CHAPTER I
INTRODUCTION

1.1 RNA interference (RNAi)

RNA interference (RNAi) has been extensively reported to be a powerful mechanism of post-transcriptional gene silencing in animals and plants as an antiviral defense mechanism. RNAi is a post-transcriptional gene silencing process whereby double-stranded RNA triggers the degradation of its homologous mRNA in a sequence-specific manner. In the initiation of RNAi mechanism, long dsRNAs are cleaved by ribonuclease III enzyme, Dicer to generate small dsRNAs of size about 21-23 nucleotides with 5' phosphates and 2 nucleotides overhang at the 3' end which is known as small interfering RNA (siRNA). The siRNA is incorporated into RNA induced silencing complex (RISC). The sense and antisense strands of siRNA are unwound. Only the antisense strand will be targeted to the complementary mRNA and leads to mRNA degradation (1). Integral to this process, initiation enzyme is the type III ribonuclease (RNase III), Dicer which is responsible for cleavage of long dsRNAs into siRNAs. Dicer can target to dsRNAs or highly structured single-stranded RNA (ssRNAs) encoded by viruses, inverted terminal repeat RNAs encoded by transposons, or experimentally delivered dsRNAs or hairpin RNAs (2). In addition, Dicer or Dicer-like enzymes also has an important role in the processing of micro RNA (miRNA) (3). Biogenesis of miRNA occurs in a two-step process. In the nucleus (*Drosophila*), the primary miRNA (pri-miRNA) transcript is processed by the RNase III, Drosha into 70-nt stem loop pre-miRNA (4). Drosha requires the company of the dsRNA-binding protein, Pasha for processing pre-miRNA. Once generated, pre-miRNA is exported to the cytoplasm and cleaved by Dicer-1 into 21–22-nt mature miRNA (4, 5). The miRNA is incorporated into RISC and perfect complementary of miRNA with target mRNA induced mRNA degradation similar to siRNA process whereas imperfect complementary will inhibit translation of target mRNA resulting in translational repression (6) (Figure 1-1).

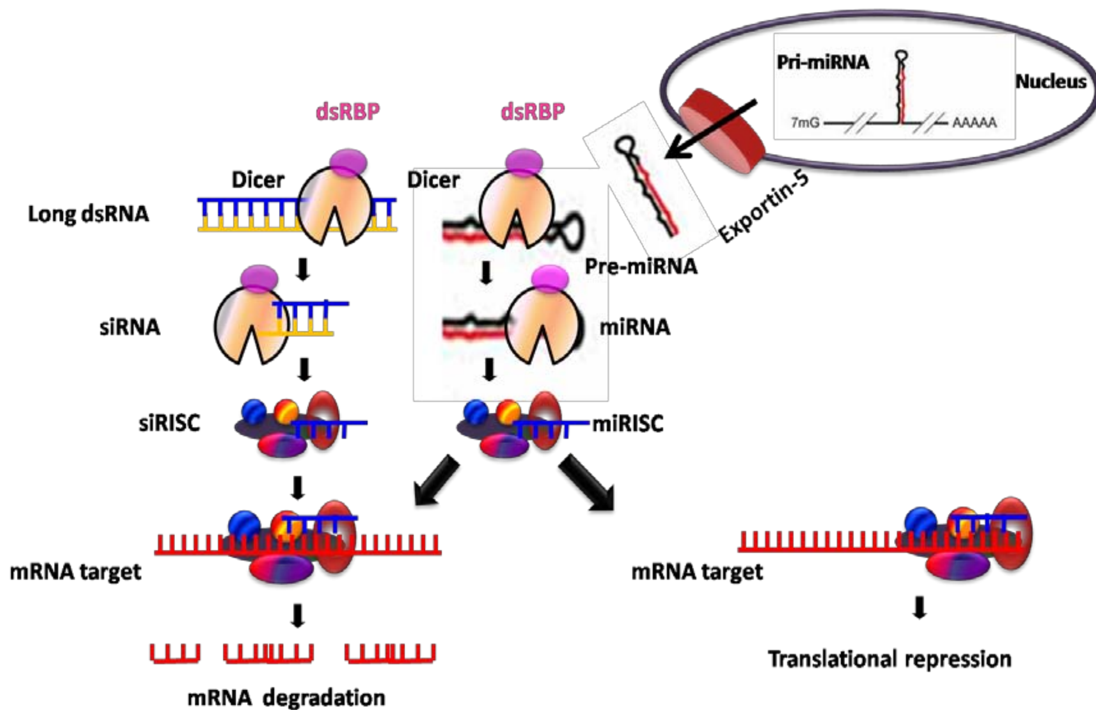

Figure 1-1 RNA interference pathway.

