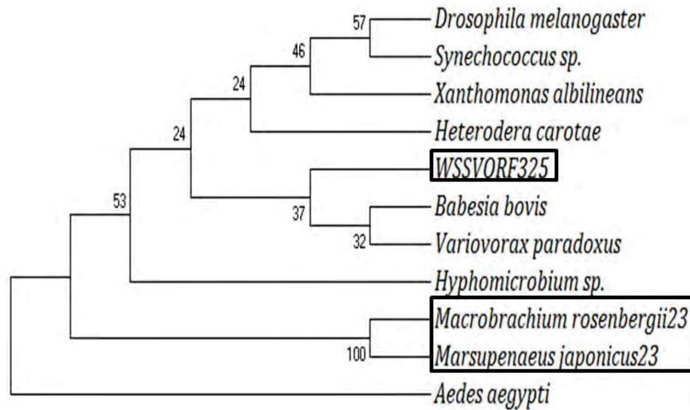

Figure 1. Molecular Phylogenetic analysis by Maximum Likelihood method of c23. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood (-3282.2441) is shown.

Expression of c23 in different tissues

Utilizing cDNAs obtained from mRNAs of a normal/healthy *M. rosenbergii*, the expression pattern of c23 in different tissues was examined through RT-PCR analysis. As shown in Figure 2, c23 is expressed in all tissues examined and were highly expressed in the gills, heart, hepatopancreas, intestine and hemocyte. Elongation Factor (EF-1α) was used as the internal reference control and was positively amplified, with similar levels, from all tissues of normal shrimp.

Interaction of c23 with WSSV infection

To elucidate the expression pattern of c23 in WSSV-infected prawns, RNA was isolated from the hemocytes and gills of three PBS-treated samples at four time intervals (Day 0, 1, 3, and 7 p.i.). cDNAs were derived from isolated RNA and were analyzed through RT-PCR. Only hemocyte and gill cDNAs were utilized as templates for the gene expression analysis because these two important tissues were essential in the progression of viral diseases and these templates showed consistent expression using normal tissues. EF-1α was used as the reference gene. WSSV specific primers from other regions with a target size of 200bp, were utilized to indicate expression of WSSV. In addition, the expression of other WSSV gene confirmed that the high mortality observed in PBS-treated shrimps was indeed due to the WSSV infection (Figure 3A).

Figure 3B shows the expression of WSSV in the GFP-dsRNA treated prawns. This further shows that the animals were still infected with WSSV despite their survival during the experiment period.

Sequence-specific silencing by c23

To further explain the reason behind the high survival rate observed in c23-dsRNA treated shrimps, the expression of c23 was also determined through RT-PCR. Using cDNAs derived from gills and hemocytes as templates, the expression level of c23 and WSSV was completely silenced starting Day 3 p.i. as shown in Figure 4. This result indicates that c23-dsRNA has a specific, inhibitory effect on the expression of the target gene, c23.

Effect of c23 on WSSV infection

To assess the effect of the WSSV homolog c23 in WSSV infection, the mortality of c23-dsRNA-injected *M. rosenbergii* after challenge against WSSV was monitored. In PBS-treated prawns, mortality was observed starting on day 6 p.i. although 30% of the stock survived on the last day of sampling. In those treated with c23-dsRNA, no mortality was observed through the sampling days (Day 0 till10 p.i.). High survival rate was also observed in GFP-dsRNA treated stocks. The dsRNA-treated stocks showed 100% survival while those that were PBS-treated showed 30% survival after Day 7 p.i. (Figure 5). Delayed onset of mortality was also observed in other species of shrimps *Penaeus (Marsupenaeus) japonicus* and *Penaeus*

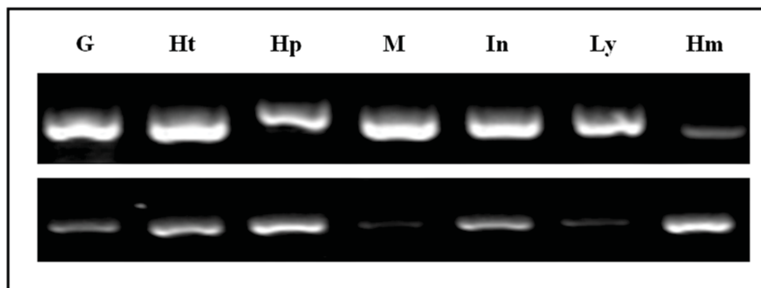

Figure 2. Expression of c23 in different tissues of healthy *Macrobrachium rosenbergii*. First lane corresponds to expression of EF-1α (reference gene) while the second lane corresponds to expression of c23. **G**: gills; **Ht**: heart; **Hp**: hepatopancreas; **M**: Muscle; **In**: intestine; **Ly**: lymphoid organ; and **Hm**: hemocyte.

