RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2007.33.035

ScienceAsia 33 (2007): **35-39**

A Simple and Cost Effective Method to Generate dsRNA for RNAi Studies in Invertebrates

Chalermporn Ongvarrasopone[a*], Yaowaluck Roshorm[a] and Sakol Panyim[a,b]

[a] Institute of Molecular Biology and Genetics, Mahidol University, Salaya, Nakhon Pathom 73170, Thailand.
[b] Department of Biochemistry, Faculty of Science, Mahidol University, Rama VI Road, Bangkok 10400, Thailand.

* Corresponding author, E-mail: mbcov@mahidol.ac.th

Received 24 Feb 2006
Accepted 21 Jul 2006

ABSTRACT: Using an *E. coli* strain deficient in ribonuclease III activity, we have developed a simple and cost effective *in vivo* bacterial expression system to generate large amounts of double-stranded RNA (dsRNA). This method, which involves *E. coli* culture, RNase A treatment of lysed cells and total RNA extraction, is easier and less expensive than traditional *in vitro* transcription techniques. The system was validated by knocking down yellow head virus replication in shrimp OKA cell cultures. Results showed that the dsRNAs prepared *in vitro* and *in vivo* possessed similar potency in inhibiting viral replication. This methodology gives an alternative means to prepare large amounts of dsRNA at low cost.

KEYWORDS: double-stranded RNA, *E. coli* expression, *in vitro* transcription, yellow head virus, shrimp.

INTRODUCTION

RNA interference is a phenomenon whereby double-stranded RNA triggers a potent and specific inhibition of its homologous mRNA and was discovered by Andrew Fire and colleagues in 1998 [1]. 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. siRNA is then incorporated into RNA induced silencing complexes (RISC), unwound, and the single-stranded antisense siRNA is targeted to a specific region of its complementary mRNA resulting in mRNA degradation. RNAi is widely used to study gene function, prevent viral infection and undertake genome wide screening for potential candidates to treat cancer and infectious diseases [2,3]. In invertebrates, long dsRNA can be efficiently used to silence gene expression without activation of dsRNA-activated protein kinase (PKR) or the interferon response that has been shown to occur in mammalian cell systems. Double-stranded RNA can be introduced into animals and cells by injection, electroporation and chemical mediated transfection. Therefore, a simple and cost effective approach to produce long dsRNA will be useful for RNAi studies, especially in invertebrates.

Several approaches can be used to synthesize long dsRNA. *In vitro* transcription can be effectively employed to synthesize a single-stranded RNA. The gene of interest can be cloned into a plasmid vector containing the T7, T3 or Sp6 RNA polymerase promoters. The plasmid DNA template is linearized and then gel purified. Single-stranded sense and antisense RNA are synthesized using the appropriate RNA polymerases and the plasmid DNA template is removed by DNase I treatment. The single-stranded RNAs are isolated from protein contamination by phenol chloroform extraction and ethanol precipitation. Then, the two single-stranded RNAs can be annealed to produce dsRNA [1,4,5]. In addition, DNA templates containing T7 promoter sequences on both ends can be produced by a PCR technique using gene specific primers containing T7 promoter sequences linked to the 5' end. After *in vitro* transcription, the dsRNA is purified using solid phase adsorption to remove protein, nucleotides and oligonucleotides (http://www.ambion.com/techlib/prot/fm_1626.pdf). These approaches give a high yield of dsRNA.

Production of dsRNA using the commercially available kits based on the principle of *in vitro* transcription on linearized DNA template or on PCR generated templates is widely used. However, it is increasingly expensive when one needs to produce large amounts of dsRNA for RNAi studies. *In vivo* production of dsRNA in the *E. coli* strain HT115, which lacks ribonuclease III (RNase III) activity, with a protocol modified from that of Timmon, et al., 2001 [6], can be used as an alternative approach to produce large amounts of dsRNA at low cost. The *E. coli* strain HT115

was modified to express the T7 RNA polymerase from an IPTG inducible promoter [6]. In this study, the effectiveness of viral derived dsRNA produced *in vivo* and *in vitro* to inhibit viral replication was compared in shrimp cells. A similar specific silencing effect to knock down viral replication was demonstrated, suggesting that dsRNA produced *in vivo* can be used as an alternative method to produce large amounts of dsRNA at low cost.

