รายงานวิจัยฉบับสมบูรณ์

โครงการเทคโนโลยี RNA interference เพื่อการควบคุมและ
ป้องกันโรคตัวแดงจุดขาว (WSSV) และ HPV
ในกุ้งกุลาดำ

โดย

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

ชุดโครงการการวิจัยแบบมุ่งเป้า “การผลิตสัตว์น้ำเศรษฐกิจ”

สารบัญ

บทคัดย่อ

Abstract

Shrimp culture in Thailand has suffered as much as 25% loss form viral diseases. Effective prevention or treatment of viral infected shrimps is not yet achievable. This research aims to develop an effective means to prevent and/or protect *Peneaus monodon* shrimp from white spot syndrome virus (WSSV) and hepatopancreatic parvovirus (HPV) infection. WSSV caused high mortality in *Peneaus monodon* whereas HPV caused loss in production yield.

RNA interference technology, using dsRNA was employed to prevent WSSV infection and shrimp mortality. Aproximately 400bp double stranded RNA corresponding to 6 important WSSV genes (ie1, ie3, pol, rr2, vp26 and vp28) was designed and produced in bacteria *E.coli*. Injection of the dsRNA into shrimp haemolymph gave varying degree of preventing WSSV infection. Double-stranded RNA corresponding to rr2 gene gave the strongest, while that of pol the least (rr2>ie3>vp26=vp28>ie1>pol) protection. rr2-dsRNA could also protect shrimp from mortality if infected with WSSV. A combination of rr2-dsRNA and Rab7-dsRNA which knocked down shrimp gene could also prevent shrimp mortality from WSSV infection.

For prevention of HPV infection, dsRNA (409bp) corresponding to non-structural protein 1 (NS1) and dsRNA (422bp) structural protein (VP) were designed and synthesized in *E.coli*. The HPV infection was experimentally achieved by feeding infected hepatopanereas since experimentally infection of HPV by injection was not achievable. Injection of NS1-dsRNA into the haemolymph protected HPV infection more effective than the VP-dsRNA. In addition, NS1-dsRNA could reduce severity of HPV in pond-infected shrimp. This should pave the way to cure HPV infection in shrimp culture.

This research has produced 8 international publications, 1 submitted manuscript and 2 M.Sc. graduates.

Key words : RNA interference, *P. monodon*, WSSV, HPV, PmDNV, double-standed RNA.

บทคัดย่อ

อุตสาหกรรมการเลี้ยงกุ้งในประเทศไทย ประสบปัญหาการสูญเสียมูลค่าการส่งออกประมาณ 25% จากการติดเชื้อไวรัส ปัจจุบันยังไม่มีวิธีการป้องกันหรือรักษาโรคติดเชื้อไวรัสในกุ้ง งานวิจัยนี้มีจุดมุ่งหมายในการป้องกันและลดการสูญเสียกุ้งจากการติดเชื้อไวรัสตัวแดงดวงขาว (WSSV) และไวรัสตับ (HPV) ในกุ้งกุลาดำWSSV เป็นไวรัสซึ่งทำให้กุ้งตายอย่างรวดเร็ว ส่วน HPV ทำให้กุ้งติดเชื้อโตช้า ส่งผลต่อการเสียหายในคุณค่า

เทคโนโลยี RNAi สามารป้องกันและรักษาการติดเชื้อไวรัสหัวเหลืองได้ดี จึงได้นำมาใช้ในการศึกษานี้ โดยในการป้องกันการติดเชื้อWSSV และการตายจากการติดเชื้อดังกล่าว ได้ออกแบบและสร้าง DNA เส้นคู่ (dsRNA) ขนาดประมาณ 400 เบส ซึ่งมีลำดับเบสตรงกับยีน 6 ชนิด (ie1, ie3, pol, rr2, vp26 และ vp28) ของ WSSV ซึ่งสำคัญในการการเพิ่มปริมาณ โดย dsRNA ดังกล่าวได้มีการออกแบบให้สร้างในแบคทีเรีย *E.coli* เมื่อสกัดแยก dsRNA จาก *E.coli* นำมาฉีดเข้าสู่เลือดกุ้งพบว่า สามารถป้องกันการติดเชื้อWSSV ได้ในระดับต่างๆ โดย dsRNA ต่อ rr2 ให้ผลยับยั้งการเพิ่มจำนวน WSSV ในกุ้งสูงสุด รองลงมาได้แก่ dsRNA ต่อ ie3, vp26, vp28, ie1 และ pol จาก WSSV (rr2>ie3>vp26=vp28>ie1> pol) เมื่อทดสอบการป้องกันกุ้งตายจาก WSSV ก็พบว่า dsRNA ต่อ rr2 สามารถป้องกันการตายจากเชื้อ dsRNAต่อRab7 (สามารถ knock-down ยีน Rab7 ของกุ้งได้) ก็สามารถป้องกันการตายของกุ้งจากเชื้อWSSV ได้ดีเช่นกัน

สำหรับการใช้ dsRNA ป้องกันการติดเชื้อHPV ได้ออกแบบและสร้างdsRNAขนาด 409bp ต่อ non-structural protein (NS1) และขนาด 422 bp ต่อ structural protein (VP) โดยทำการสร้างและผลิต dsRNA ดังกล่าว ในแบคทีเรีย*E.coli* เมื่อประสบความสำเร็จในการทำให้กุ้งกุลาดำติดเชื้อHPV ได้โดยการให้กินตับกุ้ง จึงได้ฉีด dsRNA เข้าสู่ระบบเลือดกุ้งเพื่อป้องกันการติดเชื้อHPV พบว่า dsRNA ต่อ NS1 ให้ผลในการป้องกันดีกว่า dsRNAต่อVP นอกจากนี้เมื่อใช้ dsRNA ในการรักษากุ้งติดเชื้อHPV ก็พบว่า dsRNAต่อNS1 สามารถลดปริมาณเชื้อ HPV ในกุ้งที่ติดมาแล้วจากบ่อเลี้ยงได้ ผลการทดลองนี้กำลังนำไปสู่วิธีการรักษากุ้งติดเชื้อHPV ในบ่อเลี้ยงในอนาคต

งานวิจัยนี้ได้ผลิตผลงานตีพิมพ์ในวารสารนานาชาติ 8 เรื่อง และกำลังอยู่ในระหว่างพิจารณาตีพิมพ์ 1 เรื่อง พร้อมผลิตนักวิจัยวุฒิปริญญาโท 2 คน

เนื้อหางานวิจัย

Inhibition of WSSV infection in shrimp by suppression of PmRab7 with dsRNA

Viral entry into host cells requires endocytosis machineries of the host for viral replication. PmRab7, a *Penaeus monodon* small GTPase protein was investigated for its function in vesicular transport during viral infection. The double-stranded RNA of Rab7 was injected into a juvenile shrimp prior to challenging with either white spot syndrome virus (WSSV) or yellow head virus (YHV). PmRab7 mRNA was specifically decreased at 48 hours post dsRNA-Rab7 injection. Silencing of PmRab7 dramatically inhibited WSSV-VP28 mRNA and protein expression. Unexpectedly, the silencing of PmRab7 also inhibited YHV replication in the YHV-infected shrimp. These results suggested that PmRab7 is a common cellular factor required for WSSV or YHV replication in shrimp. As PmRab7 should function in the endosomal trafficking pathway, its silencing prevents successful viral trafficking necessary for replication. Silencing of PmRab7 could be a novel approach to prevent both DNA virus (WSSV) and RNA virus (YHV) infection of shrimp.

Introduction

The black tiger shrimp (*Penaeus monodon*) is one of the most economically important crustacean species in Thailand and Southeast Asian countries. Diseases, especially those caused by viruses have been the most serious cause of shrimp production loss worldwide. White spot syndrome virus (WSSV) and yellow head virus (YHV) are two major causative agents leading to high mortality rates in cultured shrimp. WSSV is of the genus *Whispovirus*, family *Nimaviridae*. It is a large enveloped DNA virus with genome size of about 300 kb. Its morphology is an ellipsoid to bacilliform with a size of about 275 nm in length and 120 nm in width, with a tail-like appendage at one end (van Hulten et al., 2001). Whereas YHV is a positive sense, single- stranded RNA virus that belongs to a new genus *Okavirus*, within the family *Roniviridae*, in the order *Nidovirales* (Sittidilokratna et al., 2002). To date, an effective control to prevent viral infections has not been established in farmed shrimp. An understanding of viral entry and propagation in shrimp and the interaction between shrimp and viruses at the cellular and molecular levels will shed light on possible effective strategies to overcome these economically important viral infections.

Several viruses enter into host cells via an endocytosis pathway (Sieczkarski and Whittaker, 2002) and may rely on cellular machinery for viral infection and replication in the host cells. The Rab proteins (Ras related in brain) belong to the Ras superfamily of small GTPases which act as molecular switches by changes between an active, GTP bound and an inactive, GDP bound conformation. More than 60 different Rab proteins have been identified in mammals which act as important regulators of specific intracellular trafficking pathways such as cargo sorting, vesicle budding, motility and fusion (Seabra et al., 2002). Recently, *Penaeus monodon* Rab7 (PmRab7) has been identified as one of the WSSV-VP28 binding proteins by using a viral overlay protein binding assay on the haemocyte membrane protein (Sritunyalucksana et al., 2006). The full-length cDNA of PmRab7 contained 1357 bp

encoding a polypeptide of 205 amino acids. Similar to other mammalian Rab proteins, PmRab7 contained 5 conserved motifs involved in GTP-binding, GTPase activity and an isoprenylation site. Recent reports demonstrated that mammalian Rab7 is involved in late endosomal trafficking and plays an important role in fusion and transportation of cargo from early to late endosome and/or from late endosome to lysosome. Expression of the dominant negative mutant of Rab7 has been shown defective in endocytic trafficking of proteins such as LDL, mannose-6-phosphate receptor, angiotensin II type 1A receptor and vesicular stomatitis virus (Meresse et al., 1995; Feng et al., 1995; Vitelli et al., 1997; Bucci et al., 2000; Dale et al., 2004). In addition, an overexpression of the dominant negative mutant of Rab7 resulted in viral accumulation in the early endosome (Vonderheit and Helenius, 2005). Whether PmRab7 functions in the endocytic trafficking pathway during viral infection in shrimp remains to be elucidated. Nevertheless, disruption of the function of PmRab7 may prevent viral infection. In this study, RNA interference technology was employed to investigate the role of PmRab7 in viral infection.

RNA interference (RNAi) is a novel gene regulatory mechanism whereby double-stranded RNA (dsRNA) triggers a sequence-specific mRNA degradation of its homologous mRNA (Fire et al., 1998; Meister and Tuschl, 2004). Once inside the cell, double-stranded RNA (dsRNA) is cleaved by a ribonuclease III homolog or Dicer into 21-23 nucleotide long small interfering RNA (siRNA) with a 2-nucleotide overhang at the 3' end and a 5' phosphate (Bernstein et al., 2001). Small interfering RNA is then incorporated into RNA-induced silencing complexes (RISC), unwound, and the single-stranded antisense is targeted to a specific region of its complementary mRNA resulting in mRNA degradation. RNAi is a powerful experimental tool to study individual gene function, viral infection as well as for genome-wide genetic screening (Tomari and Zamore, 2005; Novina and Sharp, 2004).

In shrimp, dsRNAs corresponding to the viral genes of WSSV or YHV have been used to suppress viral replication (Yodmuang et al., 2006; Robalino et al., 2005). In addition, a number of cellular factors that are required for the viral life cycle could be used as an alternative target to inhibit viral infection in shrimp. Introduction of dsRNA corresponding to the putative endogenous YHV receptor resulted in protection against YHV infection in primary lymphoid cells (Assavalapsakul et al., 2006). PmRab7 has been demonstrated to be a VP28 binding protein involved in shrimp WSSV infection (Sritunyalucksana et al., 2006). Silencing of PmRab7 should specifically prevent WSSV attachment and infection. Unexpectedly, we found that silencing of PmRab7 by specific dsRNA, not only prevented WSSV but also YHV infection. These results suggest that PmRab7 is involved in endosomal trafficking of viral replication within the shrimp cell.

Materials and Methods

Shrimp culture

Juvenile black tiger shrimp (*Penaeus monodon*) (7-10 g) that were free from viral pathogen were obtained from commercial shrimp farms in Thailand. Shrimp (10 shrimp per tank) were reared in 50 liter tanks containing 30 liter continuous aerated artificial saltwater at 10 ppt salinity. Shrimp were acclimatized for 2-3 days before dsRNA injection and fed with commercial feed every day. Half of the saltwater was changed every day.

Virus stock

WSSV stock was kindly provided by Dr. Sritunyalucksana, BIOTEC. The viral titer (10^6 copies μl^{-1}) was determined by real-time PCR (Sritunyalucksana et al., 2006). YHV stock was kindly provided by Dr. Tirasophon, Mahidol University (Tirasophon et al., 2007). The YHV viral titer ($2x10^9$ infectious virions per ml) was determined according to the method described by Assavalapsakul, et al., 2003 (Assavalapsakul et al., 2003). Both viral stocks were stored at –80ºC. The stocks were diluted 50 fold for WSSV and 100 fold for YHV in 150 mM NaCl. Fifty µl of WSSV (~10^6 copies) or 50 µl of YHV (~10^6 virions) were used to inject into haemolymph of individual shrimp.

Production of dsRNA

Double-stranded RNAs produced by using *in vivo* bacterial expression system and by *in vitro* transcription have been shown to have no different in the silencing effect (Ongvarrrasopone, et al., 2007). A recombinant plasmid containing an inverted repeat of PmRab7 that used for *in vivo* production of dsRNA was not available. Therefore, dsRNA-Rab7 was synthesized by *in vitro* transcription. The sense and anti-sense DNA templates for *in vitro* transcription were generated by using a PCR approach. The forward primers for both DNA templates were designed to contain T7 promoter sequence at the 5 ' end (underline). RabF-sense: 5’ TAA TAC GAC TCA CTA TAG GGA TAC AGC TGG TCA AGA GAG 3’ and RabR-sense: 5’ GGT CAA TTT GAT CT GGT CTG G 3’ were used to amplify sense DNA template. RabF-antisense: 5’ TAA TAC GAC TCA CTA TAG GGT CAA TTT GAT CTG GTC TGG 3’ and RabR-antisense: 5’ GGA TAC AGC TGG TCA AGA GAG 3’ were used to amplify antisense DNA template. The plasmid pET17b-PmRab7 (kindly provided by Dr. Sritunyalucksana, BIOTEC, Thailand) encoding the full-length cDNA of the PmRab7 was used as a template for PCR. The 411 bp PCR product was excised and gel purified using Wizard® SV Gel and PCR clean-up system (Promega). DsRNA-Rab7 was synthesized by using T7 Ribomax™ Large Scale RNA Production Systems (Promaga) according to the manufacture’s instructions.

