

Therapeutic inhibition of yellow head virus multiplication in infected shrimps by YHV-protease dsRNA

Witoon Tirasophon^{a,*}, Supansa Yodmuang^a, Wanlop Chinnirunvong^a,
Nongluk Plongthongkum^a, Sakol Panyim^{a,b}

^a The Institute of Molecular Biology and Genetics, Mahidol University, Salaya, Nakhonpathom 73170, Thailand

^b Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok 10400, Thailand

Received 7 October 2006; accepted 14 November 2006

Abstract

Yellow head virus (YHV) is an invertebrate nidovirus which causes a severe mortality in cultured *Penaeus monodon*. The mortality may be prevented by prior treatment of shrimps with YHV-protease dsRNA. Whether the YHV infected shrimp might be cured by the dsRNA remains to be investigated. *P. monodon* injected with 10^{-6} YHV showed a high virus replication and mortality within 2 days. Injection of 25 μ g YHV-protease dsRNA at 3, 6, 12 or 24 h post YHV infection showed a strong inhibition of YHV replication up to 12 h. Unrelated dsRNA-GFP showed no inhibition, indicating that the inhibition was nucleic acid sequence specific through RNAi pathway. Shrimp mortality could be prevented at 3 h post YHV infection by the dsRNA, but not at 24 h. These results demonstrate that YHV-protease dsRNA gives therapeutic effect and pave the way to develop a cure for YHV-infected shrimps.

© 2006 Elsevier B.V. All rights reserved.

Keywords: Yellow head virus (YHV); RNAi; antiviral; dsRNA-protease

1. Introduction

Yellow head disease is a fatal infection in penaeid shrimp caused by yellow head virus (YHV), an invertebrate virus with positive sense single-stranded RNA genome classified as nidovirus (Cowley and Walker, 2002; Sittidilokratna et al., 2002). YHV has caused rapid mass mortality of farmed penaeid shrimp and remains a serious problem to shrimp production industry worldwide. In Thailand alone, estimated loss in shrimp production was about 1 billion U.S.\$ between years 1997 and 2000 (Flegel, 2006). Currently, there is no effective drug or vaccine to treat or prevent yellow head disease. Development of new strategy to control YHV therefore represents a great challenge in shrimp aquaculture industry.

RNA interference (RNAi) is a process by which double-stranded RNA (dsRNA) yielding siRNA induces degradation of homologous messenger RNA (mRNA) in a sequence specific mode at post-transcriptional level (Fire, 1999; Tuschl et al., 1999). Accumulated researches from diverse fields described

RNAi as a mechanism in which eukaryotes knock down expression of specific genes (Hannon, 2002). This RNA-associated mechanism allows cells to control the expression of undesirable mRNA of either exogenous or endogenous origin. Based upon this scenario, RNAi is therefore considered as a primitive form of natural defense mechanism for eliminating the intruding foreign RNA including virus (Lu et al., 2005).

Recent studies demonstrated that introduction of dsRNA into shrimp prior to viral challenge can prevent viral propagation and shrimp mortality (Robalino et al., 2004, 2005; Yodmuang et al., 2006). It is not known if dsRNA given post-viral infection is still effective. In this study, we aim to investigate whether the dsRNA-triggered RNAi is functional in a curative mode in the shrimp.

2. Materials and methods

2.1. Shrimp rearing

Ten to 12 g healthy *Penaeus monodon* juveniles were used in all experiments. Shrimps were reared in 80 l tank filled with 40 l aerated artificial seawater at 10 ppt salinity and fed with commercial diet. Water was changed every 2 days.

* Corresponding author. Tel.: +66 2 800 2732; fax: +66 2 441 9906.
E-mail address: mbwtr@mahidol.ac.th (W. Tirasophon).