Double stranded RNA (dsRNA) can come from either exogenous dsRNA (long dsRNA) or endogenous pre-miRNA (from nucleus). DsRNA was cleaved to small interfering RNA (siRNA) or mature miRNA by Dicer that incorporated with double stranded RNA binding protein (dsRBP) and loaded into RNA induced silencing complex (RISC). The siRNA and miRNA that complementary with mRNA target will guide to mRNA degradation whereas uncomplimentary miRNA leads to inhibit the translational process.

1.2 RNAi machineries

Previous studies showed that many components of RNAi were identified. Argonuate (Ago) protein plays a primary role in developmental control and also involves in mRNA degradation pathway via RNAi. In *Drosophila*, two argonuates were identified. Ago1 is required for the efficient RNAi. Mutant ago1 resulted in decrease in the ability of mRNA degradation in response to dsRNA whereas Ago2 showed biochemically to be the important component in RISC (7). In addition, a number of the associated proteins in the RISC such as VIG, dFXR and Tudor staphylococcal nuclease were identified (8, 9). In *Drosophila* S2 cells, VIG functioned in the maintaining of the silencing efficiency and the suppression of VIG expression resulted in inhibition of the RNAi activity (9). The dFXR protein functions in repressing the translation of an mRNA encoding the microtubule-associated protein (9). Tudor staphylococcal nuclease (TSN) is composed of staphylococcal nuclease domains and a tudor domain. Knockdown of TSN showed to diminish the efficiency of dsRNA-mediated gene silencing in shrimp (10). Moreover, one of the key proteins in the RNAi pathway has been identified as Dicer. Dicer protein is a member of RNase III family which recognizes dsRNA and digests into small RNA (11). Two isoforms of Dicer, Dicer-1 and Dicer-2 have been found in *Drosophila*. Genetic studies suggested that Dicer-1 and Dicer-2 are involved in miRNA and siRNA production, respectively (12). Furthermore, recent study showed that Dicer is associated with dsRNA-binding protein (dsRBP) for its efficient function in the RNAi pathway (13).

1.3 The black tiger shrimp

Penaeus monodon (black tiger shrimp) is one of the most economically important animals in Thailand. Nowadays, the production loss of shrimps caused by viral diseases are remained a major problem of shrimp culture industries worldwide. The virus pathogens such as white spot syndrome virus (WSSV) and yellow head virus (YHV) are the major cause of serious diseases that resulted in high mortality in penaeid shrimp and lead to economical loss in shrimp culture. Novel strategies to control the viral disease problem are highly desirable. One of the technologies that has been studied to solve this problem is RNA interference (RNAi). RNAi has been

employed as a tool to inhibit replication of several viruses such as YHV, WSSV, etc (14). Injection of dsRNA that corresponding to either viral genes or endogenous genes could be used to prevent or cure virus infections in shrimp (15).

1.3.1 Viral diseases in the black tiger shrimp

1.3.1.1Yellow head virus (YHV)

Yellow head virus (YHV) was first characterized in Thailand in 1993. It is an enveloped, rod-shape virus with size about 150-170 nm in length. YHV belongs to genus *Okavirus* in the order *Nidovirales*. Its genome is a positive sense, single-stranded RNA. Morphology of YHV revealed enveloped bacilliform which showed particle size about 50-60 x 190-200 nm and the particles contained the internal helical nucleocapsid (16) which was closely surrounded by an envelope studded with prominent peplomers or spikes (17). YHV complete genome contains 26,662 nt sequence that consists of ORF1a, ORF1b, ORF2 and ORF3. YHV contains three structural proteins that have two major structural transmembrane glycoproteins (gp116 and gp64) and a nucleoprotein (p20) (18). The 3'-terminal region of the YHV genome from the ORF3 termination codon to the poly A tail (677 nt) shows relatively high level of nucleotide identity with GAV ORF4 sequence. However, this region of YHV contains multiple termination codons in all three reading frames which were introduced by a series of addition, deletion and substitution of nucleotide sequences (19).

YHV is a major virulent pathogen of *P. monodon* that leads to high mortality in shrimp farm. The infected shrimp will swim erratically near the surface of the pond, refuse feed, display yellowish head and start to die. The mortality will occur within 2 to 3 days after the appearance of clinical signs (20, 21). The primary targets of YHV replication are gill and lymphoid organ. However, YHV can infect various tissues including midgut, nerve cord, heart, abdominal muscle, hepatopancreas and eyestalk (18). The moribund shrimp shows abnormal characteristic in hemocytes and cells of connective tissues including necrosis cells, vacuolated cells and densely basophilic cytoplasmic inclusions which are usually located adjacent to hypertrophied nuclei.