On the other hand, the recombinant plasmid containing an inverted repeat of stem loop GFP is available. Its dsRNA-GFP production by *in vivo* bacterial system has been used in many studies as a control for an unrelated dsRNA (Yodmuang, 2006, Tirasophon, 2007). Therefore, dsRNA-GFP was synthesized by *in vivo* bacterial expression according to the method described by Ongvarrasopone, et al., 2007 (Ongvarrasopone et al., 2007). Briefly, a recombinant plasmid (pET3a-GFP) containing an inverted repeat of stem loop GFP was transformed by heat shock method into a ribonuclease III (RNase III) mutant *E.coli* strain HT115. The expression of stem loop GFP was induced by adding 0.4 mM Isopropyl- β-D Thiogalactoside (IPTG) into bacterial culture. The culture was harvested 4 hours after IPTG induction. Bacterial single-stranded RNA (ssRNA) and loop region of stem loop GFP were digested with ribonuclease A (RNase A). Then, the dsRNA-GFP was extracted by TRI reagent (Molecular Research Center) according to the manufacture’s instructions.

Concentration of dsRNA-Rab7 and dsRNA-GFP were determined by UV-spectrophotometry at wavelength 260 nm. The quality of dsRNAs was verified by ribonuclease digestion assay. Double-stranded RNAs of Rab7 and GFP have an

expected size of 411 bp and 400 bp, respectively. Both dsRNAs can be cleaved by RNase III but not by RNase A suggesting that good quality of dsRNAs were obtained in this study.

Injection of dsRNAs and virus

Shrimp were injected with 25 μg dsRNA-Rab7 dissolved in 50 μl of 150 mM NaCl into the haemolymph using 1 ml tuberculine syringe with 26 gauge needle to investigate whether endogenous Rab7 mRNA could be knocked down. Haemolymph (250 μl) was collected once a day from the same shrimp for at least 9 days after dsRNA-Rab7 injection.

In order to study the effect of knocked-down Rab7 mRNA on viral infection, shrimp were injected with either 25 μg dsRNA-Rab7 or dsRNA-GFP dissolved in 50 μl of 150 mM NaCl into the haemolymph. Forty-eight hours after dsRNA injection, shrimp were challenged with 50 μl of either WSSV (10^6 copies) or YHV (10^6 virions). Injection of 150 mM NaCl or virus alone was used as control groups in this study. Haemolymph from individual shrimp was collected 48 hours post WSSV or YHV infection.

Total RNA extraction and RT-PCR analysis

Total RNAs from haemolymph were extracted by using TRI-LS reagent (Molecular Research Center) according to the manufacturer's instructions and 1 g of each RNA sample was used to generate first-stranded cDNA by ImPromp-II™ reverse transcriptase (Promega) and oligo-dT_{12} primer. To determine the expression level of mRNA of PmRab7, and WSSV, the primer pairs of PmRab7 (GenBank accession no. **DQ231062**) (5' ATG GCA TCT CGC AAG AAG ATT 3' and 5' TTA GCA AGA GCA TGC ATC CTG 3'), and of WSSV-VP28 gene (GenBank accession no. **AF502435**) (5' ATG AGA ATG AAC TCC AAC TTT AA 3' and 5' CAG AGC CTA GTC TAT CAA TCA T 3') were used, respectively. Actin primers: 5' GAC TCG TAC GTC GGG CGA CGA GG 3' and 5' AGC AGC GGT GGT CAT CAC CTG CTC 3' were used as an internal control to normalize for the RNA loading. PCR conditions for PmRab7 were as follows: 95ºC for 5 min, followed by 25 cycles of 95ºC for 30 sec, 53ºC for 30 sec, and 72ºC for 45 sec, with a final extension at 72ºC for 7 min. For WSSV-VP28 and actin genes, the similar PCR conditions were performed for 25 cycles except that the annealing temperature were changed to 55ºC and 52ºC, respectively. The PCR products of PmRab7 (617 bp), WSSV-VP28 (447bp), and actin (500bp) were analysed on 2% agarose gel electrophoresis. Multiplex PCR of 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') and actin was performed (Yodmuang et al., 2006). The PCR products of YHV-helicase (850 bp) and actin (500 bp) were analysed on 1% agarose gel electrophoresis.

Quantitation of RT-PCR products

The PCR products of PmRab7, WSSV-VP28 and actin were quantified using Scion image analysis program. The signal was corrected for the background. The relative expression levels of PmRab7 and WSSV-VP28 were normalized against actin and expressed in arbitary units.

Statistical analysis

The data were expressed as the mean ± standard error (SEM). The statistical analysis of the mean ± SEM was performed by using ANOVA test in the Sigma Stat program. A measurement of $p<0.05$ was accepted as statistically significant.

Western blot analysis

Gills were isolated from shrimp and kept at -80 ºC until used. Tissues were ground to powder in liquid nitrogen using mortar and pestle. The tissue powders were homogenized in buffer M (100 mM NaCl, 20 mM Tris-Cl, 2 mM $MgCl_2$, 1 mM EDTA and 1 mM PMSF) containing 0.2% Triton X. The crude protein lysate was quantitated using Bradford reagents (Bio-Rad Laboratories, Inc., USA). An equal amount of protein (5 μg) was loaded on 8% SDS-polyacrylamide gels and stained by Coomassie brilliant blue for loading control. In addition, gels run in parallel (10 μg protein per lane) were transferred onto PVDF membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with a SemiDry electrophoresis transfer apparatus (Bio-Rad Laboratories, Inc., USA). The membranes were blocked overnight at 4 ºC in phosphate buffered saline (PBS) containing 5% skimmed milk. The YHV-structural protein was detected by incubating a membrane with mouse anti-gp116 antiserum in 5% skimmed milk in PBS containing 0.2% Tween-20 (PBST) (dilution 1:2000) for 1 hour at room temperature. After washing with PBST, the membrane was incubated with horseradish peroxidase conjugated goat anti-mouse polyclonal antibodies (Sigma Chemical, St. Louis, MO, USA) (dilution 1:8000). The signal was detected by ECL Plus Western Blotting Detection Reagent (Amersham Biosciences, Buckinghamshire, UK). In addition, WSSV-VP28 protein was detected by using mouse anti-VP28 antibody (dilution 1: 1000).

Results

Suppression of endogenous PmRab7 by dsRNA

To investigate whether the endogenous PmRab7 mRNA could be knocked down, dsRNA-Rab7 was injected initially into haemolymph and then haemolymph was collected from individual shrimp every day from day 2 to day 9. The endogenous PmRab7 mRNA was almost completely knocked down on day 2. However, the expression of PmRab7 mRNA remained at a relatively low level from day 2 to day 9 when compared to the control (Figure 1A). In addition, the expression of PmRab7 mRNA remained unchanged after dsRNA-GFP (Figure 1B) or 150 mM NaCl injection (supplemented Figure 1) suggesting that knock down of PmRab7 by dsRNA-Rab7 is sequence-specific. The knock down effect of PmRab7 causes less than 5% of shrimp mortality which is similar to the NaCl and dsRNA-GFP injection group.

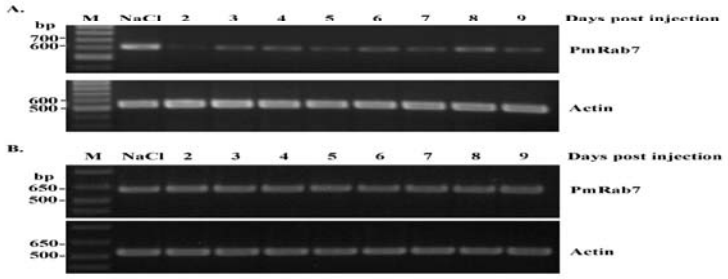
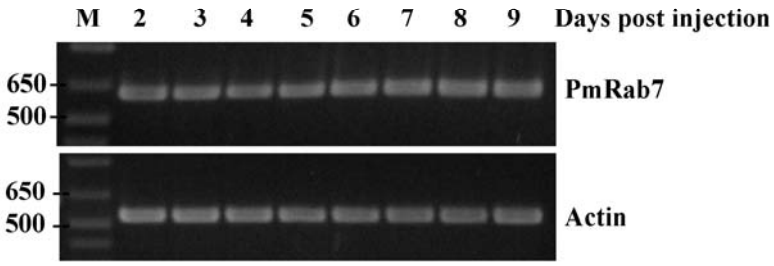


Figure 1

Figure 1 Double-stranded RNA of Rab7 inhibited endogenous PmRab7 mRNA. (A) DsRNA-Rab7 (25 μg) and (B) dsRNA-GFP (25 μg) were injected and haemolymph was collected from individual shrimp every day from day 2 to day 9 post injection. A representative gel of RT-PCR products of PmRab7and actin mRNAs from haemolymph is shown. Shrimps were injected with 150 mM NaCl in the control group. M is 1 kb plus DNA marker.



Supplemented Figure 1

Supplemented figure 1 Injection of 150 mM NaCl has no effect on endogenous PmRab7 mRNA expression. NaCl (150 mM) were injected and haemolymph was collected from individual shrimp every day from day 2 to day 9 post injection. A representative gel of RT-PCR products of PmRab7 and actin mRNAs from haemolymph is shown. M is 1 kb plus DNA marker.

Inhibition of WSSV infection in PmRab7 knocked-down shrimp

Previously, PmRab7 was identified as a WSSV-binding protein (Sritunyalucksana et al., 2006). Therefore, it was likely that knock down of endogenous PmRab7 could prevent WSSV infection. In this study, dsRNA-Rab7 or dsRNA-GFP (an unrelated dsRNA) was injected into haemolymph 48 h prior to challenging with WSSV. The haemolymph was collected at 48 h post WSSV infection in order to investigate the silencing effect of PmRab7 on WSSV infection

(Figure 2). Injection of dsRNA-Rab7 into shrimp resulted in more than 90% reduction in endogenous PmRab7 mRNA expression within 48 h whereas injection of NaCl or dsRNA-GFP had no effect (Figure 2A and 2B). No WSSV-VP28 could be observed in the NaCl group that was not injected with WSSV suggesting that shrimp in this study were virus free. WSSV-VP28 could be detected in the NaCl and dsRNA-GFP groups after challenging with WSSV. Interestingly, very low expression levels of WSSV-VP28 mRNA were observed in PmRab7 knocked-down shrimp, suggesting that silencing PmRab7 mRNA prevents WSSV replication in shrimp (Figure 2A and 2C). The relative mRNA expression of PmRab7 (Figure 2B) and of WSSV-VP28 (Figure 2C) in PmRab7 knocked-down group were significantly (*$p<0.05$) reduced when compared to NaCl and dsRNA-GFP groups. In addition, protein expression of WSSV-VP28 as determined in gill was decreased in PmRab7 knocked-down group when compared to NaCl and dsRNA-GFP groups (Figure 2D). In this study, an equal amount of the protein was loaded in each lane as shown in the coomassie staining gel (Supplemented figure 2).

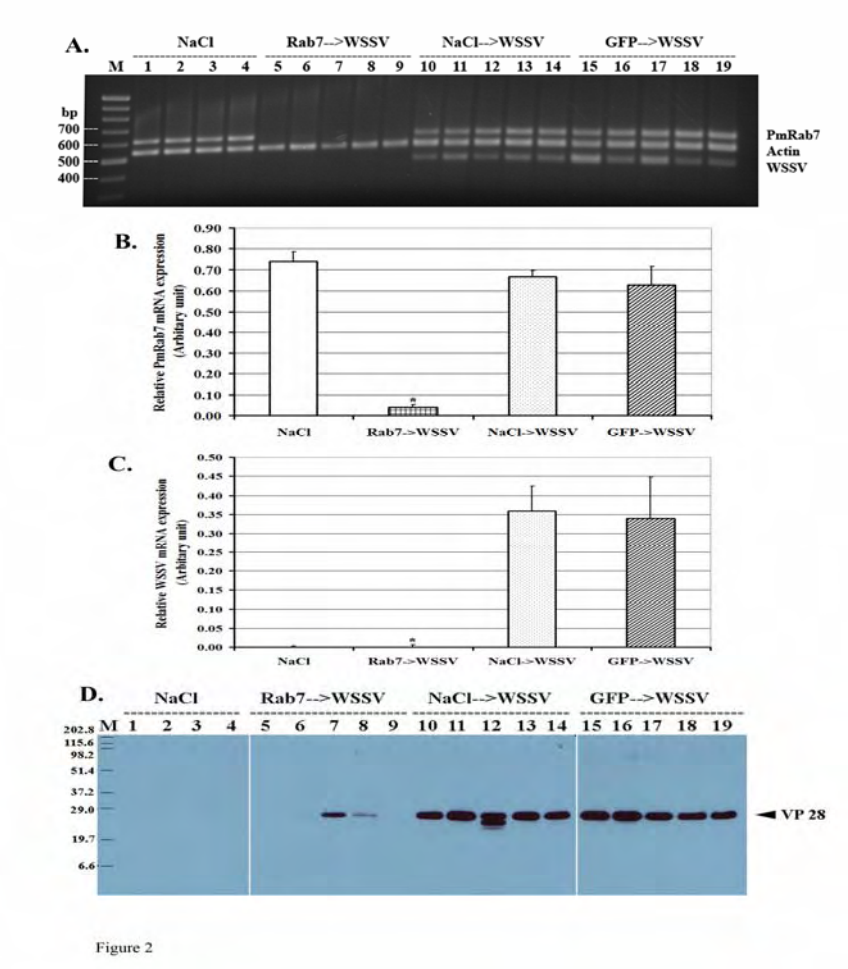
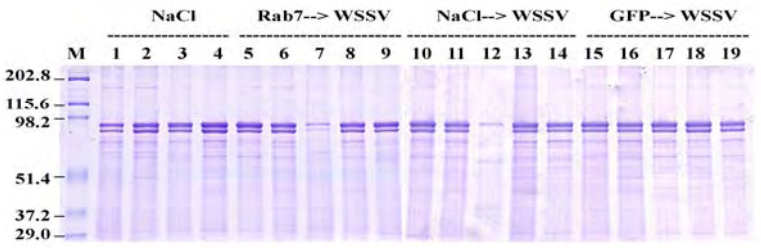


Figure 2 Suppression of PmRab7 by dsRNA-Rab7 inhibited WSSV infection. Injection of 150 mM NaCl alone (lane 1-4) followed by WSSV (lane 10-14) and injection of dsRNA-Rab7 (lane 5-9) or dsRNA-GFP (lane 15-19) 48 h prior to challenging with WSSV. Each lane represents an individual shrimp. (A) RT-PCR products of PmRab7, actin, and WSSV-VP28 mRNAs from haemolymph. M is 1 kb

plus DNA marker. Relative mRNA expression of PmRab7 (B) and of WSSV-VP28 (C) were normalized with actin and expressed as mean ± SEM from n=10 for each group. (D) WSSV-VP28 protein expression was determined by western blot analysis of protein isolated from gill samples using anti-VP28 antibody. An equal amount of the protein (10 μg) was loaded in each lane and run on 8% SDS-PAGE. M is a protein broad range marker. The Coomassie blue staining gel of the protein (5 μg each) was shown in Supplemented figure 2.