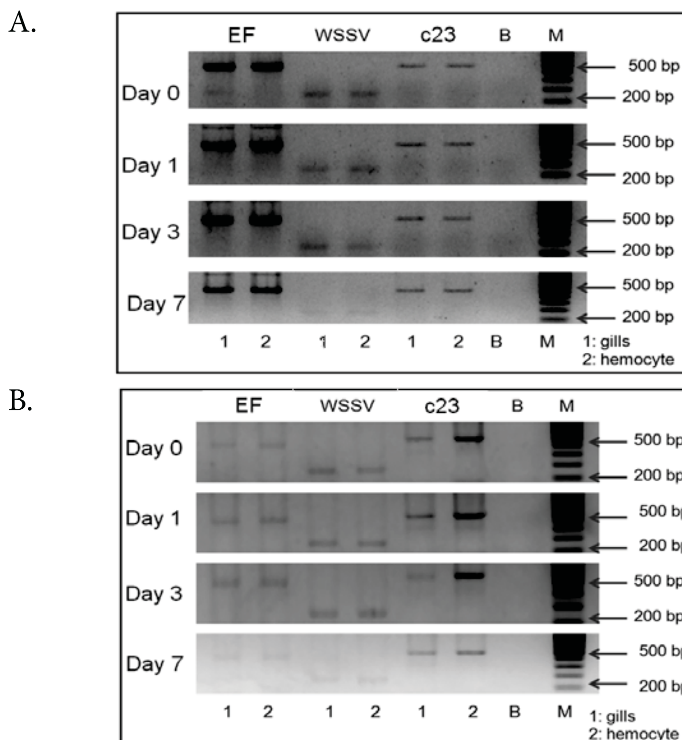

Figure 3. Expression of WSSV and c23 in gills (1) and hemocytes (2) at 0-7 dpi. A. PBS treatment; B. GFP treatment. **EF**- elongation factor was used as a reference gene. **M**-marker, **B**- blank (master mix without the DNA template).

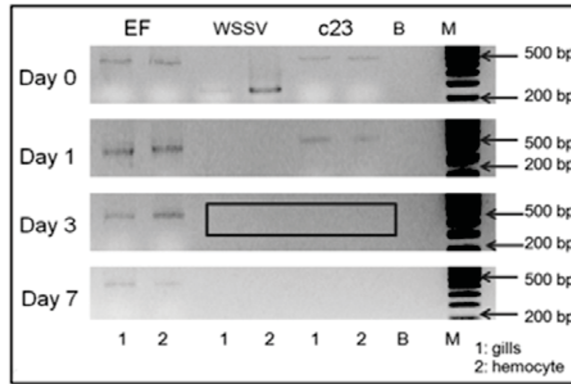

Figure 4. RT-PCR analysis. Sequence-specific gene silencing by c23-dsRNA in gills (1) and hemocytes (2) of infected *Macrobrachium rosenbergii* juveniles. **EF**- elongation factor was used as a reference gene for successful DNA amplification. **M**-marker, **B**- blank (master mix without the DNA template).

