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Comparison of Knockdown Efficiency of GW182 by dsRNAs Specific to GW182

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***Abstract*:** *Shrimp, especially Penaeus monodon and P. vannamei, plays an important role in Thai aquaculture industry. Many shrimp diseases that lead to severe mortality and subsequence economic losses are caused by viral infection, such as Yellow Head Syndrome and White Spot Syndrome. MicroRNA (miRNA) is one of the three classes of small RNAs that confer target specificity to RNA interference, a potent antiviral mechanism in shrimp. To silence its target transcript, a miRNA is loaded into Argonaute to form a miRNA-induced silencing complex (miRISC). However, the miRISC is not functionally competent, due to the imperfect complementarity between the miRNA and its target, until Argonaute interacts with its binding partner GW182. The essential role of GW182 is highlighted by reports that its depletion in many organisms resulted in derepression of miRNA targets. In shrimp, many components in miRISC have been functionally characterized in the contex of viral infection, including three Argonautes and MOV-10. However, functions of GW182 in shrimp are still unknown. This report is part of a study that aims to characterize effect of GW182 knockdown in shrimp that are infected by Yellow Head Virus (YHV). Here, two double-stranded RNAs were used to identify conditions that were most efficient at knocking down the expression of GW182 in P. vannamei. The results revealed that the dsRNA-GW182 which binds to a region in the M-domain of GW182 provides a higher knockdown efficiency at 3 days after injection. Further study of GW182 function using dsRNA-mediated knockdown is underway to determine the role of GW182 in shrimp infected with YHV in P. vannamei.*

***Keywords*:** *RNAi, GW182, miRNA, Yellow Head virus, dsRNA*

1. Introduction

Yellow head virus (YHV) has been discovered in Thailand since 1992 [1], [2]. Yellow head disease causes high cumulative mortality in shrimp culture up to 100% within three to five days [2]. As an invetebrate, shrimp solely relies an innate immune system to defend against viral infection. RNA interference (RNAi) is one of the major mechanisms in this branch of immunity [3].

RNAi is a highly conserved post-transcriptional gene silencing mechanism [4] of which specificity is guided by small RNAs. There are two small RNAs that are involved in an antiviral mechanism, namely small interfering RNA (siRNA) and microRNA (miRNA) [5], [6]. To exert its silencing function, the small RNA must be incorporated into a multiprotein complex called an RNA induced silencing complex (RISC) [7], of which core enzymatic component is a member of protein in the Argonaute (Ago) family [7].

GW182 is an Ago-binding protein that is involved in miRNA-mediated silencing by translation repression, deadenylation and mRNA decay [8]. For an effector step of miRNA-mediated silencing, the partial complementary between miRNA and mRNA target requires the Ago-GW182 interaction [9]. In many organisms, such as human and *Drosophila*, the deletion of GW182 in cell lines results in a derepression of targeting mRNAs [9], [10]. Therefore, GW182 plays a crucial role in the miRNA pathway.

Proteins in the GW182 family contain two major domains: an N-terminal Ago-binding domain (N-term) and a silencing domain (Fig. 1). The N-term domain is a glycine (G)-tryptophan (W) rich region that is required for Ago subfamily protein interaction [8]. The silencing domain functions in silencing, comprises of Mid region (M1 and M2), poly(A)-binding protein interacting motif 2 (PAM2) and RNA recognition motif (RRM).

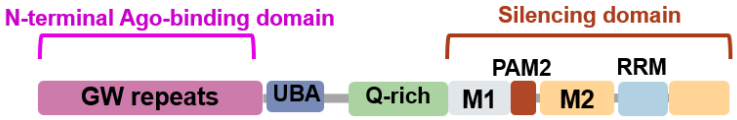

Fig. 1: A schematic diagram representing GW182 domain organization. The N-terminal Ago-binding domain characterized by glycine (G)-tryptophan (W) rich repeats is purple. The center of the protein contains a UBA-like domain (blue) and glutamine(Q) rich region (green). A silencing domain containing a Mid domain; M1 (grey) and M2 (orange), a poly(A)-binding protein interacting motif 2 (PAM2, brown), and RNA recognition motif (RRM, light blue) are located in the C-terminus.

The discovery of various RNAi components, including DGCR8/Pasha, Dicer, Ago and MOV-10 [11], [12], indicates that RNAi is functional in shrimp. As part of an investigation into how the miRNA pathway, particularly GW182, responds to viral infection, this study aims to identify methods by which the level of GW182 expression can be repressed by gene-specific dsRNAs.

2. Materials and Methods

2.1. Experimental Shrimp

1-2 g White leg shrimp (*P. vannamei*) that were free from viral pathogens were obtained from local shrimp farms in Thailand. Shrimp were reared in laboratory tanks containing artificial sea water at 10 ppt salinity, fed with a commercial feed every day and allowed to acclimatize for 1 day before injection.