Supplemented Figure 2

Supplemented figure 2 The Coomassie blue staining gel of the protein used in the western blot analysis. An equal amount of protein (5 μg) was loaded in each lane and run on 8% SDS-PAGE. M is a protein broad range marker. Injection of 150 mM NaCl alone (lane 1-4) followed by WSSV (lane 10-14) and injection of dsRNA-Rab7 (lane 5-9) or dsRNA-GFP (lane 15-19) 48 h prior to challenging with WSSV. Each lane represents an individual shrimp.

Inhibition of YHV infection in PmRab7 knocked-down shrimp

In order to test whether PmRab7 has an important role in the viral protein trafficking pathway, the effect of silencing of PmRab7 was determined in YHV infected shrimp. The result showed that dsRNA-Rab7 inhibited PmRab7 expression in haemocytes (Figure 3A, lane 5-9) to varying extents in each shrimp. No YHV-helicase mRNA (Figure 3A, lane 5-9) and YHV structural protein, gp116 (Figure 3D, lane 5-9) could be detected in PmRab7 knocked-down group infected with YHV. An

equal amount of the protein was loaded in each lane as shown in the coomassie staining gel (Supplemented figure 3). In contrast, YHV mRNA expression (Figure 3A, lane 10-18) and gp116 (Figure 3D, lane 10-18) can be detected in YHV infected shrimp which had earlier received saline (lane 10-13) or dsRNA-GFP (lane 14-18). The relative mRNA expression of PmRab7 (Figure 3B) and of YHV-helicase (Figure 3C) in the PmRab7 knocked-down group infected with YHV were significantly (*$p<0.05$) reduced when compared to NaCl and dsRNA-GFP groups. These results demonstrate that knocking down of PmRab7 prevents YHV replication.

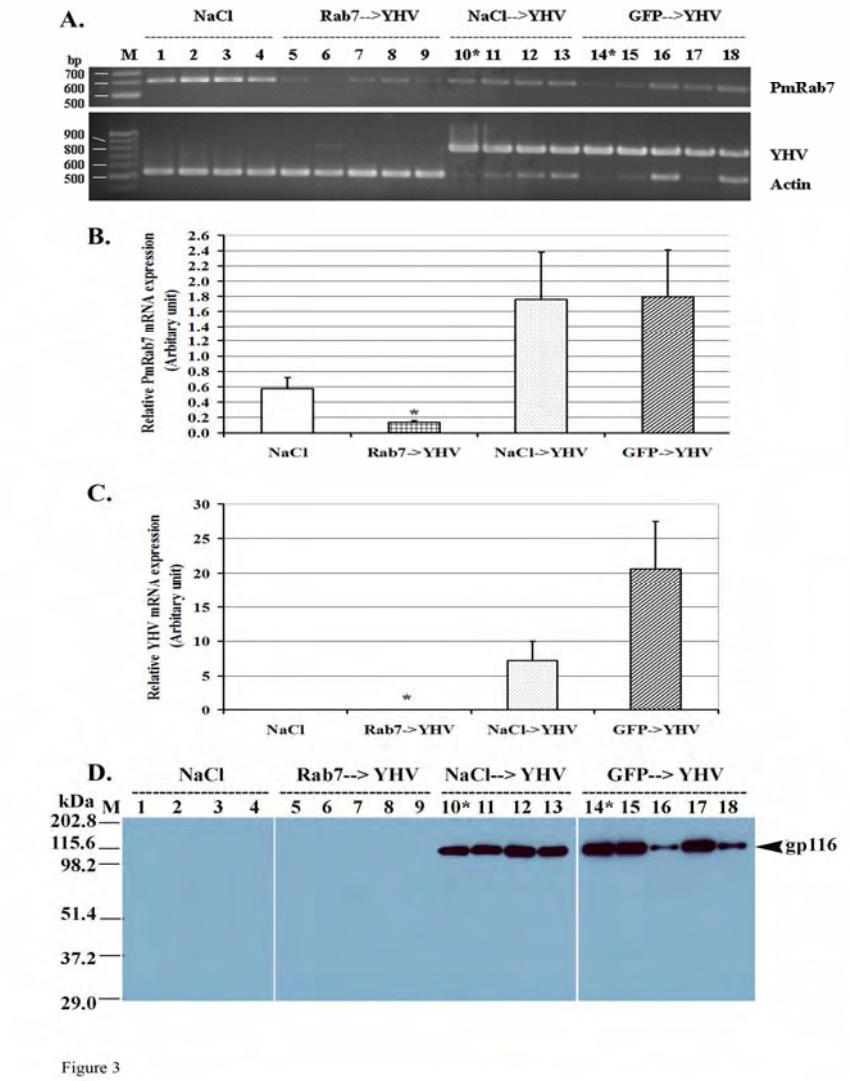
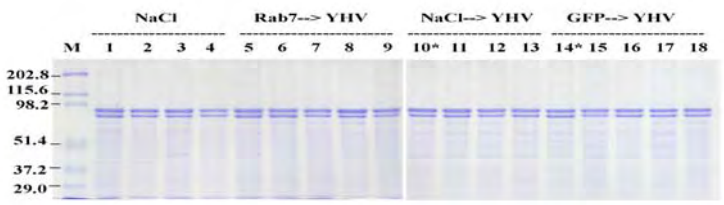


Figure 3

Figure 3 Suppression of PmRab7 by dsRNA-Rab7 inhibited YHV infection. (A) RT-PCR products of PmRab7, YHV-helicase and actin mRNAs from haemolymph. Injection of 150 mM NaCl alone (lane 1-4) and followed by YHV (lane 10-13) and injection of dsRNA-Rab7 (lane 5-9) or dsRNA-GFP (lane 14-18) 48 h prior to challenging with YHV. Each lane represents an individual shrimp. * indicates moribund shrimp. M is 1 kb plus DNA marker. Relative mRNA expression of PmRab7 (B) and of YHV-gp116 (C) were normalized with actin and expressed as mean ± SEM from n=10 for each group. (D) Western blot analysis of protein isolated from gill samples using anti-YHV-gp116 antibody. An equal amount of the protein

(10 μg) was loaded in each lane and run on 8% SDS-PAGE. M is a protein broad range marker. The Coomassie blue staining gel of the protein (5 μg each) was shown in Supplemented figure 3.



Supplemented Figure 3

Supplemented figure 3 The Coomassie blue staining gel of the protein used in the western blot analysis. An equal amount of protein (5 μg) was loaded in each lane and run on 8% SDS-PAGE. M is a protein broad range marker. Injection of 150 mM NaCl alone (lane 1-4) followed by YHV (lane 10-13) and injection of dsRNA-Rab7 (lane 5-9) or dsRNA-GFP (lane 14-18) 48 h prior to challenging with YHV. Each lane represents an individual shrimp.

Discussion

In this study we have shown that a depletion of PmRab7 by using dsRNA injection in shrimp inhibits viral replication (WSSV and YHV). Double-stranded RNA has been used to inhibit viral cognate genes in shrimp which resulted in suppression of viral replication (Yodmuang et al., 2006; Robalino et al., 2005). Shrimp may be protected from or cured of viral infection by using dsRNA targeting viral genes (Yodmuang et al., 2006; Tirasophon et al., 2007). However, it was not known if viral replication also could be inhibited by suppression of shrimp genes. We have shown that PmRab7 gene expression could be knocked down by injecting PmRab7 dsRNA which resulted in inhibition of DNA as well as RNA virus replication. This is the first demonstration in shrimp that viral replication could be suppressed by knocking down a host gene.

RNAi has been widely used to inhibit gene expression in mammalian cells. In shrimp, dsRNA or siRNA corresponding to the viral genes has been used as an

antiviral defense to inhibit viral gene expression. Double-strand RNA corresponding to YHV or WSSV genes can induce both sequence dependent and independent antiviral immunity (Robalino et al., 2005; Robalino et al., 2004; Westenberg et al., 2005; Tirasophon et al., 2007; Yodmuang et al., 2006). In addition, siRNA corresponding to VP28, a major envelop protein gene of WSSV, was able to silence the sequence specific gene (Xu et al., 2007) as well as causing a sequence independent antiviral immunity in shrimp (Westenberg et al., 2005). Viruses may escape RNAi by mutating siRNA-target sequences. Moreover, dsRNA for a specific viral gene can silence only specific corresponding virus. For example, dsRNA specific for protease of YHV can inhibit YHV gene expression whereas dsRNA specific for protease of Taura syndrome virus cannot inhibit YHV (Yodmuang et al., 2006). Therefore, to circumvent this problem, an alternative approach to target host cellular factors required for viral replication was used here. Rab7, a small GTPase protein mediates early to late endosome and from late endosome to lysosome trafficking in mammalian cells (Feng et al., 1995; Press et al., 1998; Mohrmann and van der Sluijs, 1999; Ohashi et al., 1999; Zuk and Elferink, 1999). In addition, Rab7 is involved in sorting of virus and in the formation of transport vesicles (Vonderheit and Helenius, 2005; Vidricaire and Tremblay, 2005). In *Penaeus monodon*, PmRab7 was previously identified as a WSSV- binding protein (Sritunyalucksana et al., 2006). To elucidate the importance of PmRab7 involvement in viral entry and replication in shrimp, dsRNA corresponding to PmRab7 was injected into shrimp and challenged with either WSSV or YHV, representative of DNA and RNA viruses, respectively. The dsRNA-Rab7 specifically knocked down PmRab7 mRNA 48 h post-injection. The expression of PmRab7 was recovered and remained at relatively low levels for 9 days without harm to the survival of shrimp (Figure 1). The silencing effect of PmRab7 led to the prevention of both WSSV and YHV replication. WSSV infection had no effect on the relative endogenous mRNA expression of PmRab7. A knock-down of PmRab7 almost completely inhibited WSSV-VP28 mRNA expression in hemocytes and VP-28 protein in gill samples indicating the inhibition of WSSV replication as a result of PmRab7 depletion. In addition, suppression of endogenous PmRab7 mRNA by dsRNA-Rab7 showed significant inhibition of YHV infection. No YHV- helicase mRNA and YHV-gp116 structural protein were detected in PmRab7 knocked-down shrimp. These results suggest that PmRab7 plays important roles for both WSSV and YHV entry and replication in shrimp. It is possible that upon viral infection, an active PmRab7 may associate to the membrane and recruit Rab effector protein to form a complex that may involve in sorting and endocytic trafficking of virus. Whether or not PmRab7 is localized on the cell surface needs to be elucidated.

The mechanism of virus entry into shrimp cells has not been elucidated. Since a YHV receptor has been identified, it is possible that virus is internalized into host cells via receptor mediated endocytosis. Silencing of the YHV receptor using dsRNA resulted in prevention of YHV infection in Oka cells (Assavalapsakul et al., 2006). This effect has not been verified in whole shrimp. In this study, knocked-down PmRab7 resulted in a complete inhibition of YHV replication suggesting that once internalized into host cells, YHV may rely on host cell endocytic machinery such as PmRab7 for viral replication. An overexpression of the dominant negative mutant of mammalian Rab7 resulted in Semliki forest virus accumulation in the early endosome (Vonderheit and Helenius, 2005). Similarly, suppression of PmRab7 by dsRNA may

possibly accumulate YHV in the early endosomal compartment and block virus trafficking. Therefore, the virus cannot be uncoated prior to replication in the host cell. Other Rab proteins have been shown to be involved in viral trafficking. In *Penaeus japonicus*, Rab6A was up-regulated in WSSV resistant shrimp (Wu and Zhang, 2007). SiRNA of Rab11A inhibited HIV replication by impairment of the vesicular transport from *trans* Golgi to plasma membrane (Murray et al., 2005). Silencing Rab9 expression using siRNA dramatically inhibited replication of several viruses such as HIV, enveloped Ebola, Marburg and measles viruses but not non-enveloped reovirus (Murray et al., 2005). Similar to Rab9, PmRab7 silencing should inhibit replication of other enveloped viruses in addition to WSSV and YHV. PmRab7 silencing may be protective against all viruses employing the same endocytic mechanism of entry into the cell. Further studies are needed to characterize with various types of viruses and optimize frequency and dose of injection. This could be a novel approach to prevent diverse types of virus infection of shrimp.

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Inhibition of white spot syndrome virus replication in *Penaeus monodon* by combined silencing of viral rr2 and shrimp PmRab7

Although a significant progress has been achieved on dsRNA mediated anti-virus strategy development, there is still no effective means to control the virulent white spot syndrome virus (WSSV). Six double-stranded RNAs specific to different essential genes of WSSV (ie1, ie3, pol (DNA polymerase), rr2 (ribonucleotide reductase small subunit), vp26, and vp28) were employed to suppress viral replication in shrimp. At the condition that non-specific inhibitory effect was overwhelmed, the relative protective degree of these dsRNAs against WSSV infection (rr2 > ie3 > vp26, vp28 > ie1 > pol) was observed by semi-quantitative PCR. Besides, more than one injection of dsRNA was needed for an efficient viral inhibition. To improve viral protection in *Penaeus monodon*, synchronized blocking of viral cellular transport (by dsRNA-PmRab7) and viral essential gene synthesis (by dsRNA-rr2) was first performed in this study. The suppression effects of shrimp mortality by either combined dsRNAs of rr2 and PmRab7 or dsRNA-rr2 alone was monitored for 8 days after viral challenge. Approximately 95% of shrimp survivals were detected from both combined dsRNAs and dsRNA-rr2 alone whereas all shrimp without dsRNA were dead. It revealed that there was no additive inhibitory effect of the combined dsRNAs over dsRNA-rr2 alone.

Introduction

White spot syndrome virus (WSSV) is one of the most ravaging pathogens in cultured shrimp. It can cause high mortality up to 100% within 3-10 days (Lightner, 1996). Due to its spread worldwide and extremely broad range of potential host not only shrimp but also decapods (Lo et al., 1996), therefore, the disease outbreak was rapid and difficult to arrest. Proper farm management together with efficient protection should be the strategy for control.

More recently, many researchers have put their intensive efforts on development of an approach for virus prevention in shrimp such as *vaccination* by either purified vp protein or vp expression plasmid DNA (Rout et al., 2007; Satoh et al., 2008; Ha et al., 2008), immune-stimulation by plant extracts (Citarasu et al., 2006; Balasubramanian et al., 2008), or dsRNA mediated RNA interference (RNAi) (Robalino et al., 2004, 2005; Kim et al., 2006). Among them, RNAi seems to be the most effective technique. It is a mechanism which exists in many eukaryotic organisms that can be activated by exogenous dsRNA for the destruction of invading pathogens, such as viruses, in a sequence-dependent manner (Fire, 1999; Hannon, 2002). Because of its specificity, thus, the RNAi approach is probably the most powerful technique and has been widely applied to inhibit both DNA and RNA viruses in shrimp (Robalino et al., 2005; Yodmuang et al., 2006; Tirasophon W et al., 2005, 2007). Even though an introduction of virus specific dsRNA into shrimp showed very promising results in disease prevention but the degree of inhibition was varied between targeted genes. Meanwhile, silencing of host essential gene involved in viral infection, such as PmRab7, dramatically inhibited WSSV replication (Ongvarrasopone et al., 2008).