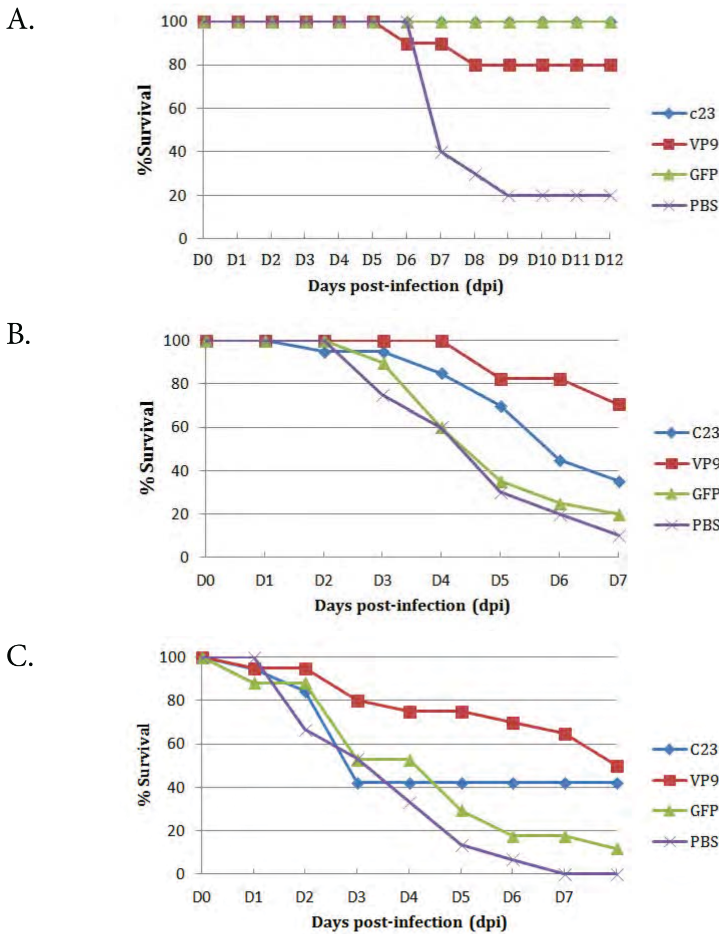

Figure 5. Survival of dsRNA-injected and WSSV-infected shrimps and prawns after WSSV challenge. A. *Macrobrachium rosenbergii* B. *Penaeus monodon*; and C. *Penaeus (Marsupenaeus) japonicus*. The animals were injected with either PBS (control) or c23-dsRNA, VP9-dsRNA, and GFP-dsRNA) and immediately challenged with WSSV (10-2 dilution from stock).The difference between the control and experimental set-ups (PBS vs GFP-dsRNA, PBS vs c23-dsRNA, PBS vs VP9-dsRNA) is statistically significant while no significant difference was observed between GFP-dsRNA vs. c23-dsRNA, based on paired t-test, ($p < 0.05$).

monodon) after c23-dsRNA treatment, highlighting the role of c23-dsRNA as a 'protective gene' for WSSV-infected shrimps. Silencing c23 significantly reduced and delayed mortality in all three species of crustaceans. Thus, the high survival rate observed in dsRNA-treated shrimps suggests that treatment of dsRNA be it specific (c23-dsRNA) or non-specific (GFP-dsRNA) in the WSSV-infected shrimp has a protective effect.

Results of the PCR assays, through the use of WSSV primers that had a 200 bp target size, confirmed that the mortality in all WSSV-challenged *M. rosenbergii* was due to WSSV infection.

Discussion

The importance of the shrimp industry in the country mandates shrimp farmers to focus on issues of high density production as well as specific pathogen-free rearing. However, like any other organisms that are reared in great numbers and produced in high densities, these cultured shrimps are easy targets of microbial and viral pathogens. In this case, one of the effective approaches is to better understand the animal's immune system (Hirono *et al.*, 2010) in order to devise successful schemes in preventing infection. This study worked on c23, a WSSV homolog previously found in Kuruma shrimp and elucidated its possible role in the infectivity of WSSV in *M. rosenbergii*.

Phylogenetic relationship of the contig 23 with other genes from other organisms showed its high homology with WSSVORF-325. Based on the tree generated, it was shown that the contig 23 from two species of crustaceans, *Penaeus (Marsupenaeus) japonicus* and *Macrobrachium rosenbergii* clustered together showing high similarity, while WSSVORF-325 branched out from them. This result is intriguing and intensifies the speculation of "viral mimicry" in WSSV. The high homology WSSVORF-325 might be one of the virus' ways of 'adapting' to its host. Thus the supposition that the virus is mimicking some genes of its host to evade immune response, as observed in other vertebrate viruses (Koyama *et al.*, 2010), is also a possibility in WSSV.

In understanding the pathogenesis of viral diseases, it is essential to have knowledge on the complex interactions between the virus and its host (Dang *et al.*, 2010). A study conducted on *M. japonicus*, revealed the presence of multiple WSSV-like genes in its genome that strongly suggests that similar mimicking mechanisms or horizontal gene transfers can also be seen in this virus group. Such information provides a good starting point for understanding unknown WSSV-host interactions since the current database does not have information on homologous proteins (Alcami and Koszinowski, 2000). The attempt to elucidate the complexity of WSSV-shrimp interaction led to the study of mechanisms mediated by RNAi in the shrimp antiviral response. RNAi has been utilized to study specific pathogens, immune related genes and antiviral mechanism in shrimp immunity (Maningas *et al.*, 2008). It comprises of a cascade of related cellular processes wherein the introduction of dsRNA suppresses the expression of the gene based on sequence homology between the dsRNA trigger and the target gene (Robalino *et al.*, 2005).