Materials and Methods

Plasmid Constructs

Recombinant plasmids expressing stem loop YHV-protease RNA were constructed in pGEM-T-easy vector (Promega, Madison, WI, USA) and pET3a vector (Novagen, Madison, WI, USA). A 400-bp cDNA fragment in the coding region of the YHV-protease gene was amplified using specific primers Pro-sense-*Nde* I (5' CAT ATG GGAATC GAC TAT CGT GAC TGC 3') and Pro-anti-*Pst* I-*Spe* I (5'CTG CAG ACT AGT ATG CCG ACG ATG TGA GCT CC 3'). The PCR fragment of the YHV-protease gene was cloned into the pGEM-T-easy vector (Promega) in the sense and antisense orientation. The pGEM-T-easy vector containing sense-YHV-protease was digested with the restriction enzymes *Spe* I and *Pst* I and ligated with a 200 bp PCR fragment of GFP sequences amplified with specific primers GFP-sense-*Spe* I (5' AAG GCA CTA GTA TGG TGA GCA AGG GCG AGG 3') and GFP-anti-*Pst* I; 5' AAT TGC TGC AGC TGC ACG CCG TAG GTC AG 3') to construct a pGEM-T-easy vector containing sense-YHV-protease linked with GFP. This clone was digested with the restriction enzyme *Pst* I and ligated with the *Pst* I - antisense-YHV-protease fragment that was digested from the pGEM-T-easy vector containing antisense-YHV-protease. Therefore, the pGEM-T-easy vector containing the entire 1 kb insert of the sense-YHV- protease, GFP and the antisense-YHV-protease was obtained and named pGEM-T-easy-Pro (Fig 1A). This plasmid was used for *in vitro* production of stem loop YHV-protease dsRNA using *in vitro* transcription[7]. The 200-bp fragment of GFP was the loop in the stem loop YHV- protease RNA. This region is cleaved after digestion with ribonuclease A (RNase A) (Fig 1C). In addition, the entire 1 kb fragment was excised from pGEM-T-easy-Pro by digestion with *Nde*I and subcloned into the *Nde*I site of pET3a to construct pET3a-Pro (Fig 1B), which was used for *in vivo* bacterial expression of the stem loop YHV-protease RNA.

Similarly, a recombinant plasmid encoding the GFP stem loop was constructed by amplifying a 400 bp GFP with the primers GFP-sense-*Nde* I (5' AAG GCA CTC ATA TGG TGA GCA AGG GCG AGG 3') and GFP-antisense-1-*Xba* I (5' TGT TCT AGA ACT CCA GCT TGT GCC 3'), and a 600 bp GFP was amplified with the primers GFP-sense-*Nde* I (5' AAG GCA CTC ATA TGG TGA GCA AGG GCG AGG 3') and GFP-antisense-2-*Xba* I (5' TGT TCT AGA TTT GCT CAG GGC GGA CTG GGT GCT CAG 3'). The two PCR fragments were ligated at