2.2. Virus stock

YHV was infected into 200 juvenile *P. monodon* shrimps with average weight ~ 15 g by intramuscular injection. Forty-eight hours post-infection, hemolymph from the shrimps was collected and pooled. YHV in the hemolymph was purified using Urografin (Schering) gradient centrifugation (Assavalapsakul et al., 2005). The purified YHV was aliquot and stored frozen at -80°C until use.

To titer the viral stock, primary culture of lymphoid (Oka) organ cells was prepared and grown to 100% confluent in 96-well plate as previously described (Assavalapsakul et al., 2005). Serial 10-fold dilutions of purified YHV were prepared in the same culture medium then inoculated into four wells of the primary cells and incubated for 90 min at room temperature. After incubation, the virus was discarded and fresh medium was added. The cells were grown at 26°C for 7 days. The virus infectivity was identified by crystal violet staining. Viral titer was determined by TCID₅₀ end points as described by Reed and Muench (1938). The viral stock used in this study was $\sim 3 \times 10^9$ infectious virions.

2.3. dsRNA preparation

dsRNA-protease and dsRNA-GFP was prepared by over-expression in *Escherichia coli* HT115 as previously described (Ongvarrasopone et al., in press). *E. coli* HT115 containing the recombinant plasmid with inverted sequence of YHV protease or of GFP was inoculated into $2 \times \text{YT}$ medium and cultured until OD₆₀₀ of 0.4 at 37°C . The expression of hairpin RNA of the corresponding gene was induced by addition of IPTG to final concentration of 0.4 mM for 4 h. One OD₆₀₀ ml of bacterial cell was pelleted by centrifugation and resuspended in 50 μl phosphate saline buffer (PBS) containing 0.1% SDS. The sample was boiled for 2 min then snapped cool on ice. Single-stranded RNA in the loop region of hairpin structure and endogenous RNA from the bacterial host strain was eliminated by addition of 1 μg RNase A in RNase A buffer (300 mM sodium acetate, 10 mM Tris-HCl, pH 8.0) and incubated for 15 min at 37°C . Double stranded RNA was extracted from bacterial lysate by TRI reagent (Molecular Research Center) according to manufacturer protocol. Each dsRNA obtained from this preparation appears as a single band in agarose gel electrophoresis corresponded to its expected size. The dsRNA integrity was confirmed by RNase III and RNase A digestion. DsRNA concentration was estimated by OD₂₆₀ and adjusted to final concentration of 1 $\mu\text{g}/\mu\text{l}$ prior to storage at -80°C until use.

2.4. Shrimp injection and sample processing

For YHV inhibition assay, shrimps were challenged with YHV 10^{-6} dilution in PBS (approximately 3×10^3 virions), by injecting 50 μl into hemolymph using 1 ml syringe with 29 gauge needle. Such injection usually resulted in total mortality within 2–3 days. The infected shrimps were then injected with 25 μg dsRNA-protease, dsRNA-GFP or 150 mM NaCl into the hemolymph at indicated time post-infection. To determine viral

load, hemolymph was collected 48 h after the viral challenge for RNA extraction and RT-PCR analysis. Gill sample from the same shrimp was collected for YHV antigen determination by Western blot analysis.

For mortality assay, healthy *P. monodon* juveniles (~ 10 g) were used (10 shrimps/group). Same procedure for YHV inoculation and dsRNA injection was performed as described above. The mortality was recorded twice a day for 10 days after YHV challenge. Statistical analysis of the mortality test was performed using Fisher's exact test.

2.5. RT-PCR analysis

Total RNA was isolated from 200 μl of hemolymph from individual shrimps using TRI-LS reagent (Molecular Research Center). First strand cDNA was prepared from 1 μg of total RNA using oligo-dT and Imprompt II reverse transcriptase (Promega) as described by manufacturer. The level of YHV in each RNA sample was monitored by multiplex PCR. Primer pairs specific to YHV helicase gene (5'-CAA GGA CCA CCT GGT ACC GGT AAG AC-3' and 5'-GCG GAA ACG ACT GAC GGC TAC ATT CAC-3') was used to monitoring YHV level whereas primer pairs for *P. monodon* actin (5'-GAC TCG TAC GTG GGC GAC GAG G-3' and 5'-AGC AGC GGT GGT CAT CTC CTG CTC-3') are for internal control. The PCR amplification was performed for 30 cycles and the PCR product was analyzed by 1% agarose gel electrophoresis.

