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NOTE

Application of YHV-protease dsRNA for protection and therapeutic treatment against yellow head virus infection in *Litopenaeus vannamei*

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ABSTRACT: While farming of the Pacific white shrimp *Litopenaeus vannamei* is well established in North and South America, the industry has more recently been introduced to Asia, and the Pacific white shrimp is now the most commonly farmed species in Thailand. However, outbreaks of yellow head virus (YHV) disease in the Pacific white shrimp have caused severe economic losses and currently there is no effective prevention or treatment of YHV infections. The YHV-protease double-stranded RNA (YHV-Pro dsRNA) can act as both a prophylactic agent and as a treatment to inhibit YHV replication in infected black tiger shrimp *Penaeus monodon*. The utility of this methodology to other shrimp species has not, however, been established. The purpose of this study was to determine whether YHV-Pro dsRNA can be applied to the Pacific white shrimp. To assess prophylactic efficiency, YHV-Pro dsRNA was injected into juvenile shrimp 24 h prior to challenge with YHV. Subsequent YHV replication was inhibited by YHV-Pro dsRNA as compared with injection of an unrelated dsRNA. For therapeutic treatment of YHV-infected shrimp, shrimp were challenged with YHV before dsRNA injection. Injection of YHV-Pro dsRNA up to 6 h post-infection resulted in the almost complete elimination of YHV replication. These results suggest that YHV-Pro dsRNA can also be broadly applied as a prophylactic agent to inhibit YHV replication and therapeutic treatment of YHV-infected Pacific white shrimp.

KEY WORDS: *Litopenaeus vannamei* · Viral disease · Yellow head virus · Double-stranded RNA · RNAi

INTRODUCTION

Farming of Pacific white shrimp *Litopenaeus vannamei* is a common industry in North and South America and the industry has more recently become established in Asian countries such as China, Taiwan, Vietnam and Thailand (Briggs et al. 2005), primarily because of the shrimp's tolerance to a wide range of salinities (0.5 to 45 ppt) and temperature (15 to 33°C). However, it grows particularly well in low salinities of around 10 to 15 ppt and at temperatures between 23 and 30°C (Wyban & Sweeney 1991). Additionally, specific pathogen free (SPF) and specific pathogen resistant (SPR) broodstock are available. These positive characteristics have resulted in a rapid expansion of Pacific white shrimp farming in Asia with *L. vannamei*

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now being the most commonly farmed shrimp species in Thailand.

Despite the success of a few farms, culturing Pacific white shrimp in low salinity inland waters has resulted in a variety of problems that need to be addressed (Davis et al. 2002). One of these issues is severe outbreaks of yellow head virus (YHV) disease in Thailand (Limsuwan et al. 2008). The symptoms and clinical signs of YHV disease in Pacific white shrimp are similar to those of the black tiger shrimp *Penaeus monodon* (Boonyaratpalin et al. 1993).

Previously, our group (Yodmuang et al. 2006, Tirasophon et al. 2007) investigated the YHV-protease double-stranded RNA (YHV-Pro dsRNA), which can act as a prophylactic agent as well as a treatment designed to inhibit YHV replication in infected black tiger shrimp. The purpose of this study is to determine whether this methodology can be applied to the Pacific white shrimp.

MATERIALS AND METHODS

Shrimp culture. Healthy Pacific white shrimp postlarvae were purchased from commercial farms in Thailand. These shrimps were cultured in 80 l tanks containing artificial seawater at 10 ppt salinity with aeration. The shrimp were acclimated for 2 to 3 d at 28 ± 1°C before use in the experiments.

Yellow head virus stock. YHV titer was determined using tissue culture infectious dose 50 ($TCID_{50}$) as described by Reed & Muench (1938) using a primary lymphoid organ cell culture of *Penaeus monodon* as described in Assavalapsakul et al. (2003). The YHV stock used in this study was 2×10^9 $TCID_{50}$ ml^{-1}.

