Silencing of yellow head virus replication in penaeid shrimp cells by dsRNA ☆

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Abstract

RNA interference (RNAi) has been shown to inhibit viral replication in some animals and plants. Whether the RNAi is functional in shrimp remains to be demonstrated. In vitro transcribed dsRNAs of YHV helicase, polymerase, protease, gp116, and gp64 were transfected into shrimp primary cell culture and found to inhibit YHV replication. dsRNA targeted to nonstructural genes (protease, polymerase, and helicase) effectively inhibited YHV replication. Those targeted structural genes (gp116 and gp64) were the least effective. These findings are the first evidence that RNAi-mediated gene silencing is operative in shrimp cells. This could be a powerful tool for studying gene function and to develop effective control of viral infection in shrimp.
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RNA interference (RNAi) is the process by which a gene is post-transcriptionally suppressed using double-stranded RNA (dsRNA) to target and then destroy their homologous mRNA in a sequence-specific manner [1,2]. This phenomenon has been observed as a natural defense against intruding RNA such as viruses or transposon in plants, nematode, insect, and mammal [2]. The most important characteristics of RNAi is that it is triggered by dsRNA, which is cleaved into 21–23 bp so-called small interfering RNA (siRNA) by RNase III-like enzyme Dicer [3]. The siRNA is subsequently loaded onto multicomponent nuclease to form a complex known as RNA-induced silencing complex (RISC).

The siRNA component in RISC hybridizes to target mRNA with a complementary region leading to cleavage of its target in the middle of 21–23 bp of the complementary without affecting the unrelated RNA [1,4]. In invertebrates such as *Drosophila* and worm, long dsRNA induced RNAi more efficiently than shorter dsRNA, whereas 29–38 bp duplexes were inefficient to induce RNAi [5]. In contrast, in mammals RNA duplexes longer than 30 bp failed to trigger specific suppression of target mRNA due to activation of interferon pathway [6]. Artificial introduction or over-expression of 25–30 mer RNA duplexes triggers RNAi more potent than the conventional 21 mer siRNA duplexes without provoking a nonspecific interferon response [7]. To date, RNAi has been widely used as a powerful strategy to investigate gene function as well as to develop antiviral agent to combat various viral infections [8].

Yellow head virus (YHV) is an invertebrate virus with positive sense, single-stranded RNA genome. It has recently been classified as a new genus, *Okavirus*, within a new family, *Roniviridae*, in the order *Nidovirales* [9,10].

In common with other nidoviruses, YHV possesses similar organization and expression of the viral replicase gene. The 5′ terminal replicase gene of YHV encodes two large overlapping open reading frames that are translated into a large polyprotein expected to function in genome replication and transcription of subgenomic mRNA encoding structural proteins [10]. Sequence analysis revealed several putative functional domains in the YHV polyprotein including helicase, polymerase, and protease ordered similarly to the cognate domain in other nidoviruses. YHV has caused mass mortality of cultured penaeid shrimp throughout Asia and remains the major problem for shrimp farming industry. To date, there is no effective means to prevent or cure YHV infection in farmed shrimp.

RNAi mechanism was also reported to contribute to antiviral defense in invertebrate organisms such as insect and nematode, however, the study is relatively limited to certain organisms. So far, there is no information concerning RNAi in other distinct organisms such as in crustacean species and what does it serve in this organism. This present study aims to investigate whether dsRNA-mediated gene silencing exists in penaeid shrimp if so would it be capable of inhibiting viral replication.

Materials and methods

Cell culture. The primary culture of *Penaeus monodon* lymphoid "Oka" cells was prepared as described [11]. Briefly, lymphoid tissues "Oka organ" collected from approximately 100 sub-adult shrimps were washed in washing medium (2× Leibovitz's L-15 medium containing 100 IU/ml penicillin, 100 mg/ml streptomycin, 15% fetal bovine serum, and 5% lactalbumin). The tissue was minced into small pieces in complete medium (2× Leibovitz's L-15 medium containing 100 IU/ml penicillin, 100 mg/ml streptomycin, 15% fetal bovine serum, 5% lactalbumin, and 15% shrimp meat extract). The minced tissue was then seeded onto a 24-well plate and allowed to grow to monolayer at 26 °C.