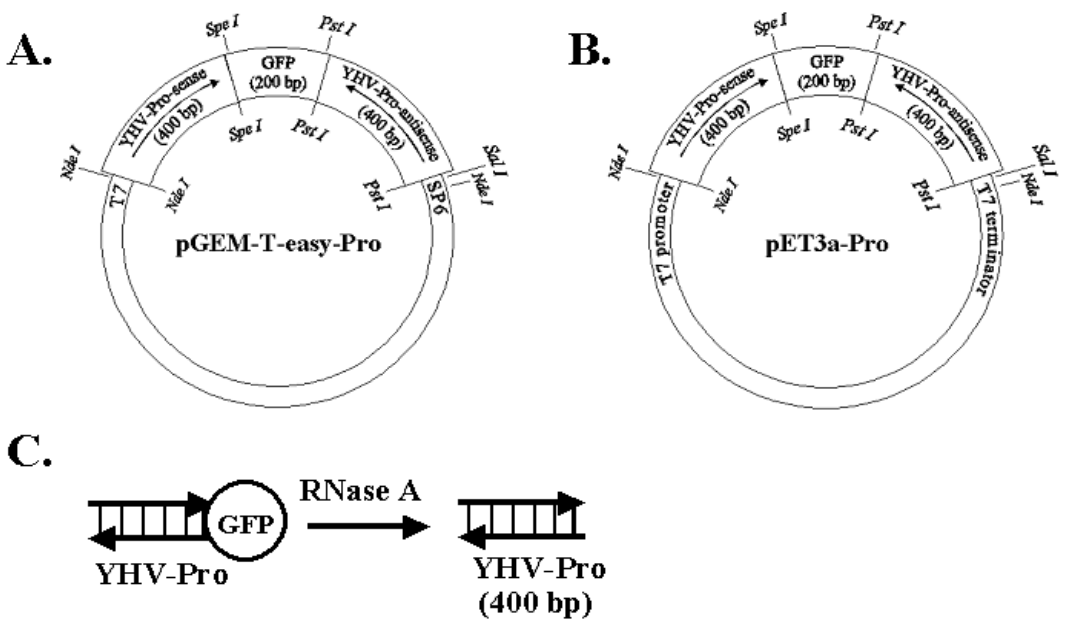

Fig 1. Diagram of plasmid DNA constructs and double-stranded RNA product after RNase A digestion. A) pGEM-T-easy-Pro for *in vitro* transcription. B) pET-3a-Pro for *in vivo* expression in HT115 bacterial host and C) dsRNA-Pro product from both methods.

the *Xba*I site and cloned into the *Nde*I site of pET3a vector to construct pET3a-GFP for an *in vivo* expression of dsRNA-GFP.

Bacterial Induction and dsRNA Purification

Plasmids, pET3a-Pro or pET3a-GFP were transformed by the heat shock method into the HT115 bacterial host, a RNase III deficient *E. coli* strain, with the RNase III gene disrupted by a Tn10 transposon which contains a tetracycline-resistance marker. The genotype of HT115 is as follows: F^-, mcrA, mcrB, IN (rrnD-rrnE)1, lambda$^-$, rnc14::Tn10(DE3) lysogen: lacUV5 promoter-T7 polymerase. The strain was modified to express T7 RNA polymerase from an isopropyl-β-D-thiogalactopyranoside (IPTG) inducible promoter [6]. Therefore, dsRNA can be produced in the HT115 bacterial host after induction with IPTG.

A single colony of the HT115 bacteria containing pET3a-Pro or pET3a-GFP was grown in 3-5 ml LB media containing ampicillin (100 μg/ml) and tetracycline (12.5 μg/ml) overnight at 37 ºC. The bacterial starter culture was diluted 100 fold with 2xYT media containing ampicillin (100 μg/ml) and tetracycline (12.5 μg/ml). The bacterial culture was inoculated in 50 ml 2xYT medium and incubated at 37 ºC until the OD_{600} reached 0.4. T7 RNA polymerase was induced to express dsRNA by the addition of 0.4 mM IPTG. The bacterial culture was further incubated at 37 ºC for 4 hours. Normally, an OD_{600} per ml reached 1 after 4 hours induction. The bacterial cells were harvested by centrifugation at 6000xg for 5 min at 4 ºC. Every one OD-ml of cell pellet was resuspended in 50 μl of 0.1% SDS and boiled for 2 min to lyse the cells. Then, one microgram of RNase A in a total volume of 65 μl buffer (300 mM sodium acetate, 10 mM Tris-Cl pH 7.5, and 5 mM EDTA) was added and incubated at 37 ºC for 5 min in order to remove the single-stranded RNA of the GFP loop (Fig 1C) and the total RNA of the bacterial host. The remaining dsRNA was purified with 200 μl of TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturers' protocol. The concentration of dsRNA was estimated by UV spectrophotometry and adjusted to a final concentration of 1 μg/μl prior to storage at –80 ºC until use.

dsRNA Production by *in vitro* Transcription

The pGEM-T-easy-Pro plasmid (harboring a stem loop construct of 400 bp of the protease gene of YHV) was purified with a QIAGEN midi prep column (QIAGEN, Hilden, Germany). In order to synthesize dsRNA *in vitro*, the plasmid DNA was linearized with *Sal* I restriction enzyme and used as the template to synthesize single-stranded RNA using a Ribomax *in vitro* transcription kit (Promega) as described by the manufacturer. After *in vitro* transcription, the synthesized RNA was annealed to produce dsRNA by incubating the mixture at 70 ºC for 15 min and gradually reducing the temperature to 22ºC[8]. The final concentration of dsRNA-Pro was determined by UV spectrophotometry at 260 nm.