2.2. Production of dsRNA using an *in vivo* Bacterial Expression System

A stem loop construct (454 bp) covering the M-domain region of GW182 (Fig. 1) was cloned into a pET28a vector to generate a pdsRNA-GW182#1 plasmid for expressing dsRNA-GW182#1. Similarly, another stem loop construct (502 bp) encoding a non-conserved region in GW182 was cloned into the pET28a vector to produce a pdsRNA-GW182#2 plasmid for dsRNA-GW182#2 expression. After confirming the accuracy of the dsRNA templates by sequencing, each plasmid was transformed into a RNase III deficient *Eschericia coli* strain, HT115(DE3). Overnight culture of the tranformed bacteria was diluted 100-fold in fresh LB media supplemented with 100 µg/ml kanamycin and 12.5 µg/ml tetracycline. The culture was incubated at 37 ˚C with constant shaking at 250 rpm until OD_{600} reached 0.4. The expression of dsRNA was induced with 0.4 mM IPTG at 37˚C for 3 hours until OD_{600} reached ~1. The bacterial culture was harvested using centrifugation at 6000g at 4˚C for 5 min. The cell pellet was resuspended with 5 ml of 75% ethanol in 1x PBS per 1 OD cell and incubated at room temperature for 5 min or at -20˚C overnight. After centrifugation at 6000g at 4˚C for 5 min, the remaining cells were resuspended in 1 mL or less of 150 mM NaCl and further incubated at room temperature for 1 hour prior to centrifugation at 8000g at 4˚C for 10 min. dsRNAs in the supernatant phase were collected [13] and verified by RNase A and RNase III enzymatic analysis.

2.3. Determination of Efficiency of dsRNA-Mediated Knockdown of GW182

Shrimp were injected with either dsRNA-GW182#1, dsRNA-GW182#2 or 1:1 mixture of the two dsRNAs at 2.5 µg/g shrimp. A control group was injected with the same volume of 150mM NaCl. Gills were collected on day 1, 3 and 6 after injection for RNA extraction by RiboZol ™ reagent (Amresco, USA) according to manufacturer's protocol. 2 µg of total RNA was used for cDNA synthesis by Improm II reverse transcriptase (Promega, USA) with oligo-dT_{20} primers following the manufacterer's protocol.

Simultaneous detection of GW182 and an internal control gene, actin, was performed by multiplex PCR. The 25 µl of PCR reaction mixture contained 0.25 µM of each GW182 primer, 0.05 µM of each actin primer, 0.2 mM dNTP, 0.5 unit of Taq DNA polymerase (New England Biolabs, USA) and 1 µl of the cDNA template. The multiplex PCR condition was performed as follows: 95˚C for 5 min, 30 cycles of 95˚C for 30 s, 60˚C for 30 s, and 68˚C for 45 s, with a final extension at 68˚C for 5 min. PCR products (650 bp for GW182 and 550 bp for actin) were analyzed on 1.5% agarose gel electrophoresis. Using a Scion Image analysis program, a relative level of GW182 expression was normalized against actin, analyzed and expressed as mean ± standard error of mean (SEM). The statistical analysis was performed by a one-way ANOVA method in the GraphPad program.

3. Results and Discussions

3.1. dsRNA specific to GW182 can be Produced by a Bacterial Expression System

With the bacterial expression system, 4 mg per 1 litre culture volume of dsRNA-GW182#1 and 5 mg per 1 litre culture volume of dsRNA-GW182#2 were obtained. To verify the hairpin structure of each dsRNA, enzymatic treatments were performed. Compared to the untreated-lane, there is a noticeable increase in mobility of the RNA band in lane U (Fig. 2). This is due to the endonucleolytic activity of RNase A which cleaves the single-stranded RNA (ssRNA) loop, at C and U residues [14], that connects the two inverted sequences constituting the stem of the dsRNA. In contrast, the dsRNA was degraded by a dsRNA-specific RNase III (Fig. 2, lane III). This shows that the dsRNA produced in this study adopted a hair-pin structure.

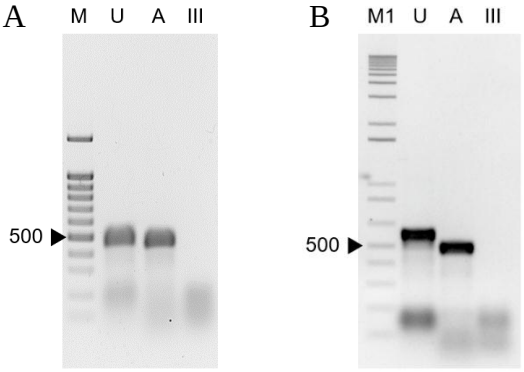

Fig. 2: Agarose gel electrophoresis of hairpin (A) dsRNA-GW182#1 and (B) dsRNA-GW182#2 expressed by the in vivo bacterial expression system and purified by the ethanol extraction method [13]. Lane M is a 100-bp DNA marker (Siberian Enzyme, Russia), M1 is a 1-kb plus DNA marker (New England Biolabs, USA), and lane U is purified dsRNA without enzymatic treatment. Lane A is dsRNA treated with RNase A. Lane III is dsRNA treated with RNase III.