WSSV, belonging to the family *Nimaviridae* (genus *Whispovirus*), contains a large circular double-stranded DNA of about 300 kb (van Hulten et al., 2001; Yang et al., 2001). According to the complete sequence of its genome, at least 181 ORFs have been identified. In general, genes of large dsDNA viruses are differentially expressed in a cascade manner; immediate early, early, and late genes (Liu et al., 2005; Marks et al., 2005). The immediate early and early genes are normally expressed prior to viral DNA replication, while late gene expression started thereafter. Immediate early genes, which can be expressed in the absence of viral proteins, have been reported to mainly encode a transcriptional regulatory protein required for other early and late genes (Kovacs et al., 1992). Early genes usually encode for an essential protein involved in DNA replication. Late expression protein or structural protein is generally participated during virus-host interactions such as cell entry and systemically spread.

In the present study, we aim to investigate the efficacy of viral protection by six dsRNAs specifically targeted to six WSSV essential genes. Two immediate early genes (ie1 and ie3) which have been identified as immediate early genes by microarray and RT-PCR analysis in protein synthesis inhibitor-treated WSSV-infected shrimp (Liu et al., 2005), early transcribed DNA polymerase (pol) and ribonucleotide reductase small subunit (rr2) genes as well as two major late structural protein genes (vp26 and vp28) were chosen. Multiple injection of dsRNA was used in order to promote the inhibitory effect. Besides targeting of the viral mRNA, an alternative way to inhibit viral replication by knocking down of the cellular gene that is critically involved in viral replication has been studied (Ongvarrasopone et al., 2008). Silencing of cellular PmRab7, a small GTPase protein involved in endocytosis pathway for virus entry into host cell, has conferred inhibition of WSSV and YHV replication in shrimp. Therefore, whether the combination of dsRNAs corresponding to shrimp cellular gene (PmRab7) and viral specific gene (rr2) would give better inhibition of WSSV infection than dsRNA-rr2 alone was elucidated in this study.

Materials and methods

Shrimp

Shrimps (*Penaeus monodon*) of approximately 300 mg body weight were selected for most experiments in this study. The post-larva shrimps were obtained from hatchery farm and then reared in 500 L tank containing artificial sea water at 10 ppt (parts per thousand) salinity with aeration until they reached juvenile stage with appropriate size. The shrimps were fed daily on pellet shrimp diet.

For mortality test, shrimps (*Penaeus monodon*) with 1-2 g were purchased from cultured shrimp farm at Nakhon Pathom province. They were reared in 500 L tank containing sea water at 10 ppt salinity with aeration until used.

Virus

The virus stock for all experiments in this study was prepared following the method of Xu et al (2006). The gill tissues from WSSV infected moribund shrimp were homogenized in TN buffer (20 mM Tris-HCl, 400 mM NaCl, pH 7.4) at 0.1 g/ml. The homogenate was centrifuged at 2 000 g for 10 min at 4°C. Then, the supernatant was diluted with 0.9% NaCl at 1:100 (v/v) and filtered through a 0.45 μm filter. The virus solution was aliquot and stored at -80°C before use. The virus at dilution 10^{-3} was injected into shrimp and it gave 100% mortality within 3-4 days.

Stem loop RNA expression plasmid construction

For viral specific dsRNA: For each recombinant plasmid, two PCR products of different sizes (400 and 600 bp) were amplified using Vent DNA polymerase to minimize an error from PCR amplification. Primer pairs specific to the WSSV gene are listed in table 1. Primers (no.1 and 2 of each gene) were used for the 600 bp fragment amplification while primers (no.3 and 4 of each gene) were used for the 400 bp fragment. Two fragments of each target gene were then cloned into a pET17b vector in an inverted manner directed by restriction enzymes flanking the fragments (*Nde*I (or *Xba*I for ie1 and pol) and *Eco*RV for 600 bp, *Eco*RV and *Xho*I for 400 bp). The recombinant clones were screened by PCR and restriction enzyme analysis. Nucleotide DNA sequence of the insert was subsequently confirmed by automated DNA sequencing.

For non-related dsRNA (gfp): The recombinant plasmid was kindly provided by Dr. Witoon Tirasophon, Mahidol University. The strategy for construction was similar to that of specific dsRNA except using pET3a as a cloning vector instead of pET17b and using *Xba*I for joining of two fragments and *Nde*I for insertion into the plasmid vector (Yodmuang et al., 2006).

Double-stranded RNA production

According to the protocol of Ongvarrasopone et al. (2007), the recombinant plasmid containing the stem loop RNA expression cassette was transformed into *E.coli* HT115. Bacterial culture was grown from a single colony in LB medium containing ampicillin and tetracyclin at 37 °C overnight. The starter was diluted 100 fold with the same medium containing antibiotic and grown at 37 °C until OD_{600} reached 0.4. In order to express each dsRNA, 0.4 mM of Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture and the culture was further incubated for 4 hours. The bacterial cells were then harvested by centrifugation at 6 000 g for 5 min at 4 °C. The cell pellet was resuspended in Phosphate buffer saline (PBS) containing 0.1% Sodium dodecyl sulphate (SDS) with a ratio of 50 µl per 1 OD. Next, the cell suspension was boiled for 2 min to lyse the cells. To remove the single-stranded RNA of the bacterial cells as well as the loop region of expressed RNA, RNaseA (one microgram per one OD cells) was added into the reaction containing 1x buffer (300 mM sodium acetate, 10 mM Tris-Cl pH 7.5). The single-stranded RNA was digested at 37°C for 30 min. The remaining dsRNA (approximately 400 bp) was sequentially purified by TRI Reagent (Molecular Research Center) following the manufacturer's protocol. Before introduction into shrimp, dsRNA was verified by treatment with RNaseA and RNaseIII. Finally, the concentration of each dsRNA was estimated by gel electrophoresis compared with a standard DNA marker.

For shrimp PmRab7 dsRNA: The dsRNA-PmRab7 was kingly provided by Dr. Chalermporn Ongvarrasopone, Mahidol University. The strategy for plasmid construction and dsRNA production was similar to that of the viral specific dsRNA (Ongvarrasopone et al., unpublished data)

Experimental condition and process

For suppression test: shrimp (300 mg) was kept in individual petridish (90 mm x 15 mm) to avoid the cannibalism and maintained in 10 ppt artificial sea water at

25-28 °C. During the experiment, the water was changed every day. Shrimp was injected with each dsRNA (corresponding to either viral specific genes or non-specific gene (green fluorescence protein, gfp)) into the haemolymph using a 0.5 ml insulin syringe 24 hours (or indicated in the experiment) prior to the challenge of WSSV (10^{-3} dilution from the stock) via the same route. At this dilution of virus (10^{-3}), 100% mortality of infected shrimp can occur within 3-4 days. Thirty six hours (otherwise indicated) post infection, the gills from each shrimp were isolated for further RNA and DNA extraction.

For mortality test: shrimp (1-2 g) was kept in a separate cage, which 20 cages were put in the same big tank (5 L), and maintained in 10 ppt artificial sea water at 25-28 °C. Approximately 50% of water in the tank was changed every 3 days. It has been shown that 0.6 µg dsRNA can be used to knock down the transcript of PmRab7 in shrimp (personal communication, unpublished data). Therefore, this amount to PmRab7 dsRNA was used to suppress its mRNA in this experiment. Shrimp was injected with dsRNA-rr2 (5 µg) or dsRNA-PmRab7 (0.6 µg) or dsRNA-gfp (5 µg) or combined dsRNA-rr2 and dsRNA-PmRab7 (5 µg of dsRNA-rr2 and 0.6 µg of dsRNA-PmRab7) into haemolymph everyday for 5 days. At the third injection, co-administration of dsRNA and WSSV at dilution $5x10^{-4}$ was done. The accumulated number of dead shrimp was recorded everyday up to 8 days post virus challenge.

RNA extraction

Total RNA was extracted from the gill tissues using TRI Reagent (Molecular Research Center). The 50-100 mg of tissue was ground in 1 ml TRI Reagent and the extraction was performed following the manufacturer's instructions. The concentration of extracted RNA was determined by spectrophotometer and the RNA solution was stored at -80ºC until required.

DNA extraction

By TRI Reagent

cording to the instructions, after removal of the RNA in the aqueous phase, DNA in the interphase and organic phase was precipitated by absolute ethanol. After washing with 0.1 M Tri-Sodium Citrate and 75% ethanol, the DNA pellet was resuspended in sterile distilled water. The DNA solution was then heated at 65ºC for 10 min to better dissolve. The absorbance at 260 nm of each DNA sample was measured in order to calculate its concentration.

By lysis buffer

In the mortality test experiment, total DNA from gill tissues of dead shrimp was extracted according to the protocol of Sukhumsirichart et al. (2002) with some modification. Ten milligrams of gill tissues were homogenized in 100 µl of lysis solution containing 50 mM NaOH and 0.025% SDS. The lysate was then boiled for 10 min before phenol/chloroform extraction. The DNA in the aqueous phase was precipitated in a new 1.5 ml tube containing 1/10 volume of 3M Sodium Acetate, pH 5.2 and 2 volume of absolute ethanol. The pellet of DNA was washed with 70% ethanol and briefly air-dried and subsequently dissolved at 65 ºC for 10 min in sterile distilled water.

Sample analysis

RT-PCR

In order to eliminate the residual DNA, extracted RNA was treated with DNaseI (RQ1, Promega) according to the manufacturer's protocol. After DNaseI inactivation at 65 ºC for 10 min, 1µg of each treated RNA sample was subjected to cDNA synthesis by random hexamer and ImProm-II™ Reverse transcriptase (Promega) following the manufacturer's instructions. Two microlitres of synthesized cDNA was used for further PCR reaction. Each viral specific gene was amplified by primer pairs corresponding to that particular gene (primer no.4 and 5 of each gene but no.5 and 6 for pol) and combined with shrimp actin gene primers (as listed in Table 1). The temperature profile for PCR amplification was performed by holding at 94ºC for 2 min, denaturation at 94ºC for 10 sec, annealing at 55ºC for 30 sec, and extension at 72ºC for 1 min. After 30 cycles (25 cycles for vp26 and vp28), the reaction was subsequently held at 72ºC for another 5 min. The PCR product was analyzed by agarose gel electrophoresis.

Semi-quantitative PCR

The 600 ng of total extracted DNA was used as a template for amplification using primer pairs of vp28 gene (primer no.3 and 4) and together with actin gene as an internal control. The PCR product, after 20 cycle amplification with the same profile as mentioned above in RT-PCR, was loaded into agarose gel electrophoresis.

Statistical analysis

Statistical analysis for PCR and mortality data was conducted using Minitab software. One way ANOVA followed by Turkey multiple comparison test was used for analysis. The data are presented as mean ± standard error (Standard error of the mean, SEM).

Results

Sequence specific silencing of WSSV gene expression by dsRNA

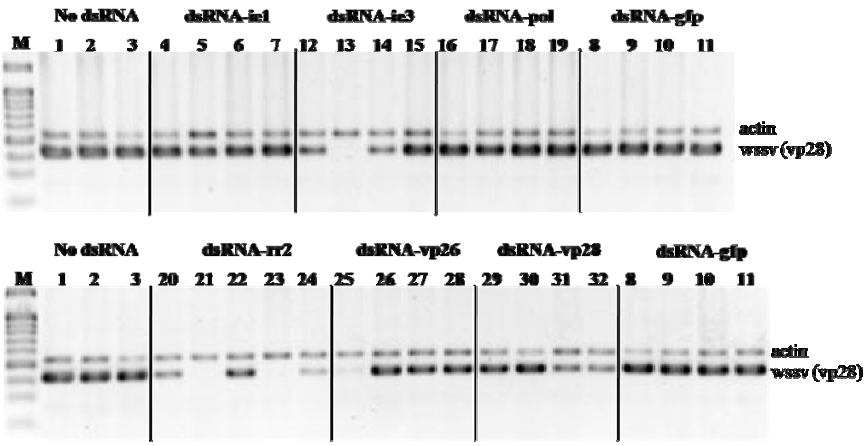
In order to determine the specificity of dsRNA for silencing the viral gene expression, the mRNA level of each viral gene target from shrimp treated with its corresponding dsRNA was measured and compared with the control after WSSV infection. Six viral essential genes, expressed at different stage of infection such as immediate early (ie1 and ie3), early (pol and rr2) and late genes (vp26 and vp28) were used. The 3.75 µg of each dsRNA was injected into the shrimp haemolymph 24 h before WSSV infection at a dilution of 10^{-3}. Thirty six hours post infection (h.p.i.), the expression of each gene in the gills was determined by RT-PCR. In this experiment, dsRNA corresponding to gfp was used as a control for specificity. The result showed that no mRNA or reduced production of mRNA of each gene was observed from shrimp receiving specific dsRNA of that particular gene (Fig. 1a). In contrast, all viral gene transcripts were clearly detected in shrimps without dsRNA treatment (control) as well as with non-specific dsRNA (gfp). It revealed that the silencing of WSSV gene expression by introduction of dsRNA was effective and sequence-specific.

Variable potency of WSSV suppression by different dsRNAs

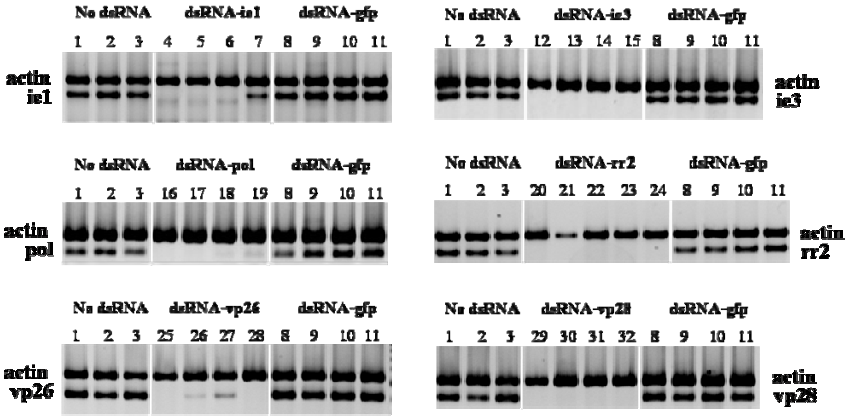
To investigate the potency of viral suppression by specific dsRNA targeted to various kinds of WSSV gene, the amount of virus in the shrimp that viral gene

expression was silenced as shown in the previous data (fig. 1a) was analyzed and compared with the control. Gills were collected from each shrimp and viral DNA was monitored by PCR 36 h post infection. The amount of WSSV vp28 PCR product of each shrimp was co-amplified with shrimp actin gene for normalization. The potency of viral inhibition from each delivered specific dsRNA was variable (Fig. 1b, 1c). High levels of viral infections were observed in all control shrimp (no dsRNA) and in shrimps with non-specific dsRNA (gfp). Reduction of WSSV in shrimp treated with dsRNA-rr2 or dsRNA-ie3 was significantly different from the control ($P = 0.0062$ and $P = 0.047$, respectively). On the other hand, the least inhibition was detected from dsRNA specific to DNA polymerase gene. Viral replication was also inhibited by dsRNA corresponding to ie1, vp26 or vp28 genes but there was no significant difference from the control (Fig. 1c). From our result, even though each viral gene transcript was completely knocked down, the dsRNA-rr2 provided the most effective viral suppression compared with the others. It indicated that the strength of the inhibitory effect of viral replication in shrimp depended critically upon the viral gene target selection.