Primary Culture of Lymphoid Cells (Oka Cells)

Primary Oka cell culture of *P. monodon* was prepared as described by Assavalapsakul, et al., 2003[9]. Briefly, lymphoid tissues were isolated from 2 kg of juvenile shrimp (10-15 g each) and washed in washing medium (2x Leibovitz's L15 medium containing 100 IU/ml penicillin, 100 mg/ml streptomycin, 15% fetal bovine serum (FBS) and 5% lactalbumin). The tissues were minced into small pieces and washed in complete medium (washing medium with 15% shrimp meat extract). The minced tissues were seeded onto a 24 well plate and incubated at 26 ºC until a monolayer formed.

Transfection of dsRNA into Primary Culture of Oka Cells

Primary cultures of Oka cells at 70% confluence in 24-well plates were transfected with 1 μg of dsRNA using the transmessenger RNA transfection kit (QIAGEN). Forty hours post-transfection, cells were infected with YHV at different dilutions from $3x10^4$ to $3x10^1$ particles. After YHV infection for 1.5 hours, the excess virus was removed. The fresh complete medium was added and was incubated at 26 ºC. The cells and culture medium were collected at 72 hours after YHV infection.

In this study, the amount of the purified YHV (150 μl per well) corresponding to $3x10^4$ to $3x10^1$ particles was calculated from 10^{-4} to 10^{-7} dilutions of the stock YHV containing $2x10^9$ $TCID_{50}$/ml. Tissue culture infectious dose 50 ($TCID_{50}$), which is the dilution of virus causing cytopathic effect in 50% of the inoculated cell culture, was determined by incubating the primary lymphoid cell culture with 10-fold serial dilutions of YHV. The number of cytopathic foci that represented YHV infection was counted after staining with crystal violet [9].

RT-PCR of the Helicase Gene of YHV and Actin

Total RNA from YHV infected Oka cells was extracted using TRI Reagent$^{@}$- LS (Molecular Research Center, Inc., Cincinnati, Ohio, USA). The first strand cDNA was synthesized using ImProm-II™ reverse transcriptase (Promega) and Oligo dT primer according to the manufacturer's instructions. Gene specific primers for the helicase gene (800 bp) of YHV (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 primers (5' GAC TCG TAC GTG GGC GAC GAG G 3' and

5' AGC AGC GGT GGT CAT CTC CTG CTC 3') were used to simultaneously amplify YHV and actin mRNAs[7]. The PCR products that used to detect the expression of YHV and actin were analysed by agarose gel electrophoresis.

Western blot Analysis

Oka cell culture medium was collected to determine the YHV replication. The medium (200 µl) was mixed with an equal volume of 4xSDS sample buffer and boiled for 10 min. Equal amounts of proteins were electrophoresed in 10% SDS-polyacrylamide gels and stained by Coomassie brilliant blue as a loading control. In addition, the proteins were transferred onto a PVDF membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with a SemiDry electrophoresis transfer apparatus (Bio-Rad Laboratories, Inc.). The membrane was blocked overnight at 4 ºC in phosphate buffered saline (PBS) containing 5% skimmed milk. The YHV-structural protein was detected by incubating the 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[10]. 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).