2.6. Western blot analysis

Detection of YHV antigen was performed similar to that previously described (Yodmuang et al., 2006). Equal amounts of protein (~ 50 μg) prepared from gills of individual shrimp in $2 \times$ sample buffer were resolved in sodium dodecyl sulfate polyacrylamide gel (8%) electrophoresis prior to transferring to PVDF membrane (BioRad). The membrane was blocked with 5% skim milk in PBS containing 0.2% Tween-20 for 1 h at room temperature. The membrane was probed with mouse antiserum raised against gp116 of YHV (dilution 1:2000) for 1 h and followed with horseradish peroxidase-conjugated goat anti-mouse polyclonal antibodies (Sigma Chemical) (dilution 1:8000). The gp116 was detected using the ECL Plus Western Blotting Detection Reagent (Amersham Pharmacia Biotech).

3. Results

3.1. dsRNA exerts therapeutic property for YHV infection in shrimp

Previously, we have shown that pre-administration of *P. monodon* with dsRNA-protease 24 h prior to YHV inoculation efficiently prevented the viral propagation and protected the shrimp from mortality (Yodmuang et al., 2006). To investigate whether the dsRNA mediated YHV inhibition is capable of inhibiting the virus in shrimps that were already infected. YHV at 10^{-6} dilution was injected into hemolymph of *P. monodon* allowing the virus to multiply for 3 h. The shrimps were then

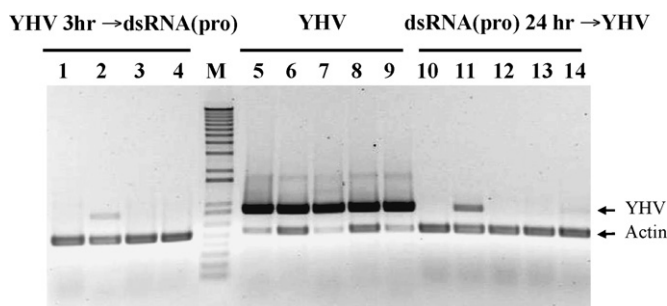


Fig. 1. Inhibitory effect on YHV replication by dsRNA. Shrimps (~10 g) were injected into hemolymph with 25 μ g dsRNA for 24 h prior to or 3 h post YHV (10^{-6}) challenge. The viral load in hemolymph of individual shrimps was determined by RT-PCR at 48 h post YHV challenge. Lanes 1–4 represent individual shrimps receiving dsRNA 3 hpi; 5–9, no dsRNA; 10–14, dsRNA 24 h prior YHV challenge. Actin was used as internal control. M is 100 bp DNA ladder.

injected with YHV specific dsRNA-protease. The YHV level in blood circulation was determined at 48 hpi by RT-PCR. Results in Fig. 1 clearly show that dsRNA-protease injected into the infected shrimps markedly reduced the viral load in the shrimps to undetected level compared to its control group. Indeed the efficiency of YHV inhibition was comparable to those observed in shrimp received the dsRNA prior to YHV challenge. These results suggest that anti-YHV activity by dsRNA-protease exerts both preventive as well as therapeutic modes in shrimps.

3.2. Duration of dsRNA therapeutic effect

To explore the effective of dsRNA mediated YHV clearance, YHV was inoculated into shrimp by injection into hemolymph for 3, 6, 12 or 24 h prior to dsRNA-protease injection (25 μ g/shrimp). As a control, shrimps were injected with 150 mM NaCl post-viral inoculation. Forty-eight hours from the onset of YHV inoculation, YHV genome in hemolymph and viral antigen in gill of infected shrimps were determined by RT-PCR and Western blot analysis respectively. YHV in shrimps injected with YHV but not with dsRNA-protease propagated efficiently to a high level in blood circulation and gill (Fig. 2).