A



B



C

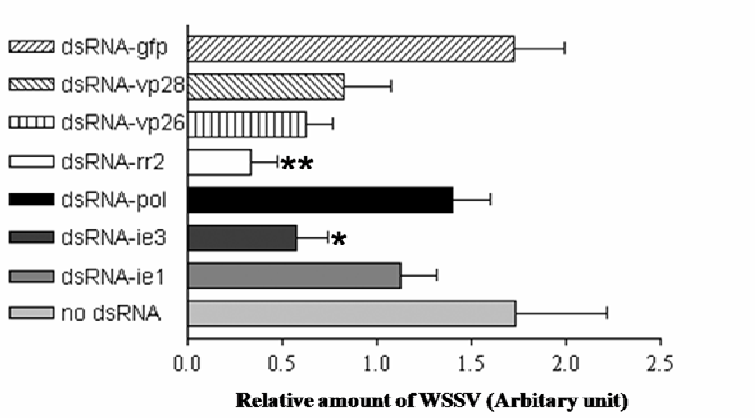


Fig. 1 Silencing of viral gene expression by specific dsRNA inhibited WSSV replication. Shrimps (300 mg) were injected with 3.75 µg dsRNA specific either to immediate early gene (ie1 or ie3) or DNA polymerase (pol) or ribonucleotide reductase small subunit (rr2) or structural protein gene vp26 or vp28 or non-related gene (green fluorescence protein, gfp) 24 h before WSSV infection at 10^{-3} dilution. Shrimp was treated with NaCl instead of dsRNA in the control group. Gills from each shrimp were collected at 36 h post challenge for RNA and DNA extraction. (a) RT-PCR product of six WSSV independent genes. The level of each gene expression from shrimp treated with that corresponded dsRNA was amplified by using primers of that particular gene. Shrimp actin gene was used for normalization. Lanes number 1-3 represent individual shrimps deriving no dsRNA; 4-7 dsRNA-ie1; 8-11 dsRNA-gfp; 12-15 dsRNA-ie3; 16-19 dsRNA-pol; 20-24 dsRNA-rr2; 25-28 dsRNA-vp26; 29-32 dsRNA-vp28. (b) Semi-quantitative PCR of WSSV vp28 DNA. The amount of WSSV in the same shrimp as indicated in the RT-PCR result was determined by multiplex PCR. M is the 100 bp DNA marker. (c) Relative amount of WSSV normalized with actin presents as mean ± SEM. *P* value less than 0.05 and 0.01 present as * and **, respectively.

Multiple injection of dsRNA for effective inhibition of WSSV

In the previous experiment, the presence of the WSSV DNA in some samples (for example; shrimp no. 20, 22, 24) could be detected despite their cognate mRNAs were being completely degraded (Fig. 1a,b). Therefore, to completely deplete the residual viral DNA, multiple injection of dsRNA was performed. To avoid an off-target effect, amount of dsRNA was reduced by half to 1.5 µg per injection per 300 mg shrimp. By using this condition, the viral transcript can completely be knocked down (data not shown). Double-stranded RNA targeting to rr2 (1.5 µg) was either single or double injected (24 h interval) into the shrimp haemolymph 24 h before viral infection. The remaining virus from gill tissues of each treated shrimp was monitored by PCR at time intervals of 24 and 48 h after WSSV infection. Reduction of the viral DNA was detected from both groups of the shrimp injected with single or double treatment of dsRNA-rr2 compared with the control (Fig. 2). Moreover, at 48 h post infection, the virus was completely depleted from shrimp receiving double injection of dsRNA whereas the virus was recovered in the single injected shrimp. It indicated that a single administration of dsRNA was insufficient to effectively inhibit WSSV replication especially in a longer period after infection.

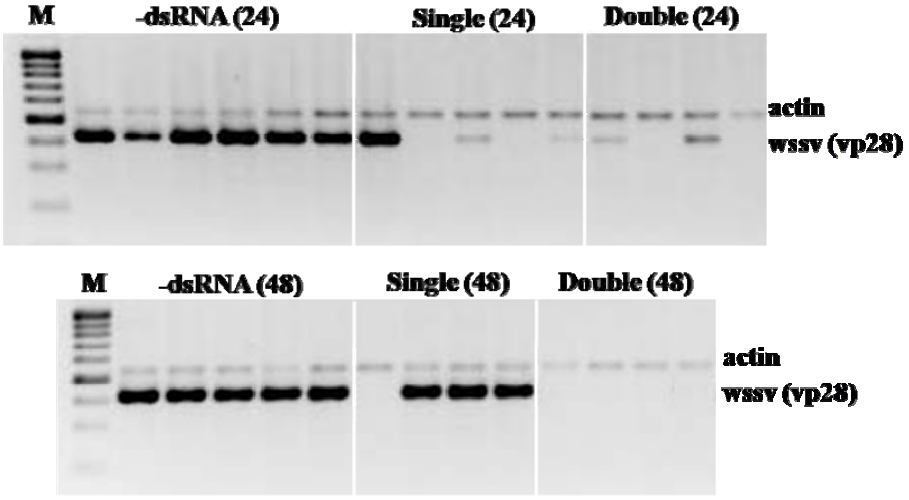


Fig. 2 WSSV was effectively inhibited by multiple injection of dsRNA. Shrimps (300 mg) were treated with dsRNA-rr2 (1.5 µg per injection) either by single or double injection (24 h interval) into haemolymph 24 h prior challenged with 10^{-3} WSSV. The remaining virus in gill tissue was determined by PCR 24 (indicated as 24) and 48 h (indicated as 48) post infection compared with the control (no dsRNA). Beta actin gene of shrimp was used as an internal control. M is the 100 bp DNA ladder.

Protection of shrimp mortality from WSSV infection by dsRNA

To examine the protective effect of dsRNA against WSSV infection, the mortality of shrimp treated with dsRNA was compared with the control after viral infection. In this experiment, the combined dsRNAs of viral specific gene (rr2) and of endogenous shrimp gene (PmRab7) was evaluated whether the combination would give some improvement of the inhibitory effect over the dsRNA-rr2 alone. Shrimps were continuously injected with dsRNAs once a day up to 5 times. At the third treatment, the virus ($5x10^{-4}$) was simultaneously injected with each dsRNA. The accumulated number of dead shrimp was recorded everyday up to 8 days post virus administration (Fig. 3). Both treatments with specific dsRNAs (combined dsRNAs or dsRNA-rr2 alone) showed similar rate of the mortality prevention (approximately 95%). However, there were no inhibition (100% mortality) was detected in the control shrimp (no dsRNA) and partial inhibition (approximately 55%) in both specific dsRNA-PmRab7 alone and non-specific dsRNA (gfp) groups. Moreover, shrimps injected with NaCl alone (no WSSV) were all alive at the end of experiment. The presences of WSSV in all dead shrimp were confirmed by PCR (data not shown). After statistical analysis by ANOVA test, there was no significant difference between combined dsRNAs and dsRNA-rr2 alone ($P = 1$) but significantly different from the control (no dsRNA) ($P = 0.0001$) and dsRNA-gfp ($P = 0.002$). It suggested that there was no additive effect on viral inhibition of the combined dsRNAs (rr2 and PmRab7) over the dsRNA-rr2 alone.

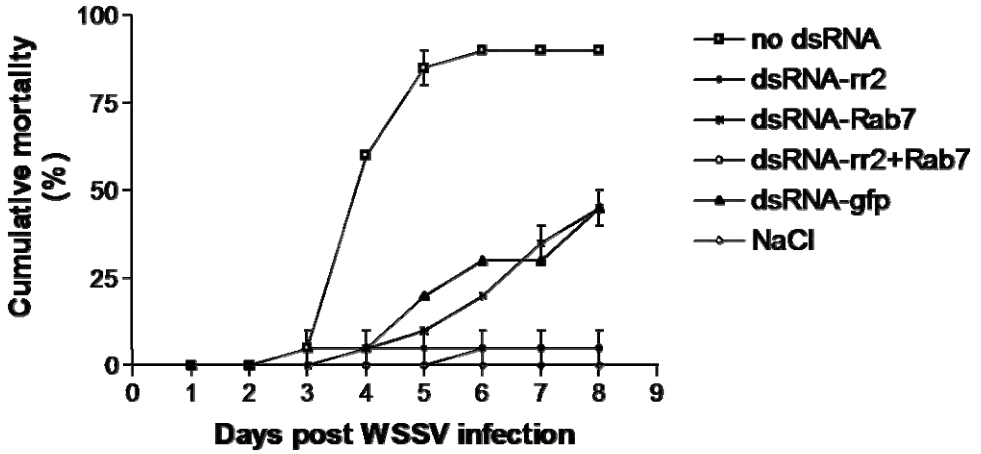


Fig. 3 Treatment of dsRNA protected shrimp from WSSV infection. Shrimp (1-2 g) were administered with either 5 µg of dsRNA-rr2 or 0.6 µg of dsRNA-PmRab7 or 5 µg of dsRNA-gfp or combined 5 µg of dsRNA-rr2 and 0.6 µg of dsRNA-PmRab7 into haemolymph everyday for 5 days. At the third injection, co-administration of dsRNA and WSSV at dilution $5x10^{-4}$ was done. The positive control shrimp (no dsRNA) was treated with NaCl instead of dsRNA whereas the negative control shrimp (NaCl) was injected with NaCl with no WSSV infection. Percent mortality of each group was recorded every day up to 8 days post virus challenge. The result was demonstrated as means with error bar of duplicate experiments. Statistic was analyzed by ANOVA test.

Discussion

Recently, a great deal of research has demonstrated the potential activity of long dsRNA against viral infection in shrimp (Robalino et al., 2004, 2005; Kim et al., 2006; Yodmuang et al., 2006). It has been suggested that this anti-viral immunity was through two pathways: RNAi and innate immunity, which were activated by sequence-dependent and sequence-independent dsRNA, respectively. However, their cooperation for the anti-viral defense response remains unclear. As mentioned by Robalino et al. (2004), the non-specific inhibition was overcome when shrimp was administered with a high dose of virus. Bearing these earlier findings in mind, this study concentrated on evaluating the protective effect of each dsRNA targeted to different essential genes of WSSV via RNAi under the subjection of a severe infection. Despite viral specific mRNA was being degraded, the different strength of protection afforded by six viral specific genes was observed (Fig. 1a). Therefore, the possible explanation for this discrepancy was related mainly to an important role of that particular gene in the viral infection cycle. Among them, a key enzyme for DNA metabolism and biosynthesis (rr2) served as the best target for WSSV inhibition (Fig. 1b). Most large DNA viruses, such as Herpes virus and pox virus (Lankinen et al., 1982; Slabaugh and Mathews, 1984), encode their own genes for nucleotide metabolism enabling their replication to occur more efficiently in resting or non-dividing cells in which rr is absent or inactive. Hence, it supported the importance of rr2 in systemically infection of WSSV in shrimp. Determination of low inhibitory

effect on viral propagation from dsRNA corresponding to pol and ie1 genes was unexpected (Fig.1b). Since WSSV DNA pol contains the conserved characteristics of the eukaryotic-type family B DNA polymerase (Chen et al., 2002), therefore, the diminished viral DNA pol activity can be compensated by that of host enzyme. For ie1, it could be suggested that ie1 protein, although its expression occurred at an early stage of infection, may act as an accessory but not an essential protein for viral replication. Therefore, knocking down of ie1 mRNA did not show any viral inhibition. Similar with the previous studies (Robalino et al., 2005; Tirasophon et al., 2005), viral inhibition observed from shrimp treated with dsRNA targeted to non-structural protein (rr2, ie3) was more potent than structural protein (vp26, vp28). In our case, however, the copy number of gene expression might not be the main factor since their specific mRNAs were completely destroyed (Fig. 1a). Taken together, the protective degree of six WSSV specific dsRNAs was correlated with the functional role of each corresponding gene in viral infection. In our study, rr2 may represent the most critical protein for WSSV replication.

Unlike RNA viruses, the dsRNA mediated silencing can only target to viral mRNA but not the viral DNA genome. Therefore, to effectively inhibit WSSV replication, both the dose and treatment frequency of dsRNA should be taken into consideration. From our data at 24 h after infection (Fig.2, upper panel), both shrimp groups receiving either single or double doses of dsRNA-rr2 illustrated a level of viral suppression by which the higher dose had a more pronounced effect than the lower one. It stated the dose-dependency of dsRNA for viral inhibition as mentioned previously by other groups (Robalino et al., 2005; Yodmuang et al., 2006). After prolonging the infection time to 48 h (Fig.2, lower panel), the remaining virus in single injected shrimps was recovered comparing to complete viral absence in double-injected shrimps. These results may be due to the depletion of dsRNA of the former group during the next round of viral replication. It can be suggested that the maintenance of dsRNA during infection period, by multiple injection of dsRNA, was required for completely inhibition of WSSV. This practical approach was quite common for effectively controlling of other DNA viruses such as Hepatitis B virus (Kayhan et al., 2007) and Human cytomegalovirus (Tao et al., 2008). Expression plasmid has been used to generate continuous production of viral specific shRNA (short hair-pin RNA) in infected cells providing an efficient inhibition of viral replication.

To improve viral protection in *Penaeus monodon*, synchronized blocking of viral cellular transport (by dsRNA-PmRab7) and viral essential gene synthesis (by dsRNA-rr2) was first performed in this study. The Rab mediated trafficking is an early event of viral lifecycle prior to reach the host nucleus to start the viral replication (Sieczkarski and Whittaker, 2002). Knocking down of Rab may result in traffic disorder leading to virus accumulation in the early endosome. Therefore, the viruses cannot replicate their genomes. During the replication of virus, the viral mRNA was being transcribed and transferred into the cytoplasm for performing a translation process. Suppression of viral gene expression by specific dsRNA led to the viral mRNA degradation in the cytoplasm. Taken together, blocking at two different essential steps of the viral infection process should give an efficient approach to inhibit WSSV infection. Our result showed high reduction rates of shrimp mortality from both dsRNA-rr2 and combined dsRNAs (specific to rr2 and PmRab7) (Fig.3)

but there is no a significant improvement of combined dsRNAs over the dsRNA-rr2 alone. It has been reported that dsRNA is up taken into Drosophila S2 cells by receptor-mediated endocytosis process (Saleh et al., 2006). Therefore, using of high amount of dsRNA-PmRab7 should be aware because if the delivery of exogenous dsRNA into shrimp cells uses the same Rab mediated endocytosis pathway, it would block the dsRNAs to enter into cells leading to loss of the inhibitory effect. Further experiments are required for optimization of the combination ratio and the injection frequency in order to promote an additive inhibitory effect.

In conclusion, the present results show that the administration of long dsRNA specific to an essential viral protein gene is effective in inhibition of WSSV replication in *P. monodon* and in prevention of shrimp mortality. At high viral titer, knocking down of viral gene can offer an opportunity for studying the function of that particular protein in the viral infection process. Finally, by the specific condition, the combined dsRNAs targeted to viral gene (rr2) and to shrimp cellular gene (Rab7) could not enhance the inhibitory effect over the dsRNA-rr2 alone.