Results and Discussion

Double stranded RNA triggers a potent and specific inhibition of its cognate mRNA in the process of RNA interference. dsRNA can be produced using both *in vitro* transcription and *in vivo* expression in bacteria. A large amount of dsRNA is required for use in gene silencing experiments to study gene function and viral defense mechanisms. In this study, we have developed a simple approach with relatively low cost to produce large amounts of dsRNA in *E. coli* strain HT115, which lacks ribonuclease III, an enzyme that normally degrades dsRNA. Induction of dsRNA production can be caused by adding IPTG to induce expression of the T7 RNA polymerase in *E.coli*. Comparison of dsRNA of the protease gene of YHV (dsRNA-Pro) produced by *in vitro* transcription and *in vivo* expression is shown in Fig 2. A major band of 400 bp of dsRNA-Pro was observed using both methods. The faint smear background of the dsRNA-Pro purified from the bacterial cells was due to non-specific contamination of the host RNA. However, *in vivo* dsRNA-Pro can be used as effectively as the *in vitro* dsRNA-Pro. In this experiment, a total of 1.5 mg was obtained from a 50 ml bacterial culture after digestion with RNase A. Therefore, one OD_{600}-ml of the bacterial cell pellet yielded 30 µg dsRNA-Pro. In this method, the production of dsRNA can be scaled up by simply growing a larger amount of the bacterial culture. On the other hand, an *in vitro* transcribed hairpin RNA requires a linearized DNA template of at least 5-10 µg in order to produce milligram amounts of dsRNA. It is time consuming, labor intensive and expensive to produce and purify a large amount of linearized DNA template. In addition, the reagents used for an *in vitro* transcription are relatively expensive.

The effectiveness of dsRNA-Pro produced by *in vitro* transcription and *in vivo* bacterial expression was compared. An unrelated dsRNA of green fluorescent protein (dsRNA-GFP) was used as a control for the specificity of dsRNA-Pro on viral replication. The result showed that the YHV genome (Fig 3) and YHV-structural protein, gp-116 (Fig 4) were detected at 72 hours post infection in the cells that were not transfected with dsRNA-Pro (mock) and in those transfected with dsRNA-GFP. The level of the YHV genome and gp-116 protein were viral dose dependent. Transfection of an *in vitro* transcribed dsRNA-Pro specifically knocked down YHV genome (Fig 3) and protein expression (Fig 4) in those cells infected with 30 to $3x10^4$ YHV particles. The dsRNA-Pro produced *in vivo* in *E. coli* showed a similar potency to the *in vitro* transcribed dsRNA-Pro in knocking down YHV replication (Fig 3 and 4). In addition, dsRNA-Pro produced *in vivo* showed no

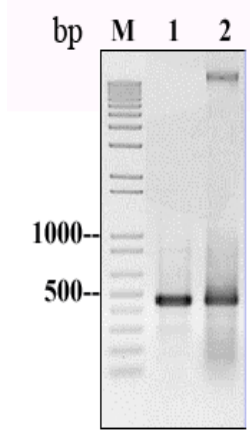

Fig 2. Double-stranded RNA of the protease gene of YHV (dsRNA-Pro) produced using *in vitro* transcription and *in vivo* expression in bacteria. dsRNA-Pro was produced as an *in vitro* transcribed hairpin RNA from a linearized pGEM-T-easy-Pro template and treated with RNase A (lane 1). The bacteria *E. coli* strain HT115 harboring pET3a-Pro was induced with 0.4 mM IPTG to express dsRNA-Pro *in vivo*. Total RNA was isolated and treated with RNase A (lane 2). DsRNAs were electrophoresed through a 1.5% agarose gel and visualized by staining with ethidium bromide. Lane M represents 1 kb Plus DNA Ladder (Invitrogen).

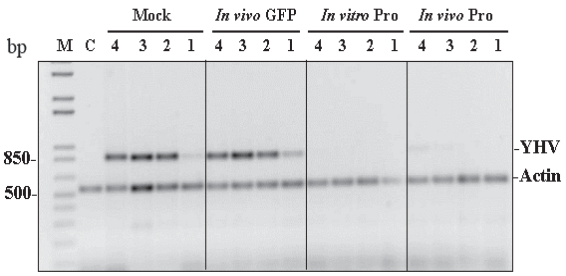

Fig 3. DsRNA-Pro produced *in vitro* and *in vivo* showed similar specific inhibition of YHV replication in Oka cells. The amount of YHV genome was determined by RT-PCR. Mock represents cells without dsRNA transfection. Control cells (C) were not infected with YHV. The numbers 4, 3, 2, and 1 represent the amounts of YHV infected cells at $3x10^4$, $3x10^3$, $3x10^2$ and 30, respectively. Actin was used as an internal control for equal loading. Lane M represents 1 kb Plus DNA Ladder (Invitrogen).