Double-stranded RNA production. Recombinant clones expressing the stem-loop of the YHV-protease (Pro) RNA (Fig. 1) or the stem-loop of green fluorescence protein (GFP) RNA were used to produce dsRNA for injection into shrimp as described in Yodmuang et al. (2006). The dsRNA concentration was estimated by spectrophotometry at OD_{260} and stored at –80°C until use.

dsRNA injection and YHV challenge. For inhibition of YHV in Pacific white shrimp, healthy Pacific white shrimp (3 g) were injected with 50 µl of control saline (150 mM NaCl) or with 2.5 µg YHV-Pro dsRNA or GFP dsRNA per gram of shrimp in 150 mM NaCl into the haemolymph through the arthrodial membrane of the fifth walking leg by means of a 1 ml syringe with a 29 gauge needle. At 24 h post-injection, shrimp were injected with 5×10^3 $TCID_{50}$ ml^{-1} of YHV in 150 mM NaCl into the haemolymph through the arthrodial membrane of the fifth walking leg. This amount of virus had been previously titrated to cause complete mortality in 2 to 4 d. To evaluate YHV presence in shrimp, gill tissues were collected 48 h after YHV challenge from individual shrimp for RNA extraction and RT-PCR analysis.

For mortality assays, healthy Pacific white shrimp (3 g) were used (8 shrimp per group). Shrimp were injected with 2.5 µg dsRNA g^{-1} shrimp in 150 mM NaCl. At 24 h post-injection of dsRNA 5×10^3 $TCID_{50}$ ml^{-1} YHV was injected into the haemolymph through the arthrodial membrane of the fifth walking leg. Mortality was recorded twice a day for 14 d after the YHV challenge. Four independent experiments were conducted.

To test the therapeutic inhibition ability of YHV-Pro dsRNA in Pacific white shrimp, initially shrimp were challenged with 5×10^3 $TCID_{50}$ ml^{-1} of YHV in 150 mM NaCl, and then injected with control saline, GFP dsRNA or YHV-Pro dsRNA at 3, 6, 9, 12 and 24 h after being challenged. After the 48 h challenge, the gills of each shrimp were collected and used for RNA extraction and YHV genome analysis by multiplex RT-PCR.

Statistical analysis. All data were analyzed using the GraphPad Prism program (GraphPad Software). All data obtained from the experiments were analyzed using 1-way ANOVA ($p < 0.05$ as significance level).

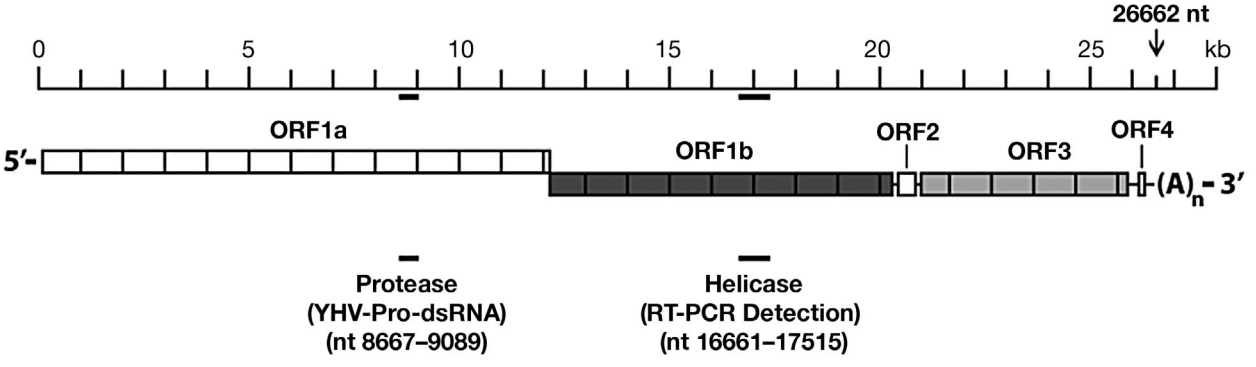

Fig. 1. Schematic representation of the YHV genome. Organization of the YHV genome is shown and the position of the YHV-Pro dsRNA (nucleotides 8667 to 9089) is indicated, as is the position of the helicase RT-PCR product (nucleotides 16661 to 17515)