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gene	Primer sequences
ie1	ie1-1: 5′ CCCTCTAGAGCCAATATGGACTTGACGGCTG 3′
	ie1-2: 5′ CCCCCCGATATCATGACCCACTCCATGGCCTTC 3′
	ie1-3: 5′ CCGCTCGAGGCCAATATGGACTTGACGGCTG 3′
	ie1-4: 5′ GAAAGTCACATCCCTAGCCATCC 3′
	ie1-5: 5′ GGCCTTTAATTTTGAAGACTC 3′
ie3	ie3-1: 5′ CGGAATTCATATGTTGCCCTTTGATAGTGCATCCC 3′
	ie3-2: 5′ CCCCCCGATATCGGCATGTGTTACAAGTTTCTGGC 3′
	ie3-3: 5′ CCGCTCGAGTTGCCCTTTGATAGTGCATCCC 3′
	ie3-4: 5′ ATCAACAAGCCTCCTCCAGCC 3′
	ie3-5: 5′ GGTAAATTGCCACATTTCTGC 3′
pol	pol-1: 5′ ATGCAACTCCGTCTAGAAAAC 3′
	pol-2: 5′ CCCCCCGATATCCGTTGATATGAAGGAAG 3′
	pol-3: 5′ CCGCTCGAGATGCAACTCCGTCTAGAAAAC 3′
	pol-4: 5′ CCCCGCTCATAAATGTGAAC 3′
	pol-5: 5′ CCGCTCGAGCCCGACCAGATATTTGTGGGAAC 3′
	pol-6: 5′ GGGCGTCCATCTCATTGTGCAC 3′
rr2	rr2-1: 5′ CGGAATTCATATGGGAGCAAGCCAACCAAGTGGC 3′
	rr2-2: 5′ CCTTCAACTGCAGCAAAGGCAAC 3′
	rr2-3: 5′ CCGCTCGAGGGAGCAAGCCAACCAAGTGGC 3′
	rr2-4: 5′ CTCCTCGCTTCTGGAATCTGG 3′
	rr2-5: 5′ GTTCACCGTTGCTGGTCTG 3′
vp26	vp26-1: 5′ CGGAATTCATATGGGCAACCTAACAAACCTGGACG 3′
	vp26-2: 5′ CCCCCCGATATCGATTTCGTCCTTGATATCGATCAC 3′
	vp26-3: 5′ CCGCTCGAGGGCAACCTAACAAACCTGGACG 3′
	vp26-4: 5′ TGGCCGAACAGTAATGTCGTTG 3′
	vp26-5: 5′ ATGGAATTTGGCAACCTAAC 3′
vp28	vp28-1: 5′ CGGAATTCATATGACTCTTTCGGTCGTGTCGGCC 3′
	vp28-2: 5′ CCCCCCGATATCGCCAGAGTAGGTGACGTGCAC 3′
	vp28-3: 5′ CCGCTCGAGACTCTTTCGGTCGTGTCGGCC 3′
	vp28-4: 5′ GGCACCATCTGCATACCAGTG 3′
	vp28-5: 5′ ATGGATCTTTCTTTCACTCTTTC 3′
actin	F: 5′ GACTCGTACGTCGGGCGACGAGG 3′
	R: 5′ AGCAGCGGTGGTCATCACCTGCTC 3′

Table 1 Primer sequences for PCR amplification in this study. Viral gene specific primers were designed according to the sequence of WSSV genome (GenBank accession no. **AF36902**

Inhibition of *Penaeus monodon* densovirus (*Pm*DNV) replication in shrimp by double-stranded RNA

Stunted shrimp caused by *Penaeus monodon* densovirus (*Pm*DNV) infection is one of the main problems leading to a significant economic loss in Thailand. To control this pandemic disease, a double-stranded RNA mediated virus-specific gene silencing approach was applied to inhibit viral replication. In this study, two dsRNAs corresponding to the non-structural protein (ns1) and the structural protein (vp) genes of *Pm*DNV were synthesized and introduced into shrimp haemolymph prior to viral challenge. After allowing viral replication for two weeks, the suppression effect by each dsRNA was evaluated by semi-quantitative PCR and compared with the control. A reduction of *Pm*DNV in shrimp treated with each dsRNA was observed. In contrast, a high level of viral infection was detected in the control group (NaCl). Based on a limited sample number, we reached the tentative conclusion that virus-specific dsRNA can inhibit *Pm*DNV replication in which the dsRNA-ns1was more effective than the dsRNA-vp.

Introduction

During the past few years the shrimp culturing industry has encountered a tremendous economic loss which is mainly from viral infectious diseases. Among the causative pathogens, *Penaeus monodon* densovirus (*Pm*DNV) (formerly hepatopancreatic parvovirus or HPV) has been reported to be associated with a slow growth rate and stunted appearance of shrimp [11]. Although it does not cause an abrupt mortality crisis of cultured shrimp as WSSV and YHV-associated outbreaks, the infected shrimp have the same value as the dead ones. This problem leads to the significant reduction of shrimp production and a consequent loss of profit. At present, there is no available treatment for this disease.

The recently described phenomenon of RNA interference (RNAi) provides a powerful means for silencing gene expression in a sequence-specific manner [12]. Moreover, it has been shown to be applicable to viral protection in a number of organisms, including shrimp, through the introduction of exogenous, specific double-stranded RNA (dsRNA). Previously published data has demonstrated that the replication of both RNA [21, 22, 25] and DNA viruses [3, 13, 19] was effectively inhibited by dsRNAs corresponding to their viral gene targets. Consequently, the shrimp mortality was significantly diminished when compared to shrimp without treatment with dsRNA in which all shrimp were dead [3, 18, 21, 24].

*Pm*DNV is a non-enveloped virus with icosahedral shaped particle [14]. It contains a linear single-stranded DNA genome with typical palindromic termini [6]. According to the report of the complete sequence of Thai isolate [20], it composes three large open reading frames (ORFs); two of non-structural protein genes (ns1 and ns2) and one structural protein gene (vp). The ns1 protein of parvovirus is the protein known to have multiple functions including ATP-dependent site-specific DNA binding and nicking and also helicase activities which are essential for viral replication [10, 16, 24]. Meanwhile, a phospholipase A2 activity of vp protein has been reported to be critical for viral infectivity [7]. However, the biological function of ns2 during the parvovirus life cycle is still obscure [9, 15].

In this study, we determined the effectiveness of dsRNA for the inhibition of *Pm*DNV in *P. monodon*. Two dsRNAs specifically targeted to essential genes for viral infection; such as ns1and vp, were synthesized in bacterial cells and subsequently injected into shrimp followed by viral challenge. The suppression of *Pm*DNV by viral specific dsRNA was then evaluated by semi-quantitative PCR analysis.

Materials and methods

Shrimp specimens

The 300 mg juvenile *P. monodon* was used for all experiments in this study. The post-larva stage shrimps (P15-20) obtained from a hatchery farm were reared in a 500 L tank containing artificial sea water at 10 parts per thousand (ppt) salinity with aeration until they reached juvenile stage with the appropriate size. The shrimps were fed daily with a pellet shrimp diet.

Virus source

Hepatopancreas isolated from naturally *Pm*DNV-infected shrimp was utilized for viral infection through an oral route. To quantify the amount of virus, the level of *Pm*DNV in each hepatopancreas was determined by PCR. Only the hepatopancreas with equal amount of virus was then selected for further experimental feeding. The tissue was kept at -20°C until required.

Stem loop RNA expression plasmid construction

For viral specific dsRNA: Two regions of *Pm*DNV genome corresponding to ns1 and vp gene shown in Fig. 1a were selected for dsRNA targeting. In order to minimize a misfolding of RNA during the dsRNA production, the Clone Manager program was used for scanning any repeats throughout the regions of ns1 and vp before selection. Moreover, survey for the specificity of the selected region by Blast program was also performed to minimize the off-target effect. PCR products (400 and 600 bp) of each targeted gene were amplified using Vent DNA polymerase and the specific primer pair shown in table 1 (no. C and D of each gene for 400 bp while no. A and B of each gene for 600 bp). These two fragments were then cloned into pET17b vector in an inverted manner directed by restriction enzymes flanking the fragments (*Nde*I and *Eco*RV for 600 bp, *Eco*RV and *Xho*I for 400 bp) (Fig. 1b). PCR and restriction enzyme analysis were then used to screen the recombinant clones. Furthermore, the nucleotide DNA sequence of the insert was confirmed by automated DNA sequencing. By this cloning strategy, the transcribed RNA can be formed the stem-loop structure with 400 bp stem and 200 bp loop by the complementary binding of both RNA ends.

For non-related dsRNA (green fluorescent protein, gfp): The recombinant plasmid was kindly provided by Dr. Witoon Tirasophon, Mahidol University. The cloning strategy was similar to that of specific dsRNA except pET3a was used as a cloning vector instead of pET17b and *Xba*I was used for the joining of the two fragments and *Nde*I for inserting into the plasmid vector [25].

Double-stranded RNA production

dsRNA was expressed in *Escherichia coli* HT115 according to the protocol of Ongvarrasopone et al. [17]. The overnight bacterial culture in LB medium with antibiotic was diluted with new medium and grown until an OD_{600} of 0.4 at 37 °C was achieved. The expression of dsRNA was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) and the culture was further incubated for 4 h. The bacterial cells were then harvested by centrifugation at 6000 x g for 5 min at 4 °C. The cell pellet was resuspended in 0.1% sodium dodecyl sulphate (SDS) with a ratio of 50 µl per 1 OD. To lyse the cells, the cell suspension was boiled for 2 min. Next, the bacterial single-stranded RNAs (ssRNAs) and the loop region (200 bp) of the expressed RNA were eliminated by incubation with RNaseA (1 µg RNaseA per 1 OD cells) in the reaction buffer (300 mM sodium acetate, 10 mM Tris-Cl pH 7.5) at 37°C for 30 min. The remaining dsRNA (400 bp) was subjected to purification by TRI reagent (Molecular Research Center) following the manufacturer's protocol. Finally, the concentration of each dsRNA was estimated by gel electrophoresis using a standard DNA marker.

Verification of dsRNA

To ensure the integrity of the synthesized dsRNA before introducing into shrimp, treatment with RNaseA (specific to ssRNA) and RNaseIII (specific to dsRNA) was carried out. An equal amount of dsRNA was separately digested with RNaseA (0.01 µg RNaseA per 2 µg dsRNA) and RNaseIII (0.5 units RNaseIII per 2 µg dsRNA) in the reaction buffer of RNaseA (300 mM sodium acetate, 10 mM Tris-Cl) and of RNaseIII (10 mM Tris-Cl, 0.1 mM $CaCl_2$, and 2.5 mM $MgCl_2$), respectively at 37 °C for 5 min. The patterns of the digested RNAs were then determined by gel electrophoresis with a standard size marker.

Experimental condition

For infectivity of PmDNV

In order to determine the infectivity profile of *Pm*DNV in shrimp, the 300 mg shrimp were kept in individual petridish (90 mm x 15 mm) in 10 ppt artificial sea water. Thirty six hours pre-fasting shrimp were fed with *Pm*DNV-infected hepatopancreas (approximately 10% of its body weight per one meal) 3 times with roughly 12 h intervals. Subsequently, the shrimp were fed daily with pellet shrimp diet up to two weeks. Sampling of 2-3 shrimp at 4, 8, and 14 days after first feeding were performed to investigate the viral production in hepatopancreas by PCR analysis.

For viral inhibition

According to the infectivity test, shrimp of the same size kept in individual petridish were injected with approximately 750 ng of each viral specific dsRNA (ns1 and vp) 24 h before viral infection by oral feeding as previously described. To maintain the effect of dsRNA during the experiment, another dsRNA administration was carried out on day 5 post the first injection. Thereafter, the shrimp were fed daily with pellet shrimp diet. Two weeks after oral feeding with *Pm*DNV-infected tissue, the hepatopancreas of each shrimp was isolated for further DNA extraction. The inhibitory effect of delivered dsRNA was then evaluated by semi-quantitative PCR.

DNA extraction

Total DNA was extracted from hepatopancreas tissues (or as indicated) using TRI reagent (Molecular Research Center). The 50-100 mg of tissue was ground in 1 ml TRI reagent. The DNA in the interphase and organic phase was precipitated by absolute ethanol. The DNA pellet was washed with 0.1 M Tri-sodium citrate and 75% ethanol and then resuspended in sterile distilled water. To increase solubilization, the DNA pellet was heated at 65 °C for 10 min. The absorbance at 260 nm of each DNA sample was measured in order to calculate its concentration.

Sample analysis

Semi-quantitative PCR

Total amount of extracted DNA (approximately 600 ng) was used for amplification of the viral specific gene (vp) using primers (no. 1 and 2 for infectivity and inhibition experiment, no. 3 and 4 for tissue distribution test) listed in table 1. To determine the relative amount of virus between samples, PCR of an internal control gene (shrimp beta actin) was included (primers no.1 and 2 for infectivity and inhibition experiment, no. 3 and 4 for tissue distribution test). The temperature profile for PCR amplification was as follows; 94 °C for 2 min, denaturation at 94ºC for 10 sec, annealing at 55ºC for 30 sec, and extension at 72ºC for 1 min. After 20 cycles (35 cycles for tissue distribution experiment), the reaction was held at 72ºC for another 5 min. The PCR product was analyzed by agarose gel electrophoresis.

Statistical analysis

The data was statistically analyzed using GraphPad software. One way ANOVA followed by Tukey-Kramer multiple comparison test was used for analysis. The data are presented as mean ± standard error (Standard error of the mean, SEM).

Results

Double-stranded RNA verification

To test whether RNA was double-stranded RNA, the RNA extracted from bacterial cells was incubated with RNaseA and RNaseIII which specifically cleaved ssRNA and dsRNA, respectively. The result showed that all synthesized RNA was digested by RNaseIII but not RNaseA indicating that it was double-stranded RNA (Fig. 1c). In contrast, the control single-stranded RNA (ss) was completely digested with RNaseA. It clearly demonstrated that the synthesized dsRNAs (ns1, vp and gfp) were suitable for injection into shrimp to trigger the anti-virus pathway.

