CHAPTER V
DISCUSSION

RNA interference (RNAi) plays an important role in animals and plants as an antiviral defense mechanism. In shrimp, RNAi technology has been extensively used for inhibition of viral replication (14, 22, 27) and studying gene function. Recently, the key proteins in the RNAi pathway of shrimp have been identified such as Dicer, Argonaute, including the proteins associated in the RNA-induced silencing complex (RISC) such as VIG, dFXR and Tudor staphylococcal nuclease (TSN) (7-10, 25, 32, 41, 42). In addition, the dsRBP, Loquacious that associates with Dicer for its function in RNAi pathway was found in shrimp. In this study, suppression of PmLoqs was used to investigate the knockdown effect of PmLoqs on RNAi efficiency in peneaid shrimp.

Recently, dsRBP in *F. chinensis* (FcTRBP) has been characterized. It consists of three different isoforms that have size about 1.5 kb and contains three dsRBD (38). In addition, TRBP plays a crucial role in the dsRNA induced RNAi silience (39). Previously, the partial cDNA sequences of three different isoforms of the putative Loqs of *Penaeus monodon* (PmLoqs) have been characterized. It consists of three dsRBD and have size about 1.1 kb. The sequence in the 5’ direction is not complete. Sequence alignments demonstrated that the putative PmLoqs is highly conserved with TRBP family of *Fenneropenaeus chinensis,* especially in the dsRBD regions but differ at the C-terminus. PmLoqs isoform I and II consist of three dsRBD whereas isoform III has only two dsRBDs and lacks the C-terminal domain. Therefore, the aim of this study is to clone the full-length cDNA encoding the *Penaeus monodon* Loquacious (PmLoqs) and characterize functions of PmLoqs that involved in the RNAi pathway in shrimp.

The first step to study the PmLoqs function by using RNAi technique is to generate long dsRNA-PmLoqs by *in vitro* transcription. DsRNA was constructed by using specific primers that designed to corresponding with PmLoqs in two regions.

First, the conserved regions between domain one and two of PmLoqs to knockdown in all isoforms. Second, the conserved region in domain three to knockdown only long isoform. Total yield of dsRNA-PmLoqs and dsRNA-PmLoqsL are 580 and 710 µg, respectively in 100 µl reactions when using equimolar amounts of 5 µg each of sense and anti-sense PCR generated templates. Both of dsRNA-PmLoqs and dsRNA-PmLoqsL showed a single band of dsRNA size approximately 400 bp. All of these bands could be cleaved by RNase III suggesting that they are dsRNA and have good quality that can be used for subsequent studies.

To investigate the efficiency of dsRNA-PmLoqs or dsRNA-PmLoqsL in suppression of PmLoqs, shrimp were injected with dsRNA-PmLoqs and dsRNA-PmLoqsL in order to knockdown PmLoqs mRNA expression. After 3 days post-injection, a partial knockdown of PmLoqs in dsRNA-PmLoqs and dsRNA-PmLoqsL injected groups can be observed when using the primers that amplified all isoforms of PmLoqs. However, the same cDNAs cannot be used to detect specific long isoforms of PmLoqs. Therefore, optimization of the PCR condition is required.

Then the other approach that was used to synthesize dsRNA is *in vivo* bacterial expression. The yield of dsRNA-PmLoqs that produced by *in vivo* bacterial expression is 45 µg /OD cell culture and also have a single band of dsRNA size approximately 400 bp that can be cleaved by RNase III similar to the dsRNA prepared by *in vitro* transcription method. The advantage of this method is it could be scaled up the amount of dsRNAs by growing a larger amount of the bacterial cell culture whereas an *in vitro* transcription requires a larger amount of DNA template. It is time consuming and expensive.

After 3 days post-injection, the dsRNA-PmLoqs that was produced by *in vivo* bacterial expression showed a similar potency to dsRNA-PmLoqs prepared by the *in vitro* transcription in suppression of PmLoq approximately 85%. This result was not different to the previous study that showed similar silencing effect to inhibit target gene by dsRNA from two approaches (47).