RT-PCR analysis. Either 2 or 3 pieces of gill tissue were collected from individual shrimp for RNA isolation using TRI Reagent solution (Molecular Research Center). Total RNA (1 µg) was used for cDNA synthesis using Improm pt II reverse transcriptase (Promega) and oligo dT_{18} primer as described by the manufacturer. Detection of YHV genomic RNA was performed by multiplex PCR. Primer pairs for YHV helicase gene (Fig. 1) (5′-CAA GGA CCA CCT GGT ACC GGT AAG AC-3′ and 5′-GCG GAA ACG ACT GAC GGC TAC ATT CAC 3′) (Yodmuang et al. 2006, Tirasophon et al. 2007) were used to monitor YHV level whereas primer pairs for *Litopenaeus vannamei* actin, based on the gene sequence available in GenBank (accession no. AF300705), (5′-GAC TCG TAC GTC GGC GAC GAG G-3′ and 5′-GCA GCG GTG GTC ATC TCC TGC TCG-3′) (Yodmuang et al. 2006, Tirasophon et al. 2007) were used for RNA normalization and internal control. The multiplex PCR conditions used were as follows: 94°C for 3 min, followed by 25 cycles of 94°C for 30 s, 53°C for 30 s and 72°C for 45 s, with a final extension at 72°C for 10 min. The PCR products were analyzed by 1.5% agarose gel electrophoresis followed by staining in 0.5 µg ml^{-1} ethidium bromide.

RESULTS

Prophylactic inhibition of YHV replication in Pacific white shrimp

To investigate whether dsRNA could provide prophylactic protection against YHV replication in Pacific white shrimp, YHV-Pro dsRNA, GFP dsRNA or control saline were injected into shrimp 24 h before the YHV challenge with 5×10^3 $TCID_{50}$ ml^{-1} per shrimp and then sampled for PCR 48 h post-YHV exposure. As illustrated in Fig. 2, the presence of the YHV genome was detected in all control saline and GFP-dsRNA-injected shrimp, while no YHV genome was detected in shrimp injected with YHV-Pro dsRNA. This suggests that prophylactic YHV-Pro dsRNA injection effectively inhibited YHV replication.

Prevention of shrimp mortality by dsRNA

From the preceding experiment it was clear that prophylactic injection of YHV-Pro dsRNA was able to reduce the YHV load to undetectable levels. To determine whether this affected shrimp mortality, the above experiment was repeated and this time shrimp were observed for a period of 14 d post-YHV infection with mortality recorded twice daily. The experiment was repeated in quadruplicate, with 8 shrimp per group. Complete mortality (100%) was observed by Day 3 post-YHV infection in the control-saline injected ($p < 0.0001$) and GFP-dsRNA-injected ($p < 0.0001$) groups (Fig. 3). In contrast, nearly 100% survival was recorded on Day 14 for the YHV-Pro-dsRNA-injected shrimp ($p < 0.0001$).

Therapeutic inhibition of YHV-Pro dsRNA

Analyses of the control saline and GFP-dsRNA-injected shrimp clearly showed the presence of the