Plasmid construct and dsRNA generation by in vitro transcription. The following DNA fragments corresponding to specific region of YHV genes were amplified from YHV cDNA template using specific primers: 0.8 kb helicase (5′CAA GGA CCA CCT GGT ACC GGT AAG AC3′ and 5′GCG GAA ACG ACT GAC GGC TAC ATT CAC3′), 0.7 kb RNA-dependent RNA polymerase (5′CCA AAG ATC TCC ATC CA ACCT GTA GAC3′ and 5′GGT GCA TTG TAC CAG AAA CCG TCC TC3′), 0.45 kb protease (5′ATC GTG CGC GTG TAT GGT GAN CGN GGN GA3′ and 5′ACC GTT GAC TGG AGG NAT NCA NGA NAT3′), 0.5 kb gp116 (5′TTC GCC AGG ATC CTA AGT GGA ATT CCT GA3′ and 5′ATA CTG AAT TCT ACA TAC AGC CAG GGA CTG3′), and 0.5 kb cDNA gp65 (5′AGC TTG GAT CCC ACG ACA GGC ACG TGT T3′ and 5′CCG AGA ATT CAG CGT GAC CAT CGT TGA TG3′). The DNA fragments were cloned into pGEM T-eazy vector (Promega). Recombinant plasmids with the same DNA fragment but opposite orientations were purified by Qiagen DNA purification column. To synthesize dsRNA, two recombinant plasmids with designate insert cDNA but with opposite orientation were linearized by appropriate restriction endonuclease and used as template for in vitro transcription using Ribomax kit (Promega). The in vitro transcription was followed as described by the manufacturer. Equal amounts of sense and antisense RNA were annealed to produce dsRNA as described by Worby et al. [12]. Double-stranded RNA was quantified by UV spectrophotometry.

RNA transfection and YHV infection. Double-stranded RNA was introduced into the primary culture of Oka cells by using Transmessenger RNA transfection kit (Qiagen). To each transfection, Oka cells at 70% confluence in 24-well tissue culture plates were transfected with 2 μg (unless specified) of indicated dsRNA formulated as described by the manufacturer and incubated for 3 h. The transfected cells were washed once with fresh complete medium and then allowed to recover for 40 h in 26 °C incubator. The cells were challenged with YHV at different dilutions for 1.5 h. Excess viruses were removed and fresh complete medium was added and then maintained at 26 °C until harvesting.

Western blot analysis. Two hundred microliters of cultured medium was mixed with equal volume of 4× SDS sample buffer and then boiled for 10 min at 100 °C. Equal amount of protein sample was resolved in 10% SDS–PAGE in Tris–glycine buffer. Protein was transferred onto a PVDF membrane (Bio-Rad) using SemiDry transblot apparatus (Bio-Rad). The membrane was incubated with anti-gp116 antiserum in 5% skimmed milk in phosphate-buffered saline containing 0.2% Tween-20 (PBST) (dilution 1:2000) for 1 h at room temperature [13]. Excess antibody was removed by successively washing with PBST and then probed with horseradish peroxidase-conjugated goat anti-mouse polyclonal antibodies (Sigma Chemical) (dilution 1:8000). The antigen–antibody complex was detected using the ECL Plus Western Blotting Detection Reagent (Amersham Pharmacia Biotech) following with exposure to X-ray film.

RT-PCR. Total RNA from YHV infected Oka cells and RNA from YHV particle in the culture medium were prepared by using Trizol and Trizol LS reagent (Gibco-BRL), respectively. First-strand cDNA was synthesized using Imprompt reverse transcriptase (Promega) and oligo(dT) primer as described by the manufacturer. Determination of YHV RNA level was performed by PCR using primer pairs for helicase gene (0.8 kb). When applied, actin cDNA was simultaneously amplified using specific primers (5′GAC TCG TAC GTG GGC GAC GAG G3′ and 5′AGC AGC GGT GGT CAT CTC CTG CTC3′) for normalization of the RNA level. The PCR product was analyzed by agarose gel electrophoresis.