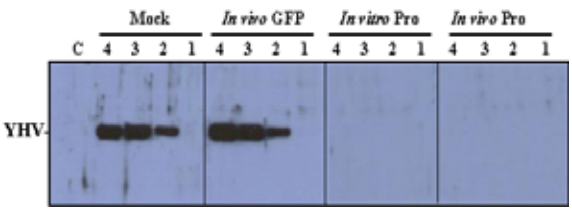

Fig 4. Bacterially expressed dsRNA-Pro inhibits YHV replication at the protein level similar to *in vitro* transcribed dsRNA-Pro. Western blot analysis was performed on the culture medium collected at 72 hr post YHV infection for analysis of YHV structural protein with anti-gp116 antibody. Mock represents cells without dsRNA transfection. Control cells (C) were not infected with YHV. The numbers 4, 3, 2, and 1 represent the amount of YHV with which the cells were infected: $3x10^4$, $3x10^3$, $3x10^2$ and 30 viral particles, respectively.

obvious toxic effect on the transfected cells, and could be injected into shrimp (data not shown). Similarly, a crude extract of the bacterially expressed virus derived dsRNA has been used to protect plants against viral infections [11].

Taken together, *in vivo* production of dsRNA using an *E.coli* bacterial system can be effectively employed to produce a large amount of dsRNA with relatively low cost for RNAi studies. Production of a large amount of dsRNA is required for silencing the expression of a gene *in vivo*. DsRNA-Pro at the dose of 25 μg per shrimp was injected into shrimp in order to knock down YHV replication and reduce mortality [12]. It should be noted that the cost of production of 30 mg dsRNA by the *in vivo* system was approximately one-third of the *in vitro* methodology. The method to generate dsRNA *in vivo* is relatively simple, involving *E. coli* culturing, RNase A treatment of the lysed cells and total RNA extraction. It thus gives an alternative means to produce a large amount of dsRNA at low cost.

Acknowledgements

We thank Wanlop Chinnirunvong for technical assistance and Prof. Duncan R. Smith for critical reading of the manuscript. This work was supported by a Thailand Research Fund (TRF) Senior Research Scholar Award to S.P., and a Commission on Higher Education and TRF grant to C.O.

References

1. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE and Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**:806-11.
2. Meister G and Tuschl T (2004) Mechanisms of gene silencing by double-stranded RNA. *Nature* **431**:343-9.
3. Novina CD and Sharp PA (2004) The RNAi revolution. *Nature* **430**:161-4.
4. Timmons L and Fire A (1998) Specific interference by ingested dsRNA. *Nature* **395**:854.
5. Betz N and Worzella T (2003) Take the RiboMax™ Express for RNAi. *Promega Note* **84**:7-11.
6. Timmons L, Court DL and Fire A (2001) Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*. *Gene* **263**:103-12.
7. Tirasophon W, Roshorm Y and Panyim S (2005) Silencing of yellow head virus replication in penaeid shrimp cells by dsRNA. *Biochem Biophys Res Commun* **334**:102-7.
8. Worby CA, Simonson-Leff N and Dixon JE (2001) RNA interference of gene expression (RNAi) in cultured *Drosophila* cells. *Sci STKE* 2001:PL1.
9. Assavalapsakul W, Smith DR and Panyim S (2003) Propagation of infectious yellow head virus particles prior to cytopathic effect in primary lymphoid cell cultures of *Penaeus monodon*. *Dis Aquat Organ* **55**:253-8.
10. Assavalapsakul W, Tirasophon W, and Panyim S (2005) Antiserum to the gp116 glycoprotein of yellow head virus neutralizes infectivity in primary lymphoid organ cells of *Penaeus monodon*. *Dis Aquat Organ* **63**:85-8.
11. Tenllado F, Martinez-Garcia B, Vargas M and Diaz-Ruiz JR (2003) Crude extracts of bacterially expressed dsRNA can be used to protect plants against virus infections. *BMC Biotechnol* **3**:3.
12. Yodmaung S, Tirasophon W, Roshorm Y, Chinnirunwong W and Panyim S (2006) YHV-protease dsRNA inhibits YHV replication in *Penaeus monodon* and prevents mortality. *Biochem Biophys Res Commun* **341**:351-6.