According to the results in this study (Figure 4-17), it is noticeable that dsRNA-GFP can efficiently knockdown PmLoqs expression. Recently, it has been reported that siRNA has an effect on unintended transcripts that contain partial complementarity. It could direct degradation of mRNAs that were only

complementary in part through “off-target” effect (43). Recently, the off-target gene regulation by RNAi had been reported. The partial identity to the dsRNA sequence could silent non-targeted transcript. This phenomenon crucially reminded when RNAi technology was used. Previous study demonstrated that dsRNA corresponding to *MAPK14* gene showed the off-target silencing of genes *KPNB3* and *FLJ20291* in mammalian cells and revealed concentration of dsRNA-dependent off-target gene expression (43). Therefore, it is possible that the non-specific inhibition may be resulted from high amount of dsRNA-GFP. In addition, the results in Figure 4-19 showed that dsRNA-GFP can efficiently knockdown PmLoqs expression similar to preliminary data but it has no effect to PmRab7 expression. Previously Lin *et al.* 2005 had reported that only a 7 nucleotide complementation between the siRNA and the target is sufficient to cause gene silencing (44). It is likely that dsRNA-GFP that introduced into the shrimp and generated siRNA population may interact non-specifically through the 7 nucleotide sequences of an endogenous gene PmLoqs but not PmRab7. However, the sequence specific inhibition by dsRNA-PmLoqs has percent inhibition more than the sequence non-specific inhibition by dsRNA-GFP. It is possible that the effect of PmLoqs knockdown is operated through both sequence specific and non-specific mechanism.

To investigate the effect of PmLoqs in the RNAi pathway, PmLoqs mRNA expression was suppressed by dsRNA-PmLoqs before injection with dsRNA-PmRab7 0.63 µg/g shrimp. From Figure 4-18, the result showed that all of shrimps that injected with dsRNA-PmLoqs showed partially knockdown of PmLoqs. Similarly, shrimp that injected with dsRNA-PmRab7 showed completely knockdown of PmRab7 mRNA expression. Therefore, semi quantitative analysis based on running the PCR products by gel electrophoresis cannot illustrate the effect of knockdown PmLoqs on the efficiency of dsRNA-PmRab7 to suppress PmRab7 expression in shrimp. Therefore, all of shrimps in groups that injected with dsRNA-PmRab7 were further characterized by using real-time PCR approach.

The result from real-time PCR revealed that shrimps injected with dsRNA-PmLoqs prior to injection of dsRNA-PmRab7 have a greater effect to knockdown endogenous PmRab7 mRNA expression approximately 85% when compared with the control group. Similar result was observed in *Drosophila.*

Depletion of *Drosophila* Loqs leads to hyper repression in RNAi efficiency. The result can be explained that in *Drosophila*, Loqs antagonizes R2D2 mediated exo-siRNA silencing and decreasing the level of either R2D2 or Loqs resulted in an increase silencing activity of the other pathway (45). In contrast, the silencing of TRBP in *M. japonicus* resulted in the impairment of the RNAi pathway (39). In Wang., *et al* study (39), dsRNA targeted prophenoloxidase gene (PPO) was used as a candidate dsRNA whereas dsRNA-PmRab7 was used in this study. PPO plays an important role in response to the innate immunity in shrimp. Injection of dsRNA is possibly triggered an increase in expression of PPO and may result in less efficient in silencing PPO expression. Therefore, suppression of PmLoqs is possibly involved in RNAi pathway by increasing the efficiency of dsRNA to inhibit gene expression. However, the PmRab7 that was used as a model in this study is an endogenous gene and is not involved in the innate immune response. Moreover, similar experiment was designed by studying the function of PmLoqs in RNAi against an exogenous gene such as a viral gene to confirm the suppression effect of PmLoqs to viral infection. Previous study showed that dsRNA targeted to the nonstructural gene such as a protease gene of YHV was effective in silencing the viral replication in shrimp. DsRHA-YHV (pro) 2.5 and 1.25 μg/g shrimp can prevent YHV replication 100 and 60 percent, respectively after 48 hours post infection (49).

The additive RNAi effect through a target gene after silencing of PmLoqs expression was studied by injection shrimp with dsRNA-PmLoqs before challenging with either YHV or co-injection of YHV and dsRNA-YHV (pro) 1.25 μg/g shrimp. After 48 and 72 hours post infection, the result revealed that suppression of PmLoqs expression increased the effect of dsRNA-YHV (pro) 1.25 μg/g shrimp to significantly reduction of YHV replication more than the control group (have PmLoqs expression) approximately 27 and 10%, respectively. This result indicated that knockdown of PmLoqs has an effect on increasing the efficiency of dsRNA-mediated gene silencing in shrimp and resulted in an increase of the ability of dsRNA-YHV (pro) in inhibition of YHV replication.