In contrast, the viral load was abruptly diminished to very low or undetected level in shrimps received dsRNA-protease between 3, 6 or 12 h post YHV challenges. Similarly, the viral protein gp116 in the gill of these shrimps was undetected. However, the viral load and gp116 viral antigen in shrimps received dsRNA at 24 hpi was clearly detected, although at relatively lower level than the control group. The result indicated that administration of dsRNA-protease into YHV infected shrimps within 12 hpi could abrogate the YHV replication.

3.3. Curative effect of YHV infection in shrimp requires YHV sequence-specific dsRNA

Previously, we have demonstrated that injection of either viral specific dsRNA or unrelated dsRNA (dsRNA-GFP) prior to YHV challenge could inhibit viral propagation with different efficacy (Yodmuang et al., 2006). We next explored whether injecting the unrelated dsRNA-GFP post YHV challenge would affect the propagation in shrimp in a similar manner to dsRNA-protease. As seen in Fig. 3 shrimps administered with dsRNA-GFP post YHV infection at any time points showed high level of YHV in both hemolymph and gill. Notably, both viral load and viral antigen in all affected shrimps was detected at high level comparable to those in the control group. This result indicated that non-specific dsRNA-GFP was unable to inhibit YHV propagation in a therapeutic mode. It is worth noting that the co-amplified RT-PCR product of actin from shrimp hemocyte was markedly reduced in shrimp with high YHV level (usually moribund). This low level of actin mRNA was confirmed by amplification using only actin primers (data not shown). We consistently observed a negative correlation of YHV level and hemocyte (the source of actin mRNA in our experiment) count due to its rapid destruction in the infected shrimps (data not shown).

3.4. Mortality reduction in YHV infected shrimps by dsRNA

Although dsRNA-protease is capable of inhibiting YHV propagation in the infected shrimps, it is desirable to investigate

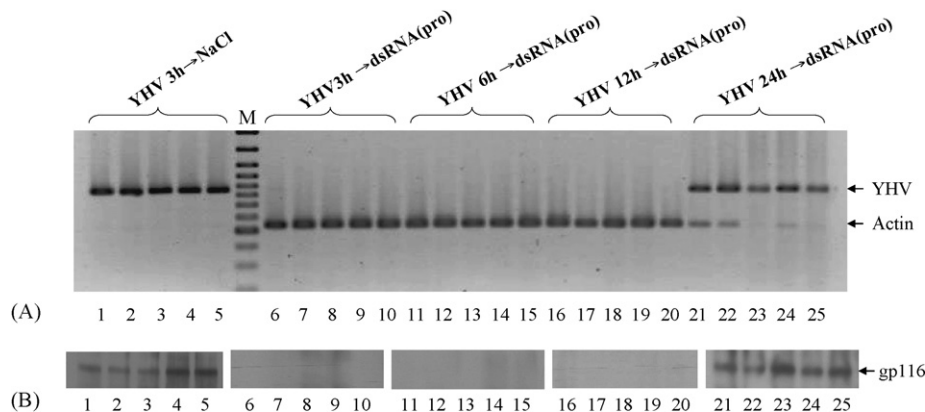


Fig. 2. The effect of time on curing YHV infected shrimp by dsRNA-protease. Shrimps (~10 g) were challenged with 10^{-6} YHV then injected into hemolymph with 150 mM NaCl (lane 1–5) or with 25 μ g dsRNA-protease 3 hpi (lane 6–10), 6 hpi (lane 11–15), 12 hpi (lane 16–20) or 24 hpi (lane 21–25). The YHV load in hemolymph of each shrimp was determined by RT-PCR (A) and the YHV structural protein gp116 in gill was determined by Western blot analysis (B). M is 100 bp DNA ladder.