1.3.2 RNA interference (RNAi) in shrimp

In shrimp, RNAi was demonstrated to have an efficiency to inhibit replication of several viruses. Inhibition of viral replications by using RNAi can be divided into 2 approaches. First, suppression of viral genes such as white spot syndrome virus (WSSV) genes including DNA polymerase, ribonucleotide reductase small subunit (rr2) (22) and vp28 resulted in reduction of WSSV replication (23, 24). Second, an endogenous gene which is a cellular factor that required for viral replication could be used as an alternative target to inhibit viral infection. Silencing of caspase-3 that involved in apoptosis process in defense mechanism against viral infection in *L.vannamei* by dsRNA resulted in delay time of death and reduce time of cumulative mortality in shrimp (25).

1.3.2.1 RNAi efficiency to inhibit YHV replication in *Penaeus monodon*.

In *Penaeus monodon*, RNAi technology was employed as a tool to defense viral replication and mortality. YHV infection could be inhibited by using either dsRNA corresponding to a protease gene of virus [dsRNA-YHV (pro)] or an endogenous gene, PmRab7 (dsRNA-PmRab7). Silencing of viral expression by using dsRNAs corresponding to a viral gene both nonstructural and structural genes resulted in inhibition of yellow head virus (YHV) replication. DsRNA corresponding to a protease gene of YHV can completely inhibit YHV replication after 2 days YHV challenge in both primary cultures of *Penaeus monodon* lymphoid (Oka) cells and in juvenile shrimp through RNAi pathway (26). In addition, YHV-protease dsRNA can cure YHV infected shrimp up to 24 hours post YHV injection and prevent mortality in juvenile shrimp (27). Moreover, suppression of an endogenous gene, PmRab7 by using dsRNA corresponding to PmRab7 gene showed the knockdown effect of PmRab7 gene in *Penaeus monodon* through RNAi process and the silencing of PmRab7 mRNA can prevent YHV replication similar to using the dsRNA targeted a viral gene and delay shrimp mortality (14). In addition, suppression of an endogenous gene, PmRab7 by dsRNA could inhibit replications of several viruses such as WSSV, YHV, Laem-singh virus (LSNV) and Taura syndrome virus (TSV) (14, 15, 28).

Therefore, identification and characterization of these RNAi machineries will be essential for understanding the RNAi mechanism in shrimp.

1.4 DsRNA-binding protein

A number of prokaryotic, eukaryotic, and even viral proteins have been demonstrated to interact with dsRNA specifically to regulate signaling and gene expression in the cell. These dsRNA binding proteins (dsRBP) that were identified in many species contain conserved dsRNA binding domain (dsRBD).

In human cells, the dsRBP that associates with Dicer is the TAR RNA binding protein (TRBP). TRBP1 and TRBP2 are isoforms of the cellular protein TRBP which was isolated by its ability to bind the human immunodeficiency (HIV)-1 TAR RNA and characterized for its stimulation of the expression of the HIV long terminal repeat in human and murine cells (29). This protein is required for RNAi function mediated by both siRNAs and miRNAs, where it acts as a biosensor in the choice of dsRNA loaded into the RISC. The TRBP-Dicer interaction has functioned as a part of the RISC and has been identified as an important component of the RNAi pathway (30).

Recently, dsRBP has been identified as an important Dicer-interacting protein in mammals (31). Previous studies have shown that knockdown of TRBP by dsRNA resulted in a general loss of RNA interference (RNAi) mediated silencing of other target mRNAs (32).

In addition, the dsRBP that associates with Dicer in *Drosophila* are Loquacious (Loqs) and R2D2. *Drosophila* Dicer-1 associated with Loqs for efficiently processing of pre-miRNAs into mature miRNA (33). Previously, mutation of Loqs in flies and depletion of Loqs in Schneider-2 (S2) cells by dsRNA-triggered RNAi disrupt normal pre-miRNA processing. Therefore, Loqs is required for robust miRNA-directed silencing and complete target gene repression directed by a transgene expressing dsRNA. Moreover, loss of Loqs function in the ovary disrupts germ-line stem cell maintenance rendering loqs mutant females sterile (34). In contrast, Dicer-2 forms a heterodimeric complex with the R2D2 which is required for production of siRNAs and loading into RISC assembly (35).