Results

Inhibition of YHV replication by long double-stranded RNA

To investigate the effect of introduction of long dsRNA into shrimp cells, cDNA corresponding to different regions of YHV genome that may exert crucial role in its replication was selected as template for synthesizing dsRNA in vitro. These regions included viral protease (0.45 kb), helicase (0.8 kb), and RNA-dependent RNA polymerase (0.7 kb). In addition, unrelated 0.7 kb dsRNA of green fluorescence protein gene was used as control. These dsRNAs (2 μg) were transfected into Oka cells and then morphological change was examined under a microscope. Both morphology and growth rate of cells transfected with these long dsRNAs were similar to the mock transfected cells, indicating that introduction of exogenous dsRNA has no deteriorate effect on these cells (Fig. 1). Next, we investigated whether the presence of dsRNA altered their susceptibility to YHV infection. At 40 h post-transfection, the cells
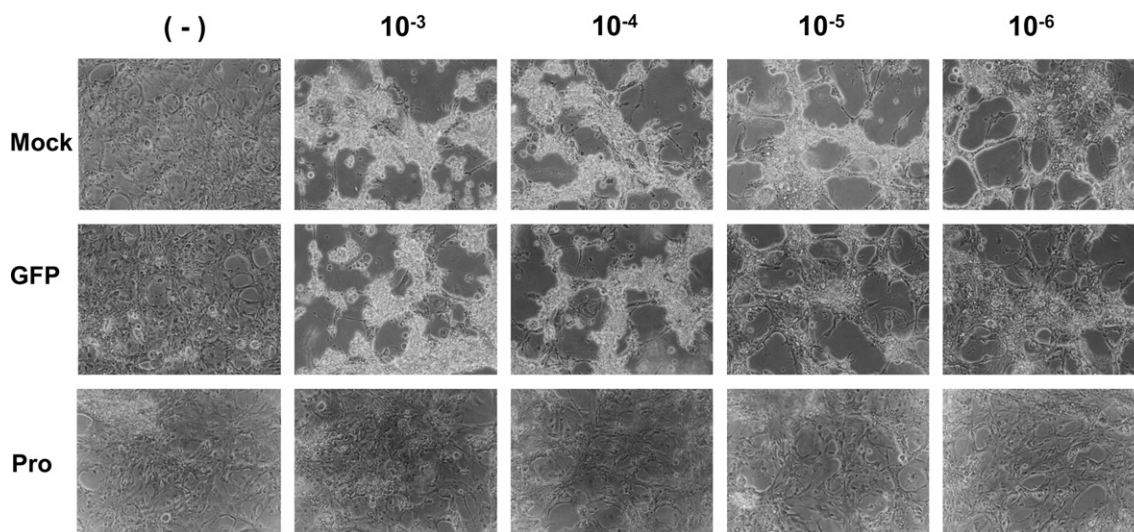
Fig. 1. Protection of YHV induced cytopathic effects by YHV-specific dsRNA. Oka cells were transfected with 2 μg dsRNA specific to YHV protease (Pro), 2 μg unrelated dsRNA for green fluorescent protein (GFP) or without dsRNA (Mock) then infected with YHV at dilution 10^{-4}–10^{-6}. Cell morphology was observed under phase contrast microscope at 48 h post-infection compared to the uninfected cells (−).

were subsequently infected with different dilutions of YHV and morphological change was observed under a microscope. Mock transfected cells were highly susceptible to YHV as cytopathic effect (CPE) was observed as early as 48 h post-infection in cells infected with high level of virus (10^{-3} dilution). CPE was more prominent at 90 h post-infection and its severity was well correlated with the amount of virus used for infection. Similar result was observed in cells transfected with GFP dsRNA although the occurrence of CPE appeared slower as compared to mock transfected cells. In contrast, cells transfected with dsRNA corresponding to helicase, polymerase or protease gene appeared more resistant to YHV. The majority of these cells remained intact

and a minor CPE was observed when compared to mock transfected cells at the same titer of infection.