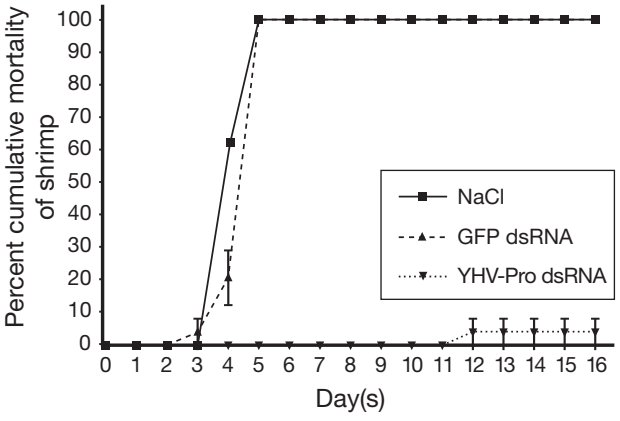

Fig. 3. *Litopenaeus vannamei.* Percent cumulative mortality of Pacific white shrimp. Shrimp (~3 g) were injected with NaCl, GFP dsRNA or YHV-Pro dsRNA for 24 h before injection challenge with a saline suspension of YHV. Shrimp mortality was recorded twice daily for 16 d. Percent mortality in each experimental group is presented as the mean of 4 replicate experiments

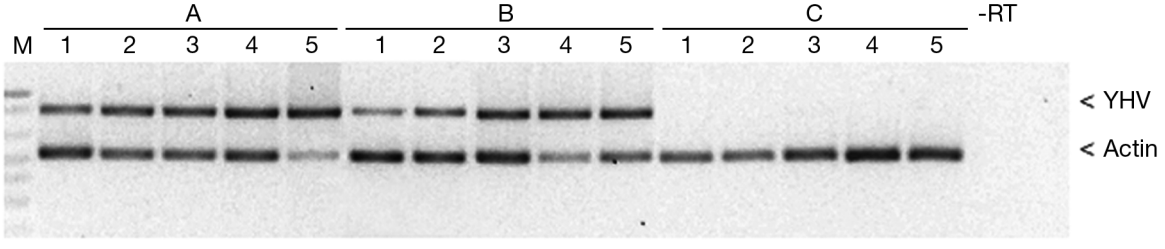

Fig. 2. *Litopenaeus vannamei.* Inhibitory effect of YHV replication in Pacific white shrimp using YHV-Pro dsRNA. Shrimp (~3 g) were injected with (A) NaCl, (B) GFP dsRNA or (C) YHV-Pro dsRNA 24 h before injection challenge with a saline suspension of YHV. The YHV load in gills of individual shrimp was determined by RT-PCR. Lane M is 1 kb Plus DNA ladder (Invitrogen)

YHV genome in all samples from all shrimp tested (Fig. 4). In contrast, no shrimp injected with YHV-Pro dsRNA at 3 h post-infection displayed the presence of the YHV genome, and only 1 shrimp out of 6 injected with YHV-Pro dsRNA at 6 h post-infection had detectable levels of the YHV genome. The majority of shrimp injected at 9 h or later post-infection displayed the presence of the YHV genome (Fig. 4).

DISCUSSION

YHV is a highly pathogenic agent that causes an acute and often fatal disease in *Penaeus monodon* (Boonyaratpalin et al. 1993). Recently, YHV has caused mass mortality of cultured *Litopenaeus vannamei* resulting in significant economic losses in Thailand (Limsuwan et al. 2008). RNAi, a sequence-specific gene-silencing mechanism that has been proposed to function as a defense mechanism of eukaryotic cells against viruses and transposons (Haasnoot & Berkhout 2006), was employed to ameliorate mass mortality of Pacific white shrimp. Robalino et al. (2005) suggested that low level viral loads in shrimp could be completely inhibited by injecting a specific dsRNA prior to viral multiplication. In the present study, an optimized 2.5 µg of dsRNA g^{-1} shrimp and approximately 5×10^3 $TCID_{50}$ ml^{-1} of YHV were used to study prophylactic and therapeutic treatment of YHV replication in Pacific white shrimp. Using these optimized conditions, non-specific inhibition of YHV by a sequence-unrelated dsRNA (GFP dsRNA) was not observed. The results showed that the YHV-Pro dsRNA could efficiently reduce YHV replication when administered prior to infection.