1.4.1 DsRNA-binding protein in shrimp

In shrimp *Fenneropenaeus chinensis*, sequences of three different isoforms of the dsRBP (FcTRBP) have been characterized. It contains three dsRBD, two N-terminal domains that mediate dsRNA binding, and a C-terminal domain that is required for interaction with Dicer (38). Both Fc-TRBP2 and Fc-TRBP3 are identical to the Fc-TRBP1, except that they lack 60 and 141 bp between dsRBD2 and dsRBD3, respectively. The isoforms result from alternative splicing of the same transcript. In addition, TRBP of *Marsupenaeus japonicus* has been studied. The result showed that RNAi against TRBP impaired the dsRNA-induced sequence specific RNAi pathway and facilitated the proliferation of white spot syndrome virus (WSSV) (39). In shrimp *Penaeus monodon*, cloning of the putative Dicer-binding protein or Loquacious of *Penaeus monodon* (PmLoqs) was performed by PCR approach or Rapid amplification of cDNAs (RACE). The primers were designed from the partial nucleotide sequences of the putative Loquacious of *Litopenaeus vannamei* that was obtained from the EST database (**MGID Number:** 4765 or **NCBI Accession:** BF024283). The partial cDNA sequences of the putative PmLoqs of size about 1156 nucleotides were obtained. The deduced amino acid sequences were blasted in the GenBank database and showed 46% similarity to R3D1 or Loquacious of *Drosophila melanogaster*. The putative sequence of PmLoqs was clarified in two major types that are long and short form. First, the long form consists of two isoforms PmLoqs(l)1 and PmLoqs(l)2. Both of them contain three dsRBDs that are identical, except that PmLoqs(l)2 lack nucleotides sequence approximately 69 bp between dsRBD2 and dsRBD3. Second, the short isoform PmLoqs(s) contains only two dsRBDs (dsRBD1 and dsRBD2). Sequence alignments demonstrated that the putative PmLoqs is highly conserved, especially in the dsRBD. It has high sequence similarity to the isoform of the TRBP family of *Fenneropenaeus chinensis* that contains three dsRBDs, two N-terminal domains and C-terminal domain but differs at the C-terminus (Figure 1-2).

However, the function of Loqs in *P. monodon* is still unclear. Whether these RNAi machineries play important roles in the shrimp RNAi pathway remains to be elucidated. Therefore, the objective of this study is to clone and characterize one of the RNAi machineries, Loquacious (Loqs) in black tiger shrimp, *Penaeus monodon.*

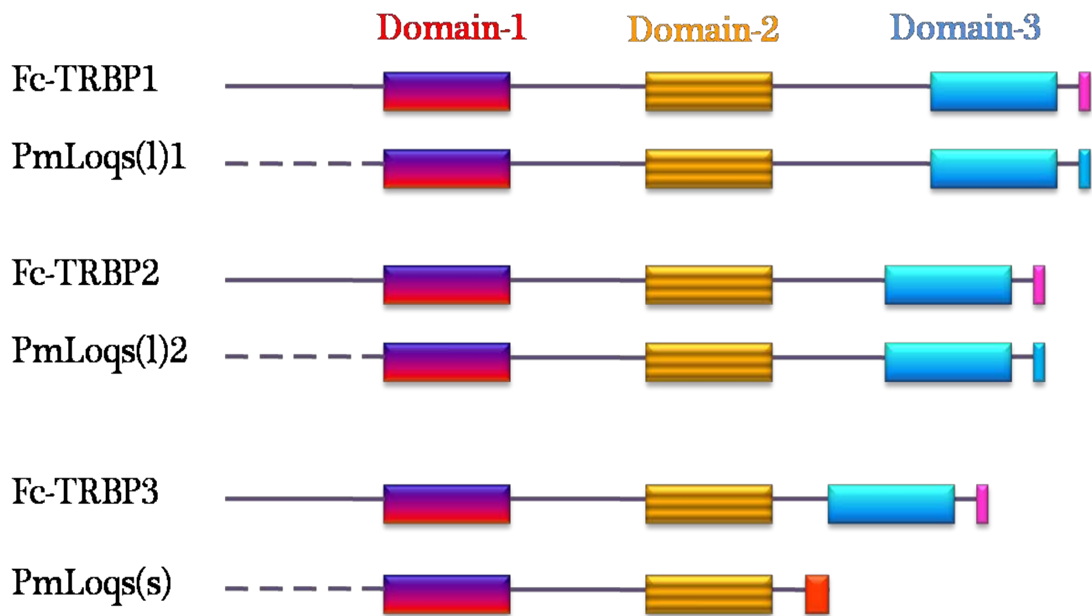

Figure 1-2 A schematic diagram of the domain structure of FcTRBP and PmLoqs.

Sequence alignments of the putative PmLoqs and FcTRBP showed highly conserved, especially in the dsRBD, domain 1, 2 and 3. Three isoforms of PmLoqs contain 2 long isoforms (PmLoqs(l)1 and PmLoqs(l)2) and 1 short isoform (PmLoqs(s)). FcTRBP has three isforms; FcTRBP1, FcTRBP2 and FcTRBP3.