In this study, gene expression analysis was done using hemocyte and gill cDNAs, since these two important tissues were

essential in the progression of viral diseases. The gills are usually the organ that is highly affected by the virus after infection while the hemocyte is an essential organ in mediating the first line of defense and plays an integral role in the overall invertebrate immune system. In invertebrates, the most important role of the circulating hemocyte is the protection of the animal against invading microorganisms by participating in recognition, phagocytosis, melanization and cytotoxicity (Hirai *et al.*, 2004).

Here, we elucidated the role of c23 earlier found in Kuruma shrimp, as a WSSV-homolog, in the infectivity of WSSV in *Macrobrachium rosenbergii*. RNAi was induced through *in vivo* experiment by injecting synthesized dsRNAs intramuscularly in the 3rd abdominal segment of WSSV-challenged prawns. Results showed that day 3 after introduction of c23-dsRNA, expression of c23 was silenced. In previous studies, the introduction of gene-specific dsRNA can cause systemic silencing in shrimps. Extracellular dsRNAs were internalized by shrimp cells *in vivo*, as evidenced by the induction of gene silencing which is known to be an intracellular phenomenon (Robalino *et al.*, 2009). This reasoning implies the presence of cell surface receptors in shrimp that mediates the uptake of dsRNA; in a previous study, it was speculated that perhaps the dsRNA is being taken up by shrimp cells (Maningas *et al.*, 2008) either by using an endocytic pathway similar to those reported for the scavenger receptor-mediated endocytosis in *Drosophila* S2 cell (Ulvila *et al.*, 2006) or the endocytic pathway mediated cell entry of dsRNA in *Caenorhabditis elegans* (Saleh *et al.*, 2006). In *C. elegans*, the RNA interference deficient-1 (sid-1) locus involved in transmitting the silencing signal between cells has been identified. SID1 encodes a protein of 11 transmembrane domains and has a structure suggestive of an import–export channel that probably functions as a receptor. In shrimp, the presence of Sid-1 homolog *(Lv-Sid-)1* has been reported,. It was revealed that *Lv-Sid-1* plays a potential role as a channel for dsRNA. In addition, knockdown experiments on *Lv-Sid-1* gene with sequence-specific dsRNA caused mortality of up to 80% death within two days post infection (Labreuche *et al.*, 2010).

In the shrimp system, researchers were able to show the effectiveness of RNAi technology in elucidating the functions of the genes, Transglutaminase (TGase) and clotting protein (CP), in the shrimp system (Maningas *et al.*, 2008). Systemic gene silencing across different tissues tested (gills, heart, hemocyte, hepatopancreas, intestine and lymphoid organ) demonstrated the efficiency of injecting naked dsRNAs into the shrimp's system. This kind of uptake was similarly observed in previous RNAi studies on different species of shrimps (Dang *et al.*, 2010; Robalino *et al.*, 2007; Kim *et al.*, 2007).

This study showed that injection of c23-dsRNA delayed and reduced the mortality due to WSSV infection; hence, it denotes the suppression of the freshwater prawn's ORF (c23) homologous to WSSV proteins specifically vp25/vp28 which are major viral envelope proteins, such further justifies the role of c23 in the infectivity in *M. rosenbergii* (also in *M. japonicus* and in *P. monodon*) to WSSV. The high % identity (100%) posed by WSSV325 with vp25/vp28 gave some definite answers on the role of this contig in the infectivity of the virus in its host. Vp28 has a signal function for transport and also responsible for the membranous structures associated with

WSSV infection *in vivo* (Durand *et al.*, 1997). In addition to this, it is noteworthy to mention that vp28 plays a crucial role in systemic WSSV infection in shrimp in that it binds to the shrimp cells in a low-pH environment and aid viral entry into the cytoplasm (Yi *et al.*, 2004). With this information at hand, it is then safe to say that c23 might be working the same way as vp28, thus explains the findings of this study. The silencing of both WSSV and c23 observed in Figure 4, demonstrated the interaction of the WSSV-homolog with WSSV, therefore suggesting that c23 is involved in the infectivity of WSSV in this species. In the study on kuruma shrimp, it was found that injecting shrimp with MjORF16-dsRNA and MjORF18-dsRNA (both ORFs are known to be homologous to WssvORF-332 and WssvORF-285, respectively) followed by WSSV challenge test delayed and reduced mortality (Dang *et al.*, 2010). These findings suggest that suppression of host ORFs homologous with predicted WSSV proteins combat spread of the virus thereby denoting that homologous MjORFs may function in the infectivity of WSSV to its host.