3.2. Investigation of the knockdown efficiency of dsRNA-GW182#1, #2 and a Mixture of both dsRNAs

After injecting shrimp with 2.5 µg/g shrimp of dsRNAs or NaCl as outlined, gills were individually collected after day 1, 3 and 6 post injection for RNA extraction and RT-PCR analysis to observe the mRNA level of GW182 (Fig. 3). The results showed that the 1:1 mixture of dsRNA-GW182#1:dsRNA-GW182#2 partially decreased the level of GW182 expression by approximately 58% after 24 hours of injection. After 3 days, injection of dsRNA-GW182#1 showed suppression of GW182 by approximately 61%, whereas

administration of dsRNA-GW182#2 or the dsRNA mixture brought about approximately 44% suppression. At day 6, the expression of GW182 returned to the pre-knockdown level. In the groups injected with dsRNA-GW182#1 or #2, however, the expression of GW182 was still downregulated by approximately 50% (Fig. 3).

These indicated that either dsRNAs targeting GW182 #1, #2 or the mixture of dsRNA-GW182 can partially knockdown GW182 expression. The dsRNA-GW182#1 provided the highest knockdown efficiency (61%) from the relative GW182 mRNA expression at day 3 after injection (Fig. 3). While the combinatorial injection approach appeared to be more efficient on day 1, individual dsRNA was more efficient at GW182 suppression at 3 and 6 days. The lower efficiency of dsRNAs mixture might be caused by the half-diluted concentration of each dsRNA.

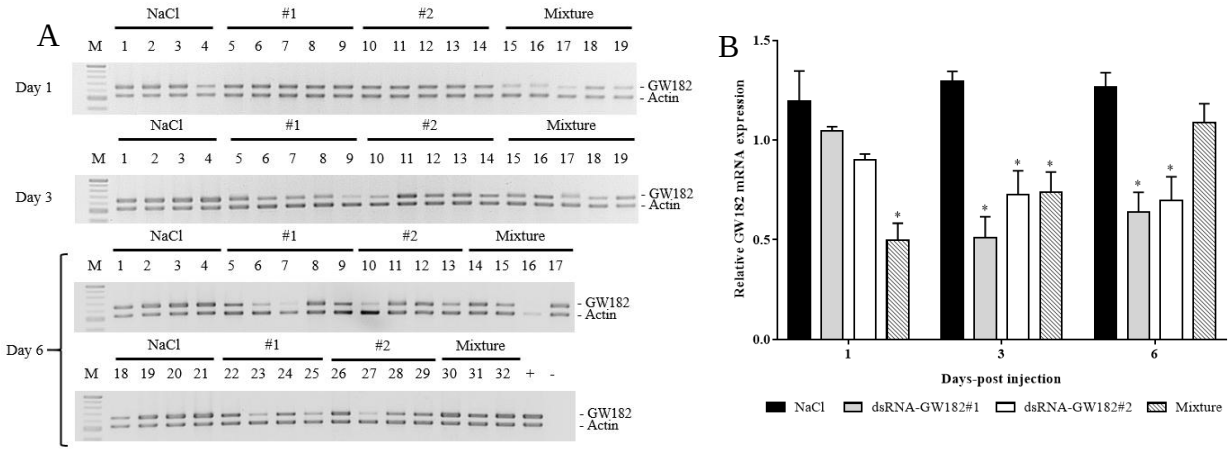

Fig. 3: The level of GW182 expression after injecting shrimp with dsRNA-GW182#1, dsRNA-GW182#2 and the 1:1 mixture of both RNA was detected by multiplex RT-PCR (A) and analyzed using actin as an internal control (B). Multiplex RT-PCR products of GW182 and actin were amplified from gill individually collected at day 1, 3 and 6 post injection with 150 mM NaCl, 2.5 µg/g shrimp of dsRNA-GW182#1, dsRNA-GW182#2 or the 1:1 mixture of both dsRNA. The relative expression of GW182 in response to dsRNA-GW182 normalized by actin. The relative GW182 expression was determined from intensity of bands on the agarose gel and normalized using the NaCl group in each time point. Error bars represent standard error of the mean (SEM) (n = 4-9). The asterisks (*) represent the significant difference ($p < 0.05$) of the relative GW182 expression comparing to the NaCl group in each time point.

4. Conclusion

This study investigated the suppression of GW182 condition using dsRNA-GW182 injection. The relative expression of GW182 was observed on day 1, 3 and 6 after injected with 2.5 µg/g shrimp of dsRNA-GW182#1, #2 or the mixture of both dsRNAs. The injection of all dsRNA-GW182 led to partially knockdown GW182 expression at approximately 40-60%. The mixture of the two dsRNAs showed no improvement in the knockdown efficiency. These results suggest that dsRNA-GW182#1 is probably more effective than dsRNA-GW182#2 and the mixture of both for GW182 suppression as, at dose 2.5 µg/g shrimp, dsRNA-GW182#1 provided the highest knockdown efficiency.

5. Acknowledgement

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