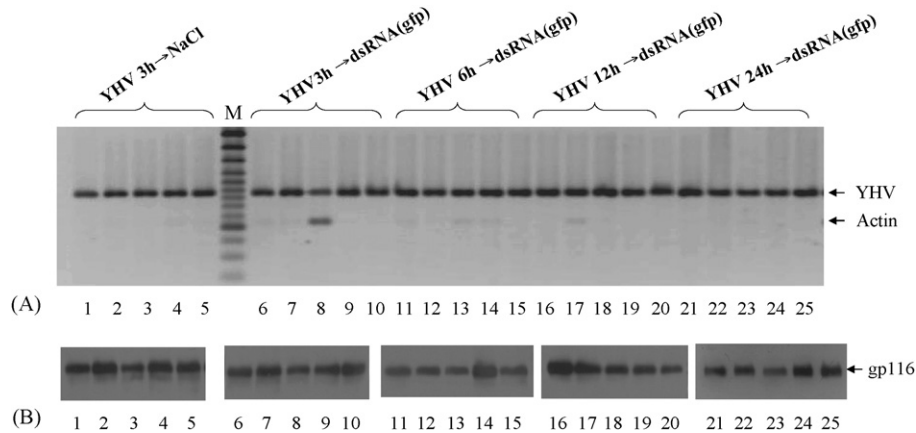


Fig. 3. Effect on YHV multiplication in infected shrimp by dsRNA-GFP. Shrimps (~10 g) were challenged with 10^{-6} YHV then injected into hemolymph with 150 mM NaCl (lane 1–5) or with 25 μ g dsRNA-GFP 3 hpi (lane 6–10), 6 hpi (lane 11–15), 12 hpi (lane 16–20) or 24 hpi (lane 21–25). The YHV load in hemolymph of each shrimp was determined by RT-PCR (A) and the YHV structural protein gp116 in gill was determined by Western blot analysis (B). M is 100 bp DNA ladder.

whether it has potential to cure those infected and prevent the shrimps from mortality. To address this possibility, shrimps were challenged with YHV for indicated time prior to injecting with dsRNA. As seen in Fig. 4, YHV infection alone led to 100% mortality within 2 days. Although mortality was observed in shrimps received YHV specific dsRNA-protease at 3 hpi, however, the cumulative mortality over 10 days period was significantly reduced compared to the untreated YHV infected group as analyzed by Fisher's exact test ($p=0.005$). The mortality rate was approximately 40% in YHV-infected shrimp administered with dsRNA-protease at 3 hpi comparable to the uninfected control group (~30%) ($p=0.825$) indicating that the mortality resulted from YHV infection could be prevented. In contrast, rapid mortality to 100% found in shrimp administered

with dsRNA-protease at 24 hpi ($p=1.000$), or dsRNA-GFP at 3 hpi ($p=1.000$) was almost identical to that of YHV infected shrimp received no dsRNA indicating that such treatments failed to rescue the shrimps from rapid mortality.

4. Discussion

Cumulative experimental data in variety of virus systems underline the fact that RNAi is a promising novel approach for antiviral development ranging from plant (Waterhouse et al., 2001), insect (Zamboni et al., 2006), nematode (Schott et al., 2005) and mammal (Morissey et al., 2005). In this study, we showed a feasibility of using RNAi approach to cure YHV infection in black tiger shrimps. Administration of YHV specific