While no mortality was observed in GFP-dsRNA treated prawns, silencing of the WSSV gene did not transpire. This raises the question: what causes the prawns to survive despite WSSV infection? In the moth system, injection of dsRNA representing GFP, a sequence foreign to the moth, was shown to reduce melanization induced by baculovirus infection (Angthong *et al.*, 2010). This implies that other than sequence-specific dsRNA-mediated gene silencing, injection of foreign dsRNA (GFP-dsRNA) may induce another type of antiviral response which helped the WSSV-infected prawns to survive. In a previous study performed in 2004, the findings suggested that exposure of marine shrimps to dsRNA triggered innate antiviral immunity in a sequence-independent manner (Robalino *et al.*, 2004). Nevertheless, the mechanisms underlying the said phenomenon as well as its occurrence in other invertebrate taxa remains unknown, but it is clearly shown that recognition of dsRNA by another pathway, RNAi, is widely distributed among invertebrates and likely an important component of the invertebrate antiviral response.

As stated above that the injection of dsRNA can induce innate antiviral immunity among shrimps in a sequence-independent manner, succeeding studies on shrimp antiviral immunity showed that same antiviral response was also observed among shrimps after the introduction of sequence-specific dsRNA. In another experiment conducted, the data collectively demonstrated that strong dsRNA-mediated antiviral immunity is dependent upon the sequence homology between the dsRNA trigger and the viral targets. The study thus proposed that partial protection induced by dsRNA of diverse length, sequence, and base composition can be observed from virus-infected shrimps (Maningas *et al.*, 2008).

In line with what were previously done on elucidating the effect of introduction of sequence-specific dsRNA, this study demonstrated that injection of c23-dsRNA was able to induce specific down-regulation of the expression of endogenous genes homologous to WSSV ORFs. Furthermore, it strengthened the fact that administration of dsRNA homologous to viral genes can induce a potent and virus-specific antiviral response that may result in a highly effective control of viral diseases, specifically WSSV.

The complexity of generating homologs by viruses is still a labyrinth; one hypothesis that can be derived from it is that the presence of this WSSV homolog actually helps the virus to evade its host's immune response through 'mimicking'. Since it is present in the host's genome, the entry of viral DNA will be 'masked' and will be considered as a 'self' instead of an antigen thus making the host susceptible to infection while it strengthens the virus' infectivity. The possibility of an enhanced viral infection is at a high stake in the presence of these homologs, since they are not considered as 'nonself' by the host, the immune response genes will not be readily released therefore giving the virus the bigger chance to continue replicating. In another perspective, the presence of these homologs in the genome of WSSV might be one of the mechanisms of WSSV infection. Mutation in the genome of viruses is very rampant and occurs rapidly. These homologs may be mutations that make the virus 'adaptable' to its host's environment, especially to the immune response machinery. In this way, entry of viral DNA will be a lot easier and viral replication will be at full speed because there will be no interference by the immune response genes of the host. As shown in this study, the silencing of c23after injection of c23-dsRNA to shrimps challenged with WSSV reduced and delayed mortality. This further supports the speculation that c23 is probably involved in the infectivity of WSSV and that the absence of this contig builds up a 'protective wall' in the host's immune system that resists the initiation of virus replication and triggers the release of immune response genes.

The biological interaction between viruses and their hosts is a delicate balance of actions and counteractions between host immune system and virus escape mechanisms. Having shown that viral immune evasion, possibly through viral mimicry by production of homologs, is observed in vertebrate viruses, this study is also directed towards the path of explaining the reason behind the presence of WSSV-homologs in the shrimp genome. It is more like a question of which is mimicking between the two organisms: is it the host mimicking the WSSV genes to evade infection or the WSSV duplicating these specific genes to escape the immune response of its host? The observed gene silencing of WSSV-homolog demonstrated in this study tends to answer the latter question: that the production of homologs by WSSV is one mechanism of the virus to be able to escape the antiviral response of its host and continue replicating as shown in Figure 1 where c23 is clearly a part of the shrimp genome. The expression of c23 in three different species (*Macrobrachium rosenbergii, Penaeus (Marsupenaeus) japonicus* and *Penaeus monodon*) further strengthens the hypothesis that the WSSV homologs are involved in the virus' mechanism by mimicking the host's gene to evade the immune response of its host during infection.

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