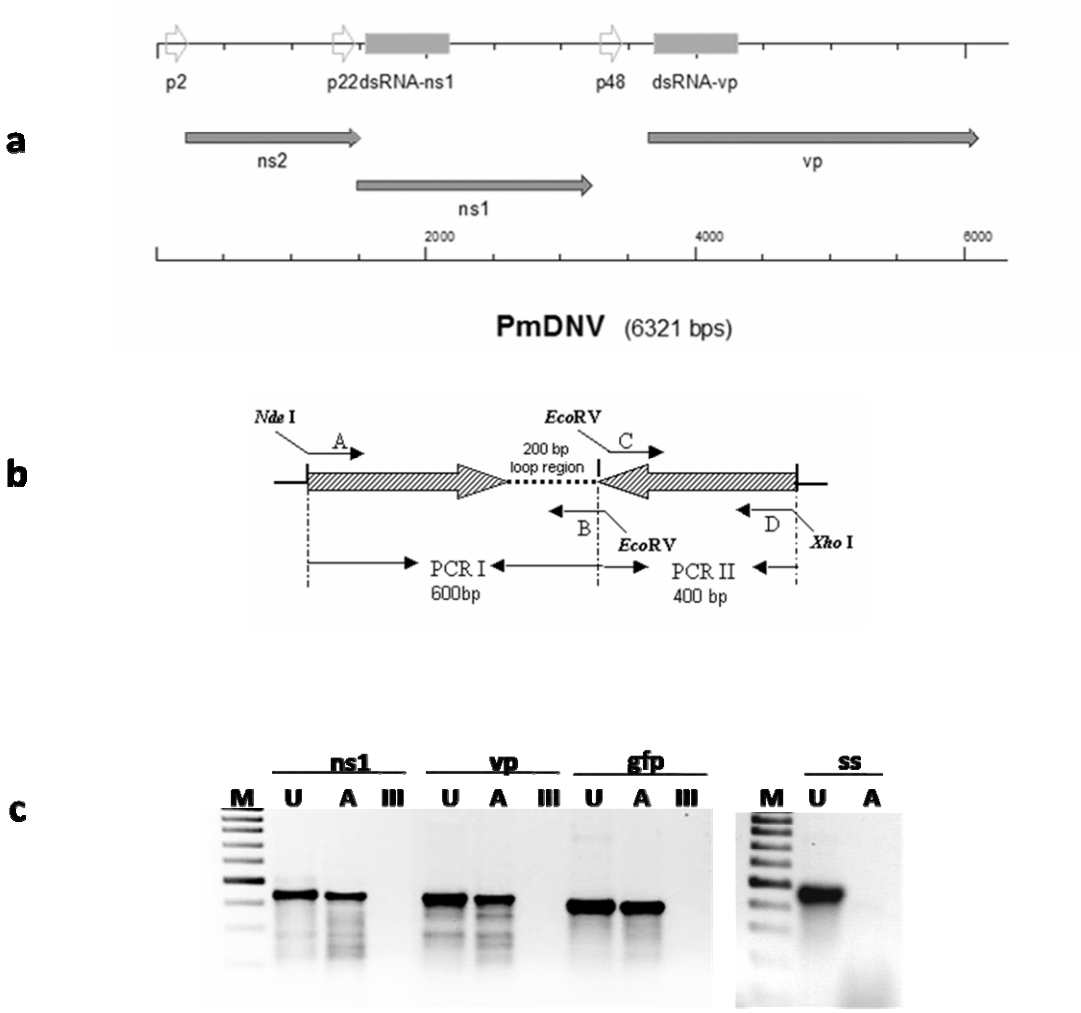

Fig. 1 Double-stranded RNAs production. (a) Schematic diagram of dsRNA targeted DNA regions on *Pm*DNV genome. Each gray arrow represents a particular gene (ns2 (nt 216-1502), ns1 (nt 1487-3226), and vp (nt 3642-6098)) lying below a line of *Pm*DNV genome. The regions targeted by dsRNA-ns1 (nt 1547-2170) and dsRNA-vp (nt 3689-4303) are shown in gray boxes while the open arrows show tentative promoters of each gene. The scale bar relative to the full-length of PmDNV genome (6321 bp) is indicated underneath. (b) Schematic diagram of stem loop RNA expression plasmid construction. Primers A+B and C+D were used for amplification of 600 bp PCR fragment (PCR I) and 400 bp fragment (PCR II) of each viral specific gene, respectively. The restriction enzyme sites providing the directional cloning are indicated at 5' end of each primer. The shaded arrow and dotted line represent an inverted repeat of target and the spacer between inverted repeat, respectively. (c) DsRNAs verification by RNase treatment. The integrity of each dsRNA targeted to ns1 gene (ns1) or vp gene (vp) or gfp gene (gfp) was confirmed by incubation with RNase A (A) and RNase III (III) which specifically digested ssRNA and dsRNA, respectively. The expected sizes of dsRNA-ns1 and dsRNA-vp are 425 bp and 409 bp, respectively. The untreated dsRNAs (U) and ssRNA (ss) were used as the control. Approximately 50 ng of dsRNA was loaded in each lane of normal 1xTBE 2% agarose gel. M is marked for the 100 bp DNA ladder

Localization of PmDNV in various tissues of shrimp

To date, the distribution of this virus in the tissues of shrimp has not been investigated. In this study, the localization of *Pm*DNV in various organs of shrimp (hepatopancreas, gill, pleopod, periopod and muscle) was determined. Semi-quantitative PCR of the specific viral vp gene was performed by using an equal amount of DNA template extracted from each tissue. By normalization with the β-actin control, it could be seen that the predominance of *Pm*DNV was in the hepatopancreas (Fig. 2). Much less was noted in the periopod, gill, pleopod and muscle. From this information, the tissue from the hepatopancreas was used for all subsequent experiments.

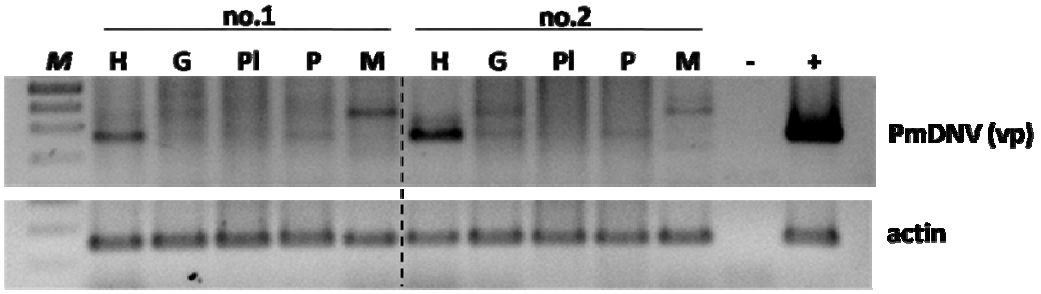

Fig. 2 Tissue distribution of *Pm*DNV in a naturally infected shrimp. Equal amount of total DNA extracted from various tissues (hepatopancreas (H), gill (G), pleopods (Pl), periopods, (P), and muscle (M)) of each naturally *Pm*DNV-infected shrimp were subjected to PCR analysis to determine the viral localization. The product was separately amplified using viral specific primers (*Pm*DNV, vp) and host beta-actin primers (actin). To control the experiment, negative (-) and positive (+) control were included. Number on the top indicates an individual shrimp. The size of PCR product was compared with the 100 bp DNA marker (*M*).

Experimental infection of PmDNV in shrimp

In order to determine an infectivity profile, shrimp were fed with *Pm*DNV-infected hepatopancreas and randomly selected for testing at three different time points (4, 8, and 14 days post first feeding). Viral replication was then assayed by PCR. A reduction of virus was observed at day 8 compared with total virus uptake at day 4. The amount of virus was then increased significantly at day 14 (Fig. 3). This indicates that the infection of shrimp with *Pm*DNV can be experimentally performed and the viral replication can also be investigated over a time period of up to 14 days.

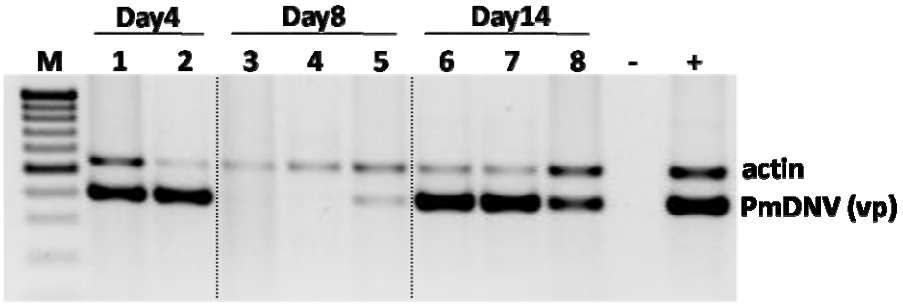

Fig. 3 Infectivity profile of *Pm*DNV in shrimp after feeding with hepatopancreas of *Pm*DNV-infected shrimp. The 300 mg shrimp were fed 3 times (approximately 30 mg each meal and 12 h intervals) with *Pm*DNV-infected hepatopanceas. Shrimp were randomly picked up and sacrificed at 4 (Day4), 8 (Day8), and 14 (Day14) days post-feeding. Total DNA extracted from hepatopancreas of each shrimp was used as a template for semi-quantitative PCR using the viral specific primers (*Pm*DNV) and the internal control actin primers (actin). Each shrimp is numbered on the top of gel while M is the 100 bp marker. Negative (-) and positive (+) control of the reactions are added.

Suppression of PmDNV replication by dsRNA

To test the efficacy of dsRNA for inhibition of viral replication, long dsRNA (approximately 400 bp) targeted to viral genes, including non-structural (ns1) and structural protein genes (vp), were introduced into the shrimp's haemolymph followed by *Pm*DNV challenge by feeding. The amount of virus in each shrimp that received viral specific dsRNA was analyzed and compared with the control (without dsRNA) and non-specific dsRNA (gfp). Fourteen days post-infection, viral DNA in the hepatopancreas of each shrimp was monitored by PCR co-amplified with shrimp actin gene for normalization (Fig. 4b). A high level of viral infection was observed in the control group. The reduction of *Pm*DNV in shrimp treated with dsRNA-ns1 was more pronounced than dsRNA-vp and significantly different from the control (no dsRNA) ($P = 0.004$) and non-related dsRNA (dsRNA-gfp) ($P = 0.02$) (Fig. 4c). These results demonstrate that the viral specific dsRNA can suppress the replication of *Pm*DNV in shrimp with the dsRNA-ns1 being more potent than the dsRNA-vp.

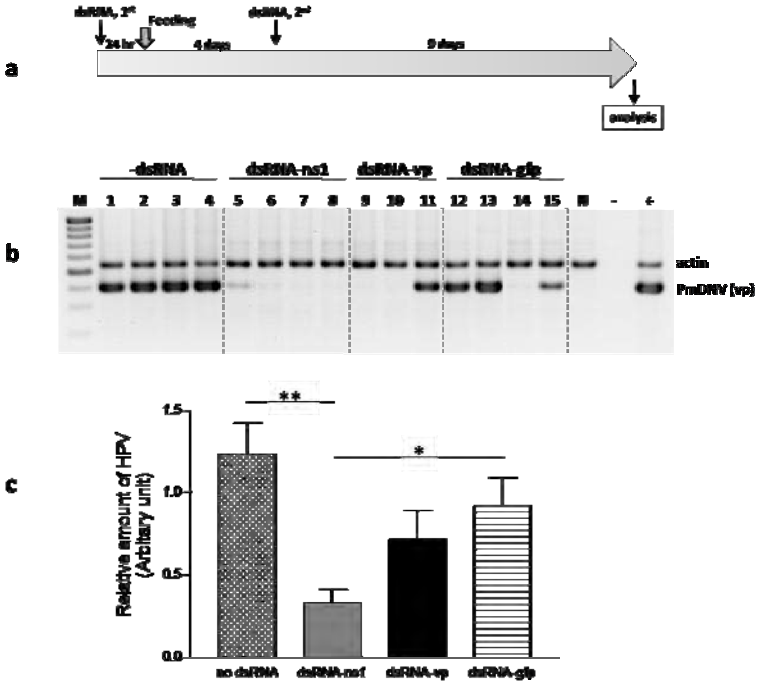

Fig. 4 Treatment of dsRNA for inhibition of *Pm*DNV in shrimp. The 300 mg shrimp were injected with 750 ng dsRNA specific either to ns1 gene or vp gene or non-related gfp gene two times (as indicated in the diagram) before challenging with *Pm*DNV by oral feeding with infected tissue. Shrimp were treated with NaCl instead of dsRNA in the control group. Fourteen days after first dsRNA administration, hepatopancreas of each shrimp was collected for DNA extraction and further PCR analysis was performed. (a) Diagram represents the experimental condition. (b) Semi-quantitative PCR of *Pm*DNV DNA. The amount of *Pm*DNV in an individual shrimp was determined by multiplex PCR using the viral specific primers (vp) together with the host control gene (actin) primers for normalization. Lanes 1-4 represent individual shrimp receiving no dsRNA; 5-8 dsRNA-ns1; 9-11 dsRNA-vp; 12-15 dsRNA-gfp. N is the normal shrimp. Negative (-) and positive (+) control of the reaction are included with the 100 bp DNA marker in lane M. (c) Relative amount of *Pm*DNV normalized with actin. The result was demonstrated as means with error bar of duplicate experiments. Statistic was analyzed by ANOVA test. *P* value less than 0.05 and 0.01 indicate as * and **, respectively.

Discussion

This study showed for the first time that the replication of *Pm*DNV in shrimp can be effectively inhibited by the administration of virus-specific long dsRNA. Our findings are in agreement with a number of previous studies demonstrating dsRNA mediated viral suppression in shrimp [3, 13, 19, 21]. It has been documented that silencing the expression of an essential viral replication protein gene can significantly prohibit the infection of that particular virus. According to the strength of the inhibitory effect, the different gene targets of dsRNA provided different degrees of effect for viral protection. The non-structural protein gene has been shown to be more potent than the structural protein gene [19, 21]. In the case of *Pm*DNV, the most effective target was probably the multi-functional ns1 protein gene. The ns1 protein has been reported to be involved in the early step of parvovirus infection via the binding and nicking of the double-stranded replicative form (RF) of DNA for generating the single-stranded DNA. This then further facilitates the replication process [2, 4, 5]. In addition to this, the ns1 of parvovirus minute virus of mice (MVM) has been shown to regulate the expression of the late protein gene (vp) through promoter activation [8]. Because of these essential functions, the ns1 of *Pm*DNV may represent a potential target for further development of anti-*Pm*DNV approach.

As mentioned recently by several research groups [3, 13, 15, 25], the anti-viral response in shrimp triggered by long dsRNA was through two pathways: sequence-dependent RNAi and sequence-independent innate immunity. In the case of non-specific inhibition from dsRNA-gfp or other irrelevant dsRNAs, the protection was not high and could be observed at low level of viral infection whereas it was overwhelmed when heavy infection. In our result, a moderate inhibition was also found in the dsRNA-gfp treated shrimp group (figure 4b, lane 14-15). It suggested that shrimp could be partially protected from *Pm*DNV infection by non-specific dsRNA. This might be due to the slow rate of its infection (approximately 2 weeks after receiving viruses as shown in figure 3); therefore, the non-specific inhibition could be effective enough to block *Pm*DNV infection.