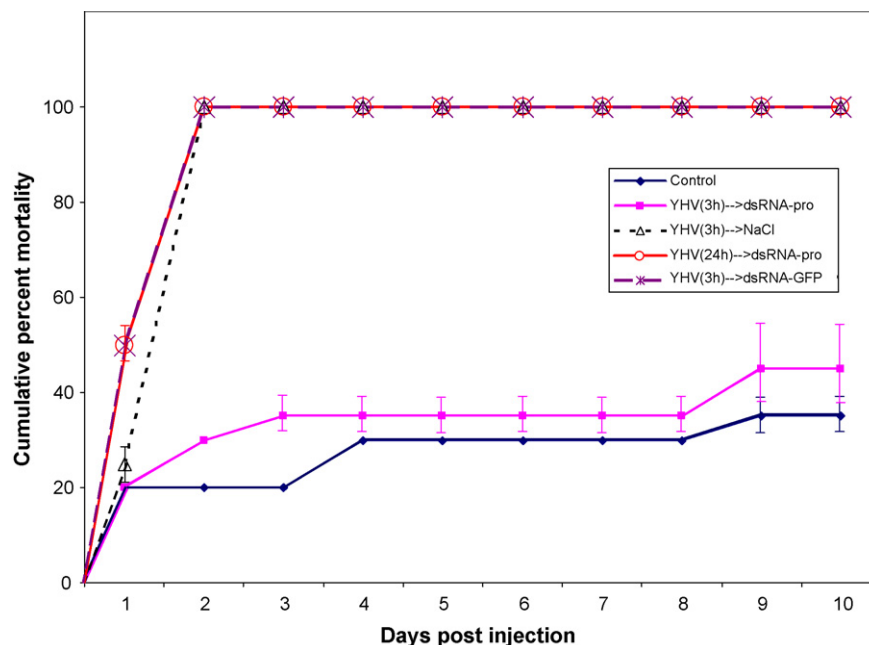


Fig. 4. Mortality of shrimps upon YHV infection. Five groups (10 each) of shrimps were infected with YHV followed by injecting into hemolymph with none, 150 mM NaCl 3 hpi, 25 μ g dsRNA-protease 3 hpi, 25 μ g dsRNA-GFP 3 hpi or 25 μ g dsRNA-protease 24 hpi. Shrimp mortality was recorded twice each day for 10 days. Percent mortality in each experimental group was presented as means with error bar of duplicate experiments. Statistical analysis was performed using Fisher's exact test.

dsRNA to infected shrimps within 12 h of the infection onset completely abrogated the viral multiplication and prevented shrimp mortality. On the other hand, the unrelated dsRNA neither suppressed the replication nor prevented mortality. Hence, these results suggest that the sequence specific pathway by RNAi mechanism is a prominent antiviral defense in shrimp that exerts both preventive and curative modes.

YHV causes systemic infection nevertheless the systemic effect of dsRNA is not known. YHV multiplication in black tiger shrimp has been demonstrated as early as 3–6 hpi (Wongteerasupaya et al., 1997). The ability of viral specific dsRNA (dsRNA-protease) to suppress YHV until 12 hpi warrants the RNAi mechanism is operative while the YHV multiplication process is active. The loss of dsRNA-protease potency at a prolonged infection (24 h) might be explained by the extremely rapid replication of the virus, therefore RNAi is overwhelmed by the YHV level. Alternatively, it is also plausible that a certain YHV protein acts as a suppressor of RNAi mechanism. Upon its accumulation to a sufficient level, the antiviral activity of RNAi is therefore shutting down. This mechanism has been reported as a counter defense of RNAi by many viruses, e.g. HCV core protein, influenza virus NS1 and Flock house virus B2 (Li et al., 2004; Lingel et al., 2005; Wang et al., 2006). Which YHV protein acts as the suppressor is being further investigated.