In contrast, silencing of other RNAi components resulted in a general loss of RNAi mechanism. In shrimp, knockdown of the core RNAi machinery, Pem-ago1 led to the impairment of RNAi to silence the endogenous gene in primary lymphoid

cell culture (40). Knockdown of PmDicer (PmDcr-1) enhanced the susceptibility of gill-associated virus infection (46). Furthermore, knockdown of PmTSN diminished the efficiency of dsRNA-mediated gene silencing in juvenile shrimp (10). Similarly, knockdown of some components which play a minor role in RNAi such as VIG found to partially reduce RNAi activity in *Drosophila* (9).

In addition, the level of PmLoqs mRNA expression in hemolymph on YHV infected shrimp did not change significantly when compared to NaCl group. PmLoqs may function as a minor component which affects to the efficiency of RNAi, but not an essential component for this mechanism. However, the result showed that it could not completely knockdown the expression of PmLoqs. Therefore, it has possibility that the residual PmLoqs in the shrimp RNAi pathway can still be functioned. This effect may interfere to the effect of PmLoqs knockdown on dsRNA mediated gene silencing in shrimp.

To identify the full-length cDNA encoding Loquacious, primers were designed from the sequence of TRBP (*Fenneropenaeus chinensis*) in the 5’ direction to amplify the sequence of PmLoq in the 5’ end. After the nucleotide sequence in the 5’ direction of PmLoqs was obtained, the primers that designed from the 5’ end were used to amplify the whole gene with oligo-dT primer. The population size of PCR products approximately 1.5 kb was observed. Mixed PCR products were purified, cloned and three recombinant plasmids that contain whole sequence of PmLoqs were subjected for sequencing.

The result showed that the full-length cDNA of PmLoqs is 1,464 bp, encoding 323 amino acids that contain three dsRBD. Analysis of the amino acid sequence of PmLoqs revealed that PmLoqs showed the domain arrangement similar to the TRBP isoform 2 of *Fenneropenaeus chinensis*. However, we cannot observe the other isoforms that are identical to isoforms 1 and 3 of the FcTRBP. It may be due to the limitation of the number of clone that subjected for sequencing. Based on phylogenetic analysis, PmLoqs could be divided into two categories. Sequence analysis showed that PmLoqs distinct from mammalian in term of amino acid sequence similarities. Moreover, phylogenetic analysis revealed that PmLoqs is closely related to the invertebrate dsRBP. It has sequence identity about 100 % with

TRBP isoform 2 of *F. chinensis*. From these results, it can be proposed that PmLoqs represent to dsRBP in arthropod.

From this study, the Dicer-binding protein, PmLoqs was identified in *P. monodon*. Suppression of PmLoqs resulted in an increase in the efficiency of dsRNA to silence gene expression. These results suggested the possible involvement of PmLoqs in the RNAi pathway in shrimp. Although, little is known about the function of the RNAi machineries in shrimp, the key proteins of the shrimp RNAi pathway have been identified including argonaute (40), Dicer-1 (46) and Dicer-2 (37). Whether or not these Dicer isoforms in shrimp play a distinct role in the RNAi pathway as have been shown in *Drosophila* remain to be investigated. The distinct role in RNAi of Dicer has been characterized in the two *Drosophila* Dicer. Dicer-1 interacts with Loqs and is involved in the processing of micro RNA (33). In contrast, Dicer-2 interacts with R2D2 and plays an essential role for loading siRNA into RISC in the siRNA pathway (36). However, the functions of Dicer-1 and Dicer-2 are redundant. Previous studies showed that Loq and R2D2 can interact at the same site of Dicer-2 and form a trimeric complex (45). Even though R2D2 has not been identified in shrimp, an existence of siRNA pathway can be found in shrimp. We can use RNAi as a tool for viral protective immunity or studying gene function (14, 15, 28). Therefore, peneaid shrimp should have the components that are essential for RNAi similar to *Drosophila*. It is possible that PmLoqs may form the complex with Dicer protein and R2D2. Depletion of PmLoqs may result in an increasing in availability of R2D2 to bind Dicer and promote loading of siRNA into the RISC. Therefore, the RNAi efficiency is increased in the PmLoqs knockdown shrimp.

Taken together, this study suggested the possible role of PmLoqs in the shrimp RNAi pathway. Suppression of PmLoqs resulted in an increase in the RNAi efficiency.