For therapeutic treatment, inhibition of YHV replication was most effective at 3 h post-infection and the effectiveness decreased after longer exposure to the virus. Thus, at 6 h post-infection approximately 83% of shrimp showed viral inhibition while inhibition disappeared at 24 h post-infection. This phenomenon suggests a competition between YHV replication and the RNAi pathway in shrimp cells. At 3 h post-infection, dsRNA should be processed to siRNA (short interfering RNA) and loaded into RISC (RNA-Induced Silencing Complex) at a sufficient level to knock out the viral protease mRNA or the genome. Without the viral protease the viral polyprotein should not be processed, making YHV unable to replicate in the shrimp cell. This study suggests that therapeutic treatment is effective as long as the infection is not too far advanced. In studies on the black tiger shrimp, YHV-Pro dsRNA was shown to be therapeutically effective up to 12 h post-infection (Tirasophon et al. 2007). This difference may result from the significantly larger shrimp used in that study (12 g versus 3 g used in the present study) or may point to species-specific differences, which would have significant implications for the therapeutic use of this technology in an aquaculture setting. The failure to cure YHV-infected shrimp beyond a certain time post-infection may result from the ability of YHV to generate antiviral RNAi suppressors including small RNAs or proteins (Silhavy & Burgyán 2004, Shi et al. 2008). Our results suggest that YHV-Pro dsRNA can be broadly applied as a prophylactic agent to inhibit YHV replication and as a therapeutic treatment of YHV-infected Pacific white shrimp. However, since vaccination of farmed shrimp populations is not practical, further experimentation is needed to determine whether oral administration of YHV-Pro dsRNA is effective in preventing or treating YHV infection.

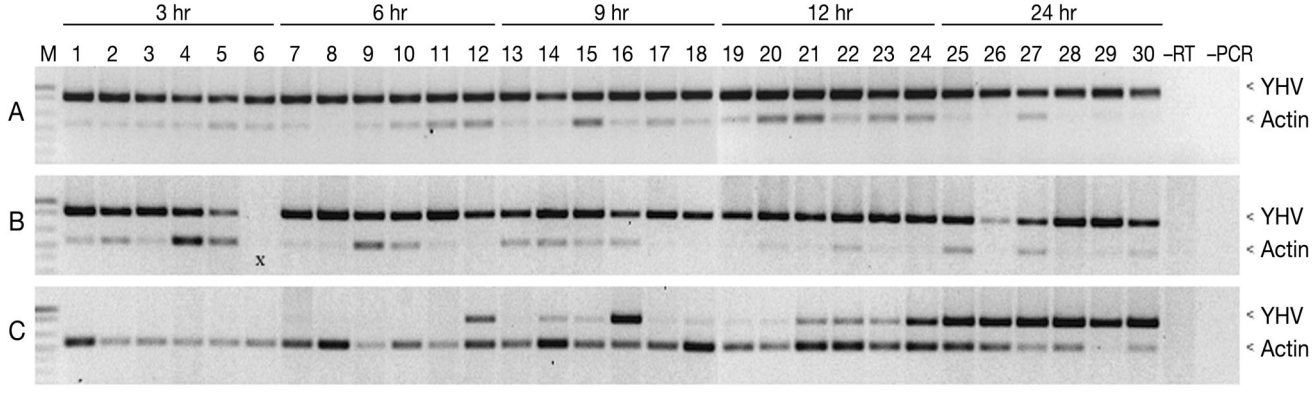

Fig. 4. *Litopenaeus vannamei.* Time effect for curing YHV-infected Pacific white shrimp using YHV-Pro dsRNA. Shrimp (~3 g) were injected with a YHV suspension followed by injection with (Panel A) NaCl, (Panel B) GFP dsRNA or (Panel C) YHV-Pro dsRNA at various time intervals; Lanes 1 to 6: 3 h post-infection; Lanes 7 to 12: 6 h post-infection; Lanes 13 to 18: 9 h post-infection; Lanes 19 to 24: 12 h post-infection; and Lanes 26 to 30: 24 h post-infection. The YHV load in gills of individual shrimp was determined by RT-PCR. Lane M is 1 kb Plus DNA ladder (Invitrogen). X in Lane 6 denotes a shrimp that died before the start of the experiment

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