An innate antiviral defense triggered by sequence independent dsRNA/siRNA has been previously reported to provide partial protection in shrimps only when receiving these dsRNAs (regardless of their sequence origin) prior to the viral challenge (Robalino et al., 2004; Westenberg et al., 2005). A synthetic dsRNA analogue poly C-G but not poly I-C was shown to induce innate antiviral activity raised a possibility that the innate immunity in shrimps required dsRNA with high G-C content. Nevertheless, we have reported that unrelated dsRNA with average G-C content of 63% in dsRNA-GFP exhibited similar partial antiviral activity only when the shrimps were received dsRNA-GFP prior to YHV infection (Yodmuang et al., 2006). This result not only confirmed the existence of sequence independent antiviral activity by dsRNA in the shrimps but also suggested its activity was not restricted to very G-C rich dsRNA. It is most likely that YHV dsRNA-protease (52% G-C content) functions through both RNAi and innate antiviral immunity. However, our finding that only unrelated dsRNA-GFP and dsRNA-helicase (42% G-C content) specific to Taura syndrome virus (data not included) failed to trigger the partial protection in shrimps with active YHV replication suggesting that the innate antiviral and the sequence specific mechanisms play distinct roles. In higher eukaryote, nonspecific dsRNA activates the antiviral response through multiple mechanisms. One of which is through Toll-Like Receptor 3 (TLR3) that prevents the virus to invade the cells (Schroder and Bowie, 2005). It is conceivable that most dsRNA including dsRNA-GFP as well as those dsRNA reported by Robalino et al. (2004) employ the similar TLR-3 mechanism. Beside the nonspecific response, the viral specific dsRNA, on the other hand, also acts through RNAi, an intracellular process where it can directly prohibit YHV replication by targeting the viral RNA for degradation.

RNAi is considered a promising strategy for viral disease control as demonstrated in many organisms including shrimp, however, many obstacles in development of RNAi-based approach into a practical means of antiviral control should be overcome. Delivery of dsRNA/siRNA to target cells or tissues is among a major bottleneck in development of RNAi-based drugs and therapeutics (Leung and Whittaker, 2005). Although dsRNA injection into hemolymph of shrimp provides efficient means to trigger the antiviral activity, it may not provide sustainable antiviral immunity in the shrimps. In addition, such delivery is not practical particularly in working with a large number of shrimps. Hence, development of a more simple but effective route of dsRNA delivery into shrimp must be further investigated. A good example is a feeding method that was successfully demonstrated in *Caenorhabditis elegans* (Timmons et al., 2001).

In summary, our result demonstrates that the antiviral defense in the shrimp through RNAi pathway is a potent mechanism to abrogate the viral multiplication both in preventing and curing aspects. It is therefore encouraging for application of dsRNA-triggered RNAi in a therapeutic modality.

Acknowledgements

This work is supported by Thailand Research Fund (TRF) Senior Research Scholars to S.P. and TRF Research Career Development Grant to W.T. and by Mahidol University Research Grant.

References

- Assavalapsakul, W., Tirasophon, W., Panyim, S., 2005. Antiserum to the Gp116 glycoprotein of yellow head virus neutralizes infectivity in primary lymphoid organ cells of *Penaeus monodon*. Dis. Aquat. Org. 63, 85–88.
- Cowley, J.A., Walker, P.J., 2002. The complete genome sequence of gill-associated virus of *Penaeus monodon* prawns indicates a gene organisation unique among nidoviruses. Arch. Virol. 147, 1977–1987.
- Fire, A., 1999. RNA-triggered gene silencing. Trends Genet. 15, 358–363.
- Flegel, T.W., 2006. Detection of major penaeid shrimps virus in Asia, a historical perspective with emphasis on Thailand. Aquaculture 258, 1–33.
- Hannon, G.J., 2002. RNA interference. Nature 418, 244–251.
- Leung, R.K.M., Whittaker, P.A., 2005. RNA interference: from gene silencing to gene-specific therapeutics. Pharmacol. Ther. 107, 222–239.
- Li, W.X., Li, H., Li, F., Dus, M., Atkinson, P., Brydon, E.W.A., Johnson, K.L., Garcia-Sastre, A., Ball, L.A., Palese, P., Ding, S.W., 2004. Interferon antagonist protein of influenza and vaccinia virus are suppressor of RNA silencing. Proc. Nat. Acad. Sci. U.S.A. 101, 1350–1355.
- Lingel, A., Simon, B., Izaurralde, E., Sattler, M., 2005. The structure of flock house virus B2 protein, a viral suppressor of RNA interference, shows a novel mode of double-stranded RNA recognition. EMBO Rep. 6, 1149–1155.
- Lu, R., Maduro, M., Li, H.W., Broitman-Maduro, G., Li, W.X., Ding, S.W., 2005. Animal virus replication and RNAi-mediated antiviral silencing in *Caenorhabditis elegans*. Nature 436, 1040–1043.
- Morrissey, D.V., Lockridge, J.A., Shaw, L., Blanchard, K., Jensen, K., Breen, W., Hartsough, K., Machemer, L., Radka, S., Jadhav, V., Vaish, N., Zinnen, S., Vargeese, C., Bowman, K., Shaffer, C.S., Jeffs, L.B., Judge, A., MacLachlan, I., Polisky, B., 2005. Potent and persistent in vivo anti-HBV activity of chemically modified siRNA. Nat. Biotechnol. 23, 1002–1007.
- Ongvarrasopone, C., Roshorm, Y., Panyim, S. A simple and cost effective method to generate dsRNA for RNAi studies in invertebrates. Science Asia, in press.