The successful infection of *Pm*DNV in juvenile *Penaeus monodon* (300 mg body weight) was firstly reported in this investigation. Interestingly, shrimp infected by feeding with virus-infected hepatopancreas had a specific infectivity profile. The amount of virus declined after initial infection but then rose significantly at a later time point. This phenomenon may be due to the cellular composition of the hepatopancreas. It is widely known that parvoviral replication depends on cellular factors associated with the S phase of the cell cycle to convert its ssDNA genome into a double-stranded RF for further genome amplification [5]. There are four main epithelial cell types present in the hepatopancreas, E (embryonic), F (fibrilar), B (blister-like), and R (absorptive) cells [1]. Out of these, only the E cells show mitotic activity. Hence, it is possible that the large number of virus particles initially entered into all of the types of epithelial cells during the feeding period but only the dividing E cells, which are a small proportion (less than 10%) of the hepatopancreatic epithelial cells [26], were able to support viral replication. On the other hand, the other differentiated epithelial cells (F, B, and R cells) would have eventually been disintegrated and extruded from the tubule epithelium releasing a digestive enzyme together with the viral particles into the lumen of hepatopancreatic tubule [23]. The virus would then later be excreted out via feces [18]. This explains the significant reduction of *Pm*DNV at day 8 compared to the initial uptake. Subsequently, propagation of the virus was promoted due to division of the infected E cells. On the basis of this finding, approximately 14 days should be the appropriate experimental time course to assess *Pm*DNV replication in 300 mg juvenile shrimp.

In conclusion, an effective tool for inhibition of *Pm*DNV replication in shrimp using virus-specific dsRNA has been demonstrated in this study. However, several aspects, such as therapeutic effect as well as methodology for continuous delivery of dsRNA into shrimp, have to be further resolved in order to apply this RNAi technology for controlling this disease in the shrimp farm.

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Table 1

gene	Primer sequences
ns1	ns1-A: 5′ CGGAATTCATATGACATTTTACTCTGGTCTCATTG 3′
	ns1-B: 5′ CCCCAGATATCGCCCTAGAACTGCTTAGTC 3′
	ns1-C: 5′ CCCCAGATATCTTGCGTATTTCCTGTCCCTG 3′
	ns1-D: 5′ CCGCTCGAGACATTTTACTCTGGTCTCATTG 3′
vp	vp-A: 5′ CGGAATTCATATGCCAAAGTAAGCGAAAGAATAAAC 3′
	vp-B: 5′ CCCCCCGATATCATTTCTTAGCGTTTTCTATGCG 3′
	vp-C: 5′ CCCCCCGATATCTCTGTGCTGTCTGAAAATCCT 3′
	vp-D: 5′ CCGCTCGAGCCAAAGTAAGCGAAAGAATAAAC 3′
	vp-1: 5′ AATCTGCAGGGTACGGAAAAAAC 3′
	vp-2: 5′ TGTGGAACCATCTCAAATGCC 3′
	vp-3: 5′ ATCTGGATAGTATACATGTC 3′
	vp-4: 5′ GGAGATATTAAGCACAGTTTC 3′
actin	actin-1: 5′ GACTCGTACGTCGGGCGACGAGG 3′
	actin-2: 5′ AGCAGCGGTGGTCATCACCTGCTC 3′
	actin-3: 5′ CAAGTGCTTCTAAGGATACTG 3′
	actin-4: 5′ CATGATTATTTTGTATATATTATCG 3′

Table 1. Primer sequences for PCR amplification in this study. Viral gene specific primers were designed according to the sequence of *Pm*DNV genome Thai-isolate (GenBank accession no. **DQ002873**). The sequence of shrimp beta actin gene (accession no. **AW600735**) was used for actin primers.

The ns1 double-stranded RNA inhibits *Penaeus monodon* densovirus amplification in infected shrimp

Penaeus monodon densovirus (*Pm*DNV) is one of the major causes of stunted shrimp in Thailand and leads to considerable economic losses in overall shrimp production. Present study shows that the double-stranded RNA corresponding to the non-structural protein gene (ns1) of *Pm*DNV inhibited viral propagation in naturally pre-infected shrimp. Multiple application of dsRNA was performed by injection into the haemolymph. The total amount of virus in hepatopancreas of treated shrimp was measured by semi-quantitative PCR. Observations indicated that *Pm*DNV was almost eradicated in comparison to the high viral propagation in the control groups (no dsRNA and non-related dsRNA-gfp). The results demonstrate the promising ability of dsRNA-ns1 to be further used as a tool for anti-*Pm*DNV therapy in shrimp.

At present, shrimp with a slow growth rate and stunted appearance are one of the main problems of the shrimp culturing industry in Thailand. Several tons of shrimp production has been lost due to improper farm management and severe infection from causative pathogenic agents. One such pathogen is *Penaeus monodon* densovirus (*Pm*DNV) (formerly hepatopancreatic parvovirus or HPV). Although, many intensive studies have developed a sensitive and efficient method for diagnosis (Rukpratanporn et al., 2005; Khawsak et al., 2008; Nimitphak et al., 2008), this disease pandemic is still seriously found. Therefore, the development of an efficient approach for *Pm*DNV treatment is needed.

RNA interference (RNAi) is a powerful technique that has been widely applied to inhibit shrimp viruses such as white spot syndrome virus (WSSV) (Robalino et al., 2005, Kim et al., 2006, Attasart et al., 2009), and yellow head virus (YHV) (Yodmuang et al., 2006, Tirasophon et al., 2007). This technique requires the activation of the exogenous dsRNA for the degradation of its complementary viral RNA (Hannon, 2002). By its sequence specificity, this method has presented a very promising strategy for the inhibition of viral replication in both preventive (Chen et al., 2006; Li et al., 2009) and curative functions (Huang, 2008).

*Pm*DNV is a single-stranded DNA non-enveloped virus (Lightner and Redman, 1985; Bonami et al., 1995). Its genome composes three large open reading frames (ORFs); two of which are non-structural protein genes (ns1 and ns2) and one which is a structural protein gene (vp) (Sukhumsirichart et al., 2006). In our previous study, we have determined the effectiveness of viral specific dsRNA for the inhibition of *Pm*DNV in shrimp (*P. monodon*) (Attasart et al., in press). It demonstrated that dsRNA corresponding to the ns1 gene (dsRNA-ns1) effectively inhibited *Pm*DNV replication in shrimp. From that result, the dsRNA-ns1 may provide a therapeutic effect for this disease in shrimp. Hence, in this work, the efficacy of dsRNA-ns1 mediated *Pm*DNV inhibition for treatment of the infected-shrimp was evaluated.

Naturally *Pm*DNV infected-shrimp (*P. monodon*) having a body weight of 300-400 mg were obtained from a hatchery farm since they were at the post-larval stage (P15-20) and reared in a tank until they reached the appropriate size. They were maintained in artificial sea water (10 parts per thousand (ppt)) supplied with oxygen, and fed daily with a commercial shrimp food pellet. Prior to testing the curative effect of dsRNA-ns1, the shrimp were sampled randomly for *Pm*DNV infection by PCR.

The total DNA of the hepatopancreatic tissue was isolated using TRI reagent (Molecular Research Center). After grinding 50-100 mg of tissue in 1 ml TRI reagent, the DNA in the inter-phase and organic phase was precipitated by using absolute ethanol and washed with 0.1 M Tri-sodium citrate and 75% ethanol. The DNA pellet was finally resuspended in sterile distilled water. The 200 ng of total extracted DNA, estimated by the measurement of UV light absorption, was used as a template for vp amplification using vp-s (5′AATCTGCAGGGTACGGAAAAAAC3′) and vp-a (5′TGTGGAACCATCTCAAATGCC 3′) primers. In each reaction, the shrimp actin primers; F (5′GACTCGTACGTCGGGCGACGAG G 3′) and R (5′AGCAGCGGTGGTCATCACCTGCTC3′), were also added for normalization between each sample. The PCR amplification procedure was carried out as follows; 94 °C for 2 min, denaturation at 94ºC for 10 sec, annealing at 55ºC for 30 sec, and extension at 72ºC for 1 min. After 20 cycles, the reaction was held at 72ºC for another 5 min. The PCR product was analyzed by agarose gel electrophoresis. As a result, the *Pm*DNV DNA was detected in every shrimp, indicating that they were already infected (data not shown).

Double-stranded RNA was produced in bacterial cells according to the protocol of Ongvarrasopone et al. (2007). The stem loop RNA expression plasmid containing the inverted repeat of the DNA region corresponding to the ns1 gene (Attasart et al., in press) was transformed into *Escherichia coli* HT115. The overnight culture was diluted 100-fold and grown at 37 ºC until OD_{600} reached 0.4. After activation with isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h, the bacterial cells were then centrifuged at 6,000 x g for 5 min at 4 °C. The cell pellet was resuspended in phosphate buffer saline (PBS) containing 0.1% sodium dodecyl sulphate (SDS) and the cell suspension was boiled for 2 min to lyse the cells. After treatment with RNaseA to destroy the bacterial single-stranded RNAs (ssRNAs) and the loop region of the expressed RNA, the remaining dsRNA (400 bp) was purified by using TRI reagent. The integrity and yield of dsRNA were then determined and compared with a standard DNA marker by agarose gel electrophoresis.

The *Pm*DNV-infected shrimp were randomly divided into three groups. Each group was separately treated with buffer (NaCl), viral specific dsRNA-ns1 or non-related dsRNA (dsRNA corresponding to green fluorescence protein gene, gfp). The shrimp were placed in individual cages and reared in 10 ppt artificial sea water during the experiment. Multiple injections of 800ng dsRNA into the haemolymph circulation occurred every 5 days. According to the diagram presented in figure 1a, 7-8 shrimp of each group were randomly selected for analysis 1 and 2 after the second and third dsRNA administration, respectively. The total DNA was extracted from the hepatopancreas of the shrimp and semi-quantitative PCR was employed to determine *Pm*DNV reduction in dsRNA-ns1 treated shrimp compared with the control (NaCl and dsRNA-gfp). The amount of DNA and conditions used for the PCR analysis were the same as described before for *Pm*DNV detection. The experiment was performed twice but the results provided here are from only one set of data.

The present study reports the ability of dsRNA-ns1 for the effective clearance of *Pm*DNV from pre-infected shrimp. We observed a significant decrease in virus production after the second and third injection of dsRNA-ns1 as shown in figure 2b and 2c. In contrast, neither the buffer nor dsRNA-gfp suppressed viral propagation indicating that the effect is sequence-specific. Notable results indicated that exogenous viral specific dsRNA-ns1 can potently suppress *Pm*DNV replication in a

curative mode whereas the non-related dsRNA cannot. Tirasophon and his colleague (2007) have also noted this phenomenon, stating that there was no curative effect against YHV infection in black tiger shrimp by non-related dsRNA-gfp. In the future, this promising ability of dsRNA-ns1 may be utilized as a tool for anti- *Pm*DNV therapy in shrimp and could be especially useful when applied to cleaning-up high value shrimp brood stock after being infected by the virus.

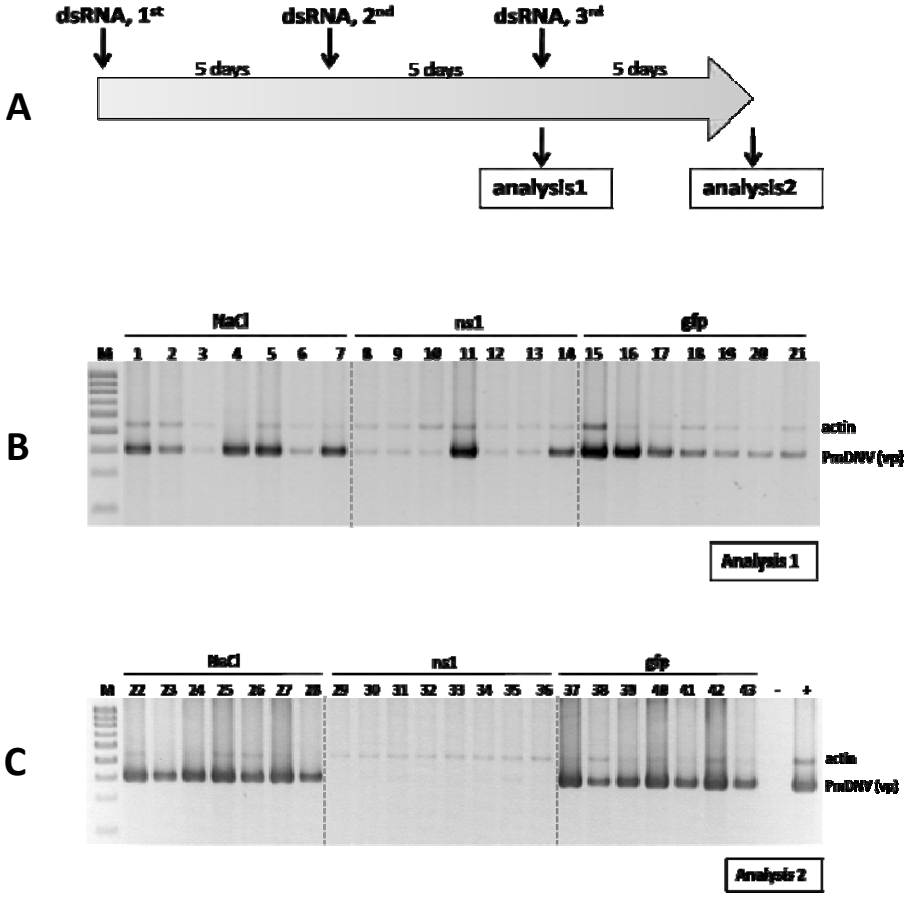

Figure Legends

Fig. 1 Treatment of naturally *Pm*DNV-infected shrimp by dsRNA-ns1. The 300-400 mg *Pm*DNV-infected shrimp were injected with 800 ng dsRNA specific to viral ns1 gene (dsRNA-ns1) three times by 5 days interval. In stead of dsRNA-ns1, shrimp were treated with NaCl in the control group or with dsRNA specific to gfp (dsRNA-gfp) in order to determine the non-specific effect. Shrimp were randomly selected for analysis 1 and 2 after the second and third administration, respectively. The hepatopancreas of each shrimp was collected for DNA extraction and further PCR analysis was performed. (a) Diagram represents the experimental condition. Semi-quantitative PCR is shown in b (analysis 1) and c (analysis 2). The amount of *Pm*DNV in the individual shrimp was determined by multiplex PCR using the viral specific primers (vp) together with the host control gene (actin) primers for normalization. Lanes 1-7 (b), 22-28 (c) represent individual shrimp receiving NaCl; 8-14 (b), 29-36 (c) dsRNA-ns1; 15-21 (b), 37-43 (c) dsRNA-gfp. Negative (-) and

positive (+) control of the reaction are included with the 100 bp DNA marker in lane M.

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Research Ouput

(จากชุดโครงการการวิจัยแบบมุ่งเป้า “การผลิตสัตว์น้ำเศรษฐกิจ)

Research Output (จากชุดโครงการการวิจัยแบบมุ่งเป้า “การผลิตสัตว์น้ำเศรษฐกิจ”)

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นักศึกษาจบปริญญาโท

1. นางสาวรสสุคนธ์ แก้วขาว (Miss Rossukon Kaekhow) หัวข้อวิทยานิพนธ์ Inhibiton of heapatopancreatic parvorirus (HPV) infection in shrimp by double-standed RNA

2. นางสาวมยุรี ชนะสกุลนิยม (Miss Mayuree Chanasakulniyom) หัวข้อวิทยานิพนธ์ Suppression of PmRab7 by dsRNA inhibits WSSV or YHV infection in *Penaeus monodon*