To test whether YHV replication could be inhibited by dsRNA, viral particle in the medium was analyzed by RT-PCR at 48 h post-infection. As shown in Fig. 2A, level of YHV progeny in the culture medium was readily detected in mock transfected cells in all dilutions of virus. The intensity of virus was correlated with the level of virus used for infection (10^{-3}–10^{-7}). On the other hand, YHV progenies in samples treated with YHV-specific dsRNAs were dramatically decreased as the viral progenies could be detected only in those samples infected with high dose of virus (10^{-3}–10^{-4}). Interestingly, the level of YHV progenies in cells treated with
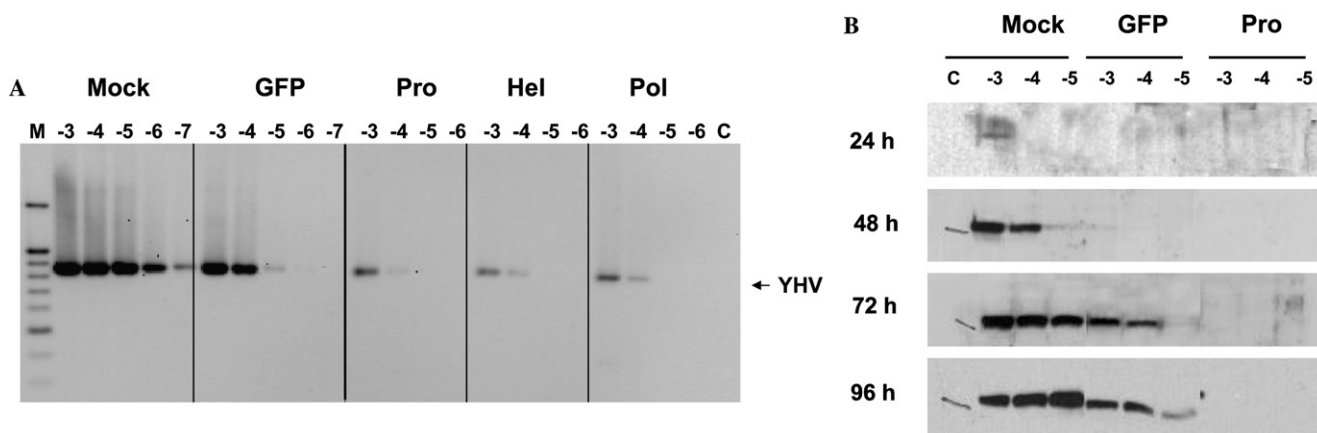

Fig. 2. dsRNA inhibits YHV replication in Oka cells. Oka cells were transfected with 2 μg dsRNA specific to gene; YHV protease (Pro), helicase (Hel) or polymerase (Pol) or unrelated dsRNA for green fluorescence protein (GFP) followed by YHV infection at dilution 10^{-3}–10^{-7} (indicated as −3 to −7) compared to no infection—C. Equal volume of culture medium was collected at 48 h post-infection for determining YHV level by RT-PCR (A). Time course of YHV inhibition by Western blot analysis using anti-gp116 of YHV (B). M, 1 kb plus DNA ladder.

irrelevant dsRNA (GFP) was clearly higher than that observed in cells treated with YHV-specific dsRNA but its level was substantial lower than that from the mock transfected cell. To confirm this result, a similar experiment was performed, however, YHV progeny in the medium was monitored at different time points post-infection (24, 48, 72, and 96 h) by Western blot analysis using antiserum against gp116 of YHV structural protein. YHV gp116 was readily detected at 48 h post-infection in mock transfected cells and its intensity was correlated well with the infection titer (Fig. 2B). The signal was further increased when the incubation time was prolonged to 72 or 96 h particularly for those infected with the lower YHV titer. Gp116 in cells treated with GFP dsRNA was detected in all infection conditions albeit with relatively lower level. Indeed, the first appearance of the YHV gp116 was observed 24 h later compared to the mock transfected control. In contrast, gp116 was not detected in cells transfected with YHV protease dsRNA even at 96 h post-infection. These data confirmed that YHV replication in Oka cells could be efficiently inhibited by dsRNA specific to its nonstructural genes.

YHV suppression by dsRNA is dose dependent

To determine the potency of dsRNA on YHV inhibition, the primary cell was treated with different amounts

Fig. 3. Dose-dependent YHV inhibition by specific dsRNA. dsRNA (0.2–1 µg) corresponding to YHV protease was transfected to Oka cells followed by YHV infection at dilution 10^{-4}–10^{-5}. Cell lysate was collected at 48 h post-infection for analysis of YHV level by RT-PCR. Actin was the internal control for normalizing the RNA level. M, 1 kb plus DNA ladder; Pro, cell transfected with dsRNA; and Mock, without dsRNA.

of dsRNA targeting protease (0.2–1 µg) prior to being infected with YHV at dilution 10^{-4} and 10^{-5}. The level of YHV RNA in the cell extract was determined and compared. The result in Fig. 3 demonstrates that the decreasing amount of dsRNA results in the increase of YHV RNA present in the cell lysate, indicating inverse correlation between YHV inhibition and the amount of dsRNA. While 1 µg of protease dsRNA showed efficient inhibition of YHV, the inhibition was drastically reduced in the presence of 0.2 µg. These data indicate that the efficiency of YHV inhibition by dsRNA occurred in a dose-dependent manner.