- Reed, L.J., Muench, H., 1938. A simple method of estimating fifty percent end points. *Am. J. Hyg.* 27, 493–497.
- 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 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 marine shrimp: convergence of RNA interference and innate immunity in the invertebrate antiviral response? *J. Virol.* 79, 13561–13571.
- Sittidilokratna, N., Hodgson, R.A.J., Cowley, J.A., Jitrapakdee, S., Boonsaeng, V., Panyim, S., Walker, P.J., 2002. Complete ORF1b-gene sequence indicates yellow head virus is an invertebrate nidovirus. *Dis. Aquat. Org.* 50, 87–93.
- Schott, D.H., Cureton, D.K., Whelan, S.P., Hunter, C.P., 2005. An antiviral role for the RNA interference machinery in *Caenorhabditis elegans*. *Proc. Nat. Acad. Sci. U.S.A.* 102, 18420–18424.
- Schroder, M., Bowie, A., 2005. TRL3 in antiviral immunity: key player or bystander? *Trends Immunol.* 26, 462–468.
- Timmons, L., Court, D.L., Fire, A., 2001. Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*. *Gene* 263, 103–112.
- Tuschl, T., Zamore, P.D., Lehmann, R., Bartel, D.P., Sharp, P.A., 1999. Target mRNA degradation by double-stranded RNA in vitro. *Genes Dev.* 13, 3191–3197.
- Wang, Y., Kato, N., Jazag, A., Dharel, N., Otsuka, M., Tanguchi, H., Kawabe, T., Omata, M., 2006. Hepatitis C virus core protein is a potent inhibitor of RNA silencing-based antiviral response. *Gastroenterology* 130, 883–892.
- Waterhouse, P.M., Wang, M., Lough, T., 2001. Gene silencing as an adaptive defense against viruses. *Nature* 411, 834–842.
- Westenberg, M., Heinhuis, B., Zuidema, D., Vlak, J.M., 2005. siRNA injection induces sequence-independent protection in *Penaeus monodon* against white spot syndrome virus. *Virus Res.* 114, 133–139.
- Wongteerasupaya, C., Tongchuea, W., Boonsaeng, V., Panyim, S., Tassanakajon, A., Withyachumnarnkul, B., Flegel, T.W., 1997. Detection of yellow head virus (YHV) of *Penaeus monodon* by RT-PCR amplification. *Dis. Aquat. Org.* 31, 181–186.
- Yodmuang, S., Tirasophon, W., Roshorm, Y., Chinnirunvong, W., Panyim, S., 2006. YHV-protease dsRNA inhibits YHV replication in *Penaeus monodon* and prevents mortality. *Biochem. Biophys. Res. Commun.* 341, 351–356.
- Zambon, R.A., Vakharia, V.N., Wu, L.P., 2006. RNAi is an antiviral immune response against a dsRNA virus in *Drosophila melanogaster* cell. *Microbiology* 8, 880–889.