YHV structural gene is an unfavorable target for suppression

In addition to dsRNA specific to nonstructural genes of YHV, we also investigated whether dsRNA targeted to other regions of YHV would block replication of YHV. 0.5 kb dsRNAs corresponding to two major structural proteins of YHV (gp116 or pg65) were designed and their inhibitory effects were validated and compared to dsRNA targeted to polymerase gene. The level of YHV in cell lysate was analyzed at 72 h post-infection by RT-PCR. As shown in Fig. 4, dsRNA targeted to gp65 or gp116 could poorly inhibit YHV replication (approximately $2 \log_{10}$ reduction) compared to the inhibition by dsRNA targeted to polymerase gene. Of the two, dsRNA targeted to gp116 region had the least inhibitory effect on viral replication. These data clearly indicate that target sequence of dsRNA strongly contributed to potency for suppression of YHV replication.

Length of dsRNA affects the RNAi efficiency in Oka cell

The observation revealed that dsRNA targeted to different regions of YHV genome, particularly nonstructural and structural genes, showed different potencies for YHV inhibition. This led us to further investigate whether size and location of dsRNA are important for this inhibition. To examine the effect of dsRNA length on YHV suppression, three shorter dsRNAs for YHV nonstructural genes with approximate size of 100 bp were generated. One of these dsRNA targeted to the 3′ end of protease, whereas the other two targeted to the region corresponding to 5′ or 3′ end of helicase gene, respectively. The activity of these dsRNAs to suppress YHV replication was determined and compared to their corresponding long dsRNAs as shown in Fig. 5. Although the 100 bp dsRNA targeted to helicase or protease gene capable of inhibiting YHV replication in Oka cells, their efficiency was relatively lower than that of longer dsRNAs targeting to the same gene. The YHV suppression was irrespective of location in the nonstructural gene these 100 bp dsRNA targeted.
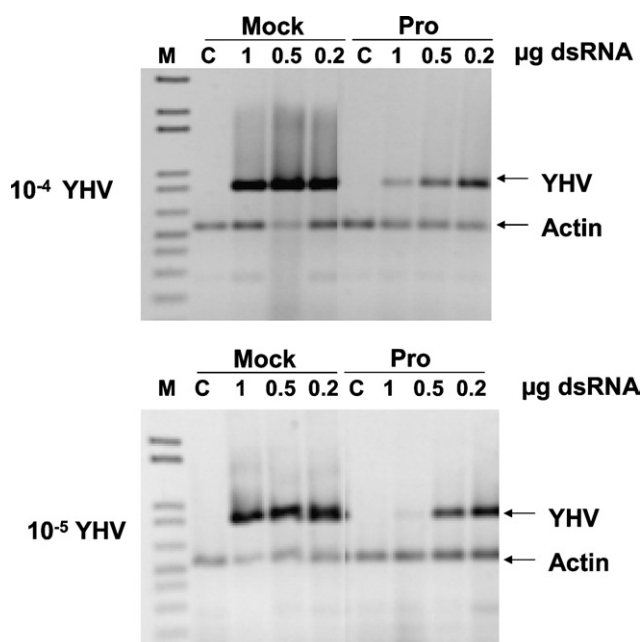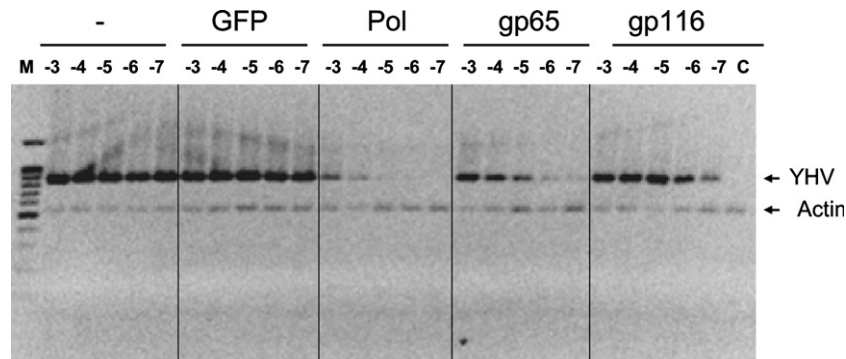
Fig. 4. Suppression of YHV replication by dsRNA targeting structural genes. Oka cells were transfected with 2 µg dsRNA specific to YHV polymerase (Pol) or structural genes gp65 or gp116 or unrelated dsRNA (GFP) followed by YHV infection at dilution 10^{-3}–10^{-7} (indicated as −3 to −7). Cell lysate was collected at 72 h post-infection for RT-PCR. Actin for normalizing the RNA level. M, 1 kb plus DNA ladder; C, without YHV.
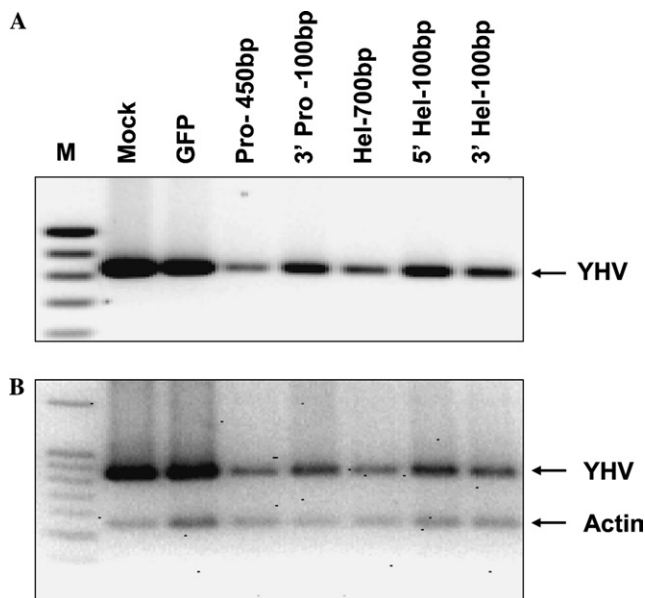

Fig. 5. Effect of dsRNA length on YHV inhibition. Prior to YHV infection (at dilution 10^{-4}) Oka cells were transfected with 2 µg of 100 bp dsRNA: 3′ Pro-100 bp, 5′ Hel-100 bp or 3′ Hel-100 bp. Protection of the cells from YHV infection was compared to its counterpart dsRNA with longer length (450 bp Pro and 700 bp Hel). The level of YHV in culture medium (A) and in cell lysate (B) was determined by RT-PCR at 72 h post-infection. Actin for normalizing the RNA level. M, 1 kb plus DNA ladder.

Discussion

The result of the present study demonstrated that dsRNA administered to the primary lymphoid cell culture (Oka cell) of black tiger shrimp gave protection against YHV infection resembled to RNA interference (RNAi) mechanism in many invertebrate species [14]. In diverse organisms such as *Caenorhabditis elegans*, fruit fly, and planaria, long dsRNA has the ability to suppress the gene function in a sequence-specific manner [14–16]. Although components required for RNAi mechanism in shrimp have yet to be identified, it is most likely that dsRNA-mediated YHV inhibition occurred through the RNAi pathway. Our finding that dsRNA targeted to nonstructural gene of YHV was more effective than structural gene in suppressing the viral replication should result from copy number of target sequence. In general, the process of viral replication requires replicating enzymes including helicase, polymerase, and protease at a lower level compared to the structural proteins thus the mRNA for nonstructural genes should exist in the infected cells at relatively low abundance [17].

Our observation demonstrated that the length of dsRNA also contributes to potency of YHV suppression in the shrimp cells. A long dsRNA could generate more diverse pool of effective siRNAs incorporated into RISC complexes than the shorter one. This makes one cognate mRNA targeted by a larger pool of siRNA. However, the bona fide of using large fragment of dsRNA should be carefully judged, as a diverse pool of siRNA could compromise the specificity of RNAi by off-target effect [18,19]. This phenomenon may explain at least in part the delay in YHV replication observed in cells transfected with irrelevant GFP dsRNA. Alternatively, GFP dsRNA may induce nonspecific antiviral immunity. Recently, Robalino et al. [20] have demonstrated that injection of dsRNA induced resistance to white spot syndrome virus and Taura syndrome virus in pacific white shrimp, *P. vannamei*, by nonspecific dsRNA. Our observation that YHV inhibition by nonspecific dsRNA (GFP) showed greater effect at 48 h than at 72 h post-infection (Figs. 2A, B and 4) was consistently observed. This should indicate that nonspecific inhibition diminished shortly after 48 h due to degradation of long dsRNA by RNase III-like, including Dicer. siRNA would be consequently generated and engaged to the RISC complex targeting YHV RNA. This should result in a longer effect of the specific dsRNA than the nonspecific one.

It would be intriguing to investigate whether our specific dsRNA could inhibit YHV replication in penaeid shrimp. If this is the case, this may pave the way for fur-

ther development of a new tool for preventing loss of shrimp culture by YHV and other viruses.

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